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# PROTEIN AND MRI PROFILING OF GENETIC FRONTOTEMPORAL DEMENTIA

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# Protein and MRI profiling of genetic frontotemporal dementia

## Thesis for Doctoral Degree (Ph.D.)

By

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*“Do. Or do not. There is no try.” - Yoda*

# Popular science summary of the thesis

Frontotemporal dementia (FTD) is one of the most common forms of dementia in people under 65 years of age. There are many different types of FTD, resulting in a great variability in clinical symptoms as well as brain pathology. There is no cure for FTD and the only treatment available is to manage the symptoms. Today the diagnosis of FTD is based on a combination of clinical symptoms, such as progressive language impairments or changes in personality and behavior. However, it can be difficult to distinguish FTD from other disorders based on symptoms alone. In situations like these biomarkers are useful to ensure correct diagnosis. A biomarker is something that can be measured in the body and that is associated with a specific biological function or a disease. It can, for example, be the concentration of a certain protein measured in a blood sample or specific changes in the brain measured via magnetic resonance imaging. However, no such biomarkers have been discovered that are specific for FTD.

The aims of the studies included in this thesis were to find new biomarkers for FTD and to further our understanding of how these markers relate to various aspects of the disease. To do this, we collected blood, skin biopsies, and cerebrospinal fluid samples from individuals with a familial form of FTD and their family members. In these samples we measured the levels of hundreds of proteins. We then determined if any of these proteins were found at different levels in individuals with FTD compared to individuals without FTD, as well as which proteins differed between different forms of FTD. Furthermore, we studied how these proteins were associated with changes in the brain, both before and after the onset of symptoms. The goal is that these proteins can serve as biomarkers for FTD and aid in its diagnosis in the future or be used in clinical trials to test if a new drug is working. Since it is unlikely that a single treatment will be able to cure all the different forms of FTD these biomarkers can also be used to ensure that only the people who will benefit from the drug are enrolled in the trial.

# Populärvetenskaplig sammanfattning

Frontotemporala demenssjukdomar (FTD) är några utav de vanligaste formerna utav kognitivsjukdom hos personer under 65 år. FTD kan ta sig många olika uttryck hos olika personer, vilket ger upphov till flera typer av både kliniska symptom och sjukliga förändringar i hjärnan. Idag diagnostiseras FTD med stöd av de observerade kliniska symptomen, så som tilltagande talsvårigheter eller personlighetsförändringar. Det kan dock vara svårt att skilja på olika typer av FTD samt att skilja på FTD och andra typer utav kognitivsjukdom som till exempel Alzheimers sjukdom. Vid dessa svårtolkade situationer kan en biomarkör vara till stor nytta, men dessvärre så finns det inga biomarkörer för FTD idag. Rent allmänt så är en biomarkör är en mätbar förändring som kan kopplas till en biologisk funktion eller en specifik sjukdom. Exempel på biomarkörer kan vara nivåer av ett protein mätt i blodplasma eller specifika förändringar i hjärnan mätt via magnetisk resonanstomografi, även kallat magnetröntgen eller MR. Biomarkörer kan i vissa fall användas för att säkerställa rätt diagnos. De kan även användas för att se om ett nytt läkemedel är effektivt eller inte i kliniska behandlingsstudier.

Syftet med studierna i den här avhandlingen var att identifiera biomarkörer för FTD samt att förstå hur dessa markörer är kopplade till olika aspekter av sjukdomsförloppet. För att uppnå detta så samlade vi in blodprover, likvorprover och hudbiopsier från personer med en ärftlig from utav FTD samt från deras familjemedlemmar. Sedan mätte vi nivåerna utav hundratals proteiner i dessa prover för att se om några utav dem var högre eller lägre hos personer med FTD jämfört med kontroller. Vi tittade även på om det fanns skillnader i proteinnivåer vid olika typer av FTD samt om några utav dessa proteiner kunde kopplas till mätbara förändringar i hjärnan. Förhoppningen är att några utav dessa proteiner ska kunna fungera som biomarkörer i framtiden för att underlätta vid diagnosticeringen utav FTD, samt kunna användas som hjälpmedel i kliniska behandlingsstudier.

# Abstract

Frontotemporal dementia (FTD) is a group of neurodegenerative diseases with a wide range of symptoms such as loss of inhibition and social cognition, language impairment and motor dysfunction. Genetic FTD, characterized by mutations in one of several disease-causing genes, accounts for 10 - 30% of all cases of FTD. The most common causes for genetic FTD are repeat expansions in *C9orf72* and mutations in *GRN* or *MAPT*, but there are also many other, rarer causes. Each mutation gives rise to a specific subtype of genetic FTD. These subtypes differ not only in clinical presentation, but also in the underlying pathophysiology. To be able to study, and eventually treat, genetic FTD a thorough understanding of the genetic subtypes is crucial.

In this thesis we characterized the effects of a p.Ala417* mutation in *TBK1*, showing that it causes haploinsufficiency as well as demonstrating systemic effects on the K63 ubiquitination system. We also analyzed blood and cerebrospinal fluid samples from carriers of pathogenic mutations associated with genetic FTD to find biomarkers that can distinguish symptomatic mutation carriers from healthy controls or distinguish between the different genetic subtypes. We also studied how these biomarker candidates correlate with cortical and subcortical atrophy in genetic FTD. The results of these studies have provided a further understanding of genetic FTD as well as new biomarker candidates for several pathological processes.

## List of scientific papers

I. Khoshnood B, **Ullgren A**, Laffita-Mesa J, Öijerstedt L, Patra K, Nennesmo I, Graff C. TBK1 haploinsufficiency results in changes in the K63-ubiquitination profiles in brain and fibroblasts from affected and presymptomatic mutation carriers. J Neurol. 2022 Jun;269(6):3037-3049.

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III. **Ullgren A***, Öijerstedt L*, Olofsson J, Bergström S, Remnestål J, van Swieten JC, Jiskoot LC, Seelaar H, Borroni B, Sanchez-Valle R, Moreno F, Laforce R, Synofzik M, Galimberti D, Rowe JB, Masellis M, Tartaglia MC, Finger E, Vandenberghe R, de Mendonça A, Tirabosch P, Santana I, Ducharme S, Butler CR, Gerhard A, Otto M, Bouzigues A, Russell L, Swift IJ, Sogorb-Esteve A, Heller C, Rohrer JD, Månberg A, Nilsson P, Graff C. Altered plasma protein profiles in genetic FTD – a GENFI study. Mol Neurodegener. 2023 Nov 15;18(1):85.

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**Scientific papers not included in the thesis:**

I. Natarajan K*, **Ullgren A***, Khoshnood B, Johansson C, Laffita-Mesa JM, Pannee J, Zetterberg H, Blennow K, Graff C. Plasma metabolomics of presymptomatic PSEN1-H163Y mutation carriers: a pilot study. Ann Clin Transl Neurol. 2021 Mar;8(3):579-591.

II. Bergström S*, Öijerstedt L*, Remnestål J, Olofsson J, **Ullgren A**, Seelaar H, van Swieten JC, Synofzik M, Sanchez-Valle R, Moreno F, Finger E, Masellis M, Tartaglia C, Vandenberghe R, Laforce R, Galimberti D, Borroni B, Butler CR, Gerhard A, Ducharme S, Rohrer JD, Månberg A, Graff C#, Nilsson P#. A panel of CSF proteins separates genetic frontotemporal dementia from presymptomatic mutation carriers: a GENFI study. Mol Neurodegener. 2021 Nov 27;16(1):79.

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## List of abbreviations

| | |
|---|---|
| AAV | Adeno associated virus |
| AD | Alzheimer disease |
| ALS | Amyotrophic lateral sclerosis |
| AMPH | Amphiphysin |
| APOA1 | Apolipoprotein A1 |
| AQP4 | Aquaporin 4 |
| bvFTD | Behavioral variant frontotemporal dementia |
| C1 – C7 | Complement 1 - 7 |
| C9orf72 | Chromosome 9 open reading frame 72 |
| CD14 | CD14 Molecule |
| CHI3L1 (YKL-40) | Chitinase 3 Like 1 |
| CHIT1 | Chitinase 1 |
| CSF | Cerebrospinal fluid |
| CTSS | Cathepsin S |
| FTD | Frontotemporal dementia |
| FTLD | Frontotemporal lobar degeneration |
| GENFI | Genetic frontotemporal initiative |
| GFAP | Glial fibrillary acidic protein |
| GRN | Progranulin |
| IL1B | Interleukin 1 beta |
| lvPPA | Logopenic variant primary progressive aphasia |
| MAPT | Microtubule associated protein tau |
| MC | Mutation carrier |
| MND | Motor neuron disease |
| MRI | Magnetic resonance imaging |
| NC | Non-carrier |
| NEFH | Neurofilament heavy chain |

| | |
|---|---|
| NEFL | Neurofilament light chain |
| NEFM | Neurofilament medium chain |
| nfvPPA | Non-fluent variant primary progressive aphasia |
| NPTX1 | Neuronal pentraxin 1 |
| NPTX2 | Neuronal pentraxin 2 |
| NPTXR | Neuronal pentraxin receptor |
| PCA | Principal component analysis |
| PDYN | Prodynorphin |
| PMC | Presymptomatic mutation carrier |
| PPA | Primary progressive aphasia |
| PTPRN2 | Protein tyrosine phosphatase receptor type N2 |
| RPH3A | Rabphilin 3A |
| ROI | Region of interest |
| S100A12 | S100 calcium binding protein A12 |
| sbvFTD | Semantic behavioral variant frontotemporal dementia |
| SEC63 | SEC63 homolog, protein translocation regulator |
| SERPINA3 | Serpin family A member 3 |
| SMC | Symptomatic mutation carrier |
| SPP1 | Secreted phosphoprotein 1 |
| svPPA | Semantic variant primary progressive aphasia |
| TBK1 | TANK binding kinase 1 |
| TDP-43 | TAR DNA binding protein 43 |
| TMEM132D | Transmembrane protein 132D |
| TNR | Tenascin R |
| TREM2 | Triggering Receptor Expressed On Myeloid Cells 2 |
| TUBE | Tandem ubiquitin binding entities |
| VGF | Neurosecretory protein VGF |

# 1 Introduction

## 1.1 Frontotemporal dementia

### 1.1.1 A brief introduction to frontotemporal dementia

Frontotemporal dementia (FTD) is a collective term for a heterogeneous group of neurodegenerative brain disorders. It is one of the most common forms of dementia in individuals under the age of 65 years. However, FTD is most commonly diagnosed in individuals above the age of 65, with peak incidence occurring at age 71 (1). Clinically, FTD generally presents in one of two forms: as behavioral variant FTD (bvFTD), characterized by changes in personality and aberrant behavior, or as primary progressive aphasia (PPA), which includes a range of speech and language related impairments. However, there can be considerable overlap of symptoms in any given individual, especially later in the course of the disease. Furthermore, FTD is often associated with motor symptoms, such as parkinsonism, as well as motor neuron disease (MND) (2). FTD is a highly heritable disorder with a positive family history of dementia reported in approximately 40% of all cases and autosomal dominant inheritance found in 10 – 30% (2–4). There is currently no disease modifying treatment nor any disease specific biomarkers available for FTD.

### 1.1.2 Clinical symptoms

#### *1.1.2.1 Behavioral variant FTD*

bvFTD is the most common form of FTD and it is characterized by personality changes, disinhibition, and apathy, all of which can manifest in many different ways (1,5). For example, social disinhibition can cause the affected individual to act inappropriately, make rude or sexually inappropriate remarks, or be overly familiar with strangers. Disinhibition can also lead to impulsive and reckless behavior, such as excessive shopping or gambling, or altered dietary habits including binge eating and/or drinking (2,6). Apathy, on the other hand, manifests as loss of interest in activities that the individual once enjoyed, like their hobbies, but also as loss of interest in more everyday activities such as household chores or personal hygiene. The symptoms can also include a loss of interest in social interactions and a

tendency to show less empathy and sympathy for family and friends (2). The apathy seen in bvFTD can be mistaken for depression; however, the individual often denies any feelings of sadness (2). In fact, individuals with bvFTD often have little to no insight into their disease and might not recognize behavioral changes reported by family members (2). Eventually, individuals with bvFTD will develop other cognitive impairments, primarily executive dysfunction, which will continue to worsen as the disease progresses (6).

#### *1.1.2.2 Primary progressive aphasia*

PPA is an umbrella term that encompasses three different variants: semantic variant PPA (svPPA), non-fluent variant PPA (nfvPPA) and logopenic variant PPA (lvPPA) (2,7). svPPA is characterized by semantic aphasia and associative agnosia. Individuals with svPPA often have word-finding difficulties and impaired single word comprehension, as well as anomia for people, places, and objects (2,7). nfvPPA is characterized by slow and effortful speech together with agrammatism and impaired word-finding while single word comprehension usually remains intact (2,7). The third variant, lvPPA, is characterized by word-finding pauses and impaired working memory. This variant, while considered a subtype of PPA, is most often caused by underlying Alzheimer disease (AD) pathology (6,7). There exists a fourth language associated subtype, semantic behavioral variant FTD (sbvFTD), which has features of both svPPA and bvFTD. sbvFTD is characterized by loss of empathy and person-specific semantic knowledge together with complex compulsions and rigid thinking (8).

#### *1.1.2.3 FTD-ALS*

FTD and amyotrophic lateral sclerosis (ALS) share many clinical, neuropathological, and genetic features, to the point where the two diseases can be considered to be part of the same spectrum. ALS is a neurodegenerative motor neuron disease characterized by progressive muscle atrophy and weakness, eventually leading to respiratory failure (9). The two different syndromes often overlap, with up to 40% of all FTD cases developing motor symptoms and 12.5% developing clinical motor neuron disease (10). Similarly, 50% of all ALS cases develop FTD-like cognitive symptoms (11). Motor symptoms in FTD are more commonly associated with bvFTD than with svPPA or nfvPPA (2).

### 1.1.3 Neuropathology

The neuropathological changes of the FTD spectrum of diseases, called frontotemporal lobar degeneration (FTLD), is divided into three main subtypes named after the characteristic protein inclusions found in neurons and/or glia: FTLD-TDP, FTLD-tau, and FTLD-FET (6,12). FTLD-TDP is named after the TAR DNA-binding protein 43 (TDP-43), a protein involved in RNA processing, stabilization, and transport, which is the main component of the characteristic intracellular protein inclusions. There are five main subtypes of FTLD-TDP pathologies named A - E (13,14). The five subtypes differ in the location of the inclusions, both by cell type and cortical layer, and if the protein aggregates are ubiquitin positive or not (14). FTLD-TDP is the most common form of FTLD comprising 50% of all cases (2). FTLD-tau is characterized by neuronal and glial filamentous inclusions of hyperphosphorylated tau and accounts for 36 - 50% of all FTLD cases (2). There are three different subtypes which are based on which tau isoforms, 3 repeat, 4 repeat or both, are present in the inclusions (6). The third pathology, FTLD-FET, is less common than FTLD-TPD or FTLD-tau, making up only 5 - 10% of all cases. It is characterized by protein aggregates of the FET family of proteins and is mainly seen in young sporadic FTD cases (12). There is also a rare subtype of FTLD called FTLD-UPS (ubiquitin/proteasome system) which is characterized by ubiquitin and p62 positive inclusions without the presence of TDP-43, tau, or FET proteins. This form of FTLD is primarily found in individuals carrying mutations in *CHMP2B* (6).

### 1.1.4 Genetics

Genetic FTD, here defined as FTD due to mutations in one of several known disease-causing genes, accounts for approximately 10% of all cases of FTD. An additional 35% of all cases have a family history of dementia (1). The inheritance pattern of genetic FTD is primarily autosomal dominant, i.e. it is enough to carry one mutated allele to develop the disease, which therefore confers a 50% risk to offspring and siblings. The majority of all genetic FTD cases are caused by mutations in one of three genes: chromosome 9 open reading frame 72 (*C9orf72*), progranulin (*GRN*) or microtubule associated protein tau (*MAPT*).

#### *1.1.4.1 C9orf72*

Although the frequency varies in different populations, repeat expansions of *C9orf72* is the most common cause for genetic FTD world-wide, accounting for approximately 4 - 29% of the cases (3). FTD due to *C9orf72* repeat expansion

(C9-FTD) is caused by a hexanucleotide G4C2 repeat expansion in a non-coding region of intron 1 of the gene. Carriers of this repeat expansion can have several hundred to several thousand G4C2-repeats, compared to 2 – 24 copies found in the general population (3,15). It has yet to be established what causes the pathogenicity of the repeat expansion, with everything from haploinsufficiency to toxic gain of function, such as accumulation of dipeptides or sequestration of RNA-binding proteins, being suggested as possible causes (3,16). The function of the C9orf72 protein remains largely unknown, though it has been suggested to be a part of endosomal trafficking via interactions with Rab proteins, and to be involved in initiation of autophagy as a part of a complex with SMCR8 (17,18). The clinical presentation of C9-FTD is heterogenous with 65% of all C9-FTD cases developing bvFTD, while 30% develop some form of PPA, most commonly nfvPPA and very rarely svPPA. Repeat expansions of *C9orf72* can also present as pure ALS or FTD-ALS, and MND symptoms are generally more common in C9-FTD compared to other forms of genetic FTD (3,17). The average age at symptom onset is at 58 years with an average disease duration of 6.4 years (19). Psychiatric symptoms are more common among individuals diagnosed with bvFTD carrying *C9orf72* repeat expansions, compared to those who do not, and psychosis can be the first symptom of the disease, manifesting years before any other symptoms are present (3). *C9orf72* mutation carriers (C9-MC) most often develop FTLD-TDP type B pathology, though they can develop FTLD type A or C as well (3).

#### *1.1.4.2 GRN*

Mutations in *GRN* are the second most common genetic cause of FTD and accounts for approximately 1 - 12% of all FTD cases, depending on the population (3). There are currently over 100 known pathogenic mutations in *GRN*, causing loss of function either via an aberrant gene transcript and subsequent mRNA degradation, or prevention of translation, resulting in *GRN* haploinsufficiency (3,19). GRN is an 88 kDa glycoprotein which is cleaved in lysosomes into smaller peptides known as granulins 1- 7. The GRN protein and its peptide products are involved in many different biological processes in the brain, including neuroinflammation, lysosomal function and axonal growth (20–22). While the exact disease causing mechanism of FTD due to *GRN* mutations (GRN-FTD) is not known, progranulin deficiency has been shown to disrupt normal lysosomal function while also inducing the transition of microglia from a homeostatic to a proinflammatory state (20,21,23). The symptom onset in GRN-FTD is on average at 61 years of age with an average disease duration of 7.1 years (19). While most *GRN* mutation carriers

| | *Neuropathology* | *Clinical phenotype* |
| --- | --- | --- |
| *C9orf72* | FTLD-TDP type A or B | ALS, FTD-ALS or bvFTD |
| *GRN* | FTLD-TDP type A | bvFTD |
| *MAPT* | FTLD-tau | bvFTD |
| *TBK1* | FTLD-TDP type A or B | ALS, FTD-ALS or bvFTD |

Figure 1: *An overview of the four most commonly mutated genes in genetic FTD and the neuropathological and clinical subtypes that they most commonly manifest as.*

(GRN-MC) develop bvFTD, the number of individuals with some form of PPA is higher compared to other forms of genetic FTD. Parkinsonism is also more common in GRN-FTD compared to other forms of FTD (3). Mutations in *GRN* primarily lead to FTLD-TDP type A pathology (3).

#### *1.1.4.3 MAPT*

Out of the three main genetic causes for FTD, mutations in *MAPT* are the least common, accounting for 5% of all FTD cases (24). In its native, unfolded state the MAPT protein is involved in microtubule assembly and stabilization as well as axonal transport. Pathogenic MAPT, however, is misfolded, hyperphosphorylated and possesses the aggregation-prone beta-sheet structure characteristic of amyloid fibrils. The resulting tau aggregates can induce neurotoxicity and are believed to be the cause of the disease (6,24). Over 60 disease-causing mutations have been identified in the *MAPT* gene (19). These mutations are

typically either missense mutations affecting the amino acid code in or around the repeat region of one of the microtubule binding domains, or splice site mutations altering the inclusion of exon 10, resulting in an increased propensity for aggregation (6,24). FTD due to mutations in *MAPT* (MAPT-FTD) most often manifests as bvFTD, though semantic impairment is not uncommon in the later stages of the disease (4). Mutations in *MAPT* can also manifest as one of the atypical parkinsonian disorders, corticobasal syndrome or progressive supranuclear palsy, or with memory impairments similar to AD (4,6). On average, MAPT-FTD has an earlier symptom onset compared to other groups of genetic FTD at around 49 years of age and a longer average disease duration of 9.3 years (19). Mutations in *MAPT* lead to FTLD-tau neuropathology (2).

##### *1.1.4.4 Other genetic causes*

Beside the three major genes causing genetic FTD, there are several rare mutations that have been shown to cause the disease. Less than 5% of all FTD cases are attributed to mutations in these additional rare genes (4). Among these, mutations in TANK binding kinase-1 (*TBK1*) are the most frequent. Mutations in *TBK1* most commonly present as ALS or FTD-ALS, rather than pure FTD. However, in FTD without ALS it is most often associated with bvFTD (3). Neuropathologically, *TBK1* mutations are associated with FTLD-TDP type A and type B (3).

## 1.2 Biomarkers

### 1.2.1 What is a biomarker?

A biomarker is a biological metric, such as proteins or metabolites measured in body fluids, which correlates with normal or pathological biological processes, or with the response to an administered treatment. An ideal biomarker should be precise, reliable, reproducible, non-invasive, cheap and easy to analyze, as well as be able to predict fundamental aspects of the pathology (25). Biomarkers can be classified in many different ways depending on their application. Diagnostic biomarkers aid in the diagnosis of a disease, including differentiation from other diseases with similar phenotypes. Prognostic biomarkers provide information regarding the course of the disease and likelihood of survival. Markers of therapeutic response aid in the determination of drug efficacy as well as pharmacokinetics and pharmacodynamics. Furthermore, biomarkers can be used

in research to study different pathological processes and to characterize subtypes of the disease.

### 1.2.2 Protein biomarkers

Proteins measured in body fluids like blood, either serum or plasma, or cerebrospinal fluid (CSF) can often provide important insight into the pathological processes of a disease. In the context of FTD, CSF is of particular importance since it surrounds the brain and spinal cord. Many of the early biomarker studies therefore focused on locating protein biomarkers in CSF. More recently, studies have shifted focus to blood-based biomarkers since blood is more readily available and the sampling procedures are less invasive. The drawback of blood-based biomarkers is that they are affected by additional factors such as possible contamination of peripheral protein production, altered renal function and diurnal variation. In the end, both fluids have their merits, and the usage should be dependent on the context.

#### *1.2.2.1 Gene-specific markers*

Carriers of pathogenic *GRN* mutations have, regardless of age or symptom onset, reduced levels of progranulin in CSF and plasma due to haploinsufficiency. Confirmation of a 50% reduction of plasma progranulin can be used to determine if a newly identified mutation is pathogenic (26). Individuals with *C9orf72* repeat expansions have dipeptide repeats in CSF originating from translation of the expanded G4C2-sequence which can be measured already at the presymptomatic stage (27). However, neither progranulin levels nor dipeptide repeats correlate to severity of disease or time to expected onset (26,27). In contrast, *MAPT* mutations do not lead to any altered levels of phosphorylated tau or total tau in CSF or blood that can be measured with the methods available today (28).

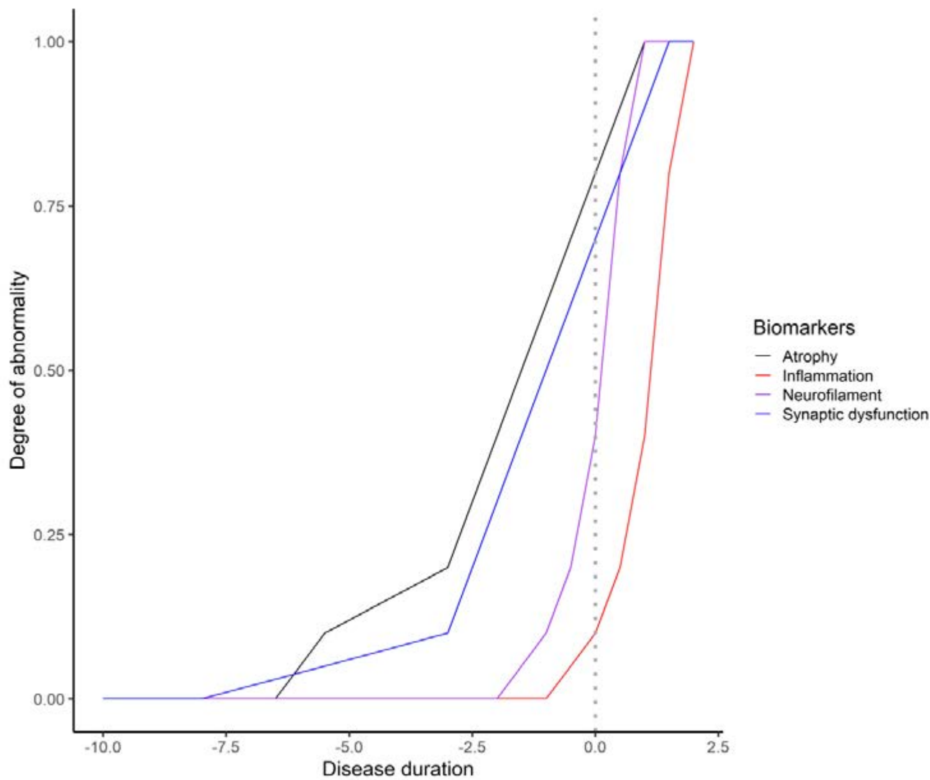


Figure 2: *Theoretical model of the temporal order of biomarker changes in FTD. The x-axis represents disease duration in years with negative numbers being before symptom onset, indicated by the dotted line, and positive numbers after symptom onset. The y-axis represents the degree of abnormality, i.e. how far the biomarker has deviated from the expected norm, ranging from 0 which indicates no abnormality, to 1 which indicates peak abnormality.*

#### *1.2.2.2 Neurofilaments*

The most promising of the emerging fluid biomarkers are the neurofilaments, a family of intermediate filaments. Neurofilaments are structural proteins in the neuronal axoskeleton and exist in three forms: light (NEFL), medium (NEFM) and heavy chain (NEFH). NEFL is elevated in both blood and CSF from patients with many different neurological and neurodegenerative diseases, and can be considered a general marker for axonal damage (29). NEFL levels also increase naturally with advancing age, with modest increases in both blood and CSF in cognitively healthy elderly individuals (29). Several studies have shown the ability of NEFL to distinguish between people with FTD and controls as well as between

individuals with FTD and individuals with primary psychiatric disorders (30–34). Differences in NEFL levels between AD, ALS and FTD have also been described, where the levels are higher in FTD than in AD and highest in ALS (28,33). Plasma NEFL levels are elevated already before symptom onset and can predict phenoconversion in genetic FTD as well as being associated with disease duration, where higher levels predict shorter survival (30). NEFH, primarily in its phosphorylated form (pNEFH), has also been investigated as a potential biomarker. It is elevated in both blood and CSF from individuals with ALS as well individuals with ALS-FTD. In FTD without MND however, the differences to controls are minor, indicating that elevated levels of pNEFH could primarily be associated with the onset of motor symptoms (31,32). NEFM is less studied compared to the other neurofilaments and its usefulness as a biomarker remained unclear until recently. NEFM will be discussed in further detail in studies II, III and IV as well as section 5.1.1 of the discussion.

#### *1.2.2.3 Synaptic markers*

Synaptic loss occurs throughout the course of FTD, making markers of synaptic function potentially useful biomarkers. The most promising markers for synaptic function in FTD are the soluble neuronal pentraxins 1 and 2 (NPTX1 and NPTX2) along with the membrane bound neuronal pentraxin receptor (NPTXR). The neuronal pentraxins are involved in the modulation of synaptic function and plasticity, as well as synaptic pruning (35). All three neuronal pentraxins are reduced in CSF from individuals with genetic FTD, independent of which mutation is causing the disease (36–38). Furthermore, NPTX2 levels in CSF correlate inversely with clinical disease severity as well as CSF NEFL levels and gray matter atrophy (38). Reductions in CSF NPTX2 levels have been suggested to be an early event in the course of the disease, preceding both increased NEFL levels and elevated markers of neuroinflammation (39). However, reduced levels of neuronal pentraxins are also seen in other neurodegenerative diseases (35). Other markers of synaptic function seem to be associated with the underlying pathology. Several proteins, including neurogranin, beta and gamma synuclein, and 14-3-3 proteins, are all elevated in CSF from *MAPT* mutation carriers (MAPT-MC), but stable in C9-MC and GRN-MC (36). Many of these proteins have also been shown to be elevated in AD, strengthening the link to tau pathology (40).

#### 1.2.2.4 Neuroinflammatory markers

Neuroinflammation is considered to be a hallmark of neurodegeneration and has been suggested to play an important role in the pathological process of FTD (41). It is a complex series of events involving several different cell types, mainly astrocytes and microglia, and cascades of proinflammatory and anti-inflammatory signals, and is normally tightly regulated in comparison to inflammatory events in peripheral tissue. Unregulated or chronic neuroinflammation can cause extensive tissue damage and eventually lead to neurodegeneration (41). Markers of neuroinflammation are therefore highly relevant in the context of FTD research. The roles of several glia-derived proteins have been investigated in FTD. Glial fibrillary acidic protein (GFAP), a marker for reactive astrocytes, have been shown to be elevated in CSF from patients with genetic FTD and in plasma from symptomatic GRN-MC (42,43). Chitinase 3 like 1 (CHI3L1, alternatively YKL-40), another marker for reactive astrocytes, is more controversial with different studies either showing elevated levels or no differences compared to controls (42,44). Similarly, the levels of both chitinase 1 (CHIT1) and soluble triggering receptor expressed on myeloid cells 2 (TREM2), both markers for microglial activation, have either been shown to be stable in genetic FTD or elevated selectively in symptomatic GRN-MC (42,44,45). These results could be due to some studies being underpowered and therefore not able to detect minor differences in protein levels. However, it could point towards heterogeneity in the neuroinflammatory profile of genetic FTD. The complement proteins, which are key components of the innate immune response, are also of interest in the context of neuroinflammation. Several of these proteins have been shown to be elevated in both CSF and plasma from individuals with genetic FTD (46). Furthermore, correlations with both reduced grey matter volume and increased levels of NEFL and GFAP were observed already at a presymptomatic stage, indicating that alterations of complement levels could precede onset of clinical symptoms (46).

### 1.2.3 Imaging biomarkers

Neuroimaging techniques, such as magnetic resonance imaging (MRI), are useful for measuring the structure and function of the brain in a non-invasive manner. MRI is commonly used for the diagnosis of major neurocognitive disorders and can be used to determine the severity of the disease and to distinguish neurodegenerative disorders from primary psychiatric disorders. FTD is associated with both gray matter atrophy and white matter abnormalities, and

different patterns of atrophy and/or abnormality can be seen in the different subtypes.

#### *1.2.3.1 Gray matter changes*

In general, FTD is characterized by frontal and/or temporal atrophy together with atrophy of the frontoinsular region (2). There are differences in the atrophy pattern between the different clinical phenotypes, although with a significant overlap. In bvFTD, atrophy most commonly affects the frontal and temporal lobes, the anterior cingulate cortex, and the insula (47). In svPPA asymmetrical, primarily left-sided, anteroinferior temporal lobe atrophy is seen, while right-sided anterior temporal lobe atrophy is associated with sbvFTD (8,47). In nfvPPA the insula and the left inferior frontal lobe are commonly atrophied (47).

In genetic FTD, the temporal and spatial patterns of atrophy depend heavily on which mutated gene is causing the disease. Gray matter atrophy pattern associated with C9-FTD tends to be widespread and symmetrical, involving the frontal and temporal cortices, insula, and cingulate cortex as well as posterior cortical regions. The atrophy is particularly prominent in the subcortical regions, with the thalamus being the most affected followed by the hippocampus, amygdala, and basal ganglia (3,48). Significant atrophy is present already in presymptomatic C9-MC, particularly of the thalamus but also of the insula, and superior temporal and inferior frontal regions (48–50). In C9-MC atrophy has been estimated to be present already 40 years before symptom onset, with a slow decline in brain volume spanning several decades (51). In contrast, GRN-FTD is associated with asymmetric atrophy patterns favoring neither hemisphere primarily affecting the frontotemporal and parietal regions (3,48). Significant atrophy is only observed a few years before symptom onset, starting in the frontal and temporal lobes, followed by a rapid decline (51). MAPT-FTD is characterized by atrophy of the temporal and orbitofrontal lobes together with the hippocampus, amygdala, and insula (48). Significant atrophy is present approximately 10 years before symptom onset starting in the hippocampus and amygdala, followed by atrophy of the temporal lobe and the insula (50,51).

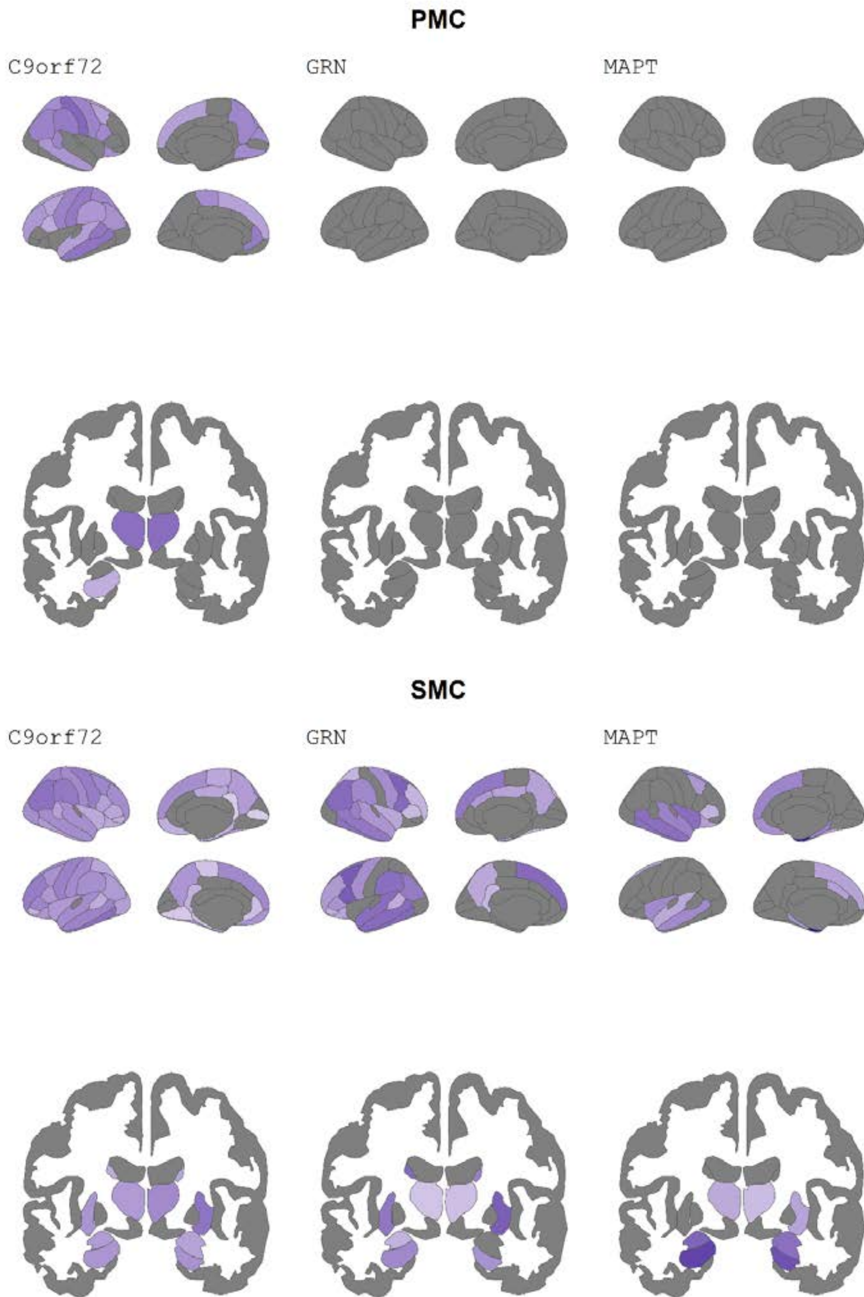


Figure 3: *Overview of cortical and subcortical atrophy patterns associated with the three main genetic subtypes of FTD. Purple color indicates areas commonly affected by atrophy, with darker shades indicating more atrophy, while grey color indicates no significant atrophy. The image is based on data from study IV. PMC: presymptomatic mutation carrier, SMC: symptomatic mutation carrier.*

#### *1.2.3.2 White matter changes*

Loss of white matter integrity is another common feature in FTD and has been reported to precede gray matter atrophy, indicating that it is the first structural change to take place (47). In general, these white matter changes are most often seen in the anterior corpus callosum, bilateral anterior and descending cingulum and the uncinate fasciculus tracts (47). Similar to the gray matter atrophy patterns, the genetic subtypes of FTD all exhibit distinct patterns of white matter degradation. C9-FTD often show changes in the posterior thalamic radiation, the posterior corona radiata and the splenium of the corpus callosum, while in GRN-FTD the degradation is most often seen in the anterior and posterior internal capsule. In MAPT-FTD loss of white matter integrity most often occurs in the uncinate fasciculus and parahippocampal cingulum (4,49). White matter hyperintensities, a feature commonly seen in other types of dementias, are rare in FTD. However, they have been observed in a subset of symptomatic and presymptomatic GRN-MC (52). Neuropathological examination of these lesions suggests that they are not of vascular origin but rather associated with pronounced microglial activation (4).

# 2 Research aims

The purpose of these studies was to examine the heterogeneity present in FTD, primarily the genetic form, and to find biomarkers that are associated with the different subtypes.

The specific aims of the projects were:

- **Study I:** To characterize the effect of a *TBK1* p.Ala417* (c.1340 + 1G > A) splice mutation.
- **Study II:** To investigate FTD-associated changes in the CSF protein profile in both genetic and sporadic FTD.
- **Study III:** To characterize the effect of FTD-associated pathogenic mutations on the plasma protein profile of the mutation carriers and to find plasma-based biomarker candidates.
- **Study IV:** To determine the association between the levels of CSF protein biomarker candidates and structural changes in the brain, including differences between genetic subtypes.

# 3 Materials and methods

Below follows descriptions of the cohorts and the most important methods used in the four studies. For a full description of all methods used in the studies see their respective manuscripts at the end.

## 3.1 Study Participants

#### *3.1.1.1 GENFI*

The genetic frontotemporal initiative (GENFI) consortium is an international collaboration with 40 sites across Europe and Canada. The study started with a handful of sites in 2012 and now includes 1491 participants who have come to a total of 4026 visits. The aim is to improve our understanding of genetic FTD including its pathogenesis, clinical markers for the disease, the discovery of biomarkers and to prepare for clinical trials.

Participants from families where pathogenic mutations segregate are invited to annual research visits. Each research visit includes clinical assessment, neuropsychological testing, tissue sampling and MRI all according to standardized protocols. During the first visit the participants are screened for the specific pathogenic mutation that is present in their family. The data from each visit is pseudonymized and uploaded into a common GENFI-database. It can be included in research projects after a data access application has been approved by the principal investigators.

All studies in this thesis include at least some samples from GENFI. Studies I and II included samples from the Stockholm site only, while studies III and IV included samples from other GENFI-sites as well.

#### *3.1.1.2 Study I*

Study I included fluid and tissue samples from a family carrying a specific mutation in *TBK1,* p.Ala417*. It included blood samples from presymptomatic mutation carriers (PMC, n = 2), and non-carriers (NC, n = 7), skin biopsies used for

**Study I**

| | Fibroblast | Blood | Formalin fixed brain | Frozen brain |
|---|---|---|---|---|
| No. of participants | 2 | 9 | 4 | 3 |
| Mutation carriers | 1 | 2 | 4 | 1 |
| Symptom status (%) | | | | |
| Presymptomatic | 1 | 2 | 0 | 0 |
| Symptomatic | 0 | 0 | 4 | 1 |

**Study II**

| | Cohort 1 | | | | |
|---|---|---|---|---|---|
| | **NC** | **PMC** | **bvFTD** | **PPA** | **Total** |
| No. of participants | 8 | 16 | 16 | 13 | 53 |
| Age, median years (range) | 52 (24-65) | 53 (31-71) | 61 (40-78) | 65 (52-79) | |
| Females (%) | 4 (50) | 10 (62) | 6 (37) | 8 (57) | 28 (53) |
| Mutated gene (%) | | | | | |
| *C9orf72* | | 8 (50) | 2 (13) | 0 | 10 |
| *GRN* | | 8 (50) | 0 | 1 | 9 |
| *VCP* | | 0 | 1 (6) | 0 | 1 |

| | Cohort 2 | | | |
|---|---|---|---|---|
| | **Control** | **FTD** | **AD** | **Total** |
| No. participants | 18 | 13 | 79 | 110 |
| Age, median years (range) | 81 (74-86) | 68 (50-83) | 72 (54-88) | |
| Females (%) | 10 (56) | 4 (31) | 49 (62) | 63 (57) |

Table 1: *Cohort demographics for study I and II.*

| Study III | | | | |
|---|---|---|---|---|
| | **NC** | **PMC** | **SMC** | **Total** |
| No. of participants | 276 | 280 | 137 | 693 |
| Age, mean years (SD) | 47 (14) | 45 (12) | 63 (9) | 49 (14) |
| Females (%) | 152 (55) | 174 (62) | 57 (42) | 383 (55) |
| Mutated gene (%) | | | | |
| *GRN* | | 133 (48) | 49 (36) | |
| *C9orf72* | | 96 (34) | 62 (45) | |
| *MAPT* | | 51 (18) | 26 (19) | |
| Age at onset, mean years (SD) | | | | |
| *GRN* | | | 61 (8) | |
| *C9orf72* | | | 60 (9) | |
| *MAPT* | | | 51 (8) | |

| Study IV | | | | | |
|---|---|---|---|---|---|
| | ***C9orf72*** | ***GRN*** | ***MAPT*** | **NC** | **Total** |
| No. of participants | 62 | 46 | 23 | 71 | 202 |
| Age, mean years (SD) | 50 (14) | 52 (14) | 47 (11) | 47 (13) | 49 (13) |
| Females (%) | 33 (53) | 26 (57) | 17 (61) | 39 (55) | 112 (55) |
| Symptom status (%) | | | | | |
| Presymptomatic | 40 (65) | 37 (80) | 16 (70) | | |
| Symptomatic | 22 (35) | 9 (20) | 7 (30) | | |
| Age at onset, mean years (SD) | 57 (8) | 60 (7) | 52 (6) | | |

Table 2: *Cohort demographics for study III and IV.*

fibroblast cultivation from PMC and NC (n = 1 and n = 1, respectively), as well as both frozen and formalin fixed brain tissue from symptomatic mutation carriers (SMC, n = 1 and n = 4, respectively) and NC (n = 2, frozen brain only). The fibroblasts and the frozen brain tissue were used in triplicates for the mass-spectrometry analysis.

#### *3.1.1.3 Study II*

Two different cohorts were used for study II, one exploratory cohort and one validation cohort (table 1). The exploratory cohort, collected at Karolinska university hospital and through GENFI, included CSF samples from 53 participants and included both genetic and sporadic FTD (n = 4 and n = 25, respectively) as well as PMC (n = 16) and NC (n = 8). bvFTD (n = 16) was the clinical subtype most common among the individuals diagnosed with FTD, followed by PPA (n = 13). The validation cohort, collected at Uppsala university hospital, included CSF samples from individuals with FTD (n = 13) and AD (n = 79) as well as controls (n = 18).

#### *3.1.1.4 Study III*

The cohort for study III consisted of plasma samples from 693 participants in the international GENFI study (table 2). The cohort included 137 samples from SMC (62 C9-MC, 49 GRN-MC and 26 MAPT-MC), 280 samples from PMC (96 C9-MC, 133 GRN-MC and 51 MAPT-MC) and 276 samples from NC. Clinically, the most common diagnosis among the SMC was bvFTD (n = 102), followed by PPA (n = 25), FTD-ALS (n = 5) and other FTD-related diagnoses (n = 5).

#### *3.1.1.5 Study IV*

Study IV included CSF samples and MRI images from 202 participants from the international GENFI study (Table 2). The cohort was based on the one used in Bergström et al (53), with the additional inclusion criteria of having a viable MRI image from the same study visit. The cohort consisted of 62 C9-MC (22 SMC, 40 PMC), 46 GRN-MC (9 SMC, 37 PMC), 23 MAPT-MC (7 SMC, 16 PMC) and 71 NC.

## 3.2 Methods

### 3.2.1 Protein profiling

#### *3.2.1.1 Mass-spectrometry*

In study I mass-spectrometry was used to analyze the effect of the *TBK1* p.Ala417* (c.1340 + 1G > A) splice mutation on K63-ubiquitination. This was done in collaboration with the core facility for mass-spectrometry based proteomics at Karolinska Institutet. The samples were first purified to only include K63-ubiquitinated proteins. This was done through the use of tandem ubiquitin binding entities (TUBE) magnetic beads, which selectively binds to K63 polyubiquitinated proteins. The magnetic K63-TUBE beads were used according to manufacturer's protocol. The TUBE purified K63 ubiquitinated proteins were analyzed in a LC-MS/MS system. Peptide identification was done through comparing the MS data to the Uniprot KB database.

Missing values were imputed through a three-step process. First the number of missing values for the protein in question was assessed. If there was more than one missing value, the protein was excluded. Second, if no signal was detected then the protein was excluded. Finally, imputation was done by setting the missing value to half of the minimum detected value.

#### *3.2.1.2 Suspension bead array*

The suspension bead array is an affinity-based proteomics method that was used to measure protein levels in studies II, III and IV. This was done in collaboration with the Peter Nilsson group at the Royal Institute of Technology. The method is fully described in Schwenk *et al* and Remnestål *et al* (54,55).

In short, the fluid samples were labeled with a ten-fold molar excess of biotin, diluted, and heat-treated. To form the suspension bead array, antibodies were conjugated onto carboxylated color-coded magnetic beads, with one bead identity per antibody, using NHS-EDC chemistry and pooled together into the array. The biotinylated fluid samples were then combined with the bead array and incubated overnight. A streptavidin conjugated fluorophore was added to allow detection and the read-out was made in a Flex-map 3D instrument. Binding events were registered as signal intensity (arbitrary units) or median fluorescent intensity.

Study II was performed using already established beadstocks with antibodies towards targets relevant for AD, ALS, and other neurodegenerative diseases. For study III, a new beadstock was designed and the protein targets were selected based on the results from study II, current scientific literature, and internal unpublished data. Study IV was based on the results from a previously published study and used the protein measurements obtained in that study (53).

#### 3.2.2 Imaging

For study IV T1 weighted 3T MRI scans were acquired from the GENFI consortium. The acquisition protocols are described in detail in Rohrer *et al* (50). The images were processed through TheHiveDB using FreeSurfer version 7.1.1 (56,57). Segmentation was done based on the Desikan-Killiany atlas resulting in extraction of cortical thickness in 68 regions of interest (ROI), 34 in each hemisphere, along with 12 subcortical volumes (right and left hippocampus, amygdala, putamen, caudate, thalamus and pallidum) (58). After segmentation the images were manually quality controlled through visual inspection. Images and segmentations that were of subpar quality were excluded from further analysis.

#### 3.2.3 Data analysis

##### *3.2.3.1 Linear and general mixed effect models*

In study III binomial generalized mixed effect models were used to determine which proteins differed between the different analysis groups, adjusted for sex and with a random intercept based on collection site. Linear mixed effect models were used to analyze how the protein levels varied with age, adjusted for sex and with a random intercept based on collection site. In study IV linear mixed effect models were used to find mutation associated atrophy patterns and to analyze how CSF protein levels were associated with cortical and subcortical atrophy. Both sets of models were adjusted for sex and had a random intercept based on collection site. In both studies linear mixed effect models were used to adjust the data for healthy ageing before analysis (see below).

##### *3.2.3.2 Age adjustment*

In studies III and IV we used a residual-based adjustment approach to account for the potential confounding effect of healthy ageing in the data. Using linear mixed effect models, we calculated the association between age and the outcome variable in question (protein levels in study III and cortical thickness/subcortical volume in study IV) in the NC group. The age-associated beta-coefficient was

then extracted and used to adjust the outcome variable in the whole cohort. The following formula was used in study III:

$$Protein_{adj.} = Protein - \beta(Age - \overline{Age})$$

where $Protein_{adj.}$ was the age-adjusted protein level, Protein was the original protein level, β was the age-associated beta coefficient, Age was the participant's age and $\overline{\text{Age}}$ the mean age in the cohort. A simpler formula, using the participants raw age instead of mean-adjusted age, was used for study IV:

$$ROI_{adj.} = ROI - \beta \times Age$$

where $ROI_{adj.}$ was the age-adjusted cortical thickness/subcortical volume in the ROI, ROI was the original cortical thickness/subcortical volume, β the age-associated beta coefficient and Age the age of the participant.

#### *3.2.3.3 Hierarchical clustering*

Hierarchical clustering was used in study I to produce heatmaps of the analyzed proteins. In study II it was used to test if the subgroups in the cohort would cluster together based on either principal components or the levels of three selected proteins.

Various metrics can be used to measure the distance between the data points used for clustering. Study I was based on the correlation between the features and study II used Euclidean distances. The linkage, i.e. the criteria on which clusters are merged, can also differ. Study I used Ward's clustering criterion which merges clusters based on minimum within cluster sum-of-squares. Study II used complete linkage which merges clusters based on the maximum distance between cluster nodes.

#### *3.2.3.4 Principal component analysis*

Principal component analysis (PCA) is commonly used to explore high dimensional data in a more manageable way since it reduces the data down to a few principal components. Every component is a linear combination of all features included in the analysis constructed to maximize the variance explained orthogonally to each other. This allows for analysis and visualization of high-dimensional and complex datasets using only a few variables.

In study II PCA was used as an exploratory analysis to find large-scale differences in the cohort. In studies I, III and IV it was used as a quality control tool to detect outliers and unwanted clusters in the data.

## 3.3 Ethical Considerations

All studies included in this thesis were ethically reviewed and approved by the Swedish ethical review authority and all participants provided written informed consents.

There are many ethical aspects to consider when working with human samples and medical data. This is especially true when working with familial diseases and genetic data. Participating in research on an inheritable, incurable, and ultimately fatal disease, such as genetic FTD, can be taxing for the participants. Ensuring access to counseling for the participants is therefore paramount. The results from the genetic screening in GENFI are never communicated in a research setting. Instead, participants who want to know their genetic status can go through presymptomatic testing via the health-care system where they get further genetic counseling and support.

Furthermore, the impact of the research results on the well-being of the participants needs to be considered. For example, finding a prognostic biomarker that can predict future conversion from the presymptomatic phase to the sympathetic phase with high accuracy would be very beneficial for participant selection in clinical trials. However, for the participants the same information could either be beneficial, giving them the opportunity to make arrangements before they turn symptomatic, or it could cause a great deal of anxiety and stress. Any such results would therefore be best communicated in a clinical setting.

The privacy of the participants is of utmost concern, and we must handle and store the data in a secure and responsible manner to make sure that it does not end up in the wrong hands. For the studies included in this thesis, all data was stored under pseudonyms in a secure, on-premise database. The key to the pseudonyms was only accessible to the lead investigator and the database manager and existed exclusively in the secure database environment, thus ensuring that the link between pseudonyms and individuals could not be inadvertently disclosed. We must also respect the participants' wishes to not know their own mutation status and must therefore not disclose this consciously

or by accident. This was ensured through blinding of the clinical personnel to the presymptomatic participants’ mutation status.

Finally, particular care needs to be taken when conducting research involving participants with neurodegenerative diseases. Being involved in a research study requires informed consent, however, the nature of these diseases might limit the participants understanding of the procedures and what they are consenting to. Despite this, individuals with neurodegenerative diseases should not be excluded from research but should be given the possibility to participate just like anyone else.

# 4 Main findings

## 4.1 Study I

The aim of this study was to characterize the effects of a recently discovered mutation in *TBK1*. The p.Ala417* (c.1340 + 1G > A) splice mutation, resulting in haploinsufficiency due to non-sense mediated decay, was first discovered in a Swedish family with a history of ALS. In the current study we describe the effect of this mutation in another, to our knowledge unrelated, family from Sweden.

Clinically, patients from this family presented with varied phenotypes within the FTD-spectrum including speech difficulties, behavioral symptoms and/or motor symptoms. Autopsy material was available for four of the individuals and neuropathological examination showed FTLD-TDP type A pathology across the individuals.

Using ddPCR we observed drastically reduced levels of *TBK1* mRNA levels in blood and fibroblasts from PMC and brain from SMC. This was followed up with a western blot analysis which showed reduced protein levels of both TBK1 and phosphorylated TBK1 in both PMC and SMC, confirming that the p.Ala417* mutation leads to haploinsufficiency.

Using mass spectrometry combined with TUBE purification we studied the effect of the p.Ala417* mutation on K63 ubiquitination in fibroblasts from PMC and brain homogenate from SMC. Using this method, we were able to identify 952 K63 ubiquitinated proteins in fibroblasts and 2234 in brain, where 371 were found in both tissues.

Next, we used hierarchical clustering to explore how the detected proteins correlated with each other. In both tissue types the samples clustered based on mutation status and the ubiquitinated proteins clustered based on if they were elevated or reduced in the mutation carriers (Figure 4). We then analyzed if the proteins were significantly differentially ubiquitinated in the mutation carriers compared to the NC. In the fibroblasts 109 proteins were differentially ubiquitinated and in brain 168 proteins were differentially ubiquitinated. A functional annotation clustering analysis of the differentially ubiquitinated

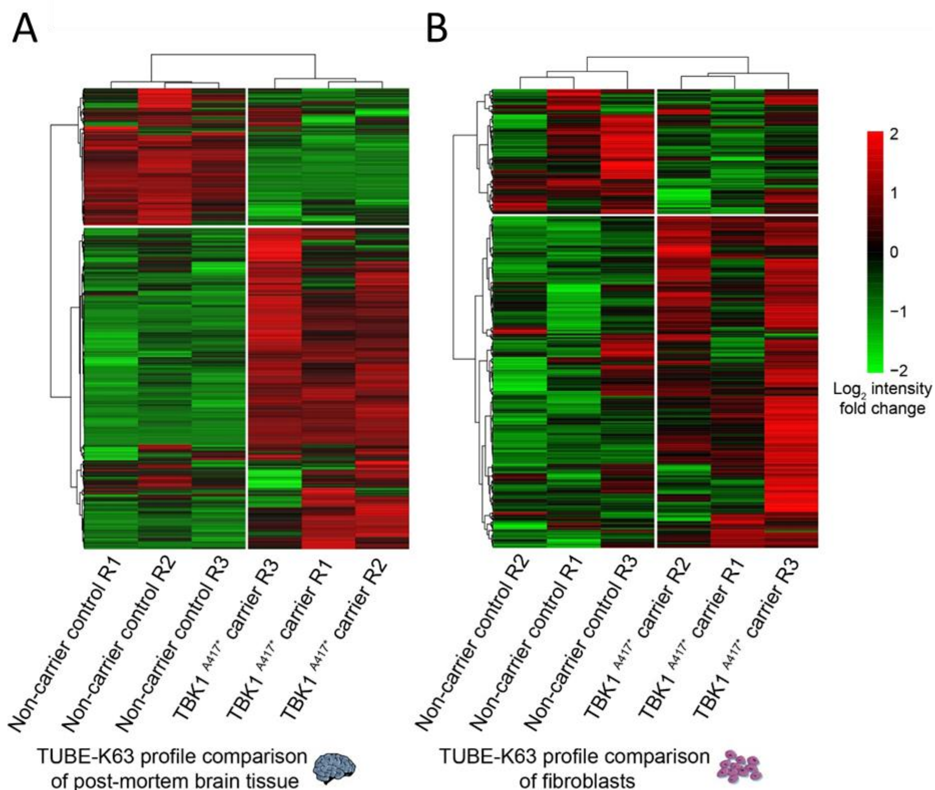


Figure 4: *Heatmap representing the differentially K63-ubiquitinated proteins in TBK1-MC samples vs. control samples in A) brain tissue from SMC and B) fibroblasts from PMC. Proteins are colored based on the intensity of the detected signal post with green representing a decrease and red representing an increase in the detected signal relative to the mean signal of the protein across all replicates of each genotype. The figure is replicated from study I (59).*

proteins showed that 21 gene-ontology terms grouped into six major clusters were affected in the fibroblasts, and in brain 19 gene-ontology terms grouped into six major clusters were affected.

Taken together our findings show that the p.Ala417* mutation leads to TBK1 haploinsufficiency already at the presymptomatic stage. The loss of TBK1 leads to disruptions in K63 ubiquitination in both brain and fibroblasts and is present before symptom onset.

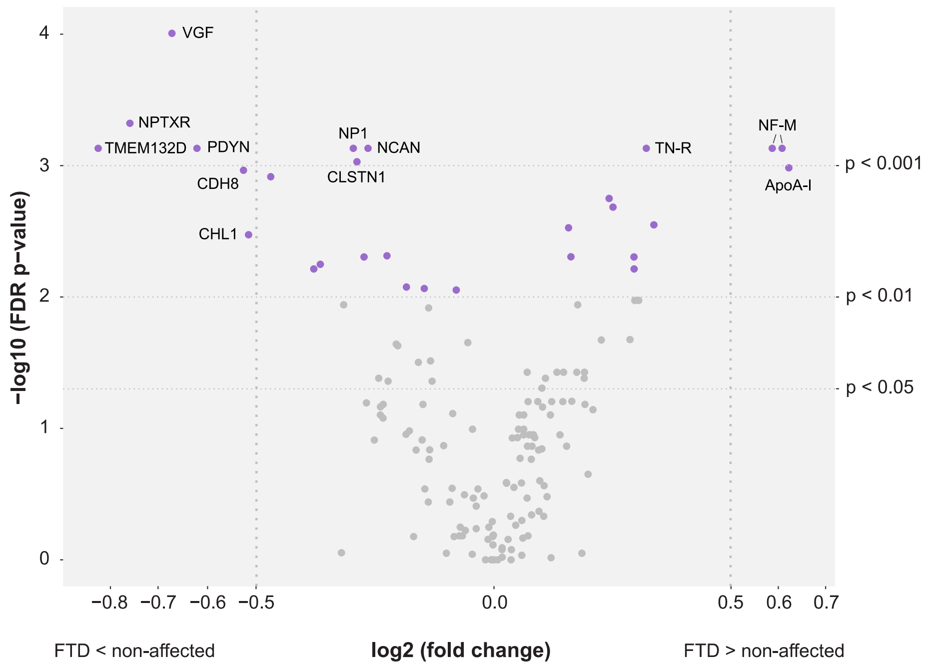


Figure 5: *Volcano plot of analyzed CSF proteins in study II. Differences in protein levels between FTD patients and unaffected individuals displayed by log2(fold change) and significance level displayed as -log10(p-value). All proteins with significant differences (FDR adjusted p < 0.01) are displayed in purple. The figure is replicated from study II (60).*

## 4.2 Study II

In this pilot study we evaluated the potential of using a multiplexed suspension bead array to discover new CSF biomarkers for FTD in two independent cohorts. The first cohort included samples from patients with both genetic and sporadic FTD, along with PMC and NC, while the second cohort included samples from patients with sporadic FTD or from patients with AD as well as from controls (Table 1).

An initial exploratory analysis of the protein level data via PCA showed potential differences between FTD cases and controls based on the first two components. Through a hierarchical clustering analysis of the first 10 components from the PCA we were able to separate FTD cases and NC into different clusters. The NC all

grouped together in one cluster (cluster 2), while the cases were spread out over three clusters (1, 3 and 4). The PMC primarily clustered with the NC, but a fraction was spread out over the other clusters.

To find which of the 70 analyzed proteins were responsible for the separation we compared which proteins were found at significantly different levels in individuals with FTD compared with asymptomatic individuals. Out of the 70 proteins 26 were found at significantly elevated or reduced levels. Five of these, neurosecretory protein VGF (VGF), NPTXR, transmembrane protein 132D (TMEM132D), prodynorphin (PDYN) and NEFM, also had an absolute log2 fold-change above 0.5 (Figure 5). By using the levels of only three different proteins, VGF, Tenascin R (TNR) and NEFM, as distance metrics for hierarchical clustering we were able to achieve a more distinct separation between FTD cases and asymptomatic individuals.

The results were then replicated in the second, independent cohort where we could show significant differences between FTD patients and controls for 10 proteins, including VGF, NPTXR, PDYN, NEFM and TNR. The validation cohort also included CSF samples from people diagnosed with AD, allowing for comparison between these two neurodegenerative diseases. Two proteins, TNR and NEFM, had significantly different levels between FTD patients and AD patients.

This pilot study showcased the potential of this method and was later on followed up in larger cohorts (study III, study IV and Bergström et al). A summary of the biomarkers analyzed in study II, III and IV is available in table 3.

## 4.3 Study III

In this study we analyzed the levels of 158 proteins in plasma samples from the GENFI cohort with the aim of finding new blood-based biomarkers (see Table 2 for more information about the cohort). We discovered 13 proteins that were elevated in SMC compared to NC and 10 proteins that were elevated in SMC compared to PMC (Figure 6). Six proteins were elevated in both comparisons. The full list of proteins with elevated levels in SMC compared to NC or PMC is available in manuscript III, table 2. Out of the 17 proteins with elevated levels in SMC compared to PMC or NC 13 had a significant association with increased age after adjusting for healthy ageing.

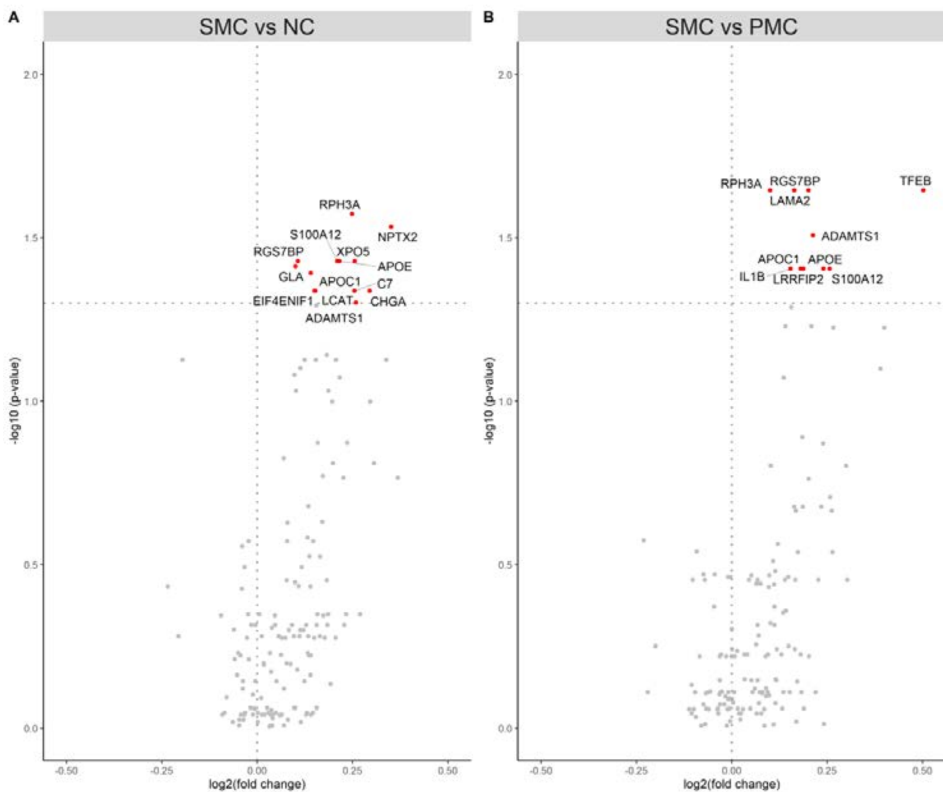


Figure 6: *Volcano plots of plasma protein levels, with -log10 (p-values) on the y-axis and log2(fold change) on the x-axis. A) Plasma protein level differences between SMC and NC. B) plasma protein level differences between SMC and PMC. Each protein is represented by a gray dot. Red dots represent proteins with levels that are increased in the SMC compared to the comparison group (NC, or PMC). Dotted horizontal line: adjusted p-value of 0.05, dotted vertical line: log2 fold change of 0. The figure is replicated from Study III (61).*

We also found some potential differences already at the presymptomatic stage. Four proteins, GRN, NEFM, NPTX2 and CHI3L1, had significantly different levels in PMC-GRN compared to NC. While only the difference in GRN levels remained significant after adjusting for multiple testing, the other three are still of interest since they all have been reported as biomarker candidates previously. NPTX2 was also one of the proteins that only was elevated in SMC compared to NC, not compared to PMC, and it did not have a significant association with increased age.

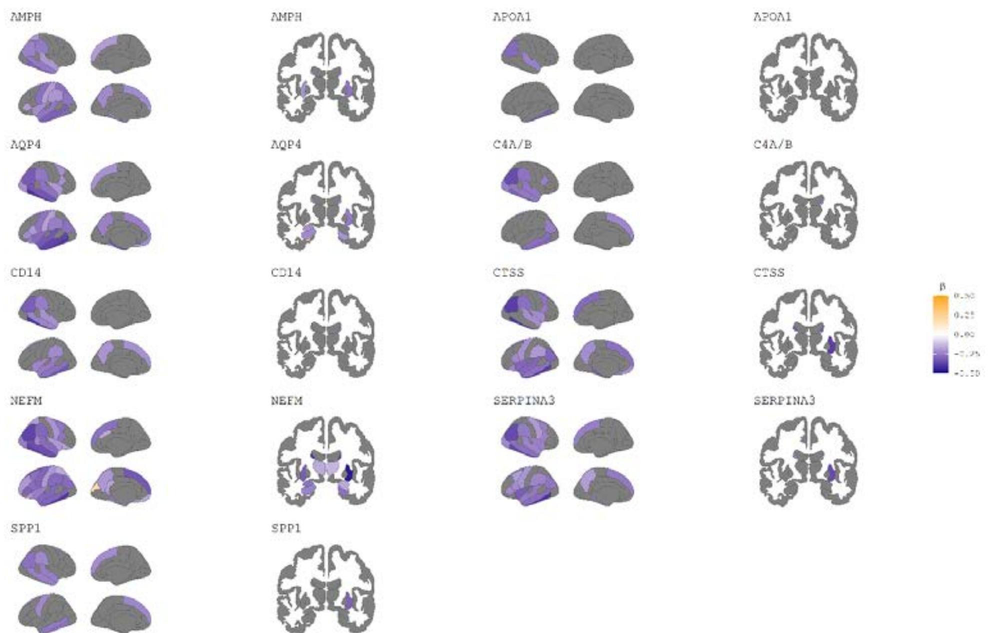


Figure 7: *Brain maps showing associations between CSF protein levels and regional cortical thickness/subcortical volume in all mutation carriers. Color scale indicates protein level associated β-values from linear mixed effect models ranging from -0.5 (dark blue) to 0.5 (orange). Only regions with adjusted p-values below 0.05 are colored. The figure is replicated from study IV.*

Together these results highlight several blood-based biomarker candidates, including markers present already at the presymptomatic stage. Some of these proteins, such as NPTX2, have previously been reported to be abnormal in CSF from people diagnosed with FTD.

## 4.4 Study IV

In a previous study we were able to identify a set of CSF proteins that could differentiate between patients with FTD and asymptomatic individuals (53). To further validate these biomarker candidates, we analyzed how their protein levels in CSF were associated with cortical and subcortical atrophy. The cohort consisted of CSF samples and MR images from 202 individuals participating in the GENFI study (Table 2).

The three genetic groups, C9-MC, GRN-MC, and MAPT-MC, all exhibited different patterns of cortical and subcortical atrophy. The C9-MC had a diffuse and wide-spread pattern of atrophy which was observable in both PMC and SMC. GRN-MC did not show any significant signs of atrophy. However, a stratified analysis based on symptom onset revealed significant atrophy patterns in the SMC, primarily involving the frontal and temporal regions. MAPT-MC had a pattern of atrophy that primarily involved temporal and subcortical regions.

When analyzing associations in the whole MC cohort, nine out of the fourteen included proteins were associated with atrophy in at least one ROI (Figure 7). Amphiphysin (AMPH), aquaporin 4 (AQP4), cathepsin S (CTSS), NEFM and serpin family A member 3 (SERPINA3) were associated with widespread, bilateral atrophy involving both cortical and subcortical regions, while CD14 molecule (CD14) was associated with bilateral medial and temporal atrophy. Furthermore, we observed asymmetry in the association patterns with apolipoprotein A1 (APOA1), complement 4 (C4) and secreted phosphoprotein 1 (SPP1) being primarily associated with right-sided temporal atrophy.

Next, we stratified the cohort by genetic group to look for mutation specific differences. In GRN-MC we observed associations between six proteins and brain atrophy, while in C9-MC and in MAPT-MC we observed associations for five proteins. In C9-MC AQP4 and CTSS were the most noteworthy proteins, exhibiting strong associations with multiple ROIs, whereas in GRN-MC and MAPT-MC, NEFM had the strongest and most widespread associations. Three other proteins of note were NPTX2, protein tyrosine phosphatase receptor type N2 (PTPRN2) and SEC63 homolog, protein translocation regulator (SEC63) which only had significant associations with brain atrophy in GRN-MC. The only protein with a significant association to brain atrophy in all three genetic groups was CTSS.

Overall, the results from this study further validate these proteins as important biomarker candidates as well as highlight the presence of mutation specific effects.

| Protein | Direction of abnormality | Group associated with abnormality | Sample type | Associated with atrophy |
|---|---|---|---|---|
| *ADAMTS1* | ↑ | SMC | Plasma | |
| *AMPH* | ↑ | SMC | CSF | Yes |
| *APOA1* | ↑ | SMC | CSF | Yes |
| *APOC1* | ↑ | SMC | Plasma | |
| *APOE* | ↑ | SMC | Plasma | |
| *AQP4* | ↑ | SMC | CSF | Yes |
| *C4* | ↑ | SMC | CSF | Yes |
| *C7* | ↑ | SMC | Plasma | |
| *CD14* | ↑ | SMC | CSF | Yes |
| *CHGA* | ↑ | SMC | Plasma | |
| *CHI3L1* | ↑ | GRN-PMC | Plasma | |
| *CTSS* | ↑ | SMC | CSF | Yes |
| *EIF4ENIF1* | ↑ | SMC | Plasma | |
| *GLA* | ↑ | SMC | Plasma | |
| *IL1B* | ↑ | SMC | Plasma | |
| *LAMA2* | ↑ | SMC | Plasma | |
| *LCAT* | ↑ | SMC | Plasma | |

Table 3: *Summary of changes in biomarkers investigated in studies II, III and IV including direction of abnormality (increased or reduced levels), in which group and sample type they were found to be abnormal and if they are associated with brain atrophy.*

| *Protein* | *Direction of abnormality* | *Group associated with abnormality* | *Sample type* | *Associated with atrophy* |
|---|---|---|---|---|
| *LRRFIP2* | ↑ | SMC | Plasma | |
| *NEFM* | ↑ | SMC and GRN-PMC | CSF | Yes |
| *NPTX1* | ↓ | SMC | CSF | |
| *NPTX2* | ↓↑ * | SMC and GRN-PMC | CSF and plasma | Only in GRN-MC |
| *NPTXR* | ↓ | SMC | CSF | |
| *RGS7BP* | ↑ | SMC | Plasma | |
| *RPH3A* | ↓↑ * | SMC | CSF and plasma | |
| *S100A12* | ↑ | SMC | Plasma | |
| *SERPINA3* | ↑ | SMC | CSF | Yes |
| *SPP1* | ↑ | SMC | CSF | Yes |
| *TFEB* | ↑ | SMC | Plasma | |
| *TMEM132D* | ↓ | SMC | CSF | |
| *TNR* | ↑ | SMC | CSF | |
| *VGF* | ↓ | SMC | CSF | No |
| *XPO5* | ↑ | SMC | Plasma | |

Table 3 - continued. **: Reduced levels in CSF and elevated levels in plasma.*

# 5 Discussion

## 5.1 Biomarkers

The study of human neurodegenerative diseases is hampered by the lack of access to the affected tissue. For many other types of diseases, tissue biopsies are readily available which allows for in-depth molecular characterization at different timepoints throughout the course of the disease. Tissue samples from human brains are almost exclusively available *post-mortem* and therefore only reflect the final stages of the disease. Research into diseases affecting the brain therefore has to rely on biomarkers from other tissues to provide information on which biological processes are affected and when they start to deviate from normal. Many of the studies included in this thesis consequently focused on investigating biomarkers for a range of biological processes that are involved in FTD.

### 5.1.1 Neurofilaments

Elevated levels of neurofilaments in CSF or plasma act as markers for axonal damage and many studies have therefore investigated the possibility of using NEFH, particularly pNEFH, and NEFL as biomarkers for FTD. While the utility of pNEFH as a biomarker for FTD is controversial, NEFL is much more promising. NEFL levels are elevated in CSF and plasma from individuals with FTD, regardless of clinical or genetic subtype, and can be used to predict phenoconversion in PMC (30,34,62). CSF levels of NEFL are also higher in FTD than most other neurodegenerative diseases, with the exception of ALS, further strengthening its potential as a biomarker (33). In our studies we focused on investigating the potential of NEFM, the less well-studied sibling of NEFL and NEFH, as a biomarker. We were able to demonstrate elevated levels of NEFM in CSF from individuals with FTD in two independent cohorts as well as elevated levels of NEFM in FTD compared to AD (study II). NEFM has also been shown to be further elevated in ALS, compared to FTD, indicating that NEFM follows the same trend as NEFL with higher levels in FTD and ALS compared to other neurodegenerative disorders (63). Furthermore, we were able to show that CSF levels of NEFM are associated with

cortical and subcortical atrophy in genetic FTD (study IV) and that NEFM levels can be used to differentiate SMC from PMC and NC (53).

However, we observed no elevated levels of NEFM in plasma from SMC compared to PMC or NC (study III). Whether this was due to an absence of a true difference or just a lack of sensitivity to detect it remains to be determined. We did, however, detect a significant elevation of plasma NEFM in presymptomatic GRN-MC compared to NC, although it lost significance after correction for multiple testing. A more sensitive measuring technique, such as single-molecule array (Simoa), is most likely required to determine if plasma NEFM has any utility as a biomarker for FTD.

Taken together, NEFM levels seem to follow the same patterns as NEFL and provide equivalent information. Both are markers for axonal damage and neurodegeneration in the different disorders along the FTD-ALS spectrum with the potential to differentiate FTD from AD and other dementias if combined with other biomarkers. NEFM can therefore be considered as an alternative to NEFL, if information about the latter is not available.

#### 5.1.2 Neuronal pentraxins

The neuronal pentraxins form a family of synaptic proteins that have shown considerable promise as biomarkers for FTD. All three neuronal pentraxins are reduced in CSF from individuals with FTD, both genetic and sporadic, compared to controls (study II and (36–38)). However, it is NPTX2 that has garnered the most attention. When measured in CSF it is correlated with severity of symptoms in SMC, becomes abnormal before NEFL, and can be used, together with a panel of other proteins, to accurately distinguish between SMC and controls (38,39,53).

A previous study have shown that CSF levels of NPTX2 correlate with brain volume in C9-MC and GRN-MC in the presymptomatic stage, and with GRN-MC and MAPT-MC at the symptomatic stage (36). In study IV we observed no association between NPTX2 levels and cortical thickness or subcortical volume in the cohort as a whole and only GRN-MC had any significant associations after stratification by mutated gene. While these contradictions are most likely due to differences in methodology, an interesting alternative explanation could be that NPTX2, and in extension synapse dysfunction, plays a different role in the different genetic sub-groups. GRN-MC seem to have an early and sustained synapse dysfunction, while in MAPT-MC it manifests later in the course of the disease. A transient reduction of NPTX2 in C9-MC during the presymptomatic stage, followed by normalization

after symptom onset, is possible and a similar phenomenon has been observed for astrocytosis in familial AD (64). However, this is just speculation at this point and further research is required to fully understand the role of neuronal pentraxins and synapse dysfunction in genetic FTD.

We also observed elevated levels of NPTX2 in plasma from SMC compared to NC, but not compared to PMC, as well as potentially elevated levels of NPTX2 in presymptomatic GRN-MC (study III). A similar pattern was observed for rabphilin 3A (RPH3A), another synaptic protein, which was reduced in CSF from individuals with FTD (study II) but elevated in plasma from SMC (study III). The cause for this inverse relationship between the levels of these two proteins in plasma and CSF is unclear. However, neither protein is expressed exclusively in the brain and the elevated levels could be due to increased production from peripheral sources. Further research is therefore necessary to establish if NPTX2 or RPH3A can be used as blood-based biomarkers for FTD.

#### 5.1.3 Inflammation

Several markers of neuroinflammation in FTD have been proposed, such as elevated levels of GFAP, CHI3L1 and TREM2, which are markers of reactive astrocytes and activated microglia (42–45). Elevated levels of complement proteins, in both CSF (C1q and C3b) and plasma (C2 and C3), have been observed as well (46). In study IV we report that an additional complement protein, C4, is associated with cortical atrophy in genetic FTD, primarily in C9-MC and GRN-MC. Additionally, in study III we observed elevated plasma levels of several proinflammatory proteins, including C7, in SMC compared to NC. The complement proteins are an integral part of the innate immune system and are involved in many different processes, including synaptic pruning (65). Synaptic dysfunction is, as described earlier, an important part of FTD pathology. It could therefore be of interest to study if the elevated complement levels correlate with markers of synaptic dysfunction, such as NPTX2, or if the complement levels become abnormal later as a part of an inflammatory response to tissue damage.

Several other proinflammatory proteins, including interleukin 1 beta (IL1B) and S100 calcium binding protein A12 (S100A12), were also elevated in plasma from SMC compared to PMC or NC, further supporting the role of inflammation in FTD. Elevated levels of CHI3L1 were also observed in plasma from presymptomatic GRN-MC, indicating that a heightened inflammatory state could be an early event

in GRN-FTD. It is, however, unclear whether the elevated levels of inflammatory proteins in plasma are due to leakage from CSF or peripheral inflammation.

We also proposed a novel marker of neuroinflammation in FTD, SERPINA3 (study IV and Bergström *et al* (53)). SERPINA3 is a serine protease inhibitor produced by astrocytes and has previously been reported as a risk-factor for AD as well as being a marker for a high-inflammation subtype of schizophrenia (66,67). In FTD, elevated CSF levels of SERPINA3 are associated with cortical and subcortical atrophy, primarily in the temporal regions (study IV).

#### 5.1.4 Imaging

Previous studies have shown that, while overlapping, there are distinct patterns of brain atrophy associated with the different genetic subtypes of FTD (48,49). In study IV we confirmed the results from these earlier studies. The C9-MC exhibited a diffuse, widespread pattern of atrophy involving regions across the whole brain, while the atrophy in MAPT-MC primarily affected the medial and temporal regions. In GRN-MC significant atrophy was only observed at the symptomatic stage, involving primarily frontal and temporal regions. The only genetic subtype where atrophy was observed before symptom onset was in C9-MC. The presymptomatic C9-MC had a similar pattern of atrophy as the symptomatic C9-MC, although not as severe. In C9-MC atrophy has been estimated to be present already 40 years before symptom onset and they have a significant reduction of brain volume already in their twenties (51,68). Interestingly, early alterations in brain structure have been reported for GRN-MC and MAPT-MC as well. However, where C9-MC have reduced brain volume, both GRN-MC and MAPT-MC have enlarged total intracranial volume compared to family controls (68). Early structural changes to the brain have been reported in genetic AD as well where PMC had increased cortical thickness compared to controls 15- 20 years before estimated onset, which was then followed by a rapid cortical thinning (69). GRN-MC and MAPT-MC could potentially follow a similar biphasic trajectory with early hypertrophy followed by atrophy. These indications of very early, potentially neurodevelopmental, effects of FTD associated mutations warrant further investigation with longitudinal studies examining structural alterations throughout the presymptomatic phase.

#### 5.1.5 Longitudinal changes

One major limitation of many biomarker studies in FTD, including the studies that are a part of this thesis, is their cross-sectional design. While cross-sectional

studies can be very useful in biomarker discovery, a lot of information can be lost if temporal changes in biomarker levels are not considered. One example is the identification of non-linear associations between biomarkers and disease progression. If the levels of such a biomarker are only transiently abnormal, or the levels fluctuate with time, they can appear to be stable at a group level, albeit with a great deal of variability. Such markers would be of particular use for the delineation of the presymptomatic phase of genetic FTD.

PMC are, in many studies, considered to be one homogeneous group. This is not due to the assumption that they truly are a homogeneous group, but rather the lack of markers that are able to distinguish between the various stages of the presymptomatic phase, such as early-stage PMC with a phenotype similar to controls, and those who are in the late stages and are about to convert to the symptomatic phase. Such distinctions are important since late-stage PMC have a biomarker profile that is more similar to SMC, with significant brain atrophy and elevated NEFL levels, than early stage PMC (30,49). While NEFL levels and brain atrophy can be used to identify the PMC for whom phenoconversion is imminent, there is still a lack of markers for early pathological changes. Longitudinal studies with multiple sampling occasions can discover such biomarkers, as well as determine at what stage and in which order they become abnormal.

## 5.2 Heterogeneity in FTD

The main genetic subtypes of FTD, C9-FTD, GRN-FTD and MAPT-FTD, have many overlapping features but there is also a great deal of heterogeneity. There are differences in neuropathology, clinical presentation, patterns of brain atrophy and protein profiles (as described above). This heterogeneity makes studies of genetic FTD complicated, since it causes a lot of variance in the data. However, it also gives rise to interesting opportunities. One goal of the studies in genetic FTD is to eventually be able to translate the findings into knowledge about the sporadic forms of FTD, which accounts for the majority of the cases. Finding the disease-associated alterations that the different genetic subtypes, including the rare ones, have in common could provide much needed insight into the pathological processes of sporadic FTD.

Mutations in *TBK1* are a rare cause of genetic FTD and their mechanisms are therefore less studied. While a few samples from TBK1-MC were available for studies III and IV, the low number of samples made between-group comparisons impossible, and these samples were therefore excluded. To be able to compare the CSF and plasma protein profiles, as well as patterns of brain atrophy, between TBK1-MC and the other genetic subtypes special efforts need to be made to tailor the studies for this purpose. For example, the studies should only include samples that are age and sex matched to the TBK1-cohort and protein profiles should be measured with high sensitivity methods.

There are, however, similarities between TBK1-FTD and the other forms of genetic FTD and lessons learned from studying TBK1-MC can potentially be informative for the more common genetic subtypes as well. In study I we examined the effects of a p.Ala417* mutation in *TBK1*, demonstrating alterations in the K63 ubiquitination system which were present already at the presymptomatic stage. The K63 ubiquitination system is involved in clearance of protein inclusions in neurodegenerative diseases and dysregulation of protein clearance has been proposed as one of the main mechanisms behind neurodegeneration in FTD (70,71). Using the same approach as we did in study I, purification of poly-ubiquitinated proteins followed by mass-spectrometry analysis, on samples from the other genetic subtypes could prove informative. The similarities and differences between the altered K63 ubiquitination profiles could give further insight into what drives the phenotypic differences between the different genetic subtypes of FTD. For example, a recent study described disruptions of the autophagy-lysosome pathway in *C9orf72* knock-out motor neurons with accumulation of p62, an autophagy receptor for ubiquitinated proteins, and elevated levels of phosphorylated TBK1 (72). If C9-MC share the same disruptions to the K63 ubiquitination system as seen in TBK1-MC, then this could be a possible explanation for their shared phenotypes, such as the presence of MND symptoms.

One complicating factor in the study of FTD is the heterogeneity not only between the genetic subtypes, but also within them. Within the same family age at onset and disease duration varies, and different members can develop very different clinical phenotypes (19). This within-subtype heterogeneity can also be seen at a protein level. Woollacott *et al* reported that while at group-level several inflammatory glia-derived proteins showed only minor differences between genetic FTD and controls, a subset of each genetic subtype had very high levels (44). Similarly, in study III we observed very wide confidence intervals for several

proteins, indicating a high within group variance, although we did not investigate this further. Additional studies into the cause of this within group variation are warranted, especially if it can be linked to clinically important metrics such as survival time or age at onset. Studies using unsupervised machine learning, and a large cohort would be ideal to identify such subtypes. Unsupervised machine learning, in contrast to the more commonly used supervised methods, does not rely on prior labels to detect differences between groups. Instead, they allow for detection, and identification, of subtypes based solely on patterns in the data. Unsupervised machine learning methods could therefore be used to detect subtypes to which no prior labels exist or where labels would be difficult to establish beforehand, for example subtypes based on a high degree of synapse dysfunction or neuroinflammation.

A subtype based on a high degree of neuroinflammation would be of particular interest. One proposed diseased modifying therapy for GRN-FTD is gene-replacement using adeno associated viruses (AAV) (73). While this treatment is well tolerated in most animal models, in some it causes a high degree of inflammation followed by neurodegeneration (74). The administration of an AAV-based therapy to an individual with a high-inflammation subtype of FTD could prove disastrous, with results similar to those seen in the latter study. This could lead to not only an adverse event for the participant, but also the termination of an otherwise beneficial therapeutic trial due to safety concerns. It is therefore crucial that the possibility of a high-inflammation subtype is investigated thoroughly, and if it exists, robust biomarkers for it are established and used in the participant selection for AAV-based gene replacement trials.

Another explanation for the observed within-group heterogeneity is that the course of FTD, including the presymptomatic phase, goes through several distinct stages. If those stages are associated with non-linear changes in biomarker levels, such as temporary elevation during a certain part of the presymptomatic phase, then they would give rise to the same type of variation in the data from a cross-sectional study as subtypes. Such an effect was demonstrated by Poulakis *et al* who described five distinct subtypes of AD based on cross-sectional measurements of atrophy (75). However, upon longitudinal reexamination only three subtypes remained, and the others were determined to be stages of these subtypes (76). A similar longitudinal analysis of biomarkers in genetic FTD could

shed light on if the within-group heterogeneity is due to subtypes or if it represents different stages of the disease.

Further heterogeneity is found in the clinical presentation of FTD. In study II and III we analyzed if there were detectable differences between individuals with bvFTD compared to individuals with PPA. While the multivariate analyses in study II indicated that there might be differences, no single protein was found at differing levels in the two groups. Neither did we find any indications of differences in the plasma protein profiles between bvFTD and PPA in study III. If differences exist, which likely is the case given the phenotypic differences, large scale studies targeting a wide range of proteins are probably necessary to find them.

# 6 Conclusion and future perspectives

In these studies, we have characterized the proteomic profile of several types of genetic FTD and validated previous biomarkers or proposed new ones for several important pathological processes including synaptic dysfunction, neuroinflammation and axonal loss. These studies have added to the wealth of knowledge that has been published on the topic of FTD in the last few years. Even though our understanding of FTD in general, and genetic FTD in particular, has come a long way, there are still many questions that are left unanswered.

While a lot of effort has gone into discovering biomarkers for FTD, it is still unclear when these markers become abnormal. To determine this, longitudinal studies covering the presymptomatic phase all the way to symptom onset are necessary. These types of studies are most likely also the only way to uncover non-linear relationships between biomarkers and pathology, such as the biphasic model of changes to cortical structure or transient upregulation of certain protein biomarkers. Natural history studies, such as GENFI and ALLFTD (the American equivalent of GENFI) provide cohorts that are suitable for such longitudinal observations and they have now gone on for over a decade, providing ample opportunity to study the temporal patterns of biomarkers.

The indications of potential neurodevelopmental effects, primarily in C9-MC but also in the other genetic subtypes, merits further investigation. If the mutations associated with FTD indeed cause neurodevelopmental changes then the carriers of these mutations, and their families, could benefit from care much earlier in life than previously thought. Studies in young adult or adolescent MC would be necessary to detect such changes. GENFI NeuroDev, an extension of the GENFI study, will soon start to recruit children of already enrolled GENFI participants, with the aim of determining if there are any neurodevelopmental effects associated with the mutations causing genetic FTD. The results from this study will hopefully provide valuable insight into the earliest pathological changes in genetic FTD.

Finally, one promising avenue of investigation into novel biomarkers is the observable effects of cryptic splicing in TDP-43 proteinopathies. Loss of nuclear TDP-43 leads to the erroneous inclusion of introns into mature RNA, giving rise to

cryptic exons (77). These cryptic exons have been detected in individuals with ALS and FTD and can potentially be used as biomarkers for TDP-43 pathology, something that is urgently needed (77). However, the presence of cryptic exons is not easily detectable and further research is necessary for them to be considered as viable biomarkers.

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