Fluid Biomarkers in the Frontotemporal Dementia Spectrum

Lieke H.H. Meeter

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Fluïde biomarkers in het frontotemporale dementie spectrum

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General Introduction

CHAPTER 1.1

Introduction to the Thesis

Frontotemporal dementia (FTD) is a heterogeneous form of dementia that characteristically presents at a presenile age with progressive behavioural disturbances (behavioural variant FTD) and/or language problems (semantic or nonfluent variant primary progressive aphasia).[1,2] Concomitant motor symptoms frequently occur, represented by FTD with motor neuron disease, corticobasal syndrome, and progressive supranuclear palsy as part of the same clinicopathological spectrum. An autosomal dominant genetic form of FTD is present in 10-20% of patients, most commonly caused by mutations in either *MAPT* (microtubule-associated protein tau), *GRN* (progranulin), or a repeat expansion in *C9orf72* (chromosome 9 open reading frame 72).[3] Postmortem brain examination shows frontotemporal lobar degeneration (FTLD) with inclusions of either tau protein (FTLD-tau), TAR DNA-binding protein 43 (TDP-43; FTLD-TDP), or FET (fused in sarcoma, Ewing's sarcoma and TAT-binding protein-associated factor 15).[4,5]

During the past decades, major advances have been made in understanding the pathology and genetics of FTD, which are now actively being translated into trials with therapeutic interventions. To appropriately select and monitor patients in these trials, robust biomarkers are urgently needed. For sporadic FTD patients, diagnostic markers that identify clinical and pathological subgroups are essential for the selection of patients, and monitoring markers to measure therapeutic effects. For genetic FTD, biomarkers are required to determine disease onset, disease progression and target engagement. For example neurofilament light chain (NfL) is a promising biomarker for neurodegenerative diseases,[6] but was at the start of this PhD project limitedly studied in FTD.

This thesis investigated the utility of biomarkers for FTD in cerebrospinal fluid (CSF) and blood, the so-called fluid biomarkers. The aims of the current thesis were twofold:

1. To investigate the application of NfL across the sporadic FTD spectrum (**Chapter 2**); and
2. To identify and study the value of blood and CSF biomarkers in genetic forms of FTD (**Chapter 3**).

Chapter 1.2 provides a general introduction into FTD and its imaging and fluid biomarkers. The utility of NfL and the phospho- to total tau ratio across the entire clinical and pathological FTD spectrum is described in **Chapter 2.1**. Next, the thesis outlines the value of NfL in two specific sporadic subtypes: semantic variant primary progressive aphasia (**Chapter 2.2**) and progressive supranuclear palsy (**Chapter 2.3**). In **Chapter 3**, the focus shifts towards fluid biomarkers in hereditary forms, starting with serum and CSF NfL and their clinical and imaging correlations in genetic FTD (**Chapter 3.1**). Subsequently poly(GP) and NfL levels were studied in relation to grey matter deficits in *C9orf72* repeat expansion carriers (**Chapter 3.2**), and progranulin protein levels were measured over time in *GRN* mutations carriers (**Chapter 3.3**). **Chapter 3.4** describes the identification of novel candidate biomarkers in *GRN* mutations carriers by proteomics on CSF. The results of this thesis are discussed in light of the current literature along with methodological considerations and future directives in **Chapter 4**. Lastly, **Chapter 5** summarizes the main results of this thesis.

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CHAPTER 1.2

Imaging and fluid biomarkers in frontotemporal dementia

Lieke H.H. Meeter; Laura Donker Kaat; Jonathan D. Rohrer; John C. van Swieten

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Abstract

Frontotemporal dementia (FTD), the second most common type of presenile dementia, is a heterogeneous neurodegenerative disease characterized by progressive behavioural and/or language problems, and includes a range of clinical, genetic and pathological subtypes. The diagnostic process is hampered by this heterogeneity, and correct diagnosis is becoming increasingly important to enable future clinical trials of disease-modifying treatments. Reliable biomarkers will enable us to better discriminate between FTD and other forms of dementia and to predict disease progression in the clinical setting. Given that different underlying pathologies probably require specific pharmacological interventions, robust biomarkers are essential for the selection of patients with specific FTD subtypes. This Review emphasizes the increasing availability and potential applications of structural and functional imaging biomarkers, and cerebrospinal fluid and blood fluid biomarkers in sporadic and genetic FTD. The relevance of new MRI modalities — such as voxel-based morphometry, diffusion tensor imaging and arterial spin labelling — in the early stages of FTD is discussed, together with the ability of these modalities to classify FTD subtypes. We highlight promising new fluid biomarkers for staging and monitoring of FTD, and underline the importance of large, multicentre studies of individuals with presymptomatic FTD. Harmonization in the collection and analysis of data across different centres is crucial for the implementation of new biomarkers in clinical practice, and will become a great challenge in the next few years.

Box 1. Main clinical characteristics of FTD

Behavioural variant frontotemporal dementia (bvFTD)
bvFTD is characterized by personality and behavioural changes (including disinhibition, apathy, loss of sympathy, perseverative behaviour, and abnormal appetite), and executive dysfunction
Primary progressive aphasia (PPA)
PPA features progressive prominent language difficulties that impair daily living. Subtypes include:

- *Semantic variant PPA (svPPA).* Fluent speech characterized by anomia and impaired single word comprehension
- *Nonfluent variant PPA (nfvPPA).* Nonfluent speech with agrammatism and/or apraxia of speech
- *Logopenic variant (lvPPA).* Nonfluent speech with word-finding difficulties in spontaneous speech and in repetition

Introduction

Frontotemporal dementia (FTD) is the second most common form of dementia in people aged under 65 years, and encompasses two main clinical manifestations: behavioural changes with executive dysfunction, so-called behavioural-variant FTD (bvFTD), or predominant language impairment, so-called primary progressive aphasia (PPA) (Box 1).[1,2] PPA can be further divided into semantic variant PPA (svPPA), nonfluent variant PPA (nfvPPA) and logopenic variant PPA (lvPPA).[2] Patients can develop concomitant parkinsonism or motor neuron disease (MND) at an early or late stage in the disease course, which results in a broad clinical phenotype that ranges from amyotrophic lateral sclerosis (ALS) to progressive supranuclear palsy (PSP) and corticobasal syndrome (Figure 1).[3] Patients who present with nfvPPA can develop characteristic features of PSP or corticobasal syndrome over time, whereas lvPPA is frequently associated with underlying Alzheimer disease (AD).

Postmortem examination of the brains of people who present clinically with FTD reveals frontotemporal lobar degeneration (FTLD) associated with inclusions of either microtubule-associated protein tau (referred to as FTLD-tau), TAR DNA-binding protein 43 (TDP-43; referred to as FTLD-TDP), or RNA-binding protein fused in sarcoma (referred to as FTLD-FUS).[3] Rare cases of FTLD are characterized by ubiquitin-positive inclusions without immunoreactivity for TDP-43 or FUS (referred to as FTLD-UPS). Correlation between the clinical presentation and specific underlying pathology is poor in bvFTD compared with svPPA and FTD associated with MND, both of which are associated with TDP-43 pathology.[4] Postmortem examination of patients who developed symptoms consistent with PSP or corticobasal syndrome often reveals FTLD-tau. In contrast to sporadic FTD, the underlying pathology in genetic FTD can be accurately predicted (Figure 1).

FTD is highly heritable, and 10–20% of all cases are caused by mutations in three genes: *MAPT* (encoding microtubule-associated protein tau), *GRN* (encoding progranulin, also known as acrogranin), and *C9orf72* (encoding protein C9orf72).[3] Other rare FTLD-associated genes include *CHMP2B* (encoding charged multivesicular body protein 2B), *VCP* (encoding valosin containing protein), *SQSTM1* (encoding sequestosome-1), *TARDP* (encoding TDP-43), and *TBK1* (encoding the serine–threonine-protein kinase TBK1) — the latter being

the most recently discovered FTLD-associated gene, identified in 2015.[5] Although some phenotypes are associated with specific mutations — for example the co-occurrence of MND with *C9orf72* mutations — genotype–phenotype correlations are generally poor, even within families.[3]

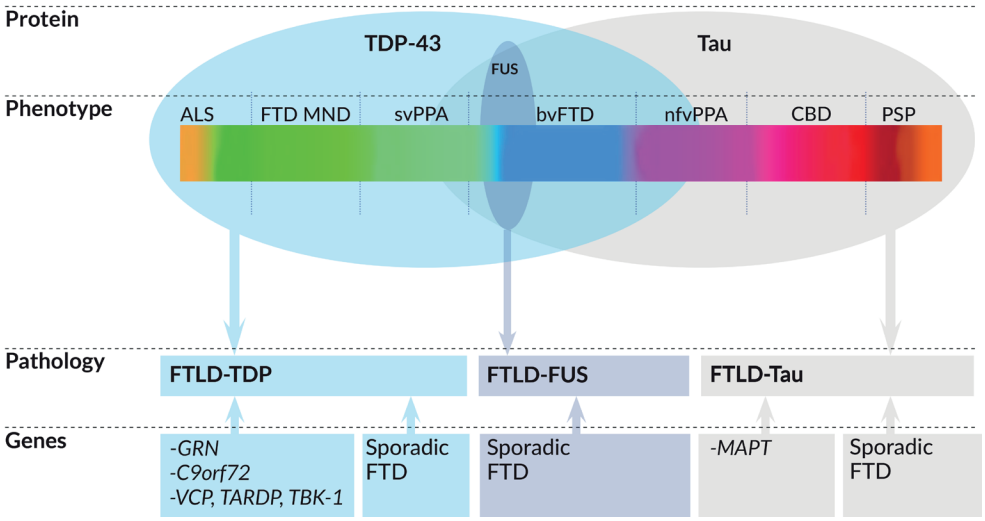

Figure 1. Clinical, pathological and genetic spectrum of FTD.
Genetic forms of frontotemporal dementia (FTD) have predictable pathology: *GRN* mutations and *C9orf72* repeat expansions result in TDP-43 pathology, whereas *MAPT* mutations result in tau pathology. By contrast, variable underlying pathologies and genetic forms are found across the clinical spectrum of FTD. Amyotrophic lateral sclerosis (ALS) and FTD with concurrent motor neuron disease (FTD–MND) phenotypes are infrequently caused by FTLD-FUS pathology or *FUS* mutations, but for simplicity this detail is not included in the figure. bvFTD: behavioural variant FTD; CBD corticobasal degeneration; FUS: RNA-binding protein FUS; nfvPPA: nonfluent variant primary progressive aphasia; PSP: progressive supranuclear palsy; svPPA: semantic variant primary progressive aphasia; TDP-43: transactive response DNA-binding protein 43. Modified with permission from BMJ Publishing Group © Seelaar, H. *et al. J. Neurol. Neurosurg. Psychiatry* **82**, 476–86 (2011).

Sensitive biomarkers for FTD are crucial owing to the heterogeneity of the disorder. Great efforts to identify these biomarkers have been made over the past two decades, with a predominant focus on fluid biomaterial and neuroimaging features. According to previous consensus, the ideal biomarker should detect a fundamental pathological feature of the disease, should be validated in pathological proven cohorts, and should be precise, reliable, inexpensive and detectable through a procedure that is noninvasive and simple to perform (Box 2).[6] Different biomarkers can be used for specific purposes, so the value of a biomarker depends on its application. In FTD, diagnostic biomarkers should discriminate between individuals with FTD, control individuals and individuals with other neurodegenerative diseases, or should differentiate between clinical, genetic or pathological subtypes. Staging biomarkers should enable us to assess disease severity and to discriminate between presymptomatic, prodromal, and early or late symptomatic stages

of the disease. Pharmacodynamic biomarkers are important for the evaluation of the biological and clinical effect of future therapeutic interventions. Prediction of the underlying pathology in FTD (tau versus TDP-43) is one of the greatest challenges, as this distinction will be essential when specific disease-modifying interventions become available. Ideally, these interventions should be applied at an early stage in the disease when only minimal neuronal damage is present, which highlights the need for early biomarkers; at-risk individuals from families with genetic forms of FTD are the ideal study population for detecting these earliest changes.

Box 2. Biomarkers: requirements and applications

Requirements: *(adapted from [6])*
- Able to detect fundamental feature of FTD pathology
- Validated in neuropathologically confirmed FTD
- Precise
- Reliable
- Noninvasive
- Simple to perform
- Inexpensive

Applications:
- Prediction
- Diagnostic
- Staging
- Monitoring of disease progression
- Monitoring of treatment response (surrogate endpoint, target engagement)
- Prognostic

In this Review, we focus on fluid and neuroimaging biomarkers in FTD. We discuss previous studies on biomarkers with their current application in clinical practice and we highlight the development of new, promising biomarkers.

Neuroimaging biomarkers

Most FTD imaging studies have focused on structural changes by assessing grey matter atrophy, but studies within the past 5–10 years have examined white matter integrity using diffusion tensor imaging (DTI); these white matter changes are probably more sensitive for the earliest changes in FTD than are grey matter changes. In neurodegenerative diseases, structural abnormalities are often preceded by functional changes; in the following sections we describe both the structural and functional changes identified with different imaging modalities. Most imaging studies have focused on group analyses, but these group-based results cannot always be translated to the individual patient, as a strong discriminative power between patient groups or outcomes is needed to apply these biomarkers in clinical practice.

Structural changes

Grey matter. The majority of imaging studies in FTD have used volumetric T1-weighted MRI to investigate changes in grey matter structure.[7–10] This technique is used to measure brain volume, the rate of brain atrophy, and the volumes of specific brain regions of interest — for example, the frontal lobe or hippocampus. Several postprocessing analytical techniques have also been applied to T1-weighted imaging; for example, investigation of changes at the voxel level (such as voxel-based morphometry) or measurement of cortical thickness (using software such as FreeSurfer), each of which provide an alternative way to investigate grey matter loss in the brain.

On an individual patient level, semiquantitative assessment of atrophy using visual rating scales, performed by experienced dementia experts, has provided good diagnostic performance in the discrimination of FTD from AD (with more posterior cortical involvement seen in the latter than the former), with a specificity of 81%.[11] Clinical, genetic and pathological syndromes of FTD can also be distinguished to some degree by distinct and dissociable patterns of grey matter atrophy at a group level (Figure 2). Clinically, bvFTD is associated with atrophy in the frontal and temporal lobes, the insula and the anterior cingulate cortex, with the earliest involvement of frontal paralimbic cortices and insula.[12–14] Cluster analyses suggest that four anatomical forms of bvFTD exist: frontal-dominant, temporal-dominant, frontotemporal and distributed frontotemporoparietal.[15,16] However, these analyses have underplayed the involvement of subcortical structures in bvFTD: atrophy of the hippocampus, amygdala, basal ganglia and thalamus clearly occur as the disease progresses.[14,17] In the PPA syndromes, svPPA is associated with asymmetrical (commonly left-sided) anteroinferiortemporal lobe atrophy, nfvPPA with predominantly left-sided inferior frontal and insula involvement, and lvPPA with left temporoparietal junction loss.[18,19] In each of the PPA syndromes, the extent of atrophy progresses over time, not only within the same hemisphere but also — later in the disease course — in the opposite hemisphere.[20–22] In the genetic forms of FTD, *GRN* mutations are associated with asymmetrical frontotemporoparietal atrophy, *MAPT* mutations are associated with relatively symmetrical involvement of the anteromedial-temporal and orbitofrontal lobes, and *C9orf72* expansions are associated with a symmetrical and widespread pattern of atrophy with involvement of the thalamus and superior cerebellum.[16,23–26] Despite the presence of group-level patterns, identification of individuals with specific pathological forms of FTD has proved difficult when structural T1-weighted imaging is used alone, and the distinction between patients with FTLD-TDP or FTLD-tau has not been possible.[9] Patients with FTLD-FUS pathology generally present with prominent caudate atrophy, accompanied by orbitofrontal, anteromedial temporal, anterior cingulate, and insula atrophy.[27,28]

Across clinical, genetic and pathological forms of FTD, less research has been conducted to assess longitudinal changes in grey matter loss than loss at single time points. However, rates of atrophy clearly vary between different groups, with some being relatively fast (for example, in patients with *GRN* mutations), and some very slow (for example, a subgroup of patients with *C9orf72* repeat expansions).[29] If longitudinal structural imaging could be

used for monitoring in clinical trials, sample size estimations show that focal atrophy rates, such as in the temporal lobe in svPPA, would enable use of a smaller sample than do whole brain atrophy rates.[7]

Findings from several small studies of individuals who are at risk of genetic FTD have been inconsistent, with some showing grey matter atrophy before onset of symptoms and others not. However, in 2015, a large multicentre analysis from the Genetic Frontotemporal Dementia Initiative (GENFI) study identified the presence of atrophy in individuals with FTD-associated mutations at least 10 years before expected symptom onset (Figure 3A), with different genetic groups showing different patterns.[30] In individuals with *MAPT* mutations, atrophy was first noted in the hippocampus and amygdala, followed by the temporal lobe and later the insula; in *GRN* mutation carriers, differences started in the insula, followed by the temporal and parietal lobes and thereafter the striatum; in the *C9orf72* group, changes were found very early (25 years before expected onset) in subcortical areas (including the thalamus), the insula and the occipital cortex, then the frontal and temporal lobes and subsequently the cerebellum.[30] In individuals with *GRN* mutations, but not in the other genetic subgroups, prominent asymmetry was found in the atrophy at 5 years before expected symptom onset. Examination of changes in this cohort over time is important, as small-scale longitudinal studies have proven more sensitive than cross-sectional studies, as illustrated by the identification of a significant reduction in left temporal cortical thickness over time in presymptomatic *GRN* carriers, with no differences found between presymptomatic individuals and noncarriers at baseline.[31]

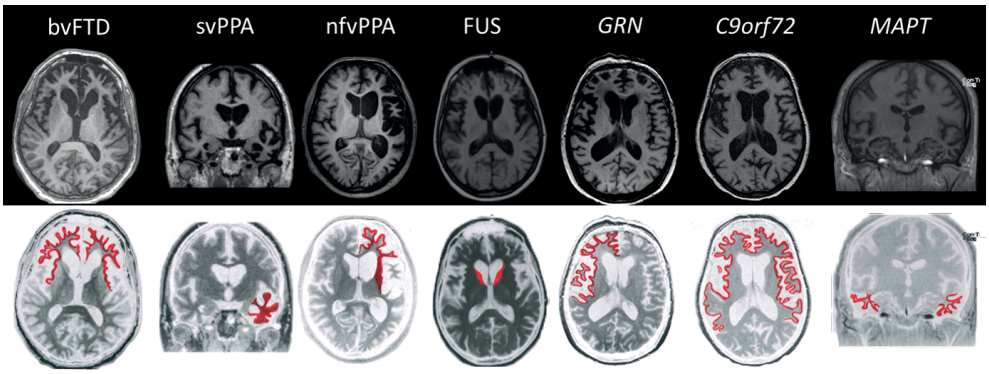

Figure 2. Grey matter atrophy in FTD.
Characteristic patterns of grey matter atrophy (highlighted in red) in different clinical and genetic subtypes of frontotemporal dementia (FTD). Patients with behavioural variant FTD (bvFTD) exhibit prominent frontal, insular and anterior cingulate atrophy. Typical temporal atrophy in semantic variant primary progressive aphasia (svPPA) is asymmetrical (most often left-sided). Patients with nonfluent variant primary progressive aphasia (nfvPPA) exhibit left frontal and insular atrophy. In patients with underlying RNA-binding protein FUS (FUS) pathology, nucleus caudatus atrophy is pronounced. Patients with *GRN* mutations often exhibit asymmetrical frontotemporoparietal atrophy. Patients with a *C9orf72* repeat expansion present mostly with a generalized symmetrical atrophy. Patients with *MAPT* mutations exhibit marked symmetrical temporal atrophy.

White matter. DTI is a valuable noninvasive imaging technique for the assessment of the white matter structure of the brain. This technique measures the microstructural integrity of white matter by determining the rate of diffusion (that is, the motion of water molecules) in different directions. Changes in different DTI metrics are thought to reflect different pathological changes in microstructure: a decrease in axial diffusivity correlates with axonal degeneration; an increase in radial diffusivity indicates myelin breakdown; and a decrease in fractional anisotropy — a composite measure of both axial diffusivity and radial diffusivity — represents a general, nonspecific loss of white matter integrity.[32] Abnormalities in white matter diffusivity have been found to precede grey matter atrophy in FTD and to have a more widespread distribution in the brain, supporting the importance of white matter involvement in FTD.[33–38] DTI findings could become valuable biomarkers, as the approach has at least four potential applications: differentiation between individuals with FTD, individuals with other types of dementia, and individuals without dementia; differentiation between subtypes of FTD; disease monitoring; and detection of early changes before disease onset. However, white matter integrity has only been investigated with DTI at a group level and not at a single-patient level.

DTI enables highly sensitive differentiation between individuals with FTD, individuals with other types of dementia (such as AD) and controls without dementia.[32–36,38–45] Evidence has shown that changes in white matter microstructure are more widespread in people with FTD than in those with AD,[32,34,39,40] and whole-brain mean fractional anisotropy enables discrimination between these conditions with a high sensitivity (78%) and moderate specificity (68%).[34] White matter degradation co-occurs with frontal, temporal and insular atrophy in FTD, and probably results from axonal degeneration associated with grey matter neuronal loss. This degeneration encompasses the anterior corpus callosum, bilateral anterior and descending cingulum, and uncinate fasciculus tracts,[42] which are part of motor, executive and language neural networks.

Although patterns of white matter damage on DTI largely overlap between subtypes of FTD, some distinctive DTI changes have been found in clinical, pathological and genetic subtypes.[33,35,36,38,40,42–44,46] The uncinate fasciculus, cingulum bundle and genu of the corpus callosum seem to be key tracts involved in the bvFTD disease process.[34,41,44] Different spatial patterns of white matter damage have been found in PPA subtypes: patients with nfvPPA show damage to the left orbitofrontal and anterior temporal white matter (superior longitudinal fasciculus); patients with svPPA show asymmetric (mostly left-sided) changes in the anterior and inferior temporal white matter (including the inferior longitudinal fasciculus), and bilateral uncinate fasciculi; and patients with lvPPA show posterior abnormalities, such as in the posterior region of the left inferior longitudinal fasciculus.[33,35,36,38,42–44] DTI might be able to differentiate FTLD-tau from FTLD-TDP *in vivo*: two studies have found more severe loss of white matter integrity in FTLD-tau than in FTLD-TDP.[33,46] This observation parallels post-mortem findings in which tau pathology is associated with marked axonal loss and glial tau inclusions, and TDP-43 pathology is associated with greater grey

matter neuronal loss than white matter pathology.[46] Larger studies are needed to enable conclusive observations to be made before this technique can be used to differentiate pathological subtypes in individual patients. DTI studies have revealed different patterns of white matter loss across patients with different genetic subtypes of FTD: patients with *MAPT* mutations have consistent alterations in the uncinate fasciculus and right parahippocampal cingulum,[34,41] whereas patients with *C9orf72*-FTD tend to have a greater amount of dorsal white matter tract pathology located in the cingulum, corpus callosum and the superior cerebellar peduncles.[34,41]

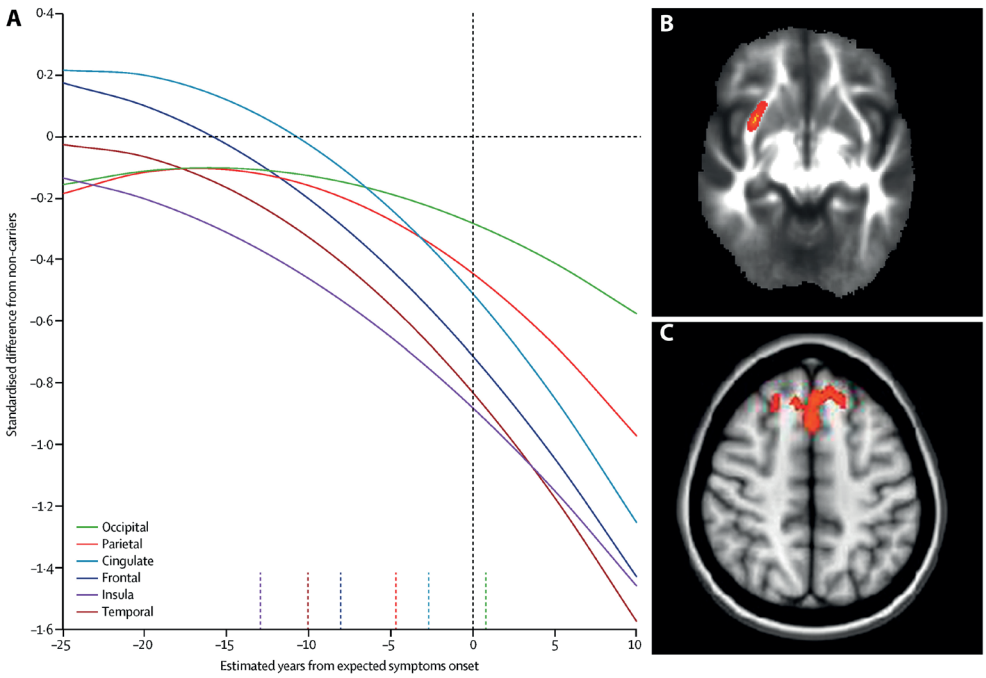

Figure 3. Imaging abnormalities in the presymptomatic stage of genetic FTD.
(A) Grey matter changes from the Genetic Frontotemporal Dementia Initiative (GENFI) study: standardized difference between all (presymptomatic and symptomatic) mutation carriers and noncarriers in cortical grey matter volumetric imaging measures, versus estimated number of years from expected symptom onset for a given difference in volume between carriers and noncarriers. Dotted lines on the x-axis show the time at which the upper 95% confidence intervals for each curve crosses zero on the y-axis (that is, the point at which a statistically significant difference exists between mutation carriers and noncarriers). (B) Changes in fractional anisotropy (red) as measured by diffusion tensor imaging. Presymptomatic carriers of *GRN* and *MAPT* mutations have decreased fractional anisotropy in the uncinate fasciculus versus control individuals. (C) Changes in brain perfusion (red) as measured by arterial spin labelling. Presymptomatic *GRN* mutation carriers have a lower cross-sectional cerebral blood flow at follow-up than in control individuals. Part A modified with permission from Elsevier © Rohrer, J. D. *et al. Lancet. Neurol.*14, 253–262 (2015) under a Creative Commons CC BY 4.0 license. Part B reproduced with permission from Lippincott Williams & Wilkins © Dopper, E. G. *et al. Neurology* 80, 814–823 (2013) http://bit.ly/2rAsnKd. Part C reproduced with permission from Elsevier © Dopper, E. G. *et al. NeuroImage. Clin*. 12, 460–465 (2016).

Longitudinal assessment of white matter changes with DTI could also be used to monitor the disease process and evaluate therapeutic effects in future clinical trials in FTD, although studies so far are limited.[41] Over time, white matter changes have been found to be more widespread than the changes in grey matter atrophy, and have shown distinct patterns between clinical and genetic FTD subtypes, which reflect different propagation of the neurodegenerative process within large-scale brain networks.[35] In bvFTD, the largest reduction in fractional anisotropy has been seen in the bilateral uncinate fasciculus and paracallosal cingulum,[35,41] whereas left-to-right sided progression is detected in both svPPA and nfvPPA.[35,43] In svPPA, longitudinal white matter changes extend to bilateral frontotemporal tracts, whereas changes in nfvPPA seem to remain comparatively focal.[35,43]

Finally, the use of white matter pathology detected by DTI as a biomarker might even enable the detection of pathological changes before the onset of clinical symptoms and before grey matter atrophy in FTD. Decreased fractional anisotropy and increased radial diffusivity have been found in the bilateral uncinate fasciculi in a group of presymptomatic carriers of *MAPT* or *GRN* mutations who did not have grey matter atrophy (Figure 3B).[37,47]

In conclusion, white matter changes detected with DTI are a promising biomarker for early diagnosis of FTD and for monitoring the effect of pharmacological interventions in the future. For DTI to be used in individual patients, reference data are essential to enable identification of abnormal changes in white matter integrity, as in automated quantitative MRI.[48] However, the assembly of such normative data is challenging, owing to variability across scanners and field strengths, and choices of DTI metric, tract to assess, and method of analysis (for example, tracking or skeletonized analysis). Region of interest analyses of specific tracts, such as the uncinate fasciculus, inferior longitudinal fasciculus and superior longitudinal fasciculus, will probably provide the best opportunity to move forward from the current group-level studies to single-patient analyses.

Functional changes

FDG-PET. The use of PET with ^{18}F-fluorodeoxyglucose as the tracer (FDG-PET) enables visualization of alterations in brain metabolism that precede grey matter atrophy in FTD and different forms of dementia.[49–52] Distinct patterns of regional hypometabolism detected with FDG-PET enables an accurate clinical diagnosis to be made at an individual patient level, both by visual inspection and especially by quantitative assessment.[53] Low glucose metabolism (often asymmetrical) in the orbitofrontal cortex, dorsolateral cortex, medial prefrontal cortex, anterior temporal poles, and basal ganglia, is highly specific for bvFTD, and differentiates patients with bvFTD from those with other dementia types and healthy controls with a sensitivity and specificity of 80–95%.[50,51,53–56] These patterns of hypometabolism are early features of symptomatic bvFTD, but also occur a few years before patients fulfil the criteria for probable bvFTD.[50] However, FDG-PET has produced false positive findings in some primary psychiatric disorders that mimic FTD, so future quantitative

assessment of metabolism patterns with PET are needed to increase the diagnostic value of the technique.[54]

The patterns of focal hypometabolism vary between subtypes of PPA and between different genetic forms of FTD, and mirror those of the structural changes described in the previous section. svPPA is characteristically associated with asymmetrical bilateral temporal hypometabolism, whereas nfvPPA is associated with a higher variability in hypometabolic patterns of the left inferior frontal gyrus, dorsolateral frontal cortex, anterior cingulate cortex, insula, and — occasionally — the parietal cortex.[57] Distinct patterns of metabolic abnormalities in PPAs might predict progression to specific dementia subtypes: evidence suggests that bilateral temporoparietal hypometabolism predicts conversion to AD, parietal hypometabolism predicts conversion to corticobasal syndrome, and involvement of the basal ganglia, midbrain and cerebellum predicts conversion to PSP.[57] Longitudinal changes of metabolism detected by FDG-PET could provide additional information about the patterns and speed of pathological spread.[31,56] For example, patients with svPPA exhibit a bilateral reduction of glucose metabolism in the temporal lobes over time, which extends to the anterior cingulate cortex and the posterior temporal lobes.[58] Regarding genetic subtypes, *GRN* mutations are associated with asymmetrical hypometabolism in frontal and temporal brain regions,[31,59] ALS and/or FTD resulting from *C9orf72* expansions is associated with hypometabolism in the limbic system, basal ganglia and thalamus,[60] and *MAPT* mutations are associated with hypometabolism in the medial temporal lobe and the frontal and parietal cortices.[24]

Interestingly, FDG-PET can reveal abnormalities in the presymptomatic stage of FTD, and could serve as a surrogate endpoint in future therapeutic trials; asymmetrical hypometabolism was found in the frontal and temporal lobes of asymptomatic *GRN* carriers before the onset of clinical symptoms and of grey matter atrophy.[31,59]

Arterial spin labelling. The MRI technique arterial spin labelling (ASL) measures brain perfusion noninvasively by magnetically labelling water protons in arterial blood, which creates an endogenous tracer of cerebral blood flow.[61] Brain perfusion measured by ASL correlates very well with metabolism measured by FDG-PET,[51,53] but ASL has several advantages over FDG-PET: ASL can be combined with other MRI techniques in a single session, is noninvasive, involves no radiation exposure, is widely available and is less costly.[62]

In patients with FTD, ASL has detected hypoperfusion in the insula, the amygdala and several parts of the medial frontal lobes, including the anterior cingulate.[51,53,63–65] ASL has also been used to differentiate bvFTD from AD at an early phase, with a diagnostic accuracy (area under the receiver operating characteristic curve) of up to 0.87 for cerebral blood flow in specific frontal or parietal regions.[51,53,63] In two comparative studies, the regions of hypoperfusion identified on ASL MRI scans largely overlapped with those identified on FDG-PET scans,[51,53] and diagnostic performance for distinguishing bvFTD from AD and controls was similar for both modalities.[53]

Brain perfusion measured by ASL could also be an early biomarker in the preclinical stage of genetic FTD. Decreases in cerebral blood flow over time are significantly larger in presymptomatic individuals who carry *GRN* or *MAPT* mutations than in control individuals (Figure 3C), independent of grey matter atrophy, in widespread frontal, temporal, parietal, and subcortical regions; the largest decline in perfusion was observed in those who converted to the disease stage.[62] Some regional changes in brain perfusion might be specific for particular gene defects, as hypoperfusion can extend into posterior temporal and parietal regions in those with a *GRN* mutation.[62]

Resting-state functional MRI. Another potential biomarker for early diagnosis and disease staging in FTD is functional connectivity measured with resting-state functional MRI (RS-fMRI). RS-fMRI measures intrinsic functional connectivity between brain regions, which can be detected as synchronous patterns of spontaneous, low-frequency fluctuations in blood oxygen level-dependent signals. RS-fMRI is a safe, noninvasive and repeatable tool that is sensitive to changes in brain functional connectivity before the onset of clinical symptoms or atrophy at the group level, as opposed to the individual level.[37,66,67] Decreased connectivity between the frontoinsula and anterior cingulate cortex, part of the salience network, is the most consistent RS-fMRI finding in patients with FTD,[67–71] but some studies have found normal or increased connectivity.[72–74] Inconsistent differences (increased and decreased connectivity) have been found in the default mode network in FTD.[67–69] These discrepancies in functional connectivity might partly be explained by differences between cohorts and scanners, and by the wide variation in analytical methods used, such as independent component analyses, seed-based or region-of-interest-based approaches, or regional homogeneity analyses.[66,68,72,74,75]

Specific network alterations are also found in different clinical and genetic subtypes of FTD. Reduced left temporal lobe connectivity is found in svPPA,[76,77] attenuated connectivity in salience and sensorimotor networks is found in patients with *C9orf72* bvFTD,[26] and reduced left frontal connectivity is found in patients with *GRN* mutations.[78] In the presymptomatic phase of FTD, RS-fMRI might be sensitive to connectivity differences: altered (reduced and increased) frontoinsula and/or anterior cingulate cortex connectivity have been reported in presymptomatic mutation carriers.[37,66,78,79]

Amyloid and tau PET tracers. Several tracers other than ^{18}F-fluorodeoxyglucose could be used to identify diagnostic biomarkers in the differential diagnosis between FTD and AD, and between different pathological subtypes of FTD. PET with an amyloid tracer, such as Pittsburgh compound B (PiB), robustly and sensitively detects amyloid-β deposits, which indicate AD pathology *in vivo*,[80] whereas bvFTD, svPPA and nfvPPA are mostly PiB-negative. Most lvPPA cases are atypical AD cases and are associated with a PiB binding pattern similar to that seen in AD,[81–83] but lvPPA with negative PiB-PET is accompanied by structural and FDG-PET abnormalities, which support an underlying FTLD pathology.[83,84] Unexpected positive PiB-PET findings in patients with FTD can result from mild coincidental AD pathology, unrelated to the clinical FTD presentation.[85]

Several tracers have been developed that visualize tau pathology *in vivo*; however, an ideal ligand that captures the wide range of tau pathology in existence has not yet been developed. Selectivity to different tau isoforms and their intracellular aggregation probably requires the application of different tau ligands.[86] In tau PET with the ^{18}F-AV-1451 ligand (also known as flortaucipir), increased uptake in the temporal cortex, frontal cortex, and basal ganglia is seen in patients with FTD who have an Arg406Trp *MAPT* mutation, which is associated with both 3-repeat and 4-repeat tau pathology. In these patients, increased regional ^{18}F-AV-1451 uptake correlated with decreased glucose metabolism and with the post-mortem burden of tau pathology.[87] However, conditions in which only 4-repeat tau pathology is present are associated with poor binding of ^{18}F-AV-1451, as illustrated by the lack of correlation between ^{18}F-AV-1451 binding and post-mortem tau pathology in PSP.[88,89] A 2017 study of post-mortem brains reported that the ligand ^{11}C-PBB3 could more robustly capture a wide range of tau pathologies than ^{18}F-AV-1451, including 3-repeat and 4-repeat tau conditions.[90] Furthermore, the ^{18}F-THK-5351 ligand produced promising results in the 4-repeat tau diseases PSP and corticobasal syndrome, in post-mortem tissue and *in vivo*.[91,92] Once validated, tau PET could become effective for the diagnosis of underlying tau pathology in FTD and could provide a surrogate marker for trials with anti-tau therapeutics.[86]

Summary of imaging biomarkers

Grey matter atrophy and hypometabolism are validated diagnostic biomarkers that show fairly consistent changes between studies at a group level, and are clinically applied at an individual level for the differentiation between FTD, AD and control individuals (Table 1). More work is required on the use of imaging modalities to distinguish FTD subtypes at an individual level, with a need for larger studies of longitudinally acquired imaging data, before these techniques can be used as an outcome measure to monitor disease progression in clinical trials.

We expect that new modalities, such as DTI, ASL and RS-fMRI, will become valuable tools for detecting biomarkers in clinical practice, especially owing to their sensitivity, and have the potential to enable early diagnosis and longitudinal monitoring of disease (Table 1). Crucial to the implementation of these techniques is the harmonization of methodology across different centers, as scanners and protocols can vary considerably. For example, the diversity of ASL scanning protocols influences perfusion quantification, which could be overcome by a proposed international standardization of protocols.[61] Additionally, the integration of different types of information through the combination of imaging modalities holds great promise for the future, as demonstrated by multimodal analyses that have improved the discrimination between FTD and AD,[40,45,52,65,93,94] and between clinical FTD subtypes.[94]

Table 1. Potential biomarkers in FTD and their application in clinical practice

Biomarker	Application					
	Ability to differentiate between diagnoses		Staging and monitoring of disease progression		Prognosis	Monitoring treatment response
	FTD versus AD	Clinical, genetic and/or pathologic subtypes of FTD	Symptomatic	Presymptomatic		
Imaging biomarkers						
Grey matter atrophy (detected by volumetric T1-weighted MRI)	++	++	+	+	NS	+
White matter integrity loss (detected by DTI)	++	+	+	+	NS	+
Brain metabolism (detected by FDG-PET)	++	++	+	+	NS	+
Tau pathology (detected by Tau-PET)	+	+	NS	NS	NS	NS
Brain perfusion (detected by ASL)	++	+	+	+	NS	NS
Functional connectivity (detected by RS-fMRI)	+	+	+	+	NS	NS
Fluid biomarkers						
p-tau, t-tau and $A\beta_{1-42}$	++	+	NS	NS	+*	NS
NfL	+	+	++	+	++	+
Progranulin	NS	++	NS	NS	NS	+
Poly(GP)	NS	+	NS	NS	NS	+

Summary of current or potential biomarkers and their applications reported thus far. *p-tau:t-tau ratio. ++: robust biomarker, replicated in independent cohorts; +: potential biomarker; $A\beta_{1-42}$: amyloid $beta_{1-42}$; AD: Alzheimer disease; ASL: arterial spin labelling; DTI: diffusion tensor imaging; FDG-PET: ^{18}F-fluorodeoxyglucose PET; FTD: frontotemporal dementia; NfL: neurofilament light chain; NS: not studied; poly(GP): glycine–proline repeating protein; p-tau: phospho-tau_{181}; RS-fMRI: resting-state functional MRI; tau-PET: tau PET; t-tau: total-tau.

Fluid biomarkers

Alterations in the concentrations of specific proteins in different human fluid compartments could reflect pathophysiological changes in disease processes. The proximity of cerebrospinal fluid (CSF) to the brain means that it is likely to contain disease-specific biomarkers in patients with neurological disease. Subsequent validation of such biomarkers in blood would be of great value, as the acquisition of blood samples is minimally invasive and would enable repeated measurements to be taken over time. Some brain-specific proteins in neurodegenerative disorders can be detected reliably in blood by novel ultrasensitive assays — such as single molecule array technology. In the next few years, the progress resulting from these developments will offer new opportunities for the diagnosis, staging

and monitoring of patients with FTD. In this section, we will first review the current CSF markers used to differentiate FTD from AD, then highlight promising CSF and blood-derived biomarkers in sporadic and genetic FTD.

CSF amyloid–β and tau

The core CSF biomarkers for AD are phospho-tau$_{181}$ (p-tau), total-tau (t-tau), and amyloid-$\beta_{1–42}$ ($A\beta_{1-42}$); these species correspond to the pathological changes that occur in AD — that is, accumulation of hyperphosphorylated tau in neurofibrillary tangles, neuronal loss (associated with increased CSF levels of t-tau), and Aβ deposition in senile plaques, respectively.[95] These biomarkers have been comprehensively validated to exclude AD in the diagnostic work-up of FTD, both in clinical cohorts and in small, pathologically confirmed case series: higher levels of p-tau and t-tau, and lower levels of $A\beta_{1–42}$ are found in patients with AD than in those with FTD.[96] A high ratio of p-tau:$A\beta_{1–42}$ or t-tau:$A\beta_{1–42}$ enables an especially accurate diagnostic performance for the differentiation of FTD from AD (p-tau:$A\beta_{1–42}$ — specificity 80% and sensitivity 87%; t-tau:$A\beta_{1–42}$ — specificity 79% and sensitivity 89%). The use of ratios of other Aβ isoforms could improve diagnostic accuracy, especially when differentiating between AD and vascular dementia or dementia with Lewy bodies, but also for distinguishing AD from FTD.[97,98]

The core AD biomarkers are also valuable for differentiating between underlying AD or FTLD pathology in the differential diagnosis of PPA, in which an AD-like CSF profile is often found in patients with clinically diagnosed lvPPA, but not in patients with svPPA or nfvPPA.[99–102] An AD-like CSF profile occasionally occurs in patients with FTD, even in pathologically proven cases, which could partly be explained by the co-occurrence of AD pathology with FTLD in these patients.[103] Moreover, decreased $A\beta_{1–42}$ levels (compared with reference ranges) were found in up to 25% of patients with the *C9orf72* repeat expansion in a Finnish cohort, but not in patients with a *GRN* mutation; additional clinicopathological and genetic studies are required to elucidate the pathophysiological relevance of $A\beta_{1–42}$ in these cases.[104–106]

CSF levels of tau are not increased in patients with FTD with underlying tau pathology or in patients with *MAPT* mutations, compared with patients with tau-negative or sporadic FTD.[107,108] The ratio of p-tau:t-tau is lower in FTLD-TDP than in FTLD-tau, and enables a specific differentiation between these subtypes; however, this relationship seems to be driven by the presence of concomitant MND in some individuals with FTLD-TDP.[109–112] Whether the lower ratio of p-tau to t-tau is the result of an increase in t-tau owing to neuronal loss or a reduction of p-tau is not completely clear. Interestingly, in line with the hypothesis of neuronal damage, one study found an association between reduced p-tau:t-tau ratio and survival in patients with FTD.[111]

Neurofilament proteins

Neurofilament light chain (NfL) probably has the most promising short-term prospects of all fluid biomarkers for FTD disease monitoring and prognosis. Neurofilaments are the major constituent of the neuroaxonal cytoskeleton and play an important part in axonal transport and in the synapse.[113] NfL is the most abundant and soluble neurofilament subunit, and increased levels are thought to reflect axonal damage.

Blood and CSF levels of NfL are 2.5–11-fold higher in patients with FTD than in control individuals, and the clinical value of this protein lies in its correlation with disease severity and progression, survival, and cerebral atrophy (Figure 4).[111,114–119] CSF NfL is also increased, although to a lesser extent, in several other neurodegenerative diseases (such as ALS, AD, PSP and vascular dementia), and should, therefore, be combined with disease-specific biomarkers.[114,117,120–122] Levels of NfL are equally elevated among the FTD subtypes bvFTD, nfvPPA and svPPA, and are strongly increased in FTD with MND.[111,114–116,118] High CSF levels of NfL were found in patients with TDP-43 pathology compared with tau pathology, a difference that was largely driven by ALS co-occurrence.[111,118] Among the genetic subgroups, particularly high NfL levels were found in FTD patients with *GRN* mutations, levels varied greatly in patients with *C9orf72* expansions (ranging from high levels in concomitant MND to low levels in patients who progress slowly), and patients with *MAPT* mutations had comparatively low levels (Figure 4C).[115] Interestingly, presymptomatic individuals with FTD-associated mutations have normal levels of NfL in CSF and blood, with a sharp increase reported after conversion to the disease stage in two individuals (Figure 4C).[115] Whether and in what manner NfL levels fluctuate over time in FTD is unknown, but longitudinal data in ALS have shown stable NfL levels or a minor increase over time.[120,123] A strong correlation has been shown between NfL levels in CSF and serum, which makes this biomarker measurable in blood and, therefore, especially suitable for repeated measurements.[115,119]

In mouse models of neurodegenerative diseases that exhibited tau, Aβ or α-synuclein pathology, an increase in blood and CSF levels of NfL coincided with the onset and progression of brain pathology; inhibition of Aβ production — thus, reducing Aβ lesions — attenuated the NfL increase.[124] This observation suggests that we can use NfL to monitor treatment response in neurodegenerative diseases. In conclusion, NfL is a promising, noninvasive biomarker for disease staging, monitoring and prognosis in FTD. Longitudinal studies in FTD need to be conducted to better understand the role of NfL as a marker of disease progression.

Gene–specific biomarkers

Progranulin. The multifunctional protein progranulin plays an important part in neurite outgrowth and inflammation.[125] Pathogenic loss-of-function mutations in *GRN* reduce the blood and CSF levels of progranulin to 25–40% of normal levels, owing to haploinsufficiency.[125–129] Blood or CSF levels of progranulin are diagnostic biomarkers of pathogenic *GRN* mutations, as they enable the discrimination of presymptomatic and symptomatic

GRN mutation carriers from noncarriers with high sensitivity (96–100%) and specificity (93–100%)(Figure 5A).[128,129] Consequently, blood levels of progranulin can help to assess the pathogenicity of unclassified variants in *GRN*. Currently, therapeutic trials are focusing on interventions that increase progranulin expression, such as histone deacetylase inhibitors.[130] In these trials, target engagement is assessed using blood progranulin levels, as they seem to be constant over time.[129,131] However, blood and CSF levels of progranulin are differentially regulated, as demonstrated by the moderate correlation between these compartments in *GRN* mutation carriers; therefore, CSF should also be sampled.[129] Progranulin levels thus provide a good pharmacodynamic biomarker, but do not reflect the extent of neurodegeneration in the brain, for which additional biomarkers are needed as surrogate endpoints.

Dipeptide-repeat proteins translated from the C9orf72 repeat expansion. C9orf72 repeat expansions are transcribed to G_4C_2 repeat RNA, which forms RNA foci. In parallel, this RNA is translated into proteins of repeating dipeptides (dipeptide-repeat (DPR) proteins) by repeat-associated nonATG-initiated translation.[132] RNA foci and DPRs are both thought to have a key role in the pathophysiology resulting from the G_4C_2 expansion.[132–134] Elevated levels of glycine–proline-repeating protein (poly(GP)), one of the DPR proteins, have been found in the CSF of patients with *C9orf72* repeat expansions, and also in presymptomatic carriers of the expansion (Figure 5B+C).[134,135] Moreover, poly(GP) levels remained fairly constant over time, which supports the use of poly(GP) as a potential pharmacodynamic biomarker in future therapeutic trials.[135] In human cell models of *C9orf72* FTD– ALS, antisense oligonucleotides that bind to G_4C_2 RNA reduce the levels of extracellular poly(GP), and in mice harbouring a G_4C_2 expansion, they reduce the number of RNA foci and total levels of DPR proteins, as well as CSF levels of poly(GP).[133–135] These findings indicate that poly(GP) is a potential biomarker for therapeutic target engagement and enables the measurement of biochemical responses to treatment with agents such as antisense oligonucleotides.[134,135] As CSF levels of poly(GP) did not correlate with age at onset, disease duration, symptom severity or survival in patients with *C9orf72* repeat expansions, future clinical trials in these patients could benefit from the combination of poly(GP) levels as a pharmacodynamic marker and NfL levels as a prognostic marker.

Potential fluid biomarkers

As FTLD with phosphorylated TDP-43 (pTDP-43) aggregates constitutes one of the major pathological subgroups of FTLD, levels of pTDP-43 protein in CSF or blood would be an interesting biomarker. However, to date, results have been contradictory. Strongly elevated CSF levels of pTDP-43 have been found in a small series of patients with *C9orf72* or *GRN* mutations, but did not differ between FTD with TDP-43 or tau pathology in a pathology-proven cohort.[112,136] Quantification of levels of pTDP-43 in CSF is challenging owing to low concentrations, the presence of different isoforms, and various antibodies that recognize

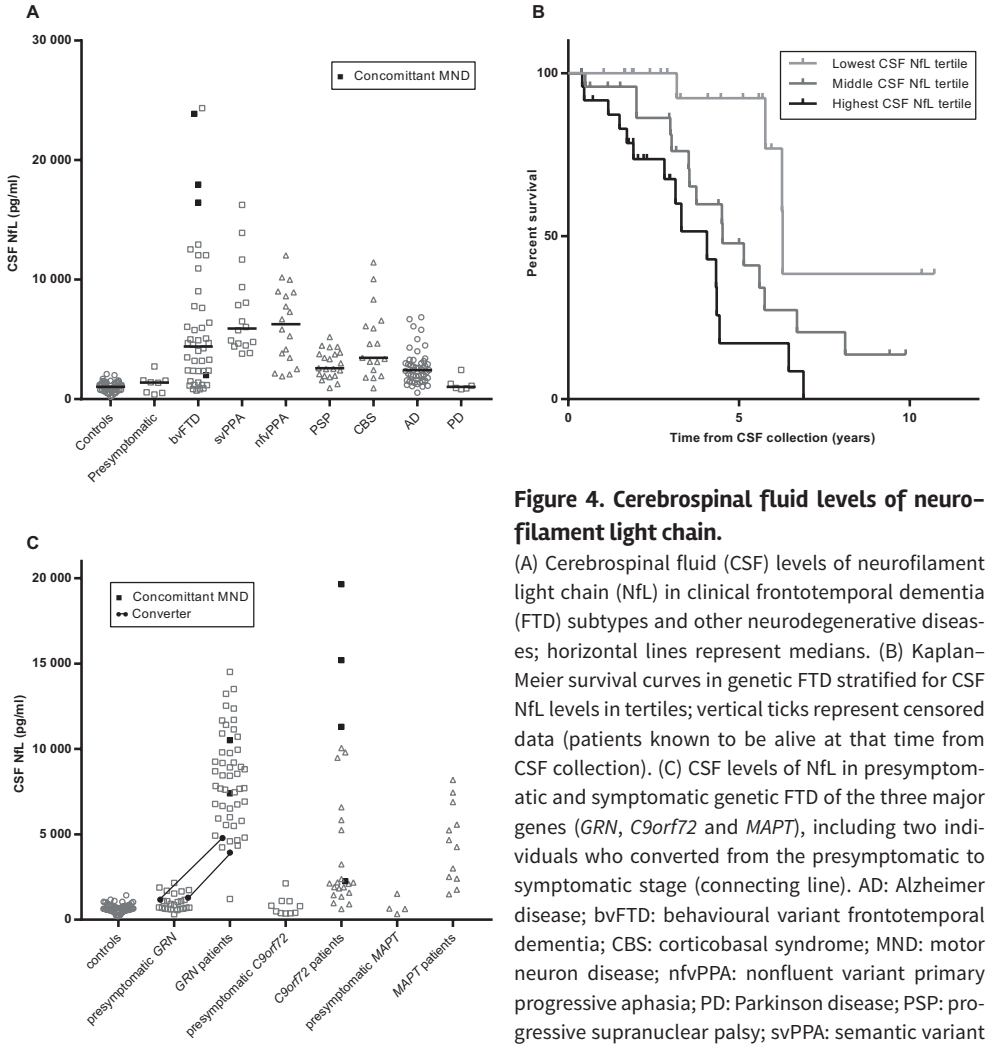

Figure 4. Cerebrospinal fluid levels of neurofilament light chain.

(A) Cerebrospinal fluid (CSF) levels of neurofilament light chain (NfL) in clinical frontotemporal dementia (FTD) subtypes and other neurodegenerative diseases; horizontal lines represent medians. (B) Kaplan–Meier survival curves in genetic FTD stratified for CSF NfL levels in tertiles; vertical ticks represent censored data (patients known to be alive at that time from CSF collection). (C) CSF levels of NfL in presymptomatic and symptomatic genetic FTD of the three major genes (*GRN*, *C9orf72* and *MAPT*), including two individuals who converted from the presymptomatic to symptomatic stage (connecting line). AD: Alzheimer disease; bvFTD: behavioural variant frontotemporal dementia; CBS: corticobasal syndrome; MND: motor neuron disease; nfvPPA: nonfluent variant primary progressive aphasia; PD: Parkinson disease; PSP: progressive supranuclear palsy; svPPA: semantic variant primary progressive aphasia. Part A modified with permission from Wiley & Sons © Scherling, C. S. *et al. Ann. Neurol.* 75, 116–126 (2014). Parts B+C modified with permission from Wiley & Sons © Meeter, L. H. *et al. Ann. Clin. Transl. Neurol.* 3, 623–636 (2016) under a Creative Commons license.

different regions of pTDP-43 and vary in specificity;[112,137] the development of better TDP-43 assays is warranted.

Neuroinflammation plays an important part in FTD and other neurodegenerative diseases, as it is a consequence and a trigger of pathology.[138] Microglia are the major immune component of the CNS, and are activated by damaged neurons and misfolded proteins, resulting in the initiation of a chronic inflammatory response.[138] A study of patients with

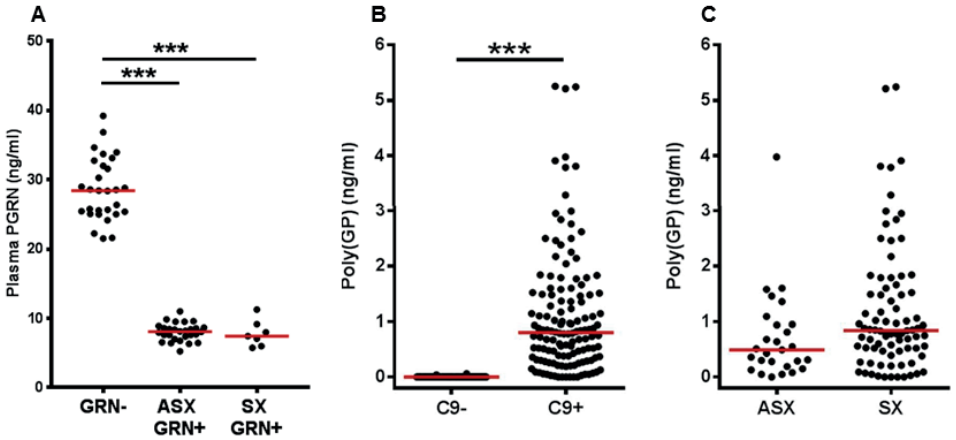

Figure 5. Gene–specific fluid biomarkers.
Horizontal red lines represent the sample medians in a given group. (A) Plasma levels of progranulin (PGRN) are significantly lower (***: $p<0.001$), without overlap, in individuals with *GRN* mutations (including both presymptomatic (ASX) and symptomatic (SX) carriers) than in control individuals. (B) Cerebrospinal fluid (CSF) levels of glycine–proline repeating protein (poly(GP)) are significantly higher in carriers of a *C9orf72* repeat expansion than in noncarriers. (C) CSF levels of poly(GP) are already raised in the presymptomatic stage when compared to the symptomatic stage. Part A modified with permission from Karger © Meeter, L. H. H. *et al. Dement. Geriatr. Cogn. Dis. Extra* 6, 330–340 (2016) under a Creative Commons license. Part B modified with permission from the American Association for the Advancement of Science © Gendron, T. F. *et al. Sci. Transl. Med.* 9, eaai7866 (2017).

sporadic FTD or AD found reduced levels of soluble triggering receptor expressed on myeloid cells 2 (TREM2), a protein involved in inflammation and phagocytosis and mainly expressed by microglia.[139] CSF levels of chitinase-3-like protein 1 (also known as YKL-40 or cartilage glycoprotein-39), an inflammatory protein produced by astrocytes, were found to be elevated in pathologically proven FTD, but also in AD, vascular dementia, normal ageing and other neurological disorders, such as multiple sclerosis.[140–142] Similarly, glial fibrillary acidic protein, an astrocytic cytoskeletal protein, was found to be increased in FTD and other dementia types.[143] In the past few years, a strong link between *GRN* mutations and microglial activation has been established, with excessive complement production leading to synaptic pruning.[144] Promisingly, data suggest that proteins involved in complement activation are potential biomarkers of disease progression in *GRN* mutation carriers.[144]

Various changes in CSF and/or blood levels of cytokines (primarily pro-inflammatory cytokines, such as MCP-1, IL-6 and TNF) have been found in FTD, but these changes seem to reflect nonspecific mechanisms, as they are also present in AD.[145–150] The role of several neuropeptides in FTD has been extensively reviewed elsewhere;[151] for example, levels of neurogranin, a postsynaptic protein involved in synaptic plasticity, were lower in patients with FTD than in control individuals and patients with AD.[141] Larger cohorts with pathologically proven and genetically determined disease are needed for validation of these cytokines and neuropeptides.

Novel approaches are focusing on enriched protein fractions and microRNAs in exosomes as potential biomarkers. Exosomes are vesicles secreted from cells; they facilitate intercellular communication and are enriched sources of biomolecules. The value of the examination of exosomes is supported by a small study that reported reduced levels of synaptic proteins in blood-derived exosomes in FTD.[152] microRNAs regulate gene expression, and seem to have a role in TDP-43 and FUS pathology, but have not yet been reported as biomarkers in FTD.[153]

Summary of fluid biomarkers

Several fluid biomarkers for FTD are currently usable (for example, core AD biomarkers such as tau and Aβ levels) or show promise (for example, levels of NfL) (Table 1). Combinations of metabolites in the CSF are likely to yield more information than single markers; for example, one biomarker panel enabled highly sensitive differentiation between TDP-43 pathology and tau pathology.[145] Generally, more validation and longitudinal data are needed to determine the full potential of fluid biomarker candidates. Lastly, harmonization of fluid biomarker collection and analysis is important, as levels of the markers can be influenced by multiple pre-analytical and analytical factors, including sampling and storage methods, and choice and implementation of assays.[154] Multicentre standardization of these procedures and the establishment of quality control programmes will facilitate collaborative research and the implementation of new fluid biomarkers in clinical practice.

Conclusions

Neuroimaging and fluid biomarkers are becoming increasingly important in the context of future therapeutic interventions in sporadic and genetic forms of FTD. Several imaging and CSF biomarkers (such as grey matter atrophy, FDG-PET findings and CSF biomarkers of AD) are already established and being used in clinical practice, often in the differential diagnosis of FTD versus AD. Progress is being made in the identification of gene-specific markers and the discovery of new biomarkers for disease staging, the prediction of underlying pathology and monitoring of treatment responses. For example, DTI has performed well in discriminating between FTD and AD, and in demonstrating early pathological changes; NfL can be used to differentiate patients with FTD from control individuals and is a promising staging and prognostic biomarker for FTD; and genetic-specific biomarkers (such as progranulin and DPR proteins) could be valuable for the assessment of target engagement in therapeutic trials. Importantly, combinations of biomarkers will be valuable in enabling the accurate definition of FTD subtype and disease onset, and for the monitoring of disease progression and, eventually, treatment response. For example, in a trial in which the aim is to increase progranulin production, target engagement could be assessed by progranulin levels, but additional surrogate endpoints would be needed to assess the physiological effect (that is, the reduction of neurodegeneration).

Most alterations of these novel biomarkers have been demonstrated at a group level and need to be validated for individual patients, which is challenging because FTD is fairly rare. Multicentre research can help to increase statistical power and prove clinical utility; prime examples of longitudinal observational cohorts include GENFI, ARTFL (Advancing Research and Treatment for Frontotemporal Lobar Degeneration Consortium), LEFFTDS (Longitudinal Evaluation of Familial Frontotemporal Dementia Subjects), and a collaboration including these consortia in the FPI (FTD Prevention Initiative). Research in genetic FTD provides a unique opportunity to study the earliest disease effects and consequently offers good prospects for the identification of valuable biomarkers. Despite similarities between genetic and sporadic FTD, biomarkers identified in genetic cases require validation for use in sporadic cohorts, as biomarker profiles and trajectories can differ, as they do in AD.[155]

Interestingly, researchers are now emphasizing that FTD, which has typically been considered an early-onset dementia, frequently manifests after the age of 65 years and can include clinical features suggestive of AD.[156,157] This finding stresses the need for diagnostic biomarkers that are specific for FTD, as the co-occurrence of AD pathology with FTD increases with age. The value of FTD biomarkers in different age groups with comorbidities remains to be elucidated. Additionally, future research should focus on the combination of different biomarkers (both fluid and imaging) to make optimal use of these modalities, as well as on harmonization of collection and analysis protocols to facilitate dissemination in research and clinical practices.

Key points

- Most of the validated biomarkers in frontotemporal dementia (FTD) are used to differentiate patients with FTD from patients with Alzheimer disease or from control individuals
- Currently validated biomarkers in FTD include grey matter atrophy, alterations in brain metabolism as detected by ^{18}F-fluorodeoxyglucose-PET and cerebrospinal fluid levels of amyloid-β_{1-42}, phospho-tau$_{181}$ and total-tau.
- New imaging biomarkers, detected via techniques such as arterial spin labelling and diffusion tensor imaging, are sensitive to the subtle changes that precede grey matter atrophy in FTD, potentially enabling use in diagnosis and disease monitoring
- Promising fluid biomarkers include neurofilament light chain (for staging, monitoring and prognosis in all FTD subtypes) and dipeptide-repeat proteins and progranulin (for target engagement in gene-specific forms of FTD)
- Reliable biomarkers that differentiate between tau pathology and TDP-43 pathology are still needed, to facilitate trials of disease-modifying treatments
- Future research should focus on the multimodal combination of fluid and imaging biomarkers, as well as the harmonization of biomarker collection and analysis protocols

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Authors contributions

All authors contributed equally to the preparation of the manuscript.

Competing interests statement

The authors declare no competing interests.

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CHAPTER 2

The application of neurofilament light chain across the sporadic FTD spectrum

CHAPTER 2.1

Clinical value of neurofilament and phosphotau/tau ratio in the frontotemporal dementia spectrum

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Abstract

Objective: To examine the clinical value of neurofilament light chain (NfL) and the phospho-tau/total tau ratio (p/t-tau) across the entire frontotemporal dementia (FTD) spectrum in a large, well-defined cohort.

Methods: CSF NfL and p/t-tau levels were studied in 361 patients with FTD: 179 behavioural variant FTD (bvFTD), 17 FTD with motor neuron disease (FTD-MND), 36 semantic variant primary progressive aphasia (PPA), 19 non-fluent variant PPA, 4 logopenic variant PPA (lvPPA), 42 corticobasal syndrome, and 64 progressive supranuclear palsy. Forty-five cognitively healthy controls were also included. Definite pathology was known in 68 patients (49 frontotemporal lobar degeneration [FTLD]-TDP, 18 FTLD-tau, 1 FTLD-FUS).

Results: NfL was higher in all diagnoses, except lvPPA (*n*=4), than in controls, equally elevated in behavioural variant FTD, semantic variant PPA, nonfluent variant PPA, and corticobasal syndrome, and highest in FTD-MND. The p/t-tau was lower in all clinical groups, except lvPPA, than in controls and lowest in FTD-MND. NfL did not discriminate between TDP and tau pathology, while the p/t-tau ratio had a good specificity (76%) and moderate sensitivity (67%). Both high NfL and low p/t-tau were associated with poor survival (hazard ratio on tertiles 1.7 for NfL, 0.7 for p/t-tau).

Conclusion: NfL and p/t-tau similarly discriminated FTD from controls, but not between clinical subtypes, apart from FTD-MND. Both markers predicted survival and are promising monitoring biomarkers for clinical trials. Importantly, p/t-tau, but not NfL, was specific to discriminate TDP from tau pathology *in vivo*.

Classification of Evidence: This study provides Class III evidence that for patients with cognitive issues, CSF NfL and p/t-tau levels discriminate between those with and without FTD spectrum disorders.

Introduction

Frontotemporal dementia (FTD) is a heterogeneous disease encompassing behavioural FTD (bvFTD) and the primary progressive aphasias (PPAs): the semantic variant (svPPA), the nonfluent variant (nfvPPA) and the logopenic variant (lvPPA).[1,2] Motor neuron disease (MND), progressive supranuclear palsy (PSP), and corticobasal syndrome (CBS) are considered as part of the FTD spectrum.[3] The underlying pathology, frontotemporal lobar degeneration (FTLD), is heterogeneous with tau (FTLD-tau), TAR DNA-binding protein 43 (FTLD-TDP), or fused in sarcoma inclusions (FTLD-FUS).[4] Underlying pathology can only be predicted in genetic FTD: microtubule-associated protein tau *(MAPT)* mutations cause FTLD-tau, and progranulin *(GRN)* and chromosome 9 open reading frame 72 (*C9orf72*) mutations cause FTLD-TDP.[5] The svPPA and FTD-MND are often associated with FTLD-TDP, and PSP with FTLD-tau, whereas underlying pathology can poorly be predicted in bvFTD.[6]

Disease-modifying therapies against FTD are currently under development, thus robust markers to track disease progression are essential. Neurofilament light chain (NfL), reflecting neuroaxonal damage, is a promising monitoring biomarker for FTD and other neurodegenerative diseases.[7–12] For pathology-specific therapies, biomarkers predicting pathology are paramount, and lower phospho-tau$_{181}$ to total tau (p/t-tau) ratios were found in small series of patients with FTLD-TDP compared to FTLD-tau.[10,13,14] However, both biomarkers have scarcely been validated and directly compared in large series across the entire FTD spectrum, which is needed before implementation in practice. In the current study, we compared the clinical value of NfL versus the p/t-tau ratio across all clinical and pathological subtypes of the FTD spectrum.

Methods

Subjects

From our previously described cohorts from the Erasmus Medical Center and the VU University Medical Center,[15,16] we selected 361 patients based on the following criteria: (1) clinical diagnosis of bvFTD, nfvPPA, svPPA, FTD-MND, PSP, CBS, or lvPPA with a probable underlying FTLD[1,2,17,18]; (2) CSF available for research. Patients with CSF results suggesting Alzheimer disease (AD) (low CSF amyloid-$\beta_{1\text{-}42}$ [$A\beta_{42}$] and high p- or t-tau level, applying local laboratory standards) were not included, unless a definite FTD diagnosis was established (*n*=3).[1,19] As control group (*n*=45), we used controls and participants with subjective memory complaints with normal CSF $A\beta_{42}$ levels (>550 pg/ml) from previous studies.[9,10] A definite diagnosis was ascertained in 68 patients based on known FTD-causing mutations (genotyping was performed when family history was positive) or autopsy-confirmation: underlying FTLD-TDP pathology in 49 patients (12 *GRN*, 26 *C9orf72* [of whom 7 underwent autopsy], 1 *optineurin*, 10 FTLD-TDP), FTLD-tau pathology in 18 patients (11 *MAPT* [of whom 1 underwent autopsy], 5 FTLD-tau, 1 CBD and 1 PSP) and 1 patient with autopsy-confirmed

FTLD-FUS. For subanalyses on suspected pathology, patients with svPPA and FTD-MND were added to the FTLD-TDP group and patients with PSP to the FTLD-tau group. Concomitant AD pathology in autopsied patients was scored by an experienced neuropathologist (A.J.R.) as low (*n*=21, ABC score "not" or "low") or high (*n*=4, ABC score "intermediate" or "high").[20] For 1 of 26 autopsied patients, insufficient information was available for this scoring.

Disease onset was defined as the time of first symptoms (e.g. first personality change or language difficulties) noted by a caregiver. Age at death was acquired by web-based consultation of the Dutch municipal personal records database (not available for one patient). The Mini-Mental State Examination (MMSE) was used to examine global cognition; the Frontal Assessment Battery (FAB) for executive function; and the Clinical Dementia Rating scale (CDR), including Sum of Boxes (CDR-SB) if available, for disease severity.

Standard Protocol Approvals, Registrations, and Patient Consents

All patients (or legal representatives) provided written informed consent, and this study was approved by the local ethics committees.

CSF analyses

CSF was collected and stored at -80 °C until analyses according to international consensus protocols.[21] All measurements were performed blinded to clinical information and in one single center. CSF NfL was determined with the enzyme-linked immunosorbent assay (ELISA) of UmanDiagnostics (Umeå, Sweden) in duplicate, according to the manufacturer's instructions over 5 different batches. Mean intra-assay coefficient of variation (CV) was 1.2% (±1.2% standard deviation), interassay CV ranged from 6.1% to 16.7%. Samples with too low volume for a duplicate (*n*=9), were included in the analysis as overall intra-subject CV was low. CSF phospho-tau$_{181}$ (p-tau) and total tau (t-tau) were measured by commercial ELISAs (Innotest, Fujirebio, Ghent, Belgium) and remeasured when CV was >15%.

For controls, all 3 biomarkers were measured, but for some patients insufficient CSF volume was available; NfL levels were available in 335 patients, the p/t-tau ratio in 352 patients, and both in 324 patients.

Statistical analyses

Statistical analyses were performed in SPSS 21.0 for Windows (IBM Corp., Armonk, NY) applying a significance level of $p<0.05$, and graphs were drafted with GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Demographic data were compared by Kruskal-Wallis tests with post-hoc Dunn tests (Bonferroni corrected), or chi-square tests. Both CSF NfL and p/t-tau ratio were nonnormally distributed (Shapiro-Wilk test) and normalized after log transformation. Biomarker levels across (clinical or pathological) groups were compared on log-transformed data with correction for age by analyses of covariance with post-hoc Šidák tests. In case of missing data, patients were removed from respective

subanalyses. Diagnostic performance was assessed by areas under the curve (AUCs) with 95% confidence intervals (CIs) obtained by receiver operating characteristic analyses, with optimal cutoff levels at the highest Youden index.[22] Diagnostic performance combining the 2 markers was analyzed by receiver operating characteristic analyses on probabilities obtained by binary logistic regression when comparing 2 groups (patients vs controls, TDP vs tau pathology) or multinominal logistic regression for different clinical diagnoses. Diagnostic performance was compared between the markers by the Hanley&McNeil method in MedCalc (Ostend, Belgium).[23] Biomarkers were correlated to clinical variables using Spearman correlation coefficient (r_s). Survival was examined by Kaplan-Meier curves on NfL tertiles with living patients as censored data, and Cox regressions on tertiles or continuous biomarker levels, adjusted for age, sex, disease duration (time between onset and CSF collection), and presence of MND. Five-year survival rates were extracted from the Kaplan-Meier curves. Multivariate regression examined the following influencing factors in the association between logNfL levels (dependent variable) and log-transformed p/t-tau ratio: age, disease duration, CSF $A\beta_{42}$ as proxy for AD co-pathology, and MND (covariates entered in second block).

Classification of Evidence

This case-control study provides Class III evidence that patients across the entire FTD spectrum can be discriminated from healthy controls by high CSF NfL levels (sensitivity 79%, specificity 89%, $p<0.001$) or by low p/t-tau levels (sensitivity 73%, specificity 93%, $p<0.001$).

Results

Subject characteristics

Demographic characteristics of the 361 patients and 45 controls are displayed in Table 1. Patients with CBS or PSP were older than controls, and patients with PSP were older than patients with bvFTD. Sex did not differ among the diagnostic groups. Age at onset, disease duration at CSF collection, and survival per clinical diagnosis is displayed in Table 1. In total, 195 patients had deceased and their mean survival after CSF collection was 3.7 (±2.6) years. Survival did not differ between patients with definite TDP versus tau pathology ($p=0.44$, log-rank test).

Discrimination of clinical diagnosis

All clinical diagnoses, except for lvPPA ($n=4$), had higher CSF NfL levels than controls (Figure 1A, *p*-values and median differences are displayed in Supplementary Table 1), with the strongest elevation in patients with FTD-MND. CSF NfL was lower in patients with PSP than in those with bvFTD. No differences were found among the other clinical subgroups.

Table 1. Participant characteristics

	Controls	bvFTD	FTD-MND	svPPA	nfvPPA	lvPPA	CBS	PSP	*p*-value
Number	45	179	17	36	19	4	42	64	
Age at CSF collection, years	60 (53-65)	61 (55-67)	63 (56-69)	62 (58-65)	62 (52-66)	64 (51-69)	65 (60-73)[a]	66 (62-70)[b]	<0.001
Male sex (% within clinical group)	26 (58%)	101 (56%)	9 (53%)	19 (53%)	9 (47%)	1 (25%)	28 (67%)	28 (44%)	0.35
Age at onset, years	-	58 (51-64)[c]	61 (55-67)	57 (55-62)	60 (50-65)	62 (50-67)	62 (56-71)	63 (57-67)	0.001
Time between onset and CSF collection, years	-	2.5 (1.5-4.6)	1.3 (0.7-2.1)[d]	2.7 (1.9-5.0)	2.1 (1.2-3.2)	1.9 (1.6-2.9)	2.2 (1.2-3.3)	2.2 (1.5-3.7)	0.009
Survival after CSF collection in deceased patients[e], years	-	3.9 (2.0-5.9)	1.0 (0.5-1.2)	5.3 (4.5-8.9)	6.2 (1.9-8.2)	3.2	1.8 (1.2-3.4)	3.0 (1.9-4.0)	<0.001
CSF NfL available	45	164	14	36	19	4	40	58	
p/t-tau ratio available	45	174	16	34	19	4	42	63	
CSF NfL, pg/ml	974 (616-1357)	3168 (1752-4818)	19232 (10094-27016)	3151 (1906-4802)	2345 (1956-2957)	1731 (1181-2472)	2664 (1715-4158)	1907 (1474-2755)	<0.001
CSF p-tau, pg/ml	44 (37-56)	42 (34-54)[f]	43 (33-50)	40 (36-53)	51 (31-66)	54 (33-73)	47 (38-57)[f]	37 (28-44)	0.01
CSF t-tau, pg/ml	243 (197-308)	342 (271-467)[g]	488 (322-623)[g]	333 (265-454)[g]	361 (205-442)	427 (246-583)	336 (246-446)[g]	237 (176-315)	<0.001
CSF amyloid-β_{1-42}, pg/ml	987 (874-1142)	893 (726-1072)	985 (732-1162)	877 (728-1048)	969 (840-1219)	861 (486-1033)	810 (607-999)[h]	826 (665-1003)[h]	0.001
p/t-tau ratio	0.18 (0.17-0.20)	0.12 (0.10-0.15)	0.09 (0.07-0.10)	0.12 (0.11-0.15)	0.14 (0.12-0.17)	0.13 (0.12-0.14)	0.13 (0.11-0.16)	0.15 (0.13-0.17)	<0.001

Values are displayed as median (interquartile range), continuous variables are compared by Kruskal-Wallis tests. [a]Older than controls. [b]Older than controls and bvFTD. [c]Younger than CBS and PSP. [d]Shorter than svPPA and bvFTD. [e]0 controls, 88 bvFTD, 13 FTD-MND, 18 svPPA, 6 nfvPPA, 1 lvPPA, 24 CBS and 46 PSP patients were known to be deceased; CSF NfL was available in 178 and the p/t-tau ratio in 189 of them. [f]Higher than in patients with PSP. [g]Higher than in controls and patients with PSP. [h]Lower than in controls. bvFTD: behavioural variant frontotemporal dementia; CBS: corticobasal syndrome; CSF: cerebrospinal fluid; FTD-MND: frontotemporal dementia with concomitant motor neuron disease; lvPPA: logopenic variant primary progressive aphasia; NfL: neurofilament light chain; nfvPPA: non-fluent variant primary progressive aphasia; PSP: progressive supranuclear palsy; p-tau: phospho-tau$_{181}$; p/t-tau: phospho-tau/total tau; svPPA: semantic variant primary progressive aphasia; t-tau: total tau.

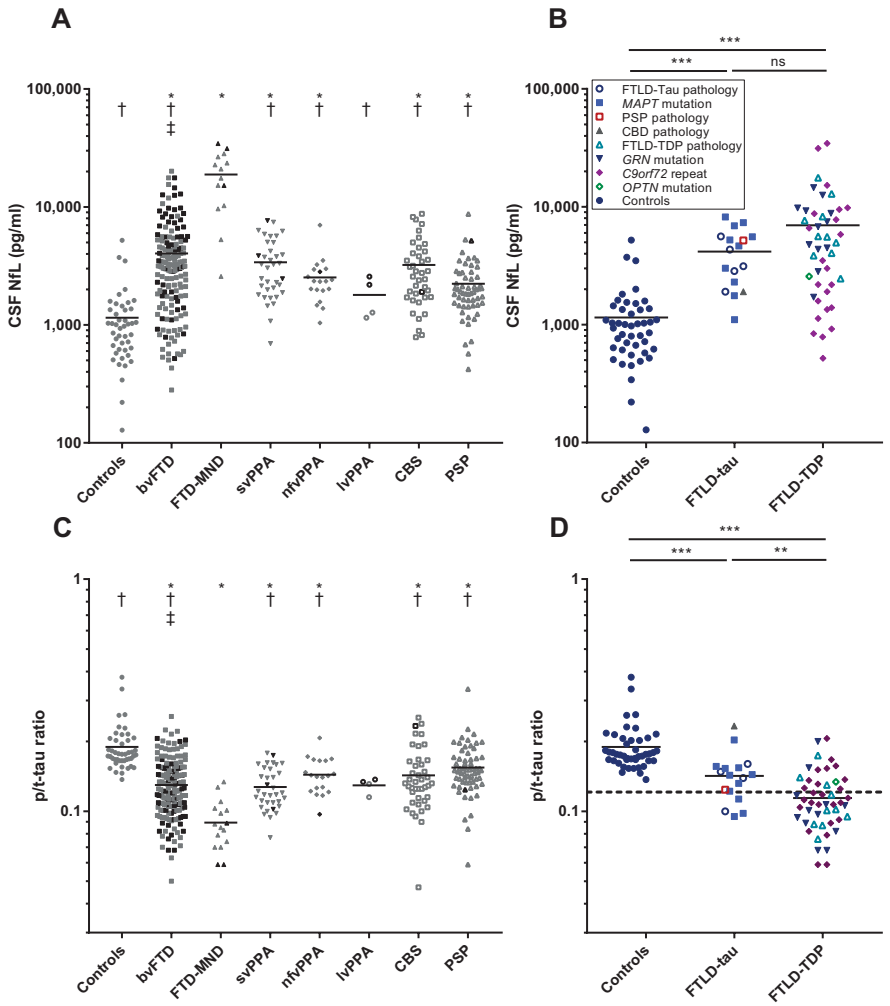

Figure 1. NfL and p/t–tau ratio levels by clinical diagnosis and by underlying pathology.
(A) NfL levels and (C) the p/t-tau ratio in clinical FTD subgroups; patients with confirmed underlying pathology are depicted in black. (B) NfL levels and (D) the p/t-tau ratio in patients with known underlying pathology based on autopsy-confirmed pathology (FTLD-tau: blue circles; PSP: red squares; CBD: grey filled upward triangles; FTLD-TDP: acqua upward triangles) or a known pathogenic mutation (*MAPT*: filled light-purple squares, *GRN*: filled blue downward triangles, *C9orf72* repeat expansion: purple filled diamonds; *OPTN*: green diamonds); a low p/t-tau ratio (cut-off ≤0.121, dashed line) discriminated FTLD-TDP from FTLD-tau with a specificity of 76% and a sensitivity of 67%. Horizontal lines represent means.
***: $p<0.001$; **: $p<0.01$; *:(A) higher NfL or (C) lower p/t-tau ratio compared with controls; **†:** (A) lower NfL or (C) higher p/t-tau ratio compared with FTD-MND, **‡:** (A) higher NfL or (C) lower p/t-tau ratio compared with PSP.
bvFTD: behavioural variant frontotemporal dementia; CBD: corticobasal degeneration; CBS: corticobasal syndrome; CSF: cerebrospinal fluid; FTD-MND: frontotemporal dementia with concomitant motor neuron disease; FTLD-tau: frontotemporal lobar degeneration with tau inclusions; FTLD-TDP: frontotemporal lobar degeneration with TAR DNA-binding protein 43 inclusions; lvPPA: logopenic variant primary progressive aphasia; NfL: neurofilament light chain; nfvPPA: non-fluent variant primary progressive aphasia; n.s.: not significant; PSP: progressive supranuclear palsy; p/t-tau: phospho/total-tau; svPPA: semantic variant primary progressive aphasia.

The p/t-tau ratio mirrored the results of NfL, as it was lower in all clinical diagnoses compared to controls, except for lvPPA (*n*=4), and FTD-MND patients had the lowest values (Figure 1C, Supplementary Table 2). In addition, patients with bvFTD had lower levels than patients with PSP. The decreased p/t-tau ratio in clinical patients compared to controls was driven by elevated t-tau levels in patients (p<0.001, for p-tau: p=0.52).

Diagnostic performance: clinical diagnosis

To discriminate patients from controls, CSF NfL achieved an AUC of 0.87 (95% CI 0.81-0.92, p<0.001), with a sensitivity of 79% and specificity of 89% (cut-off ≥1,613 pg/ml, Supplementary Table 3, including negative and positive predictive values). A low p/t-tau ratio showed a similar performance (AUC 0.86 [0.83-0.90], p<0.001, sensitivity 73%, specificity 93% at ratio≤0.153; compared to AUC NfL: p=0.74). Combining these 2 markers by logistic regression yielded a higher AUC of 0.91 (0.88-0.95, p<0.001, Nagelkerke R^2=0.45, sensitivity 80%, specificity 93%; compared to AUC NfL: p=0.03, compared to AUC ratio: p<0.01).

Discrimination of pathologic diagnosis

When NfL levels were analyzed based on definite pathology, no difference between FTLD-tau and FTLD-TDP was found (p=0.96, Figure 1B). However, when suspected pathologies were added in the analysis (i.e. PSP in tau group; svPPA and FTD-MND in TDP group), higher levels were observed in patients with suspected TDP than in patients with suspected tau (p<0.001, Supplementary Figure 1A). NfL levels were similar in autopsied patients with low versus high concomitant AD pathology (p=0.83, Mann Whitney test).

The p/t-tau ratio was lower in definite or suspected TDP pathology than in those with definite or suspected tau pathology (p=0.005 and p<0.001 respectively, Figure 1D and Supplementary Figure 1B). The decreased ratio in FTLD-TDP was driven by lower p-tau levels (median 38 pg/ml [interquartile range 29-44]) than in FTLD-tau (54 [42-67] pg/ml), while t-tau levels were similar (335 [252-448] pg/ml and (408 [310-522] pg/ml, respectively). The ratio did not differ between low and high concomitant AD-pathology (p=0.78, Mann-Whitney test).

Diagnostic performance: pathologic diagnosis

NfL did not differentiate between underlying TDP and tau pathology (p=0.26), while the p/t-tau ratio did (AUC 0.73 [0.60-0.87], p=0.005, sensitivity 67%, specificity 76% at ratio≤0.121, Figure 1D and Supplementary Table 3). Combining NfL and p/t-tau ratio did not improve the differentiation on underlying pathology (AUC 0.75 [0.62-0.88], p=0.004; p=0.94 versus AUC of ratio alone).

Associations between biomarkers and clinical parameters

NfL correlated moderately with t-tau (r_s=0.51, p<0.001), weakly with p-tau (r_s=0.13, p=0.02) and moderately with the p/t-tau ratio (r_s=-0.62, p<0.001, Figure 2). This association between

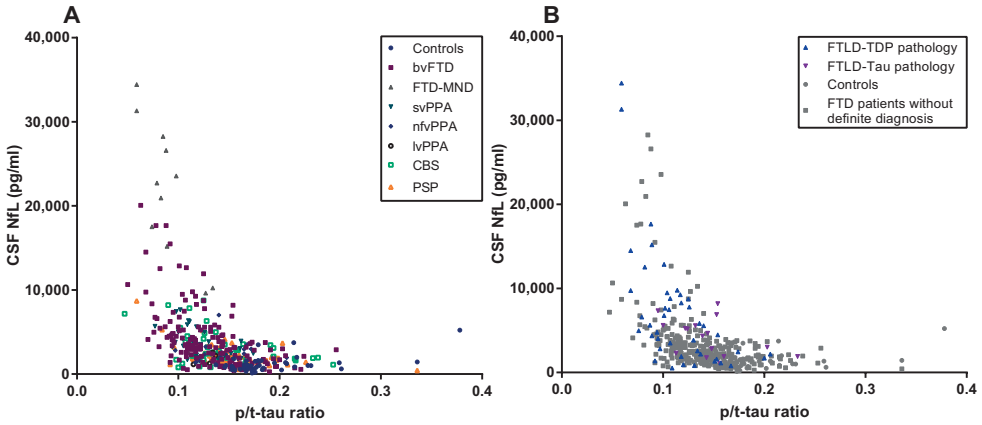

Figure 2. Association between NfL and the p/t–tau ratio.
In (A) the clinical diagnoses are marked: controls (blue filled circles), bvFTD (purple filled squares), FTD-MND (grey filled upward triangles), svPPA (acqua filled downward triangles), nfvPPA (blue filled diamonds), lvPPA (black circles), CBS (green squares) and PSP (orange triangles). In (B) the association in definite diagnoses is shown: FTLD-TDP pathology (blue upward triangles), FTLD-tau pathology (purple downward triangles), controls (grey circles), and patients without a definite diagnosis (grey squares).
bvFTD: behavioural variant frontotemporal; CBS: corticobasal syndrome; CSF: cerebrospinal fluid; FTD: frontotemporal dementia; FTD-MND: frontotemporal dementia with motor neuron disease; FTLD-tau: frontotemporal lobar degeneration with tau inclusions; FTLD-TDP: frontotemporal lobar degeneration with TAR DNA-binding protein 43 inclusions; lvPPA: logopenic variant primary progressive aphasia; NfL: neurofilament light chain; nfvPPA: non-fluent variant primary progressive aphasia; PSP: progressive supranuclear palsy; p/t-tau: phospho-tau/total tau; svPPA: semantic variant primary progressive aphasia.

the p/t-tau ratio and NfL was influenced by MND and disease duration (ΔR^2=0.11; MND: β=0.29, p<0.001; disease duration: β=-0.09, p=0.04), but not by age or CSF $A\beta_{42}$. In definite patients, this association was not influenced by type of pathology (TDP versus tau, p=0.25).

NfL associated weakly with disease duration, MMSE, and FAB, moderately with CDR-SB, but not with age, sex, or global CDR; the p/t-tau ratio associated weakly with age and disease duration, moderately with CDR-SB, but not with sex, MMSE, FAB or global CDR (Supplementary Table 4). Patients with *GRN* mutation had higher NfL levels and lower p/t-tau ratios than those with *C9orf72, MAPT,* or no known mutations (Supplementary Table 4).

Prediction of survival

Five-year survival was 73% in patients with low CSF NfL levels, 55% in moderate levels, and 36% in high levels (Figure 3A; estimated hazard ratio 1.7 [95% CI 1.3-2.1], p<0.001). After stratification on diagnosis, this association between high NfL levels and poor survival was confirmed in bvFTD (p<0.001), CBS (p=0.001), and PSP (p<0.001; Cox regression on continuous NfL levels).

For the p/t-tau ratio, 5-year survival was 37% in patients with a low ratio, 56% in moderate ratios, and 63% in high ratios (Figure 3B, estimated hazard ratio 0.70 [95% CI 0.56-0.86], p=0.001). Subanalyses by clinical diagnosis showed associations of lower p/t-tau ratios

with a poorer survival in bvFTD and PSP (p<0.001 and p=0.04 respectively, Cox regression on continuous p/t-tau ratios).

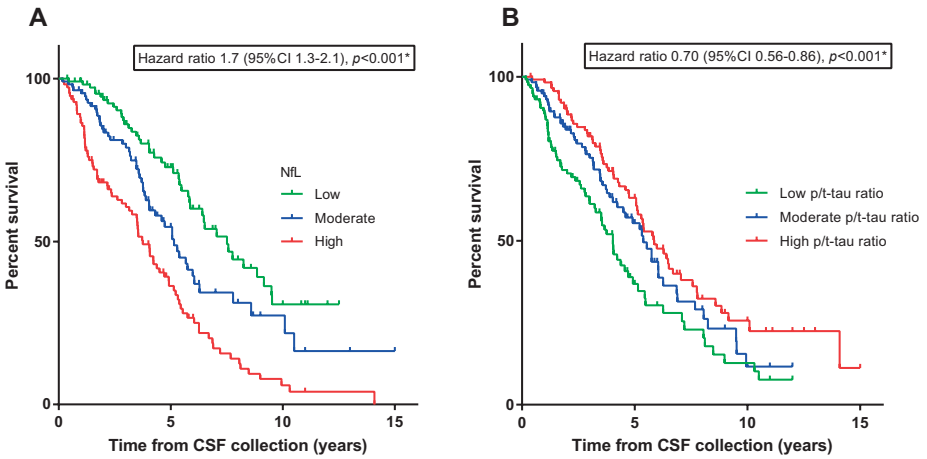

Figure 3. Association of NfL and the p/t–tau ratio with survival.
Kaplan-Meier curves of (A) NfL levels stratified to tertiles: lowest (green line; NfL < 1989 pg/ml), middle (blue line; NfL 1989-3675 pg/ml) or highest (red line, NfL > 3675 pg/ml), and (B) the p/t-tau ratio stratified into lowest (green line, ratio < 0.115), middle (blue line, ratio 0.115-0.146) or highest (red line, ratio > 0.146) tertiles; vertical ticks represent living patients. *Corrected for age, sex, disease duration, and motor neuron disease.
CI: confidence interval; CSF: cerebrospinal fluid; MND: motor neuron disease; NfL: neurofilament light chain; p/t-tau: phospho-tau/total tau.

Discussion

This study compared the clinical value of CSF biomarkers NfL and p/t-tau ratio in a large cohort of FTD patients. We showed that both biomarkers (1) discriminate patients with FTD from controls, (2) are altered in FTD-MND versus other clinical FTD subtypes, and in PSP versus bvFTD, but not between the other clinical FTD subtypes, and (3) predict survival, and that (4) p/t-tau ratio differentiates underlying TDP from tau pathology.

In clinical practice, there is a need for diagnostic markers in FTD. Prior research on NfL in CSF[9,24–26] and in blood[9,24,27] demonstrated a good discrimination between FTD and controls or non-neurodegenerative diseases including primary psychiatric disorders, while diagnostic performance analyses on p/t-tau ratio to discriminate patients with FTD from controls are rare. Our results show that both biomarkers, and their combination, have a good specificity to discriminate patients with clinical FTD from controls, at the drawback of a poorer sensitivity. Few false-positive results were found, but a considerable number of false negatives, and thus these markers support – but cannot exclude – underlying neuronal damage. Both markers are indeed involved in neuronal loss: NfL is increased and overlaps in various neurodegenerative syndromes (e.g. AD, PSP, and vascular dementia),

but increases are most pronounced in FTD and amyotrophic lateral sclerosis;[7,8,28–30] p/t-tau is decreased in various diseases characterized by marked neuronal loss, including Creutzfeldt-Jakob disease, amyotrophic lateral sclerosis, AD, and FTLD-TDP.[10,13,14,31–33] Overlap in underlying pathophysiology is supported by our results showing correlation between the 2 markers.

Since both NfL and the p/t-tau ratio are aspecific neurodegeneration markers, we anticipated a limited role in subtyping different phenotypes. Indeed, NfL levels were comparable across bvFTD, nfvPPA, svPPA and CBS, in concordance with prior results in other series.[7,27,30,34] This study demonstrates a similar pattern for the p/t-tau ratio for the first time. Meanwhile, both CSF markers discriminated FTD-MND from other subtypes as supported by previous research;[10,24] in the clinical practice, the added value of these markers may seem limited because MND is ascertained by clinical and electromyographic examination, but they could warn for subclinical MND. NfL and the p/t-tau ratio also differed between patients with PSP and bvFTD; nevertheless, levels considerably overlapped, yielding a diagnostic performance that is insufficient to implement in clinical practice. The lack of higher NfL and lower p/t-tau ratio in lvPPA compared with controls is likely explained by the small subgroup (*n*=4) and not by underlying AD pathology because they had a definite FTD diagnosis (one *C9orf72* repeat expansion, one *optineurin* mutation) and/or normal AD CSF biomarkers (*n*=2).

NfL did not discriminate underlying TDP from tau pathology, or improve the performance of the ratio, which contrasts to smaller studies showing high NfL levels in FTLD-TDP.[10,30] In our sample NfL levels strongly varied in patients with *C9orf72* repeat expansions, including a considerable number of patients with low levels. Including patients with suspected pathology did yield higher NfL levels in FTLD-TDP, but this seems to result from very high levels in patients with FTD-MND. These findings underline the heterogeneity in pathophysiology in FTLD.

In concordance with earlier reports,[10,13,14] we show that the p/t-tau ratio is specific to differentiate TDP from tau pathology, which can enable the application of disease-modifying agents that target a specific underlying pathology. Compared with previous studies,[13,35] we report different absolute tau levels and ratios because of different measurement platforms; this impedes direct comparison of the studies and illustrates the need for local cutoffs or multicenter evaluation.

The pathophysiological background of a low p/t-tau ratio is not entirely clear; the p/t-tau ratio differed between patients and controls because of elevated t-tau levels – representing neuronal loss – in concordance with prior studies.[10,33,36] In contrast, the observed differences between underlying TDP and tau pathology were mainly driven by low p-tau levels in FTLD-TDP. Prior studies have reported conflicting results: p-tau was decreased in TDP pathology,[13,32,35] t-tau was increased,[14] or no differences were found.[10,33] These differences may be explained by variation in cohort compositions, especially regarding the proportion of genetic patients, of concomitant MND, and of AD copathology. We found similar p/t-tau

ratios in patients with low versus high AD copathology, but it is not possible to draw firm conclusions since the group with copathology was too small and mostly excluded because of the study design. A recent study observed an association of antemortem CSF p-tau, and not t-tau, with post mortem cerebral tau pathology, suggesting that low p-tau levels reflect the low tau burden in FTLD-TDP.[35]

In the day-to-day practice, prognostic markers are important to inform patients and caregivers and to customize treatment plans. The present study corroborates the association of NfL and the p/t-tau ratio with survival,[9,10,37] and shows 5-year survival rates that can be applied to clinical patients, if replicated. In clinical trials, this can aid in sample size estimations that will facilitate trial efficiency.

Lastly, these biomarkers – especially NfL – could serve as surrogate endpoints in therapeutic trials, supported by the association with disease severity we show, as in earlier reports.[7,9,27] For example, in multiple sclerosis, a dynamical decrease of CSF NfL was observed after treatment intervention.[38] A similar application in dementia is endorsed by an amyloidosis mouse model, in which BACE1-inhibitor treatment reduced the amyloid-β deposits along with CSF NfL levels.[39] Furthermore, CSF NfL strongly correlates with serum NfL in FTD,[9,24] implicating that NfL can be determined in a less invasive way, enabling repeated sampling. In this clinical study, we have investigated CSF, because its collection (and not serum) is an integral part of our diagnostic process to exclude AD, and has the advantage of allowing measurement of NfL simultaneously with markers unmeasurable in blood (i.e. p/t-tau ratio). In future trial settings, one could envision a CSF measurement of the ratio and NfL at baseline, to stratify on suspected underlying pathology and disease progression, and subsequent serum NfL measurements for monitoring.

Strengths of this study include a large, well-characterized, cohort with a large number of definite FTD diagnoses (*n*=68). This study was an important head-to-head comparison of NfL versus the p/t-tau ratio across the entire FTD spectrum, including PSP and CBS, which is representative of a memory clinic population. We also show the differences in p/t-tau ratio across clinical diagnoses. Our information can aid in moving these biomarkers from benchside to clinical practice. The exclusion of patients with a low CSF $A\beta_{42}$ and high tau may have excluded some patients with FTLD,[3] however it ensures that no concomitant AD pathology is causing alterations in the studied biomarkers and therefore enabled a pure study cohort. A limitation is the retrospective design, which resulted in missing data and the lack of FTD-specific scales (e.g. Frontotemporal Dementia Rating Scale or FTD-CDR-SB),[40] and the fact that we did not include longitudinal samples, which are necessary to determine the usability in trial settings.

Both NfL and the p/t-tau ratio thus show similar patterns in discriminating clinical FTD groups and predicting survival. This implies that the markers are interchangeable for these applications, and NfL has the advantage of being measurable in blood (although the performance in blood remains to be proven). However, to stratify underlying TDP from tau pathology, the p/t-tau ratio should be used since it outperforms NfL.

Authors contributions

L.M., E.V, A.R., W.v.d.F., C.T., J.v.S. and Y.P. designed the study. L.M. performed the statistical analyses. All authors worked on data collection, interpreting the data, and drafting or revising the manuscript.

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Disclosures

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Supplementary material

Supplementary Table 1. Differences of CSF NfL between the patient and control groups

	bvFTD	FTD-MND	svPPA	nfvPPA	lvPPA	CBS	PSP
Controls	2194	18258	2177	1371	757	1690	933
p-value	<0.001	<0.001	<0.001	<0.001	0.97	<0.001	<0.001
bvFTD		16064	-17	-823	-1437	-504	-1261
p-value		<0.001	1.00	1.00	0.98	1.00	0.002
FTD-MND			-16081	-16887	-17501	-16568	-17325
p-value			<0.001	<0.001	<0.001	<0.001	<0.001
svPPA				-805	-1419	-487	-1244
p-value				1.00	0.99	1.00	0.10
nfvPPA					-614	319	-439
p-value					1.00	1.00	1.00
lvPPA						933	175
p-value						1.00	1.00
CBS							-757
p-value							0.61

Differences between medians are displayed (column minus row). Significances (corrected for multiple comparisons) of the ANCOVA analysis with correction for age are displayed.

bvFTD: behavioural variant FTD; CBS: corticobasal syndrome; CSF: cerebrospinal fluid; FTD: frontotemporal dementia; FTD-MND: FTD with concomitant motor neuron disease; lvPPA: logopenic variant PPA; NfL: neurofilament light chain; nfvPPA: non-fluent variant PPA; PPA: primary progressive aphasia; PSP: progressive supranuclear palsy; svPPA: semantic variant PPA

Supplementary Table 2. Differences of CSF p/t–tau ratio between the patient and control groups

	bvFTD	FTD-MND	svPPA	nfvPPA	lvPPA	CBS	PSP
Controls	-0.052	-0.089	-0.057	-0.033	-0.043	-0.044	-0.026
p-value	<0.001	<0.001	<0.001	0.003	0.17	<0.001	<0.001
bvFTD		-0.037	-0.005	0.019	0.009	0.008	0.026
p-value		<0.001	1.0	0.75	1.0	0.95	0.001
FTD-MND			0.032	0.056	0.046	0.045	0.063
p-value			<0.001	<0.001	0.16	<0.001	<0.001
svPPA				0.024	0.014	0.013	0.031
p-value				0.91	1.0	1.0	0.054
nfvPPA					-0.010	-0.010	0.007
p-value					1.0	1.0	1.0
lvPPA						-0.001	0.017
p-value						1.0	1.0
CBS							0.017
p-value							0.89

Differences between medians are displayed (column minus row). Significances (corrected for multiple comparisons) of the ANCOVA analysis with correction for age are displayed.

bvFTD: behavioural variant FTD; CBS: corticobasal syndrome; CSF: cerebrospinal fluid; FTD: frontotemporal dementia; FTD-MND: FTD with concomitant motor neuron disease; lvPPA: logopenic variant PPA; NfL: neurofilament light chain; nfvPPA: non-fluent variant PPA; PPA: primary progressive aphasia; PSP: progressive supranuclear palsy; svPPA: semantic variant PPA

Supplementary Table 3. Diagnostic performance of CSF NfL, p/t–tau ratio and the combination

	AUC	95% CI AUC	*p*-value	Cut-off	Sensitivity	Specificity	NPV	PPV
Case vs control								
NfL	0.87	0.81-0.92	<0.001	≥1613	78.5%	88.9%	35.7%	98.1%
p/t-tau ratio	0.86	0.83-0.90	<0.001	≤0.153	73.3%	93.3%	30.9%	98.9%
Combined	0.91	0.88-0.95	<0.001	≥0.860	79.8%	93.3%	38.9%	98.9%
FTLD-TDP vs FTLD-tau								
NfL	0.59	0.45-0.73	0.26	n.s.	n.s.	n.s.	n.s.	n.s.
p/t-tau ratio	0.73	0.60-0.87	0.005	≤0.121	66.7%	76.5%	44.8%	88.9%
Combined	0.75	0.62-0.88	0.004	≤0.212	59.1%	87.5%	43.8%	92.9%

Diagnostic performance per application and per biomarker or combination. To discriminate cases from controls, the combination outperformed NfL (p=0.04 compared to AUC of combination) and the ratio (p<0.01) alone. To discriminate TDP from tau pathology, the ratio performed similar to the combination of NfL and the ratio (p=0.95).

AUC: area under the curve from the receiver operating curve analysis; CI: confidence interval; CSF: cerebrospinal fluid; FTLD-tau: frontotemporal lobar degeneration with tau inclusions; FTLD-TDP: frontotemporal lobar degeneration with TAR DNA-binding protein 43 inclusions; NfL: neurofilament light chain; NPV: negative predictive value; n.s.: not significant; PPV: positive predictive value.

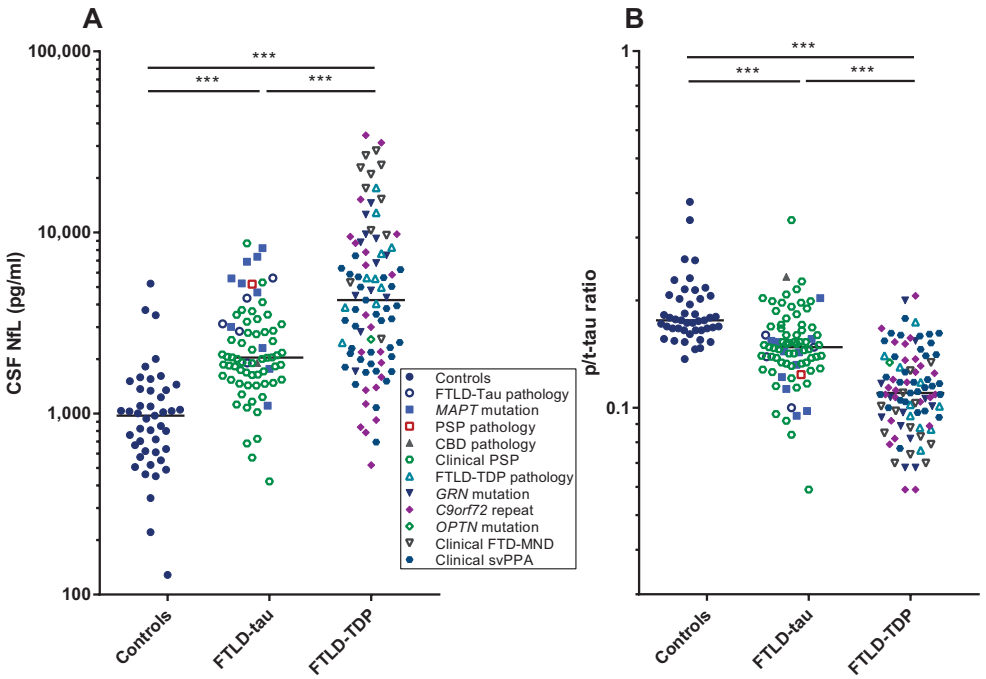
Supplementary Table 4. Association of NfL and p/t–tau ratio with demographic and clinical variables.

	NfL			p/t-tau ratio		
	r_s	*n*	*p*-value	r_s	*n*	*p*-value
Age at CSF collection	-0.03	335	0.65	0.12	352	0.03
Sex	n/a	335	0.38	n/a	352	0.53
Disease duration at CSF collection	-0.22	335	<0.001[a]	0.15	352	0.006[b]
Genetic statusc	n/a	334	0.001[c]	n/a	351	0.005[c]
MMSE	-0.15	273	0.01[d]	0.10	291	0.08
FAB	-0.15	201	0.03[a]	0.12	219	0.07
CDR	0.08	208	0.25	-0.08	227	0.25
CDR-SB	0.38	51	0.005[a]	-0.31	51	0.03[a]

All clinical patients and no controls were included in the correlation analysis. For the cognitive scales, only assessments within 6 months of CSF collection were considered.

[a]After stratification on clinical diagnosis, an association was confirmed in bvFTD. [b]Stratification by clinical diagnosis did not yield an association. [c]The following groups were compared: patients with a mutation in *GRN*, *C9orf72*, *MAPT*, and patients without a known mutation, one patient with a *optineurin* mutation was excluded; post-hoc analysis showed higher NfL levels and lower p/t-tau ratios in *GRN* mutations than in those without a known mutation. [d]After stratification on clinical diagnosis, an association was confirmed in CBS.

bvFTD: behavioural variant frontotemporal dementia; CBS: corticobasal syndrome; CDR: clinical dementia rating scale; CDR-SB: clinical dementia rating scale sum of boxes; CSF: cerebrospinal fluid; FAB: frontal assessment battery; MMSE: Mini-Mental State Examination; n/a: not applicable; NfL: neurofilament light chain; r_s: Spearman's correlation coefficient.



Supplementary Figure 1. NfL and p/t–tau ratio levels by suspected pathology.

(A) NfL levels and (B) the p/t-tau ratio in patients with suspected FTLD-TDP or FTLD-tau pathology. Patients with suspected pathology [Clinical PSP (green hexagons) in FTLD-tau group; clinical FTD-MND (grey downward triangle) and clinical svPPA (blue filled hexagon) in FTLD-TDP-43 group] were combined in this analysis with those with known underlying pathology [FTLD-tau pathology (blue circles); *MAPT* mutation (filled light-purple squares); PSP pathology (red squares); CBD pathology (grey filled upward triangles); FTLD-TDP pathology (aqua upward triangles); *GRN* mutation (filled blue downward triangles); *C9orf72* repeat expansion (purple filled diamonds); *OPTN* mutation (green diamonds)]. Horizontal lines represent medians.

The median of p-tau levels in suspected FTLD-TDP pathology was 39 pg/ml (interquartile range 33-49) and 38 pg/ml (30-49) in suspected FTLD-tau pathology; median t-tau level was 352 pg/ml (271-471) for suspected FTLD-TDP, and 267 pg/ml (177-353) for suspected FTLD-tau pathology.

***: $p<0.001$; CBD: corticobasal degeneration; CSF: cerebrospinal fluid; FTD-MND: frontotemporal dementia with concomitant motor neuron disease; FTLD-tau: frontotemporal lobar degeneration with tau inclusions; FTLD-TDP: frontotemporal lobar degeneration with TAR DNA-binding protein 43 inclusions; NfL: neurofilament light chain; n.s.: not significant; PSP: progressive supranuclear palsy; svPPA: semantic variant primary progressive aphasia.

CHAPTER 2.2

Cerebrospinal fluid neurofilament light chain has a limited value in semantic variant primary progressive aphasia

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Submitted

Abstract

Background: Semantic variant primary progressive aphasia (svPPA) is a neurodegenerative disorder characterized by progressive language problems falling within the clinical spectrum of frontotemporal lobar degeneration (FTLD). The development of disease-modifying agents may be facilitated by its relative clinical and pathological homogeneity, but requires robust monitoring biomarkers to measure their efficacy. In different FTLD subtypes, neurofilament light chain (NfL) is a promising marker and we aimed to investigate the utility of cerebrospinal fluid (CSF) NfL in svPPA.

Methods: This large retrospective multicenter study compared CSF NfL levels of 162 svPPA patients with 65 controls. CSF NfL levels of patients were correlated with clinical parameters (including survival), neuropsychological test scores, and regional grey matter atrophy (including longitudinal data in a subset).

Results: CSF NfL levels were significantly higher in svPPA patients (median: 2326 pg/mL, interquartile range: 1628-3593) than in controls (577 [446-766], $p<0.001$). Higher CSF NfL levels were moderately associated with naming impairment, as measured by the Boston Naming Test (r_s= -0.32, p=0.002), and with smaller grey matter volume of the parahippocampal gyri (r_s= -0.31, p=0.004). However, CSF NfL levels were not associated with progression of grey matter atrophy, and did not predict survival.

Conclusions: CSF NfL was elevated in svPPA, but concentrations were only moderately associated with language impairment and regional grey matter atrophy. Unlike in other FTLD subtypes, baseline NfL in svPPA was not related to progression of atrophy or survival in svPPA, which suggests a limited utility of cross-sectional CSF NfL in svPPA.

Introduction

Semantic variant primary progressive aphasia (svPPA) is a slowly progressive, sporadic neurodegenerative disorder characterized by loss of semantic knowledge, impaired naming and word comprehension, with preserved speech production.[1] The estimated prevalence of svPPA is 4.2-7.6/100,000,[2] and compared to other disorders within the frontotemporal lobar degeneration (FTLD) spectrum, it is relatively homogeneous because of the typical clinical presentation and uniform neuroimaging signature of asymmetrical (most often left-sided) anteroinferior temporal atrophy.[1,3,4] Also, autopsy studies show a predominant clinicopathological concordance with type C frontotemporal lobar degeneration with TAR DNA binding protein 43kDa inclusions (FTLD-TDP).[1] This clinical and pathological homogeneity provides opportunities for the development of disease-modifying agents, for which reliable biomarkers are essential to measure the efficacy of potential new therapeutics.

A promising cerebrospinal fluid (CSF) biomarker in frontotemporal dementia (FTD) is neurofilament light chain (NfL), which is a major component of the neuronal cytoskeleton involved in axonal and dendritic growth, signaling and transport.[5] Previous studies have demonstrated elevated CSF NfL levels across the FTD spectrum, which associated with disease severity, brain atrophy and survival.[6–9] In addition, CSF NfL levels strongly correlate to serum NfL levels, enabling repeated measurements to track disease progression or therapy responses.[6,10] Small series have shown that svPPA features high CSF and serum NfL concentrations, similar to other FTD subtypes,[8,11–13] but small cohorts may lack the power to detect meaningful or distinctive associations with clinical variables. Since high NfL levels are associated with a short survival,[6,7,14,15] and svPPA is a rather slowly progressive disorder compared to other FTD subtypes,[2,16] questions arise regarding whether NfL concentrations in svPPA are reflective of alternate phenotypic variables, such as disease severity, degree or localization of atrophy, or a unique and unrecognized underlying pathogenic process.

In a large series of svPPA patients from 14 different centers in Europe and the United States, we aimed to investigate our hypothesis that CSF NfL levels in svPPA patients are elevated and correlate with disease severity, atrophy and clinical progression.

Methods

Subjects

In total, 168 svPPA patients from 14 different centers in Europe and the Unites States (numbers per site in Supplementary Table 1) were retrospectively included in this study. Patients with a CSF profile suggestive of Alzheimer's pathology (a combination of low amyloid-$\beta_{1\text{-}42}$ and high phospho- and/or total-tau,[18] according to local references at time of CSF collection), were excluded from the study (*n*=6). Patients were diagnosed with svPPA according to international consensus criteria at the time of inclusion.[4,17] Thus, patients presented initially with language difficulties, characterized by a fluent speech with impaired naming and

comprehension. Asymmetric temporal atrophy or hypometabolism on imaging was used as supportive feature, and was present in 143 patients (with frontoparietal involvement in a minority of patients). The remaining 19 patients fulfilled clinical diagnostic criteria (of whom 3 were autopsy-proven FTLD-TDP), but no neuroimaging (*n*=18) was performed or atrophy was mild (*n*=1). In a subset of patients (*n*=63), a variable degree of behavioural disturbances was present, however all patients initially presented with language problems as most prominent and functionally impairing feature. Inclusion criteria, in addition to clinical diagnosis of svPPA, were availability of CSF NfL concentrations (*n*=162), and survival (*n*=157), neuropsychological (*n*=147) and/or neuroimaging data (*n*=87). To compare NfL levels between controls and svPPA patients, 65 sex- and age-matched healthy controls from our previous studies were included.[6,8] Controls had normal CSF amyloid-$\beta_{1\text{-}42}$ levels and were either subjects with normal neurological examinations, neuropsychological testing scores, and Clinical Dementia Rating (CDR) scores of 0 (*n*=44), or cognitively healthy family members without a mutation or spouses from patients with genetic FTD or a different neurodegenerative dementia (*n*=21).

Disease duration was defined as time between first symptoms noted by a caregiver (onset) and CSF collection. Survival was defined as time between CSF collection and death.

The local ethics committees approved the study and all subjects (or legal representatives) provided written informed consent.

Neuropsychological assessment (NPA)

Most subjects (*n*=147) underwent global cognitive screening and/or NPA at the local study site (for numbers per test, see Supplementary Figure 1); only assessments within six months of CSF collection were analyzed. Screening instruments included the Mini-Mental State Examination (MMSE), global Clinical Dementia Rating scale (CDR), CDR - sum of boxes (CDR-SB) and the FTD-CDR-SB. Annual progression rate after CSF collection was calculated by the change in MMSE, CDR, CDR-SB, or FTD-CDR-SB divided by the number of years between baseline and follow-up (at least 6 months).

NPA batteries differed across the sites, and specific tests were included when available in at least 30 patients (Supplementary Figure 1). A proportion of the test scores were transformed to uniformly and meaningfully combine different tests or versions and thus use the maximal amount of neuropsychological data. Short versions of the Boston Naming Test (BNT) were multiplied to match the total possible score of the full 60-item version. Trail-making Test-part A (TMT-A), and part B (TMT-B) were truncated to 300 seconds for patients that exceeded the time limit of 300 seconds. For the Stroop Color-Word Test (SCWT), we transformed versions that scored the number of correct answers into seconds needed to complete a 100-item version: number of seconds allowed * 100 / number correct. Next, the interference score (score on interference card / score on color naming) was calculated for all SCWTs and used for analysis. Different word-list learning tasks were transformed into a percentage correct items (Rey Auditory Verbal Learning Test *n*=34, California Verbal

Learning Test *n*=24, CERAD word list memory test *n*=2), and also scores on different versions of the Clock Drawing Test (CDT) were converted to percentages. Non-transformed tests included categorical fluency (animal naming), verbal fluency (three letters), digit span (forward+backward), and the Rey complex figure test.

CSF analyses

CSF was collected and stored according to standardized local procedures. NfL was measured by the enzyme-linked immunosorbent assay (ELISA) of Uman Diagnostics (Umeå, Sweden) according to the manufacturer's instructions in duplicates. Measurements were performed in three different laboratories: the VU medical center (135 svPPA patients, 21 controls), Bristol Myers Squib, Wallingford, CT (19 svPPA patients, 44 controls), and the Washington University School of Medicine (8 svPPA patients). All laboratories used the same ELISA, but the latter two added a dilution step (1:3 diluted, instead of 1:1 as the manual stipulates). Thus, considering the optimal linearity of the assay used,[19] a correction factor of two was used for all NfL levels determined at these sites, resulting in comparable CSF NfL levels in patients across the different laboratories (p=0.09). Controls from laboratory 2 had slightly lower NfL concentrations, but with overlapping ranges (laboratory 1 [VUmc]: median 800 pg/ml, range 548-1093 pg/ml; laboratory 2 [Bristol Myers Squib]: median 511 pg/ml, range 99-1047 pg/ml). We covaried analyses for laboratory and performed a subanalysis on NfL levels from laboratory 1, where most samples were measured. Within all laboratories, the interassay coefficient of variation was within acceptance criteria (≤20%). Mean intra-assay coefficient of variation was 1.4% (range 0-11.3%, unavailable in 25 patients).

Magnetic Resonance Imaging (MRI)

In 87 patients, structural T1-weighted (T1w) 3T MR-images within 6 months of CSF collection were available for neuroimaging analysis (mean CSF-MRI interval 0±1 months), and in 32 patients a follow-up scan after ≥6 months was available (mean interval between scans 13.5±7.0 months). Brain images were acquired locally, and scans from a scanner that only contributed one or two scans were excluded, resulting in datasets from 9 different scanners; detailed information on numbers and reasons for exclusion are presented in Supplementary Figure 1.

The T1w images were processed according to earlier described procedures.[20] In short, a multi-atlas approach was used to calculate grey matter volume (mL) in 83 regions of interest (ROIs) from each subject's T1w images. The unified tissue segmentation method[21] of SPM8 (Statistical Parametric Mapping, London, UK) was used to segment T1w images into grey matter (GM), white matter, and CSF. Then, regions of interest were defined for each subject by using a multi-atlas approach containing 30 labeled T1w images with each 83 ROIs.[22,23] A rigid, affine and non-rigid B-spline transformation model was used to register the atlas' T1w images to the subjects' T1w images. All images were masked with the brain extraction tool[24] and non-uniformity corrected after which a majority-voting algorithm was

used to fuse ROI labels. From the 83 ROIs, we selected 11 specific cortical ROIs that have previously been implicated in svPPA (Supplementary Table 2) for further analysis.[1,25,26] Processed images were visually inspected for each subject and each processing step; six scans had extensive segmentation errors and were therefore excluded (Supplementary Figure 1). For the 11 ROIs, processed images with outlying volumes (<25th percentile–1.5*IQR, or > 75th percentile+1.5*IQR; IQR: interquartile range) were closely inspected by three raters; when consensus was reached that the outlier was caused by a segmentation error, the specific ROI was excluded from analysis.

Since svPPA is an asymmetric disorder, GM ROIs of the dominant brain side (most atrophied, based on the smallest temporal lobar GM volume for each patient) were used for analysis; secondary analyses were undertaken on bilateral GM ROIs. All ROIs were corrected for head size by normalizing to intracranial volume (ICV), and reported as percentage of ICV. The degree of asymmetry was assessed by calculating the ratio of temporal lobar GM volume from the dominant side, to that of the non-dominant side, yielding a smaller ratio when more asymmetry is present. For follow-up images, progression of atrophy was assessed by change in volume (mL) per year uncorrected for ICV: (volume follow-up - volume baseline) / interval between scans (years).

Statistical analyses

SPSS Statistics 21.0 for Windows (Armonk, NY, USA) was used to analyze the data. Statistical significance was set at $p<0.05$ and Bonferroni correction for multiple testing was used when appropriate. Continuous data were compared between two groups by Mann-Whitney U or t-tests where appropriate; categorical variables were compared by Chi-Square tests. CSF NfL levels were normalized by log transformation and compared between svPPA patients with controls using analysis of covariance (ANCOVA), correcting for sex, age at CSF collection and laboratory of NfL measurement.

Spearman's correlation coefficient (r_s) was used to correlate NfL levels with age at CSF collection, disease duration at CSF, cognitive screening scales (MMSE, CDR-SB and FTD-CDR-SB), neuropsychological tests, and GM ROIs. Next, multivariate linear regression analysis (β) was also used to assess the association between (1) NfL levels (independent variable) and neuropsychological tests (dependent variable), correcting for age, sex, and laboratory; and (2) NfL levels and GM ROIs, correcting for age, sex, laboratory and scanner. Correction for multiple comparisons was done with the Bonferroni method. NfL as predictor for survival (either after CSF collection or after onset) in patients was analyzed using the Log Rank test and Kaplan-Meier curves comparing NfL tertiles, and a Cox regression with correction for age, sex, and laboratory, both on tertiles and NfL as continuous variable.

To test the consistency of our results, we performed two subanalyses by repeating the analyses described above in: 1) in patients with diagnostic certainty supported by imaging and/or pathology; and 2) only in the measurements from laboratory 1.

Results

Demographical and clinical data

In total, 162 svPPA patients and 65 healthy controls were studied, who did not differ in age or gender (Table 1). Median disease duration at CSF collection was 3.3 years, ranging from

Table 1. Subject characteristics.

	svPPA patients, *n*=162	Controls, *n*=65	*p*-value
Sex, *n* male (%)	75 (46%)	32 (49%)	0.69
Age at CSF collection, years	64 (58-68)	65 (60-70)	0.43
Age at onset, years	60 (54-65)[1]	n/a	n/a
Age at death, years	69 (65-74)[2]	n/a	n/a
MMSE score	25 (21−28)[3]	n/a	n/a
CDR-SB score	3.5[4] (2.5−4.8)	n/a	n/a
FTD-CDR-SB score	4.0[5] (2.0−6.0)	n/a	n/a
CSF NfL, pg/mL	2326 (1628-3593)	577 (446-766)	<0.001
MRI available at baseline (at follow-up), *n*	87 (32)	n/a	n/a

Continuous variables are presented as medians (IQR). [1]Unknown in 5 patients. [2]30 patients were known to be deceased at time of data analysis. [3]Available in 135 patients. [4]Available in 65 patients. [5]Available in 34 patients. IQR: interquartile range; CDR-SB: clinical dementia rating scale sum of boxes; CSF: cerebrospinal fluid; FTD-CDR-SB: frontotemporal dementia CDR-SB; MMSE: mini-mental state examination; MRI: magnetic resonance imaging; n/a: not applicable; NfL: neurofilament light chain; svPPA: semantic variant primary progressive aphasia.

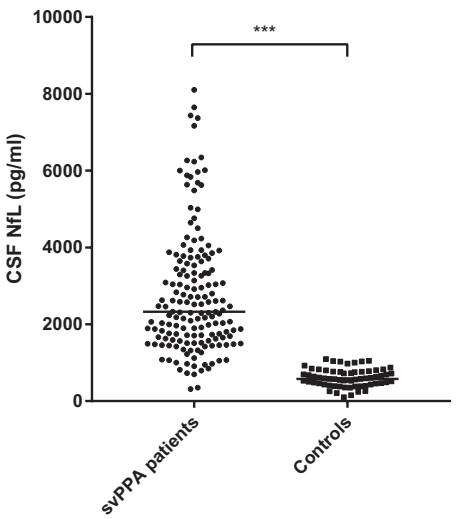

Figure 1. CSF NfL concentrations in svPPA patients and controls.
The horizontal lines represent the median per group. CSF: cerebrospinal fluid; NfL: neurofilament light chain; svPPA: semantic variant primary progressive aphasia; ***: *p*<0.001.

0.3 to 15.2 years. Of the patients with a usable MRI-scan (*n*=87), 65 had left-sided dominant atrophy and 22 right-sided dominant atrophy. There was a strong temporal asymmetry in patients with MRI (mean ratio temporal atrophy of 0.76 (±0.08)).

NfL in relation to clinical characteristics

CSF NfL levels were higher in svPPA patients than in controls ($p<0.001$, corrected for age, sex and laboratory, Table 1, Figure 1). In patients, CSF NfL did not correlate with age or disease duration at CSF collection (Table 2), nor was there a difference between patients with or without concomitant behavioural symptoms at CSF collection ($p=0.61$).

Association between NfL and neuropsychological data

NfL was not associated with MMSE, global CDR, CDR-SB or FTD-CDR-SB at CSF collection (Table 2). Progression rate, as measured by annual change of MMSE – but not of CDR, CDR-SB or FTD-CDR-SB change – after CSF collection, was associated with NfL, but did not survive correction for multiple comparisons (uncorrected $p=0.04$, Table 2). NfL was associated with the BNT only ($r_s= -0.32$, $p=0.002$), but not with any other neuropsychological test scores (Figure 2A, Table 3; the TMT-A and TMT-B showed an association that did neither survive multiple testing, nor correction for covariates). Additional correction for disease duration (next to age, sex, and laboratory), yielded similar results.

Table 2. Association between neurofilament light chain and clinical characteristics or global cognitive scales in semantic variant primary progressive aphasia patients.

	Cross-sectional			Longitudinal		
	n	r_s	*p*	*n*	r_s	*p*
Age at CSF collection	162	-0.002	0.98	n/a	n/a	n/a
Age at onset	157	-0.06	0.46	n/a	n/a	n/a
Disease duration at CSF collection	157	0.03	0.68	n/a	n/a	n/a
MMSE	135	-0.11	0.20	51	-0.29	**0.04**
Global CDR	78	0.15	0.18	33	-0.03	0.86
CDR-SB	65	0.23	0.07	33	-0.01	0.97
FTD-CDR-SB	34	0.05	0.79	10	0.33	0.37

For longitudinal analysis, annualized change of the scores was used. *p*-values <0.05 are bolded, none survived Bonferroni correction for multiple testing. CDR: global score of clinical dementia rating scale; CDR-SB: clinical dementia rating scale sum of boxes; FTD-CDR: frontotemporal dementia clinical dementia rating scale; CSF: cerebrospinal fluid; MMSE: mini-mental state examination; n/a: not applicable.

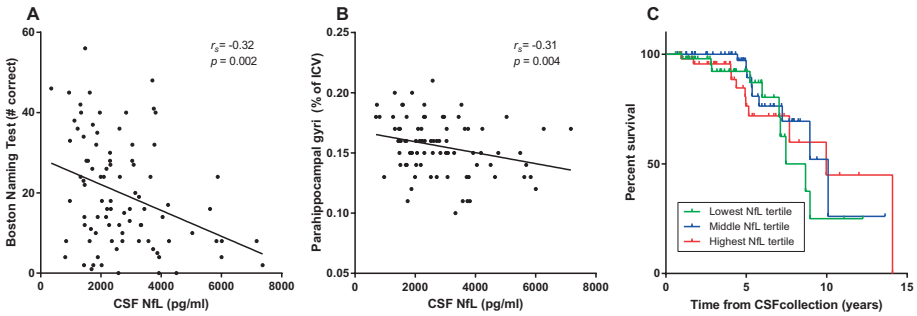

Figure 2. Relationship of CSF NfL with language impairment, parahippocampal atrophy, and survival in semantic variant primary progressive aphasia patients.

(A) Association between NfL and the Boston Naming test as measure for naming impairment. (B) Association between NfL and grey matter volume of the parahippocampal gyrus at the dominant side, displayed as percentage of ICV. (C) NfL was not associated with survival after CSF collection as exemplified by this Kaplan-Meier curve of NfL levels stratified to lowest (green line), middle (blue line), and highest tertiles (red line). Vertical ticks represent living patients.

CSF: cerebrospinal fluid; ICV: intracranial volume; NfL: neurofilament light chain.

Table 3. Associations between neurofilament light chain and neuropsychological test scores.

		Correlation		Multivariate regression	
Test	*n*	r_s	*p*	β	*p*
BNT	89	-0.32	**0.002***	-0.34	**0.002***
Categorical fluency	110	-0.17	0.08	-0.14	0.14
Letter fluency	66	-0.03	0.83	-0.14[1]	0.28
Digit span	107	0.07	0.47	0.04	0.71
TMT-A	84	-0.23	**0.04**	-0.20[1]	0.07
TMT-B	78	-0.30	**0.007**	-0.19[1]	0.09
SCWT interference ratio	52	0.12	0.39	0.08[2]	0.57
Word list immediate recall	60	-0.17	0.20	-0.22[3]	0.08
Word list delayed recall	60	-0.23	0.07	-0.24[3]	0.08
Clock drawing test	30	0.23	0.23	0.21[1]	0.30
Rey figure copy	50	-0.05	0.71	0.04[1]	0.76
Rey figure delayed recall	44	-0.17	0.27	-0.24[1]	0.10

Multivariate regression is corrected for age, gender, and laboratory. *p*-values <0.05 are bolded, *survived Bonferroni correction for multiple testing (p<0.004). [1]Not corrected for laboratory, since all samples were measured in the same laboratory for these tests. [2]After additional correction for version seconds to complete versus number correct: β=0.15, p=0.27. [3]Similar results with additional correction for test version.

BNT: Boston Naming Test; SCWT: Stroop Color-Word Task; TMT: Trail-making Test.

Association between NfL and regional GM volumes

CSF NfL levels were negatively associated with GM volume of the parahippocampal gyrus on the dominant atrophic side (Table 4, Figure 2B). Also, a negative association was seen for the medial and inferior temporal gyri, only when correction for covariates and multiple correction was not applied. When the analyses were repeated with bilateral volumes (Supplementary Table 3), there was again a negative association of NfL with the parahippocampal gyri. Additionally, the bilateral medial and inferior temporal gyri, temporal pole and hippocampus were negatively associated with NfL, however after correction for covariates, the association did not withstand Bonferroni correction.

In patients with a follow-up scan available (*n*=32), no association between NfL and progression of atrophy in the selected ROIs was found (Table 4 and Supplementary Table 3). Additional correction for disease duration yielded similar results after Bonferroni in all imaging analyses.

Table 4. Associations between neurofilament light chain and grey matter regions of interest of the dominant side.

	Cross-sectional (*n*=87)				Longitudinal (*n*=32)			
	Correlation		Multivariate regression		Correlation		Multivariate regression	
Region of interest	r_s	*p*	β	*p*	r_s	*p*	β	*p*
Temporal pole	-0.13	0.24	-0.13	0.27	-0.11	0.57	-0.11	0.63
Medial and inferior temporal gyri	-0.28	**0.009**	-0.21	0.06	0.03	0.88	-0.29	0.16
Superior temporal gyrus, central part	-0.17	0.11	-0.16	0.16	-0.01	0.94	-0.07	0.72
Fusiform gyrus	-0.16	0.14	-0.18	0.12	-0.33	0.07	-0.34	0.08
Parahippocampal gyrus	-0.31	**0.004***	-0.33	**0.003***	0.08	0.67	-0.04	0.85
Hippocampus	-0.20	0.07	-0.20	0.08	0.02	0.93	0.06	0.80
Amygdala	-0.18	0.09	-0.19	0.09	0.12	0.54	0.08	0.69
Insula	-0.19	0.07	-0.17	0.13	-0.09	0.62	0.04	0.82
Orbitofrontal cortex	-0.05	0.62	-0.05	0.67	0.10	0.59	0.17	0.40
Inferior frontal gyrus	-0.03	0.78	0.04	0.76	0.09	0.61	0.03	0.87
Anterior cingulate gyrus	-0.02	0.89	0.07	0.56	0.00	1.00	-0.02	0.91

Association of neurofilament light chain with grey matter regions of the dominant side on cross-sectional scanning, and with change in volume between follow-up and baseline scan.

For cross-sectional associations, intracranial volume-corrected volumes were used; for longitudinal associations, the change of volume per year was used. Multivariate regression was corrected for age, gender, laboratory, and scanner (the latter only for cross-sectional scans); *p*-values <0.05 are bolded, *survived Bonferroni correction for multiple testing (p<0.0045).

Survival analyses

The median survival after CSF collection of the 30 deceased patients was 5.3 years (range 1.0-14.1) and the median follow-up of living patients was 4.1 years (range 0.6-13.6, *n*=127; 5 patients were lost to follow-up). Autopsy was performed on seven of the deceased patients, all showing TDP-pathology. Estimated median time between onset and death for all patients was 14.5 years (95% confidence interval [CI] 12.2-16.9). In each CSF NfL tertile, a similar number of patients had deceased (*p*=0.86) and follow-up time was comparable. NfL tertiles did not associate with survival after CSF collection (Figure 2C, *p*=0.66 log rank test, *p*=0.80 Cox regression with correction for sex, age and laboratory). CSF NfL as a continuous variable only showed a trend for association (*p*=0.06, corrected Cox regression, Hazard Ratio 1.22 (95% CI 0.99-1.51). Neither did CSF NfL associate with total disease duration (time between onset and death; *p*=0.97 for NfL tertiles, *p*=0.36 for continuous NfL levels).

Subanalyses

Subanalysis of patients with a diagnosis supported by imaging and/or pathology showed similar NfL levels to those without (*p*=0.49). Similar results were found when comparing these patients to controls, and for associations with neuropsychological measures (significant for the BNT after correction for covariates and multiple testing). The Cox regression on continuous NfL levels and survival after CSF collection became significant (Hazard Ratio 1.25 for increase of 1ng/ml, *p*=0.04), but remained not significant when using NfL tertiles, or total disease duration.

In the second subanalysis on NfL levels only measured in laboratory 1, svPPA patients again had higher levels than controls (*p*<0.001). There were no associations with clinical characteristics, scales, changes in scales over time, or survival. The association between NfL and the BNT became a trend (*n*=66, correlation: r_s=-0.24, *p*=0.05; regression: β=-0.30, *p*=0.02), different neuropsychological tests showed similar results as the entire cohort. For the imaging analyses (*n*=63), no significant associations were found for the dominant side; bilaterally, the temporal pole, medial and inferior temporal gyri and the parahippocampal gyrus were significantly associated with NfL (regression analysis), but did not survive after multiple testing.

Discussion

This large international multicenter svPPA study shows that CSF NfL has a different and limited utility in svPPA compared to what is previously reported for other FTD subtypes. Increased CSF NfL levels in svPPA patients as compared to controls were associated with more severe naming impairment and smaller GM volume of the parahippocampal gyrus, both with only a medium effect size (correlation coefficient of -0.3). In contrast to other FTD subtypes, NfL did not associate with progression of GM atrophy or survival.

This first large international multicenter svPPA study on CSF NfL showed that NfL is elevated in comparison to controls, which agrees with blood or CSF measurements in previous smaller svPPA series.[8,10–12]

The association we show between higher NfL levels and worse performance on the BNT, indicates that the variation in NfL is partly explained by disease severity. Naming problems are one of the hallmarks of svPPA, and poor performance on language tests addressing semantic knowledge – such as the BNT – are typical for svPPA patients, while other domains remain mostly intact.[27–29] Although categorical fluency also assess semantic knowledge, this was not associated with NfL in our study. This may be explained by the fact that fluency tasks do not purely measure language impairment, as they also assess executive control ability and speed of performance.[30]

The present study did not find an association with CDR, while previous studies in FTD (different subtypes combined) showed a correlation between NfL and disease severity as measured by the CDR.[6,8,11] A possible explanation is that svPPA remains long confined to the language domain – which is not included in the global CDR – in the first period of disease. In line, it has been reported before that the conventional CDR is not an ideal proxy for disease severity nor progression in svPPA.[31] The FTD-CDR-SB does include a language domain, but the number of patients with data on this scale was too small to draw strong conclusions.

Our imaging analyses show an association between higher CSF NfL levels and smaller parahippocampal GM volume, which is in line with previous studies that found correlations of NfL with temporal GM as well as other cortical regions in FTD subtypes combined.[6,8] This is congruent with a large body of evidence that NfL levels can reflect the extent of neuronal loss.[6,8,32] However, a previous study did not find such association with serum NfL in svPPA patients,[12] which could be explained by the larger power of the current study, or CSF NfL might be more sensitive than serum NfL.[32]

In svPPA, the earliest changes in GM volume include loss of the anterior temporal lobe, in particular the temporal pole, fusiform gyrus, parahippocampal and entorhinal cortex, but also the hippocampus and amygdala. In more advanced disease stages, the atrophy extends anteriorly into the orbitofrontal, inferior frontal, insular and anterior cingulate cortices, as well as posteriorly to temporoparietal regions and into homologous areas of the contralateral hemisphere.[1,25,26] The parahippocampal gyrus is located in the core of the neurodegenerative process, it is therefore logical that we found an association between NfL and that region. More regions were (borderline) significantly associated when analyzing bilaterally rather than at the dominant side only. This may point towards an important difference between these markers: GM atrophy represents cumulative injury, while CSF NfL measures the current balance between release and clearance of NfL, and thus reflects ongoing neuronal loss. A previous report on neurodegenerative mouse models showed that increases in CSF and blood NfL indeed concurred with the onset and progression of protein inclusions.[33] Moreover, studies in other neurologic diseases as multiple sclerosis

and traumatic brain injury, have found high NfL levels during active periods while they normalize afterwards or after treatment.[34,35] If this hypothesis on NfL release is correct, the change of NfL over time would associate with progression of atrophy, as has previously been shown for PPA or FTD subtypes combined.[6,11,12] However, we did not observe an association between baseline NfL and progression of GM atrophy, perhaps due to the smaller longitudinal cohort. Larger series of longitudinal NfL levels with corresponding MRI scans are needed to elucidate these discrepancies, preferably including patients who are early in their disease process with multiple time-points and by taking a possible non-linear relationship into account.

Neurofilaments are especially abundant in axons,[36] and in subsets of autopsied svPPA patients a considerable degree of corticospinal tract degeneration has been reported.[37] It was not possible to study whether this associated with NfL levels in our svPPA patients since only few patients were autopsied (*n*=7) and uniform imaging data on white matter tracts were not available. However, this could be interesting for future studies since most of the variability in NfL levels between our patients were not explained by the variables we investigated.

Our estimated median total disease duration of 14.5 years is in line with a meta-analysis, that showed the longest median survival in svPPA (12 years) compared to different FTD subtypes and AD.[16] In different neurodegenerative disorders including FTD, AD and amyotrophic lateral sclerosis, NfL is associated with survival, which contrasts to the finding of our study that NfL cannot predict survival in svPPA patients.[6,7,14,15] These prior studies on FTD included heterogeneous groups of different clinical, genetic or pathological FTD forms, in which other subtypes than svPPA may have driven those associations. In our study, the follow-up time (median 4 years) may have been too short to capture the differences in survival, since average survival ranges from 9 to 12 years in svPPA.[2,16] Even so, we show that CSF NfL does not predict survival in 4-5 years in svPPA patients. This illustrates that NfL in svPPA has a distinctive utility when comparing to other dementia types.

A major strength of this study was the large international series of CSF samples from patients with this rare disease. A second strength was the multimodal approach in correlating NfL to clinical, neuropsychological and imaging data. Moreover, this study included only one FTD subtype – after exclusion of patients with CSF profiles suggestive of AD, making the results specific and not influenced by heterogeneity in clinical presentation and progression of different subtypes or AD pathology. Since svPPA is one of the most homogeneous subtypes of FTD, it is a promising target to develop novel treatments. Our results are important to for future trials as they suggest a limited role for cross-sectional CSF NfL as a monitoring or progression marker in svPPA.

The multicenter approach of this study also caused some limitations, especially in heterogeneity of collected data. First, NfL levels were measured in 3 centers, which we accounted for by normalization and laboratory-correction since the rareness of these samples did not allow remeasuring them. Neuropsychological test batteries differed per

center and we transformed different versions of the same test to analyze them together, but this might have introduced variability and loss of sensitivity. Also, scanners and parameters of the T1-weighted imaging varied. We tried to limit this variability by only including 3T data, excluding data from scanners on which <3 datasets were acquired, and correcting for scanner in the analysis. Additionally, we did not study different primary progressive aphasia subtypes and did not include serum - which will likely replace CSF measurements in the near future. The lack of longitudinal data did not allow to draw conclusions about NfL and clinical or imaging markers over time; it might be conceivable that a plateau phase or even a decrease of NfL may occur over time.

In conclusion, the results of this study show that cross-sectional CSF NfL may be a useful biomarker for the neurodegenerative process in svPPA, but not for monitoring disease severity, prediction of progression of atrophy or survival in svPPA. This is distinctive from previous reports on NfL in other neurodegenerative diseases, and should thus be taken into account when interpreting studies that combined different FTD subtypes. Recently, it became clear that NfL in blood and CSF strongly correlate,[6,10] facilitating longitudinal monitoring. More longitudinal studies are needed to assess how NfL levels fluctuate over time in relation to clinical and imaging changes in svPPA, and thereby their potential utility.

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Disclosures

The authors report no conflicts of interest relevant to this study.

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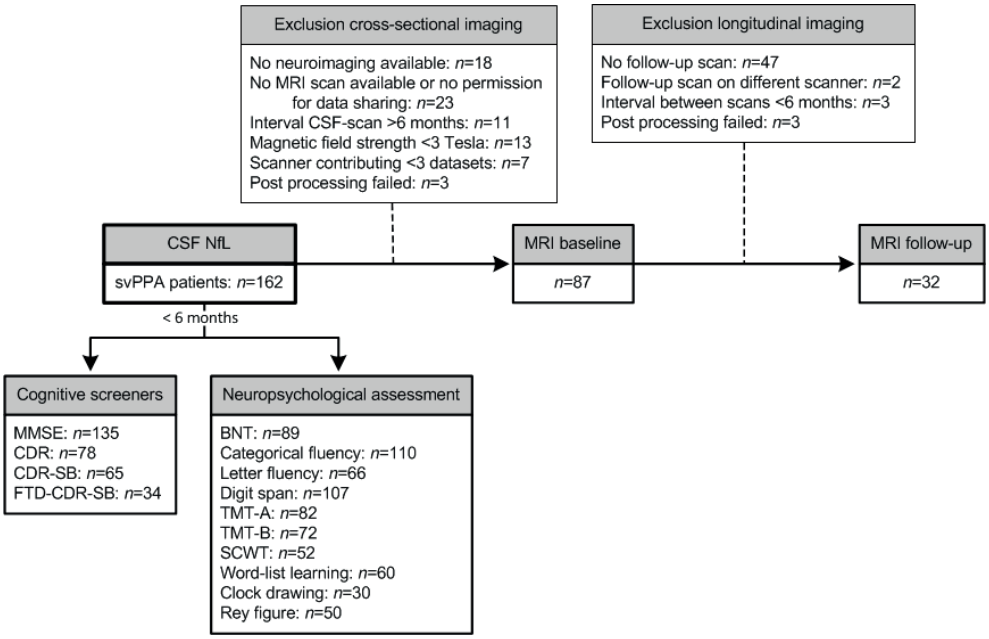
Supplementary material



Supplementary Figure 1. Flowchart of SD patients in– and excluded per analysis.
In total, 162 SD patients were studied (after exclusion of 6 patients with a CSF profile suggestive of Alzheimer's disease), of whom 87 were included in the cross-sectional imaging associations and 32 in the longitudinal imaging associations; reasons for exclusion are displayed in the upper boxes. Number of patients included in the associations with cognitive screeners and neuropsychological tests are displayed in the lower boxes and are based on the availability of these measures within 6 months of CSF sampling.
BNT: Boston Naming Test; CDR: clinical dementia rating scale; CDR-SB: CDR sum of boxes; CSF: cerebrospinal fluid; FTD: frontotemporal dementia; MMSE: Mini-Mental state examination; SCWT: Stroop Color-Word Task; svPPA: semantic variant primary progressive aphasia; TMT: Trail-making Test.

Supplementary Table 1. Number of subjects included per site.

Site	svPPA patients	Controls
University of Pennsylvania	31	0
University of California, San Francisco	27	44
Hospital Clínic Barcelona	14	13
Erasmus Medical Center	17	8
VU University Medical Center	22	0
Tehnical University of Munich	13	0
Lund University	11	0
University of Brescia	7	0
University of Milan	6	0
University College London	6	0
University of Tübingen	3	0
University of Lisbon	2	0
University Hospital Leuven	2	0
IRCCS Centro San Giovanni di Dio Fatebenefratelli	1	0

svPPA: semantic variant primary progressive aphasia

Supplementary Table 2. Studied grey matter regions of interest based on previous literature

Studied region of interest	ROI(s) from Hammers[1] atlas
Medial and inferior temporal gyri	Medial and inferior temporal gyri
Superior temporal gyrus, central part	Central part of the superior temporal gyrus
Temporal pole	Medial anterior temporal lobe + lateral anterior temporal lobe + anterior part of the superior temporal gyrus
Fusiform gyrus	Fusiform gyrus
Parahippocampal gyrus	Parahippocampal gyrus
Hippocampus	Hippocampus
Amygdala	Amygdala
Insula	Insula
Orbitofrontal cortex	Orbital gyri: anterior + medial + lateral + posterior
Inferior frontal gyrus	Inferior frontal gyrus
Anterior cingulate gyrus	Anterior cingulate gyrus: supragenual + subgenual + pre-subgenual part

[1]Hammers A, Allom R, Koepp MJ, et al. Three-dimensional maximum probability atlas of the human brain, with particular reference to the temporal lobe. Hum. Brain Mapp. 2003;19(4):224–247.

Supplementary Table 3. Associations between neurofilament light chain and bilateral grey matter region of interest volumes.

	Cross-sectional (*n*=87)				Longitudinal (*n*=32)			
	Correlation		Multivariate regression		Correlation		Multivariate regression	
Region of interest	r_s	*p*	β	*p*	r_s	*p*	β	*p*
Temporal pole	-0.22	**0.04**	-0.24	**0.04**	-0.14	0.45	-0.19	0.39
Medial and inferior temporal gyri	-0.32	**0.002***	-0.29	**0.008**	-0.08	0.64	-0.33	0.07
Superior temporal gyrus, central part	-0.21	0.05	-0.17	0.12	0.07	0.72	-0.17	0.43
Fusiform gyrus	-0.16	0.15	-0.15	0.19	-0.24	0.19	-0.28	0.09
Parahippocampal gyrus	-0.32	**0.003***	-0.35	**0.002***	0.05	0.79	-0.11	0.60
Hippocampus	-0.25	**0.02**	-0.25	**0.02**	0.01	0.94	0.05	0.82
Amygdala	-0.18	0.10	-0.20	0.08	-0.03	0.89	-0.01	0.95
Insula	-0.18	0.09	-0.19	0.11	-0.18	0.33	0.02	0.92
Orbitofrontal cortex	-0.07	0.54	-0.07	0.49	0.11	0.54	0.14	0.49
Inferior frontal gyrus	-0.06	0.58	-0.01	0.91	0.15	0.40	0.10	0.59
Anterior cingulate gyrus	-0.01	0.92	-0.02	0.87	-0.07	0.70	0.03	0.87

Association of neurofilament light chain with bilateral grey matter regions on cross-sectional scanning, and with change in volume between follow-up and baseline scan.

For cross-sectional associations, intracranial volume-corrected volumes were used; for longitudinal associations, the change of volume per year was used. Multivariate regression was corrected for age, gender, laboratory, and scanner (the latter only for cross-sectional scans); *p*-values <0.05 are bolded, *survive Bonferroni correction for multiple testing.

CHAPTER 2.3

Serum neurofilament light chain in progressive supranuclear palsy: an indicator for disease severity and survival

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Abstract

Background: Neurofilament light chain (NfL) is a promising biomarker in neurodegenerative diseases. Elevated NfL levels in CSF and blood have been observed in a growing number of neurodegenerative disorders, including frontotemporal dementia and Alzheimer's disease. We studied serum NfL levels in patients with progressive supranuclear palsy (PSP) in relation to disease severity and survival.

Methods: Serum NfL levels were determined in a retrospective cohort of 131 patients with PSP and 95 healthy controls. Detailed clinical examination was performed and disease severity was assessed by several rating scales.

Results: We found that serum NfL levels in PSP were twice as high as those in controls, and that NfL levels correlated with worse functional, motor and cognitive functioning. During follow-up, 119 PSP patients had died, and higher NfL levels were associated with a shorter survival.

Conclusions: This study provides evidence that serum NfL is a relevant and promising biomarker in PSP for disease severity, and may be used as a prognostic tool to predict survival in clinical practice.

Introduction

There is an urgent need for sensitive, easily accessible biomarkers in neurodegenerative disorders to monitor both disease progression and the effects of treatment. Elevated levels of neurofilament light chain (NfL), a neuronal cytoskeletal protein, reflect neuronal injury and have been found in the cerebral spinal fluid (CSF) of patients with several neurodegenerative disorders.[1] An important recent finding is that NfL levels can be reliably measured in blood, and that these correlate well with NfL levels in CSF.[2]

Progressive supranuclear palsy (PSP) is a neurodegenerative disorder characterized by parkinsonism, frequent falls, supranuclear gaze palsy and widespread tau positive inclusions at brain autopsy. Elevated NfL levels have been found in PSP patients' CSF.[3–6] Blood NfL has recently been shown to be a useful biomarker for assessing disease severity and predicting disease progression in PSP patients.[7] In addition, blood NfL has a good diagnostic performance in the discrimination of atypical parkinsonism (including PSP) from Parkinson's disease.[8] NfL levels have been associated with survival in frontotemporal dementia (FTD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS).[9–11] This prognostic value has not been studied in PSP, except for a small study of 12 patients where mortality at 24-month follow up was associated with high CSF NfL levels.[3]

In this retrospective case-control study, we investigated serum NfL levels in a large and well characterized cohort of PSP patients, demonstrating its relationship with disease severity and survival.

Methods

Subjects

PSP patients were enrolled between 2003 and 2014 as part of a Dutch genetic epidemiological PSP study.[12] The study was approved by the Medical Ethical committee of Erasmus MC; all participants or legal representatives signed informed consent. Inclusion in the study took place at the out-patients clinic, or by visiting patients at home or in nursing homes. At study entry, detailed clinical assessment was performed, including an interview with the caregiver and an extensive neurological examination with the assessment of the following rating scales: PSP-rating scale (PSP-RS), Mini Mental State Examination (MMSE), Frontal Assessment Battery (FAB), Hoehn and Yahr (HY) scale and Schwab and England Activities of Daily Living (SEADL). In a consensus meeting, medical records and MR images were reviewed, and the diagnosis PSP was established according to the National Institute for Neurological Disorders and Society for PSP (NINDS-SPSP) criteria. Controls were healthy spouses or caregivers from stroke patients, above 55 years of age and randomly selected from a large database.

NfL measurements

Serum samples from PSP patients and controls were centrifuged the same day and stored at -80°C. We used a recently developed, ultrasensitive Simoa assay to measure the sample NfL levels.[2] The measurements were performed in one round of experiments using one batch of reagents by board-certified technicians who were blinded to clinical data. For a quality control sample with a concentration of 16.8 pg/ml, repeatability was 7.36% and intermediate precision was 7.43%. For a quality control sample with a concentration of 127 pg/ml, repeatability was 6.69% and intermediate precision was 8.24%.

Statistical analysis

Differences in demographics between cases and controls were analyzed using parametric and non-parametric statistics as appropriate (SPSS version 21.0).

Six subjects (2 controls and 4 cases) with extremely high NfL values (>2 SD above the mean) were considered as outliers and their NfL values were replaced by values corresponding to the upper 2 SD cut off. Because of non-normality, log transformation of NfL data was performed. Age was correlated with NfL levels in both cases (Spearman's rho=0.37, p<0.001) and controls (Spearman's rho=0.55, p<0.001). We conducted therefor an ANCOVA to analyze NfL levels between cases and controls adjusted for age. The diagnostic accuracy of NfL was examined using receiver operating characteristic (ROC) curve analysis, with optimal cut off according to Youden's index. Correlations between NfL and disease severity (measured by PSP-RS, MMSE, FAB, SEADL and HY scale) were studied using linear regression analyses, with age and gender as covariates.

During follow up, 119 PSP patients had died and survival time was calculated from the moment of serum collection till date of death. In twelve patients the deceased status was unknown and in these subjects survival time was calculated from the moment of serum collection till last contact alive and entered as censored data. Survival in PSP patients was compared between NfL tertiles by Kaplan-Meier curves with log-rank tests and Cox regressions adjusted for age and gender.

Results

Serum NfL levels were determined in 131 PSP patients and 95 controls. PSP patients showed significantly higher NfL levels compared to controls (Table 1 and Figure 1A). This significance held after correction for age and sex on log-transformed NfL (p<0.001). We were able to accurately distinguish PSP patients from controls using serum NfL (area under the curve 0.87, 95% confidence interval 0.83-0.92). A cutoff value of 38.3 pg/ml provided a sensitivity of 90% and specificity of 63%. During follow-up, the diagnosis PSP was confirmed at autopsy in 23 subjects (filled black circles in Figure 1A).

Significant correlations were found between NfL levels and PSP-RS sum scores (β= 0.37, p<0.001), SEADL scores (β= -0.32, p=0.001), HY scale (β=0.30, p=0.001), FAB scores (β= -0.29,

Table 1. Demographic and clinical characteristics of PSP patients and controls.

	PSP patients (*n*=131)	Controls (*n*=95)	*p*-value
Gender, % female	45	54	0.23[a]
Age at serum sampling, years	71.3 (7.7)	68.5 (6.3)	0.003[b]
Age at disease onset, years	66.4 (7.6)	n/a	
Disease duration at serum sampling, years	4.9 (2.9)	n/a	
Median NfL level, pg/ml (interquartile range)	64.2 (42.4-86.1)	30.6 (20.1-41.1)	<0.001[c]
NINDS-SPSP criteria, number possible/probable/definite	49/59/23	n/a	
PSP-RS [*n*=113]	47.2 (15.5)	n/a	
Hoehn and Yahr scale [*n*=116]	4.3 (0.8)	n/a	
MMSE [*n*=107][d]	23.9 (4.6)	n/a	
FAB [*n*=92]	10.0 (3.5)	n/a	
Deceased subjects, *n*	119	n/a	
Disease duration from onset till death, years	7.8 (3.3)	n/a	

Values are presented as means (standard deviations) unless otherwise stated. [a]Chi-square test, [b]Student's T test, [c]Mann-Whitney Test, [d]In 20 subjects, the maximal possible score was less than 30 due to severe motor disability. FAB, Frontal Assessment Battery; MMSE, Mini-Mental state examination; n/a, not applicable; NfL, neurofilament light chain; PSP, progressive supranuclear palsy, PSP-RS, pogressive supranuclear palsy rating scale.

p=0.004) and MMSE scores (β= -0.18, *p*=0.05), indicating that higher NfL levels are associated with more severe motor, functional and cognitive disability. No associations were found between NfL levels and age at symptom onset or disease duration from onset till serum collection.

PSP-patients with higher NfL levels showed worse survival (Log Rank test *p*<0.001, Figure 1B). After adjusting for age and sex, NfL levels remained significantly associated with worse survival (Hazard Ratio 1.5 [1.1-1.9], *p*=0.003).

Discussion

This study shows that serum NfL levels are elevated in PSP patients, and that these are associated with disease severity and survival. Serum NfL is thus a promising biomarker in PSP with the major advantages over CSF that sample collection is minimally invasive and can be easily repeated over time. Because of the association with disease severity and survival, serum NfL may serve as a prognostic tool in clinical practice. Additionally, it can facilitate study design for therapeutic trials and genetic studies to categorize patients into disease subtypes.

The NfL concentrations in PSP patients were twice as high as those in controls, which is similar to the results from two previous studies on blood-based NfL in PSP.[7,8] We further demonstrate that NfL levels correlate with motor, cognitive and functional disability in PSP. Comparably, a previous study showed that blood NfL levels were correlated with HY stage

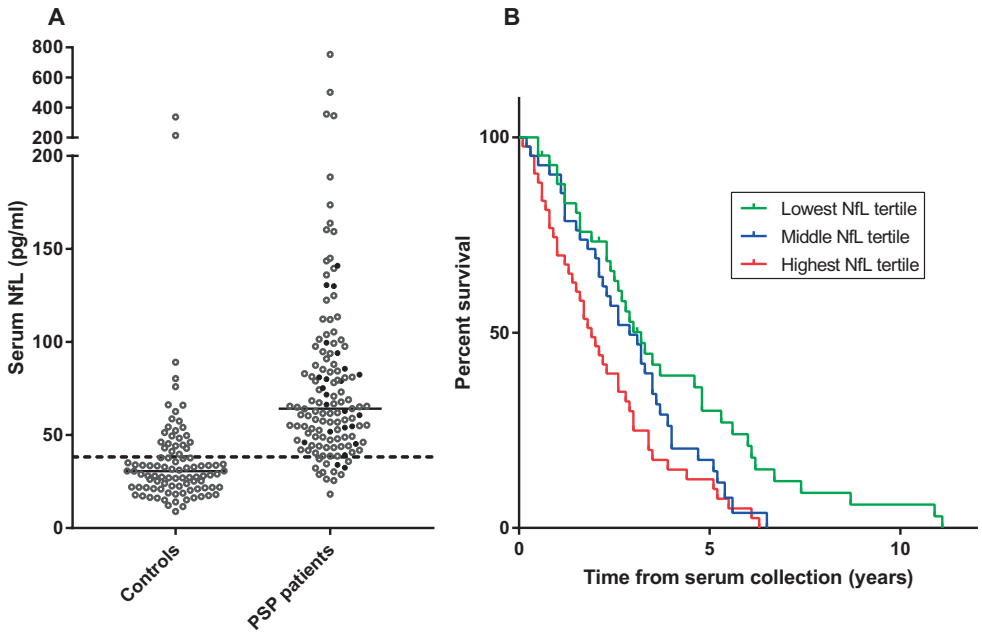

Figure 1. Serum NfL levels are elevated in PSP patients versus controls and are associated with survival.

(A) Serum NfL levels in PSP patients and controls. Horizontal solid lines represent median values. Dotted line (i.e. at 38.3 pg/ml) corresponds to the optimal cut off to discriminate PSP patients from controls (sensitivity of 90% and specificity of 63%). Filled black circles indicate PSP patients with pathological confirmation. (B) Survival curves for PSP patients with low (green line), intermediate (blue line), and high (red line) NfL levels.

and UPDRS-III (Unified Parkinson's disease rating scale III) motor scores,[8] while in another study, high baseline NfL levels were associated with a more rapid neurological, functional and neuropsychological decline.[7] In addition to this, we show that high serum NfL levels are associated with shorter survival. This prognostic value, has been demonstrated for NfL in a few other neurodegenerative diseases, but usually measured in CSF,[9–11] whereas in blood, this association has only been demonstrated in ALS and FTD patients so far.[9,13,14]

Absolute NfL concentrations differed between our cohort and previous studies, which has been observed before.[8] There are many factors which may explain this difference such as age, disease severity, sampling or storage effects, inter-laboratory variation and the application of serum versus plasma. Before the implementation of NfL in clinical practice, standardized procedures with uniform protocols across sites and the establishment of cut-off levels are necessary.

In 23 individuals (18%), the diagnosis PSP was confirmed at autopsy and these patients showed mild to strongly increased NfL levels, not different from clinically diagnosed PSP patients (Figure 1A). A few PSP patients showed extremely high NfL levels. It is possible that other comorbidities (such as a minor stroke or head trauma) may have influenced NfL lev-

els at that time in these patients. The presence of high NfL values in a few control subjects remains unresolved, as we have no additional clinical information or neuroimaging data.

The strengths of the current study are the large sample size with substantial pathological confirmation, a clinically well characterized cohort with the assessment of several rating scales, and the long period of follow-up, including information on survival. We also acknowledge some limitations. First, this study relies on cross-sectional acquired data, with many patients at an advanced disease stage. Longitudinal data would have been valuable in helping to determine whether and how NfL concentrations in PSP patients change over time. Studies in ALS patients have shown stable NfL levels over time,[13,14] while in patients with primary progressive aphasia, serum NfL increased after one year follow-up.[15] Secondly, many samples were stored at – 80°C for more than 10 years. While the effect of long storage on serum NfL levels is unknown, the NfL concentrations in CSF appeared stable under pre-analytical variations, and no negative effect was observed due to long-term storage or delayed processing.[16] In summary, we demonstrate that serum NfL is a promising, easily accessible biomarker for monitoring disease severity and survival in PSP.

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CHAPTER 3

Blood and CSF biomarkers in genetic forms of FTD

CHAPTER 3.1

Neurofilament light chain: a biomarker for genetic frontotemporal dementia

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Abstract

Objective: To evaluate cerebrospinal fluid (CSF) and serum neurofilament light chain (NfL) levels in genetic frontotemporal dementia (FTD) as a potential biomarker in the presymptomatic stage and during the conversion into the symptomatic stage. Additionally, to correlate NfL levels to clinical and neuroimaging parameters.

Methods: In this multicenter case-control study, we investigated CSF NfL in 174 subjects (48 controls, 40 presymptomatic carriers and 86 patients with microtubule-associated protein tau *(MAPT)*, progranulin *(GRN),* and chromosome 9 open reading frame 72 (*C9orf72*) mutations), and serum NfL in 118 subjects (39 controls, 44 presymptomatic carriers, 35 patients). In 55 subjects both CSF and serum was determined. In two subjects CSF was available before and after symptom onset (converters). Additionally, NfL levels were correlated with clinical parameters, survival and regional brain atrophy.

Results: CSF NfL levels in patients (median 6762 pg/ml, interquartile range 3186-9309 pg/ml) were strongly elevated compared with presymptomatic carriers (804 pg/ml, 627-1173 pg/ml, $p<0.001$), resulting in a good diagnostic performance to discriminate both groups. Serum NfL correlated highly with CSF NfL ($r_s=0.87$, $p<0.001$) and was similarly elevated in patients. Longitudinal samples in the converters showed a three- to four-fold increase in CSF NfL after disease onset. Additionally, NfL levels in patients correlated with disease severity, brain atrophy, annualized brain atrophy rate and survival.

Interpretation: NfL in both serum and CSF has the potential to serve as a biomarker for clinical disease onset and has a prognostic value in genetic FTD.

Introduction

Mutations in the microtubule-associated protein tau *(MAPT),* progranulin *(GRN)* or chromosome 9 open reading frame 72 *(C9orf72)* genes are major causes of genetic frontotemporal dementia (FTD) and are associated with considerable clinical heterogeneity.[1–5] The presymptomatic stage offers a unique window to study the earliest disease stages.[6] Changes in neuroimaging biomarkers have been found in presymptomatic FTD, similar to findings in familial Alzheimer's disease (AD) and Huntington's disease.[6–9] However, fluid biomarkers determining disease onset and progression are lacking, which are essential for forthcoming trials on disease modifying treatments. Neurofilament light chain (NfL) in cerebrospinal fluid (CSF) is elevated in FTD, and other neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), AD, and vascular dementia, and dynamically decreases in response to anti-inflammatory treatments in multiple sclerosis.[10–14] In contrast, small series of presymptomatic carriers of FTD-causing mutations have shown low CSF NfL levels.[10,15] NfL is one of the three subunits of neurofilaments, which are the major constituent of the neuroaxonal cytoskeleton and are essential for axonal growth, transport, and signalling pathways.[16,17] CSF NfL has been correlated with disease severity, disease progression, and brain atrophy in neurodegenerative diseases.[10,13,18] Blood-derived NfL levels have proven to highly correlate with CSF NfL in ALS.[18] An important question is whether NfL levels may serve as a biomarker for conversion from presymptomatic to symptomatic genetic FTD and be useful in tracking disease severity and progression. To evaluate the potential of NfL levels as a biomarker in genetic FTD, we determined CSF and serum NfL in presymptomatic carriers and patients with pathogenic mutations in *MAPT, GRN* or *C9orf72*, and correlated these levels with clinical and neuroimaging measures.

Methods

Subjects

For this study, three subject groups were included from 11 centers collaborating in the Genetic FTD Initiative (GENFI)[19]: (1) patients with FTD caused by a pathogenic mutation in *GRN, MAPT* or *C9orf72* (*n*=102); (2) presymptomatic carriers of a pathogenic mutation (*n*=63); and (3) cognitively healthy subjects without mutation (controls, *n*=73). A pathogenic *C9orf72* expansion was defined as more than 30 repeats.[5] For *GRN*, only nonsense mutations were included (Table 1), for *MAPT*, published pathogenic mutations and those predicted as pathogenic were taken into account (software package Alamut v2.6.1, Interactive Biosoftware, Rouen, France; Table 1). Participants were recruited as part of GENFI (*n*=126) or ascertained before participation in GENFI (*n*=112). Participants were either patients with a mutation, or known presymptomatic carriers, or 50% at-risk individuals (presymptomatic carriers and controls), or cognitively healthy family members without a mutation (controls). At-risk individuals are first-degree relatives of a known carrier of a pathogenic

mutation. Genotyping of all participants was performed at local sites and clinical investigators were blinded for the mutation status of at-risk individuals. At-risk individuals and control subjects underwent neuropsychological examination. Subjects were categorized as presymptomatic or symptomatic according to criteria at the time of inclusion.[20–22] At-risk individuals were followed yearly or two yearly to assess conversion into symptomatic FTD. We defined conversion as the presence of symptoms of behavioural variant FTD (bvFTD), primary progressive aphasia (PPA) or amnestic FTD as reported by informants and supported by neuropsychological assessment and neuroimaging. Disease onset in patients (*n*=102) and converters (*n*=4) was defined as the moment of first symptoms noted by a caregiver. In presymptomatic carriers, estimated time from onset was calculated as age at sample collection minus mean familial age at onset, resulting in a negative measure in carriers younger than the estimated onset derived from onset ages in their family.[6] Mini-Mental State Examination (MMSE) was used to measure global cognition,[23] disease severity was assessed by the Clinical Dementia Rating scale (CDR) including, if available, the sum of boxes (CDR-SB);[24] we only considered scale measurements within 90 days of biosample collection. In seven subjects (five CSF, one serum, one both; five *C9orf72*, two *GRN* mutations)[25] ALS-symptoms were present at sample collection; five of them met El Escorial criteria at collection, the other two 6 months after collection.[26] No ALS-patients without FTD symptoms were included. Local ethics committees at each site approved the study and all participants (or a legal representative) provided written informed consent at enrollment.

Procedures

CSF (*n*=179) was collected according to standardized local procedures. Serum samples were collected from Dutch participants only (*n*=120). Both CSF and serum collection within 1 year were available in 57 out of 61 subjects with both CSF and serum (same day *n*=37, range 0-360 days). Longitudinal CSF samples were available in five subjects, including converters; in one converter a third CSF sample was available. T1-weighted MRI-images within 6 months of CSF collection were available in 101 subjects and a follow-up scan in 22 subjects. Detailed data on available biosamples and MRI scans in the three subgroups after exclusion of outliers (see statistical analysis) are presented in Supplementary Figure 1. Grey matter volumes were determined by anatomical parcellation of the whole brain, using a multiatlas segmentation propagation approach,[27,28] with the anatomical definitions following the brainCOLOR protocol for the cortical regions and Neuromorphometrics protocol for subcortical regions and other structures.[29,30] Regions-of-interest were combined to calculate grey matter volumes of the frontal, temporal, parietal, occipital, cingulate, and insular cortices.[28] Whole-brain volumes were calculated by combining all regions from the automated brain segmentation method.[30] All volumes are presented as percentage of total intracranial volume (TIV). Atrophy rates were calculated as the percentage decrease in volume per year relative to baseline.

Laboratory methods

Measurements of NfL (in CSF and serum) were performed in one laboratory (of CET respectively JK), blinded to clinical information and mutation status. CSF NfL was measured in duplicates using the enzyme-linked immunosorbent assay (ELISA) of Uman Diagnostics (Umeå, Sweden), according to the manufacturer's instructions over four different batches. Median intra-assay coefficient of variation (CV) was 0.8% (range 0-66.5%), inter-assay variability was below 20%. Serum NfL concentrations were measured in duplicate by an earlier described, slightly modified, electrochemiluminescence immunoassay with antibodies identical to those used in the CSF ELISA (Supplementary Methods).[31,32]

Statistical analyses

Statistical analyses were performed in SPSS 21.0 for Windows (Armonk, NY, USA) and GraphPad Prism 6 (La Jolla, California, USA) applying a significance level of $p<0.05$. NfL values with an intra-assay CV of >20% ($n=1$) and outliers (values > three standard deviations from the mean: four CSF and two serum samples) were excluded. CSF and serum NfL were analyzed using non-parametric tests (Mann-Whitney *U* tests and Kruskal-Wallis with Dunn's post-hoc tests). Since the data were not normally distributed, and log-transformation did not normalize the data, square root transformed CSF and serum NfL were used to correct for age in all subjects and disease duration in patients using analysis of covariance (ANCOVA) with post-hoc Bonferroni corrections where appropriate. Spearman's correlation coefficient (r_s) was used to correlate serum with CSF NfL, NfL levels with clinical measures and CSF NfL with brain volumes, the latter also with correction for gender and study site (partial rank correlations). Diagnostic performance was assessed by areas under the curve (AUC) with 95% confidence intervals (CIs) obtained by receiver operating characteristic (ROC) analyses, with optimal cut-off levels at the highest Youden's index (sensitivity+specificity-1).[33] In analogy to the study of Lu *et al*.,[18] survival in patients was compared between NfL tertiles by Kaplan-Meier curves and Cox regressions adjusted for age and disease duration. NfL concentrations are described as medians.

Results

Demographic and clinical data

The total group of 234 subjects consisted of 101 patients (53 *GRN*, 29 *C9orf72,* 19 *MAPT*), 62 presymptomatic carriers (34 *GRN,* 14 *C9orf72,* 14 *MAPT)* and 71 controls (Table 1, Supplementary Figure 1). Patients were older than presymptomatic carriers ($p<0.001$) and controls ($p<0.001$). *GRN* and *C9orf72* patients were older than *MAPT* patients ($p=0.01$ and $p=0.04$ respectively). The age at onset in patients was highly variable ranging between 39 and 76 years and several presymptomatic carriers were past their estimated age at onset. However, 50% percent of the patients had an onset between 52 and 62 years; and the age

of both converters was close to the estimated onset age. The disease duration in *C9orf72* patients was longer than in *GRN* patients (p=0.007). The clinical presentation was bvFTD (n=60), PPA (n=17), FTD-ALS (n=7), predominant memory phenotype (n=4), mild cognitive impairment (n=4), progressive supranuclear palsy or corticobasal syndrome (n=2), and dementia not otherwise specified (n=7).

Table 1. Subject characteristics

	Controls	Presymptomatic carriers			Patients			*p*-value
Number	71	62			101			
Male gender	29 (41%)	23 (37%)			49 (49%)			0.32
Age at collection, years (IQR)	54 (43-61)	49 (42-57)			59 (56-65)			<0.0001
Age at onset, years (range)	-	55 (46-70)[a]			56 (39-76)[b]			0.84
Disease duration, years (IQR)	-	-			2.0 (1.3-3.4)			
Time to onset or estimated onset, years (IQR)	-	7.3 (2.5 – 13.2)[a]			-			
MMSE (IQR)	29 (29-30)	30 (29-30)			25 (21-28)			<0.0001
Concomitant ALS	0	0			7			0.005
Gene specific information		*GRN*	*C9orf72*	*MAPT*	*GRN*	*C9orf72*	*MAPT*	
Number per gene		34[c]	14	14[d]	53[e]	29	19[f]	
Age at collection, years (IQR)		55 (48-58)	45 (42-49)	41 (36-49)	60 (57-65)	61 (55-68)	57 (53-59)	
Age at onset, years (range)					58 (47-76)	55 (39-75)	53 (42-70)	
Disease duration, years (IQR)					1.8 (1.1-2.6)	3.0 (2.0-5.0)	2.1 (1.5-3.7)	0.008
Time to onset or estimated onset, years (IQR)		5.8 (1.6-11.0)	11.5 (5.9-14.8)	7.3 (3.3-15.8)				0.19

Values are displayed as median (IQR). In case of multiple samples in one subject, characteristics at first collection are displayed. [a]Four presymptomatic subjects converted during follow-up into symptomatic stage after collection (2 with CSF, 1 with serum and 1 with CSF and serum). [b]In 2 patients the age at onset was unknown. [c]17 Ser82fs, 8 Gln125X, 5 Gly35fs, 2 Val411fs, 2 Cys416fs. [d]8 Pro301Leu, 3 Gly272Val, 1 Arg406Trp, 1 Leu135Arg, 1 Ser320Phe; [e]16 Thr272fs, 7 Ser82fs, 4 Gly35fs, 4 IVS1+5G>C, 3 Cys366fs, 3 Tyr294X, 2 Gln125X, 1 c.708+6+9delTGAG, 1 Gln257fs, 1 Val279fs, 1 Gln341X, 1 Thr278fs, 1 Cys314X, 1 c.709-3C>G homozygous, 1 Gln130fs, 1 Cys149fs, 1 Cys157fs, 1 Cys315X, 1 Asn188fs, 1 Val200fs, 1 Pro127fs. [f]10 Pro301Leu, 2 Gly272Val, 3 Arg406Trp, 1 Leu315Arg, 1 Val337Met, 1 Val287Ile, 1 Ser305Thr.
IQR: interquartile range; ALS: amyotrophic lateral sclerosis; MMSE: mini-mental state examination.

NfL in CSF and in serum

CSF NfL levels in patients (6762 pg/ml) were more than eight times higher than in presymptomatic carriers (804 pg/ml) and controls (650 pg/ml, both p<0.001, Figure 1A), without a difference between the latter two groups (p=0.46, Supplementary Figure 2A). The eleva-

tion was confirmed after genetic stratification (Figure 1B). *GRN* patients had higher CSF NfL levels than *C9orf72* and *MAPT* patients (p<0.001 and p=0.004 respectively, Figure 1B). CSF NfL did not differ between the three presymptomatic groups (p=0.17, Supplementary Figure 2B). Correction for age in all subjects and disease duration in patients on square root transformed CSF NfL yielded similar p-values as without transformation, except for presymptomatic *C9orf72* cases versus *C9orf72* patients (before correction p<0.001, after correction p=0.04, all corrected p-values are displayed in Figure 1 and transformed data is presented in Supplementary Figure 3). NfL levels in serum showed a similar pattern as in CSF, with higher levels in patients (31.5 pg/mL) than in presymptomatic carriers (3.5 pg/mL, p<0.001) and controls (2.9 pg/mL, p<0.001, Figure 1C), without a difference between the latter two groups (Supplementary Figure 2C). Consistently, the elevation was confirmed after genetic stratification. *GRN* patients had higher serum NfL levels than *MAPT* patients *(*p=0.03, Figure 1D), both did not differ from *C9orf72* patients. Serum NfL did not differ between the three presymptomatic groups (p=0.76, Supplementary Figure 2D). Correction for age and disease duration showed similar results, except for the difference between presymptomatic carriers and patients which showed only a trend for the *MAPT* and *C9orf72* mutations (both p=0.11, Figure 1D and Supplementary Figures 3C and 3D), probably due to the small groups.

Correlation between CSF and serum NfL

CSF NfL correlated strongly with serum NfL (Figure 2A, entire group r_s=0.87, p<0.001). The correlations were strongest in carriers (patients r_s=0.77*,* p<0.001 and presymptomatic carriers r_s=0.83, p<0.001), whereas controls showed only a trend *(*r_s= 0.50, p=0.06). Sample sets collected on the same day showed slightly, although not significantly, stronger correlations (n=37, entire group r_s=0.90, presymptomatic carriers r_s=0.90, and patients r_s=0.86, all p<0.001).

Correlation with demographical and clinical characteristics

Age correlated with CSF NfL levels in presymptomatic carriers and controls (r_s=0.79, p<0.001 respectively r_s=0.58, p<0.001), but not in patients (r_s=0.13, p=0.22). In serum, a similar pattern was found (presymptomatic carriers r_s=0.46, p<0.002, controls r_s=0.70, p<0.001, patients r_s=0.23, p=0.19). Females and males showed similar NfL levels (CSF p=0.18, serum p=0.08). CSF NfL levels in patients correlated positively with CDR and CDR-SB, but not with MMSE or disease duration (Table 2, Figure 2B); serum NfL correlated positively with CDR-SB and not with MMSE or disease duration (Table 2). CSF NfL in four of the six patients with concomitant ALS fell in the highest 20% (Figure 1A). Associations between NfL concentrations and estimated onset in presymptomatic carriers are displayed in Supplementary Figure 2E and 2F.

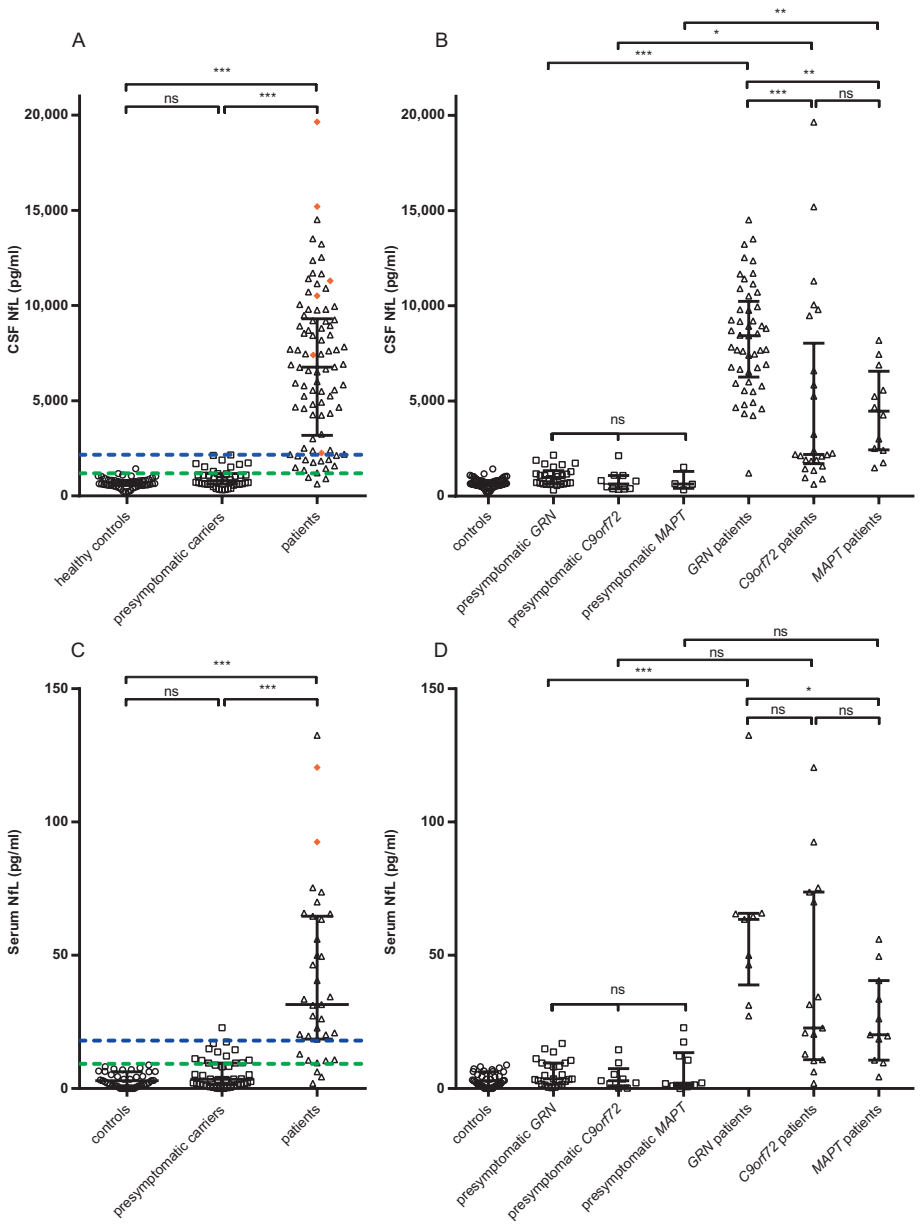

Figure 1. Neurofilament light chain (NfL) levels in presymptomatic carriers and patients.

NfL in (A) cerebrospinal fluid (CSF) and (C) serum by controls, presymptomatic carriers and patients; patients with concomitant amyotrophic lateral sclerosis are displayed as filled orange diamonds. Upper blue dashed lines represent the cut-off line to separate presymptomatic carriers from patients at 2165 pg/ml for CSF (sensitivity 84%, specificity 100%) and at 18.0 pg/ml for serum (sensitivity 77%, specificity 98%). Lower green dashed lines represent the cut-off line to separate controls from patients at 1190 pg/ml for CSF (sensitivity 97%, specificity 98%) and at 9.3 pg/ml for serum (sensitivity 91%, specificity 100%). NfL levels in (B) CSF and (D) serum specified by genetic group and clinical stage. Significances from the analysis of covariance analyses are displayed (corrected for age in all comparisons and additionally for disease duration in the comparisons between affected genes in patients). In Supplementary Figure 3, graphs of the transformed data are shown. ns: not significant; *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

Table 2. Association between neurofilament light chain levels (in cerebrospinal fluid [CSF] and serum) and clinical characteristics or scales in patients.

	CSF			Serum		
	r_s	*p*	*n*	r_s	*p*	*n*
Disease duration	-0.07	0.50	84[a]	-0.33	0.06	34[a]
MMSE	-0.19	0.14	66	-0.28	0.13	30
CDR	0.33	0.04	40	0.36	0.08	25
CDR-SB	0.60	0.001	27	0.53	0.02	19

[a]In two patients with CSF and one patient with serum, disease onset was unknown.
MMSE: Mini-Mental State Examination; CDR: Clinical Dementia Rating scale; CDR-SB: Clinical Dementia Rating scale sum of boxes.

CSF NfL levels versus brain volumes

Whole-brain volume as a percentage of TIV was lower in patients than in presymptomatic carriers ($p<0.001$) and lower in presymptomatic carriers than in controls ($p=0.04$). Cortical volumes were lower in patients than in presymptomatic carriers in all investigated areas, except for occipital (all areas $p<0.001$), without differences between controls and presymptomatic carriers.

CSF NfL in carriers negatively correlated with whole-brain volume (Figure 2C) and with frontal, temporal, parietal, insular and cingulate cortices (Table 3 and Supplementary Figure 4), indicating smaller volumes in case of higher CSF NfL. The analysis of patients only ($n=28$) yielded significant negative correlations for whole brain, frontal cortex, and insular cortex. In presymptomatic carriers, negative correlations were found for whole brain and frontal, temporal, and parietal cortices. Subgroup analyses of scans from presymptomatic and symptomatic carriers combined and a CSF-MRI interval of 90 days or less, as well as correction for gender and age showed similar patterns, albeit with lower correlation coefficients in the latter. Similar results were obtained after correction for study site. Unexpectedly, a positive correlation between NfL CSF and occipital cortex volume was found in the patient group.

In the subgroup of carriers with a follow-up scan after CSF collection (7 patients and 10 presymptomatic carriers, median time between scans 1.1 years [interquartile range 1.0-2.1]) we found significant correlations between CSF NfL and annualized rate of atrophy for whole brain, frontal, temporal, parietal, cingulate, and insular cortices (Table 3 and Figure 2D).

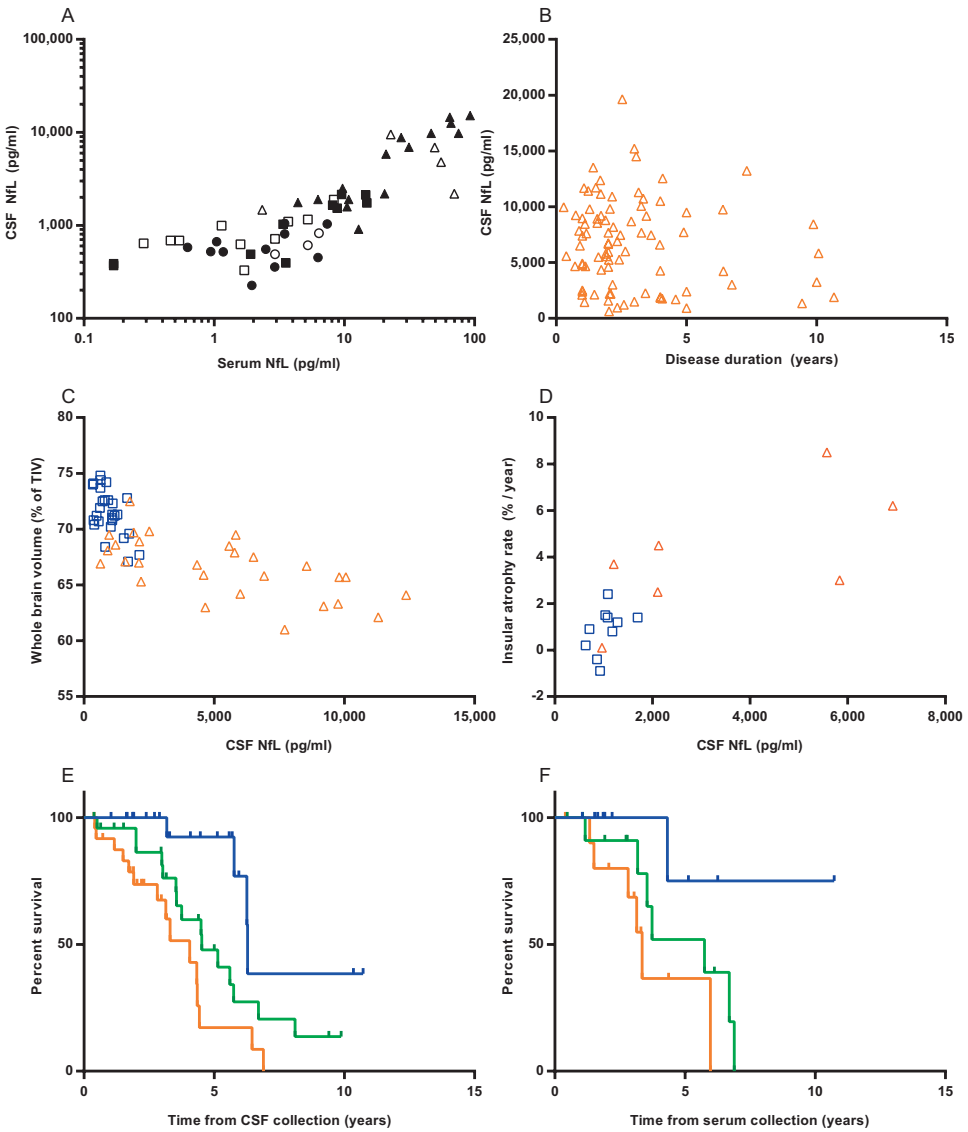

Figure 2. Correlations between neurofilament light chain (NfL) levels and clinical or imaging data.
(A) Correlation between serum and cerebrospinal fluid (CSF) NfL, circles represent controls, squares represent presymptomatic carriers and triangles represent patients; filled data points are collected on the same day; a log-scale is used for display purposes, one sample had a serum NfL of 0 pg/ml and is thus excluded from the graph, but not from the analysis. (B) Correlation of CSF NfL with disease duration in patients (orange triangles). Correlations between CSF NfL and (C) whole-brain volume and (D) insular annualized atrophy rate in presymptomatic carriers (blue squares) and patients (orange triangles). Kaplan-Meier curves of (E) all patients with CSF available and (F) all patients with serum available; NfL levels were stratified into tertiles: the blue upper lines represent the lowest tertiles, the green middle lines the middle tertiles and the orange lower lines the highest tertiles; information on survival was available in 72 out of 86 patients with CSF and all patients with serum (*n*=35).

Table 3. Correlations of cerebrospinal fluid (CSF) neurofilament light chain (NfL) with MRI volumes.

		Cross-sectional MRI			Longitudinal MRI
Region of interest		All carriers, *n*=55	Patients, *n*=28	Presymptomatic carriers, *n*=27	Annualized atrophy rate, all carriers, *n*=17
Whole-brain	r_s	-0.78	-0.66	-0.43	0.79
	p	<0.001	<0.001	0.03	<0.001
Frontal	r_s	-0.72	-0.54	-0.59	0.64
	p	<0.001	0.003	0.001	0.006
Temporal	r_s	-0.51	-0.001	-0.50	0.74
	p	<0.001	1.00	0.008	0.001
Parietal	r_s	-0.67	-0.24	-0.41	0.76
	p	<0.001	0.23	0.03	<0.001
Occipital	r_s	0.004	0.56	-0.28	0.48
	p	0.98	0.002	0.16	0.05
Cingulate	r_s	-0.43	-0.32	-0.21	0.72
	p	0.001	0.10	0.29	0.001
Insula	r_s	-0.63	-0.59	-0.24	0.83
	p	<0.001	0.001	0.23	<0.001

Correlations of CSF NfL with whole-brain and grey matter volumes at baseline are displayed in the first three columns. Correlations of CSF NfL with annualized atrophy rate from longitudinal scans are displayed in the last column. MRI: magnetic resonance imaging.

Diagnostic performance

ROC analyses on CSF NfL levels showed a high AUC to separate patients both from controls (AUC 0.99 [95% CI: 0.97-1.00]) and from presymptomatic carriers (AUC 0.97 [0.94-0.99]), with a sensitivity of 84% and specificity of 100% for a cut-off level of 2165 pg/ml (Figure 1A). Lower AUCs, although not significantly lower, were found for serum NfL (patients versus controls 0.97 [0.93-1.00], patients versus presymptomatic carriers 0.93 [0.87-0.98]). Serum NfL had a sensitivity of 77% and a specificity of 98% to separate patients from presymptomatic carriers (cut-off level of 18.0 pg/ml, Figure 1C). To separate presymptomatic carriers from controls, CSF NfL levels showed an AUC of 0.65 (0.53-0.77) with a sensitivity of 40% and a specificity of 94% for a cut-off level of 1066 pg/ml (Supplementary Figure 2A); the AUC of serum NfL for controls versus presymptomatic carriers was 0.63 (95% CI: 0.51-0.75, sensitivity 34% and specificity 97% at a cut-off level of 8.3 pg/ml, Supplementary Figure 2C).

Survival analyses

The median survival after CSF collection of deceased patients was 3.6 years (range 0.4-8.1, *n*=34), the median follow-up of alive patients was 2.8 years (range 0.4-10.7, *n*=38). High CSF NfL levels were associated with a poor survival (estimated hazard ratio (HR) of 2.21 (95% CI: 1.30-3.77), *p*=0.004, corrected for age and disease duration, Figure 2E). This association

was most prominent in *C9orf72* cases, even after correction for ALS (estimated HR 24.59, p=0.02, corrected for ALS, age, and disease duration). Dividing the cohort into two groups gave similar results; dividing into four groups gave major overlap in CIs. Cox-regressions on 'raw' CSF NfL confirmed the association with mortality: hazard ratio 1.02 for each increase in 1000 pg/ml (p<0.001). Serum NfL was also associated with survival (estimated HR on tertiles 3.10, 95% CI: 1.09-8.76, p=0.03, Figure 2F, 14 deceased and 21 alive; estimated HR on 'raw' serum NfL 1.02, p=0.02); gene-specific analyses in serum yielded no significant results.

Longitudinal samples

Longitudinal CSF samples of the two *GRN* converters, showed a three- to four-fold increase in NfL levels over conversion into the symptomatic stage (interval 3.1 and 2.0 years respectively; Supplementary Figure 5), with a 5.8-fold increase (from 9.5 to 55.3 pg/ml) in serum samples available in one converter. A decrease (-48% in one year) in CSF NfL of the third relative to the second sample was seen in the symptomatic stage of one converter. Longitudinal CSF samples of one patient and two presymptomatic carriers showed a 0.8-1.5-fold change (Supplementary Figure 5); the CVs of all described longitudinal samples were below 5%.

Discussion

The present study on a large cohort of carriers of pathogenic *GRN, MAPT* or *C9orf72* mutations showed eight-fold higher CSF NfL levels in patients than in presymptomatic carriers and controls. CSF NfL discriminated presymptomatic carriers from patients and might be useful to determine conversion. Serum NfL correlated highly with CSF NfL and showed a similar elevation in patients. Additionally, NfL levels in patients correlated with disease severity, brain atrophy, annualized brain atrophy rate, and survival. Hence, NfL in CSF or blood has the potential to serve as a biomarker for clinical disease onset and severity with a prognostic value.

The finding of elevated CSF and serum NfL levels in patients, with a good diagnostic performance to separate them from presymptomatic carriers, confirms the earlier findings in small series of presymptomatic carriers.[10,15] The strong correlation between CSF and serum NfL levels, alike in ALS,[15,18] suggests a promising role for serum NfL as a biomarker, as blood collections are more patient friendly than lumbar punctures. The trend for a lower correlation between serum and CSF NfL in controls than in mutation carriers is probably explained by the small group in combination with a suboptimal sensitivity of the serum assay in the lower range of values. Forthcoming new platforms have a higher sensitivity in this lower range; however, this will probably not influence the conclusions of the current study, as genetic FTD patients showed high serum NfL levels.

The higher NfL levels in *GRN* patients than in *MAPT* patients are supported by earlier findings of higher CSF NfL in cases with TDP-43-pathology than with tau-pathology,[34] and suggest mutation-specific underlying mechanisms. An intriguing question is whether NfL levels merely reflects the extent of neuronal cell death or white matter involvement, as has been reported in FTD-*GRN*.[35] Correlation of NfL levels with white matter damage has been found in FTD, AD, vascular dementia, and ALS.[10,36,37] On the other hand, neurofilament proteins are also integral components of synapses with an important role in receptor-specific synaptic plasticity.[17] Therefore, mutation-specific NfL elevation may reflect distinct pathophysiological mechanisms with a more white matter and/or synaptic origin of the disease process in *GRN* mutations. The wide range of NfL levels in our *C9orf72* patients correlated with the clinical phenotype, with mostly high levels in subjects with concomitant ALS and/or fast progression and low levels in patients with a slow progression. This is in line with high NfL levels in genetic ALS (half *C9orf72* carriers) and sporadic ALS, the latter correlating with a fast progression and DTI abnormalities.[15,18,37] Although DTI analyses across multiple centers are at the moment challenging to harmonize, future DTI studies combined with NfL levels in the different genetic subtypes of FTD, may elucidate the relationship with white matter integrity.

The identified correlations of NfL levels with disease severity and survival in genetic FTD patients are also in line with earlier reports in sporadic FTD, AD, and ALS.[10,11,13,18] Specifically, the association of high NfL levels with a poor survival could serve as a meaningful prognostic clinical tool. The lack of correlation between NfL levels and age in patients as opposed to the controls and presymptomatic carriers, is likely explained by the magnitude of the disease effect outweighing the effect of age.

The negative correlation between CSF NfL and brain and cortical volumes is in line with findings in a cohort of mainly sporadic FTD patients in which a negative correlation with grey and white matter volume was found.[10] This supports the hypothesis that NfL levels reflect the extent of neurodegeneration.[16] So far, the positive correlation between CSF NfL and occipital volume in our patients is difficult to explain. Perhaps gene-specific differences are underlying, since the occipital lobe is often affected in *C9orf72*,[38] which is associated with relatively low NfL levels in current study, and spared in *GRN*, showing high NfL levels; however groups were too small for gene-specific analyses. The correlation between CSF NfL and annualized rate of atrophy in the subset of carriers with two consecutive scans, supports the observed prognostic value of NfL levels in the cross-sectional analysis. Larger future studies are needed to determine whether gene-specific rates of atrophy, as found in the study by Whitwell et al., could be correlated to corresponding NfL levels.[39]

The observed three- to fourfold increase in CSF NfL levels within three years in our converters and normal levels over the entire presymptomatic phase in our large series of healthy mutation carriers, gives a first indication of the time period in which NfL increases. Although the time to onset is difficult to estimate, due to varying age at onset among families, we showed only a small increase in asymptomatic subjects approaching their

estimated onset. The elevation in NfL levels suggests a rather explosive nature of the disease process, at least for *GRN* mutations, in which a rapid breakdown of the neuroaxonal compartment takes place, instead of a more linear disease progression. Similar dynamics are suggested in ALS.[40] NfL levels in CSF and, according to our data, likely also in serum may thus serve as a biomarker for an active disease process coinciding with the onset of clinical symptoms in genetic FTD.

Major strengths of our study are the large series of presymptomatic carriers and patients with genetic FTD and the multimodal approach in correlating clinical and imaging data with a fluid biomarker. NfL determinations were performed in one laboratory which excludes an important source of variability.[41] Additionally, studying genetic FTD allows us to investigate the earliest disease processes in subjects with a known underlying pathology, which is ideal to identify biomarkers. An important weakness in our study was the interval between collection of CSF, serum, and MRI scanning. However, results were similar in the carriers with an shorter interval between CSF sampling and MRI scanning as well as similar correlations in serum and CSF samples collected on the same day. Secondly, combining subjects from multiple centers resulted in variability regarding sample collection, however NfL measurements in CSF are known to be robust to preanalytical variables.[42] Lastly, too few samples were available to draw conclusions on longitudinal dynamics and the meaning of the decrease in CSF NfL in one converter at a third time point. The relatively stable NfL levels over time in ALS might indicate that release and accumulation of NfL is counterbalanced by clearing mechanisms.[18] Additionally, in multiple sclerosis CSF NfL have shown to dynamically decrease after therapeutical interventions, which suggests a potential to serve as a pharmacodynamic biomarker in FTD as well.[14] Longitudinal NfL studies in CSF and serum in FTD are needed to determine (1) whether yearly NfL measurements are a robust biomarker for conversion; (2) changes throughout the disease process; and (3) the potential to measure pharmacodynamic response to interventions. In our opinion however, our cross-sectional results clearly discriminated presymptomatic carriers from patients, making longitudinal studies interesting, but not necessary before the application in the clinic.

In conclusion, NfL in both CSF and serum is a promising biomarker for disease onset, severity, and survival in genetic FTD. Longitudinal studies are warranted to assess dynamics over time and thereby the usefulness of NfL for clinical trials in FTD.

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Author contributions

LHM contributed to the study design, in the acquisition and interpretation of data, performed the statistical analysis and drafted the manuscript and figures. CB, JK, CET contributed to the study design, in the acquisition of data (i.e. NfL determinations), and revised the manuscript. JCvS contributed to the study design and supervision and to the interpretation of data, and drafted the manuscript. All other authors (EGD, LCJ, RS-V, CG, LB, RG, YAP, BB, DG, RL, MM, RV, IL, MO, JMP, RvM, SAR, MB, LO, VJ, DMC, SH, MJC, SO, KD, MNR, AP, ES, CF, MCT, FL, JDR) contributed to the study design, in the acquisition of data and in study coordination, and revised the manuscript.

Conflicts of interest

Dr. Cardoso reports grants from UK Medical Research Council (Centres of Excellence in Neurodegeneration grant), from Alzheimer's Research UK, Brain Research Trust, The Wolfson Foundation, during the conduct of the study. Dr. Cash reports grants from UK Medical Research Council (MR/M023664/1), Alzheimer's Research UK (ARUK-PG2014-1946), Brain Research Trust, The Wolfson Foundation, Anonymous Charity, during the conduct of the study. Dr. Teunissen reports personal fees from advisory board of Fujirebio and Roche, non-financial support from research consumables from Euroimmun, IBL, Fujirebio, Invitrogen and Mesoscale Discovery, other from performed contract research for IBL, Shire,

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Supplementary material

Supplementary methods

Serum NfL concentrations were measured in duplicate by an earlier described, slightly modified, electrochemiluminescence (ECL) immunoassay with antibodies identical to those used in the CSF ELISA.[1,2] The ECL assay was slightly modified: coating was done with 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 4°C. Non-specific binding sites were blocked with 100 µl of TBS, containing 3% milk powder, per well for 1h. We used 25 µl of TBS containing 1% milk powder, 0.1% Tween 20 and 600/300 µg/ml HeteroBlock® (Omega Biologicals, Bozeman, MT, USA) as sample diluent. Calibrators were prepared in TBS containing 1% milk powder, 0.1% Tween 20 and 300 µg/ml HeteroBlock®. Samples below the lowest standard but above the signal of the blank were extrapolated from the standard curve, otherwise assigned a concentration of 0 pg/ml.[3] Intermediate precision/repeatability were 6.1%/3.7%, respectively (sample with mean concentration 72.8 pg/ml), 8.9%/7.1% (52.3 pg/ml) and 14.9%/9.8% (9.1 pg/ml) for the ECL assay.[4] All sample CVs of duplicate measurements were below 20.0% (median 4.7%).

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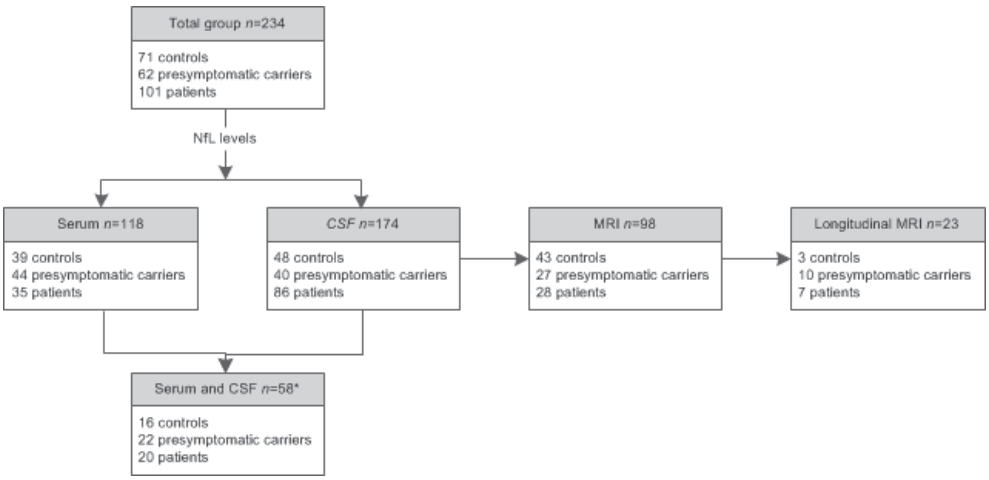
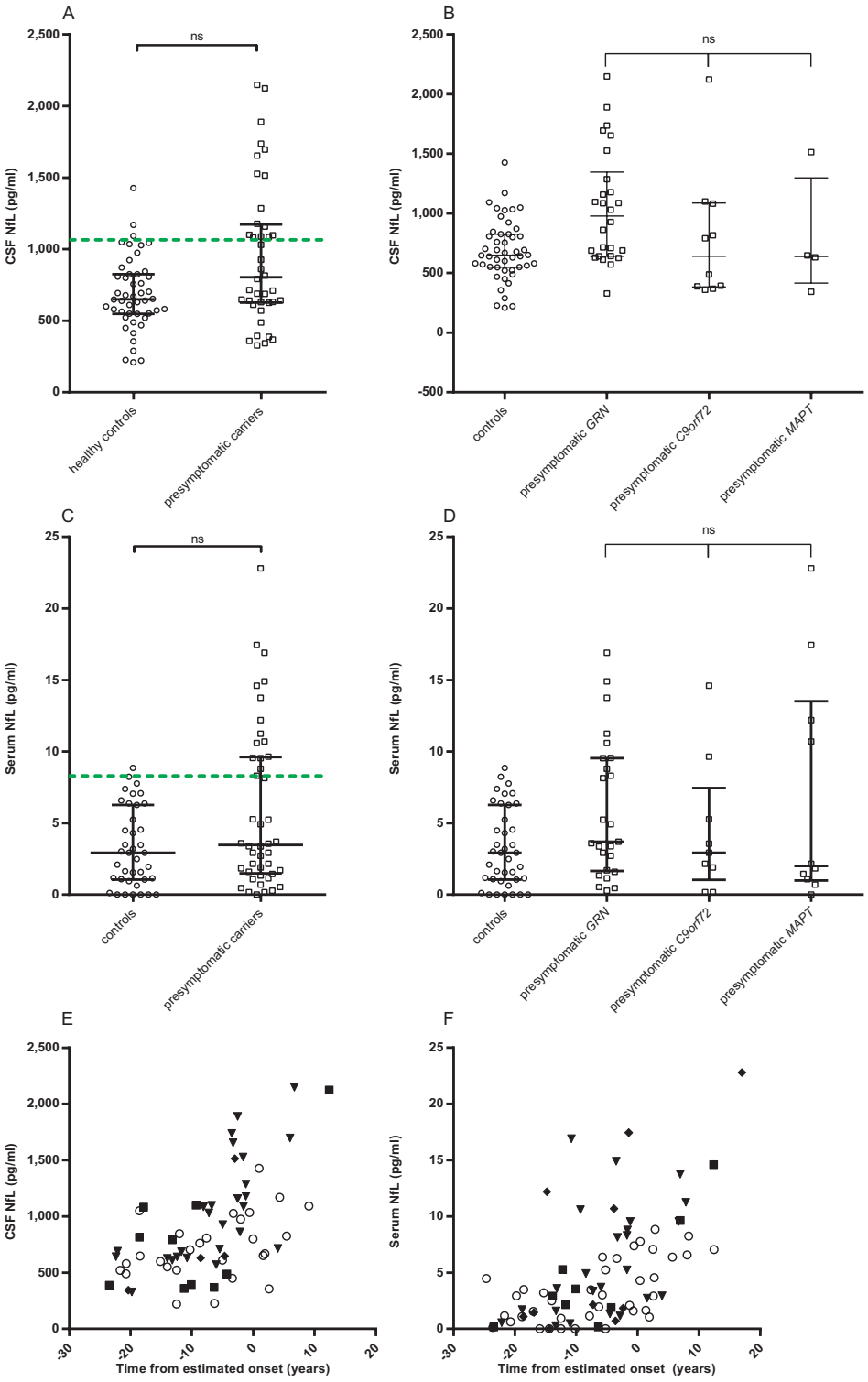
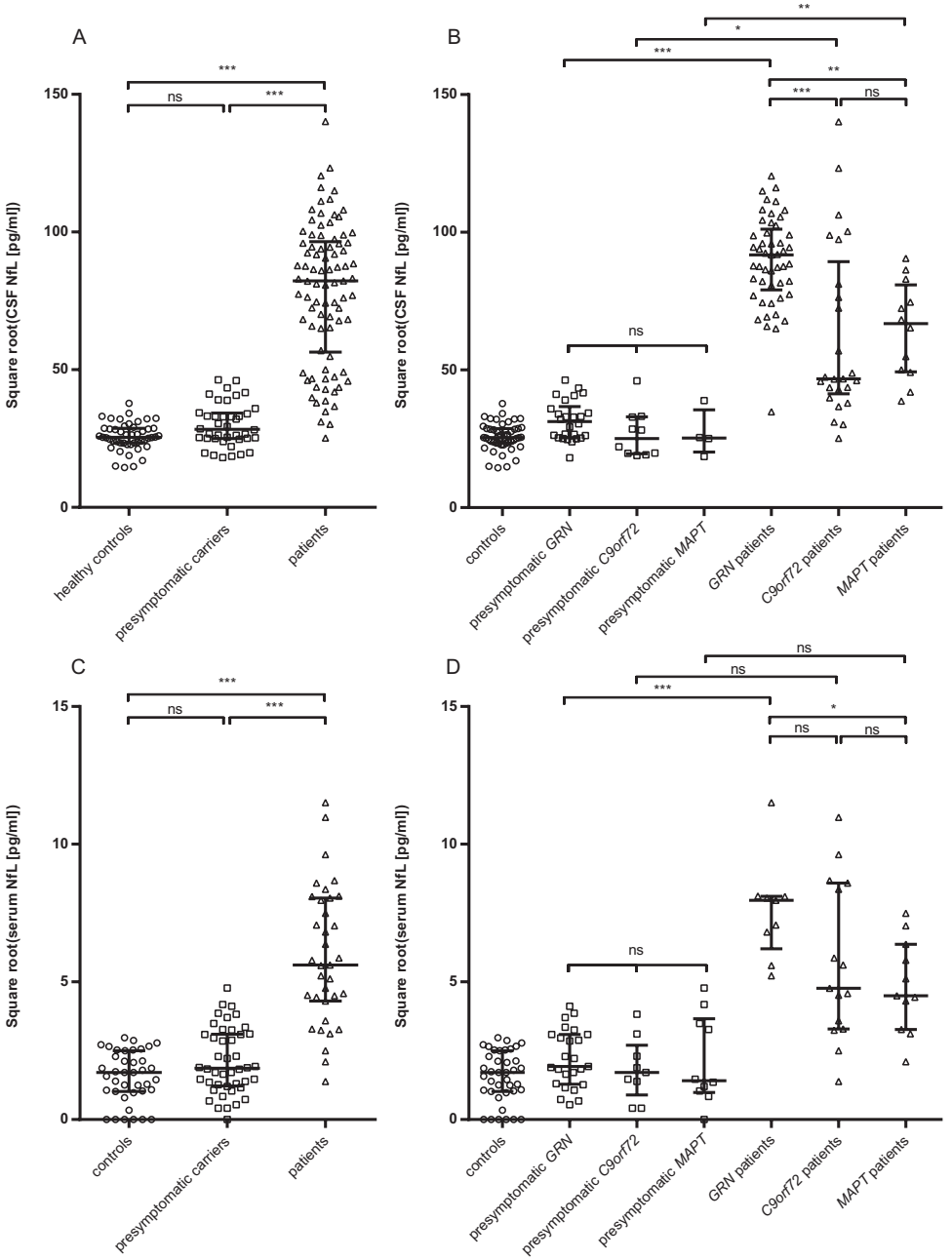
Supplementary figures



Supplementary Figure 1. Patient numbers per collected material and available MR–imaging.
Displayed numbers are after exclusion of outliers. *Three subjects were excluded from the analysis on the correlation between serum and CSF because the interval between serum and CSF collection was longer than one year (1 control, 2 presymptomatic carriers).
CSF: cerebrospinal fluid; MRI: magnetic resonance imaging.

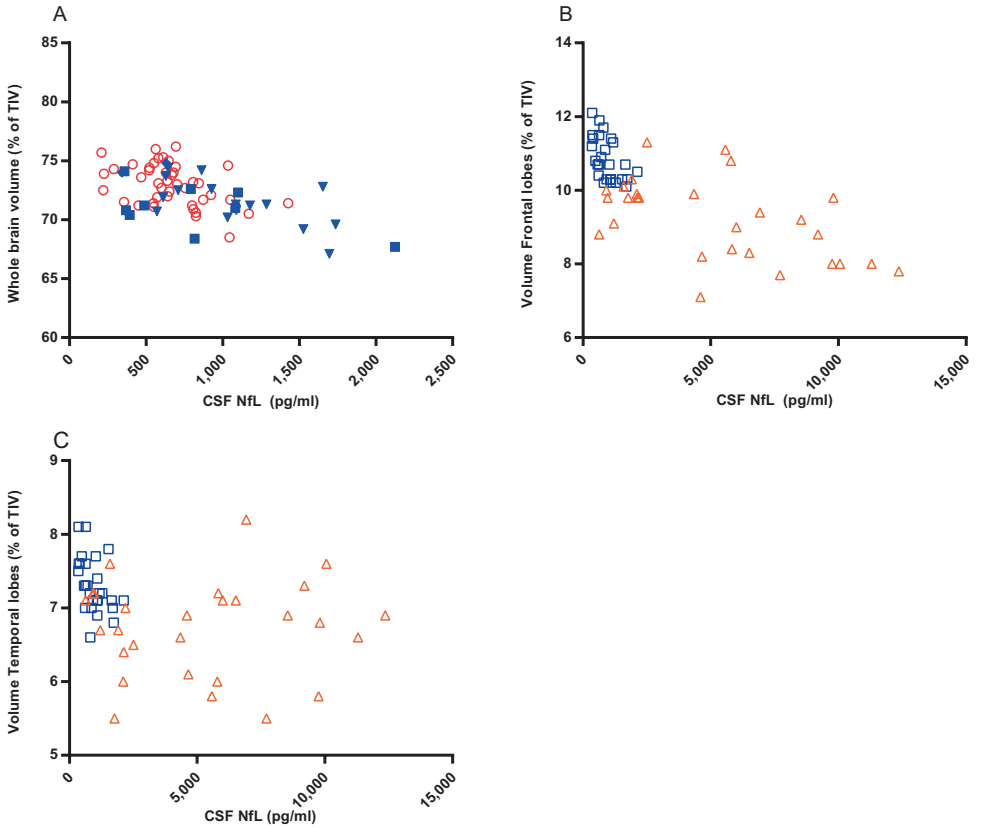

A
B
C
D
E
F
ns
CSF NfL (pg/ml)
Serum NfL (pg/ml)
healthy controls
presymptomatic carriers
controls
presymptomatic GRN
presymptomatic C9orf72
presymptomatic MAPT
Time from estimated onset (years)

Supplementary Figure 2. NfL levels in presymptomatic carriers and controls.
NfL levels in (A) CSF and (C) serum by controls and presymptomatic carriers. Green dashed lines represent the cut-off line to separate controls from presymptomatic carriers at 1066 pg/ml for CSF (sensitivity 40%, specificity 94%) and at 8.3 pg/ml for serum (sensitivity 34%, specificity 97%). NfL levels in (B) CSF and (D) serum in controls and presymptomatic carriers specified by genetic group (*GRN, C9orf72* and *MAPT*). Significances from the ANCOVA analyses are displayed (corrected for age). Association between (E) CSF NfL and (F) serum NfL and time from estimated onset in controls (red circles) and presymptomatic carriers (*GRN* filled blue triangles, *C9orf72* filled blue squares, *MAPT* filled blue diamonds). One young individual is omitted from the graphs, but not from the analyses, to prevent disclosure of the genetic status. Presymptomatic carriers with CSF NfL values (n=9) and serum NfL values (n=14) of >2SD above the mean of controls were closer to or beyond the estimated onset (CSF mean 1,1 years and serum mean 0,8 years after estimated onset) than the presymptomatic carriers below that cut-off (CSF mean 10,2 years and serum 9,1 years to estimated onset, both p<0.001). In presymptomatic carriers, both CSF and serum NfL significantly correlated with time to onset or estimated onset (CSF r_s=0.69, p<0.001 and serum r_s=0.57, p<0.001). CSF: cerebrospinal fluid; NfL: neurofilament light chain; ns: not significant.

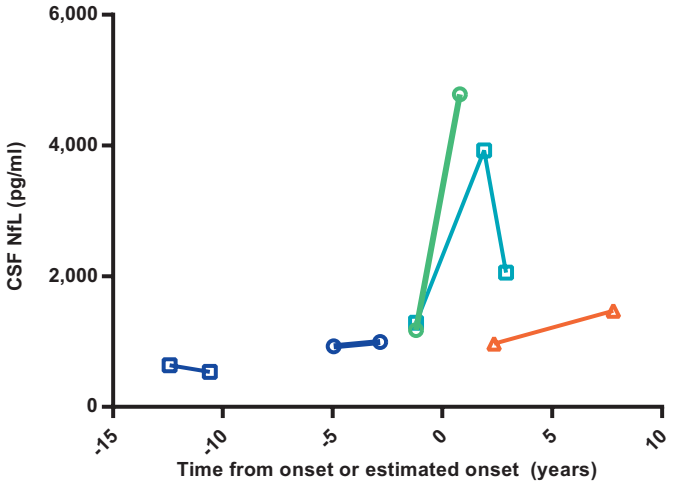


Supplementary Figure 3. Square root transformed NfL levels in presymptomatic carriers and patients.

Square root of NfL in (A) CSF and (C) serum by controls, presymptomatic carriers and patients. Additionally, square root of NfL levels in (B) CSF and (D) serum specified by genetic group and clinical stage. Significances from the ANCOVA analyses are displayed (corrected for age in all comparisons and additionally for disease duration in the comparisons between affected genes in patients). CSF: cerebrospinal fluid; NfL: neurofilament light chain; ns: not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.



Supplementary Figure 4. Correlation between CSF NfL and MR–imaging data.
(A) Correlation of whole brain volume with CSF NfL in controls (red circles) and presymptomatic carriers (*GRN* blue filled triangles, *C9orf72* blue filled squares, *MAPT* blue filled diamonds). Correlations between CSF NfL and (B) frontal lobe volume and (C) temporal lobes volume in presymptomatic carriers (blue squares) and patients (orange triangles). CSF: cerebrospinal fluid; NfL: neurofilament light chain.



Supplementary Figure 5. Longitudinal CSF NfL samples.
Longitudinal samples of two converters (green and light blue lines), two presymptomatic carriers (dark blue lines) and one patient (orange line), plotted by time from onset or estimated onset in years. CSF: cerebrospinal fluid; NfL: neurofilament light chain.

CHAPTER 3.2

Poly(GP), neurofilament and grey matter deficits in *C9orf72* expansion carriers

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**These authors contributed equally to this work.*

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Abstract

Objective: To evaluate poly(GP), a dipeptide repeat protein, and neurofilament light chain (NfL) as biomarkers in presymptomatic *C9orf72* repeat expansion carriers and patients with *C9orf72*-associated frontotemporal dementia. Additionally, to investigate the relationship of poly(GP) with indicators of neurodegeneration as measured by NfL and grey matter volume.

Methods: We measured poly(GP) and NfL levels in cerebrospinal fluid (CSF) from 25 presymptomatic *C9orf72* expansion carriers, 64 symptomatic expansion carriers with dementia, and 12 non-carriers. We explored associations with grey matter volumes using region of interest and voxel-wise analyses.

Results: Poly(GP) was present in *C9orf72* expansion carriers and absent in non-carriers (specificity 100%, sensitivity 97%). Presymptomatic carriers had lower poly(GP) levels than symptomatic carriers. NfL levels were higher in symptomatic carriers than in presymptomatic carriers and healthy non-carriers. NfL was highest in patients with concomitant motor neuron disease, and correlated with disease severity and survival. Associations between poly(GP) levels and small grey matter regions emerged but did not survive multiple comparison correction, while higher NfL levels associated with atrophy in frontotemporoparietal cortices and the thalamus.

Interpretation: This study of *C9orf72* expansion carriers reveals that: (1) poly(GP) levels discriminate presymptomatic and symptomatic expansion carriers from non-carriers, but are not associated with indicators of neurodegeneration; and (2) NfL levels associate with grey matter atrophy, disease severity and shorter survival. Together, poly(GP) and NfL show promise as complementary biomarkers for clinical trials for *C9orf72*-associated frontotemporal dementia, with poly(GP) as a potential marker for target engagement and NfL as a marker of disease activity and progression.

Introduction

Frontotemporal dementia (FTD) is a neurodegenerative disorder characterized by early progressive behavioural and/or language deficits.[1,2] Up to 15% of patients with FTD concomitantly develop motor neuron disease (MND), and 10-20% of patients with MND develop FTD,[3] suggesting that the two disorders lie on a clinical continuum. Pathogenic G_4C_2 repeat expansions in chromosome 9 open reading frame 72 (*C9orf72)* are the most common genetic cause of autosomal dominant FTD and amyotrophic lateral sclerosis (ALS).[4,5] Potential pathomechanisms include the loss of function of normal C9orf72 protein, and/or toxicity resulting from the accumulation of G_4C_2 transcripts that form RNA foci, interact with RNA-binding proteins, and impair RNA processing.[6] Expanded G_4C_2 transcripts also lead to the production of five dipeptide repeat (DPR) proteins through repeat-associated non-ATG (RAN) translation.[7,8] RAN translation of sense transcripts of the repeat produces poly(GA), poly(GR) and poly(GP), while RAN translation of antisense repeat transcripts produces poly(PA), poly(PR) and poly(GP).

Although promising drugs for *C9orf72* expansions have emerged in preclinical studies, biomarkers for evaluating their efficacy have not been adequately assessed in humans. Disease-modifying therapies advancing towards clinical trials include antisense oligonucleotides (ASOs) and small molecules that target G_4C_2 transcripts and consequently reduce G_4C_2 RNA foci and DPR proteins in *C9orf72* patient-derived cell models and animal models.[9–13] In parallel with the rapid development of these potential therapeutics, biomarkers that measure target engagement, disease onset, and disease progression must be established for clinical trials to be successful. Previous studies suggest that poly(GP) is a promising marker of target engagement. This protein is detectable in the cerebrospinal fluid (CSF) of presymptomatic and symptomatic *C9orf72* expansion carriers,[10,11,14] and poly(GP) levels in CSF from $(G_4C_2)_{66}$-expressing mice correlate with ASO-induced decreases in G_4C_2 RNA expression, RNA foci burden, and DPR protein levels within their brain.[11] In addition, neurofilament light chain (NfL) is a potential marker of disease severity and progression for ALS, FTD, as well as other neurodegenerative diseases, including Alzheimer's disease.[15–17] This marker for axonal injury is also increased in symptomatic but not presymptomatic *C9orf72* expansion carriers, and correlates with prognosis and disease severity in genetic FTD.[18,19]

Prior studies found that poly(GP) in CSF did not correlate with indicators of disease progression or neurodegeneration, yet these studies were largely conducted in patients with *C9orf72*-associated ALS.[11,14,20] In addition, no imaging data were available to assess potential relationships between CSF poly(GP) and grey matter atrophy. In the present study, we investigated the clinical correlates of poly(GP) and NfL levels, and we explored associations between these biomarkers and grey matter volume in a large cohort of presymptomatic *C9orf72* expansion carriers and patients with *C9orf72*-associated dementia.

Materials and methods

Subjects

We examined CSF from 101 subjects from *C9orf72* families, which was collected among eight sites (five sites participating in the Genetic FTD Initiative (GENFI), the University of California, San Francisco, the VU University Medical Center, and IRCCS Fatebenefratelli; Supplementary Table 1). These CSF samples were obtained from 64 patients with dementia caused by the *C9orf72* repeat expansion (symptomatic carriers), and 37 healthy first-degree family members of *C9orf72* expansion carriers. The unaffected family members consisted of 25 presymptomatic *C9orf72* expansion carriers and 12 non-carriers, and clinical investigators were blinded to mutation status. Family members were defined as unaffected if they had an absence of motor deficits, behavioural changes, and cognitive changes, as assessed by neurological examination, neuropsychological testing and structured informant interviews (e.g., with a spouse or sibling). The presence of a pathogenic *C9orf72* repeat expansion, defined as more than 30 repeats,[4] was ascertained at the local sites. Symptomatic *C9orf72* expansion carriers were diagnosed according to criteria for behavioural variant FTD (bvFTD, *n*=47; 9 with concomitant MND)[1] or primary progressive aphasia (PPA, *n*=6; 2 with concomitant MND)[2] at time of inclusion. Subjects with mild cognitive or behavioural symptoms who did not meet these diagnostic criteria for FTD were classified as having mild impairment (*n*=9), and were included in the symptomatic carrier group. Among these nine subjects with mild symptomatology, seven had behavioural symptoms and two had memory symptoms. Lastly, two patients had dementia with a predominant memory presentation, without known behavioural or motor changes. ALS patients without cognitive or behavioural symptoms were not included in the present study because the number of available subjects was too small to perform statistical analyses on this subgroup (*n*=2). Of the 101 subjects, 33 were included in our previous study on CSF NfL.[18]

Age at disease onset was defined as the age when caregivers first noted a behavioural, motor or cognitive change, and disease duration was defined as the interval between the age at disease onset and CSF collection. Mini-Mental State Examination (MMSE) was used to measure global cognition,[21] and the Clinical Dementia Rating scale (CDR) was used to assess symptom severity.[22] All cognitive test scores were collected within 90 days of CSF collection.

Local ethics committees approved the study and all participants (or their legal representative) provided written informed consent.

CSF analyses

CSF was collected according to standardized local procedures and longitudinal samples were available from 10 *C9orf72* expansion carriers who remained in the same clinical stage (presymptomatic or symptomatic) as when the baseline CSF was collected. Measurements of poly(GP) and NfL were performed blinded to clinical information. We performed each

biomarker measurement in one laboratory to eliminate variability caused by testing at multiple sites. Poly(GP) was measured at the Mayo Clinic in Jacksonville, FL, and NfL was measured at the VU University medical center. CSF poly(GP) concentrations were measured in duplicate wells using a previously described immunoassay.[11]

CSF NfL was measured in duplicate using the enzyme-linked immunosorbent assay of Uman Diagnostics (Umeå, Sweden), according to the manufacturer's instructions. Median intra-and interassay coefficient of variation were 1.5% (range 0-11%), and 6.3% (range 6.1-16.7%) respectively. Two samples had NfL levels that exceeded the upper limit of quantification of the assay (10,000 pg/ml). Since there was insufficient CSF available to measure NfL upon the dilution of these samples, they were excluded from the NfL analyses. Among the longitudinally collected samples, NfL measurements were not performed on two because of insufficient CSF volumes.

For a given clinical subgroup (non-carriers, presymptomatic carriers, and symptomatic carriers), CSF NfL and poly(GP) levels did not significantly differ among the different centers at which CSF was collected.

MRI acquisition and preprocessing

T1-weighted MRI-images (1,5 or 3 Tesla) captured within 3 months of CSF collection were included for imaging analyses (*n*=72). After excluding poor quality scans (e.g. motion artefact, *n*=6) and scans from subjects with structural abnormalities (including extensive white matter hyperintensities or lacunar infarcts, *n*=3), scans were available for 63 subjects (11 non-carriers, 24 presymptomatic carriers, and 28 symptomatic carriers) from 11 different scanners across seven sites. The NfL level was unavailable for one symptomatic carrier. MRI images were analyzed using two methods: region of interest (ROI) analysis and voxel-based morphometry (VBM). For ROI analysis, scans were parcellated into brain regions as previously described,[23] using an atlas propagation and label fusion strategy,[24] combining bilateral ROIs to calculate grey matter cortical (frontal, temporal, parietal, occipital, cingulate, insular), subcortical (hippocampus, amygdala, caudate, putamen, thalamus), and cerebellar volumes.[25,26] Whole brain volumes were calculated by combining all grey and white matter regions extracted from the automated brain segmentation method. All volumes were expressed as percentage of total intracranial volume (TIV), computed with SPM12 (Statistical Parametric Mapping, Wellcome Trust Centre for Neuroimaging, London, UK) running under Matlab R2014b (Math Works, Natick, MA, USA).[27] For the VBM preprocessing, T1 images were normalized using standard spatial normalization in SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/), modulated, corrected for non-linear warping, then segmented into grey and white matter images. Grey matter images were smoothed using a half-maximum isotropic Gaussian kernel with a size of 8mm full-width chosen due to the heterogeneity of scanners in the study.

Statistical analysis

Statistical analyses were performed in SPSS 21.0 for Windows (Armonk, NY, USA) and graphs were drafted with GraphPad Prism 7 (La Jolla, California, USA). Test statistics were considered significant at $p<0.05$ (two-tailed). Since the poly(GP) concentrations were non-normally distributed, a log-transformation was applied after adding a constant of 0.1 to all values to avoid values of zero. CSF NfL was log-transformed to normalize the data; three samples with levels >10,000 pg/ml [all had concomitant MND with either bvFTD (n=2) or nonfluent variant PPA (n=1)] continued to skew the data and were therefore set at 10,000 pg/ml prior to the transformation to allow further parametric analysis. Group comparisons of poly(GP) and NfL were first performed by Kruskal-Wallis (with Dunn's post-hoc tests) or Mann-Whitney tests on raw data, followed by ANCOVAs on log-transformed data with correction for age and gender. When longitudinal CSF samples were available, only the time-point close to MRI or the first time point was used in group comparisons. Area under the curve (AUC) with a 95% confidence interval (CI), obtained by receiver operating characteristic analyses, was used to examine diagnostic performance, with optimal cut-off levels at the highest (sensitivity+specificity-1). Correlations with age at CSF collection, age at disease onset, disease duration and cognitive scores were assessed with Spearman correlations on non-transformed data. Survival in patients was compared among tertiles of poly(GP) and NfL, by Kaplan-Meier curves and Cox regressions adjusted for age, gender, presence of MND and disease duration, with living patients included as censored data. The Cox regressions were also performed using poly(GP) or NfL as a continuous variable. Although we had a relatively small number of carriers with longitudinal samples (n=10), exploratory analyses on poly(GP) and NfL change were undertaken by calculating the annual change [(second concentration – first concentration) / interval between time-points] and testing these values against zero using a one-sample Wilcoxon signed rank test.

All imaging analyses were controlled for age at CSF collection, gender, scanner and TIV. Linear regressions were used to explore the associations between transformed poly(GP) or NfL and the ROIs. VBM analyses were conducted in SPM12 using subjects' smoothed, modulated grey matter segments. Within SPM's general linear model framework, we used one sample t-test designs in two separate analyses to correlate either log-transformed poly(GP) levels or NfL with grey matter volume among all *C9orf72* expansion carriers. We repeated these correlations between poly(GP) and NfL with grey matter in presymptomatic and symptomatic expansion carrier subgroups. Results were regarded as significant at $p<0.05$ family-wise error corrected for multiple comparisons (p_{fwe}). When associations were not significant at this threshold, a less stringent threshold of $p<0.001$ was used. Mean raw grey matter intensities were extracted from regions showing significant results at $p<0.001$ using the MARSBAR toolbox for SPM8,[28] and plotted against poly(GP) and NfL concentrations for visualization purposes.

Results

Demographic and clinical data

In total, 64 symptomatic *C9orf72* repeat expansion carriers, 25 presymptomatic expansion carriers, and 12 healthy non-carriers were included in our study (Table 1). The clinical phenotypes of the symptomatic carriers were: bvFTD (*n*=38), bvFTD-MND (*n*=9), subjects with mild impairment (*n*=9), PPA [*n*=6; four nonfluent variant PPA (two had concomitant MND), one with semantic variant PPA, and one with logopenic variant PPA], and dementia with a memory presentation (*n*=2). For presymptomatic carriers, the clinical diagnoses of affected family members included: dementia only (*n*=16), dementia and/or MND (*n*=8) or MND only (*n*=1). As expected, symptomatic carriers were older, performed worse on the MMSE, and had higher CDR sum of boxes (CDR-SB) scores compared to both non-carriers and presymptomatic carriers (Table 1). The median age at disease onset in symptomatic carriers was 56 years but varied widely (17-76 years). The median disease duration at CSF collection was 2.8 years. Twenty-four symptomatic carriers died during follow-up with a median survival of 2.1 years after CSF collection (IQR 1.0–4.2); the median follow-up interval of patients living at follow up was 2.8 years (IQR 1.2–4.5).

Table 1. Demographic, clinical and biochemical characteristics of non–carriers, presymptomatic and symptomatic C9orf72 repeat expansion carriers

	Non-carriers, *n*=12	Presymptomatic carriers, *n*=25	Symptomatic carriers, *n*=64	*p*-value
Male : female, *n*	7 : 5	8 : 17	35 : 29	0.13
Age at CSF collection, years (IQR)	44 (34-53)	47 (41-57)	60 (55-66)[a]	<0.001
Age at onset, years (range)	n/a	n/a	56 (17-76)	n/a
Disease duration, years (range)	n/a	n/a	2.8 (0.5-28.9)	n/a
Concomitant MND, *n*	n/a	n/a	11	n/a
MMSE (IQR)	30 (28-30)	29 (29-30)	25 (22-28)[b]	<0.001
CDR[c] (IQR)	0 (0-0)	0 (0-0)	1 (0.5-1)[a]	<0.001
CDR-SB[d] (IQR)	0 (0-0)	0 (0-0)	6 (3-8)[a]	<0.001
CSF Poly(GP), ng/ml (IQR)	0.00 (0.00-0.00)	0.75 (0.33-1.50)[e]	1.44 (0.49-2.51)[e]	<0.001
CSF NfL[f], pg/ml (IQR)	333 (212-536)	429 (336-830)	1885 (848-2841)[a]	<0.001
MRI available, *n*	11	24	28	n/a

Medians are displayed for continuous variables, with according IQRs unless otherwise specified.
[a]higher than non-carriers and presymptomatic *C9orf72* expansion carriers, [b]lower than in non-carriers and presymptomatic *C9orf72* expansion carriers, [c]available in 9 non-carriers, 18 presymptomatic *C9orf72* expansion carriers and 32 symptomatic carriers, [d]available in 9 non-carriers, 18 presymptomatic and 28 symptomatic *C9orf72* expansion carriers, [e]higher than in non-carriers, [f]available in 12 non-carriers, 25 presymptomatic and 62 symptomatic *C9orf72* repeat expansion carriers. CSF: cerebrospinal fluid; IQR: interquartile range; MND: motor neuron disease; MRI: magnetic resonance imaging; n/a: not applicable; NfL: neurofilament light chain.

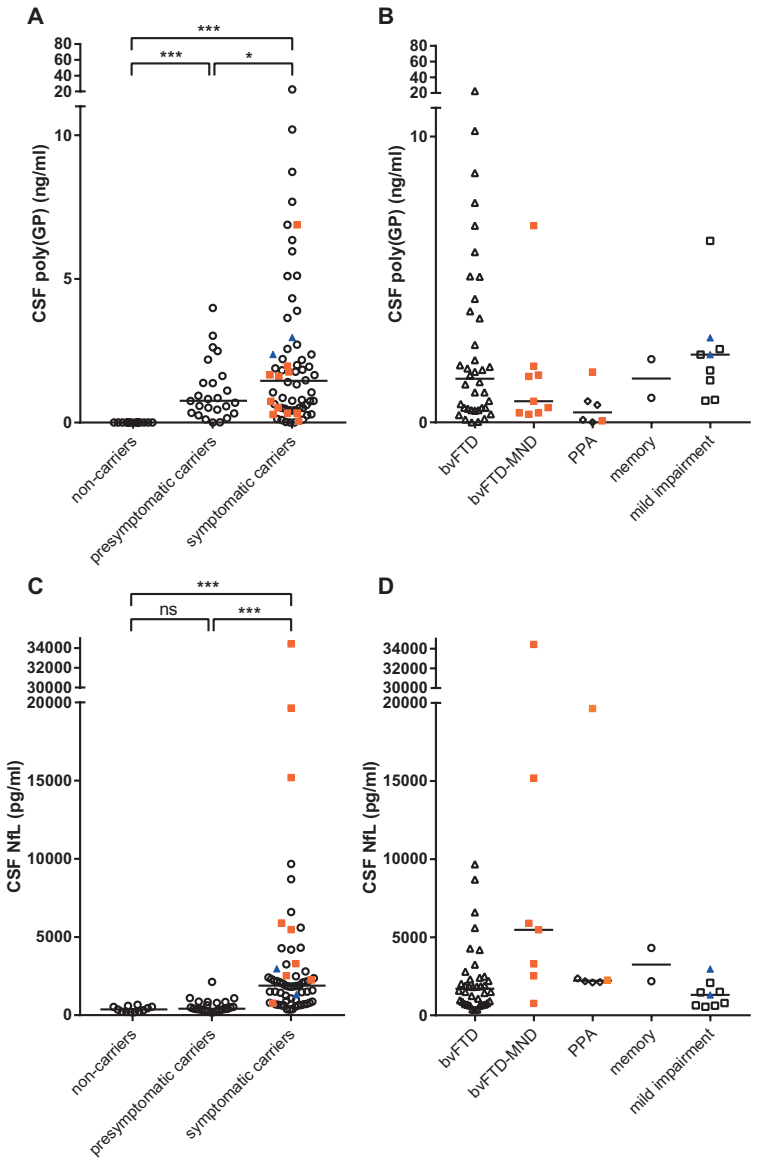

Figure 1. Poly(GP) and NfL levels by clinical stage and diagnosis. (A) Poly(GP) levels were higher in presymptomatic and symptomatic *C9orf72* expansion carriers than in healthy non-carriers, and higher in symptomatic carriers than in presymptomatic carriers after correction for age and gender. (B) Poly(GP) levels did not differ between different diagnoses. (C) NfL levels were elevated in symptomatic *C9orf72* repeat expansion carriers when compared to non-carriers and presymptomatic carriers. (D) NfL levels were highest in symptomatic carriers with concomitant MND.

Patients with concomitant MND at CSF collection are displayed as orange filled squares, those who developed MND after collection are displayed as blue filled triangles. Horizontal lines represent group medians. *P*-values from the ANCOVA analyses are displayed (corrected for age and gender) as follows: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ns: not significant.

bvFTD: behavioural variant frontotemporal dementia; CSF: cerebrospinal fluid; MND: motor neuron disease; NfL: neurofilament light chain; PPA: primary progressive aphasia.

Poly(GP) levels

C9orf72 expansion carriers had higher poly(GP) levels than non-carriers (p<0.001, Figure 1A), which discriminated carriers from non-carriers with high accuracy (AUC 0.98, p<0.001), with a specificity of 100%, and a sensitivity of 96.6% (cut-off of > 0.00 ng/ml, three expansion carriers fell below this cut-off). Poly(GP) levels trended higher in symptomatic carriers compared to presymptomatic carriers (p=0.10), and this observation became statistically significant upon correction for age and gender (post-hoc Bonferroni corrected p=0.04). Poly(GP) levels did not differ between males and females, among presymptomatic carriers grouped by their relatives' diagnoses, among clinical diagnoses of the symptomatic carriers, nor between patients with or without concomitant MND (Figure 1A and 1B). No significant associations were found between poly(GP) and age at CSF collection, age at disease onset, disease duration at time of CSF collection, MMSE, CDR, or CDR-SB. No association between poly(GP) and survival was found.

In our exploratory longitudinal analysis of 10 *C9orf72* expansion carriers, a modest but significant increase in poly(GP) was observed, and this was especially evident in the four presymptomatic carriers (p=0.03, Figure 2A). The median annual change in poly(GP) was 0.04 ng/ml, and the median time between the first and second samples was two years (range 1.0-5.4 years).

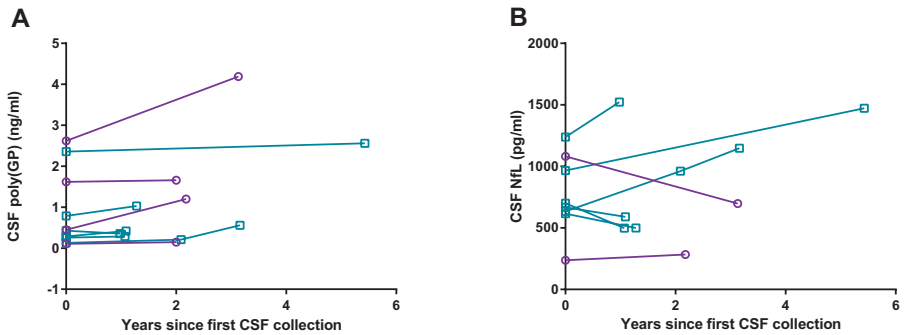

Figure 2. Longitudinal CSF poly(GP) and NfL levels. Longitudinal poly(GP) (A), but not NfL levels (B), increased significantly over time (p=0.03 and p=0.89, respectively) in presymptomatic (purple circles and connecting lines) and symptomatic (blue squares with connecting lines) *C9orf72* repeat expansion carriers. CSF: cerebrospinal fluid; NfL: neurofilament light chain.

NfL levels

CSF NfL levels were significantly higher in symptomatic carriers than in presymptomatic carriers and non-carriers (both p<0.001, Figure 1C, Table 1), and did not differ between the latter two groups. High CSF NfL levels differentiated symptomatic from presymptomatic carriers with a specificity of 96.0% and sensitivity of 65.4% (cut-off at 1169 pg/ml, AUC 0.89, p<0.001). Patients with concomitant MND at the time of CSF collection had higher NfL levels

(median 5468 pg/ml) than those without concomitant MND (median 1819 pg/ml, p=0.001, Figure 1C and 1D). NfL did not differ between males and females, but did correlate with age in the total group and in each subgroup (entire cohort r_s=0.60, p<0.001, carriers r_s=0.53, p<0.001). NfL did not correlate with age at onset or disease duration at CSF collection, but negatively correlated with MMSE, and positively correlated with CDR and CDR-SB in all carriers combined (MMSE r_s=-0.57, p<0.001, CDR r_s=0.73, p<0.001, CDR-SB r_s=0.72, p<0.001), and in symptomatic carriers after stratification into presymptomatic versus symptomatic stage (MMSE r_s=-0.42, p=0.01, CDR r_s=0.39, p=0.03, CDR-SB r_s=0.43, p=0.03). High NfL levels associated with a poorer prognosis in terms of survival [Figure 3A, hazard ratio on NfL tertiles of 4.2 (95% CI 2.0-8.6), p<0.001].

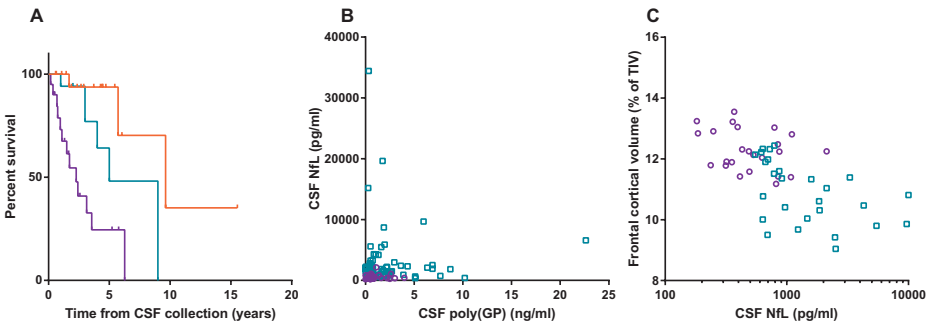

Figure 3. NfL predicts survival, does not correlate with poly(GP), and negatively correlates with frontal cortical volume.

(A) Kaplan-Meier curve representing survival of symptomatic *C9orf72* expansion carriers based on NfL levels; NfL levels were stratified into lowest (orange upper line), middle (blue middle line) and highest (purple lower line) tertiles; vertical ticks represent living patients censored at the date the patient was last known to be alive. Patients in the highest NfL tertile had the shortest survival. (B) NfL does not correlate with poly(GP) in pre-symptomatic (purple circles) or symptomatic (blue squares) *C9orf72* expansion carriers, and (C) higher NfL levels significantly associated with lower frontal cortical volumes across presymptomatic (purple circles) and symptomatic (blue squares) *C9orf72* expansion carriers combined (ROI analysis).

CSF: cerebrospinal fluid; NfL: neurofilament light chain; ROI: region of interest; TIV: total intracranial volume.

NfL did not correlate with poly(GP) in all carriers combined (p=0.33, Figure 3B), nor in presymptomatic or symptomatic carriers separately (p=0.58 and p=0.85 respectively). In an exploratory longitudinal analysis, NfL levels increased over time in some individuals but decreased in others, resulting in no significant longitudinal change at the group level (p=0.89, n=8, Figure 2B). The different trajectories were not explained by evident differences in clinical characteristics.

Table 2. Associations between poly(GP) or NfL and grey matter regions of interest in C9orf72 expansion carriers

		poly(GP)			NfL		
ROI		All carriers, *n*=52	Presymptomatic carriers, *n*=24	Symptomatic carriers, *n*=28	All carriers, *n*=51	Presymptomatic carriers, *n*=24	Symptomatic carriers, *n*=27
Whole brain	β	-0.11	-0.22	-0.09	0.19	-0.22	0.38
	p	0.52	0.35	0.73	0.41	0.54	0.21
Frontal	β	-0.25	-0.35	-0.17	-0.60	-0.28	-0.70
	p	**0.045**	0.10	0.48	**<0.001**[a]	0.38	**<0.001**[a]
Temporal	β	-0.20	-0.49	-0.02	-0.42	-0.45	-0.19
	p	0.12	**0.02**	0.92	**0.01**	0.17	0.43
Parietal	β	-0.16	-0.08	-0.06	-0.43	-0.40	-0.35
	p	0.24	0.69	0.78	**0.01**	0.17	0.12
Occipital	β	-0.19	0.07	-0.18	-0.32	0.06	-0.34
	p	0.19	0.73	0.38	0.09	0.84	0.14
Cingulate	β	-0.31	-0.05	-0.51	-0.07	0.04	-0.07
	p	**0.02**	0.81	**0.02**	0.69	0.88	0.79
Insula	β	0.01	-0.07	0.09	-0.15	-0.02	-0.13
	p	0.97	0.76	0.63	0.31	0.94	0.53
Cerebellum	β	-0.19	-0.31	-0.01	0.04	-0.36	0.32
	p	0.24	0.19	0.98	0.87	0.30	0.26
Hippocampus	β	-0.02	-0.23	-0.01	0.03	0.10	-0.05
	p	0.87	0.35	0.97	0.97	0.80	0.83
Amygdala	β	-0.23	-0.24	-0.88	-0.35	0.10	-0.32
	p	0.15	0.29	0.39	0.10	0.77	0.28
Caudate nuclei	β	-0.25	-0.35	-0.23	-0.10	-0.21	-0.27
	p	0.07	0.05	0.30	0.62	0.45	0.30
Putamen	β	-0.24	-0.12	-0.31	-0.33	-0.27	-0.41
	p	0.06	0.62	0.11	0.05	0.53	0.06
Thalamus	β	-0.08	-0.09	-0.16	-0.02	0.05	0.07
	p	0.52	0.68	0.49	0.91	0.89	0.95

Associations between poly(GP) (first three columns) and NfL (last three columns) concentrations and different brain and grey matter ROIs in *C9orf72* repeat expansion carriers, by means of linear regression corrected for age, gender and scanner. *P*-values below 0.05 are bolded.
[a]significant after correction for multiple testing (Bonferroni corrected *p*-value: $p<0.004$).
NfL: neurofilament light chain, ROI: region of interest

Imaging associations

Associations of poly(GP) with grey matter volume: Although none of the correlations between grey matter volume and poly(GP) reached significance when corrected for multiple comparisons, trends of interest were noted. Across all *C9orf72* expansion carriers

combined, higher levels of poly(GP) trended with lower frontal and cingulate grey matter volumes from the ROI analysis (Table 2); this trend remained after excluding FTD-patients with concomitant MND. The voxel-wise grey matter analysis showed poly(GP) tended to associate with regions of bilateral dorsomedial prefrontal and medial frontal cortices and lateral temporal cortex at p<0.001 (Figure 4A, Supplementary Table 2). Within these grey matter regions, bvFTD-MND and bvFTD showed the lowest grey matter intensities (Figure 4B). For the presymptomatic carriers, higher poly(GP) levels tended to associate with lower temporal ROI volume (Table 2), and lower volume in medial prefrontal cortex and scattered

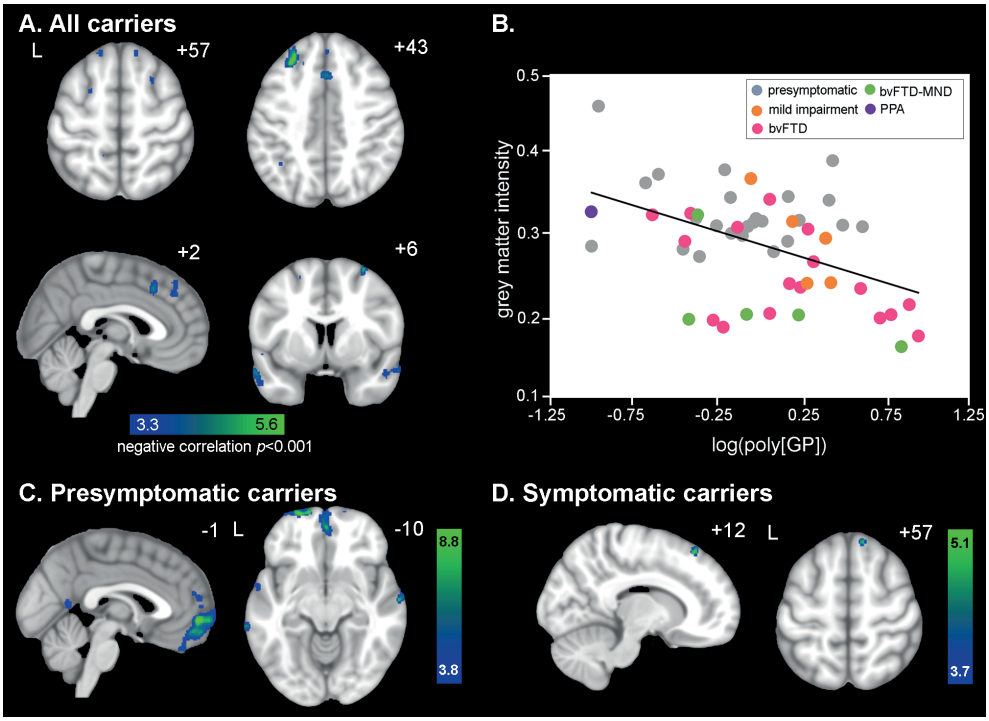

Figure 4. Voxel–wise associations of grey matter deficits with higher poly(GP) levels.
(A) Cross-sectionally, regions in bilateral dorsolateral prefrontal and medial frontal cortices, and lateral temporal cortex showed lower grey matter volume associated with higher poly(GP) levels in the voxel-wise analysis of *C9orf72* repeat expansion carriers. Significant clusters were defined at a t-threshold of p<0.001 uncorrected, no significant clusters were found at p_{fwe}<0.05. Color bars represent t-scores, and statistical maps are superimposed on the Montreal Neurological Institute template brain. The left side of the axial and coronal images corresponds to the left (L) side of the brain. (B) Mean grey matter intensity versus log-transformed poly(GP) within the p<0.001 map in (A), for 24 presymptomatic *C9orf72* expansion carriers (grey dots), 4 mild impairment (orange dots), 17 bvFTD (pink dots), 5 bvFTD-MND (green dots), and 1 PPA (purple dot) plotted for visualization purposes only. In general, FTD-MND showed the lowest grey matter intensities compared to the other diagnostic groups. (C) For presymptomatic carriers, grey matter volume was negatively correlated with poly(GP) in medial prefrontal cortex and scattered regions within lateral temporal cortices. (D) For symptomatic carriers, a small dorsomedial frontal cluster showed lower grey matter volume associated with higher poly(GP) levels. bvFTD: behavioural variant frontotemporal dementia; fwe: family-wise error correction; MND: motor neuron disease; PPA: primary progressive aphasia.

regions within lateral temporal cortices in the VBM analysis (p<0.001, Figure 4C, Supplementary Table 2). For symptomatic carriers, higher poly(GP) levels trended with lower cingulate grey matter ROI volume (Table 2), and with lower volume in a small dorsomedial frontal cluster in the VBM analysis (p<0.001, Figure 4D).

Associations of NfL levels with grey matter volume: Higher CSF NfL levels were associated with lower frontal (Figure 3C), temporal and parietal ROI grey matter volumes in all carriers combined, but the latter two ROIs did not survive multiple comparisons correction (Table 2). In the VBM analysis, higher NFL levels were associated with lower grey matter volumes in the ventral and dorsomedial prefrontal cortex, ventral and dorsal insula, anterior cingulate, caudate, medial thalamus, and several other frontotemporoparietal regions (p<0.001, Figure 5A and 5B, Supplementary Table 2). At p_{fwe}<0.05, higher NfL levels were associated with lower grey matter volume in small regions of dorsolateral prefrontal cortex, dorsal posterior insula, and the left caudate. In a subgroup analysis of presymptomatic carriers only, no significant correlations between grey matter ROIs and NfL emerged, and voxelwise, NfL levels correlated with grey matter deficits in the inferior and middle frontal gyrus, pre-and post-central gyrus, operculum, superior temporal gyrus, lateral parietal regions, and the caudate only at p<0.001 (Figure 5C, Supplementary Table 2) with no significant regions at p_{fwe}<0.05. For symptomatic carriers, NfL significantly correlated with frontal cortex in the ROI analysis, and the VBM showed associations with bilateral dorsolateral prefrontal cortex, anterior and mid cingulate cortex, dorsal insula, pre-and post-central gyrus, medial parietal regions and the caudate (p<0.001, Figure 5D), with no significant regions at p_{fwe}<0.05.

Discussion

In this study, CSF poly(GP), NfL and grey matter volumes were determined in a cohort of 89 *C9orf72* repeat expansion carriers to examine associations among these measures. Poly(GP) was detected in CSF of both presymptomatic and symptomatic *C9orf72* expansion carriers, and not detected in non-carriers. In contrast, we found high NfL levels exclusively in symptomatic carriers, while levels in presymptomatic carriers remained similar to healthy non-carriers. Higher NfL levels correlated with greater disease severity as well as shorter survival. In addition, higher NfL levels associated with lower grey matter volumes in regions known to show smaller grey matter volume in presymptomatic and symptomatic carriers, but for poly(GP), only trends were observed. As such, CSF NfL and complementary biomarkers for disease detection and future treatment monitoring.

Poly(GP) is a highly sensitive and specific biomarker for C9orf72 expansion carriers

We showed that CSF poly(GP) has high power to differentiate *C9orf72* expansion carriers and non-carriers, consistent with previous reports.[10,11,14] Interestingly, poly(GP) levels were undetectable in three *C9orf72* expansion carriers, who had various clinical presentations,

including a presymptomatic individual, a bvFTD patient, and a PPA patient. Future studies may inform whether alternative detection strategies with increased sensitivity would detect poly(GP) in these individuals, or whether their poly(GP) levels are truly negligible. Should post-mortem tissue become available for these individuals and others, it would be of particular interest to evaluate how CSF poly(GP) levels compare to the frequency of DPR protein pathology and levels of repeat-containing transcripts in the brain. As in previous studies,[11,14] we found that poly(GP) was detectable in CSF from presymptomatic *C9orf72* expansion carriers, suggesting that DPR protein production emerges prior to neurodegeneration and that poly(GP) can be actively released from putatively healthy neurons. This notion is supported by *in vitro* experiments that show that DPR proteins are secreted from

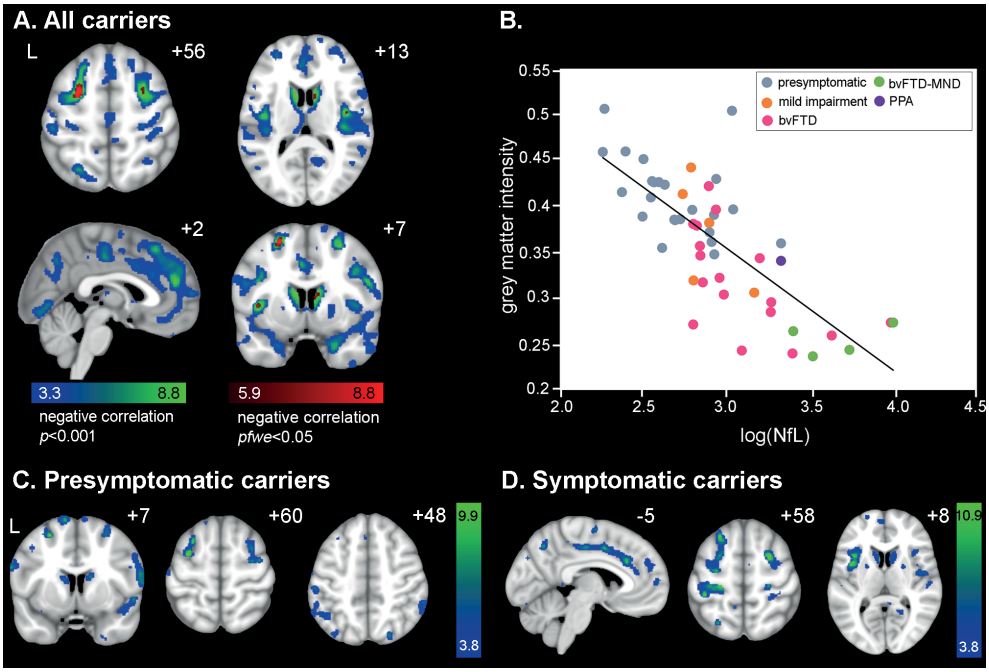

Figure 5. Voxel-wise associations of grey matter deficits with higher NfL levels. (A) Widespread regions including ventral and dorsomedial prefrontal cortex, ventral and dorsal insula, anterior cingulate, caudate, and medial thalamus showed lower grey matter volume (voxel-wise analysis) associated with higher NfL levels. Significant clusters were defined at a t-threshold of $p<0.001$ uncorrected (blue-green colored) and $p_{fwe}<0.05$ (red colored). Color bars represent t-scores, and statistical maps are superimposed on the Montreal Neurological Institute template brain. The left side of the axial and coronal images corresponds to the left (L) side of the brain. (B) Mean grey matter intensity versus log-transformed NfL levels within the $p<0.001$ map in (A), for 24 presymptomatic *C9orf72* expansion carriers (grey dots), 4 mild impairment (orange dots), 17 bvFTD (pink dots), 4 bvFTD-MND (green dots), and 1 PPA (purple dot) plotted for visualization purposes only. Both presymptomatic (C) and symptomatic carriers (D) show associations of NfL with grey matter atrophy in bilateral dorsolateral prefrontal cortex, parietal regions and caudate. Symptomatic carriers (D) additionally show grey matter atrophy associated with NfL in key hubs targeted in bvFTD, including anterior and mid cingulate cortex and insula.
bvFTD: behavioural variant frontotemporal dementia; fwe: family-wise error correction; MND: motor neuron disease; NfL: neurofilament light chain; PPA: primary progressive aphasia.

cultured cells.[11,29] Reports of autopsy studies in *C9orf72*-patients have also described widespread DPR protein pathology prior to the formation of TDP-43 inclusions and neuronal loss.[30–32] These studies provide converging evidence that poly(GP) expression arises early in the lifespan of *C9orf72* expansion carriers.

Poly(GP) levels are higher in symptomatic C9orf72 expansion carriers compared to presymptomatic carriers

Symptomatic carriers had higher poly(GP) levels compared to presymptomatic carriers after correcting for age and gender. In parallel, we found a modest increase of CSF poly(GP) over time in a small subset of 10 *C9orf72* expansion carriers, most frequently in the presymptomatic subjects. In contrast, other studies had not identified a significant difference in poly(GP) levels between presymptomatic and symptomatic carriers,[11,14] nor an increase in poly(GP) levels over time.[11] The time interval between repeated CSF collections was longer in the present exploratory analysis, suggesting that changes in poly(GP) might only emerge over longer periods. Measuring poly(GP) in larger cohorts over extended time periods as individuals convert from the presymptomatic to symptomatic phase will further help elucidate the temporal trajectory of poly(GP), and whether levels change relative to symptom onset.

We found that poly(GP) levels did not differ among clinical phenotypes, and did not correlate with age at disease onset or survival. Although there is a possibility of a type II error (i.e., a false-negative association) given that several clinical subgroups had a small sample size, these data are in line with the lack of associations between clinical phenotypes and other *C9orf72*-associated features, such as repeat size and RNA foci burden.[33,34] While we were unable to examine correlations between CSF poly(GP) and repeat length in blood because repeat length data were not available, such an analysis would likely be complicated given the substantial variation of repeat sizes among various tissues from the same individual.[33] Furthermore, a previous study has shown no association between CSF poly(GP) and repeat length in blood,[14] and we observed no association between poly(GP) levels in the cerebellum or frontal cortex and repeat length in these regions.[35] Nonetheless, examining associations between antemortem CSF poly(GP) and repeat size, poly(GP) levels, and levels of other DPR proteins in various neuroanatomical regions will be of interest when postmortem tissue becomes available from a suitable number of cases. Also, as arginine-containing DPR proteins, poly(GR) and poly(PR), are considered to be highly toxic,[6] it is possible that elevated levels of these DPR proteins may correlate with clinical features and measures of neurodegeneration. The development of immunoassays quantifying these proteins remains technically challenging, but may lead to more insights into *C9orf72* disease mechanisms.

NfL is normal in presymptomatic C9orf72 expansion carriers and is elevated in symptomatic carriers

Determining symptom onset in *C9orf72* repeat expansion carriers is notoriously challenging. Presymptomatic carriers have a high incidence of psychiatric symptoms overlapping with bvFTD symptomatology,[36] and some carriers have a mild, slowly progressive prodromal phase spanning several decades.[37] Consequently, surrogate endpoints reflecting neurodegeneration are critical for assessing disease onset and the efficacy of therapeutic interventions. Our data show that NfL is elevated during the symptomatic phase of *C9orf72*-associated dementia and correlate with indicators of disease severity (i.e., MMSE, CDR, and CDR-SB), survival and grey matter atrophy, consistent with other studies of NfL in sporadic and genetic FTD.[18,38,39] In mouse models, NfL also correlates with disease severity, specifically with the burden of α-synuclein, tau, or β-amyloid inclusions, and NfL levels are attenuated with treatment.[40] Thus, NfL could be utilized in clinical trials to stratify patients into more homogeneous groups with respect to disease severity and to assess the neuroprotective effect of therapeutic interventions. Furthermore, the strong association between CSF NfL and survival supports the use of NfL as prognostic marker. NfL can now be reliably measured in serum and plasma, which is collected less invasively than CSF, making it promising for clinical use, especially when frequent measures are needed.[17,18,41]

Our small longitudinal study of CSF NfL levels did not show consistent changes over the time intervals assessed. Previous studies on ALS patients reported stable NfL levels, but showed an increase over time for a subset of patients with a rapid disease progression.[17,42,43] Serum NfL levels have also been shown to progressively increase in sporadic PPA patients.[44] The discrepancy between our and previous findings could be attributable to the small size of our longitudinal cohort, and the range of clinical phenotypes and disease durations among individuals at the time of sample collection. Given that the rate of neurodegeneration can differ not only throughout the course of disease but also among patients, additional longitudinal studies on larger cohorts of *C9orf72* expansion carriers are needed to fully understand the temporal trajectory of NfL in relation to clinical changes.

Relationships between poly(GP) and indicators of neurodegeneration

The present study did not show a significant relationship between poly(GP) levels and indicators of neurodegeneration, as reflected by NfL levels and grey matter volumes. Similarly, previous studies show no correlation between poly(GP) and NfL,[14] nor neurofilament heavy chain (a different neurofilament subunit) in *C9orf72*-associated ALS.[20] Because previous studies have shown lower grey matter volumes in both presymptomatic and symptomatic carriers compared to controls,[23,36,45,46] we had hypothesized that higher levels of poly(GP) might be associated with lower grey matter volumes in *C9orf72*-targeted regions. We found a trend toward higher poly(GP) with lower frontal and cingulate volume in our ROI analysis. In parallel with the ROI analysis, the voxel-wise analysis showed that certain sparse regions of lower grey matter volumes tended to associate with higher poly(GP), which included

regions in the bilateral dorsolateral prefrontal, medial frontal, and lateral temporal cortices. Although higher poly(GP) levels showed a relatively weak association with lower grey matter volumes in our analyses, the regions identified include those atrophied in *C9orf72*-associated FTD patients,[45,47–49] and show reduced volume in presymptomatic *C9orf72* expansion carriers.[23,36]

Interestingly, our subgroup analysis of presymptomatic carriers showed a trend for association between higher poly(GP) and reduced bilateral medial orbitofrontal cortex, but symptomatic carriers showed only sparse regions that trended towards an association with high poly(GP). One potential explanation for this result is that DPR accumulation may arise focally during the presymptomatic phase and become widespread during the symptomatic phase, thus attenuating any potential relationship between grey matter atrophy and poly(GP) during the symptomatic phase. Overall, our results suggest that higher poly(GP) levels may be associated with some key foci in *C9orf72*-associated regions, particularly for presymptomatic carriers, but poly(GP) levels are not a marker for neurodegeneration *per se*.

Higher NfL levels are associated with grey matter deficits

Elevated NfL levels were associated with lower grey matter volume based on both types of analysis. These data reveal vulnerable neuroanatomical structures during the presymptomatic and symptomatic stages, which include ventral and dorsomedial prefrontal cortex, ventral and dorsal insula, anterior cingulate, caudate, and the medial thalamus. These regions are highly anatomically congruent with atrophy patterns found in *C9orf72*-associated frontotemporal dementia.[45,47–50] Notably, several studies confirmed that the medial thalamus is a region affected across *C9orf72* expansion carriers,[50] even during the presymptomatic phase.[23,36,46] Interestingly, our subgroup analysis of presymptomatic carriers showed that higher NfL levels were associated with smaller bilateral frontoparietal and caudate volumes, which may indicate that these regions are among the earliest regions of neurodegeneration, but longitudinal presymptomatic studies are needed to test this hypothesis.

Conclusion

The strength of the present study is the use of a multimodal approach combining two CSF biomarkers with quantitative structural imaging metrics to investigate the relationship between poly(GP) and indicators of neurodegeneration in a large cohort of presymptomatic and symptomatic *C9orf72* expansion carriers. To the best of our knowledge, this is the largest study thus far on poly(GP) in *C9orf72*-associated FTD, and the first in examining associations between DPR protein levels and grey matter volume. We used two neuroimaging methods: ROI analyses, the metrics of which are reliable and can be readily analyzed by clinical groups, and voxel-wise analyses which can refine grey matter deficits in a more granular fashion. We show that poly(GP) and NfL are promising complementary biomark-

ers that capture the effects of the *C9orf72* repeat expansion during different phases, and demonstrate different relationships with grey matter volume. While poly(GP) may display less utility as a prognostic or staging biomarker, it shows great promise as a pharmacodynamic biomarker for therapeutic approaches that target G_4C_2 RNA in preclinical models.[11] Importantly, poly(GP) levels are detectable in presymptomatic carriers and would thus make the inclusion of this population in clinical trials more feasible. Because NfL reflects neurodegeneration and is associated with grey matter atrophy, it will be most useful for monitoring disease severity, and predicting disease progression and survival.

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Author contributions

L.H.M., T.F.G. and S.E.L. contributed to the study design, in the acquisition, analysis and interpretation of data, drafted the manuscript and figures. A.C.S., L.C.J., S.R., contributed to data acquisition and analysis, and drafting of manuscript and figures. A.L.B., L.P., J.D.R., and J.C.v.S. contributed to the study design, in the acquisition and interpretation of data, and revised the manuscript. All other authors (L.D.K., J.M.P., J.L.P., E.L.v.E., E.G.D., S.F., C.G., H.J.R., R.S.V., D.G., Y.A.P., L.B., R.G., B.B., R.J.L., M.D.C., C.E.T., R.v.M., J.C.R., G.C., D.H.G., R.R., A.M.K., L.O., E.S., G.B., A.P., D.M.C., K.M.D., M.B., B.L.M.) contributed to the acquisition of data and/or in study coordination, and revised the manuscript.

Potential conflicts of interest

T.F.G. and L.P. have a US patent on methods and materials for detecting *C9orf72*-associated ALS and FTD using poly(GP) proteins (European patent filed). All other authors report no conflicts of interest relevant to this work.

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Supplementary material

Supplementary Table 1. Number of subjects per site.

Site	Non-carriers	Presymptomatic carriers	Symptomatic carriers
Karolinska Institutet, Stockholm	3	6	19
University of California, San Francisco	0	6	20
Erasmus Medical Center, Rotterdam	7	9	7
Hospital Clinic Barcelona	2	3	5
University of Milan	0	0	7
VU University Medical Center, Amsterdam	0	0	4
IRCCS Fatebenefratelli, Brescia	0	0	2
Université Laval	0	1	0

Supplementary Table 2. Negative correlations between grey matter volume and poly(GP) or NfL for all, presymptomatic and symptomatic C9orf72 repeat expansion carriers – peak voxel regions from the voxel–based morphometry analysis.

Region Containing peak voxel	BA	x,y,z	T	# of voxels	At p<0.001	At p_{fwe}<0.05
Grey matter versus poly(GP) in all *C9orf72* expansion carriers						
L Middle Frontal Gyrus	9	-28,33,46	5.62	417	X	
R Middle Temporal Gyrus	21	66,0,-15	4.64	349	X	
R Superior Frontal Gyrus	8	32,8,64	4.27	118	X	
R Inferior Occipital Gyrus	17	26,-100,-4	4.26	152	X	
R Superior Parietal Gyrus	5	20,-50,68	4.25	34	X	
L Middle Temporal Gyrus	21	-57,-3,-18	4.18	205	X	
R Superior Frontal Gyrus	6	20,-4,69	4.15	99	X	
R Mid Cingulate Cortex	32	2,21,42	4.02	187	X	
R Superior Frontal Gyrus, medial	9	3,39,38	3.97	88	X	
L Superior Frontal Gyrus	8	-15,33,58	3.93	23	X	
L Precuneus	n/a	-12,-44,51	3.92	64	X	
L Superior Temporal Gyrus	22	-63,-9,6	3.88	38	X	
L Middle Temporal Gyrus	21	-66,-24,-6	3.85	44	X	
R Superior Frontal Gyrus, medial	10	6,75,3	3.73	23	X	
R Superior Frontal Gyrus	8	14,33,58	3.69	24	X	
L Superior Frontal Gyrus, medial orbital	10	-2,66,-2	3.59	21	X	
L Superior Frontal Gyrus	6	-21,4,57	3.57	21	X	
Grey matter versus poly(GP) in presymptomatic *C9orf72* expansion carriers						
L Middle Frontal Gyrus, orbital	11	-21,64,-12	8.76	372	X	
L Superior Frontal Gyrus. orbital	10	-2,56,-4	7.91	1142	X	
R Middle Temporal Gyrus	21	64,-10,-12	6.27	120	X	

Supplementary Table 2. Negative correlations between grey matter volume and poly(GP) or NfL for all, presymptomatic and symptomatic C9orf72 repeat expansion carriers – peak voxel regions from the voxel–based morphometry analysis. (continued)

Region Containing peak voxel	BA	x,y,z	T	# of voxels	At p<0.001	At p_{fwe}<0.05
L Middle Temporal Gyrus	21	-69,-34,-14	5.91	133	X	
R Thalamus	n/a	12,-4,15	5.45	23	X	
L Middle Temporal Gyrus	21	-64,-9,-18	5.38	36	X	
R Middle Temporal Pole	38	57,16,-26	5.30	106	X	
L Mid Cingulate Cortex	n/a	-16,-34,45	5.27	109	X	
R Precentral Gyrus	4	46,-9,60	5.16	36	X	
R Superior Frontal Gyrus, medial	10	3,60,10	5.15	153	X	
L Middle Frontal Gyrus	9	-34,32,45	4.98	101	X	
R Superior Frontal Gyus, orbital	11	16,64,-14	4.96	75	X	
L Calcarine Fissure and surrounding cortex	17	-4,-57,8	4.85	92	X	
L Precentral Gyrus	6	-52,10,46	4.77	56	X	
L Superior Temporal Gyrus	21	-57,2,-12	4.61	63	X	
L Middle Temporal Gyrus	21	-58,4,-33	4.52	48	X	
L Middle Frontal Gyrus	10	-32,64,9	4.31	36	X	
R Middle Temporal Pole	21	57,3,-16	4.24	36	X	
R Middle Frontal Gyrus, orbital	10	34,62,-2	4.06	32	X	
Grey matter versus poly(GP) in symptomatic *C9orf72* expansion carriers						
R Superior Frontal Gyrus, medial	8	12,33,56	5.11	47	X	
Grey matter versus NfL in all *C9orf72* expansion carriers						
L Middle Frontal Gyrus	6	-24,2,57	8.84	1603	X	X
R Insula	48	44,-6,10	6.83	36960	X	X
R Inferior Frontal Gyrus	48	45,14,26	6.81	77	X	X
R Caudate Nucleus	n/a	9,10,12	6.16	1167	X	X
R Middle Frontal Gyrus	8	28,4,56	6.01	2491	X	X
L Caudate Nucleus	25	-9,10,12	5.82	1177	X	
R Anterior Cingulate and Paracingulate Gyri	32	2,46,21	5.78	7937	X	
R Middle Frontal Gyrus, orbital	47	34,39,-12	5.43	542	X	
R Calcarine Fissure and surrounding cortex	17	21,-56,8	5.38	1220	X	
R Precuneus	7	14,-64,63	5.04	515	X	
L Middle Frontal Gyrus	46	-36,33,38	4.98	690	X	
L Middle Frontal Gyrus	46	-42,48,8	4.92	1259	X	
L Lingual Gyrus	18	-10,-58,3	4.81	835	X	
L Frontal Middle Gyrus, orbital	11	-26,34,-14	4.60	402	X	
L Supplementary Motor Area	6	-3,-6,66	4.57	269	X	
R Lingual Gyrus	17	2,-72,-2	4.54	639	X	
R Superior Frontal Gyrus, dorsolateral	11	16,66,20	4.43	197	X	

Supplementary Table 2. Negative correlations between grey matter volume and poly(GP) or NfL for all, presymptomatic and symptomatic C9orf72 repeat expansion carriers – peak voxel regions from the voxel–based morphometry analysis. (continued)

Region Containing peak voxel	BA	x,y,z	T	# of voxels	At p<0.001	At p_{fwe}<0.05
R Inferior Frontal Gyrus, orbital	47	46,48,-8	4.34	608	X	
L Fusiform Gyrus	30	-15,-32,-15	4.30	124	X	
L Hippocampus	37	-34,-33,-8	4.25	101	X	
L Inferior Occipital Gyrus	19	-50,-72,-8	4.24	89	X	
R Inferior Temporal Gyrus	20	39,6,-45	4.15	88	X	
R Lenticular Nucleus, putamen	11	20,12,-2	4.14	220	X	
R Hippocampus	37	30,-36,0	4.11	64	X	
L Supramarginal Gyrus	42	-58,-45,26	4.09	47	X	
L Middle Temporal Gyrus	37	-48,-64,12	3.99	43	X	
L Superior Frontal Gyrus, medial orbital	11	-6,68,-6	3.97	87	X	
L Middle Temporal Gyrus	21	-54,-58,21	3.95	45	X	
L Superior Parietal Gyrus	1	-24,-45,70	3.94	268	X	
R Temporal Pole, Middle Temporal Gyrus	20	39,15,-40	3.92	64	X	
R Superior Frontal Gyrus, dorsolateral	6	21,-6,70	3.84	58	X	
L Postcentral Gyrus	4	-38,-33,68	3.82	58	X	
L Fusiform Gyrus	20	-36,-18,-33	3.80	39	X	
R Inferior Temporal Gyrus	37	50,-54,-22	3.72	35	X	
R Supplementary Motor Area	6	16,6,69	3.69	37	X	
L Caudate Nucleus	25	0,9,3	3.68	26	X	
L Lenticular Nucleus, putamen	n/a	-21,4,6	3.65	44	X	
L Calcarine Fissure and surrounding cortex	27	-10,-46,58	3.61	25	X	
R Middle Frontal Gyrus, orbital	11	22,64,-12	3.60	35	X	
R Gyrus Rectus	11	6,42,-28	3.54	74	X	
R Precuneus	5	4,-38,56	3.54	34	X	
L Inferior Frontal Gyrus, triangular part	45	-56,38,2	3.46	54	X	
Grey matter versus NfL in presymptomatic *C9orf72* expansion carriers						
L Inferior Frontal Gyrus, triangular part	45	-57,27,10	9.94	244	X	X
R Inferior Frontal Gyrus	44	58,12,24	8.11	1524	X	
L Lenticular Nucleus, putamen	48	-30,0,60	8.04	488	X	
R Superior Temporal Gyrus	21	68,-27,10	7.75	282	X	
L Supplementary Motor Area	6	-10,3,70	6.97	406	X	
L Postcentral Gyrus	43	-63,-10,21	6.86	646	X	
L Middle Temporal Gyrus	22	-64,-12,-3	6.65	76	X	
L Angular Gyrus	39	-48,-57,42	6.44	572	X	
R Caudate Nucleus	n/a	15,14,14	6.38	407	X	
R Superior Frontal Gyrus, medial	9	6,54,36	6.29	346	X	

Supplementary Table 2. Negative correlations between grey matter volume and poly(GP) or NfL for all, presymptomatic and symptomatic C9orf72 repeat expansion carriers – peak voxel regions from the voxel–based morphometry analysis. (continued)

Region Containing peak voxel	BA	x,y,z	T	# of voxels	At p<0.001	At p_{fwe}<0.05
L Middle Occipital Gyrus	19	-34,-72,45	6.13	164	X	
R Superior Frontal Gyrus, dorsolateral	6	33,-3,68	6.02	711	X	
L Superior Parietal Gyrus	5	-21,-54,66	5.96	200	X	
R Middle Frontal Gyrus	46	32,42,33	5.92	61	X	
L Lingual Gyrus	17	-6,-78,-8	5.90	122	X	
L Lingual Gyrus	18	-21,-69,-10	5.74	246	X	
R Superior Parietal Gyrus	7	24,-75,50	5.60	97	X	
R Supramarginal Gyrus	40	58,-40,46	5.46	191	X	
L Supramarginal Gyrus	40	-63,-39,33	5.40	130	X	
L Inferior Parietal, excluding Supramarginal and angular	2	-52,-30,50	5.40	141	X	
R Superior Frontal Gyrus, medial	10	12,69,18	5.39	152	X	
R Fusiform Gyrus	19	22,-64,-10	5.30	83	X	
L Superior Temporal Gyrus	48	-58,3,0	5.28	198	X	
R Calcarine Fissure and surrounding cortex	19	26,-52,63	5.26	32	X	
L Middle Frontal Gyrus	46	-33,42,34	5.23	78	X	
L Inferior Temporal Gyrus	20	-52,-8,-32	5.18	105	X	
L Caudate Nucleus	n/a	-12,3,16	5.09	316	X	
L Middle Frontal Gyrus	46	-42,48,9	5.07	23	X	
L Superior Frontal Gyrus, dorsolateral	8	-14,28,57	5.01	40	X	
L Middle Temporal Gyrus	22	-69,-38,10	5.00	57	X	
R Cuneus Cortex	19	16,-82,40	5.00	50	X	
L Superior Temporal Gyrus	22	-64,-24,12	4.91	79	X	
L Insula	48	-42,8,44	4.89	35	X	
R Middle Temporal Gyrus	21	58,2,-24	4.82	52	X	
L Fusiform Gyrus	19	-36,-68,-16	4.78	24	X	
L Middle Temporal Gyrus	21	-62,-54,9	4.77	40	X	
R Superior Temporal Gyrus	22	69,-12,10	4.75	39	X	
R Insula	48	40,-6,-6	4.74	47	X	
R Inferior Temporal Gyrus	20	51,-16,-34	4.69	69	X	
L Calcarine fissure and surrounding cortex	17	4,-98,6	4.69	42	X	
R Precentral Gyrus	6	21,-20,74	4.62	25	X	
L Olfactory Cortex	25	-2,18,-10	4.60	29	X	
R Caudate Nucleus	n/a	9,10,70	4.59	131	X	
R Crus 1 Cerebellum	19	33,-80,-24	4.49	52	X	
R Lingual Gyrus	27	9,-42,2	4.48	24	X	

Supplementary Table 2. Negative correlations between grey matter volume and poly(GP) or NfL for all, presymptomatic and symptomatic C9orf72 repeat expansion carriers – peak voxel regions from the voxel–based morphometry analysis. (continued)

Region Containing peak voxel	BA	x,y,z	T	# of voxels	At p<0.001	At p_{fwe}<0.05
R Inferior Temporal Gyrus	36	30,10,-44	4.39	59	X	
L Superior Frontal Gyrus, orbital part	11	-32,58,0	4.38	20	X	
R Precuneus	7	14,-63,63	4.38	20	X	
R Fusiform Gyrus	18	22,-81,-14	4.38	28	X	
L Superior Frontal Gyrus, medial	8	-3,34,54	4.36	104	X	
R Superior Frontal Gyrus, dorsolateral	10	18,62,28	4.36	27	X	
L Postcentral Gyrus	3	-50,-18,62	4.33	31	X	
R Calcarine Fissure and surrounding cortex	18	16,-96,6	4.31	21	X	
L Superior Frontal Gyrus, medial	10	-6,66,4	4.21	27	X	
R Precentral Gyrus	6	30,-22,70	4.13	31	X	
R Hemispheric Lobule VI of Cerebellum	n/a	9,-70,-20	4.04	20	X	
Grey matter versus NfL in symptomatic *C9orf72* expansion carriers						
L Superior Frontal Gyrus, dorsolateral	8	-20,22,54	10.93	1579	X	X
L Precentral Gyrus	6	-20,-26,62	9.46	2232	X	X
R Superior Frontal Gyrus, dorsolateral	6	24,4,60	8.62	1302	X	
L Middle Occipital Gyrus	7	-34,-66,39	8.24	198	X	
R Cuneus Cortex	19	14,-81,36	7.86	306	X	
R Middle Frontal Gyrus, orbital part	47	34,39,-12	7.61	193	X	
L Insula	48	-42,6,6	7.4	1308	X	
L Superior Frontal Gyrus, dorsolateral	11	-28,51,2	6.34	130	X	
L Precuneus	7	-6,-64,45	6.29	109	X	
L Superior Frontal Gyrus, dorsolateral	9	-18,42,38	6.16	138	X	
R Middle Frontal Gyrus	9	27,33,34	6.13	169	X	
L Anterior Cingulate and Paracingulate gyri	24	-4,26,30	6.12	1484	X	
L Inferior Frontal Gyrus, triangular part	47	-36,40,10	6.05	44	X	
L Supplementary Motor Area	6	-3,-8,63	6.04	92	X	
R Superior Temporal Gyrus	48	50,-8,34	6.02	328	X	
L Superior Parietal Gyrus	7	-24,-62,58	5.89	64	X	
R Middle Frontal Gyrus	9	21,48,34	5.57	48	X	
R Postcentral Gyrus	6	24,-26,60	5.54	274	X	
R Lenticular nucleus, pallidum	48	24,0,-6	5.48	32	X	
L Superior Frontal Gyrus, dorsolateral	11	-20,63,4	5.47	91	X	
L Superior Frontal Gyrus, dorsolateral	11	-21,54,16	5.36	150	X	
L Rolandic Operculum	48	-51,4,21	5.31	234	X	
L Cerebellum Hemispheric Lobule IV/V	n/a	-6,-42,3	5.29	18	X	
L Superior Parietal Gyrus	7	-16,-69,48	5.26	145	X	

Supplementary Table 2. Negative correlations between grey matter volume and poly(GP) or NfL for all, presymptomatic and symptomatic C9orf72 repeat expansion carriers – peak voxel regions from the voxel–based morphometry analysis. (continued)

Region Containing peak voxel	BA	x,y,z	T	# of voxels	At p<0.001	At p_{fwe}<0.05
L Middle Frontal Gyrus, orbital part	47	-32,38,-12	5.09	217	X	
L Caudate Nucleus	n/a	-10,14,14	5.02	452	X	
R Precuneus	23	16,-63,27	5.01	173	X	
R Angular Gyrus	39	39,-62,40	5.01	48	X	
R Rolandic Operculum	48	48,-6,8	4.98	242	X	
R Superior Frontal Gyrus, dorsolateral	9	20,40,42	4.95	58	X	
R Lingual Gyrus	27	9,-39,4	4.93	77	X	
R Middle Temporal Gyrus	37	50,-58,14	4.85	76	X	
L Cuneus Cortex	19	-16,-84,34	4.79	21	X	
L Superior Frontal Gyrus, medial	10	-4,58,8	4.78	81	X	
R Middle Frontal Gyrus	47	42,46,3	4.77	30	X	
R Middle Frontal Gyrus	46	36,42,28	4.69	62	X	
R Postcentral Gyrus	3	27,-39,56	4.68	91	X	
L Superior Parietal Gyrus	5	-21,-48,74	4.65	67	X	
R Superior Occipital Gyrus	7	24,-69,48	4.63	28	X	
L Superior Frontal Gyrus, dorsolateral	9	-24,34,33	4.63	51	X	
R Caudate Nucleus	25	10,14,8	4.62	564	X	
R Insula	48	39,21,6	4.53	168	X	
L Anterior Cingulate and Paracingulate gyri	10	-4,48,28	4.51	277	X	
R Superior Temporal Gyrus	48	54,-20,9	4.5	56	X	
R Superior Frontal Gyrus, medial	9	3,39,40	4.45	65	X	
R Median Cingulate and Paracingulate Gyri	32	8,16,46	4.42	26	X	
L Precuneus	5	-14,-50,63	4.42	63	X	
R Calcarine Fissure and surrounding cortex	19	20,-51,6	4.38	57	X	
R Superior Parietal Gyrus	5	18,-58,66	4.31	37	X	
R Median Cingulate and Paracingulate Gyri	n/a	9,-40,52	4.31	70	X	
R Supramarginal Gyrus	2	56,-36,36	4.23	25	X	
R Lenticular nucleus, putamen	n/a	18,10,0	4.23	35	X	
L Lingual Gyrus	17	-14,-51,4	4.21	43	X	
R Superior Frontal Gyrus, medial	8	2,28,52	4.19	38	X	
R Inferior Temporal Gyrus	20	63,-21,-27	4.18	21	X	
L Lenticular Nucleus, putamen	n/a	-21,3,8	4.16	23	X	
L Middle Temporal Gyrus	21	-66,-32,-4	4.16	25	X	
R Lenticular nucleus, putamen	n/a	24,2,9	4.14	39	X	

BA: Brodmann area; fwe: family wise error corrected; NfL: neurofilament light chain in cerebrospinal fluid; T: t-score

CHAPTER 3.3

Progranulin levels in plasma and cerebrospinal fluid in granulin mutation carriers

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Abstract

Background: Pathogenic mutations in the granulin gene (*GRN*) are causative in 5-10% of patients with frontotemporal dementia (FTD), mostly leading to reduced progranulin protein (PGRN) levels. Upcoming therapeutic trials focus on enhancing PGRN levels.

Methods: Fluctuations in plasma PGRN (*n*=41) and its relationship with cerebrospinal fluid (CSF, *n*=32) and specific single nucleotide polymorphisms, were investigated in pre- and symptomatic *GRN* mutation carriers and controls.

Results: Plasma PGRN levels were lower in carriers than in controls and showed a mean coefficient of variation of 5.3% in carriers over one week. Although plasma PGRN correlated with CSF PGRN in carriers (r=0.54, p=0.02), plasma only explained 29% of the variability in CSF PGRN. Rs5848, rs646776 and rs1990622 genotypes only partly explained the variability of PGRN levels between subjects.

Conclusions: Plasma PGRN is relatively stable over one week and therefore seems suitable for treatment monitoring of PGRN-enhancing agents. Since plasma PGRN only moderately correlated with CSF PGRN, CSF sampling will additionally be needed in therapeutic trials.

Introduction

Frontotemporal dementia (FTD), a common type of presenile dementia, shows an autosomal dominant inheritance in 20-30%.[1] Pathogenic mutations in granulin (*GRN)* are a major cause of heritable FTD and mostly reduce progranulin protein (PGRN) levels in blood and cerebrospinal fluid (CSF) by haploinsufficiency.[2–6] PGRN plays an important role in neurite outgrowth and inflammation, which may be the link to neurodegeneration.[7]

As PGRN levels vary greatly between individuals, various genetic and environmental regulators may play a role.[5,8–10] A number of single nucleotide polymorphisms (SNPs) have been associated with altered CSF or plasma PGRN levels: rs5848 (*GRN),* rs646776 (near sortilin 1 [*SORT1*]) and rs1990622 (near transmembrane protein 106B [*TMEM106B*]).[10–14]

Current FTD research is shifting towards disease-modifying agents, and sensitive biomarkers are essential to evaluate these potential agents in the clinic. Histone deacetylase inhibitors, alkalizing reagents and inhibitors of vacuolar ATPase, have been shown to enhance PGRN expression in carrier-derived cells and might therefore inhibit the disease process.[15,16] Although blood PGRN poorly correlates to CSF PGRN in healthy controls and Alzheimer's disease, sporadic FTD and amyotrophic lateral sclerosis,[10,17,18] this information is lacking in *GRN* mutation carriers. To use PGRN levels as biomarkers in therapeutic trials on PGRN-enhancing agents, a better understanding in *GRN* mutation carriers is needed on correlations between levels in blood and CSF, fluctuations over time and variability between subjects.

In this study patients and presymptomatic carriers of pathogenic *GRN* mutations were studied to investigate (1) the correlation between plasma and CSF levels in *GRN* mutation carriers, (2) the fluctuations of plasma PGRN over time, and (3) the associations between three SNPs (rs5848, rs646776 and rs1990622) and PGRN levels in plasma and CSF.

Methods

Subjects

A group of 57 (37 women, 20 men) asymptomatic first-degree relatives of patients with FTD caused by a pathogenic *GRN* mutation (at-risk group), was selected from our longitudinal neuropsychological and MRI study in genetic FTD.[19] Participants were selected depending on the availability of plasma and/or CSF (no biosample available: *n*=10). The participants originate from three different families with *GRN* mutations (p.Ser82Valfs, p.Gln125* or p.Val411Serfs mutation). After screening of these *GRN* mutations,[20] participants were divided into those with (presymptomatic carriers, *n*=28) and those without a pathogenic *GRN* mutation (controls, *n*=29); investigators and at-risk individuals remained blinded to the individual carrier status. Plasma was available from all 57 at-risk individuals; a lumbar puncture was carried out in 28 of them (16 presymptomatic *GRN* carriers and 12 controls, see Supplementary Figure 1). Longitudinal blood samples for the determination of vari-

ability of plasma PGRN were collected in 37 at-risk subjects (18 presymptomatic *GRN* carriers and 19 controls), and the other 20 subjects did not participate mainly because of logistical reasons (e.g. long travel distance) or a lack of motivation.

Additionally, ten patients with a pathogenic *GRN* mutation (p.Ser82Valfs, p.Gln125*, p.Val411Serfs and p.Gln130Serfs) were included from our previously described cohorts.[21,22] Plasma was available from 7 patients (longitudinal sampling in 4) and CSF from 7 (plasma also available in 4).

The study was approved by the medical ethics committee. All participants or legal representatives provided written informed consent for the blood and/or CSF collections.

Plasma and CSF Collection

Blood collections were performed according to standard procedures. For longitudinal analyses, blood was collected in a local hospital or nursing home at five time points during one week: 0 hours (h), 6 h, 12 h, 24 h and 7 days (d). The collections at the time points 0 h, 24 h and 7 d were performed in the morning, while the 6 h time point was performed after noon and the 12 h time point in the evening. To diminish the burden for FTD patients resident in a nursing home, the venipuncture at 12 h was omitted (*n*=3). To assess the influence of fasting on plasma PGRN, participants fasted for a minimum of 8 h before the venipuncture at 24 h. Plasma was isolated from K_2EDTA coated tubes (Becton Dickinson) by direct centrifugation at 1300 relative centrifugal force (RCF) for 20 minutes at room temperature, aliquoted and directly stored at -80°C.

CSF was collected using standard procedures into polypropylene tubes. The first 1-3 ml were discarded and samples were centrifuged in the polypropylene tube at 2000 RCF for 10 min at +4°C. CSF was aliquoted into polypropylene vials and immediately stored at -80°C.

Laboratory Methods

All measurements were performed blinded to the mutation status and clinical stage. PGRN concentrations in plasma and CSF samples were determined using a qualified immunoassay based on an enzyme-linked immunosorbent assay (ELISA) kit from BioVendor (Brno, Czech Republic) following kit instructions. Low, medium and high PGRN concentration quality control samples were run on each assay plate with an acceptance coefficient of variation (CV) cut-off of ≤20%. PGRN replicates with a CV of >20% were excluded from the analyses. CSF was diluted 1:2 and plasma 1:40. All reported concentrations fell within the qualified range of the assays. PGRN concentrations were determined on a standard curve by plotting optical density versus concentration, using four-parameter logistic curve-fitting.

DNA was extracted from whole blood following standard procedures. SNPs rs5848 (NM_002087.2(*GRN*):c.*78C>T), rs646776 (NM_001408.2(*CELSR2*):c.*1859C>T, near *SORT1*) and rs1990622 (NM_000007.13:g.12283787A>G, near *TMEM106B*) were genotyped using Taqman assays (respective assay numbers C_7452046_20, C_3160062_10 and C_11171598_10; Life Technologies, Carlsbad, USA) on a 7900HT Fast Real-Time PCR System

(Applied Biosystems, Carlsbad, USA). Genotypes were assigned using SDS v3.1 software (Applied Biosystems, Carlsbad, USA).

Statistical analysis

Statistical analyses were performed in SPSS 21.0 for Windows (Chicago, USA) and GraphPad Prism 6 (La Jolla, USA), applying a significance level of $p<0.05$. Comparisons between two groups were made by Mann-Whitney U tests because of non-normally distributed data; multiple groups were compared by Kruskal-Wallis tests. Pearson's correlation coefficient (r) was used to correlate two normally distributed variables; otherwise Spearman's correlation coefficient (r_s) was used. To assess the variability (variance) in CSF PGRN levels accounted for by plasma PGRN, the coefficient of determination ($R^2 = r^2$) was used.[10] Age at onset (AAO) was defined as the age of first symptoms noted by a caregiver. For the longitudinal blood withdrawals, repeated measures ANOVAs were used and variability per individual was assessed by the CV (ratio of the standard deviation to the mean) and compared between the groups with *t*-tests. In the case of longitudinal withdrawals, the median of each individual was used for comparisons of single plasma PGRN values; for plasma-CSF-correlations, we used the plasma sample closest in time to the lumbar puncture. Associations between SNP genotypes and PGRN levels were analyzed by multivariate regression with *GRN* mutation status, age and gender as covariates.

Table 1. Subject characteristics.

	Plasma available (n = 64)		CSF available (n = 35)	
	Controls	*GRN* mutation carriers	Controls	*GRN* mutation carriers
Number (male)	29 (11)	35[a] (12)	12 (4)	23 (9)
Number of symptomatic subjects	-	7	-	7
MMSE (range)	30 (25-30)[c]	30 (0-30)[b]	30 (28-30)	29 (18-30)
Age at onset, years (IQR)	-	59.1 (57.6-65.8)[c]	-	57.6 (55.1-65.5)[c]
Age at collection, years (IQR)	58.6 (51.8-63.5)	58.1 (53.4-64.3)	58.1 (50.8-64.0)	57.4 (54.7-61.0)
Time between onset and collection, years (range)	-	2.3 (-1.2-5.3)	-	2.0 (-1.2-5.0)
Time between plasma and CSF collection, days (range)	35 (0-265)	19 (0-597)	35 (0-265)	19 (0-597)
PGRN level, ng/ml (range)	28.5 (21.5-39.2)	8.0 (5.2-11.3)	0.76 (0.60-1.25)	0.29 (0.15-0.46)

Values are displayed as medians; [a]Mutations: n=23 p.Ser82Valfs; n=9 p.Gln125*; n=2 p.Val411Serfs; n=1 p.Gln130Serfs; [b]presymptomatic *GRN* mutation carriers median MMSE 30 (range 26-30), symptomatic carriers median MMSE 18 (range 0-26); [c]only known age at onset in 7 patients and one presymptomatic carrier who converted after collection. CSF: cerebrospinal fluid; IQR: interquartile range; MMSE: Mini-Mental State Examination: PGRN: progranulin.

Results

Cohort Characteristics

The baseline characteristics of the cohort are displayed in Table 1. Presymptomatic carriers, controls and patients did not differ in age (p=0.08). Mini Mental State Examination (MMSE) scores were lower in patients than in at-risk individuals (p<0.01); no differences were found between controls and presymptomatic carriers (p=0.51).

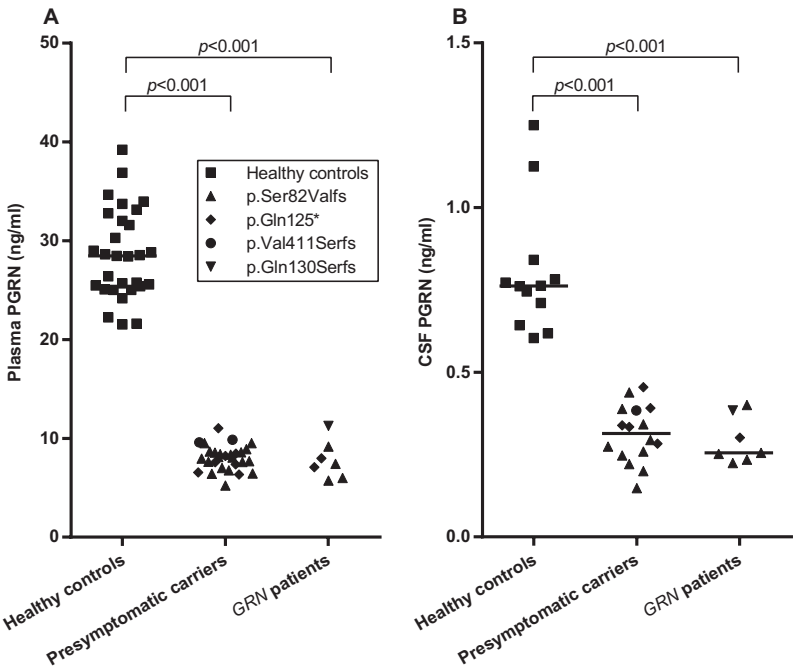

Figure 1. PGRN levels in plasma and CSF.

PGRN in (A) plasma from healthy controls (n=29), presymptomatic *GRN* mutation carriers (n=28) and *GRN* patients (n=7) and in (B) CSF from healthy controls (n=12), presymptomatic *GRN* mutation carriers (n=16) and *GRN* patients (n=7). Each individual is represented as a data point and labeled by type of mutation. Horizontal lines are median plasma levels per group.

CSF: cerebrospinal fluid; PGRN: progranulin.

Plasma PGRN Levels

GRN mutation carriers had lower plasma PGRN levels than controls, without any overlap between the groups (p<0.001, Table 1 and Figure 1A). Median level in carriers was 28% of that in the controls. Maximum variation between subjects was a factor of 1.8 in controls and 2.2 in carriers (ratio of the highest to the lowest expression per group). PGRN levels did not differ between presymptomatic carriers and patients (p=0.51). Differences between various mutations did not reach significance (p=0.06), and limited numbers across the mu-

tations did not allow for post-hoc testing. Plasma PGRN did not correlate with age (Figure 2A), gender or AAO.

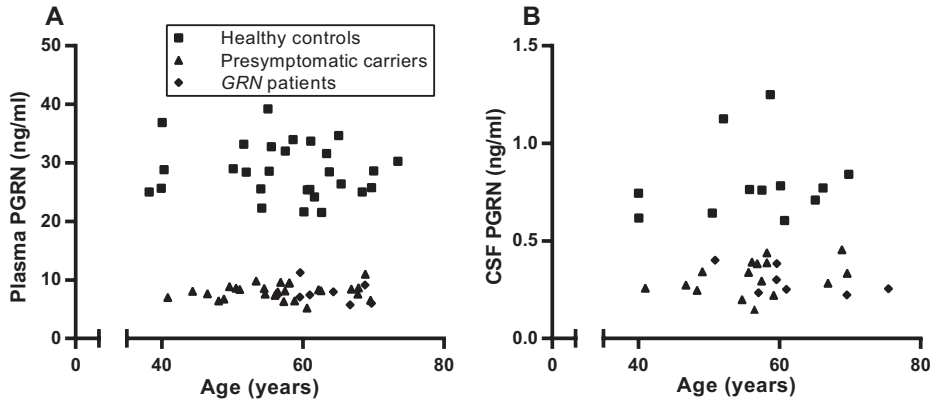

Figure 2. PGRN levels as a function of age.

Scatterplots of PGRN levels in plasma (A) and CSF (B) by age at collection in healthy controls (squares), presymptomatic *GRN* mutation carriers (triangles) and *GRN* patients (diamonds). To prevent disclosure of genetic status, a 23-year-old subject was excluded from the graph (but not from the analyses). CSF: cerebrospinal fluid; PGRN: progranulin.

CSF PGRN Levels

Median CSF PGRN in carriers was 39% of that in controls, without overlap of the levels between the groups (p<0.001, Table 1 and Figure 1B). In carriers, the lowest expression level of CSF PGRN was 3.1 times lower than that of the highest expression level; in controls this was a 2.1-fold difference. CSF PGRN did not differ between presymptomatic carriers and patients (p=0.58), or between various *GRN* mutations (p=0.16). CSF PGRN did not correlate with age (Figure 2B), gender or with AAO.

Correlation between Plasma and CSF

Within the group of subjects with both plasma and CSF samples (16 presymptomatic carriers, 4 *GRN* patients and 12 controls), PGRN plasma levels correlated significantly with CSF levels (r_s=0.80, p<0.001, Figure 3). The median interval between blood and CSF collection was 32 days, range 0–597 (also see Table 1). The correlation between PGRN levels in CSF and plasma collected on the same day in 13 subjects (6 presymptomatic carriers, 3 *GRN* patients and 4 controls) was similar to the total group (r_s=0.78, p=0.002). The correlation between PGRN levels in plasma and CSF was weaker when both subgroups were separately analyzed: r=0.54 (p=0.02) in carriers and r=0.21 (p=0.51) in controls. The coefficient of determination, R^2, was 0.29 in carriers; this means that in our group of carriers, 29% of the variability in CSF PGRN levels is explained by the variability in plasma PGRN levels and the remaining 71% is still unaccounted for.

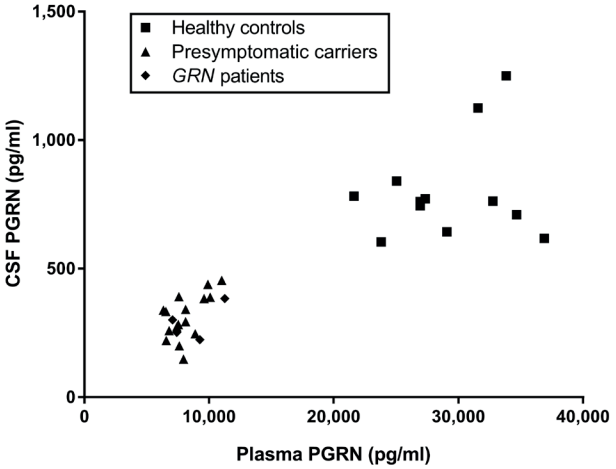

Figure 3. Correlation plasma versus CSF PGRN.
Scatterplot of the correlation (r_s=0.80, p<0.001) between PGRN levels in plasma and CSF in 12 healthy controls (squares), 16 presymptomatic *GRN* mutation carriers (triangles) and 4 *GRN* patients (diamonds).
CSF: cerebrospinal fluid; PGRN: progranulin.

Longitudinal Plasma PGRN Levels

Plasma PGRN levels were found to be stable over one week in carriers and controls (Figure 4), with a mean CV in carriers of 5.3% (range 2.3-9.7%) and in controls of 4.7% (range 1.4-8.6%; not statistically different, p=0.32). Repeated measures analyses showed no significant differences over the five time points (p=0.13 in carriers and p=0.053 in controls); the trend for a difference in controls was caused by a non-significant lower plasma PGRN 6 h vs 0 h (p=0.09 Bonferroni post hoc test). The fasting PGRN plasma levels (24 h time point) were not significantly different from other time points (e.g. fasting PGRN level versus time point 0 h in carriers p=0.18, paired t-test).

Associations between SNPs and PGRN Levels

The associations of the SNPs with PGRN levels are displayed in Supplementary Table 1 and Supplementary Figure 2. *GRN* mutation carriers had lower PGRN plasma levels with each additional minor allele (A) of rs5848 (*GRN)* without an effect in CSF. In contrast, in the control group an effect was found in CSF only. The minor allele (G) of rs646776 (near *SORT1*) was associated with lower PGRN levels in plasma of *GRN* mutation carriers, without a significant effect in controls or in CSF. For rs1990622 (near *TMEM106B*), the minor allele (C) was associated with lower plasma PGRN levels in the entire group of subjects; no significant associations were observed for CSF.

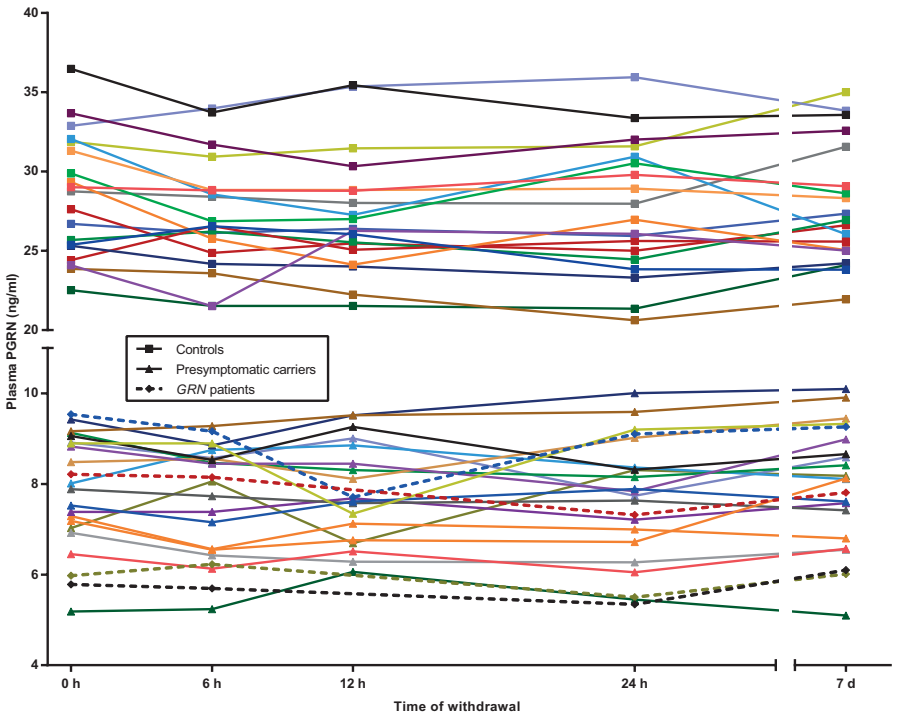

Figure 4. Longitudinal plasma PGRN.
PGRN levels over time in plasma of healthy controls (n = 19, squares), presymptomatic *GRN* mutation carriers (n = 18, triangles) and *GRN* patients (n = 4, diamonds). Each withdrawal is represented with a data point; lines connect data points of each individual. At the 12-hour time point, 3 data points are missing (all *GRN* patients).

Discussion

This study showed that CSF PGRN levels in a large series of presymptomatic *GRN* mutation carriers were already lower than in age-matched controls. Plasma PGRN levels strongly correlated with CSF levels, however, this was largely explained by the difference in *GRN* carrier status and in carriers, only 29% of the variability in CSF PGRN was explained by plasma PGRN. Plasma PGRN levels within subjects fluctuated by 5% over a one-week period, which has major implications for the clinical trial design aiming at PGRN restoration. Known SNPs only partly explained variation in plasma and CSF PGRN levels between subjects.

The significantly lower plasma PGRN levels in *GRN* mutation carriers than in healthy controls found in this study, and the similar levels in presymptomatic carriers and patients, are in line with previous reports.[4,5,8,9,11] The gap between plasma PGRN levels in our carriers versus non-carriers was even larger and the spread of PGRN levels was smaller than in previous studies.[4,5,8] This might be explained by a standardized collection of samples and the use of a new qualified ELISA assay with strict performance acceptance criteria and

quality control samples on each plate. As previously suggested, plasma PGRN can serve as a reliable screening tool for pathogenic *GRN* mutations in patients with seemingly sporadic FTD or with an unspecified early onset dementia.[4,5,9] Plasma PGRN can also serve as a rapid *ex vivo* screening tool in patients with variants of unknown significance in *GRN*. This identification of pathogenic *GRN* mutations becomes even more important with forthcoming potential treatments increasing PGRN expression.

PGRN levels in CSF of presymptomatic carriers and patients did not show overlap with controls at all ages, which is in line with earlier reports in small series of *GRN* patients.[4,6] With the development of PGRN-enhancing therapies, a crucial question remains whether and at what age enhancing PGRN will have an effect on disease course. As this study showed for the first time that CSF PGRN levels are already reduced in the presymptomatic stage, additional biomarkers (clinical, neuroimaging and/or biochemical) are needed to determine disease onset and to track disease progression in therapeutic trials.

The strong correlation between peripheral and CSF PGRN levels contrasts with a weaker correlation found in studies in healthy controls (partial r=0.17 and r=0.36) and no significant correlation in sporadic FTD.[10,17,18] This could be explained by the clear dichotomy in PGRN levels between *GRN* mutation carriers and non-carriers; our observed correlation was mainly driven by *GRN* genotype, as subgroup analyses by carrier status showed lower correlations. In our opinion, plasma PGRN levels can serve as an easily accessible biomarker to assess target engagement for potentially disease-modifying agents. However, since plasma PGRN levels in carriers explained only 29% of the variability of CSF PGRN levels, plasma PGRN cannot predict CSF PGRN. Moreover, these and previous data suggest a differential regulation of PGRN in plasma/serum versus CSF and likely the majority of CSF PGRN is synthesized in the central nervous system,[10,18,23] therefore it could also be that PGRN-enhancing agents have differential effects on different tissues. Although it is unknown whether CSF indeed reflects PGRN levels in the brain and/or interstitial space,[18] drug effects could be missed in the worst case if solely relying on plasma PGRN, and CSF sampling will additionally be needed to evaluate effects of pharmacological interventions. The performance of plasma versus CSF PGRN as a surrogate biomarker depends on effect place and –size of potential PGRN-restoring agents, and remains to be investigated in longitudinal trials.

This first observation of stable PGRN levels over a one-week period in carriers has important implications for therapeutic trials of pharmacological agents aiming to restore PGRN levels. It is in line with the findings of no significant differences over a longer time period in mainly non-carriers.[23] The mean observed CV over a week of 5% can be technically explained by the assay variability. Plasma PGRN did not alter under fasting conditions, which additionally indicates that plasma PGRN levels can be a suitable biomarker of target engagement. Furthermore, our data can support sample size estimations greatly facilitating clinical trial design: using intra-subject over inter-subject variability, significantly reduces trial size in this rare disease.

It was recently published that more commonly used PGRN ELISA kits (Adipogen and R&D) detected CSF PGRN in non-*GRN*-carriers only at the lower detection range of the kits.[24] Because CSF PGRN of *GRN*-carriers is at least 50% lower, we opted for the more sensitive protocol of Biovendor; R&D and Biovendor yielded similar PGRN concentrations, additionally, Biovendor was qualified to detect low ranges of CSF PGRN [qualified range: 0.018-2.1 ng/ml, data not shown]. A limitation is that comparison with studies that used Adipogen and meta-analyses for cut-off levels would be complicated, due to different normality values among the kits.

PGRN levels are known to vary widely among subjects and several factors have been found which partly explain this variability. In this study, neither age at collection, nor gender were correlated with PGRN levels, in contrast to some earlier reports in non-carriers.[4,5,10,12,13] This might be explained by the limited age range and the small sample size. However, in carriers, previous studies did not detect a correlation with age, similar to our result.[8,9] The same holds true for AAO, for which conflicting results have been reported and this study did not find a correlation.[5,8,9]

Several SNPs have been found to influence PGRN levels: rs5848 in *GRN*, rs646776 near *SORT1,* and rs1990622 near *TMEM106B*. The correlation between lower PGRN levels and the minor allele of rs5848 in our series is in line with findings from previous studies.[10,12,25] Probably microRNA-659 binds more efficiently to this minor allele, resulting in translational suppression of PGRN.[26] The significantly lower plasma PGRN levels with each minor allele in our *GRN* mutation carriers, suggest that the translational suppression also takes place when there is only one functional *GRN* allele.

In our *GRN* mutation carriers and in previous studies, rs646776 (near *SORT1*) correlated significantly with plasma PGRN levels, but not with PGRN CSF levels.[10,13,27] This SNP is probably a liver-specific regulator of *SORT1* and therefore only a peripheral modifier.[10,28]

The minor allele (C) in rs1990622 (*TMEM106B)* was not associated with higher plasma PGRN levels, in contrast to the strong association in *GRN* patients and healthy controls found in previous studies.[11,14] Such an association was supported by a proposed functional link between *TMEM106B* and PGRN and by a delay in AAO with each minor allele.[11,14,29–31] Our findings might be explained by analysis in a few families resulting in genetic bias. Larger cohorts are needed in order to investigate the exact role of *TMEM106B* on PGRN and AAO in various genetic backgrounds.

Major strengths of our study include longitudinal plasma collections over a week within the same individuals, uniformly performed by a single investigator and collection protocol. Furthermore, presymptomatic carriers and controls were well matched since they originate from the same families. A methodological weakness of this study is the interval of more than one day between plasma and CSF collection in half of the cases. However, a subgroup analysis of samples collected on the same day showed a correlation comparable to that in the entire group. This might be expected given the demonstrated low variability in plasma PGRN levels over time in this study and in CSF PGRN levels recently reported.[23] Future

studies are warranted to analyze variations between plasma sampled more than one week apart and fluctuations in PGRN CSF over time in *GRN* mutation carriers. Additionally, the population was too small to get robust conclusions on the effect of the studied SNPs on PGRN levels in *GRN* mutation carriers and should be studied in larger cohorts.

To conclude, PGRN levels in plasma and CSF were already low in presymptomatic *GRN* mutation carriers and separated completely from non-carriers. Although PGRN levels in plasma and CSF strongly correlated, plasma PGRN levels only explain 29% of the variability of CSF PGRN levels in *GRN* mutation carriers, and therefore both blood and CSF sampling is needed in PGRN-enhancing trials. Plasma PGRN levels can serve as biomarker of target engagement for potentially disease-modifying agents addressing PGRN steady-state, as they were relatively stable over one week. Further research is required to elucidate which other factors are associated with PGRN regulation with emphasis on the differences between the regulation in plasma and CSF.

Acknowledgments

This project was supported by FORUM Pharmaceuticals Inc. (who is developing a PGRN-enhancing therapy) and grants from 'Nationaal Initiatief Hersenen en Cognitie', Alzheimer Nederland (grant nr 056-13-018) and Van Leersumfonds (grant nr VLF2013220). We are greatly indebted to Dorothy Flood (FORUM Pharmaceuticals Inc., USA) for critically reviewing the manuscript. We would like to thank all study participants for taking part in this study.

Authors disclosures

This research project was supported by FORUM Pharmaceuticals Inc. (who is developing a PGRN-enhancing therapy) and grants from 'Nationaal Initiatief Hersenen en Cognitie', Alzheimer Nederland (grant nr 056-13-018) and Van Leersumfonds (grant nr VLF2013220). H.H. Meeter received support from Alzheimer Nederland (grant nr. WE.09-2014-04). H. Patzke and G. Loewen are employees of FORUM Pharmaceuticals Inc. E.G.P. Dopper received support from Dioraphte Foundation grant 09-02-03-00, the Association for Frontemporal Dementias Research Grant 2009, The Netherlands Organization for Scientific Research (NWO) grant HCMI 056-13-018 and Netherlands Alzheimer Foundation. Y.A.L. Pijnenburg, R. van Minkelen and J.C. van Swieten report no disclosures.

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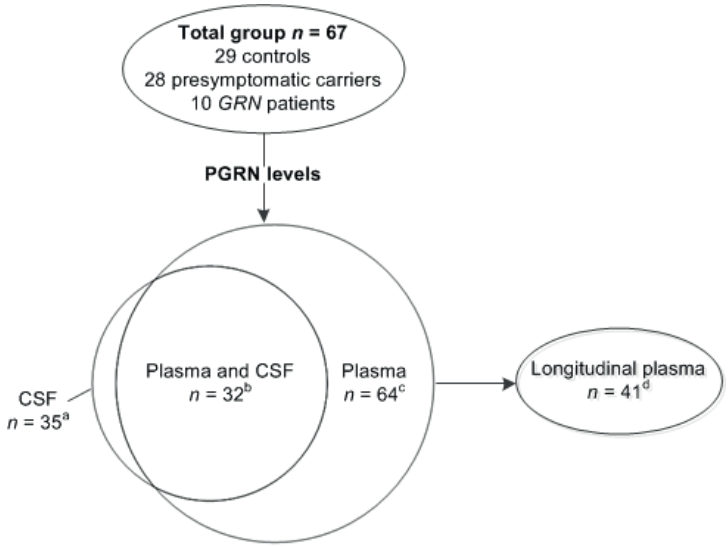
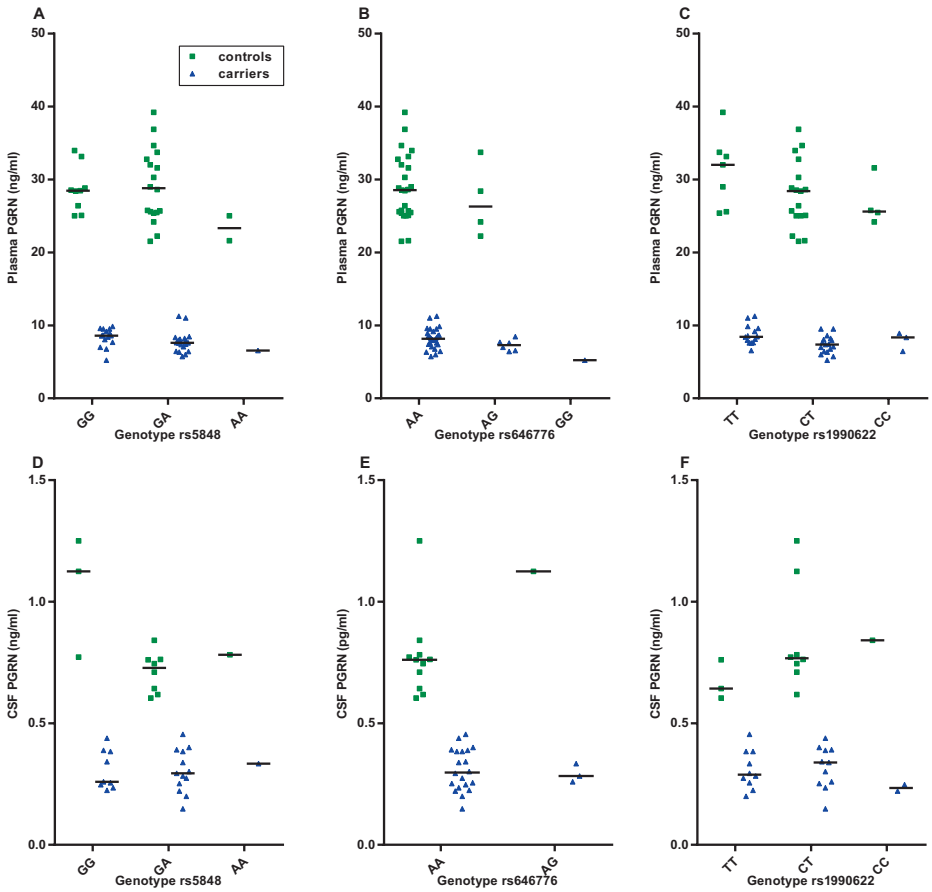
Supplementary material



Supplementary Figure 1. Number of subjects per type of collected sample.
[a]12 controls, 16 presymptomatic carriers and 7 *GRN* patients; [b]12 controls, 16 presymptomatic carriers and 4 *GRN* patients; [c]29 controls, 28 presymptomatic carriers and 7 *GRN* patients; [d]19 controls, 18 presymptomatic carriers and 4 *GRN* patients. CSF: cerebrospinal fluid.



Supplementary Figure 2. Associations of SNPs versus PGRN levels.

Scatterplots of rs5848, rs646776 and rs1990622 genotype with plasma PGRN (A, B and C) and with CSF PGRN (D, E and F) , data points represent individual values and horizontal lines are median PGRN levels per group (carriers of a pathogenic *GRN* mutation versus controls). Genotyping for rs1990622 failed in one presymptomatic *GRN* mutation carrier and one control.

CSF: cerebrospinal fluid; PGRN: progranulin.

Supplementary Table 1. PGRN levels by SNP genotype

SNP (minor allele)		genotype			β[a]	*p*-value[a]
rs5848 (A), *GRN*		GG	GA	AA		
Plasma	Entire group	9.5 (5.2-34.0)	11.3 (5.7-39.2)	21.6 (6.5-25.0)	-.050	0.25
	n=	24	37	3		
	Carriers	8.6 (5.2-9.8)	7.6 (5.7-11.3)	6.5[b]	-.399	0.04
	n=	15	19	1		
	Controls	28.5 (25.0-34.0)	28.8 (21.5-39.2)	23.3 (21.6-25.0)	-.078	0.72
	n=	9	18	1		
CSF	Entire group	0.36 (0.22-1.25)	0.39 (0.15-0.84)	0.56 (0.33-0.78)	-.165	0.06
	n=	12	21	2		
	Carriers	0.26 (0.22-0.44)	0.29 (0.15-0.46)	0.33[b]	.024	0.91
	n=	9	13	1		
	Controls	1.13 (0.77-0.13)	0.73 (0.60-0.84)	0.78[b]	-.794	0.03
	n=	3	8	1		
rs646776 (G), near *SORT1*		AA	AG	GG		
Plasma	Entire group	11.0 (5.7-39.2)	8.1 (6.4-33.7)	5.2[b]	-.051	0.19
	n=	53	10	1		
	Carriers	8.2 (5.7-11.3)	7.3 (6.4-8.4)	5.2[b]	-.423	0.01
	n=	28	6	1		
	Controls	28.6 (21.5-39.2)	26.3 (22.2-33.7)	-	-.098	0.61
	n=	25	4	0		
CSF	Entire group	0.39 (0.15-1.25)	0.31 (0.26-1.13)	-	.100	0.25
	n=	31	4	0		
	Carriers	0.30 (0.15-0.46)	0.28 (0.26-0.33)	-	-.048	0.83
	n=	20	3	0		
	Controls	0.76 (0.60-1.25)	1.13[b]	-	.562	0.10
	n=	11	1	0		
rs1990622 (C), near *TMEM106B*		TT	TC	CC		
Plasma	Entire group	9.6 (6.5-39.2)	15.5 (5.2-36.9)	24.2 (6.5-31.6)	-.095	0.02
	n=	21	34	7		
	Carriers[c]	8.4 (6.5-11.3)	7.4 (5.2-9.5)	8.4 (6.5-8.9)	-.347	0.06
	n=	14	17	3		
	Controls[c]	32.0 (25.4-39.2)	28.4 (21.5-36.9)	25.6 (24.2-31.6)	-.337	0.08
	n=	7	17	4		
CSF	Entire group	0.33 (0.20-0.76)	0.40 (0.15-1.25)	0.25 (0.22-0.84)	.065	0.47
	n=	13	19	3		
	Carriers	0.29 (0.20-0.46)	0.34 (0.15-0.44)	0.23 (0.22-0.25)	-.106	0.66
	n=	10	11	2		
	Controls	0.64 (0.60-0.76)	0.77 (0.62-1.25)	0.84[b]	.367	0.32
	n=	3	8	1		

Comparisons of median (range) PGRN levels (plasma and CSF in ng/ml) within genotype of rs5848, rs646776 and rs1990622. [a]Multivariate regression with correction for age and gender; [b]*n*=1, therefore no range is displayed; [c]genotyping for rs1990622 failed in one presymptomatic carrier and one control.
CSF: cerebrospinal fluid; SNP: single nucleotide polymorphism.

CHAPTER 3.4

Identification of candidate CSF biomarkers by proteomics in presymptomatic and symptomatic *GRN*-associated frontotemporal dementia

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In preparation

Abstract

Objective: To identify candidate cerebrospinal fluid (CSF) biomarkers in presymptomatic and symptomatic *GRN*-associated frontotemporal dementia using unbiased proteomics.

Methods: CSF samples of 19 presymptomatic *GRN* mutation carriers, 9 symptomatic *GRN* mutation carriers and 24 non-carriers were included. Abundant proteins were depleted and CSF proteomes were investigated using advanced liquid chromatography and high resolution mass spectrometry. Peptide and protein abundancies were compared between groups, and candidate biomarkers were selected for further validation based on the number of identified peptides (≥4), and a fold change of ≤0.5 or ≥2.0.

Results: No differences were found between presymptomatic carriers and non-carriers. In the comparison between presymptomatic and symptomatic carriers, multiple proteins were differentially abundant, of which neuronal pentraxin receptor fulfilled criteria for a candidate biomarker. In the comparison between symptomatic and non-carriers the following candidates were identified: neuronal pentraxin receptor, receptor-type tyrosine-protein phosphatase N2, Ig alpha-1 chain C region, neurosecretory protein VGF, chromogranin-A, V-set and transmembrane domain-containing protein 2B, and complement component C8 gamma chain.

Conclusion: We have identified seven candidate CSF biomarkers in presymptomatic and symptomatic *GRN* mutation carriers. Our results show that synaptic, secretory vesicle and inflammatory proteins are dysregulated in the symptomatic stage of *GRN* mutation carriers and provide new insights into the pathophysiology, which is important for development of therapeutics. Further validation is needed to investigate the applicability as a diagnostic or monitoring biomarker.

Introduction

Frontotemporal dementia (FTD) is the second most common form of presenile dementia and autosomal dominant inheritance is present in 20-30% of cases.[1] Pathogenic mutations in granulin (*GRN)* are a major cause of hereditary FTD with underlying transactive response DNA-binding protein (TDP-43) pathology. Most mutations reduce progranulin (PGRN) protein levels by haploinsufficiency.[2–7] PGRN plays an important role in neurite outgrowth and inflammation.[8] However, the exact mechanism by which PGRN reduction leads to neurodegeneration is thus far unknown. With upcoming therapeutic trials, markers that help in determining the appropriate moment to start treatment – likely already in the presymptomatic stage when damage is minimal – and to measure treatment effect are critically needed.[9] PGRN levels in plasma and cerebrospinal fluid (CSF) are reduced in carriers of *GRN* mutations regardless of the presence of symptoms. PGRN levels can therefore serve as a biomarker for target engagement in PGRN enhancing trials, but not to predict or measure disease onset.[7] Biomarkers that reflect disease severity and activity (e.g. neurofilament light chain) are emerging as surrogate endpoints for clinical trials. However, increased neurofilament light chain is not a specific marker for FTD, as it is elevated in many diseases (e.g. other dementia types, multiple sclerosis, traumatic brain injury) and reflects general neuronal damage.[9]

Most studies on fluid biomarkers in FTD have used targeted approaches,[9,10] with as major disadvantage that only known protein candidates are measured. In contrast, unbiased approaches – including proteomics – enabling discovery of new biomarkers and pathophysiological insights, have scarcely been performed in CSF of FTD patients.[11,12] This approach has been used in the presymptomatic stage of autosomal dominant Alzheimer's disease (AD), uncovering early changes in the proteome.[13]

In the present study, we investigated CSF proteomics in *GRN* carriers with an unbiased approach using high resolution mass spectrometry (MS). We aimed to identify novel proteins that 1) reflect disease activity and/or 2) give insight into the pathophysiology of *GRN*-associated FTD. Both presymptomatic and symptomatic carriers of pathogenic *GRN* mutations were included in this study, enabling investigation of the earliest changes.

Methods

Subjects

The current discovery cohort consisted of 52 CSF samples from 9 symptomatic *GRN* mutation carriers, 19 healthy presymptomatic *GRN* mutation carriers, and 24 healthy non-carriers (control group), who participate in the Dutch longitudinal FTD Risc Cohort of individuals at-risk for genetic FTD.[14] In this study, healthy first-degree relatives of carriers of FTD-causing mutations are followed on a 2-yearly basis by means of neurological examination, structured informant interviews (e.g., with a spouse or sibling), extensive neuropsycho-

logical testing, MRI scanning, collection of blood, and CSF. In these at-risk subjects, DNA is screened for the familial mutation at enrollment, resulting in presymptomatic carriers and healthy controls (non-carriers) and study participants and clinical researchers are blinded to the individual carrier status. Symptomatic carriers were diagnosed with FTD according to international criteria,[15,16] and include patients diagnosed at the outpatient clinic and participants who converted to the symptomatic stage during the study. Age at onset was defined as the moment that caregivers first noted cognitive or behavioural changes.

Local ethics committees approved the study and all participants (or a legal representative) provided written informed consent.

Sample preparation and LC–MS/MS measurements

CSF was collected and processed according to standard protocols and stored at -80°C.[17] In order to maximize the number of peptides that could be detected by the LC-MS/MS system, Pierce™ Top 2 Abundant Protein Depletion Spin Columns were used to remove highly abundant proteins albumin and IgG. In short, the spin columns were equilibrated to room temperature for 20' and subsequently incubated with 50 µl CSF (thawed on ice) for 30' with vortexing every 5'. The bottom closure was removed, the cap was loosened, spin columns were placed into clean 2 ml Eppendorf tubes and centrifuged at 1000 g for 2'. The filtrate contained a final volume of 500 µl in 10 mM PBS, 0.15 M NaCl, 0.02% Na azide, pH 7.4. Reproducibility was assessed by depleting and measuring 1 pooled CSF sample in triplicate. Samples were subsequently reduced by DTT and alkylated by IAA.[18] Samples were dried using a SpeedVac concentrator, reconstituted in 50 µl 0.1% RapiGest and digested overnight using 500 ng trypsin (expected ratio trypsin:protein = 1:20) in a heat block (37°C) under continuous shaking (350 rpm). To stop digestion, 4 µl 50% TFA/50% water solution was added. The sample was then incubated for 45' at 37°C and spun down using a table top Eppendorf centrifuge at maximum rpm (approximately 10,000 g). Samples were analyzed by LC-MS/MS in a randomized order using an Ultimate 3000 nano RSLC system (Thermo Fischer Scientific, Germering, Germany) coupled online to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific). Twelve microliter of each tryptic digest was loaded onto a C18 trap column (C18 PepMap, 300 µm ID x 5 mm, 5 µm particle size, 100 Å pore size (Thermo Fisher Scientific) and desalted for 10' using 0.1% TFA in water at a flow rate of 20 µl/min. The trap column was switched online with the 50 cm long analytical column (PepMap C18, 75 µm ID x 500 mm, 2 µm particle size, 100 Å pore size (Thermo Fisher Scientific)) and peptides were eluted using a 90' binary gradient of eluent A (0.1% formic acid in ultrapure water) and eluent B (80% acetonitrile and 0.08% formic acid in ultrapure water). The column flow rate was set to 300 nL/min (oven temperature: 40°C) and a spray voltage of 1.5 kV was applied. For MS/MS analysis a data dependent acquisition method was used; a high resolution survey scan from 400 - 1600 m/z was performed in the Orbitrap. Based on a survey scan the 5 most intense ions were consecutively isolated and fragmented for MS/MS analysis. Precursor masses within a tolerance range of +/- 3 ppm

that were selected once for MS/MS were excluded for subsequent MS/MS fragmentation for 60 seconds.

Peptides were identified and assigned to proteins by exporting features, for which MS/MS spectra were recorded (Bioworks software package (version 3.3; Thermo Fisher Scientific, peak picking by Extract_msn, default settings)) and submitting the resulting .mgf file to Mascot Daemon (version 2.5, Matrix Science, London, UK) for interrogation of the UniProt database (release v151112; taxonomy: Homo sapiens, containing 20,194 sequences). Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine as a variable modification, allowing a maximum of 1 missed cleavage. Mass tolerance for precursor ions was set to 10 ppm and for fragment ions at 0.5 Da. Mascot Daemon .DAT files were loaded into Scaffold Proteome Software (version 4.8.3) and a Scaffold peptide export file (1% FDR), containing peptide identification data, was generated. Mascot Daemon search results and Scaffold peptide/protein identification files were imported into Progenesis QI software to link the identified peptides to the detected abundances. Only ions with charge states between +2 and +8 were considered and only proteins with at least two unique peptides (Mascot ions sore >25, (i.e. a peptide probability cut off value of 0.01)) assigned to them were accepted as true identifications. The peptide and protein abundances were normalized to the total ion current to compensate for experimental variations using an algorithm available in the analysis software. Subsequently, the data were exported in Excel format and analyzed using Excel and SPSS statistical software.

Statistical analysis

Demographic and clinical data were compared by Kruskal-Wallis tests for continuous data and Chi-Square tests for categorical data.

For the peptide analysis, a non-parametric test (Wilcoxon rank-sum test) was used since the raw peptide abundances were not normally distributed. The following group comparisons were performed: 1) presymptomatic versus non-carriers; 2) symptomatic versus non-carriers; 3) symptomatic versus presymptomatic carriers. Corresponding proteins were regarded as significantly differentially abundant when they satisfied the following criteria, as described before[19], with minor adjustments: 1) the protein is identified by 2 or more peptides; 2) 25% or more of the peptides of the protein are significant at $p<0.01$; 3) 50% or more of the peptides of the protein are significant at $p<0.05$; 4) 75% or more of the peptides are changed in the same direction between the groups (i.e. up- or downregulated). Statistical background levels were determined by permutation tests on all samples and all identified peptides/proteins. For each of the three group comparisons, the permutation test was performed on the according dataset by random assignment of sample group and repeated 1000 times, after which the resulting thresholds were saved (mean ± standard deviation). On protein level, similar permutation tests were performed and repeated 10 times. The number of differentially abundant proteins was regarded as significant when the observed number in the true analysis exceeded the threshold from the permutation analysis: mean

+ three times the standard deviation. Fold changes based on median abundances were calculated for all group comparisons on peptide levels and peptides with a median of zero were excluded. Next, protein fold changes were calculated by the mean of corresponding peptide fold changes. Candidate proteins for further validation were selected on the number of identified peptides (≥4), combined with the fold change of ≤0.5 or ≥2.0.

Results

Subject characteristics

Symptomatic, presymptomatic- and non-carriers did not differ in age at CSF collection or gender (Table 1). Symptomatic carriers had a median age at onset of 58 years, and the median time between onset and CSF collection was 2.3 years (range 0.8-6.4 years).

Table 1. Subject characteristics

	Symptomatic *GRN* mutation carriers[a]	Presymptomatic *GRN* mutation carriers	Non-carriers	*p*-value
Number	9	19	24	n/a
Gender, male (%)	3 (33%)	9 (47%)	14 (58%)	0.42
Age at CSF collection, years (IQR)	58 (53-60)	56 (47-60)	51 (40-58)	0.09
Age at symptom onset, years (IQR)	57 (51-58)	n/a	n/a	n/a
Time between onset and CSF collection, years (IQR)	2.3 (1.5-3.6)	n/a	n/a	n/a

[a]Seven symptomatic carriers presented as a behavioural variant frontotemporal dementia and two as a non-fluent variant primary progressive aphasia. Continuous variables are presented as medians.
CSF, cerebrospinal fluid; IQR, interquartile range; n/a, not applicable.

LC–MS/MS proteome profiling

A total of 4539 unique peptides were identified within the entire dataset, corresponding to 572 unique proteins, of which 503 were identified by 2 or more peptides (Figure 1). The results of the permutations and differentially abundant peptides are presented in Supplementary Table 1.

No significant differences were found between presymptomatic carriers and non-carriers, as the number of differentially abundant proteins did not exceed the number of background hits.

Comparison of data from symptomatic with presymptomatic carriers (volcano plot of peptides in Figure 2) resulted in nine differentially expressed proteins (background 1.4 ± 2.2), with higher abundancies in four and lower abundancies in five (Figure 1, Table 2). Neuronal pentraxin receptor (NPTXR) was measured by six peptides and had a fold change of 0.39, and thus fulfilled the proposed criteria for further validation (Table 2, Figure 3).

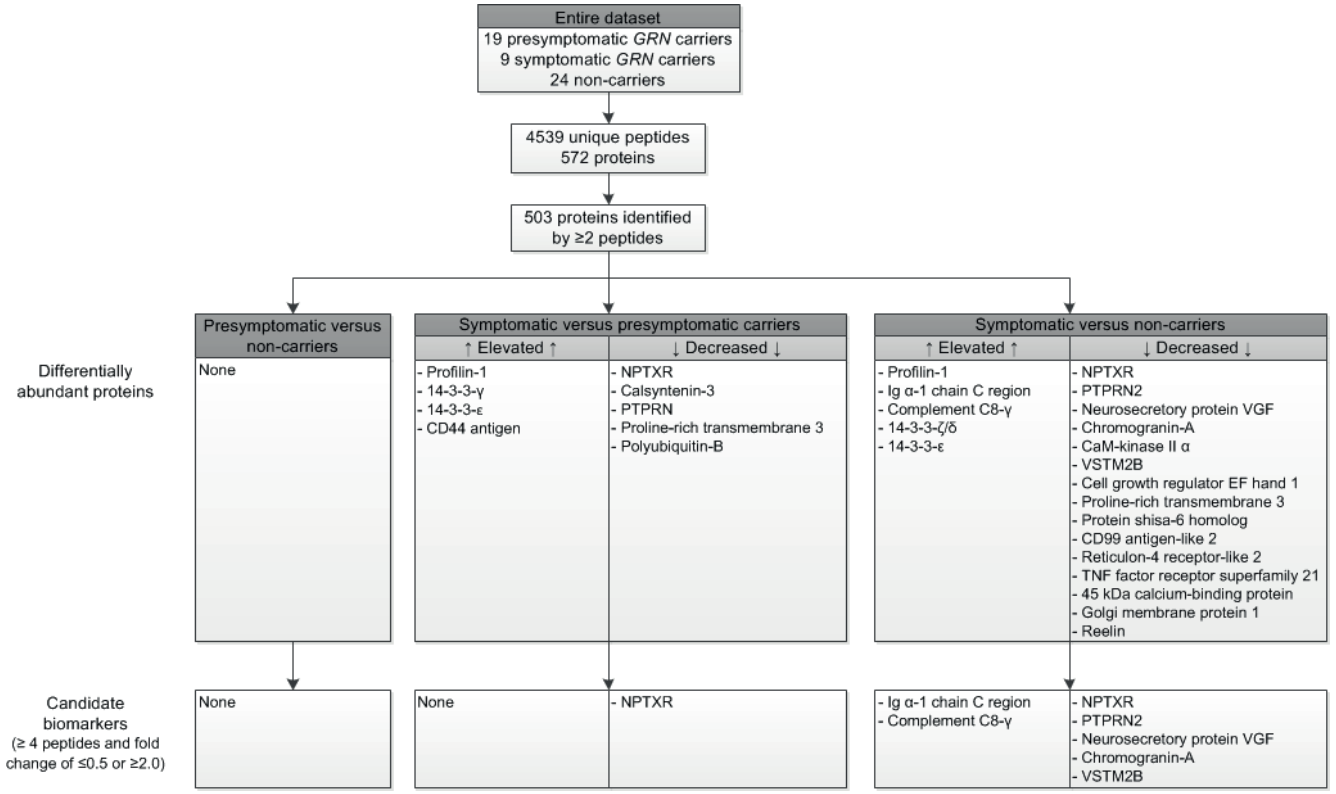

Figure 1. Flowchart of differentially abundant proteins.

The number of identified peptides and proteins are displayed and are then split to the differentially abundant proteins per group comparison: 1) presymptomatic versus non-carriers, 2) symptomatic versus presymptomatic carriers, and 3) symptomatic versus non-carriers. In the lower row, proteins are displayed that fulfill the proposed criteria for a candidate biomarker and thus warrant further validation.

CaM: Calcium/calmodulin-dependent; NPTXR: neuronal pentraxin receptor; PTPRN: receptor-type tyrosine-protein phosphatase-like N; PTPRN2: receptor-type tyrosine-protein phosphatase N2; TNF: tumor necrosis factor; VSTM2B: V-set and transmembrane domain-containing protein 2B.

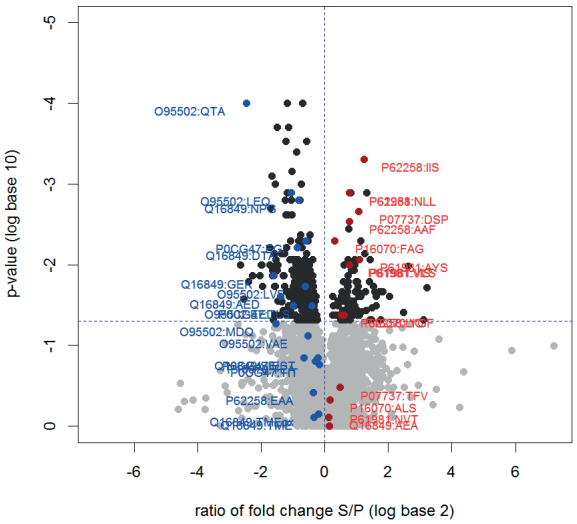

Figure 2. Vulcano plot showing differentially abundant peptides in symptomatic compared to presymptomatic carriers.

Significance of individual peptides (y-axis, log-10 scale) plotted by fold change (x-axis, log-2 scale) when comparing symptomatic (S) to presymptomatic carriers (P). Peptides from proteins that are significantly differentially abundant based on the described criteria are labeled (protein primary accession number and first three aminoacids of the corresponding peptide). Positive fold changes mean higher peptide levels in symptomatic carriers than presymptomatic carriers (peptides in red) and vice versa (peptides in blue are higher abundant in presymptomatic carriers). The dashed horizontal line shows the threshold for significant results (*p*-value=0.05).

Table 2. Differentially abundant proteins in symptomatic versus presymptomatic carriers

Protein (accession number)	Peptides, *n*	*p*-value <0.01, %	*p*-value <0.05, %	Peptides changed in same direction, %	Fold change (symptomatic / presymptomatic)
Higher abundant in symptomatic than presymptomatic carriers					
Profilin-1 (P07737)	2	50	50	100	1.68
14-3-3 protein gamma (P61981)	5	40	80	100	1.59
14-3-3 protein epsilon (P62258)	5	60	80	80	1.40
CD44 antigen (P16070)	4	25	50	75	1.11
Lower abundant in symptomatic than presymptomatic carriers					
Neuronal pentraxin receptor (O95502)	6	33	67	100	0.39
Calsyntenin-3 (Q9BQT9)	2	50	100	100	0.45
Receptor-type tyrosine-protein phosphatase-like N (Q16849)	8	25	50	88	0.56
Proline-rich transmembrane protein 3 (Q5FWE3)	2	50	50	100	0.68
Polyubiquitin-B (P0CG47)	4	25	50	100	0.78

Proteins are displayed that were significantly abundant between symptomatic and presymptomatic carriers, based on: ≥2 peptides identified, ≥25% of the peptides with a $p<0.01$, ≥50% of the peptides with a $p<0.05$, ≥75% of the peptides changed in the same direction.

Bolded are proposed candidate proteins for further validation (based on: at least 4 identified peptides and a fold change of ≤0.5 or ≥2.0).

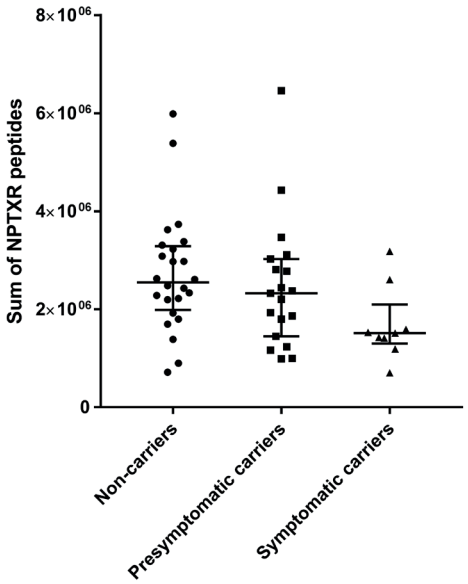

Figure 3. Neuronal pentraxin receptor (NPTXR) levels.
The sum of the six individual peptide abundancies of NPTXR in cerebrospinal fluid are plotted by non-carriers, presymptomatic carriers, and symptomatic carriers. Horizontal lines represent the median and interquartile range.

Comparing symptomatic to non-carriers showed 20 differentially abundant proteins (background 1.5 ± 2.0). Five proteins were more abundant in symptomatic carriers, of which Ig α-1 chain C region and complement component C8 γ-chain are proposed as candidate biomarkers since they had a fold change ≥2.0 and at least four identified peptides (Figure 1, Table 3). Fifteen proteins were less abundant in symptomatic carriers than in non-carriers, including NPTXR, receptor-type tyrosine-protein phosphatase N2 (PTPRN2), neurosecretory protein VGF, chromogranin-A, and V-set and transmembrane domain-containing protein 2B as most promising candidate biomarkers (Figure 1, Table 3).

Two proteins, NPTXR and profilin-1, were differentially abundant in both comparisons (symptomatic versus presymptomatic carriers, and symptomatic versus non-carriers), although the latter did not fulfill our arbitrary criteria for a candidate marker, since only two unique peptides were identified.

Discussion

In this discovery approach using high resolution proteomics, we identified several differentially expressed proteins in CSF of *GRN*-associated FTD-patients. These proteins are involved in different pathways, such as synaptic plasticity, secretory processes, immunity and cytoskeletal structures, and are candidate biomarkers to measure disease activity.

The most interesting candidate is NPTXR, identified by six unique peptides, which was decreased in symptomatic versus presymptomatic carriers, ánd versus non-carriers. According to the proposed criteria (i.e. ≥4 identified peptides and a fold change of ≤0.5 or

Table 3. Proteins significantly differentially abundant in symptomatic versus non–carriers

Protein (accession number)	Peptides, *n*	*p*-value <0.01, %	*p*-value <0.05, %	Peptides changed in same direction, %	Fold change (symptomatic/ non-carriers)
Higher abundant in symptomatic than non-carriers					
Profilin-1 (P07737)	2	100	100	100	2.47
Ig alpha-1 chain C region (P01876)	6	33	67	100	2.39
Complement component C8 gamma chain (P07360)	4	25	50	100	2.00
14-3-3 protein zeta/delta (P63104)	6	67	83	100	1.98
14-3-3 protein epsilon (P62258)	5	40	60	80	1.22
Lower abundant in symptomatic than non-carriers					
Neuronal pentraxin receptor (O95502)	6	67	100	100	0.34
Receptor-type tyrosine-protein phosphatase N2 (Q92932)	5	60	80	100	0.35
Neurosecretory protein VGF (O15240)	21	38	90	100	0.45
Chromogranin-A (P10645)	18	44	61	100	0.46
Calcium/calmodulin-dependent protein kinase type II alpha (Q9UQM7)	2	50	100	100	0.48
V-set and transmembrane domain-containing protein 2B (A6NLU5)	4	50	50	100	0.49
Cell growth regulator with EF hand domain protein 1 (Q99674)	4	50	50	100	0.54
Proline-rich transmembrane protein 3 (Q5FWE3)	2	50	100	100	0.57
Protein shisa-6 homolog (Q6ZSJ9)	2	50	100	100	0.60
CD99 antigen-like protein 2 (Q8TCZ2)	8	38	50	100	0.65
Reticulon-4 receptor-like 2 (Q86UN3)	4	25	50	75	0.69
Tumor necrosis factor receptor superfamily member 21 (O75509)	5	40	60	80	0.70
45 kDa calcium-binding protein (Q9BRK5)	2	50	50	100	0.73
Golgi membrane protein 1 (Q8NBJ4)	8	25	50	88	0.75
Reelin (P78509)	11	27	55	82	0.76

Proteins are displayed that were significantly abundant between symptomatic and presymptomatic carriers, based on: ≥2 peptides identified, ≥25% of the peptides with a $p<0.01$, ≥50% of the peptides with a $p<0.05$, ≥75% of the peptides changed in the same direction.
Bolded are proposed candidate proteins for further validation (based on: at least 4 identified peptides and a fold change of ≤0.5 or ≥2.0).

≥2.0), it was the only candidate biomarker discriminating between the presymptomatic and symptomatic stage of *GRN* mutations. NPTXR is involved in synaptic plasticity,[20] and has been identified in proteomics studies as a progression biomarker in AD, with elevated levels in mild cognitive impairment and low levels in AD patients.[21–24] Similar dynamics seem to take place in autosomal dominant AD since NPTXR levels were higher in presymptomatic- compared to non-carriers,[13] an effect we did not observe in our presymptomatic *GRN* carriers. This discrepancy may result from differences in underlying pathophysiology, the lower sensitivity of our unbiased semi-quantitative proteomics approach, or because we studied presymptomatic carriers of all ages and thus of varying time from onset.

Neurosecretory protein VGF and chromogranin A were lower in symptomatic carriers than non-carriers, and belong to the granin protein family, involved in large dense-core vesicle biogenesis and regulated secretion.[25] These proteins are precursors of peptides with numerous biological functions, including microglial activation (chromogranin A) and synaptic plasticity.[26,27] Decreases of both proteins were also found in proteomics studies in AD, likely reflecting synaptic degeneration.[13,21–24,28] The current observed decrease in synaptic markers is an interesting finding, since there is increasing evidence that synaptic dysfunction may contribute to the pathogenesis of FTD, especially in *GRN* mutations,[8,29,30] a pathophysiological concept that previously was primarily recognized for AD.[29,31] In rat hippocampal neurons, knocking down PGRN decreases synapse density,[32] and in *GRN*-knockout mice, PGRN-deficiency causes synaptic dysfunction,[33] likely through activation of microglia which leads to synaptic pruning.[30]

We found lower levels of receptor-type tyrosine-protein phosphatase-like N (PTPRN, alias: IA-2) in symptomatic compared to presymptomatic carriers. PTPRN is a transmembrane protein present in dense-core vesicles, implicated in secretory processes in the pancreatic islets, but also in the brain,[34] and is also decreased in AD patients.[22] Although strictly PTPRN did not fulfill proposed candidate criteria because of a fold change of 0.56, a protein family member (PTPRN2) was also lower in symptomatic carriers versus non-carriers. These proteins also play more general roles in secretion of hormones and neurotransmitters, and knock-down of these proteins result in behavioural and learning impairments in mice.[34]

Ig α-1 chain C region and complement component C8 γ-chain, both implicated in immunity, were elevated in symptomatic carriers compared to non-carriers. The first protein is part of immunoglobulin heavy chains of antibodies and thus involved the adaptive immunity,[35] but this might be an artificial finding, since most immunoglobulins were removed by the depletion step. The latter protein is a constituent of the innate immunity by being part of the membrane attack complex.[36] An important role for inflammatory pathways in FTD is supported by prior studies that identified YKL-40, complement factors and interleukines as candidate biomarkers for FTD.[11,30,37–42] For *GRN*-mutations specifically, PGRN is implicated as an anti-inflammatory protein and haploinsufficiency results in lysosomal dysfunction, complement production and microglial activation.[30]

The last candidate protein we identified is V-set and transmembrane domain-containing protein 2B, this is a membrane protein but its exact function has scarcely been studied.

Two protein groups did not fulfill our criteria for candidate markers, but are interesting based on their function, and will therefore be shortly discussed. First, profilin-1 was found to be elevated in symptomatic carriers, both in comparing to presymptomatic and to non-carriers. Profilin-1 binds to actin, is essential for the structure of the cytoskeleton and mutations in the encoding gene are a cause of familial amyotrophic lateral sclerosis.[43] However, only 2 peptides of this protein were identified in our experiment, arguing the robustness of this finding. Secondly, we found several 14-3-3 protein isoforms to be higher abundant in symptomatic carriers. 14-3-3 is a known biomarker for Creutzfeldt-Jacob's disease, however this is an aspecific marker reflecting rapid neuronal loss and not specifically prion pathology, and can thus be increased in different neurodegenerative diseases as well.[44,45]

Methodological considerations and future directives

In this study, we investigated CSF because of its direct contact with the central nervous system, as opposed to for example blood or urine. The protein content of CSF is known to be unevenly distributed: a small number of proteins constitute the largest proportion of proteins present in CSF, and this hinders the detection and quantification of low-abundant proteins.[46] The depletion step removing albumin and IgG has considerably improved the detection of low-abundant proteins, however pathophysiological relevant proteins may bind to the column or to the depleted proteins, thereby impeding the detection of those proteins.[47] *GRN*-mutation carriers are known to have low PGRN levels, both in blood and CSF,[2,5–7] and symptomatic carriers have increased neurofilament levels.[48] We did not identify these proteins, which is likely explained by the low abundancy of these proteins in CSF (on average below 10 ng/ml), that cannot be identified by our unbiased proteomics approach even after a depletion step.[47]

Strengths of this study include the unique sample set with a large group of presymptomatic *GRN* mutation carriers, especially considering that these originate from a single center. The unbiased proteomics approach has, to the best of our knowledge, not been performed in presymptomatic versus symptomatic *GRN* mutation carriers before, and allowed identification of novel biomarkers and early pathophysiological mechanisms without predefined hypotheses, which is important for development of therapeutics. A limitation is that our symptomatic carrier group was relatively small, resulting in unbalanced numbers per group, which we overcame by applying non-parametric statistical tests. We did not identify novel biomarkers in the presymptomatic phase, despite the relatively large group. Studying presymptomatic mutation carriers close to disease onset would be interesting, albeit challenging since disease onset in these individuals is not yet known, and difficult to estimate due to large variations even within families.

The next step for these candidate markers, is verification by a second technique to eliminate false-positive results and confirm the differential abundance in a more quantitative way.[49] As described, we applied criteria based on the effect size and the number of detected unique peptides, to select candidate markers for this next phase. We expect that applying these quite stringent criteria results in less false-positive proteins, than when we would verify all proteins identified with the shotgun proteomics. Verification can be performed by ELISA or parallel reaction monitoring (PRM) – a targeted proteomics approach – and the absolute quantification makes it possible to explore correlations with clinical features (e.g. survival, disease severity, age as proxy for approaching symptom onset in presymptomatic carriers).

The protein signature we identified in symptomatic *GRN* mutation carriers in this discovery approach largely overlaps with those previously identified in AD proteomics studies.[13,21–24,28] This may imply that overlapping candidate markers reflect neuronal and synaptic loss as opposed to FTD-*GRN* specific pathophysiological changes. Therefore – if future verification is successful – it will be important to validate these candidates in broader patient groups, including AD patients, presymptomatic and symptomatic carriers of other FTD-causing mutations. Further steps in the process of implementing these candidates to the clinical practice, will be validation in independent cohorts and to assess longitudinal changes.

To conclude, we have identified seven candidate CSF markers in a unique cohort of presymptomatic and symptomatic *GRN*-associated FTD. These candidates are functionally implicated in various processes, including synaptic function (e.g. NPTXR), secretory processes (e.g. PTPRN2), and immunity. Further verification and validation is needed to determine whether these candidates can be developed into clinically useful biomarkers.

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Supplementary material

Supplementary Table 1. Differentially abundant peptides versus permutation test per group comparison.

	Observed, *n*	Permutation, mean ± standard deviation
Presymptomatic versus non- carriers	54	41 ± 58
Symptomatic versus presymptomatic carriers	66	35 ± 48
Symptomatic versus non-carriers	194	45 ± 65

Presented are the observed number of differentially abundant peptides per group comparison (first column), and the background hits derived by 1000x permutation (second column).

CHAPTER 4

General Discussion

Frontotemporal dementia (FTD) is an early-onset dementia, predominantly characterized by behavioural disturbances (behavioural variant FTD [bvFTD]) and/or language deterioration (primary progressive aphasia [PPA], including semantic variant PPA [svPPA] and nonfluent variant PPA [nfvPPA]). The clinical spectrum also comprises phenotypes with prominent motor problems: FTD with concomitant motor neuron disease (MND), corticobasal syndrome (CBS), and progressive supranuclear palsy (PSP). FTD is highly heritable and 10–20% of all cases are caused by autosomal dominant mutations in either *MAPT* (microtubule-associated protein tau), *GRN* (progranulin), or *C9orf72* (chromosome 9 open reading frame 72) repeat expansions.[1] Postmortem brain examination shows frontotemporal lobar degeneration (FTLD) with inclusions of either tau protein (FTLD-tau), TAR DNA-binding protein 43 (TDP-43; FTLD-TDP), or FET (fused in sarcoma, Ewing's sarcoma and TAT-binding protein-associated factor 15; FTLD-FET).

This disease is devastating for patients and their relatives, and therefore therapeutic interventions are critically needed. Currently, only symptomatic treatments are applied that have not been designed for FTD, nor properly studied with placebo-controlled trials.[2] Key problem in FTD is its heterogeneity on multiple levels: in symptomatology, age at onset, underlying genetics and underlying pathology. As we enter an era of disease-modifying interventions for neurodegenerative diseases, it is of utmost importance to overcome this heterogeneity and be able to appropriately select and monitor patients in treatment trials. Various biomarkers can aid in this process by virtue of their different applications (contexts of use), e.g. as diagnostic, staging, pharmacodynamic or prognostic markers. In this thesis, we have investigated the utility of biomarkers in cerebrospinal fluid (CSF) and blood – fluid biomarkers – across the FTD spectrum. Specifically, the aims were to:

1. investigate the application of neurofilament light chain (NfL) across the sporadic FTD spectrum (**Chapter 2**); and
2. identify and study the value of blood and CSF biomarkers in genetic forms of FTD (**Chapter 3**).

In Table 1, the different fluid biomarkers studied in this thesis are summarized by their application in specific subtypes within the FTD spectrum. This chapter presents the results of our studies in perspective of existing knowledge, discusses methodological considerations and suggests future directives.

1. Neurofilament light chain – a multipurpose biomarker

Neurofilaments are specific to neurons, constitute the main components of their cytoskeleton, and are composed of light, medium and heavy subunits.[3] Beside having important structural and functional roles in maintaining axonal integrity and transport, neurofilaments also play important roles in the synaps.[4] The results of this thesis illustrate different potential applications for NfL (Table 1), as discussed below.

Table 1. Biomarkers studied in this thesis summarized by their application in specific FTD subtypes

		Diagnostic				Staging		Prognostic	Pharmacodynamic
Biomarker	FTD subtype	Vs controls	Clinical subtypes	Pathological subtypes	Genetic subtypes	Disease severity	Pre- versus symptomatic	Survival	Target engagement
NfL	Entire spectrum	+	±	-	±	+		+	
	svPPA	+				±		-	
	PSP	+				+		+	
	Genetic FTD[a]	+			±	+	+	+	
p/t-tau ratio	Entire spectrum	+	±	+	±	+		+	
PGRN	*GRN* mutation carriers	+					-		+
Poly(GP)	*C9orf72* expansion carriers	+	-			-	±	-	+

The differential diagnosis with different dementia's was outside the scope of this thesis, and thus not included.
+: can be used for this application; ±: differences were found, but no or limited applied value; -: cannot be used for this application; blank cells are not studied in this thesis or not applicable.
[a]Including subjects with *C9orf72* repeat expansions who were also studied separately.
C9orf72: chromosome 9 open reading frame 72 gene; FTD: frontotemporal dementia; *GRN:* granulin gene; NfL: neurofilament light chain; PGRN: progranulin protein; PSP: progressive supranuclear palsy; p/t-tau: phospho-tau$_{181}$ to total-tau; svPPA: semantic variant primary progressive aphasia

1.1 NfL in blood strongly correlates with CSF

We showed a strong correlation between NfL levels in serum and CSF, in line with other reports in FTD and different neurodegenerative diseases.[5–10] The discovery of a blood-based biomarker is exciting and stimulating for research, since blood markers are highly advantageous over those in CSF; their collection is less invasive and therefore more patient-friendly and applicable for frequent measurements, collection is cost- and time-effective and feasible at the population level.[11]

Since most studies have shown similar performances between blood- and CSF-derived NfL, results from these compartments are discussed together below. Of note however, a few studies suggested that CSF NfL levels are elevated earlier than blood – since it is hypothesized that most blood NfL derives from the central nervous system – and therefore slightly more sensitive, especially in subjects with narrow ranges of levels (e.g. controls).[12,13]

1.2 NfL as a diagnostic biomarker

Throughout this thesis it becomes clear that elevated NfL levels, either in blood or CSF, can discriminate all different FTD subtypes from controls. In our large clinical cohort comprising the entire FTD spectrum as encountered in the day-to-day practice, we show that patients with FTD-MND can be distinguished by their extraordinary high CSF NfL levels, but other

clinical forms show similar levels. Although patients with bvFTD had higher CSF NfL levels than PSP patients, and *GRN*-patients higher NfL levels than *C9orf72*- and *MAPT*-patients, the overlap in concentrations does not allow diagnostic differentiation of these subtypes using NfL. In PPAs, serum NfL may have a diagnostic role since svPPA and nfvPPA (variants caused by FTLD) patients had higher levels than those with the logopenic variant (often caused by Alzheimer's disease [AD] pathology).[14] Additionally, high blood NfL distinguishes atypical parkinsonian disorders – including PSP – from patients with Parkinson's disease;[10] and high CSF NfL differentiated bvFTD from primary psychiatric disorders, often presenting with similar symptoms.[15]

NfL is a rather aspecific neuronal injury marker and elevated in a range of neurological diseases, including (but not limited to) FTD, AD, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and traumatic brain injury.[7,16–19] NfL will therefore be of limited value – apart from the above-mentioned exceptions – in the differential diagnosis between different dementia types, but will be rather used as a staging and prognostic marker (see below). In addition, one could envision using serum NfL as a screening tool to identify patients with neurodegeneration, but first longitudinal population-based series would be needed to determine cut-offs.[20]

1.3 NfL increases with symptom onset in genetic FTD

Predicting and establishing disease onset in genetic FTD is notoriously challenging due to the high variability in onset age and the presence of early subtle symptoms. It is therefore of great help that CSF and serum NfL levels were normal in our presymptomatic carriers and elevated in the symptomatic stage, and thus can serve as a staging marker of disease onset. These results are in line with those in presymptomatic genetic ALS,[21] but contrast to three more recent reports.[12,22,23] First, slightly elevated serum NfL levels were shown in presymptomatic carriers of AD-causing mutations, and NfL associated with estimated years to onset.[22] This difference might be explained by the underlying pathophysiology in AD, characterized by a more gradually progressive preclinical phase with declining CSF amyloid-β levels 20-25 years before estimated onset and brain atrophy at 15 years before estimated onset.[24,25] While in FTD – at least for *GRN* mutations – a rather explosive start of disease is suggested, as exemplified by the steep increase of NfL levels in our two converters, and the emergence of white matter integrity and grey matter volume loss between 4 and 2 years before symptom onset (manuscript under review).[26] Secondly, a study in *CHMP2B* (a rare FTD-causing gene) mutation carriers, found elevated CSF NfL levels in presymptomatic compared to non-carriers, however this finding did not persist after correction for age, a known confounding factor for NfL.[23] Thirdly, in a mouse model of three neurodegenerative proteopathies (tau, amyloid-β and α-synuclein pathology), CSF NfL increased in parallel or slightly before protein deposits became visible, and increased before symptom onset.[12] Again, this difference might be explained by the underlying pathophysiology: a model

for TDP-43 pathology was not studied, while most of our mutation carriers were *GRN* or *C9orf72* carriers.

In presymptomatic *C9orf72* expansion carriers, grey and white matter damage has been shown particularly early,[27–30] even before the age of 40.[31] Moreover, several symptomatic *C9orf72* expansion carriers with a remarkable slow disease progression have been described (so-called phenocopies),[32–34] leading to the hypothesis that *C9orf72*-mediated pathology has a neurodevelopmental origin, with early deficits that progress very slowly. However, some cross-sectional studies do suggest a specific time point in onset of grey matter loss, after the age of 40 in one study,[29] and 25 years before estimated onset in the Genetic FTD Initiative (GENFI).[27] To elucidate this matter, it will be interesting to investigate longitudinal NfL measurements together with neuroimaging, to determine whether NfL remains stable over time which would support the developmental hypothesis.

1.4 NfL as a disease severity biomarker

NfL associated with disease severity in sporadic FTD, PSP, and genetic FTD. In line with this, we showed in genetic FTD that NfL associates with severity of atrophy, as also reported for sporadic FTD.[16] This makes NfL a promising staging biomarker to quantify severity both in clinical practice and research setting, and use as a monitoring marker/surrogate endpoint. Although we also found associations of NfL with severity of atrophy and naming impairment in svPPA, these correlations were only moderate and may therefore be of limited use. Measuring disease severity and progression is difficult in the individual patient because of the phenotypical heterogeneity in the FTD spectrum, and therefore we used different assessments across this thesis. For svPPA, the Boston Naming Test was used, since naming difficulties are the hallmark of svPPA;[35,36] PSP is characterized by parkinsonism, frequent falls and more global cognitive disturbances[37] and thus we studied corresponding functional, motor and cognitive scales; lastly, we used the Clinical Dementia Rating Scale – a more general measure – in the large genetic and sporadic cohorts with mixed phenotypes. Clinical trials should always measure clinically meaningful outcomes. Although FTD-focused scales have been developed (e.g. the Frontotemporal Dementia Rating Scale[38] and the FTLD -modified Clinical Dementia Rating[39]), it is still challenging to capture the entire spectrum of FTD symptoms due to the phenotypical heterogeneity . Since NfL seems to be a uniform severity marker – except for svPPA – it could complement clinical assessments as outcome measure.

1.5 NfL as a prognostic biomarker

In sporadic FTD, PSP, and genetic FTD, we have shown in well-characterized large cohorts that higher NfL levels (either in serum or CSF) are associated with a shorter survival after biomarker collection. For the FTD spectrum, we also presented 5-year survival rates that are valuable information for individual patients and their relatives to customize treatment plans. Additionally, NfL associated with progression of atrophy in genetic FTD, as has been

shown in sporadic FTD as well,[40] and NfL levels predicted clinical disease progression in PSP.[6,41]

However, svPPA is the exception to this rule; we did not find an association with survival or progression of atrophy, suggesting that (cross-sectional) NfL may not predict clinical progression in svPPA patients, at least not in the short term. The pathophysiological background is completely unclear; a possible explanation might be that a plateau phase of language impairment and atrophy is reached, resulting in stable or even decreasing NfL levels unrelated to the relatively long survival in svPPA.[42,43] Or perhaps changes in NfL are indicative of disease progression.

1.6 NfL as a monitoring biomarker

The associations of NfL with disease severity, atrophy, and survival showed that NfL is a promising monitoring biomarker, which is supported by two neurodegenerative mouse models.[12,44] Blocking amyloidβ-lesions in a β-amyloidosis model attenuated the increase in NfL levels.[12] In a different model, further increases of NfL were prevented when neurodegeneration was switched off after initial induction.[44] Furthermore, in human intervention studies, a dynamic decrease in both serum and CSF NfL after treatment has been proven, for example in MS.[18,45] Future trials can thus benefit from this biomarker, not only as surrogate endpoint to track disease progression, but also by creating homogeneous study populations regarding disease severity and progression and therefore better powered clinical trials.

1.7 Application of NfL

Overall, in the current clinical setting I envision measuring NfL at time of diagnosis 1) to support a neurodegenerative cause of disease and help in the differential diagnosis of e.g. psychiatric disorders, parkinsonism and PPAs, and 2) to predict survival (with the exception of svPPA patients). NfL levels can be measured in serum when underlying AD pathology is not suspected, otherwise in CSF along with the core AD biomarkers. Serum NfL measurements can readily be used in clinical trials, however not yet in the clinical practice since the added clinical value remains to be proven (see Future directions).

2. The phosphotau/tau ratio to predict underlying proteinopathy *in vivo*

Apart from using NfL as biomarker, future clinical trials will likely focus on a specific proteinopathy, and therefore predicting underlying pathology *in vivo* is paramount. We showed in 67 patients with known pathology based on autopsy or genetic mutation, that the phospho- to total tau (p/t-tau) ratio discriminated FTLD-TDP from FTLD-tau with a sensitivity of 67% and a specificity of 76%, while NfL levels did not. In addition, the p/t-tau ratio discriminated FTD patients from controls, associated with disease severity and pre-

dicted survival; however, these findings mirrored those in NfL, and since NfL can reliably be measured in blood, the p/t-tau ratio will unlikely have added value in those applications.

The pathophysiological mechanism underlying decreased p/t-tau ratios in FTLD-TDP remains to be elucidated, since the driving factor of a lower ratio (elevated total tau, or decreased phospho-tau levels), is inconsistent between previous reports.[46–51] Total tau levels can be elevated due to extensive neuronal loss, especially in the FTD-MND patients; on the other hand, low p-tau levels (the driving factor in our study) may be explained by a low tau burden in FTLD-TDP.[48]

It should be noted that p/t-tau ratio's in FTLD-TDP considerably overlapped with the FTLD-tau group. Both sensitivity and specificity should exceed 85% to be classified as a valid biomarker in AD,[11] which was not reached in our cohort, nor in three different cohorts.[47,49,50] Thus, more candidate biomarkers to discriminate underlying proteinopathy are needed. CSF tau levels do not aid in this discrimination: they are not increased in FTLD-tau patients compared with patients with tau-negative or sporadic FTD.[52,53] Plasma tau levels are elevated in FTD, but application for identifying proteinopathy remains to be investigated.[54] TDP-43 is a candidate marker being higher in both FTD and ALS than controls;[55] however up till now, conflicting results are found and quantification remains challenging.[50,56–58]

3. Genetic–specific biomarkers

In addition to accurate prediction of underlying pathology, genetic FTD offers the key advantage to study presymptomatic subjects. Evidence from autosomal dominant AD and Huntington's disease suggested that biomarkers change years before symptom onset, implying that the disease starts before onset and treatment should be initiated prior to symptoms when neuronal damage is still limited.[25,59] Our FTD Risk Cohort at the Erasmus MC and the international GENFI consortium are designed to study biomarkers in genetic FTD from the presymptomatic to the symptomatic stage, by longitudinally following first-degree relatives of mutation-carriers. A presymptomatic phase is now convincingly proven in FTD, providing information on the earliest pathophysiological changes and facilitating the search for biomarkers identifying pathology onset and the optimal therapeutic window.[60] Different genotypes cause specific biomarker alterations, which will be discussed in this section.

3.1 Dipeptide repeat proteins in C9orf72 repeat expansions

In Chapter 3.2, we studied biomarkers in *C9orf72* repeat expansion carriers, who mainly present with FTD and/or ALS. Patients typically have hundreds to thousands hexanucleotide repeats, but repeat size is variable across different tissues within the same person, and it is not possible to exactly quantify large expansions.[61,62] Three mechanisms have been postulated how *C9orf72* expansions are pathogenic:

1. a loss-of-function through haploinsufficiency of the C9orf72 protein;

and/or a toxic gain-of-function through:

2. RNA toxicity from bidirectional transcripts of the repeat that form RNA foci, or
3. repeat-associated non-ATG (RAN) translation resulting in five different dipeptide repeat proteins (DPRs): poly(GA), poly(GR), poly(GP), poly(PA), and poly(PR) – where G refers to glycine, A to alanine, R to arginine, and P to proline.

To discern these pathogenic mechanisms is important, as most therapeutic options currently under development target the gain-of-function mechanisms.[63] Loss-of-function now seems the most unlikely all-explaining hypothesis, since corresponding models show immune related problems, but no neurodegeneration.[64] The sequestration of RNA foci impairs the normal function of various RNA-binding proteins in *C9orf72* repeat expansions.[65] However, no association has been shown between the location of RNA foci and the clinicopathological variability like disease subgroup (FTD and/or ALS).[66] On the other hand, DPR accumulation is widespread across the brain of *C9orf72*-associated FTD and ALS, even before symptom onset,[67–70] and is unrelated to neuronal loss or clinical symptoms.[71,72] In line with this, we showed that elevation of CSF poly(GP) – one of the DPRs – is an exclusive and early phenomenon in *C9orf72* expansion carriers. Also poly(GP) was not associated with indicators of neurodegeneration (i.e. NfL and grey matter atrophy). Although these observations may point toward no pathological effect of the DPRs, in functional studies especially the arginine containing DPRs were toxic,[65] and a human autopsy study identified an association of poly(GR) with disease anatomy including TDP-pathology.[63] Perhaps early effects are caused by DPR and/or RNA toxicity, followed by TDP-43 dysfunctioning and neurodegeneration in a later stage.[73] It would be ideal if DPRs in the brain could be visualized *in vivo*, for example by PET ligands, to investigate cause and consequence in the pathomechanisms of *C9orf72* expansions,[74] since post-mortem tissue only allows studying end-stage features.

In summary, there are supporting arguments for all three hypotheses, and perhaps a combination of these and other factors causes the disease.[74] Although the gain-of-function mechanisms are difficult to study separately, antisense oligonucleotides (ASOs) and other small molecules will block both. To test the efficacy of these therapeutic agents, it is paramount to measure target engagement after blocking the formation of repeat RNA and RAN translation. Previously, ASO-treatment was shown to alleviate pathological *C9orf72*-features along with CSF poly(GP) levels in mice and human cell models of *C9orf72* expansions, and thus poly(GP) is a promising pharmacodynamic biomarker.[75] We showed that poly(GP) is elevated in *C9orf72*-associated FTD patients, and also in presymptomatic expansion carriers, in line with two prior reports.[75,76] This is important for the design of future trials and enables including presymptomatic carriers, who likely benefit the most of disease modifying interventions. The variation in poly(GP) levels between investigated subjects could not be explained by clinical features. Poly(GP) levels may depend on vari-

ous factors, including the size of the repeat expansion, the efficacy of RAN translation and clearance mechanisms. Therefore, poly(GP) will especially be useful to monitor poly(GP) expression intra-individually as a read out of RAN translation activity over time.

3.2 Progranulin levels in GRN mutation carriers

Autosomal dominant *GRN* mutations causing FTLD lead to haploinsufficiency and therefore reduced progranulin protein (PGRN) levels in both blood and CSF. PGRN controls neuronal function, suppresses neuroinflammation and has an important role in lysosomes.[77] Various agents can elevate PGRN levels in experimental models through different ways including gene therapy, increasing PGRN transcription from the wildtype allele, or by enhancing the bioavailability of PGRN by preventing its degradation.[77] A recent *GRN*$^{+/-}$ mice study has shown that boosting PGRN levels corrected behavioural deficits.[78] Until now, two agents advanced to clinical trials (as registered in ClinicalTrials.gov): both nimodipine and a histone deacetylase inhibitor (FRM-0334) did not elevate PGRN levels in *GRN* mutation carriers,[79] although the results of the latter have not yet been published.

The trial design for PGRN enhancing agents will be facilitated by our findings of stable plasma PGRN levels over one week time, and only a moderate correlation between plasma and CSF PGRN levels. Previous studies in subjects without *GRN* mutations found similar results,[80–83] but it is essential to assess this biomarker in *GRN* mutation carriers. The consistent finding of differentially regulated PGRN levels between blood and CSF, means that blood sampling could measure systemic target engagement, but CSF collections are needed to measure target engagement in the central nervous system.

Additionally, we showed that PGRN levels can detect *GRN* mutations, which is especially important when effective treatments come to hand. Although genotyping remains the golden standard, plasma PGRN levels can serve as a quick and cheap tool to screen large groups of patients, or to assess the pathogenicity of variants of unknown significance in *GRN*.

Much is still unknown about why PGRN levels greatly vary between individuals. In subjects without *GRN* mutations, PGRN levels seem to increase with age, and differ between males and females;[81] an effect we and others did not find in *GRN* mutation carriers.[84,85] Single nucleotide polymorphisms as rs5848, rs646776, and rs1990622 have proven to influence PGRN levels both in controls and *GRN* mutation carriers. The latter two are located near *SORT1* and *TMEM106B* respectively, two genes implicated in lysosomal function and affecting PGRN pathways. Furthermore, the rs1990622 major allele is associated with inflammation and neuronal loss especially in the frontal cortex, and with cognitive deficits, even in controls.[86] It is therefore surprising that we found an association between the minor allele of rs1990622 and lower plasma PGRN levels, while before higher levels have been reported.[87,88] A recent large study did not find an association between plasma PGRN levels and rs1990622 in 141 subjects, illustrating the complexity of the regulation of PGRN levels.[89]

3.3 Discovery of novel biomarkers by proteomics in GRN mutation carriers

Although major advances have been made towards discovering biomarkers, we still cannot identify disease onset on a pathophysiological level in presymptomatic mutation carriers before symptom onset. Therefore, we also aimed to identify novel biomarkers by applying a proteomics approach on CSF from presymptomatic and symptomatic *GRN* mutation carriers. Proteomics' major advantages are the unbiased investigation, which has proven to be successful in FTD before,[90] and measuring analytes directly, overcoming the pitfalls of indirect measuring with antibodies.[11]

We identified multiple proteins that were differentially abundant between symptomatic *GRN* carriers and either presymptomatic- or non-carriers; seven fulfilled our proposed criteria for a candidate biomarker. The most interesting candidate was neuronal pentraxin receptor, being downregulated in patients compared to presymptomatic- ánd non-carriers. This protein regulates signaling in excitatory and inhibitory synapses and is thus implicated in synaptic plasticity.[91] It is also a potential progression biomarker in AD, even in the pre-dementia stage,[92–95] and although we did not find altered levels in presymptomatic carriers, this will be interesting to further investigate with more sensitive techniques. Also neurosecretory protein VGF and chromogranin A, both involved in synaptic plasticity, were decreased supporting an important role for synaptic dysfunction in *GRN* mutations. Furthermore, we detected proteins involved in secretory processes, immunity and cytoskeletal structures, reflecting the pathophysiology of *GRN* mutations, which may provide new targets for treatment. It is now important to verify and validate these results by a second technique and in different patient cohorts, to determine the specificity of our findings and whether these proteins can function as monitoring biomarkers.

4. Towards implementing fluid biomarkers

In general, strengths of the studies in this thesis are well-characterized large cohorts, encompassing the entire FTD spectrum, and the multimodal approach combining biomarkers. Specific strengths and limitations are discussed in the respective chapters. This paragraph describes - using experiences from our studies - how I envision the ideal study design for FTD biomarkers and what should be studied to move forward in the transition from bench to bedside.

4.1 The ideal study design

4.1.1 Sample size and multicenter cohorts

Since FTD is a rare disease, it is difficult to collect sufficient data in studies of clinical or genetic subtypes. Multicenter cohorts are needed to overcome these power problems, but we have to face a number of challenges. The ideal multicenter study should use harmonized protocols for prospective and longitudinal data collection that can be easily shared,

encompassing clinical and neuropsychological data, fluid and neuroimaging biomarkers, and also autopsy data. Biomarkers should be measured in one laboratory, preferably in one batch. It is of great importance that FTD consortia (e.g. GENFI, ARTFLL and LEFFTDS) are now operating and use harmonized research protocols, enabling large multimodal studies and validation of biomarkers. Also, these cohorts form valuable platforms for upcoming clinical trials, providing a large ready cohort of diverse subjects who can potentially participate in trials (see below).

4.1.2 Sampling methods and timing

The FTD research can learn much from the AD and MS fields, in which major strides in standardizing and harmonizing biomarker procedures have been made(e.g. the BiomarkAPD, SOPHIA and BioMS projects). Collection methods, storage tubes and temperature, diurnal variation, and number of freeze-thaw cycles can influence biomarker levels,[11,96] underscoring the importance of standardized sampling methods. Also the interval between different procedures (collection of fluid biomarkers, neuroimaging and neuropsychological data) should be as short as possible and it is at least recommended to collect blood and CSF samples on the same day.

4.2 What should be studied?

4.2.1 Verification and clinical validation of fluid biomarkers

In order to implement a novel biomarker, four steps are needed: (1) candidate discovery, (2) qualification, (3) verification, and (4) clinical validation.[97,98] To determine which novel candidates from Chapter 3.4 justify the effort and expense of full validation, next steps are qualification, using a targeted technique to confirm the differential expression, followed by verification in larger cohorts including different genetic forms and dementia subtypes.[97]

The studied biomarkers NfL, p/t-tau ratio, PGRN and poly(GP) are in the phase of clinical validation, a complex and time consuming process. Reference intervals of healthy individuals and identification of factors influencing biomarker measurement (e.g. preanalytical factors, and biological variability within- and between-subjects like age or fasting) are needed to develop cutoff criteria that can be used in individual patients.[97] Furthermore, the results should be replicated in independent study cohorts and multi-center studies, before its possible clinical implementation.[98,99] In the research setting of clinical trials, NfL, PGRN and poly(GP) can be readily applied; however, implementation in the clinical practice first requires prove of an added value for individual patients. Preventing asynchronous research can accelerate this clinical validation by aligning and prioritizing research projects, for example by creating an overview of gaps in evidence for FTD biomarkers, using a framework as recently proposed for AD.[99]

4.2.2 Longitudinal studies

Knowledge on fluctuations of biomarkers over time are necessary for future application in clinical trials. For NfL, a few longitudinal studies in ALS, PSP and PPA, have shown either increasing or stable levels over time,[7,100–103]while these data in bvFTD and genetic FTD are still lacking. Longitudinal serum NfL studies in presymptomatic genetic FTD – including converters – would answer whether changes in yearly NfL levels predict the onset of symptoms and indeed measure conversion to the dementia stage on an individual level. By studying biomarker changes within a person (delta's), sensitivity might be increased by filtering out interpersonal differences, also it could yield important insights into disease biology. It will also be informative to know whether the increasing poly(GP) levels found in a few presymptomatic *C9orf72* expansion carriers could be replicated, and to establish whether these subjects are closer to disease onset.

To detect non-linear changes it is important to study three or more time points in longitudinal studies. Linear models are not applicable to most neurodegenerative disease,[104] as illustrated by a sigmoid model in AD that showed a deceleration of atrophy after the initial acceleration.[105] For FTD, acceleration of neuronal loss is supported a dramatic NfL increase in two converters (Chapter 3.1), and by the only subtle presymptomatic neuroimaging and neuropsychological abnormalities that become prominent in the symptomatic stage of genetic FTD.[29,106–108] More longitudinal multimodal data on non-converters, converters, as well as sporadic FTD, will help elucidating the sequence of pathological events approaching symptom onset and thereafter.

4.2.3 Combining biomarkers

The association of fluid biomarkers with different modalities as clinical or neuroimaging data allows us to study the clinical value of individual biomarkers (e.g. NfL as proxy for disease severity) and provides valuable insights into underlying pathophysiological mechanisms (e.g. poly(GP) does not associate with indicators of neurodegeneration).

The pathophysiological complexity of FTD implicates that a single biomarker cannot capture all changes, thus multiple biomarkers reflecting different disease processes should be studied in concert. In *C9orf72*-associated FTD, CSF poly(GP) might be used as a target engagement marker, complementary with NfL as a surrogate endpoint. In sporadic FTD, a lumbar puncture early in the process can exclude AD pathology and may predict underlying pathology by determining the p/t-tau ratio, followed by less invasive serum NfL measurements to monitor disease progression over time. The most common hypothesis is that a specific sequence of biomarker abnormalities occurs – at least for genetic FTD – starting with fluid biomarkers, followed by connectivity imaging markers, grey matter atrophy, and finally clinical metrics.[60] Combining these modalities will likely improve pinpointing the disease stage of a gene carrier facilitating timing of treatment, and improve diagnostic accuracy (clinically and pathologically) in sporadic FTD, especially in the early stages of disease when current clinical tools are often insufficient.[109]

4.2.4 Studying treatment options

Two tau-directed treatments, davunetide in PSP[110] and TRx0237 in bvFTD[111], were unfortunately unsuccessful, but have proven that large international trials are feasible in these rare diseases.[2]

Recent advances in understanding FTLD biology have led to the identification of new treatment targets, that hopefully lead to disease modification and prevent or stop disease progression. ASOs are promising and feasible agents for various targets, already used in clinical practice for spinal muscular atrophy.[112] An tau targeting ASO with the goal to reduce human tau expression (thus not directed to mutant tau), prevented disease progression in mice.[113] This ASO is currently under study in mild AD patients,[114] and if this approach proves to be successful, it will hopefully be a treatment option for other tauopathies, including FTLD-tau and PSP.

The genetic forms of FTD may provide a proof-of-principle of treatability of neurodegenerative diseases, since treatment can be initiated before extensive neuronal damage (i.e. presymptomatically). Neurodegenerative diseases resulting from gain-of-function mutations, are ideal targets for gene suppression therapy, a fast progressing field. Post-transcriptional inhibition may modify disease in *C9orf72* repeat expansion carriers, since a single-dose ASO injection reduced RNA foci, DPR accumulation and behavioural deficits in mice,[64] and first clinical trials are expected to start in 2018.[115] The discovery of CRISPR/Cas9 is a major recent breakthrough in medicine, enabling accurate edits of the genome and perhaps correction of mutations in the future.[112]

4.2.5 The importance of pathological confirmation

An important problem remains that underlying pathology in sporadic FTD patients cannot be accurately predicted, while treatments will focus on specific molecular pathologies. Diffusion tensor imaging (DTI) shows more severe loss of white matter integrity in tau than in TDP pathology,[116,117] but this is not yet applicable in individual patients. Although tau PET (positron emission tomography) imaging is a promising strategy to discriminate tau from TDP and other proteinopathies, off-target binding to non-tau proteins is a problem and further development of specific ligands is needed.[118,119] New approaches should thus be employed to identify this “holy grail”, and may be found in combining known neuroimaging (e.g. grey matter and DTI), fluid (e.g. NfL, p/t-tau) and genetic biomarkers to multimodal predictors. Unbiased approaches by means of proteomics or microRNA sequencing,[120] especially in exosomes in blood or CSF that are regarded as enriched sources of biomolecules, may also be helpful to identify new biomarkers .

Another issue is that co-pathology frequently occurs and relevant levels of AD co-pathology are reported in 10-20%.[48,121–123] Co-pathology with TDP-43, tau and α-synuclein does not only occur in older populations, but is already present in young (≤65 years) patients.[123] Co-pathology is important for two reasons: first, it can influence biomarkers that cause misinterpretations. For example, patients with FTD ánd AD co-pathology may have

abnormal core AD biomarkers that lead to a misdiagnosis of frontal AD.[122,124] Secondly, it has important implications for future treatments, since targeting a single protein may not be effective in patients with co-pathology.[124] The effort to inform patients about the importance of brain autopsy for research, should be encouraged since the knowledge we acquire from pathological studies remains invaluable.

5. Conclusions

We are on the verge of an exciting era for neurodegenerative diseases, since the pathophysiological knowledge acquired in the past is actively translated to developing therapeutic interventions. To expedite the development and testing of these disease-modifying agents, biomarkers that aid in selecting appropriate patients and measure efficacy are paramount. This thesis has shown that NfL is a promising biomarker in CSF and in blood across the entire FTD spectrum, both in sporadic and genetic forms. NfL levels are higher in patients than controls and can thus be used to ascertain disease. Also, NfL could monitor disease severity and progression and predicts survival – valuable information for the individual patient – in most subtypes within the FTD spectrum, except for svPPA. To select patients on underlying proteinopathy, the p/t-tau ratio may be useful being decreased in FTLD-TDP versus FTLD-tau, but more discriminative markers are needed. Our CSF proteomics study in *GRN* mutations identified potential novel biomarkers and provides knowledge of the pathophysiology of *GRN* mutations. Lastly, for FTD caused by *GRN* mutations or *C9orf72* repeat expansions, PGRN and poly(GP) respectively identify mutation carriers and are target engagement markers for clinical trials that are underway. Our results facilitate the implementation of these biomarkers for clinical or research purposes, and provide additional understanding of the pathophysiological process underlying FTD, opening new avenues towards treating FTD.

6. References

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CHAPTER 5

Summary & Samenvatting

Summary

Frontotemporal dementia (FTD) is a heterogeneous neurodegenerative disorder, comprising a spectrum of subtypes that are different on the clinical, genetic and pathological level. Clinically, symptoms typically present before the age of 65 and include behavioural and/or language disturbances, yet also motor problems frequently occur. FTD can be heritable, and 10-20% of the patients have an autosomal dominant form, which is most frequently caused by a mutation in granulin (*GRN*, also known as progranulin), in microtubule-associated protein tau *(MAPT),* or a repeat expansion in chromosome 9 open reading frame 72 *(C9orf72)*. Pathological examination shows frontotemporal lobar degeneration (FTLD) with inclusions of either TAR DNA-binding protein 43 (FTLD-TDP), tau (FTLD-tau), or FET (fused in sarcoma, Ewing's sarcoma and TAT-binding protein-associated factor 15).

Currently, major advances are being made to develop therapeutic interventions for FTD. However, the heterogeneity of this disorder hampers the diagnostic process, tracking of disease progression, and the appropriate selection of patients for clinical trials. Reliable biomarkers are therefore critically needed. This thesis investigated the utility of biomarkers in cerebrospinal fluid (CSF) and blood, so-called fluid biomarkers, across the entire FTD spectrum.

Chapter 1 introduces the aims and studies of the thesis (**Chapter 1.1**), and reviews current knowledge on neuroimaging and fluid biomarkers in FTD (**Chapter 1.2**). In the past decade, major strides have been made to identify biomarkers for FTD, which are increasingly important for upcoming treatment trials. Magnetic resonance imaging of grey matter atrophy, PET-imaging of cerebral hypometabolism, and CSF biomarkers are currently used in the clinical practice to diagnose FTD versus other types of dementia. Progress is being made in the identification of genetic specific markers and biomarkers for disease staging, prediction of underlying pathology, and monitoring of treatment response. Yet, harmonized multicenter studies are important to validate these new biomarkers before they can be used in individual patients.

Chapter 2 describes the utility of neurofilament light chain (NfL), a promising biomarker reflecting neuronal damage and loss, in sporadic forms of FTD. In **Chapter 2.1**, we studied the clinical value of NfL and the phospho- to total tau ratio (p/t-tau) in CSF, in a well-defined cohort of 361 patients. The entire FTD spectrum was investigated: behavioural variant FTD, semantic variant of primary progressive aphasia (svPPA), non-fluent variant PPA, logopenic variant PPA, FTD with motor neuron disease (FTD-MND), corticobasal syndrome, and progressive supranuclear palsy (PSP). NfL concentrations were high in all FTD patients compared with controls, highest in FTD-MND, but did not differentiate between other clinical subtypes. The p/t-tau ratio mirrored the results, being lower in patients than controls, and lowest in FTD-MND. Both high NfL and low p/t-tau ratio levels predicted a poor survival. Importantly, the p/t-tau ratio, but not NfL, discriminated FTLD-TDP from FTLD-tau pathology.

In **Chapter 2.2**, we assessed the utility of NfL specifically for svPPA. CSF NfL was measured in 162 patients with svPPA from 14 international sites, and correlated to clinical characteristics, neuropsychological metrics, grey matter atrophy on MRI-scans, and survival. CSF NfL was higher in patients with svPPA than in controls, and high levels were only moderately associated with more severe naming problems and smaller grey matter volumes of the parahippocampal gyri. Unlike in other FTD subtypes however, CSF NfL concentrations were not related to progression of grey matter atrophy and did not predict survival, which suggests a limited utility of NfL in svPPA.

In **Chapter 2.3**, we describe the value of NfL in PSP, by investigating serum NfL and clinical characteristics of 131 patients. NfL concentrations were twice as high in patients with PSP than in controls, and correlated with worse functional, motor and cognitive performance. Additionally, higher NfL levels were associated with shorter survival. These results indicate that serum NfL is a promising monitoring and prognostic biomarker for patients with PSP.

Chapter 3 focuses on blood and CSF biomarkers in genetic forms of FTD. **Chapter 3.1** provides evidence that CSF NfL levels reflect disease onset in genetic FTD, since levels were low in a large international group of 40 presymptomatic mutation carriers and strongly elevated in 86 patients with genetic FTD. Moreover, longitudinal samples in two subjects who converted from the presymptomatic to symptomatic stage showed a three- to four-fold increase of CSF NfL after symptom onset. Importantly, serum NfL correlated strongly with CSF NfL, and was similarly elevated in patients compared to presymptomatic carriers. This implicates that blood sampling might replace lumbar punctures for this biomarker. Additionally, NfL concentrations in patients correlated with disease severity, brain atrophy, progression of atrophy, and survival.

Chapter 3.2 reports an international study on CSF NfL and poly(GP) in 25 presymptomatic and 64 symptomatic *C9orf72* repeat expansion carriers, and 12 non-carriers. Poly(GP) is one of the five dipeptide repeat proteins that are translated from the *C9orf72* repeat expansion and may therefore be an interesting pharmacodynamic marker. Poly(GP) levels were present in carriers and absent in non-carriers, and were slightly higher in symptomatic than presymptomatic carriers. NfL levels were higher in symptomatic than presymptomatic carriers, and correlated with disease severity, grey matter atrophy, and survival. Poly(GP) did not associate with disease severity, survival, or indicators of neurodegeneration (i.e. NfL and grey matter volume). This study showed that NfL and poly(GP) are promising complementary biomarkers for clinical trials in *C9orf72* repeat expansion carriers, where poly(GP) can measure target engagement and NfL disease activity and progression.

In **Chapter 3.3**, we measured progranulin protein levels in plasma and CSF in families with *GRN* mutations, since these mutations reduce progranulin levels and increasing progranulin is thus a promising treatment strategy. Plasma progranulin levels were lower in all *GRN* mutation carriers than in controls, already in the presymptomatic stage. Progranulin in plasma was relatively stable over five measurements in one week, and therefore seems suitable to monitor the effect of progranulin-enhancing medications. However, plasma

could not accurately predict CSF progranulin, and therefore lumbar punctures are additionally needed in clinical trials for *GRN* mutation carriers.

In **Chapter 3.4** we aimed to identify novel candidate biomarkers for FTD caused by *GRN* mutations, by means of proteomics on CSF. We found no differences between 19 presymptomatic carriers and 24 healthy non-carriers, but identified seven candidate biomarkers for the symptomatic stage (9 patients were included). The candidates were neuronal pentraxin receptor, receptor-type tyrosine-protein phosphatase N2, Ig alpha-1 chain C region, neurosecretory protein VGF, chromogranin-A, V-set and transmembrane domain-containing protein 2B, and complement component C8 gamma chain. These proteins have roles in synaptic plasticity, secretory processes and inflammation. Further studies are needed to validate the results in larger and more diverse sample sets.

Overall, this thesis has expanded the knowledge on how different fluid biomarkers can be applied across the FTD spectrum. **Chapter 4** places the main results of this thesis in perspective of existing literature, and provides suggestions for future research.

Samenvatting

Frontotemporale dementie (FTD) is een heterogene neurodegeneratieve aandoening en omvat een spectrum van subtypes die verschillen op klinisch, genetisch en pathologisch niveau. Wat betreft het klinische beeld, treden symptomen van gedragsveranderingen en/of taalproblemen typisch op voor het 65e levensjaar, maar ook motorische problemen komen vaak voor. FTD kan erfelijk zijn en 10-20% van de patiënten met FTD heeft een autosomaal dominante vorm, welke meestal wordt veroorzaakt door een mutatie in granuline (*GRN,* ook bekend als progranuline), in microtubule-associated protein tau (*MAPT*) of een repeat expansie in chromosome 9 open reading frame 72 (*C9orf72*). Pathologisch onderzoek toont frontotemporale lobaire degeneratie (FTLD) met eiwit-inclusies van TAR DNA-binding protein 43 (FTLD-TDP), tau (FTLD-tau), of FET (fused in sarcoma, Ewing's sarcoma and TAT-binding protein-associated factor 15).

Momenteel wordt er grote vooruitgang geboekt in het ontwikkelen van therapieën voor FTD. Echter, de heterogeniteit van het ziektebeeld belemmert het diagnostische proces, het meten van ziekteprogressie, en het identificeren van geschikte patiënten voor medicijnonderzoek. Daarom zijn betrouwbare meetinstrumenten, biomarkers, hard nodig. Dit proefschrift onderzocht het gebruik van biomarkers in hersenvocht en bloed, zogenaamde fluïde biomarkers, voor het gehele FTD spectrum.

Hoofdstuk 1 introduceert de doelen van dit proefschrift (**Hoofdstuk 1.1**), en vat de huidige kennis op het gebied van imaging en fluïde biomarkers samen (**Hoofdstuk 1.2**). Het afgelopen decennia zijn er grote stappen gemaakt in het identificeren van biomarkers voor FTD, welke van toenemend belang zijn voor opkomende behandelingsonderzoeken. Magnetische resonantie beelden (MRI) van grijze stof atrofie, PET-scans en hersenvocht biomarkers worden gebruikt in de huidige klinische praktijk, om de diagnose FTD te stellen en onderscheiden van andere vormen van dementie. Vooruitgang wordt geboekt in het identificeren van genetisch-specifieke markers, biomarkers voor ziektestadiëring, het vaststellen van onderliggende pathologie, en het monitoren van behandelingseffect. Nu zijn geharmoniseerde multicenter studies belangrijk om deze nieuwe biomarkers te valideren, voordat ze kunnen worden toegepast in individuele patiënten.

Hoofdstuk 2 beschrijft het nut van neurofilament light chain (NfL), een veelbelovende biomarker die zenuwschade en –verlies meet, in sporadische (niet-genetische) vormen van FTD. In **Hoofdstuk 2.1** onderzochten wij de klinische waarde van NfL en de phospho- op totaal-tau ratio (p/t-tau) in hersenvocht, in een goed gekarakteriseerd cohort van 361 patiënten. Het gehele FTD spectrum werd bekeken: gedragsvariant FTD, semantische variant van primair progressieve afasie (svPPA), niet-vloeiende variant PPA, logopenische variant PPA, FTD met motorneuronziekte (FTD-MND), corticobasaal syndroom, en progressieve supranucleaire parese (PSP). NfL concentraties waren hoog in alle patiënten in vergelijking met controles, het hoogste in FTD-MND, maar kon andere klinische subtypes niet onderscheiden. De p/t-tau ratio toonde gespiegelde resultaten, zijnde lager in patiënten dan controles en het laagst in FTD-MND. Zowel hoge NfL als lage p/t-tau gehaltes konden

overleving voorspellen. Een belangrijke bevinding tot slot was dat p/t-tau, maar niet NfL, het onderscheid tussen FTLD-TDP en FTLD-tau pathologie kon maken.

In **Hoofdstuk 2.2** beoordeelden we het nut van NfL specifiek voor svPPA. NfL werd gemeten in hersenvocht van 162 patiënten met svPPA uit 14 internationale centra, en we onderzochten de samenhang met klinische karakteristieken, neuropsychologische maten, grijze stof atrofie op MRI-scans, en overleving. NfL was hoger in patiënten met svPPA dan in controles, en hogere concentraties hingen slechts matig samen met ernstigere benoemproblemen en kleinere grijze stof volumes van de parahippocampale gyri. Anders dan in ander FTD subtypes waren NfL gehaltes in hersenvocht niet gerelateerd aan toename van atrofie of aan overleving, dit geeft aan dat NfL een beperkt toegepast nut heeft in svPPA.

In **Hoofdstuk 2.3** beschrijven we de waarde van NfL in PSP, door het bloed van 131 patienten te onderzoeken in relatie tot klinische eigenschappen. NfL gehaltes waren twee keer zo hoog in patiënten met PSP dan in controles, en correleerden met slechtere functionele, motorische en cognitieve prestaties. Bovendien waren hoge NfL gehaltes geassocieerd met een kortere overleving. Deze resultaten laten zien dat NfL in bloed een veelbelovende biomarker is voor monitoring van ziekte-ernst en prognose in patiënten met PSP.

Hoofdstuk 3 richt zich op biomarkers in bloed en hersenvocht in erfelijke vormen van FTD. **Hoofdstuk 3.1** toont bewijs dat NfL concentraties in hersenvocht de start van ziekte kunnen meten in erfelijke FTD, aangezien gehaltes laag waren in een grote internationale groep van 40 presymptomatische mutatiedragers en sterk verhoogd in 86 patiënten met erfelijke FTD. Bovendien toonden longitudinale samples in twee personen die converteerden van de presymptomatische naar de symptomatische fase, een drie- tot viervoudige toename van NfL na het begin van de symptomen. Een zeer belangrijke bevinding is dat NfL in bloed sterk correleerde met NfL in hersenvocht, en vergelijkbaar verhoogd was in patiënten ten opzichte van presymptomatische dragers. Dit duidt aan dat bloedafnames mogelijk ruggenprikken kunnen vervangen voor deze biomarker. Tot slot correleerden NfL gehaltes in patiënten met ziekte-ernst, atrofie van de hersenen, progressie van atrofie, en overleving.

Hoofdstuk 3.2 beschrijft een internationale hersenvochtstudie naar NfL en poly(GP) in 25 presymptomatische en 65 symptomatische *C9orf72* repeat expansie dragers, en 12 niet-dragers. Poly(GP) is een van de vijf dipeptide repeat eiwitten die gevormd worden door de *C9orf72* repeat expansie, en zou daarom een interessante farmacodynamische biomarker kunnen zijn. Poly(GP) gehaltes waren aanwezig in dragers en afwezig in niet-dragers, en iets verhoogd in symptomatische ten opzichte van presymptomatische dragers. NfL concentraties waren hoger in symptomatische dan presymptomatische dragers, en hingen samen met ziekte-ernst, grijze stof atrofie en overleving. Poly(GP) correleerde niet met klinische eigenschappen, survival of maten van neurodegeneratie (NfL en grijze stof volume). Deze studie toont dat NfL en poly(GP) veelbelovende biomarkers zijn die elkaar aan kunnen vullen in klinische trials voor *C9orf72* repeat expansie dragers, waarbij poly(GP) target engagement (het biologische effect) kan meten en NfL ziekteactiviteit en –progressie.

In **Hoofdstuk 3.3** hebben we concentraties gemeten van het progranuline-eiwit in bloed en hersenvocht van families met *GRN* mutaties, aangezien deze mutaties de progranuline gehaltes verlagen en daarom is het verhogen van deze gehaltes een veelbelovende behandelingsoptie. Bloed progranuline concentraties waren lager in alle *GRN* mutatiedragers dan in controles, reeds in de presymptomatische fase. Progranuline in bloed was relatief stabiel over vijf meetmomenten in één week, en lijkt daarom geschikt om het effect van progranuline-verhogende middelen te monitoren. Progranulinegehaltes in bloed konden die in hersenvocht niet betrouwbaar voorspellen, en daarom zijn ruggenprikken ook nodig in medicijnonderzoek bij *GRN* mutatiedragers.

Hoofdstuk 3.4 had tot doel nieuwe kandidaat-biomarkers te identificeren voor FTD veroorzaakt door *GRN* mutaties, met behulp van proteomics op hersenvocht. Wij vonden geen verschillen tussen 19 presymptomatische dragers en 24 gezonde niet-dragers, maar vonden zeven kandidaat-biomarkers voor de symptomatische fase (9 patiënten werden onderzocht). De kandidaten zijn neuronal pentraxin receptor, receptor-type tyrosine-protein phosphatase N2, Ig alpha-1 chain C region, neurosecretory protein VGF, chromogranin-A, V-set and transmembrane domain-containing protein 2B, en complement component C8 gamma chain. Deze eiwitten spelen een rol in synaptische plasticiteit, secretoire processen en ontsteking. Verdere studies zijn nodig om deze resultaten te valideren in grotere en meer diverse patiëntengroepen.

In het algemeen heeft dit proefschrift de kennis uitgebreid over hoe verschillende fluïde biomarkers kunnen worden toegepast in het gehele FTD spectrum. **Hoofdstuk 4** plaatst de belangrijkste bevindingen van dit proefschrift in het perspectief van de bestaande literatuur, en geeft suggesties voor toekomstig onderzoek.

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Curriculum Vitae

Hielkje Heleen Meeter (Lieke) was born on April 15, 1987 in Pijnacker. She graduated 'cum laude' from secondary school at the Dalton in Voorburg. In 2005, she started Medical school at the University of Utrecht, and she undertook internships to Singapore and Peru. After obtaining her medical degree in 2011, she worked at the Diakonessenhuis in Utrecht and in Zeist, as medical doctor in Neurology (2012-2013) and in Internal Medicine and Geriatrics (2013-2014). Her interest was drawn most to patients with neurodegenerative disorders, and therefore she applied for a research project at the Alzheimer Center ErasmusMC (department of Neurology), Rotterdam. In 2014 she started her PhD project on fluid biomarkers in Frontotemporal Dementia, under supervision of Prof. dr. J.C. van Swieten, dr. L. Donker Kaat, and dr. Y.A.L. Pijnenburg. In her research projects, she collaborated with the VU medical center and the Genetic Frontotemporal Dementia Initiative (GENFI). Lieke currently lives in Rotterdam with her partner Imre, and started as a Neurology resident at the ErasmusMC in april 2018.

PhD Portfolio

1. PhD training	Year	ECTS
General courses		
Biostastical Methods 1: Basic Principles part A (NIHES)	2014	2
Good Clinical Practice	2014	1.5
Biomedical English Writing and Communication	2015	3
Research Integrity	2015	0.3
Course on R	2015	1.4
Personal Efficacy Training	2016	1
Specific courses		
CME course Neuropathology of Neurodegenerative Diseases	2014	1
Principles of Genetic Epidemiology (NIHES)	2014	0.7
SNPs and Human Diseases (MolMed)	2014	2
Biomedical research Techniques (MolMed)	2014	1.5
Conferences and seminars		
9th International Conference on Frontotemporal Dementias (poster presentation)	2014	1
European Human Genetics Conference (poster presentation)	2015	1
Mix & Match meeting Alzheimer Nederland, Utrecht	2014-2017	1
Dutch Neuroscience Meeting	2015	0.3
GENFI investigators meeting (oral presentation)	2016	1
10th International Conference on Frontotemporal Dementias (poster presentation)	2016	1
3rd Congress of the European Academy of Neurology (oral presentation)	2017	2
Alzheimer's Association International Conference 2017 (poster and oral presentation)	2017	2
Other		
Research meeting, weekly	2014-2018	4
Study coordination medication trial FRM-0334	2015-2016	14
Member of the Dutch "FTD Expertgroep"	2016-2018	1
Journal club, monthly	2017	1
2. Teaching		
Lectures		
Dutch FTD Caregivers (FTD Lotgenoten), 3 presentations	2014-2016	1
Symposium frontotemporale dementie, de Bilt	2015	1
Presentations department Neurology	2016-2018	2
Presentation Rotary Nieuwkoop	2018	0.3
Supervising Master's theses		
Three students	2014-2018	4.5
TOTAL		52.5

List of Publications

L.H.H. Meeter, R.M.E. Steketee, D. Salkovic, M. Vos, M. Grossman, C. McMillan, D.J. Irwin, A.L. Boxer, J.C. Rojas, N.T. Olney, A. Karydas, B. Miller, Y.A.L. Pijnenburg, F. Barkhof, R. Sanchez-Valle, Albert Lladó, S. Borrego-Écija, J. Diehl-Schmid, T. Grimmer, O. Goldhardt, A.F. Santillo, O. Hansson, S. Vestberg, B. Borroni, A. Padovani, D. Galimberti, E. Scarpini, J.D. Rohrer, I.O.C. Woollacott, M. Synofzik, C. Wilke, A. de Mendonça, R. Vandenberghe, L. Benussi, R. Ghidoni, G. Binetti, W. Niessen, H. Seelaar, L.C. Jiskoot, F.J. de Jong, L. Donker Kaat, M. del Campo, C.E. Teunissen, E.E. Bron, E. van den Berg, J.C. van Swieten. Cerebrospinal fluid neurofilament light chain has a limited value in semantic variant primary progressive aphasia. *Submitted.*

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List of Abbreviations

AAO	age at onset
Aβ	amyloid-β
$Aβ_{1–42}$ or $Aβ_{42}$	amyloid-$β_{1–42}$
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ARTFL	Advancing Research and Treatment for FTLD
ASL	arterial spin labeling
ASO	antisense oligonucleotide
AUC	area under the curve
BNT	Boston Naming Test
bvFTD	behavioural variant FTD
C9orf72	chromosome 9 open reading frame 72 gene
CaM	Calcium/calmodulin-dependent
CBD	corticobasal degeneration
CBF	cerebral blood flow
CBS	corticobasal syndrome
CDR	Clinical Dementia Rating scale
CDR-SB	Clinical Dementia Rating scale sum of boxes
CDT	Clock Drawing Test
CHMP2B	charged multivesicular body protein 2b gene
CI	confidence interval
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats-Cas9
CSF	cerebrospinal fluid
CV	coefficient of variation
CVLT	California Verbal Learning Test
DNA	deoxyribonucleic acid
DPR	dipeptide repeat
DTI	diffusion tensor imaging
ECL	electrochemiluminescence
e.g.	example given
ELISA	enzyme-linked immunosorbent assay
FA	fractional anisotropy
FAB	Frontal Assessment Battery
FDG-PET	^{18}F-fluorodeoxyglucose positron emission tomography
FLAIR	fluid-attenuated inversion recovery
FPI	FTD Prevention Initiative
FRS	frontotemporal dementia rating scale
FTD	frontotemporal dementia

FTD-CDR	FTD-modified Clinical Dementia Rating scale
FTD-CDR-SB	FTD-modified Clinical Dementia Rating scale sum of boxes
FTD-MND	frontotemporal dementia with concomitant motor neuron disease
FTLD	frontotemporal lobar degeneration
FTLD-FET	frontotemporal lobar degeneration with fused in sarcoma, Ewing's sarcoma or TAT-binding protein-associated factor 15 inclusions
FTLD-FUS	frontotemporal lobar degeneration with fused in sarcoma inclusions
FTLD-tau	frontotemporal lobar degeneration with tau inclusions
FTLD-TDP	frontotemporal lobar degeneration with TDP-43 inclusions
FTLD-UPS	frontotemporal lobar degeneration with ubiquitin proteasome system inclusions
FUS	fused in sarcoma gene
FUS	fused in sarcoma protein
GENFI	Genetic Frontotemporal dementia Initiative
GM	grey matter
GRN	granulin gene, also known as progranulin gene
HC	healthy control
HY	Hoehn and Yahr
ICV	intracranial volume
i.e.	id est
IL	interleukin
IQR	interquartile range
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LEFFTDS	Longitudinal Evaluation of Familial FTD Subjects
lvPPA	logopenic variant primary progressive aphasia
MAPT	microtubule-associated protein tau gene
MCI	mild cognitive impairment
MCP	monocyte chemotactic protein 1
MMSE	Mini-Mental State Examination
MND	motor neuron disease
MRI	magnetic resonance imaging
MS	mass spectrometry
MS	multiple sclerosis
NfL	neurofilament light chain
nfvPPA	non-fluent variant primary progressive aphasia
NINDS-SPSP	National Institute for Neurological Disorders and Society for PSP
NPA	neuropsychological assessment
NPTXR	neuronal pentraxin receptor
ns	not significant
PD	Parkinson's disease

PET	positron emission tomography
PGRN	progranulin protein
PiB	Pittsburgh compound B
Poly-GP	glycine-proline-repeating protein
PPA	primary progressive aphasia
PSP	progressive supranuclear palsy
PSP-RS	progressive supranuclear palsy rating scale
p-tau	phospho-tau_{181}
pTDP-43	phosphorylated TDP-43
PTPRN	receptor-type tyrosine-protein phosphatase-like N
PTPRN2	receptor-type tyrosine-protein phosphatase N2
p/t-tau	phospho-tau_{181} to total-tau ratio
R^2	coefficient of determination
RAVLT	Rey Auditory Verbal Learning Test
RAN	repeat-associated non-ATG
RD	radial diffusivity
ROC	receiver operating characteristics
ROI	region of interest
RNA	ribonucleic acid
r_s	Spearman's correlation coefficient
RS-fMRI	resting-state functional magnetic resonance imaging
SCWT	Stroop Color-Word Test
SEADL	Schwab and England Activities of Daily Living
Simoa	single molecule array
SNP	single nucleotide polymorphism
SOD1	superoxide dismutase 1 gene
SORT1	sortilin 1 gene
SPECT	single-photon emission computed tomography
SPSS	Statistical Package for the Social Sciences
svPPA	sematic variant primary progressive aphasia
SQSTM1	sequestosome-1 gene
T1w	T1-weighted
TARDP	TAR-DNA binding protein gene
TBK1	TANK-binding kinase 1 gene
TDP or TDP-43	transactive response DNA-binding protein 43
TIV	total intracranial volume
TMEM106B	transmembrane protein 106B gene
TMT-A	Trail-making Test part A
TMT-B	Trail-making Test part B
TNF	tumor necrosis factor

TREM2	triggering receptor expressed on myeloid cells 2
t-tau	total-tau
UPDRS-III	Unified Parkinson's disease rating scale III
YKL-40	chitinase-3-like protein 1
VBM	Voxel-Based Morphometry
VCP	valosin-containing protein gene
VSTM2B	V-set and transmembrane domain-containing protein 2B