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PhD Thesis

***CHMP2B*-mediated Frontotemporal Dementia**

Markers, Models, and Mechanisms

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Preface and acknowledgments

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The research conducted in this project, along with the preceding studies it builds upon, is made possible by the consistent dedication of a Danish family living with a rare, hereditary form of dementia. During the inclusion of participants, I met with members from the millennial generation of the family. Despite starting families of their own and understanding the potential consequences of genetic testing, they choose to engage in research. Their commitment deserves recognition.

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List of manuscripts included in the thesis

- I. Toft A, Roos P, Jääskeläinen O, Museaus CS, Henriksen EE, Johannsen P, Nielsen TT, Herukka S, Simonsen AH and Nielsen JE. Serum neurofilament light in patients with frontotemporal dementia caused by *CHMP2B* mutation. *Dement Geriatr Cogn Disord* 2020. 49(6):533-538.
- II. Toft A, Sjödin S, Simonsen AH, Ejlerskov P, Roos P, Musaeus CS, Henriksen EE, Nielsen TT, Brinkmalm A, Blennow K, Zetterberg H, Nielsen JE. Endo-lysosomal protein concentrations in CSF from patients with frontotemporal dementia caused by *CHMP2B* mutation. *Alzheimers Dement (Amst)* 2023. 15(1):e12402.
- III. Vardheim E*, Toft A*, Nielsen JE, Hasselbalch SG, Simonsen AH. Cerebrospinal fluid ubiquitin as a biomarker for neurodegenerative diseases: a systematic review. *Neuroscience Applied* 2023. 2(4):102438.

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Abstract

Frontotemporal dementia (FTD) represents a group of syndromes characterized by progressing behavioral changes, language impairments, and cognitive decline. Presently, there is a lack of disease-modifying treatment options and specific biomarkers for these conditions. FTD is a common cause of early-onset dementia and is distinguished by high heritability. While hereditary dementias are rare diseases, they offer significant research value, as presymptomatic carriers of pathogenic genetic variants present a unique opportunity to investigate the underlying pathological mechanisms.

This PhD project is based on a Danish family with a disease-causing genetic variant in the *CHMP2B* gene, serving as a model for a broader spectrum of neurodegenerative diseases. Given the growing evidence linking disruptions in cellular proteostasis and abnormal immune activation to FTD pathogenesis, our primary objective was to identify biomarkers and potential treatment avenues for *CHMP2B*-FTD associated with these processes. To this end, patient-derived biofluid samples and cell models were analyzed. The thesis comprises 3 studies, complemented by a screening of the cerebrospinal fluid (CSF) proteome as well as experiments on the established cellular models.

The initial study examined serum levels of the neurodegenerative marker neurofilament light chain (NfL), demonstrating correlations with the CSF concentrations and the included measure of disease burden. The CSF/serum albumin ratio was employed as an indicator of blood-brain barrier permeability, with elevated values reaching statistical significance for symptomatic carriers of the genetic variant. However, this did not explain the increased serum quantities of NfL when both variables were included in a generalized linear model.

Subsequently, a panel of CSF proteins related to the endo-lysosomal system, innate immunity, and synaptic function was investigated. The analysis did not suggest an overall dysregulation of proteostatic markers but rather highlighted elevated immune markers in *CHMP2B*-FTD and significantly reduced CSF levels of ubiquitin. The latter may be associated with *CHMP2B*

dysfunction, which has been shown to cause an intracellular build-up of enlarged multivesicular bodies that sort ubiquitinated proteins.

The utility of CSF ubiquitin as a biomarker in neurodegenerative diseases was explored in a systematic review. The review found that this marker has been most extensively investigated in Alzheimer’s disease, consistently demonstrating increased levels. Only a limited number of studies have been published on CSF ubiquitin in the other included diseases, showing an overall increase in dementia with Lewy bodies and Huntington’s disease, unchanged levels in amyotrophic lateral sclerosis, and, interestingly, unaltered or significantly decreased concentrations in frontotemporal dementia and Parkinson’s disease. The review concluded that these divergent levels must be validated for the biomarker to hold potential for clinical application.

An unbiased quantification of the CSF proteome in members of the Danish *CHMP2B*-FTD family revealed enrichment of pathways related to the complement system, lipid metabolism, and innate immune function. The downregulation of processes linked to cell adhesion and tight junctions may reflect the blood-brain barrier dysfunction observed in the first study. The analysis indicated a disturbance in the classical complement pathway among presymptomatic carriers and an overactivation of the complement cascade in symptomatic carriers. The microglia-secreted protein AIM (CD5L) ranked among the most prominent, and its involvement in various immune functions, lipid metabolism, and complement activation calls for a more detailed examination of this protein in *CHMP2B*-FTD.

Induced pluripotent stem cells (iPSCs) derived from tissue samples from carriers of the *CHMP2B* variant and healthy controls were differentiated into microglia and neurons. Functional assays on microglia did not show differences in phagocytosis, while cells carrying the *CHMP2B* variant produced lower levels of pro-inflammatory cytokines. Induced microglia were responsive to oxidized low-density lipoprotein (oxLDL) stimulation, which increased the secretion of AIM protein. Preliminary data related to AIM is presented in the results section.

In conclusion, while no clear indications of endo-lysosomal dysregulation or a pro-inflammatory profile were observed in *CHMP2B*-FTD, these studies provided new insight into other potentially

underlying mechanisms involving the blood-brain barrier, ubiquitin, the complement cascade, lipid metabolism, and AIM. The outcomes of this PhD project, along with its provided resources, will hopefully serve as a valuable foundation for further research aimed at comprehending the pathological mechanisms of the disease, developing biomarkers, and exploring disease-modifying therapies.

Resumé (Danish abstract)

Frontotemporal demens (FTD) udgør en gruppe af syndromer kendetegnet ved progredierende adfærdsændringer, sprogforstyrrelser og kognitive deficit. Der mangler sygdomsmodificerende behandlingsmuligheder samt specifikke biomarkører for disse tilstande. FTD er en hyppig årsag til tidligt indsættende demens, og udviser en høj grad af arvelighed. Arvelige demenstilfælde er sjældne, men har stor forskningsmæssig værdi, da præsymptomatiske bærere af patogene genetiske varianter giver en unik mulighed for at undersøge de underliggende patologiske mekanismer.

Dette ph.d.-projekt tager udgangspunkt i en dansk familie med en sygdomsfremkaldende genetisk variant i *CHMP2B*-genet. Denne særlige genotype er tiltænkt at fungere som model for et spektrum af neurodegenerative sygdomme. Eftersom stigende evidens har påvist at forstyrrelser i cellulær proteostase og abnorm immunaktivering er en del af patogenesen ved FTD, var formålet med nærværende projekt at identificere biomarkører og potentielle behandlingsveje for *CHMP2B*-FTD, associeret med disse processer. Til dette formål analyserede vi cerebrospinalvæske (CSV) og blodprøver fra familiemedlemmerne, samt cellemodeller genereret ud fra hudbiopsier. Afhandlingen omfatter 3 studier, suppleret med en screening af CSV-proteomet og eksperimenter på de etablerede cellemodeller.

I det første studie blev serumniveauerne af neurodegenerations-markøren neurofilament light chain (NfL) undersøgt. Serum-NfL korrelerede med CSV-koncentrationerne og det anvendte mål for sygdomsbyrde. CSV/serum-ratioen af albumin blev inkluderet som en indikator for blod-hjernebarrierens permeabilitet, og de forhøjede værdier opnåede statistisk signifikans hos symptomatiske bærere af genvarianten. Dette kunne dog ikke forklare de øgede serumniveauer af NfL, ved inkludering af begge variable i en generaliseret lineær model.

Efterfølgende blev et panel af CSV-proteiner relateret til det endo-lysosomale system, innat immunitet og synapsefunktion analyseret. Der var ikke tegn på generel dysregulering af de proteostatisk markører, men undersøgelsen fremhævede derimod forhøjede niveauer af immunmarkører i *CHMP2B*-FTD, og signifikant reducerede CSV-niveauer af ubiquitin.

Sidstnævnte kan være associeret med dysfunktionelt CHMP2B, der er vist at medføre intracellulær akkumulering af forstørrede multivesikulære legemer, som sorterer ubiquitinerede proteiner.

Anvendeligheden af CSV-ubiquitin som biomarkør for neurodegenerative sygdomme blev evalueret i et systematisk litteraturstudie. Gennemgangen viste, at markøren er mest velundersøgt i Alzheimers sygdom, hvor der konsekvent er påvist forhøjede niveauer. Kun få studier er blevet publiceret om CSV-ubiquitin i de øvrige inkluderede sygdomsgrupper, og de viste, at koncentrationen er øget i Lewy body demens og Huntingtons sygdom, uændret i amyotrofisk lateral sklerose, og signifikant lavere eller uændret i frontotemporal demens og Parkinsons sygdom. Studiet konkluderede at disse divergerende niveauer skal kunne valideres hvis biomarkøren skal have nogen klinisk anvendelighed.

En utargeteret kvantificering af CSV-proteomet hos medlemmerne af den danske FTD-familie viste overrepræsentation af signaleringsveje relateret til komplement-systemet, lipid-metabolisme og innat immunfunktion. Nedregulering af processer forbundet med celleadhæsion og "tight junctions" afspejler muligvis dysfunktion af blod-hjerne-barrieren, svarende til fundet fra det første studie. Analysen viste tegn på forstyrrelse i den klassiske komplement-pathway hos præsymptomatiske bærere og en overaktivering af komplement-kaskaden hos symptomatiske bærere. Det mikrogliasecernerede protein AIM (CD5L) var blandt de mest forhøjede i analysen, og proteinets involvering i immunfunktioner, lipid-metabolisme og komplement-aktivering giver anledning til nærmere undersøgelse i *CHMP2B*-FTD.

Inducerede pluripotente stamceller (iPSCs), genereret ud fra vævsprøver fra bærere af *CHMP2B*-varianten samt raske kontroller, blev differentieret til inducerede mikrogli og neuroner. Funktionelle undersøgelser af mikrogli viste ingen forskelle i fagocytose, mens produktionen af pro-inflammatoriske cytokiner var lavere hos mikrogli med *CHMP2B*-varianten. AIM-proteinet blev secerneret af de inducerede mikrogli og dette blev fremmet af stimulering med humant oxideret low-density lipoprotein (oxLDL). De præliminære undersøgelser af AIM er præsenteret i resultatafsnittet.

Sammenfattet viser undersøgelserne ikke tydelige tegn på endo-lysosomal dysregulering eller en pro-inflammatorisk profil i *CHMP2B*-FTD, men flytter derimod fokus mod andre potentielt underliggende processer som involverer blod-hjerne-barrieren, ubiquitin, komplement-kaskaden, lipid-metabolisme og AIM. Resultaterne fra dette ph.d.-projekt, og de ressourcer som det har bidraget med, vil forhåbentlig udgøre et værdifuld grundlag for kommende forskning rettet mod at opnå forståelse for de patologiske mekanismer i sygdommen, udvikle biomarkører og udforske sygdomsmodificerende behandlingsveje.

List of abbreviations

A β 42	Beta-amyloid peptide 1-42
ACE	Addenbrooke's cognitive examination
AD	Alzheimer's disease
AIM	Apoptosis inhibitor of macrophage
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
ALP	Autophagy-lysosomal pathway
β -III-tub	Tubulin beta 3 class III
bvFTD	Behavioral variant FTD
C9	Complement C9
C9orf72	Chromosome 9 open reading frame 72
CCL4	C-C motif chemokine ligand 4
CCNF	Cyclin F
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain containing 10
CHMP2B	Charged multivesicular body protein 2B
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
CSF	Cerebrospinal fluid
DLB	Dementia with Lewy bodies
EB	Embroid body
ELISA	Enzyme-linked immunosorbent assay
ESCRT-III	Endosomal sorting complex required for transport III
FOXP1	Forkhead box G1
FTD	Frontotemporal dementia
FTD-3	Frontotemporal dementia linked to chromosome 3
FTLD	Frontotemporal lobar degeneration
FUS	Fused in sarcoma
GAG	Glycosaminoglycan
GC	Gene-corrected
GENFI	Genetic frontotemporal dementia initiative
GFAP	Glial fibrillary acidic protein
GRN	Progranulin
HD	Huntington's disease
IBA1	Ionized calcium-binding adapter molecule 1
IF	Immunofluorescence
IFN- γ	Interferon gamma

IL-1 β	Interleukin 1 beta
LC3	Microtubule associated protein 1 light chain 3 alpha
LPS	Lipopolysaccharide
iMicroglia	Induced microglia
NfL	Neurofilament light chain
iPSCs	Induced pluripotent stem cells
MAP2	Microtubule-associated protein 2
MAPT	Microtubule-associated protein tau
MMSE	Mini-mental state examination
MS	Multiple sclerosis
MVB	Multivesicular body
MS	Multiple sclerosis
NfL	Neurofilament light chain
nfvPPA	Non-fluent variant primary progressive aphasia
NGN2	Neurogenin 2
OPTN	Optineurin
oxLDL	oxidized low-density lipoprotein
PD	Parkinson's disease
PRM-MS	Parallel reaction monitoring-mass spectrometry
qPCR	Quantitative polymerase chain reaction
RCAs	Regulators of complement activators
ROS	Reactive oxygen species
Simoa	Single molecule array
svPPA	Semantic variant primary progressive aphasia
SEM	Standard error of the mean
SQSTM1	Sequestosome-1
TBK1	TANK binding kinase 1
TARDBP	TAR DNA binding protein
TDP-43	TAR DNA-binding protein 43
TIA1	T-cell intracellular antigen-1
TGF- β	Transforming growth factor beta
TNF- α	Tumor necrosis factor alpha
TREM2	triggering receptor expressed on myeloid cells-2
UPS	Ubiquitin-proteasome system
UBQLN2	Ubiquilin 2
VCP	Valosin-containing protein
VGLUT1	Vesicular glutamate transporter 1
WT	Wild-type

1. Introduction

1.1. Frontotemporal dementia

Dementia is a complex neurodegenerative syndrome involving progressive decline in cognition, executive functions, language abilities, and activities of daily living. The etiologies include a broad spectrum of reversible and irreversible conditions, impacted by modifying factors¹. The most common underlying cause is neurodegenerative disease, encompassing Alzheimer’s disease (AD), Parkinson’s disease (PD), dementia with Lewy bodies (DLB), Huntington’s disease (HD), amyotrophic lateral sclerosis (ALS), and frontotemporal dementia (FTD).

While FTD only accounts for a small percentage of all-cause dementia in the elderly population, it is one of the most common contributors to early-onset dementia, defined as the appearance of symptoms before the age of 65 years². FTD comprises a group of neurological diseases characterized by selective degeneration of the frontal and temporal cortices. Disease onset is typically in the sixth decade of life. In about 12 % of patients, it presents before 50 years of age, and around 25 % are late-onset cases³. The focal losses of neurons lead to insidious syndromes initially dominated by behavioral changes or language deficits. Accordingly, patients can present with one of three FTD variants based on their clinical features – reflecting the profile of focal frontotemporal atrophy^{4,5}: In behavioral variant FTD (bvFTD), the most frequent phenotype, the symptomatology includes disinhibition, executive dysfunction, emotional blunting, and alterations in personality traits. Loss of semantic memory in semantic variant primary progressive aphasia (svPPA) impairs the patient’s ability to understand the meaning of concepts and words, causing problems with naming, word-finding, and communication. Nonfluent/agrammatic variant primary progressive aphasia (nfvPPV) involves slow and labored speech production, along with difficulties in constructing grammatically and syntactically correct sentences. The diagnostic criteria for FTD rely on these clinical characteristics, as well as exclusion of alternative neurological or psychiatric conditions that could more accurately explain the observations^{6,7}. Supportive imaging showing corresponding atrophy raises the diagnostic certainty from possible to probable, with a definite diagnosis requiring either histopathologic evidence or identification of a known pathogenic variant. As the focal atrophy develops into regional neurodegeneration, the clinical overlap between the three FTD entities increases, and patients gradually develop more global dementia in addition to motor impairment: Upon neurological examination, early parkinsonism is present in a considerable number of FTD

cases, particularly in bvFTD⁸. In some individuals, a combination of FTD and an atypical parkinsonian disorder (progressive nuclear palsy or corticobasal syndrome) is observed. Approximately 15 % of bvFTD patients exhibit symptoms of amyotrophic lateral sclerosis (FTD-ALS), and vice versa⁹. These two conditions are recognized as part of a common disease spectrum due to overlapping clinical presentations and shared neuropathologic features and genetic causes. In late-stage FTD, patients experience a profound decline in cognitive and functional abilities, including behavioral disturbances, psychiatric symptoms, emotional distress, and a significant loss of independence, necessitating comprehensive care and support. The survival time from clinical diagnosis ranges widely from 3 to 12 years, depending on the phenotype, with the shortest duration associated with FTD-ALS^{5,10}.

At present, there is no approved disease-modifying treatment available for patients to prevent or slow down the progression of FTD. Despite being widely used treatments for AD, cholinesterase inhibitors and NMDA-receptor antagonists demonstrate no efficacy in FTD and can even exacerbate neuropsychiatric symptoms in some patients¹¹. However, drug development for heritable FTD is a rapidly expanding and promising field of preclinical and clinical research: The pharmacological approaches include targeting aberrant mRNA using antisense oligonucleotides (ASOs); introducing functional gene copies via adeno-associated viral vectors; immunotherapy with monoclonal antibodies and vaccines against accumulating proteins; and administration of anti-aggregating or stabilizing agents¹¹. The choice of strategy depends on the particular disease mechanism and, by extension, the FTD genotype.

1.2. Genetic FTD

The etiology of FTD includes a substantial genetic component, with 30 % of patients presenting a strong family history, while 70 % of cases arise sporadically¹². From 1998 to 2017, disease-causing genetic variants have been identified in 14 genes located on 11 different chromosomes: *MAPT*, *GRN*, *VCP*, *CHMP2B*, *TARDBP*, *FUS*, *UBQLN2*, *SQSTM1*, *C9orf72*, *CHCHD10*, *OPTN*, *TBK1*, *CCNF*, and most recently *TIA1*¹³. Globally, the leading cause of genetic FTD is pathogenic variants in *C9orf72*, followed by *GRN* and *MAPT*. Pathogenic variants in these genes account for around 80 % of the heritable cases¹⁴, and up to 30 % of all FTD¹⁵, while the remaining genes are responsible for less than 5 % of FTD¹².

Frontotemporal dementia, as well as other neurodegenerative diseases such as AD, PD, HD, and ALS, are commonly referred to as proteinopathies due to the occurrence of proteins that have misfolded and aggregated, leading to a loss of their original, physiological functions and acquisition of neurotoxic properties. Neurons are particularly susceptible to the harmful effects of these protein deposits owing to their longevity and incapability to divide, whereby other cell types might “dilute” the level of toxic proteins. Different brain regions are vulnerable to certain protein accumulations, and this ultimately produces the disease phenotype¹⁶.

In genetic FTD, the disease-causing variants result in aberrant aggregation of specific proteins in neurons and glia cells (Figure 1): Disease-causing variants in *MAPT* drive the formation of cytoplasmic inclusions of the microtubule-associated protein, tau. Pathological tau conformers can be observed as rounded Pick’s bodies or neurofibrillary tangle-like structures¹⁷. This histopathology – frontotemporal lobar degeneration with tau deposition – is termed FTLD-tau. As *MAPT* was the first causative gene to be discovered, FTD was initially considered a tauopathy¹⁸.

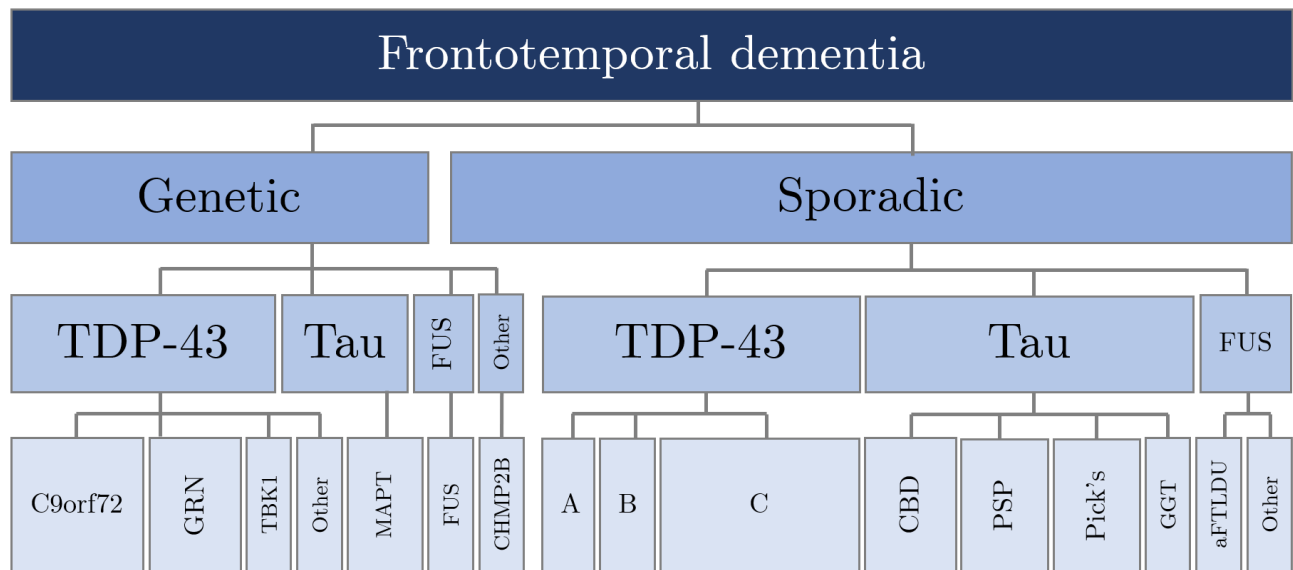


Figure 1. Etiology and neuropathological hallmarks of frontotemporal dementia. In approximately one-third of FTD cases, a strong family history is evident, with TDP-43 proteinopathies being the most prevalent neuropathology due to disease-causing genetic variants in *C9orf72* (typically TDP-43 type A or B), *GRN*, *TBK1*, *VCP*, *SQSTM1*, or *TARDBP*. The second-most common are tauopathies due to pathogenic *MAPT* variants and then FUSopathies resulting from *FUS* variants. *CHMP2B* variants are rare causes of genetic FTD, as described in the following section. Sporadic FTD is characterized by an equal distribution of TDP-43 proteinopathies (usually type C) and tauopathies (including corticobasal degeneration, progressive supranuclear palsy, Pick’s disease, and globular glial tauopathy), and a smaller proportion of FUSopathies, encompassing atypical frontotemporal lobar degeneration with ubiquitin inclusions (aFTLDU). The figure is adapted from Greaves et al.¹⁵

The identification of neuronal inclusions composed of the TAR DNA-binding protein 43 (TDP-43), encoded by the *TARDBP* gene, marked a shift towards associating FTD with ALS – as TDP-43 aggregations are detected in more than 90 % of ALS cases¹⁹. Since then, it has been established that pathogenic variants in 12 of the 14 genes mentioned above are linked to both FTD and ALS¹³. The two most commonly implicated genes in familial FTD, *C9orf72* and *GRN*, were also found to induce TDP-43 pathology. Depending on whether TDP-43 is accumulated predominantly in the nucleus, the cytosol, or in neuritic processes, FTLD-TDP can be sub-classified into types A to D¹⁷. Around 5 % of FTD cases present with aberrant intraneuronal or intranuclear deposits of another RNA- and DNA-binding protein, Fused in sarcoma (FUS). The frequent presence of FUS and TDP-43 aggregation in FTD and ALS indicates a shared pathogenic mechanism related to deficient RNA metabolism or DNA repair¹⁷. In relation to pathogenesis, it remains unclear whether these aggregating protein types should be considered the primary driving force behind neurodegeneration, or if this should be ascribed to defects in the protein clearance systems, or a mutually reinforcing interaction²⁰.

Following the identification of FUS pathology, most FTD histologies that were negative for tau and TDP-43 could be classified as FTLD-FUS upon reassessment²¹. Such specimens were previously labeled FTLD-UPS, given that the observed inclusions were only immunoreactive for ubiquitin – a small protein with an essential function in tagging other proteins for degradation via the ubiquitin-proteasomal system (UPS) or the autophagy-lysosomal system. Thus, only few cases of FTD remain in the FTD-UPS category, such as those caused by *CHMP2B*.

1.3. *CHMP2B*-mediated FTD

In 2005, a genetic variant in the *CHMP2B* gene was identified as disease-causing in a Danish family with bvFTD²². As the genetic locus is positioned on chromosome 3, the disease was termed “FTD-3”. In the following, the somewhat more intuitive designation “*CHMP2B*-FTD” will be used. Of note, a few patients with FTD due to other *CHMP2B* variants have since been presented^{23,24}.

The neuropathological features of *CHMPB*-FTD involve symmetrical and generalized atrophy of the frontal and temporal cortices²⁵. Small, round inclusions have been observed in the cytosol of neurons, particularly in the hippocampus, and to a lesser extent in the frontotemporal cortex. Immunohistochemical stainings for tau, TDP-43, and FUS were found negative, while the inclusions showed immunoreactivity for ubiquitin and p62²⁶. The latter is encoded by the *SQSM1*

gene – which has also been linked to FTD and ALS, as listed above. Inside the cell, p62 functions as a scaffold that anchors ubiquitinated proteins to the autophagosomal isolation membrane, thereby promoting their degradation through the autophagy system²⁷. In contrast to pathogenic *SQSMT1* variants, depositions of ubiquitin and p62 are not specific to the FTD-ALS spectrum. In fact, co-localization of ubiquitin with neurotoxic protein accumulations is a common feature across neurodegenerative diseases²⁰, while p62 positivity has been demonstrated in tau, α -synuclein, and huntingtin aggregates in AD, PD, and HD, respectively²⁷. It remains unknown whether a ubiquitinated, pathognomonic protein exists in *CHMP2B*-FTD. Given the lack of effective antibodies against CHMP2B, it has not been investigated if it forms part of the observed inclusions in patient tissue. The presence of ubiquitin and p62 deposits might otherwise indicate a more general proteolytic dysfunction related to the effects of the *CHMP2B* variant, which are addressed below.

The Danish *CHMP2B*-family comprises one of the most comprehensively explored pedigrees in genetic FTD: The disease has been traced back six generations to a woman born in 1876²⁸. She is likely to be the first noted case of *CHMP2B*-FTD, as her parents were reported not to show any signs of dementia. As the pattern of inheritance was found to be autosomal dominant with full penetrance – as is most frequent in genetic FTD – the disease phenotype manifests in every generation, affects half of the offspring with equal distribution among males and females, and eventually develops in every individual who inherits the pathogenic variant. More than 500 family members have been registered, with over 50 symptomatic and a smaller number of presymptomatic carriers of the *CHMP2B* variant. The age of onset is highly variable and unpredictable, with an average of 58.9 years. The clinical presentation typically involves apathy, disinhibited behavior, loss of empathy and insight, executive dysfunction, non-fluent aphasia, and extrapyramidal symptoms, while memory is often preserved. Modifiable risk factors do not convincingly influence the disease course, which leads to institutionalization in half of cases and a reduction of life expectancy by 13 years²⁸.

The underlying disease-causing variant was found to be a single base substitution – a guanine-to-cytosine transition – within the splice site of the sixth exon of the *CHMP2B* gene (c.532-1-G>C)²² (Figure 2). Aberrant splicing generates transcripts that either include the intronic region between exons 5 and 6, or lack 10 base pairs of exon 6 due to the emergence of a cryptic splice site. Translation produces respective protein products that are truncated in their C-terminus by 36

amino acids, replaced by either a single valine residue (CHMP2B^{Intron5}) or a 29 amino acid nonsense sequence (CHMP2B^{Δ10}). Through overexpression experiments and co-localization with endosomal markers, CHMP2B^{Intron5} proteins were observed to accumulate on the outer membrane of large, abnormal vesicular structures pertaining to the endo-lysosomal system²⁹, and this protein has since been considered the disease-causing factor.

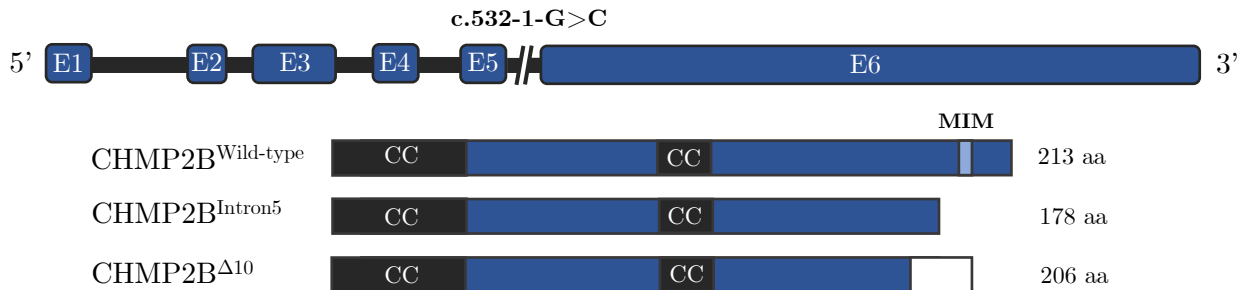


Figure 2. The pathogenic *CHMP2B* variant and its protein products. Schematic showing the *CHMP2B* gene, including the genetic variant inherited in the Danish *CHMP2B*-FTD family, namely a guanine-to-cytosine substitution at the splicing site of exon 6. The wild-type CHMP2B protein consists of 213 amino acids (aa) and contains two coiled-coil domains (CC) and a C-terminal MIT-interacting motif (MIM). The latter is necessary for the recruitment of CHMP2B's binding partner VPS4 and the subsequent membrane fission³⁰. The pathogenic variant leads to the formation of two truncated protein products that lack the MIM domain, with CHMP2B^{Intron5} recognized as the pathogenic form. Adapted from Ugbo and West³⁰.

CHMP2B is a central component of the cellular membrane remodeling machinery ESCRT-III (“endosomal sorting complex required for transport III”). This protein complex catalyzes the fission of membrane necks from the luminal side³¹, which is a central event in processes such as cell division, membrane repair, and internalization of cargo-filled endosomes into multivesicular bodies (MVBs). MVBs are large organelles that sort and transport its intraluminal vesicles for recycling to the plasma membrane, secretion as exosomes, or degradation in lysosomes³². CHMP2B^{Intron5}-mediated impairments of MVB biogenesis and endo-lysosomal function align with the initially observed, enlarged and aberrant endosomal structures. This phenotype, along with accumulations of ubiquitin and p62, has subsequently been recapitulated in cell models^{33,34}; fly, rat, and mouse models^{35–37}; as well as patient fibroblasts and cerebral cortex^{38,39}. As recently reviewed^{40,41}, these and similar CHMP2B^{Intron5} models have also demonstrated the build-up of autophagosomes resulting from impaired fusion with lysosomes. The deficiencies in endocytosis and autophagy may lead to the formation of protein inclusions, in addition to an observed delay in surface receptor

degradation, which is likely to affect certain signaling pathways^{38,42}. Lastly, evidence from post-mortem studies, along with animal and cellular models, have associated *CHMP2B*-FTD with immune activation in the central nervous system (CNS) and substantial alteration of immune cell phenotypes, as addressed in the following.

1.4. Innate immunity in genetic FTD

Recent years have seen a growing focus on the role of chronic neuroinflammation and other maladaptive immune responses in neurodegenerative diseases such as FTD. Abundant lines of evidence, including imaging studies, autoimmunity research, and genome-wide association studies, have established immune system involvement as a hallmark of FTD⁴³. However, whether it is a primary or secondary event – or, more interestingly, whether it contributes significantly to clinical onset or progression and thus presents a target for pharmacological intervention – remains undetermined and is a matter of extensive debate. The potentially pathogenic immune mechanisms are mediated by the principal innate immune cells of the CNS, microglia and astrocytes, which also contain the characteristic protein inclusions observed in several genetic FTD subtypes⁴⁴. Microglia function in immune surveillance, inflammation regulation, and clearance of pathogens, debris, and extracellular protein aggregates by phagocytosis. Astrocytes support neuronal metabolism, maintain neurotransmitter levels, and uphold blood-brain barrier integrity⁴⁵. Accordingly, there are numerous mechanisms by which glia-mediated processes might initiate or exacerbate neurodegeneration. Their exploration has been limited in familial FTD, but an increasing number of studies are directed toward it. For instance, a study using *GRN* knockout mice demonstrated that progranulin deficiency leads to excessive activation of microglia, with overproduction of complement proteins⁴⁶. The complement system, a major branch of innate immunity comprising more than 50 soluble and cell-bound components, is primarily known for its role in recognizing and eliminating pathogenic microorganisms, mediated by the pore-forming C5b-C9 membrane attack complex. Insertion of the complex into the cell membrane triggers lysis of pathogenic microbes, but excessive production or abnormal targeting can also lead to host cell death⁴⁷. In the study, complement proteins C1 and C3 were found to accumulate in synaptosomes in an age-dependent manner. In line with the well-recognized process of complement-mediated synaptic pruning by microglia⁴⁸, the authors detected a marked increase in the elimination of inhibitory synapses, leading to increased excitability in the thalamocortical circuit, behavioral deficits in the mice, and

premature mortality. Deletion of complement protein C1qa could mitigate, but not rescue, the observed pathology. The findings were interpreted as indicating that aberrant microglial phagocytosis is a potential driver of neurodegeneration in progranulin-deficient mice⁴⁶.

In *CHMP2B*-FTD, indications of innate immune engagement were first demonstrated in the histopathological assessments showing gliosis in the frontal neocortex^{26,49}. However, this unspecific reaction involving activated astrocytes and microglia has proven to be a consistent marker of chronic degenerative changes in genetic FTD and neurodegenerative diseases in general⁵⁰. *In vivo* studies investigating genetic modifiers of *CHMP2B*^{Intron5} in genome-wide screenings of *Drosophila* identified the serine protease inhibitor Serpin5 and the endosomal GTPase Rab8 as potent enhancers of toxicity^{51,52}: Serpin5-related effects were found to be conveyed through activation of the Toll signaling pathway – an essential innate immune pathway in *Drosophila* and counterpart to the human Toll-like receptor/NF- κ B pathway – which might contribute to sustained disruption of innate immune activity⁵¹. *Rab8* variants exacerbated toxicity by perturbing endosomal receptor trafficking, which led to upregulation of TGF- β and JNK signaling, involved in modulating immune responses⁵². This association between protein degradation pathways and innate immune activation is substantiated by the recent discovery that the *Drosophila* counterpart of Tank-binding kinase 1 (TBK1), a key regulator in both processes, is an additional potent modifier of *CHMP2B*^{Intron5}⁵³.

Following the initial *Drosophila* model, three mouse models have been established, employing three different promoter systems for driving heterozygous or homozygous expression of *CHMP2B*^{Intron5} with predilection for different neuronal populations^{37,54,55}. In all three studies, gliosis was observed in mice expressing the mutant protein⁴¹. In the first model, presented by Ghazi-Noori et al. in 2012, the authors additionally noted the occurrence of autofluorescent aggregates only in *CHMP2B*^{Intron5} mice from 3 months of age⁵⁶. These aggregations grew progressively in size and quantity over time and did not resemble the commonly observed, aging-related depositions of fluorescent lipofuscin pigment. The autofluorescent aggregates were evaluated to be part of a lysosomal storage pathology developing independently of p62 accumulations, given the absence of co-localization. Interestingly, by 18 months, the pathology was not detected in astrocytes, but in half of neurons, and nearly all microglia. In a follow-up study centered on neuroinflammation, the group identified early microglial proliferation in the *CHMP2B*^{Intron5} mice, which progressed into a pro-inflammatory phenotype marked by increased IL-1 β and TNF- α expression, accompanied by neurodegeneration

and behavioral changes⁵⁷. The presence of autofluorescent aggregates and an inflammatory profile could be confirmed in the cerebral cortex of *CHMP2B*-FTD patients^{56,57}.

These findings, associating the pathogenic *CHMP2B* variant with aberrant innate immune activation, have prompted a search for signs of inflammation in the blood and cerebrospinal fluid (CSF) of the patients, among a range of other potential disease biomarkers.

1.5. Fluid biomarkers in genetic FTD

A biomarker is defined as any quantifiable indicator of a biological process, condition, or response to an exposure or intervention⁵⁸. In FTD, there is a lack of disease-specific biomarkers, which are needed to inform clinicians about expected disease onset, severity, rate of progression, and response to pharmacological intervention. This poses a challenge for both patient care and clinical trials. The difficulty in identifying markers arises from the heterogeneity among FTD subtypes and their overlap with other disease entities in terms of both clinical manifestation and pathomechanisms, as well as modest patient cohort sizes⁵⁹. Additionally, a self-limiting effect might result from the lack of clinically useful biomarkers from sources such as CSF because it reduces the rationale for performing lumbar punctures in the first place. However, in parallel with the advancement of disease-modifying therapies, biomarker research in genetic FTD is rapidly evolving.

Neurofilament light chain (NfL) has been at the center of attention for the last decade. The cytoskeletal protein is highly expressed in large, myelinated axons, and soluble levels are used as a sensitive but unspecific marker of neuronal injury or degeneration⁶⁰. In the major genetic FTD subtypes, plasma and CSF concentrations of NfL have been shown to correlate closely and reflect the rate of clinical progression, making the biomarker valuable for therapeutic trials^{61–63}. Despite relatively high levels in FTD compared to other neurological disorders with increased NfL, as well as in *GRN*-FTD compared to the other genotypes, the current diagnostic value of NfL remains limited^{61,63–66}. At present, the biomarker’s utility lies in distinguishing neurodegenerative disease from conditions such as psychiatric disorders, and in the early detection of emerging axonal degeneration – which may prove to be a favorable timing for intervention in presymptomatic carriers approaching phenoconversion^{61,62,66,67}.

Other humoral biomarkers with prognostic and perhaps pharmacodynamic potential in clinical trials are CSF or blood levels of progranulin and CSF poly-GP DPRs (“poly-glycine-proline dipeptide repeat proteins”), which are respectively reduced and elevated in *GRN*- and *C9orf72*-

FTD^{15,68}. Quantification of CSF TDP-43, the hallmark protein of both genotypes, has demonstrated limited utility, as high concentrations in the bloodstream appear to emanate into the CSF across the blood-brain barrier⁶⁹. More promising measures include plasma levels of phosphorylated TDP-43 or oligomeric conformers^{70,71}; TDP-43 content in CNS-derived exosomes⁶⁹; and assessment of seeding activity using real-time quaking-induced conversion (RT-QuIC)⁷².

bvFTD represents the neurodegenerative disease with the lowest occurrence of positive fluid biomarkers for AD pathology – i.e., increased CSF tau (phospho-tau, total-tau) and reduced beta-amyloid (A β 42, A β 42/40)⁷³. The differential diagnosis between FTD and AD can be improved by combining these markers with NfL and possibly CSF levels of secreted A β precursor protein (sAPP), with NfL being relatively higher in FTD, in contrast to sAPP⁷³. Of note, patients with disease-causing *MAPT* variants may have increased concentrations of phosphorylated tau variants in CSF, which can serve to distinguish between FTLD-tau and FTLD-TDP^{74,75}.

Of the different genotypes, *GRN* variants have most often been associated with immune activation^{76,77}. Accordingly, measurements of soluble TREM2 (“triggering receptor expressed on myeloid cells-2”) and GFAP (“glial fibrillary acidic protein”), indicating microglial and astrocytic activity, have distinguished *GRN*-FTD patients from the other subtypes^{78,79}. However, available data on inflammatory biomarkers in genetic FTD are sparse, and findings show varying patterns, likely reflecting complex underlying mechanisms, small cohorts, and mixed etiologies⁷⁷. To date, efforts to establish an inflammatory biofluid signature in *CHMP2B*-FTD have been unproductive. Rostgaard et al. did not detect any difference in the quantities of the glia-derived inflammatory marker YKL-40 in CSF between 16 patients and 14 healthy controls from the Danish family⁸⁰. Similar results in the major genetic subtypes were recently presented by the Genetic Frontotemporal dementia Initiative (GENFI)⁸¹. In a follow-up study by Roos and colleagues, the CSF levels of 30 cytokines and chemokines were either unaltered or below the detection range of the V-PLEX panels used⁸². In serum, the only significantly increased protein was CCL4 – a chemoattractant involved in inflammatory signaling in the periphery as well as the CNS, and possibly in affecting blood-brain barrier integrity⁸³.

Cohorts like the Danish *CHMPB*-FTD family, with a known disease-causing variant conferring complete penetrance, provide favorable settings for exploring preclinical biomarkers, which are essential for early diagnosis and clinical trial design. An additional advantage lies in the possibility

of transferring and manipulating the genetic variant from patient samples within an *in vitro* system, such as induced cellular models.

1.6. Patient-derived cell models of *CHMP2B*-FTD

Disease models based on induced pluripotent stem cells (iPSCs) have emerged as a valuable alternative to animal studies for investigating neurodegenerative diseases. While lacking the complexity and aging aspect of *in vivo* systems, using human material in this manner circumvents inherent translational and ethical challenges of animal experiments⁸⁴. Moreover, it provides a means to study genetic variants unique to patients in cell types of interest, avoiding the reliance on overexpression or knockout disease models, which might cause pathology that differs fundamentally.

In 2017, Zhang et al. presented the first *CHMP2B*-FTD study employing iPSC-derived neurons reprogrammed from patient-fibroblasts using non-integrative episomal plasmids⁸⁵. In addition to cell lines harboring the disease-causing variant, control lines were generated from non-carrier family members and by CRISPR/Cas9-mediated gene correction of the genetic variant (isogenic controls). *CHMP2B*^{Intron5} neurons displayed enlarged endosomes, resembling the endosomal pathology observed in patient fibroblasts and brain tissue⁸⁶. Functional studies showed signs of abnormal cristae formation and perinuclear mislocalization of mitochondria; a perturbed mitochondrial functional profile with increased production of reactive oxygen species (ROS); and significant reductions in neurite lengths and soma sizes compared to control neurons⁸⁵. Notably, the applied neuronal protocol also led to the generation of astrocyte-like cells in the examined cell population, as indicated by the dysregulation of apolipoprotein E (*APOE*), a gene predominantly expressed by astrocytes, ranking among the top differentially regulated genes.

A subsequent study using iPSC-derived astrocytes linked the *CHMP2B* variant to compromised autophagy based on increased expression of p62 and LC3; elevated ratios of LC3-II/I; and the accumulation of double-membraned organelles with electron-dense contents, which remained unaffected by the autophagy-promoting agent, rapamycin⁸⁷. Again, the variant was reported to impede cellular respiration and heighten oxidative stress levels. Through isotopic labeling experiments, indications of hypometabolism were detected in the form of decreased glycolysis and tricarboxylic acid (TCA) cycle activity. Perhaps secondary to these changes, the authors observed increased expression of the commonly used activation markers GFAP and lipocalin-2 (LCN2),

along with the production of complement protein C3 and inflammatory cytokines. The findings were interpreted as suggesting a reactive astrocytic phenotype triggered by the *CHMP2B* variant. Most recently, Haukedal et al. introduced a disease model comprising microglia differentiated from iPSCs that were genetically edited to carry either a heterozygous or homozygous *CHMP2B* variant⁸⁸. The biallelic variant gave rise to “hyperactive” microglia exhibiting distinct characteristics: Excessive phagocytosis of labeled *E. coli* bioparticles, accompanied by a build-up of electron-dense vesicles upon transmission electron microscopy; increased TCA metabolism and a tendency towards mitochondrial fission; elevated cytokine secretion in unstimulated conditions, with an inflammatory profile; and a neurite-reducing effect of its conditioned medium on iPSC-derived neurons, which was aggravated by priming with low levels of interferon-gamma (IFN- γ). Conversely, microglia with the heterozygous *CHMP2B* variant accumulated cargo-filled organelles in the resting state, while phagocytosis was only modestly increased compared to wild-type lines. Signs of hypometabolism and mitochondrial fusion were observed in this phenotype, and basal cytokine production levels were downregulated relative to healthy cells. This led the authors to designate the phenotype as “unresponsive”. Conditioned medium from heterozygous *CHMP2B* microglia had no effect on neurites, while pre-treatment with a low concentration of IFN- γ was associated with greater neurite lengths than preconditioned medium from wild-type microglia. Besides providing a basis to study the cellular consequences of pathogenic variants, patient-derived cell models can also be utilized to validate findings from other sources, such as biofluids, and investigate the markers mechanistically. This holds particular relevance for genetic FTD subtypes, considering that patient group sizes are often modest and recruitment of validation cohorts challenging, increasing the necessity for alternative approaches to replicate findings.

2. Objectives and hypotheses

Diseases falling under the collective term FTD are characterized by a seemingly paradoxical combination of significant commonalities – both among FTD subtypes and in relation to other forms of dementia – alongside considerable heterogeneity. This reflects the intricate nature of neurodegenerative diseases arising from a complex interplay of genetic, molecular, and environmental factors. While specific disease-causing genetic variants or subtypes may give rise to unique phenotypes, they still share fundamental mechanisms that place them within the FTD spectrum. Hence, this PhD project centers on a single Danish family to gain a clear picture of the pathomechanism caused by the *CHMP2B* variant, while also aiming to use the genotype as a model for more commonly occurring FTD and dementias. The project was motivated by the lack of applicable biomarkers and disease-modifying treatment, as well as an unclear role of the innate immune system in FTD and in *CHMP2B*-mediated FTD specifically – despite efforts from animal studies, biofluid analyses, and cell models. Accordingly, the overall objective was to identify biomarkers and potential therapeutic avenues for *CHMP2B*-FTD by investigating biofluid proteins related to endo-lysosomal function and innate immunity, followed by a functional investigation of candidate proteins using relevant patient-derived cell models.

Study 1

Objective: To evaluate the utility of serum NfL as a biomarker to differentiate between patients with clinical *CHMP2B*-FTD, presymptomatic carriers of the pathogenic variant, and healthy individuals. A secondary objective was to assess any differences in blood-brain barrier permeability, as indicated by the CSF/serum albumin ratio.

Hypothesis: Serum NfL can serve as an equivalent to CSF-NfL, offering comparable discrimination of the clinical groups.

Study 2

Objective: To investigate whether symptomatic or presymptomatic carriers of the *CHMP2B* variant exhibited significant changes in the CSF levels of potential biomarkers linked to the endo-lysosomal system, innate immunity, and synaptic function.

Hypothesis: Examination of the marker panel provides indications of impaired proteolytic function and innate immune activation due to the genetic variant.

Study 3

Objective: Given that altered CSF-ubiquitin levels were a significant finding in Study 2, the objective of this systematic literature review was to elucidate if CSF ubiquitin has been investigated as a biomarker in FTD and comparable neurodegenerative diseases, and assess the concordance between any alterations in ubiquitin concentrations across the disease categories.

Hypothesis: CSF ubiquitin levels can distinguish between groups of neurodegenerative diseases.

Generation of iPSC-derived microglia and neurons

Objective: The primary objectives of establishing patient-derived cell models were to explore the effects of the pathogenic *CHMP2B* variant on glia and neurons, and to investigate identified CSF biomarker candidates mechanistically. To direct the search for key mechanisms and ensure the clinical significance of *in vitro* findings, the cell work was complemented by an unbiased proteomics analysis of CSF from members of the Danish *CHMP2B*-FTD family.

Hypothesis: Markers associated with dysfunctional innate immune activation can be identified in CSF from carriers of the *CHMP2B* variant, and the mechanisms involved can be elucidated using the CHMP2B^{Intron5} cell model.

3. Methods

3.1. Study population: The Danish *CHMP2B*-FTD family

For nearly three decades, the Danish *CHMP2B*-FTD family has been actively involved in research through the Frontotemporal dementia Research in Jutland Association (FReJA), which includes basic and clinical scientists from Denmark as well as international groups. The collaboration led to the discovery of the pathogenic *CHMP2B* variant in 2005²² and has since provided substantial insights into the clinical aspects and underlying cellular mechanisms of the disease. In our multidisciplinary team at the Danish Dementia Research Centre, Rigshospitalet, the family members are offered clinical evaluation and follow-up, as well as genetic counseling, psychological support, and testing for the variant, along with the option of prenatal diagnostics. Predictive genetic testing among at-risk family members has identified an increasing number of presymptomatic carriers. Approximately every five years, researchers from Rigshospitalet convene with the family to inform about ongoing research and encourage participation. Recruitment for the studies is facilitated via the family contact group, comprised of voluntary family members who assist in organizing meetings, updating the pedigree, and conveying research projects to various branches of the family.

To meet the inclusion criteria for the present cross-sectional studies, participants had to be at least 18 years of age and members of the Danish *CHMP2B*-FTD family to ensure a genetically homogenous study population. Additionally, they needed to be verified carriers or non-carriers of the disease-causing variant, representing the study population and control group, respectively. For patients affected by FTD, their inclusion also depended on the disease stage not compromising the validity of informed consent.

A total of 43 members of the Danish family were included in the cross-sectional analyses, comprising 12 symptomatic *CHMP2B*-FTD patients, 13 presymptomatic carriers of the genetic variant, and 18 healthy controls. Thirty-eight participants took part in Study 1, 31 participants in Study 2, and 40 in the subsequent untargeted CSF proteomics analysis. All participants underwent an initial evaluation at the Danish Dementia Research Centre, Rigshospitalet, which encompassed a physical examination, measurement of vital signs, a neurological assessment, and cognitive testing in the form of Mini-Mental State Examination (MMSE) and Addenbrooke's Cognitive Examination (ACE). Biosampling comprised the collection of blood samples, lumbar punctures, and, in a subset

of cases, skin biopsies intended for reprogramming of fibroblasts into iPSCs. All content in the studies and supplementary analyses were approved by the regional scientific ethical committee (H-1-2012-041).

3.2. Biosamples and analysis of biomarkers

Lumbar punctures were performed as per standard protocol in the L3/L4 or L4/L5 interspace, and CSF was collected in polypropylene tubes. According to consensus guidelines⁸⁹, the samples were centrifuged at 2000 x g for 10 minutes at 4 °C, and 250 uL aliquots were stored immediately at -80 °C, pending biochemical assays. Blood samples were acquired through venipuncture using EDTA tubes, followed by centrifugation at the settings stated above. Plasma and serum fractions were frozen at -80 °C. Full blood was maintained at -20 °C and used for genotyping at the Molecular Genetics Laboratory, Department of Clinical Genetics, Rigshospitalet.

Serum concentrations of NfL were quantified using the NF-Light Advantage Kit for the Quanterix single-molecule array (Simoa) at the Institute of Clinical Medicine-Neurology, University of Eastern Finland. The data were analyzed in Study 1 and compared with corresponding levels in CSF, which were measured with a sandwich enzyme-linked immunosorbent assay (ELISA) as part of a previous study by Rostgaard et al.⁸⁰. Measurements of serum and CSF albumin were included in the routine laboratory testing at the Department of Clinical Biochemistry, Rigshospitalet.

For Study 2, a panel of 47 pre-specified peptides representing 20 different proteins (Table 1) were quantified in CSF samples using solid-phase extraction and parallel reaction monitoring-mass spectrometry (PRM-MS).

Protein target (UniProtKB entry)	Gene	Function
AP-2 complex subunit beta (P63010)	<i>AP2B1</i>	AP2 complex subunit, central in formation of clathrin-coated vesicles in clathrin-mediated endocytosis ⁹⁰
Amyloid beta A4 protein (P05067)	<i>APP</i>	Transmembrane protein with several suggested functions ⁹¹ . Processed by secretases into fragments, e.g., A β peptides ⁹²
Complement component C9 (P02748)	<i>C9</i>	Innate immunity component of the membrane attack complex ⁹³
Cathepsin B (P07858)	<i>CTSB</i>	Lysosomal cysteine protease with carboxydipeptidase activity ⁹⁴
Cathepsin D (P07339)	<i>CTSD</i>	Lysosomal aspartic protease ⁹⁵
Cathepsin F (Q9UBX1)	<i>CTSF</i>	Lysosomal cysteine protease ⁹⁴
Cathepsin L1 (P07711)	<i>CTSL</i>	Lysosomal cysteine protease ⁹⁴
Cathepsin Z (Q9UBR2)	<i>CTSZ</i>	Lysosomal cysteine protease with carboxymonopeptidase activity ⁹⁴
Dipeptidyl peptidase 2 (Q9UHL4)	<i>DPP7</i>	Lysosomal protease producing dipeptides from tripeptides ⁹⁶
Ganglioside GM2 activator (P17900)	<i>GM2A</i>	Extracts lipids from membranes. Required for ganglioside GM2 degradation ⁹⁷
Beta-hexosaminidase subunit β (P07686)	<i>HEXB</i>	HEXB is the β -subunit of lysosomal Hex A, a heterodimeric complex that hydrolyzes ganglioside GM2, with cofactor GM2A ^{97,98}
Lysosome-associated membrane glycoprotein 1 (P11279)	<i>LAMP1</i>	Lysosomal transmembrane protein important in vesicle fusion ⁹⁹ , preserving lysosomal integrity, and potentially lysosomal exocytosis ¹⁰⁰
Lysosome-associated membrane glycoprotein 2 (P13473)	<i>LAMP2</i>	Lysosomal transmembrane protein important in vesicle fusion ⁹⁹ , chaperone-mediated autophagy, and preserving lysosomal integrity ¹⁰⁰
Lysozyme C (P61626)	<i>LYZ</i>	Glycan cleaving polypeptide. Produced and found in secretory vesicles of neutrophils, monocytes, macrophages, and epithelial cells ¹⁰¹
Tissue alpha-L-fucosidase (P04066)	<i>FUCA1</i>	Lysosomal fucosidase hydrolyzing fucose containing glycoconjugates ¹⁰²
Transcobalamin-2 (P20062)	<i>TCN2</i>	Delivers cobalamin to lysosomes where it is released ¹⁰³
Tripeptidyl-peptidase 1 (O14773)	<i>TPP1</i>	Lysosomal aspartic or serine protease producing tripeptides ¹⁰⁴
Ubiquitin (P0CG48)	<i>Ubiquitin</i>	Labels proteins for degradation by the proteasome ¹⁰⁵ . Has a multitude of functions as a post-translational modification ¹⁰⁶
Synaptotagmin-1 (P21579)	<i>SYT1</i>	Ca ²⁺ sensor that triggers vesicle fusion and neurotransmitter release ¹⁰⁷
Synaptosome-associated protein of 25 kDa (P60880)	<i>SNAP25</i>	Presynaptic plasma membrane protein and key part of the SNARE complex, enables vesicle fusion and neurotransmitter release ¹⁰⁸

Table 1. Proteins represented by the peptides quantified in the study, along with their primary cellular functions. The table is adapted from the paper of Sjödin et al. 2019, which utilized the same peptide panel as in Study 2, except for the synaptic markers included here, SYT1 and SNAP25 (Sjödin et al., *Alzheimer's Research & Therapy*, doi: 10.1186/s13195-019-0533-9)¹⁰⁹.

Concentrations of the two synaptic markers synaptotagmin-1 (SYT-1) and synaptosome-associated protein of 25 kDa (SNAP-25) were assessed by enrichment with immunoprecipitation, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The protein quantifications were

carried out by the group of Prof. Henrik Zetterberg and Prof. Kaj Blennow at the Department of Psychiatry and Neurochemistry, Sahlgrenska Academy, University of Gothenburg.

Subsequently, the group performed an additional, untargeted proteomics analysis employing tandem mass tags (TMT) for the chemical labeling of CSF peptides, which were identified and measured by LC-MS/MS. The analysis provided quantitative information on 2477 proteins. Pathways that were overrepresented in the dataset were explored using the enrichment analysis tools KEGG pathways¹¹⁰, WikiPathways¹¹¹, and Biological Processes¹¹², integrated in the Enrichr platform¹¹³. Separate analyses were conducted for symptomatic and presymptomatic carriers versus controls, and the most significantly enriched pathways were selected according to the adjusted p -values (Appendix IV, Table S1).

The data from Study 1 and 2 were processed using R statistical Software v3.6.1. Normal distribution of biomarkers was assessed with histograms, QQ-plots, and Shapiro-Wilk tests. Logarithmic transformation was utilized, when appropriate, to achieve normally distributed data prior to performing parametric tests. Fisher’s exact test was used to calculate descriptive statistics. Biomarker levels among symptomatic carriers of the *CHMP2B* variant, presymptomatic carriers, and healthy controls were compared using general linear models (Study 1) or ANOVA with post-hoc Tukey tests (Study 2), with age included as a covariate. Correlations between continuous variables were evaluated using Spearman’s rank correlation coefficient. Protein levels differing between the clinical groups in the untargeted CSF proteomics data were determined using Wilcoxon Rank Sum test with a significance threshold of 5 %.

3.3. Search strategy and selection criteria for systematic review

Literature searches were performed on April 1, 2023, using the PubMed MeSH database to identify studies comparing CSF ubiquitin levels between a neurodegenerative disease group and a control group. The original, full-text articles had to be written in English and comprise at least 10 participants. Six search strings were employed in the format “Ubiquitin AND (Frontotemporal dementia OR FTD) AND (Cerebrospinal fluid OR CSF)” for the diseases AD, ALS, DLB, FTD, HD, and PD. Two authors independently screened abstracts for relevance and extracted data from studies fulfilling the inclusion criteria. A total of 171 abstracts were evaluated, and 17 studies were ultimately included.

3.4. Cell culturing

Forearm skin biopsies were obtained from presymptomatic and symptomatic carriers of the *CHMP2B* variant, as well as healthy controls. Fragments of the tissue samples were distributed into flat-bottom tubes, and fibroblasts were isolated by two-week incubation in complete DMEM 1885 medium with NaHCO_3 , followed by medium change every two days and expansion in T25 and T75 flasks.

Fibroblasts were transfected with episomal plasmids pCXLE-hOCT4, pCXLE-hSK, and pCXLE-hUL (Addgene) to initiate reprogramming into iPSCs, as detailed in Okita et al. 2011¹¹⁴. In short, fibroblasts cultured on Matrigel-coated plates were transfected with 1 $\mu\text{g}/\mu\text{L}$ of the plasmid mixture using X-tremeGENE Transfection Reagent (Roche) in accordance with the manufacturer's instructions. The following day, the fibroblast medium was supplemented with 5 ng/mL basic fibroblast growth factor (bFGF), and the medium was changed to mTeSR1 Plus Medium with 100 μM sodium butyrate on day 3. Media changes were performed every other day until week 3 or 4, at which point iPSC colonies were ready for picking and plating on laminin-coated plates, followed by expansion and cryopreservation in liquid nitrogen tanks. Isogenic control lines were generated through CRISPR/Cas9-mediated correction of the guanine-to-cytosine substitution in iPSC lines harboring the *CHMP2B*-variant by Bioneer A/S, Hørsholm, Denmark. Stemness was immunohistochemically verified using the markers NANOG, OCT3/4, TRA-1-60, TRA-1-81, SSEA-3, and SSEA-4. Pluripotency of the iPSCs was confirmed by culturing cells in media inducing ectodermal, endodermal, or mesodermal tissue (Appendix IV, Table S2), followed by germ layer staining for TUBB3, SMA, and SOX17. To ensure that no chromosomal alterations had been introduced, fixed cell samples were sent for karyotyping at the Institute for Medical Genetics and Applied Genomics, University Hospital of Tübingen, Germany.

The detailed protocols for the generation of induced microglia and neurons can be found in Appendix IV (Protocol S1 and S2). To generate microglia-like cells, the protocols by Haenseler et al. 2017 and van Wilgenburg et al. 2013 were adapted^{115,116}. The approach aims to mimic the distinctive embryological development of microglia from primitive myeloid progenitor cells in the yolk sac during early fetal development. Embroid bodies (EBs) were formed from iPSCs, and mTeSR Plus Medium was supplemented with bone morphogenetic protein 4 (BMP4), vascular endothelial growth factor (VEGF), and stem cell factor (SCF) to guide cell differentiation toward the mesodermal germ layer and to induce endothelial and hematopoietic precursors. On day 7, EBs

were plated unto Matrigel-coated dishes, and the culture medium was changed to contain interleukin 3 (IL-3) and macrophage colony-stimulating factor (M-CSF) to drive myelopoiesis. Over 4 weeks, EBs adhered to the dish, put out surrounding stromal tissue, and transformed into large cystic structures resembling yolk sacs, from which a uniform population of free-floating or attaching microglial precursors were produced. These cells were harvested weekly or biweekly and plated for maturation in Advanced DMEM/F12 medium with cytokines IL-34, granulocyte M-CSF (GM-CSF), and transforming growth factor- β (TGF- β) for 1-2 weeks to promote differentiation of homeostatic microglia and ensure engagement of the CSF1 receptor, required for their continued development and survival¹¹⁷. Characterization of the induced microglia included immunostaining for ionized calcium-binding adapter molecule 1 (IBA1), purinergic receptor P2RY12, and transmembrane protein 119 (TMEM119), and quantitative PCR (qPCR) for expression of *P2RY12*, CX3C motif chemokine receptor 1 (*CX3CR1*), and *CD45*.

Neuronal differentiation was performed by modifying the protocol by Chen et al. 2020¹¹⁸. Initially, iPSCs were transfected with a doxycycline-inducible cassette containing Neurogenin 2 (*NGN2*) – a transcription factor whose overexpression promotes neurogenesis¹¹⁹. The construct was integrated into a “safe harbor” locus using the genome-editing technique TALEN (transcription activator-like effector nucleases) and included positive selection markers in the form of genes encoding the mCherry fluorescent protein and resistance to puromycin and neomycin antibiotics. After antibiotic selection, mCherry-expressing clones were isolated using fluorescence-activated cell sorting (FACS). An additional sorting step involved transfection with a GFP-tagged Cre recombinase expression plasmid to excise mCherry, followed by FACS to isolate the transiently GFP-positive/mCherry-negative cells. To promote a neural cell fate, the cells were cultured for 4 days in an induction medium containing the small molecules SB431542 and LDN193189, inhibiting both TGF- β and BMP signaling, known as dual SMAD inhibition¹¹⁹. Neural progenitor cells were transferred to plates coated with poly-L-ornithine and laminin, and terminal differentiation in growth factor-enriched medium was carried out for 2-3 weeks. Immunofluorescence-based characterization of the induced neurons included markers of neuronal lineage (β -tubulin III and microtubule-associated protein 2, MAP2), forebrain patterning (Forkhead Box G1, FOXG1), and neuronal maturation (NeuN, vesicular glutamate transporter 1, VGLUT1, and Synapsin 1).

3.5. Experimental procedures

Immunofluorescence staining

Cells were fixed in 4 % paraformaldehyde (PFA) for 10 minutes, blocked with blocking buffer (5 % normal goat serum, 2 % BSA, and 0.25 % Triton X-100) for 30 minutes, and then incubated with primary antibodies (see Appendix IV, Table S3) in blocking buffer overnight at 4 °C. The next day, cells were washed three times in PBS and incubated with secondary antibodies (Appendix IV, Table S3), diluted in blocking buffer, for 1 hour at room temperature. Subsequently, nuclei were stained for 5 minutes with Hoechst, washed three times in PBS, and mounted on glass slides with Fluorescence Mounting Medium (Agilent) before image acquisition using an EVOS M7000 microscope.

Western blot

Cells were lysed in a Triton X-100 buffer containing 1 % Triton X-100, protease inhibitor cocktail, and phosphatase inhibitor cocktail 2 and 3 (Sigma) for 5 minutes at room temperature and then kept on ice. The cell lysates were centrifuged at 17,000 x g for 5 minutes at 4 °C. Supernatants were collected, and protein content was determined with the DC protein assay Kit (Bio-Rad). Protein samples were prepared by adding Laemmli Sample Buffer (250 mM Tris-HCl, 8 % SDS, 40 % Glycerol, 0.005 % bromophenol blue, and 100 mM dithiothreitol) and boiled at 100 °C for 5 minutes, followed by brief centrifugation and vortex. Equal amounts of sample (20-40 µg) were loaded onto 4-12 % or 16 % NuPage polyacrylamide gels (Invitrogen) using appropriate running buffer. To size separate the proteins, electrophoresis was carried out in a Mini Gel Tank Blot Module Set (Invitrogen) at 90 V for 15 minutes, followed by 135 V or 160 V until the protein marker reached the bottom of the gel (around 40-60 minutes). Subsequently, the proteins were transferred to a nitrocellulose membrane using 20-22 V for 70-75 minutes. The membranes were blocked for 1 hour while shaking in either TBS with 0.1 % Tween 20 (TBS-T) and 5 % bovine serum albumin (BSA) or PBS with 0.1 % Tween 20 (PBS-T) and 5 % skimmed milk and then incubated overnight with primary antibodies (Appendix IV, Table S3) diluted in TBS-T or PBS-T at 4 °C on a shaker. The following day, membranes were washed three times in TBS-T or PBS-T before and after 1-hour incubation at room temperature with secondary antibodies (Appendix IV, Table S3) diluted in TBS-T/milk or PBS-T/BSA. Blots were visualized with the LI-COR Odyssey

M Imaging System, and mean fluorescence band intensities were quantified with Empiria Studio Software v2.2.

qPCR analysis

TRIzol Reagent (Invitrogen) was utilized for RNA extraction, followed by RNA purification using RNeasy mini kit (Qiagen). Concentrations of total RNA were measured with a NanoDrop 2000 spectrophotometer. Reverse transcription of 200 ng mRNA samples to cDNA was performed with Superscript IV First-Strand cDNA Synthesis Reaction (Invitrogen), with a 1:1 ratio of Oligo(dT) primers and random hexamers. Transcript levels were determined using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent), with primer pairs against P2RY12, CX3CR1, and CD45. RNA expression levels were normalized to the reference gene RPL13A.

Phagocytosis assays and flow cytometry

Induced microglia were seeded at a density of 100.000 cells/well on CC2-treated 8-well glass chamber slides (Thermo Fisher Scientific). After 2 weeks of maturation, the cells were incubated at 37 °C with Alexa Fluor 488-labeled Zymosan A (*S. cerevisiae*) bioparticles (Thermo Fisher Scientific) for 0.5, 1, 2, and 3 hours. Cells were washed three times in PBS, fixed, and counterstained with Hoechst for immunofluorescence imaging as described. The number of internalized bioparticles was quantified using ImageJ and normalized to the cell count, which was determined from the nuclear staining.

To test the phagocytosis of apoptotic neurons, iPSC-derived neurons were plated at 500.000 cells/well in a 12-well plate. After 4 weeks of cultivation, live cells were loaded with 1:100 Vybrant CM-Dil dye (ThermoFisher Scientific) for 1 hour and UV-irradiated (500 J/m²) for 20 minutes. Dye-labeled apoptotic neurons were counted, and apoptosis was confirmed with viability staining using Solution 13 (ChemoMetec). The cells were centrifuged at 1000 x g for 10 minutes and resuspended in Microglia Differentiation Medium (Appendix IV, Protocol S1). A total of 200.000 dye-labeled apoptotic neurons were added to each well of induced microglia (seeded at 400-000 cells/well in 12-well plates for 2 weeks) for 30 or 90 minutes. The microglia were washed with PBS, kept on ice, and detached using a cell scraper. The cell suspensions were analyzed with an Attune Nxt Acoustic Focusing Cytometer (Invitrogen), using the yellow laser (561 nm) for excitation and Attune Nxt Software v.4.2.0 for data analysis.

Cytokine assay and ELISA

Media from induced microglia, plated at 400.000 cells/well in fibronectin-coated 12-well plates, were collected, centrifuged at 1000 x g for 10 minutes at 4 °C to remove dead cells and debris, and used for cytokine screening and measurement of AIM concentrations ("Apoptosis Inhibitor of Macrophage" or CD5L). For the respective assays, cells were stimulated after 2 weeks of terminal differentiation with either 100 ng/ml lipopolysaccharide (LPS) or 50 ug/ml human oxidized low-density lipoprotein (oxLDL) for 4 hours. Cytokine content was screened using the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems) with medium applied to the kit membranes following the manufacturer's recommendations. Fluorescence from RDye 800CW Streptavidin was captured using the LI-COR Odyssey M Imaging System and quantified in ImageJ. AIM levels were determined with the Human CD5L ELISA Kit (Invitrogen). Briefly, standards and undiluted samples were prepared as per the provided instructions and added to microtiter plates precoated with AIM capture antibodies. Cell medium concentrations were determined by measuring absorbance at 450 nm. The kit was also employed to analyze CSF from members of the Danish *CHMP2B*-FTD family. CSF samples were thawed from -80 °C and diluted 1:5 in assay diluent before the analysis.

4. Results and discussion

4.1. Study 1: Serum neurofilament light

The study explored the potential of neurofilament light as a blood-based biomarker in *CHMP2B*-FTD for distinguishing between symptomatic, presymptomatic, and healthy individuals. To this end, cross-sectional analysis was performed on serum and CSF samples obtained from 38 members of the Danish *CHMP2B*-FTD family, encompassing 22 carriers of the pathogenic variant and 16 non-carriers. Serum NfL levels were tested for association with the clinical groups, CSF NfL concentrations, the ratio of albumin in CSF and serum, and clinical variables, including age, disease duration, and ACE score.

The quantities of NfL in serum closely correlated with those in CSF, which were approximately 50 times higher. The clinical groups could be separated based on serum NfL measurements, showing similar fold changes to CSF concentrations (Figure 3). The generalized linear model employed was adjusted for age, given the expected age gap between the presymptomatic carriers and the other participants.

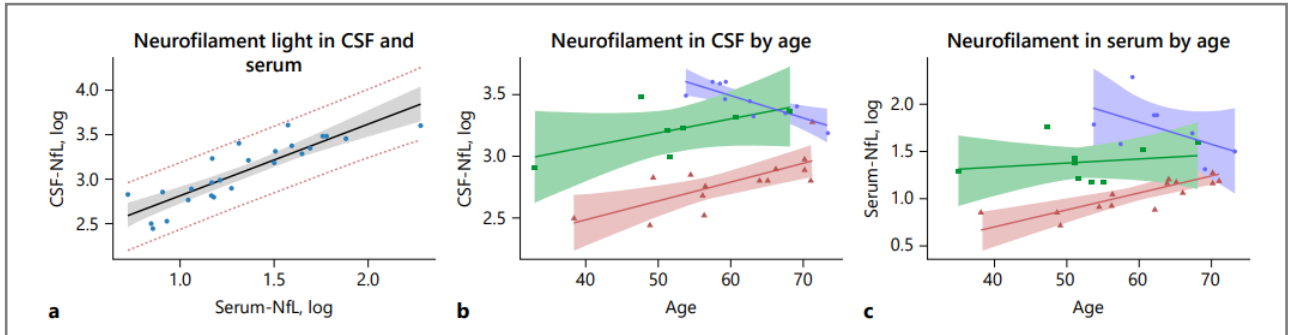


Figure 3. CSF-serum correlation and NfL levels in CSF and serum by age. (a) Depiction of the linear relationship between measurements of neurofilament light protein in CSF and serum. 95 % confidence interval and prediction limits are shown. (b) and (c) represent generalized linear models of associations between age and NfL level in symptomatic (blue), presymptomatic (green), and non-carrier (red) participants. Neurofilament data are logarithmically transformed.

The CSF/serum albumin ratio – commonly used as a measure of blood-brain barrier permeability given that albumin is exclusively synthesized in the liver – was found to be elevated in *CHMP2B*-FTD patients compared with controls and correlated positively with serum NfL levels. When including the ratio in the linear model, NfL remained significant, except for the comparison between presymptomatic participants and controls ($p = 0.079$). While this suggests a potential

interaction between these variables, the interaction term was not statistically significant and was excluded from the final model.

While acknowledging the study’s cross-sectional design and the limited number of data points in the plots, the data indicate that serum NfL levels in presymptomatic carriers start to deviate from those in the controls at an early time point. This corresponds with findings in the major genetic subtypes, caused by variants in *C9orf72*, *GRN*, and *MAPT*, where plasma NfL is the earliest biomarker to change, alongside regional brain volume loss on MRI¹²⁰: Mean plasma NfL values in *C9orf72* carriers appear to begin increasing approximately 30 years before estimated onset. However, considerable variation in NfL dynamics has been observed between the FTD genotypes during the preclinical phase. These differences can be of great significance for designing preventive clinical trials, particularly when seeking participants nearing phenoconversion and when utilizing blood NfL levels as a time-to-event endpoint, with the aim of delaying disease onset^{63,120}.

In the present study, serum NfL concentrations were found to correlate negatively with ACE scores in *CHMP2B*-FTD. Similarly, the marker has been demonstrated to reflect the rate of clinical progression in the other subtypes and, thus, the prognosis^{61–63}. The plasma levels of NfL have been shown to predict the likelihood of progression or phenoconversion in asymptomatic individuals⁶³, which can be leveraged for stratification when presymptomatic carriers are included in clinical trials.

A certain degree of within-group variability in NfL quantities is observed in the current study and elsewhere⁶³. Upcoming studies should prioritize the identification and adjustment for factors that might influence the intra- and inter-individual variability in NfL levels, such as inflammatory conditions, genetic modifiers, medications, and blood-brain barrier integrity. Nonetheless, the data support the use of serum NfL as a valuable fluid biomarker in *CHMP2B*-FTD to monitor the progression of the neurodegenerative process.

4.2. Study 2: Endo-lysosomal CSF biomarkers

This study assessed a panel of peptides linked to endo-lysosomal function, innate immunity, and synaptic function in CSF samples from 18 carriers of the disease-causing *CHMP2B* variant and 13 controls. A total of 47 peptides representing 20 proteins were analyzed using a combination of solid-phase extraction and parallel reaction monitoring mass spectrometry (PRM-MS). The method was recently established by our collaborators at the Department of Psychiatry and Neurochemistry

at Sahlgrenska Academy, University of Gothenburg^{109,121,122}. The selection of peptides for the panel was informed by a prior exploratory TMT-MS analysis of human CSF¹⁰⁹, prioritizing high prominence, stability, and derivation from functionally significant regions of proteins linked to the mentioned cellular processes of interest. Significant findings were tested for correlation with CSF-NfL levels, albumin ratios, age, and ACE scores. The proteins corresponding to the analyzed peptide panel are listed in Table 1 (Methods, section 3.2). The quantities of 6 proteins were significantly altered in carriers of the *CHMP2B* variant compared with healthy controls, including increased complement C9, lysozyme, and transcobalamin-2 (TCN2); and decreased ubiquitin, cathepsin B, and amyloid precursor protein (APP). When dividing *CHMP2B* variant carriers into symptomatic and presymptomatic participants, 10 of the 14 peptides derived from these proteins were significantly associated with the clinical groups (Figure 4).

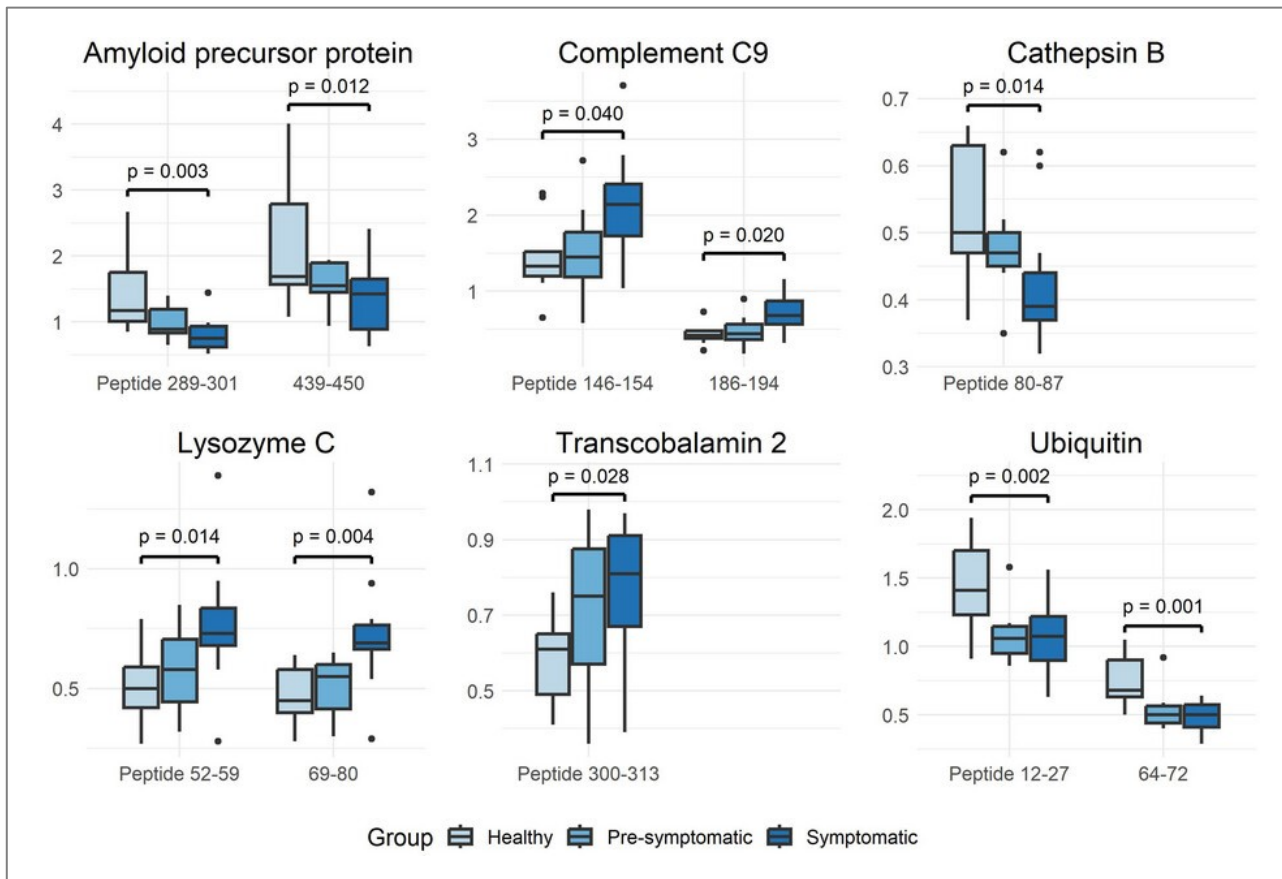


Figure 4. Significantly altered CSF peptide levels. The plots include comparisons among the three clinical groups: Healthy participants, presymptomatic carriers of the *CHMP2B* variant, and symptomatic carriers. The Y-axis represents a ratio between tryptic peptide and an internal standard. ANOVA with post-hoc Tukey multiple comparisons of means test was used for the statistical analysis, and the data were adjusted for participant age at the time of sampling.

The main findings included a significant reduction in CSF ubiquitin levels in *CHMP2B*-FTD. This was interesting, given that deposits of ubiquitin and p62 are the only immunohistochemical features identified in postmortem brain tissue²⁵, and their concentrations in CSF have not previously been investigated. The lower levels of ubiquitin contrast with results from other neurodegenerative diseases, as elaborated upon in the discussion of Study 3.

Two proteins involved in innate immune function, complement C9 and lysozyme, were found increased in *CHMP2B*-FTD patients. Elevated levels of complement proteins in the bloodstream have been observed across various neurological conditions, including stroke, epilepsy, and traumatic brain injury¹²³. CSF concentrations have been less thoroughly investigated and the focus has mostly been on the opsonizing and chemotactic C3 proteins, which serve as the convergence point for all three complement activation pathways. C9 has previously been shown to be reduced in CSF of multiple sclerosis (MS) patients and increased in plasma and astrocyte-derived exosomes from patients with AD, while a study employing the same peptide panel as here did not observe any changes in C9 quantities in CSF from AD or PD patients compared with healthy controls^{109,123}. In a recent GENFI study, complement activation was observed in the symptomatic stage of *C9orf72*-FTD, along with indications that levels of complement proteins might increase before disease onset. Complement C9, however, was not among the CSF analytes and was excluded from plasma analyses as the concentrations were below the quantification limits¹²⁴. Although practically all complement proteins are produced locally in the CNS by neurons and glial cells, the potential influence of peripherally circulating levels on the heightened CSF content of C9 (and lysozyme) cannot be disregarded, as the innate immune markers correlated significantly with the albumin ratios in the present dataset.

The levels of soluble APP were significantly lower in *CHMP2B*-FTD in comparison to the control group. The included peptides originate from the non-amyloidogenic cleavage products sAPP α and sAPP β . While the CSF concentrations of these markers appear unchanged in patients with AD¹²⁵, several studies indicate reductions in FTD cohorts, including behavioral and language variants^{126–128}. The mechanisms underlying these findings are unknown. Since the intracellular processing of APP involves internalization in MVBs in a process involving ESCRT-III before sorting to lysosomes¹²⁹, the decreased CSF levels in *CHMP2B*-FTD could be speculated to stem from entrapment of APP within the aberrant vesicular structures that characterize the disease. This could reduce the availability of APP for cleavage into soluble forms at the cell surface.

Additionally, the concentrations of cathepsin B were decreased, suggesting possible disturbances in lysosomal protein degradation. However, other cathepsins and lysosomal proteins remained unaltered when compared with controls. Hence, the screening did not reveal a substantially disrupted proteolytic function based on the examined peptides. In a recent study using the same panel, this was notably more apparent in patients with *nfvPPA*, where lower levels of 10 out of 18 examined proteins indicated dysfunctional endocytosis and chaperone-mediated autophagy¹³⁰. In contrast, the study only observed a few changes in *svFTD* and none in logopenic variant *FTD*. Collectively, the studies show varying endo-lysosomal marker profiles across *FTD* variants, potentially reflecting the underlying pathology, as the most commonly associated proteinopathies differ among the variants. Clearly, these screenings only represent an initial step toward understanding the endo-lysosomal changes in the subtypes of *FTD*.

4.3. Study 3: CSF ubiquitin in neurodegenerative diseases

This systematic review investigated the extent to which CSF levels of ubiquitin have been studied as a fluid biomarker in neurodegenerative diseases, including *FTD*. The objective was to evaluate and discuss the marker's clinical relevance and, given the reduced ubiquitin concentrations observed in the previous study, compare the direction of any changes among the included disease entities. Through systematic literature searches, 171 studies were identified that compared CSF ubiquitin levels in cohorts of *AD*, *ALS*, *DLB*, *FTD*, *HD*, or *PD* patients against control groups. Following the screening of abstracts, a total of 17 studies meeting the eligibility criteria were included, with several encompassing multiple disease groups.

Reviewing the studies revealed a broad spectrum of analytical methods employed for measuring CSF ubiquitin, primarily divided between immunoassays and, especially in more recent studies, mass spectrometry. The use of several different techniques in combination with the presence of ubiquitin in various states – free, conjugated, monomeric, or polymeric – could potentially complicate the detection of significant changes in ubiquitin levels.

However, among 13 studies involving *AD* patients, 12 observed elevated CSF ubiquitin, with 9 reporting statistically significant findings. In this instance, the diverse analytical approaches served as a supporting factor, countering measurement-related biases and indicating a well-founded association between increased CSF ubiquitin levels and *AD* pathology. Similar conclusions cannot be drawn for the remaining disease groups, as available evidence is too limited. One study included

DLB patients and another HD patients, with both demonstrating significantly increased ubiquitin levels compared with controls. Although most studies investigating ALS, FTD, or PD did not detect any differences in CSF ubiquitin, two studies presented significant alterations, with the lowest levels in patients within a PD cohort and an FTD cohort – the latter being Study 2.

The review highlights that the polyubiquitination of neurotoxic protein inclusions is a common feature in neurodegenerative diseases. As this process sequesters intracellular ubiquitin, it might reasonably be expected that CSF-ubiquitin should generally be lower in the patients compared to the healthy controls. The evident increase in AD may result from the substantial load of extracellular protein aggregates in the form of plaques and “ghost” tangles. The presence of AD pathology in the majority of DLB cases, coupled with higher NfL levels and greater disease propagation than in PD, could account for the greater influx of ubiquitin into the CSF in DLB compared with PD. In *CHMP2B*-FTD, the accumulation of MVBs carrying ubiquitinated proteins might form an additional reservoir of ubiquitin, possibly not reaching the CSF. Alternatively, reduced CSF ubiquitin levels could relate to compromised secretion of ubiquitin-containing extracellular vesicles, a process potentially reliant on CHMP2B functionality.

The study concluded that elevated levels of CSF ubiquitin is a consistent finding in AD, while there is insufficient evidence to support significant changes in the other disease groups. Particularly, the divergent levels found in FTD, ALS, and PD require further study to establish the biomarker’s potential for offering disease-specific information rather than representing all-cause neurodegeneration. Otherwise, it is challenging to envision any clinical utility of the marker in clinical practice, given the existing usage and more feasible quantification of other neurodegenerative markers such as NfL.

4.4. Untargeted CSF proteomics

To uncover dysregulated, pathological mechanisms in *CHMP2B*-FTD, an untargeted proteomics analysis was performed on CSF samples obtained from 10 symptomatic *CHMP2B*-FTD patients, 12 presymptomatic carriers, and 18 healthy controls, yielding information on 2477 proteins. Following the exclusion of data with half of the measurements or more missing in one clinical group (467 proteins), the levels of the remaining proteins were compared across the groups. The most dysregulated proteins were identified based on fold changes and significance levels, and overrepresented pathways were explored through enrichment analyses.

The latter demonstrated that the proteomic signature in CSF from symptomatic participants was predominantly characterized by complement system pathways, as detailed in Table S1 of Appendix IV. Furthermore, significant enrichment was observed in pathways associated with lipid metabolism and various infections (e.g., staphylococcus, coronavirus, etc.). Additional overrepresented processes were the regulation of peptidase activity and coagulation, while the downregulated processes included cell adhesion, glycosaminoglycan (GAG) synthesis, and axon guidance. The profile of presymptomatic carriers resembled that of symptomatic individuals, albeit based on only 197 significant proteins compared to 669. The most enriched pathways were linked to lipid metabolism (cholesterol production, lipoprotein particle remodeling, hyperlipidemia) and the complement cascade, while downregulation was observed in cell adhesion, axonogenesis, and several nonspecific processes. Other selected points of interest included upregulated lysosome pathways and downregulated glial cell differentiation, although these pathways only just reached significance in the analysis (Appendix IV, Table S1).

Examination of proteins with significantly different levels and the highest fold changes consistently identified NfL and neurofilament medium chain (NfM) as the most prominent when comparing either symptomatic carriers, presymptomatic carriers, or both groups combined against controls (Figure 5). CSF concentrations of other neurodegenerative markers, namely the neuronal pentraxins (NPTX1, NPTX2, and NPTXR), were significantly reduced only in symptomatic carriers. This aligns with the findings from a recent study on the major genetic subtypes of FTD¹³¹. Intriguingly, in a subsequent plasma profile screening employing the GENFI cohort, NPTX2 emerged as one of the proteins with significantly elevated levels, suggesting its potential as a blood-based biomarker¹³². In this context, rabphilin 3A (RPH3A) – involved in synaptic vesicle trafficking – and chromogranin A (CHGA) – present in neuronal secretory vesicles – both exhibited significant decreases in CSF from *CHMPB*-FTD patients and significant increases in plasma in the GENFI study¹³². Taken together, this could indicate synaptic loss or impaired vesicle trafficking, with proteins either leaking into the bloodstream or being upregulated by peripheral sources. Further exploration is necessary to understand these dynamics, ideally through concurrent measurement of biomarker levels in multiple biofluids.

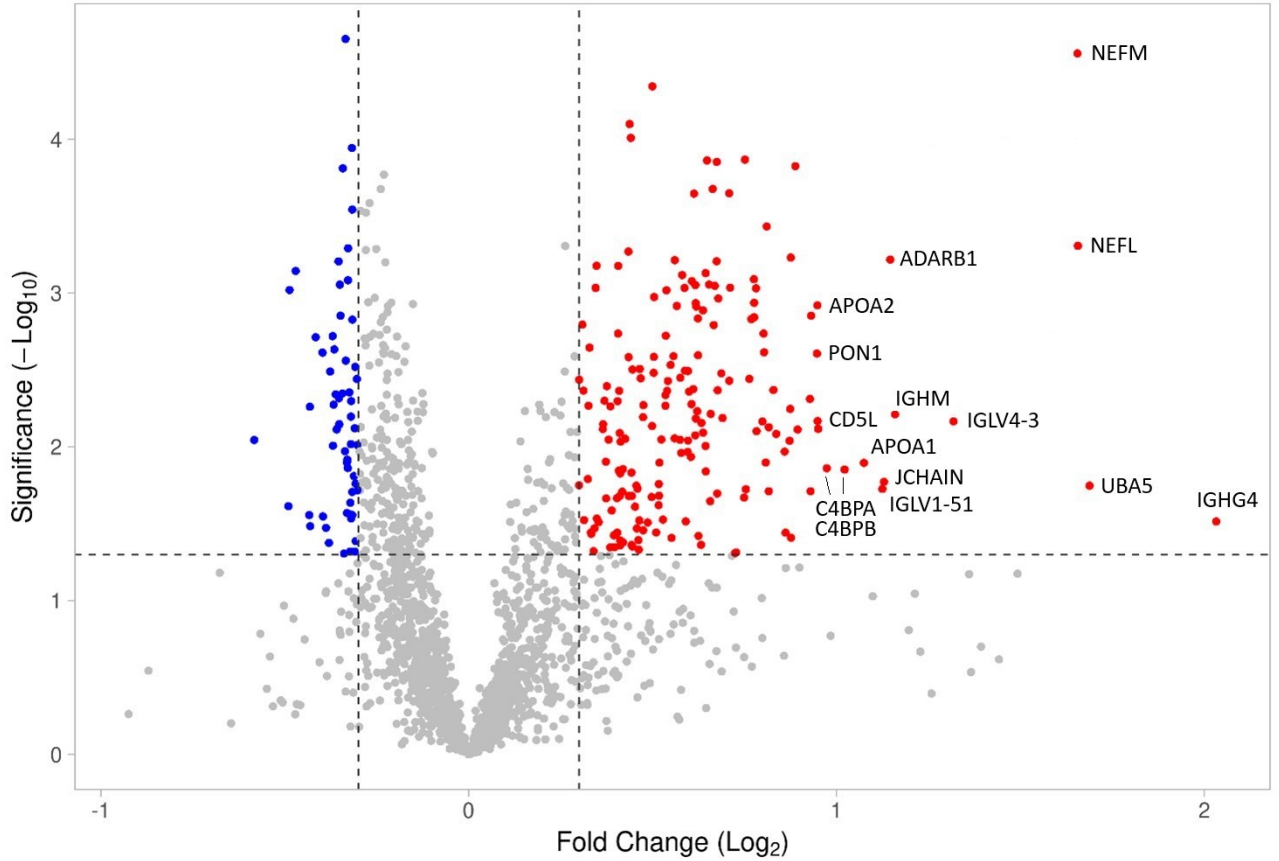


Figure 5. Volcano plot visualizing the data from the untargeted CSF proteomics analysis. All *CHMP2B* variant carriers are compared with healthy controls. Data points with positive fold changes represent proteins with higher relative levels in carriers and vice versa. Red and blue data points represent significantly different protein levels with unadjusted p -values below 0.05 and fold changes above or below 1.23 and 0.81, respectively. The top 15 proteins with the highest fold change values are labeled. The scatterplot was created using the web app VolcaNoserR¹³³.

In relation to the complement system, symptomatic participants exhibited significantly elevated levels of numerous cascade proteins, including C3, Factor B (CFB), Factor D (CFD), Factor P (CFP), C4A, C4B, C5, C6, C7, C8A, C8B, C8G, C9, as well as the regulators of complement activators (RCAs) Factor H (CFH), Factor I (CFI), and CD46. C2 showed an unadjusted p -value of 0.0537, and the key RCAs CD55 and CD59 remained unchanged compared with controls. While the levels of the C1 complex (C1q, C1r, C1s) were not increased, the C1 inhibitor Serpin G1 was – which functions to inhibit C1r and C1s. Interestingly, C1r and C1s were significantly downregulated in presymptomatic carriers, whereas all other mentioned proteins remained unaltered. The findings might indicate an early dysregulation of the classical pathway of the complement cascade, perhaps followed by compensatory activation of the alternative or lectin pathways to maintain overall complement activity.

Consistent with the findings of Study 2, complement C9 was significantly increased in the untargeted proteomics dataset, as was lysozyme, and the functionally associated innate immune components lactoferrin and alpha-defensin 1 – the latter by fivefold in symptomatic participants. As in Study 2, APP was found to be significantly reduced along with the other members of the APP family, namely APLP1 and APLP2. While cathepsin B was also found to be decreased, it did not reach statistical significance, which was the case for all the 10 included cathepsins.

The exploration of the most prominent proteins also aimed to identify candidate targets for mechanistic investigation in our established iPSC models. Ideally, proteins of interest would be present at abnormal CSF levels already in the presymptomatic stage and functionally bridge the significantly enriched processes.

The initial candidate selected for in-depth investigation was the protein CD5L (CD5 Molecule Like), otherwise denoted as AIM (Apoptosis Inhibitor of Macrophage). AIM ranked among the significant proteins exhibiting the highest fold changes in symptomatic as well as presymptomatic carriers of the *CHMP2B* variant (including the combined groups versus controls, as shown in Figure 5). The elevated levels were verified by ELISA using CSF samples from 36 members of the Danish *CHMP2B*-family (Appendix IV, Figure S1).

The main source of AIM is macrophages, and in the CNS, it is secreted by microglia^{134,135}. AIM is upregulated under inflammatory conditions, and its roles lie at the intersection between innate immune response and lipid homeostasis. Briefly, AIM functions as a soluble pattern recognition receptor (PRR), recognizing pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) for phagocytes during infections; increases the uptake of oxidized lipids by macrophages and counteracts its apoptotic effects in atherosclerosis; induces lipolysis and Toll-like receptor 4 (TLR4) inflammatory signaling in obesity-associated autoimmunity; and is a master regulator of a “metabolic switch” in CD4⁺ T helper 17 cells (Th17), restraining their pathogenicity by altering the cholesterol and fatty acid metabolism¹³⁶. Apart from its various pro- and anti-inflammatory effects, recent findings have demonstrated that accumulation of AIM on the cell surface can trigger complement-dependent cytotoxicity by interacting with membrane-bound RCAs¹³⁷.

Regarding the elevated AIM concentrations in carriers of the *CHMP2B* variant, different hypotheses can be considered: Firstly, the potential combination of neurodegeneration, dysregulated lipid metabolism, and a pro-inflammatory microenvironment may lead to upregulation

of AIM. This might confer benefits by enhancing glial phagocytosis of DAMPs, regulating lipid contents, and stimulating the release of anti-inflammatory cytokines. Secondly, the beneficial effects of AIM may be attenuated due to dysfunctional internalization via its binding receptor CD36, the recycling of which may depend on ESCRT-III. As described in the following section, there are indications that AIM might accumulate on the surface of iPSC-derived cells carrying the *CHMP2B* variant. In this scenario, the increased AIM levels could be compensatory or indicative of impaired uptake. Thirdly, AIM may contribute to a cytotoxic mechanism by triggering complement cascade activation. This could explain the elevated levels of terminal complement components observed in the CSF. To test these working hypotheses, the protein and its associated pathways were examined in the generated cell models.

4.5. Cell models

Fibroblasts isolated from skin biopsies of 6 members of the Danish *CHMP2B*-FTD family were reprogrammed into iPSCs as described, generating 3 cell lines carrying the genetic variant, 3 gene-corrected isogenic control lines, and 3 wild-type lines derived from healthy controls. Differentiation of iPSCs into microglia and neurons followed the protocols outlined in Appendix IV. The overall procedural efficiency, from iPSC thawing to terminally differentiated cell cultures, averaged approximately 80 %. When successful, both protocols yielded morphologically homogenous cell populations that were pure upon immunofluorescence-staining (IF). Prolonged neuronal cultivation (> 4 weeks) could result in the emergence of dividing cells, and during the harvesting of microglial precursors, inadvertent transfer of stromal tissue to the plating for maturation could occur – leading to the discard of affected cell cultures. Western blot analysis was used for the detection of the truncated CHMP2B proteins in the induced cell types, which were more readily detectable in microglia than neurons. Microglia were further characterized using qPCR for P2RY12, CX3CR1, and CD45 expression, along with a scratch-wound assay to assess basic cell migration. Appendix IV, Figures S2 and S3, provide an overview of the characterization of induced microglia and neurons.

As ESCRT-III may be involved in the process of phagocytosis¹³⁸, an examination of potential differences in phagocytic capacity between cell lines with and without the *CHMP2B* variant was performed. Mature microglia were exposed to fluorescent yeast particles and then fixed and stained

at different time points. The assay confirmed the phagocytic competence of the induced microglia but did not reveal any distinctions between the cell lines (Figure 6).

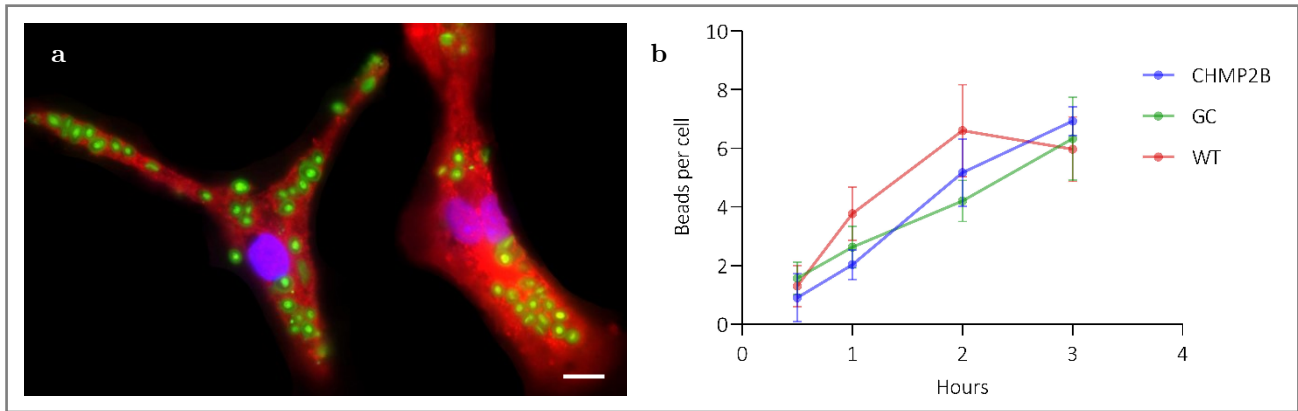


Figure 6. Phagocytosis of fluorescently labeled Zymosan A bioparticles. (a) iPSC-derived microglia (60x) taking up Alexa Fluor 488-conjugated yeast bioparticles (green). Cells are immunostained with IBA1 antibody (red) and nuclei with DAPI (blue). Scale bar, 10 μm (b) Quantitative assessment of phagocytosis over 3 hours (n = 3, using *CHMP2B* cell lines (CHMP2B), corresponding gene-corrected control lines (GC), and wild-type control lines (WT). Error bars represent SEM.

As a recent study using the same protocol to generate iPSC-derived microglia carrying *TREM2* variants¹³⁹ demonstrated unaltered phagocytosis of yeast bioparticles, but clear deficits in the uptake of apoptotic neurons, we attempted a similar setup: Stained and UV-irradiated iPSC-derived neurons were introduced to induced microglia generated from the same patient, and the results quantified with flow cytometry (Appendix IV, Figure S4). While more apoptotic neurons had been taken up by *CHMP2B* microglia compared with wild-type after 90 minutes, no differences between *CHMP2B* and gene-corrected lines were observed.

The microglial medium was semiquantitatively screened for 105 cytokines to identify potential indicators of a pro-inflammatory signature associated with the *CHMP2B* variant. Surprisingly, the media from the disease cell line contained lower levels of nearly all pro-inflammatory markers. LPS stimulation increased overall cytokine secretion and generally evened out differences between the cell lines (Appendix IV, Figure S5). Repetition of the experiment is required to confirm these findings.

Expression of AIM and its main binding receptor, CD36, was confirmed in induced microglia through IF and western blot analyses (Appendix VI, Figure S6). iPSC-derived neurons did not produce AIM, as expected, since neurons have not been described as a source of the protein. Stimulation of microglia with human oxidized low-density lipoprotein (oxLDL) led to several-fold

increases in secreted AIM, as measured by ELISA, while not affecting intracellular levels on western blot. No clear differences were observed between the microglial cell lines. Based on visual inspection, initial AIM stainings indicated that oxLDL increased the staining intensity at the plasma membrane in *CHMP2B* microglia, which was only observed to a minor extent in wild-type microglia and unstimulated *CHMP2B* microglia (Figure 7). A similar effect was observed when stimulating *CHMP2B* microglia with conditioned medium from *CHMP2B* neurons but not from wild-type neurons (Appendix IV, Figure S7). Further experiments are required to validate these observations and determine whether they arise from inherent mechanisms in *CHMP2B* microglia or components in the neuronal medium, triggering a response similar to that of oxLDL.

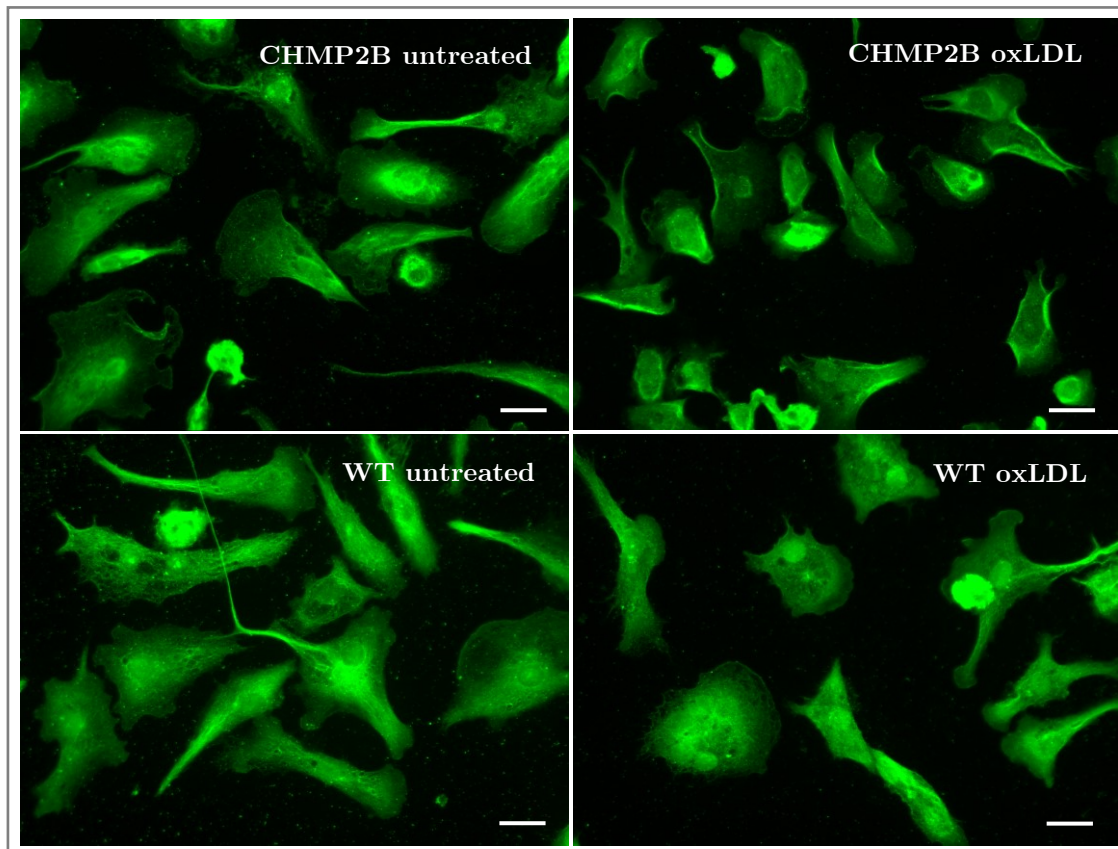


Figure 7. Immunofluorescence staining for AIM. Induced microglia carrying the *CHMP2B* variant (CHM2PB) and wild-type cells from healthy individuals (WT) stained for AIM with or without prior stimulation using 50 µg/ml human oxidized LDL. Increased staining intensity associated with the plasma membrane is observed when *CHMP2B* microglia are stimulated with oxLDL. Scale bar, 20 µm.

The preliminary assays suggest that the generated microglia are mature and capable of responding to stimulation without immediate indications of hyperactive phagocytosis or pro-inflammatory cytokine production. The cells express AIM and CD36 at comparable levels across cell lines, and

AIM secretion increases with oxLDL stimulation. The initial experiments also suggest an increased presence of AIM at the plasma membrane on microglia carrying the *CHMP2B* variant. Beyond replication of the assays, the next steps include examining whether conditioned medium from *CHMP2B* neurons upregulates AIM production in microglia and assessing AIM mislocalization in neurons after treatment with recombinant AIM or conditioned medium from microglia. Furthermore, efforts are directed toward the development of a co-culture model involving these two cell types as a supportive platform for the investigations.

4.6. Overall discussion

This project investigated biomarkers and innate immunity in *CHMP2B*-mediated FTD. We examined fluid biomarkers related to neurofilament and the endo-lysosomal system, including CSF levels of ubiquitin in FTD and other neurodegenerative diseases. Broad screening of the CSF proteome highlighted changes in the complement system, lipid metabolism, and innate immune activation. We established patient-derived cellular models to explore alterations in these systems and initiated investigations into target proteins potentially central to the disease mechanisms.

Initially, the utility of serum NfL was confirmed in *CHMP2B*-FTD (Study 1). Despite its unspecific reflection of axonal degeneration, CSF measurements of NfL are among the few fluid biomarkers routinely used in FTD diagnostics. The study demonstrated a close correlation between serum and CSF values – a consistent finding across other genetic subtypes and neurodegenerative diseases⁶¹. Published data indicate that NfL dynamics are dependent on the FTD genotype¹²⁰, emphasizing the importance of investigating the trajectory of the biomarker before and after symptom onset in *CHMP2B*-FTD through longitudinal assessments. However, the current data suggest early disparities in NfL levels between carriers of the *CHMP2B* variant and controls, as particularly noticeable in *C9orf72*-FTD¹²⁰. The sensitive detection of neurodegeneration makes NfL highly valuable in the presymptomatic stages. In contrast, the utility of the marker diminishes in late-onset cohorts, as exemplified by its lower performance in AD compared to FTD patient groups, due to the positive correlation with age^{64,65,140}. The study findings support the applicability of blood tests for quantifying NfL in *CHMP2B*-FTD, offering a less invasive alternative to lumbar punctures for repetitive sampling and monitoring of disease progression in future clinical trials. Further

clarification of the course of NfL is necessary, as this cannot be definitively determined using cross-sectional data.

Another notable finding from the study is the elevated CSF/serum albumin ratio in *CHMP2B*-FTD patients, indicating a potential compromise in blood-brain barrier integrity. In presymptomatic carriers of the genetic variant, increased albumin ratios were not statistically significant (unpublished data). However, our previous observations of reduced regional cerebral capillary blood flow in these participants¹⁴¹, combined with the significant downregulation of processes related to “Cell adhesion molecules”, “Tight junction”, and “Maintenance of blood-brain barrier” in presymptomatic carriers in the untargeted proteomics (Appendix IV, Table S1), suggest the possibility of localized or transient blood-brain barrier defects in this group as well.

Altered albumin ratios were not detected in patients diagnosed with all-cause FTD or AD at our institution¹⁴². Thus, elevated blood-brain barrier permeability may be associated with the *CHMP2B* variant and could play a central role in the pathomechanism. This is currently being investigated in other neurodegenerative diseases such as AD, ALS, HD, MS, and PD¹⁴³, and should be further explored in genetic FTD, especially as limiting dysfunction of the blood-brain barrier offers an attractive therapeutic target.

With increasing evidence substantiating the role of endo-lysosomal dysfunction as a pathogenic mechanism in FTD and a convergence point for the genetic subtypes¹⁴⁴, we proceeded to investigate a set of proteins related to endo-lysosomal function in the CSF from members of the Danish *CHMP2B-FTD* family (Study 2). This analysis was subsequently complemented by an untargeted mass spectrometric quantification of the CSF proteome.

Overall, Study 2 did not show evident disruption of proteostasis based on the examined peptides. Instead, the analysis highlighted abnormalities in CSF concentrations of ubiquitin, with the lowest values observed in *CHMP2B*-FTD patients compared with controls. Although reduced levels were also detected in presymptomatic carriers, statistical significance was not retained in this group after adjusting for age. Neither ubiquitin nor associated enzymes were part of the untargeted CSF analysis, while the concentrations of the other proteins identified in Study 2 were found to be similarly altered.

Verification of the decreased quantities of ubiquitin should involve measuring the full-length protein, for instance, through immunoassays. The applied PRM-MS technique only detects certain

peptide fragments, which may not represent the entire protein. Notably, in the systematic review of studies on CSF ubiquitin in neurodegenerative diseases (Study 3), the only two studies demonstrating reduced levels (in *CHMP2B*-FTD and PD) relied on the same PRM-MS method. This was also the case in the more recent study on primary progressive aphasia variants¹³⁰, where lower CSF ubiquitin levels were found in nvPPA patients compared with controls, which underscores the importance of considering methodological influence. However, Sjödin et al. not only identified reduced concentrations in PD but also observed increased levels in AD using this approach¹⁰⁹. Thus, it is possible that the ubiquitin peptides convey different information than full-length mono-ubiquitin, and they may perform better as biomarkers.

Irrespective of the technique, it is interesting that heightened levels of CSF-ubiquitin have not been reported in FTD subtypes, as opposed to AD. This indicates an alternative mechanism in FTD, and possibly PD, where lesser amounts of ubiquitin reach the CSF, as discussed previously. The identification of significantly altered levels of ubiquitin, rather than the other endo-lysosomal markers in Study 2, directs attention toward the UPS. This system has been less studied than the autophagy-lysosomal pathway (ALP), partially because the UPS is responsible for clearing smaller protein oligomers, while the ALP degrades higher-order protein aggregates¹⁴⁵. Ubiquitination, however, serves as a recognition signal in both the UPS and the ALP – and the pathways interact¹⁴⁶. Besides the roles of ubiquitin in the major intracellular processes for clearing neurotoxic proteins, Study 3 provided insights into the association of this protein with all investigated neurodegenerative diseases. Thus, strategies aimed at reinforcing the proteostatic systems emerge as promising treatment modalities, capitalizing on the overlap between disease entities.

The only proteins associated with innate immunity in Study 2 were found to be elevated in *CHMP2B*-FTD patients, prompting the investigation of a broader selection of markers. Untargeted CSF-proteomics revealed a profile linked to complement system pathways, lipid metabolism, and immune activation. Yet, there was no apparent pro-inflammatory signature related to the most prominent proteins, except for the overrepresentation of complement proteins, particularly the terminal components. Given that disturbance in cellular cholesterol and GAG metabolism, both highlighted in the enrichment analysis, can activate the complement cascade¹⁴⁷, there may be a connection between the processes. However, as mentioned earlier, the increased concentrations of complement in the CSF might arise from passage across the blood-brain barrier, aligning with the

elevated albumin quotient identified in Study 1. Conversely, aberrant activation of the complement cascade intrathecally may impact the integrity of the barrier, with indications of perturbations in the classical complement pathway already apparent in the presymptomatic stage. Harmful effects of complement activation on the blood-brain barrier have been observed in various neurological conditions, including neurodegenerative diseases¹⁴⁸, and a positive correlation has been found between CSF levels of the soluble C5b-C9 complex and the extent of post-injury barrier dysfunction following traumatic brain injury¹⁴⁹. Nevertheless, knowledge is lacking regarding the timing and exact contribution of the complement system to the neurodegenerative pathogenesis. While a few complement inhibitors are currently on the market, and several more are underway, caution is necessary due to potential influences on the beneficial effects of the complement system, such as immune defense and microglia-mediated synaptic pruning and clearance of debris.

The microglia-secreted protein AIM was considered a target for further examination, given its upregulation in symptomatic and presymptomatic carriers of the *CHMP2B*-variant, along with its involvement in innate immune function and lipid metabolism. The protein remains largely unexplored in neurodegenerative disease. A recent study on cerebral amyloid angiopathy demonstrated an abundance of AIM-filled migrasomes adhering to vessel walls in cerebral and peripheral tissues, observed in both patients and transgenic mice¹⁵⁰. These migrasomes were produced by microglia and macrophages, and AIM was found to induce complement-mediated injury to the blood-brain barrier in mice, increasing barrier permeability as well as levels of NfL. In the study, amyloid-beta protein 1-40 ($A\beta_{1-40}$) initiated the generation of migrasomes loaded with AIM, and it would be interesting to investigate whether the *CHMP2B*-variant elicits a similar effect. Others have analyzed AIM in age-related macular degeneration (AMD), reporting its secretion from retinal microglia, elevated levels in plasma, and the presence of circulating autoantibodies against it¹⁵¹. The authors conclude that AIM and the autoantibodies might be central in the pathogenesis of AMD involving inflammation, oxidative stress, and damage to macular tissue. Peripheral circulating levels of AIM and the potential occurrence of autoantibodies should be elucidated in *CHMP2B*-FTD, especially considering the possible dysfunction of the blood-brain barrier.

As part of the project, we generated microglia and neurons from iPSCs to investigate the effects of the *CHMP2B*-variant and explore CSF proteins of interest. Preliminary assays have not indicated a hyperactivated or overtly neurotoxic microglial phenotype caused by the variant, when compared with control lines. Instead, our findings seem to align with a recently described “unresponsive” phenotype observed in iPSC-derived microglia following the introduction of a heterozygous *CHMP2B*-variant⁸⁸. Thus, the pro-inflammatory phenotype observed in homozygous cell lines and mice is unlikely to be recapitulated. This is in line with previous observations of more evident neuroinflammation in murine models with homozygous *GRN* or *C9orf72* knockout than in heterozygous models^{152,153}.

The *CHMP2B*-variant does not appear to induce overproduction of AIM in iPSC-derived microglia, but it may cause the protein to mislocalize on the surface of cells carrying the variant. This is interesting, given its association with complement activation¹³⁷. However, these experiments require replication. The next steps involve examining whether AIM mislocalizes on neurons and if it interacts with RCAs on the cell membrane, which are expected to be less abundant on neurons than other cell types¹⁴⁷. Since stimulation with oxLDL clearly enhances AIM secretion in our induced microglia, measuring oxidized lipid levels in CSF samples from the cohort would be relevant, as group differences may explain the elevation of CSF AIM. Serum AIM levels should also be determined, considering potential blood-brain barrier passage as well as its viability as a blood-based biomarker. This could be complemented by investigating circulating autoantibodies against AIM. Additionally, the effect of the *CHMP2B*-variant on microglial production of extracellular vesicles, including migrasomes, will be explored, and their contents of AIM will be quantified. Lastly, stimulation of microglia with CSF will more accurately reflect the fluid environment that cells are exposed to *in vivo*, and the establishment of 2D and 3D co-culture models will provide a more physiologically representative platform for exploring interactions between microglia and neurons.

4.6.1. Strengths and limitations

The work presented here benefits from the genetic homogeneity of the cohort but is limited by its size. Although the Danish family offers advantages in avoiding pitfalls related to mixed FTD cohorts, the low number of participants restricts the analytical power. This limitation is particularly significant for the biomarker investigations, i.e., Study 2 and the untargeted CSF

proteome analysis, increasing the risk of false positive findings. Adjustment for multiple testing was omitted in these analyses. In Study 2, lower levels of ubiquitin would be the only significant finding under Bonferroni correction or less conservative measures. Hence, the exploratory utility of the analyses was prioritized.

The presymptomatic carrier group is a mixed study population, with some participants likely approaching phenoconversion while others are far from it. Notably, elevated NfL concentrations were observed in the youngest participant (mid-twenties), suggesting that they may all contribute information about the pathogenesis of the disease. A larger dataset would allow for the categorization of this group based on NfL levels, as practiced in other studies, and make it more advantageous to incorporate variables such as comorbidity and medication usage.

ACE score was utilized as an indicator of disease burden. More comprehensive scales have been developed to assess global cognitive impairment, incorporating behavioral and language deficits. However, because some of the data were derived from earlier studies, it was deemed most practical to adhere to the ACE score when including new participants.

The identification of an increased CSF/serum albumin ratio in Study 1, serving as an indicator of blood-brain barrier compromise, could potentially confound the CSF analyses. This should be specifically investigated in symptomatic and presymptomatic carriers of the *CHMP2B*-variant. Simultaneous examination of proteins in CSF and blood, along with correlation analysis, would both address this issue and clarify the potential as blood-borne biomarkers. Alternative measures, such as CSF/serum indexes of other proteins than albumin; imaging techniques like dynamic contrast-enhanced MRI; or other exogenous markers, could also provide valuable insight into the influence of the blood signature on CSF.

The primary limitation in the systematic review was the scarcity of studies investigating CSF ubiquitin levels as a biomarker in neurodegenerative diseases other than AD. Due to the prevalence of AD and the availability of validated biomarkers such as beta-amyloid and tau, more lumbar punctures are performed, providing ample research material. Consequently, the evidence supporting an association with CSF ubiquitin is strongest for AD patients, with included studies reporting near-consistent findings. However, the potential impact of publication bias, making studies with negative findings less likely to be published, cannot be dismissed. Another notable limitation is the heterogeneity in analytical methods employed across studies, reflecting the absence of a gold standard for measuring CSF ubiquitin, which may affect the generalizability of the findings.

The strengths of the established cell models lie in the use of patient-derived material rather than genetically engineered disease cell lines, along with access to healthy controls. Evaluating the maturity of generated cell types, especially microglia, would have benefitted from the inclusion of a positive control, such as a commercially available, differentiated cell line. Electrophysiological verification of the neurons is pending, delayed after the initial patch-clamp recordings due to the relocation of our collaborative partner. Finally, it is important to keep in mind that cell cultures represent artificial and detached systems, lacking the complexity, the compensatory mechanisms, and the aging aspects of brain tissue. Conversely, isolating a cell type from its environment may be necessary to expose a pathological mechanism.

5. Concluding remarks and perspectives

Frontotemporal dementias are incapacitating and fatal neurodegenerative diseases, representing common causes of early-onset dementia. Currently, treatment options and clinically relevant biomarkers are lacking. However, there is a growing focus on FTD syndromes, with research in these areas advancing swiftly.

This PhD project centers around one specific family and genetic subtype, serving as a model for a broader spectrum of diseases. The overarching aim is to enhance our understanding of FTD by investigating *CHMP2B*-FTD, with the hope that findings will extend to other subtypes or neurodegenerative phenotypes sharing a similar genetic background, such as familial ALS. Despite achieving several milestones in *CHMP2B*-FTD over the past years, the critical mechanisms driving neuropathology remain unclear.

Building on previous investigations into modifiers, biomarker panels, fibroblasts, transgenic mice, and more, this project exploited new strategies utilizing iPSC-derived CNS cell types and high-throughput techniques. The access to patient material as input for these methodologies provides a unique opportunity to study *CHMP2B*-FTD pathology. The cell models have been established, and efforts are directed towards their application in co-cultures and brain organoids. Transplantation of stem cell-derived neural progenitors is already a reality in clinical trials, and personalized cell models could become an integral part of treatment screening in the future.

The search for disease-related biomarkers is complex due to temporal and spatial variability, underlining the importance of longitudinal sampling and multimodal approaches. Going forward, our group will prioritize high-throughput analyses of various tissue combinations, such as blood, CSF, brain tissue, induced cells, and their secretomes. This strategy is intended to yield deeper insights into the involved mechanisms, although careful handling is required for large datasets from a small cohort. The imminent inclusion of the Danish *CHMP2B*-FTD family in the GENFI study will increase its research value by facilitating direct comparison with other genotypes, offering a means to assess the generalizability of findings from this cohort.

The high-throughput analysis and generation of induced cell types proved to be unexpectedly time-consuming in this project. Consequently, certain experimental series presented here are still

ongoing, and the CSF proteomics dataset has not yet been thoroughly processed. Despite the incomplete status of these investigations, their inclusion in this PhD thesis contributes to a more complete understanding of the work carried out in the project.

While the presence of a driving neuroinflammatory component in the pathogenesis – as observed in the homozygous animal and cell models – could open new avenues for biomarker discovery and pharmaceutical intervention, there are no clear indications that this holds true in *CHMP2B*-FTD. It is possible that neuroinflammation has peaked before the examination of CSF, blood, and brain tissue, or that loss of certain protective immunological functions is more relevant than actively harmful ones. Instead, this project emphasizes alternative, potentially underlying processes related to the blood-brain barrier, ubiquitin, the complement system, lipid metabolism, and AIM.

My continued involvement in the investigation of these processes, including the completion and further development of the ongoing cellular work, is facilitated by a 2-year post-doctoral program funded by the Novo Nordisk Foundation.

The project has provided input to the Danish Dementia Biobank for future use. The development of iPSC-derived models expands the foundation for upcoming *CHMP2B*-FTD research in our group, extending to in-house projects related to AD and HD. Our recent CSF proteomics analysis stands as the most comprehensive biomarker study in *CHMP2B*-FTD to date and may be leveraged for further investigations. Thus, the findings and resources from this PhD project can serve as a valuable springboard for further work aimed at understanding the biological mechanisms of the disease, developing clinically useful biomarkers, and exploring disease-modifying treatments.

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7. Appendices

Appendix I: Study 1

Toft A, Roos P, Jääskeläinen O, Museaus CS, Henriksen EE, Johannsen P, Nielsen TT, Herukka S, Simonsen AH and Nielsen JE. Serum neurofilament light in patients with frontotemporal dementia caused by *CHMP2B* mutation. *Dementia and Geriatric Cognitive Disorders* 2020. 49(6):533-538.

Appendix II: Study 2

Toft A, Sjödin S, Simonsen AH, Ejlerskov P, Roos P, Musaeus CS, Henriksen EE, Nielsen TT, Brinkmalm A, Blennow K, Zetterberg H, Nielsen JE. Endo-lysosomal protein concentrations in CSF from patients with frontotemporal dementia caused by *CHMP2B* mutation. *Alzheimers & Dementia (Amst)* 2023. 15(1):e12402.

Appendix III: Study 3

Vardheim E*, Toft A*, Nielsen JE, Hasselbalch SG, Simonsen AH. Cerebrospinal fluid ubiquitin as a biomarker for neurodegenerative diseases: a systematic review. *Neuroscience Applied* 2023. 2(4):102438.

* Equal contribution

Appendix IV: Supplementary information for cell culturing and experimental procedures

Serum Neurofilament Light in Patients with Frontotemporal Dementia Caused by CHMP2B Mutation

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Keywords

CHMP2B · Frontotemporal dementia · Neurofilament light · Presymptomatic

Abstract

Introduction: The potential of neurofilament light (NfL) as a blood-based biomarker is currently being investigated in autosomal dominant neurodegenerative disease. This study explores the clinical utility of serum-NfL in frontotemporal dementia due to *CHMP2B* mutation (FTD-3). **Methods:** This cross-sectional study included serum and CSF data from 38 members of the Danish FTD-3 family: 12 affected *CHMP2B* mutation carriers, 10 presymptomatic carriers, and 16 non-carriers. Serum-NfL levels measured by single-molecule array (Simoa) technology were tested for associations with the clinical groups and clinical parameters. Serum and CSF data were compared, and CSF/serum-albumin ratio was included as a measure of blood-brain barrier (BBB) function. **Results:** Serum-NfL concentrations were significantly increased in symptomatic *CHMP2B* mutation carriers compared to presymptomatic carriers and in both groups compared to healthy family controls. Serum-NfL levels appear to increase progressively with age in presymptomatic carriers, and this is per-

haps followed by a change in trajectory when patients become symptomatic. Measurements of NfL in serum and CSF were highly correlated and fold-changes in serum and CSF between clinical groups were similar. Increase in serum-NfL levels was correlated with reduced ACE-score. Higher CSF/serum-albumin ratios were demonstrated in FTD-3 patients, but this did not affect the significant associations between serum-NfL and clinical groups. **Conclusion:** Serum-NfL could be utilized as an accurate surrogate marker of CSF levels to segregate symptomatic *CHMP2B* carriers, presymptomatic carriers, and non-carriers. The observed indication of BBB dysfunction in FTD-3 patients did not confound this use of serum-NfL. The results support the occurrence of mutation-related differences in NfL dynamics in familial FTD.

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Introduction

Frontotemporal dementia (FTD) comprises a group of neurodegenerative disorders, with clinical syndromes encompassing behavioral changes, language impairment, and general cognitive decline. Familial aggregation is frequent, and high-risk variants have presently been identi-

fied in 11 genes [1]. In a large Danish family, a protein-truncating variant in the *CHMP2B* gene (c.532-1G > C) on chromosome 3 was discovered as the cause of early-onset autosomal dominant FTD, termed FTD-3 [2]. More than 50 members of this family have been diagnosed with clinical FTD-3, and a number of mutation carriers are pre-symptomatic. Studies indicate that the neuropathological process in genetic FTD is ongoing several years prior to symptom onset [3, 4]. As early detection of developing pathology may hold significance for treatment timing in upcoming therapeutic trials, there is an unmet need for validated biomarkers to identify this preclinical stage.

One relevant biomarker in neurodegenerative disease is neurofilament light (NfL) protein; a low-molecular-weight cytoskeletal subunit exclusively expressed in neurons [5]. Considered a sensitive and nonspecific biomarker of axonal damage, NfL has been shown to increase with age in healthy participants, and relatively higher CSF levels have segregated patients from healthy controls in a range of neurodegenerative disorders including ALS, Parkinson's disease, Alzheimer's disease, and vascular dementia [6]. A high correlation between CSF-NfL and the many fold lower concentration in serum has been reported for several of these conditions [7]. In genetic FTD caused by mutation in the *GRN*, *C9orf72*, or *MAPT* gene, serum-NfL was recently found to increase in presymptomatic individuals just prior to symptom onset and remain elevated in symptomatic patients compared to presymptomatic carriers and noncarriers [8]. Increase in serum-NfL was associated with reduced Mini-Mental State Examination (MMSE) score and gray matter atrophy, in line with findings from similar studies [9, 10]. Interestingly, serum-NfL dynamics were shown to differ based on the type of FTD mutation [8].

In familial FTD due to *CHMP2B* mutation, we have recently demonstrated significantly different levels of NfL in CSF from symptomatic carriers compared to presymptomatic carriers and in both groups compared to noncarrier family controls [4]. However, the capability of serum-NfL to separate clinical groups and its correlation with CSF levels and clinical parameters have not been investigated in FTD-3. Additionally, as we have previously observed reduced regional cerebral blood flow in presymptomatic *CHMP2B* mutation carriers, as an indicator of potential capillary pathology [11], we included a measure of blood-brain barrier (BBB) dysfunction, to control for a possible confounder of an association between levels of NfL in serum and CSF. This study represents a cross-sectional analysis of fluid biomarker data obtained from 38 members of the Danish FTD-3 family.

Methods

Study Population

For 6 generations, the Danish FTD-3 family has been studied through the FTD Research in Jutland Association (FReJA). Four hundred members are alive and at risk of harboring the pathogenic *CHMP2B* variant. Diagnostic workup is performed at symptom onset, and if consented to, biofluid samples are stored in the Danish Dementia Biobank from clinically affected patients, presymptomatic carriers, and non-carrier family members.

This study included data from 38 individuals: 12 affected *CHMP2B* mutation carriers, 10 presymptomatic carriers, and 16 noncarriers. All participants were clinically evaluated at the time of sampling, and participant age was based on this time point. Disease duration was defined as the time from diagnosis to biofluid sampling. Addenbrooke's Cognitive Examination (ACE) score was employed as a measure of cognitive impairment.

Laboratory Procedures

CSF samples were collected by standardized lumbar puncture. CSF-NfL concentrations were analyzed by sandwich ELISA, as previously described [4]. Serum-NfL was measured according to the instructions of the manufacturer using the NF-Light Advantage Kit for the Quanterix single-molecule array (Simoa, Lexington, MA, USA) at the Institute of Clinical Medicine-Neurology, University of Eastern Finland. Measurements of albumin concentrations were performed on Cobas 8,000 instruments (Roche diagnostics) at the Department of Clinical Biochemistry, Rigshospitalet, using commercial kits.

Statistics

Data were analyzed using R, version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). NfL and albumin values were logarithmically transformed to obtain normal distribution. For descriptive statistics, comparisons between groups were performed with Kruskal-Wallis test (untransformed data) and ANOVA with post hoc Tukey test (transformed data). Variables were subsequently analyzed by general linear modeling and multinomial logistic regression, adjusted for age and significant interactions. Correlations were calculated using Pearson's correlation coefficient (normally distributed data) or Spearman's Rank Correlation Coefficient (ACE-score data). *p* values below 5% were considered significant.

Results

CSF and serum data were available for 32 and 31 participants, respectively. Patient characteristics and distribution of fluid biomarker data are presented in Table 1.

Group demographics showed no difference in distribution of sex, whereas presymptomatic participants constituted a significantly younger age-group. ACE-scores were expectedly lower in clinically affected FTD-3 patients, while levels of CSF-NfL, serum-NfL, and albumin ratios were significantly higher (shown in Table 1).

Table 1. Patients characteristics and levels of fluid biomarkers

Clinical group	Noncarriers	Presymptomatic <i>CHMP2B</i> mutation carriers	Symptomatic <i>CHMP2B</i> mutation carriers	<i>p</i> value
Participants, n	16	10	12	
Sex, females/males	8/8	6/4	3/9	0.267
Age, years, mean (range)				0.011
Symptomatic versus noncarriers				0.701
Symptomatic versus presymptomatic	60.1 (38.1–71.1)	50.5 (32.7–68.1)	62.8 (53.6–73.3)	0.007
Presymptomatic versus noncarriers				0.029
ACE, mean (range) (CI)				<0.001
Symptomatic versus noncarriers	93.6 (87–100)	90.4 (81–96)	70.6 (41.0–89)	<0.001
Symptomatic versus presymptomatic	(91.4–95.9)	(86.9–94.0)	(60.1–81.2)	<0.001
Presymptomatic versus noncarriers				0.715
CSF-NfL, pg/mL, mean (CI)				<0.001
Symptomatic versus noncarriers	686.5 (464.4–899.6)	1,790.9 (1,230.7–2,351.0)	2,915.0 (2,371.9–3,458.6)	<0.001
Symptomatic versus presymptomatic				0.005
Presymptomatic versus noncarriers				0.003
Serum-NfL, pg/mL, mean (CI)				<0.001
Symptomatic versus noncarriers	13.6 (7.2–20.0)	26.8 (16.9–36.7)	67.0 (33.5–100.5)	<0.001
Symptomatic versus presymptomatic				0.015
Presymptomatic versus non-carriers				0.503
CSF/serum-albumin ratio (CI)				0.054
Symptomatic versus noncarriers	5.2 (4.1–6.3)	6.1 (4.5–7.7)	8.2 (6.1–10.2)	0.020
Symptomatic versus presymptomatic				0.223
Presymptomatic versus noncarriers				0.742

ACE, Addenbrooke's Cognitive Examination; NfL, neurofilament light; CI 95%, confidence interval.

In this cohort, mean concentration of NfL in serum (30.1 pg/mL, range 5.3–187.8 pg/mL) was 54 times lower than the corresponding value in CSF (1,654.8 pg/mL, 273.4–4,009.9). FTD-3 patients had 2.4-fold and 4.7-fold higher median serum-NfL than presymptomatic and healthy individuals, respectively, and between the latter groups, the fold change was 2.0 (for CSF-NfL the corresponding fold-changes were 1.7, 4.5, and 2.7). Measurements of NfL in CSF and serum were highly correlated ($R^2 = 0.76$, $p < 0.001$, Pearson's correlation coefficient = 0.87) (shown in Fig. 1). ACE-scores decreased with increasing serum-NfL levels (Spearman's correlation coefficient = -0.70 , $p < 0.0001$), also when controlling for age in multiple regressions ($p < 0.001$). In a general linear model adjusted for age, serum-NfL values differed between symptomatic and presymptomatic *CHMP2B* mutation carriers ($p = 0.019$); symptomatic and healthy participants ($p < 0.001$); as well as presymptomatic and healthy participants ($p = 0.040$) (shown in Fig. 1). This model included age ($p = 0.052$) and a significant interaction between the effect of clinical status and age on NfL.

In clinically affected FTD-3 patients, neither serum-NfL nor albumin ratio was significantly associated with disease duration. For the entire population, serum-NfL levels were positively correlated with increasing albumin ratio ($p = 0.021$). When adding the albumin ratio to the general linear model, the statistical significance of the group differences in serum-NfL persisted, except for the comparison between presymptomatic and healthy participants ($p = 0.079$). In a multinomial logistic regression analysis including the same factors (with clinical group as a 3-level outcome variable), all groups differed significantly from 1 another. This was also the case when separating groups based on CSF-NfL instead of serum-NfL in an otherwise similar linear regression model.

Discussion

As focus has shifted towards identification of more readily accessible, blood-based biomarkers, this study explores the clinical utility of serum-NfL in familial FTD caused by *CHMP2B* mutation. CSF and serum data from

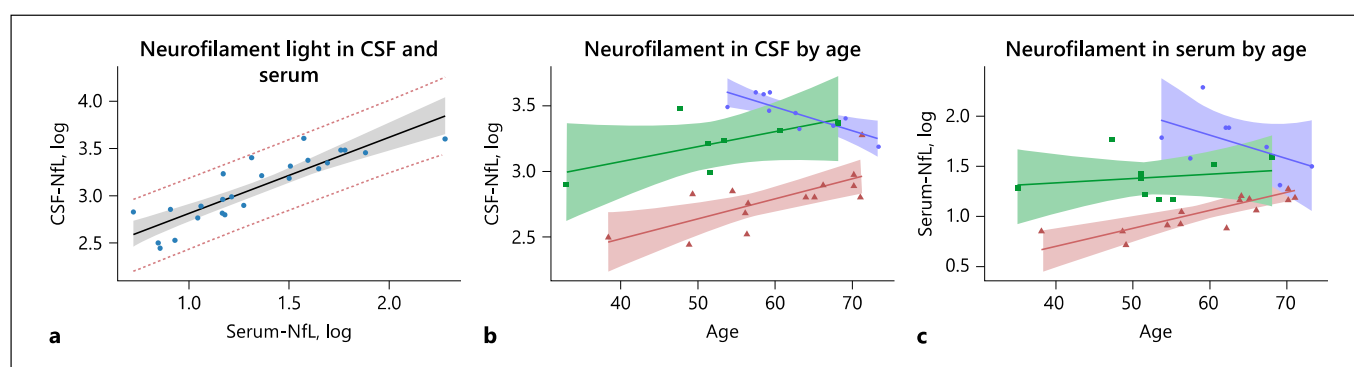


Fig. 1. CSF-serum correlation and NfL levels in CSF and serum by age. **a** depicts the linear relationship between measurements of neurofilament light protein in CSF and serum. 95% confidence interval and prediction limits are shown. **b** and **c** represent generalized linear models of associations between age and NfL level in symptomatic (blue), presymptomatic (green) and non-carrier (red) participants. Neurofilament data are logarithmically transformed.

12 clinically affected patients, 10 presymptomatic *CHMP2B* mutation carriers, and 16 noncarriers from the Danish FTD-3 family were included.

Serum-NfL levels were highly correlated with the 54-fold higher CSF-values. Fold-changes between the clinical groups were similar for serum and CSF. Based on serum-NfL concentrations, FTD-3 patients could be separated from presymptomatic carriers, and both groups from noncarrier family controls. These differences remained statistically significant when adjusted for age. Elevated serum-NfL levels were detectable several years before expected age of symptom onset (shown in Fig. 1), plausibly reflecting the early neurodegenerative process. In line with this, previous studies have demonstrated pathologic rates of cerebral atrophy in presymptomatic *CHMP2B* mutation carriers [12, 13], but whether this is preceded by or coincides with the changes in serum-NfL remains to be investigated. In familial Alzheimer's disease, progressively rising serum-NfL has been observed more than a decade before symptom onset, prior to incipient changes in structural imaging and cognition [14, 15]. A recent cross-sectional study by the GENFI consortium in FTD due to mutation in the *C9orf72*, *MAPT*, or *GRN* gene showed that accelerated brain atrophy and certain neuropsychological deficits were present 10 and 5 years before expected symptom onset, respectively [3]. This led to the prediction that serum-NfL levels would start to increase around 10 years before age of onset [10], but in a subsequent longitudinal study, presymptomatic mutation carriers grouped with the healthy controls in terms of serum-NfL level, and only symptomatic FTD patients had significantly increased values [8]. Post hoc an-

alyzes, however, showed that presymptomatic individuals who became symptomatic within 1–2 years had higher baseline serum-NfL than those not approaching conversion. An unaffected level of NfL in presymptomatic participants or an abrupt increase immediately preceding the symptomatic stage has also been observed in similar studies [9, 14, 16]. Thus, it is still unclear whether NfL is a marker of imminent phenocconversion or if it does reflect the onset and progression of neurodegeneration over several years. The latter capability would seem to best enable the proposed future use of serum-NfL as a timing marker of potential disease-modifying treatment in the prodromal stage before neuropathology becomes too manifest. As the dynamics of NfL in this regard appear to differ among neurodegenerative disease entities as well as FTD subtypes, the biomarker may only prove useful in some of these disorders. To accurately determine the preclinical course of NfL in genetic subtypes of FTD, additional longitudinal studies with serial measurements of serum-NfL are needed. This is also a prerequisite for the potential use of serum-NfL as a basis of estimating individual time points of phenocconversion in presymptomatic subjects.

The depiction of our serum-NfL data as a function of age gives the impression that the increase in presymptomatic carriers is followed by a decrease in symptomatic patients (shown in Fig. 1). This subsequent decline or stabilization, which may perhaps be explained by protein aggregation or the presence of autoantibodies against NfL [17], has also been noted in FTD patients with *C9orf72* or *MAPT* mutation – whereas levels in *GRN* mutation carriers have been found to persistently rise or fluctuate, per-

haps due to a higher degree of neuroinflammation affecting rates of axonal degeneration [8]. Clarification of disorder-specific trajectories and variability of NfL in the symptomatic phase is important for it to function as a surrogate marker of treatment effect – another potential of the biomarker currently being studied. In multiple sclerosis, reductions in serum-NfL have been demonstrated after administration of disease-modifying therapies [18, 19]. Naturally, the use of serum-NfL as a pharmacodynamic biomarker in clinical trials is dependent on correlation with clinical parameters. In this study, we found that increasing serum-NfL levels were associated with decline in ACE-score, independent of age. Similarly, increasing levels or rates of change have been shown to correlate with MMSE-score, disease severity, brain atrophy rates, and survival in FTD [8–10, 16]. Numerous studies have evaluated the diagnostic performance of serum-NfL: significant differences have been found among neurodegenerative disease categories – such as higher serum-NfL in FTD than Alzheimer’s disease [20] – as well as between genetic FTD subtypes [8]. Nevertheless, this usage of serum-NfL is clearly limited by its nonspecificity, why genetic cohorts with known pathogenicity provide more suitable target groups, in the presymptomatic stage. Another limitation is the lack of knowledge about which confounding factors, in addition to sex, age, and neurologic comorbidity, that cause the intra- and interindividual variability in serum-NfL. Furthermore, how the NfL protein is transported from CNS to the bloodstream is undetermined. One mechanism that has been discussed in particular is leakage across the BBB. In presymptomatic FTD-3 family members, we have previously observed reduced regional cerebral blood flow compared to noncarriers, on contrast-enhanced MRI [11]. The spin-echo sequences utilized were sensitive to signal from the capillaries, indicating involvement of capillaries in the pathophysiology of FTD-3. For this reason, we included a well-established measure of BBB integrity, namely CSF/serum-albumin ratio, to control for a potential confounder of the association between NfL levels in serum and CSF. The analyses showed higher albumin ratio in *CHMP2B* mutation carriers than noncarriers (not shown), as well as in FTD-3 patients than healthy family controls, while statistical significance was not reached for comparisons with presymptomatic carriers. In a recent study of BBB integrity in dementias of different etiologies, we did not find the albumin ratio to be elevated in patients with (all-cause) FTD or Alzheimer’s disease [21], suggesting that the potential BBB dysfunction is related to the *CHMP2B* mutation. This should be investigated specifically, with techniques such as dynamic con-

trast-enhanced MRI. However, when controlling the association between serum-NfL and clinical status for differing albumin ratios, it did not confound the significant correlations. This is in line with a recent meta-analysis in Alzheimer’s disease [22], where several studies have found elevated serum-NfL levels in the absence of significantly increased albumin ratios. This indicates that transport of NfL to the bloodstream occurs via different pathways, which should be investigated further, as the concentration in serum may be confounded by varying degrees of release from the CNS [23].

Collectively, it is shown that serum-NfL can be used as a surrogate marker of CSF levels to trace progressive neurodegeneration years before expected debut of symptoms in FTD-3. Serum-NfL levels could effectively separate symptomatic *CHMP2B* mutation carriers, presymptomatic carriers, and noncarriers. An elevated CSF/serum-albumin ratio was found in symptomatic FTD-3 patients as an indication of BBB dysfunction. This finding, however, did not confound the associations between NfL and clinical groups. The apparent linear course of serum-NfL in FTD caused by *CHMP2B* mutation supports the occurrence of gene-specific differences in NfL dynamics in familial FTD. Follow-up studies will determine if conversion to the symptomatic stage does in fact change the trajectory of serum-NfL and if the rate of change is a better performing biomarker than the absolute levels in this disorder.

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Statement of Ethics

This study was approved by the Ethics Committee of the Capital Region of Denmark (H-1-2012-041). All participants provided written informed consent. Clinically affected FTD-3 patients were included in early disease stages to ensure legitimate informed consent.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

RESEARCH ARTICLE

Endo-lysosomal protein concentrations in CSF from patients with frontotemporal dementia caused by *CHMP2B* mutation

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Abstract

Introduction: Increasing evidence implicates proteostatic dysfunction as an early event in the development of frontotemporal dementia (FTD). This study aimed to explore potential cerebrospinal fluid (CSF) biomarkers associated with the proteolytic systems in genetic FTD caused by *CHMP2B* mutation.

Methods: Combining solid-phase extraction and parallel reaction monitoring mass spectrometry, a panel of 47 peptides derived from 20 proteins was analyzed in CSF from 31 members of the Danish *CHMP2B*-FTD family.

Results: Compared with family controls, mutation carriers had significantly higher levels of complement C9, lysozyme and transcobalamin II, and lower levels of ubiquitin, cathepsin B, and amyloid precursor protein.

Discussion: Lower CSF ubiquitin concentrations in *CHMP2B* mutation carriers indicate that ubiquitin levels relate to the specific disease pathology, rather than all-cause neurodegeneration. Increased lysozyme and complement proteins may indicate innate immune activation. Altered levels of amyloid precursor protein and cathepsins have previously been associated with impaired lysosomal proteolysis in FTD.

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KEYWORDS

amyloid precursor protein, biomarkers, cathepsin B, cerebrospinal fluid, complement C9, frontotemporal dementia, lysozyme, proteomics, transcobalamin II, ubiquitin

Highlights

- CSF markers of proteostasis were explored in *CHMP2B*-mediated frontotemporal dementia (FTD).
- 31 members of the Danish *CHMP2B*-FTD family were included.
- We used solid-phase extraction and parallel reaction monitoring mass spectrometry.
- Six protein levels were significantly altered in *CHMP2B*-FTD compared with controls.
- Lower CSF ubiquitin levels in patients suggest association with disease mechanisms.

1 | INTRODUCTION

Frontotemporal dementia (FTD) comprises a group of early-onset dementia syndromes characterized by high heritability. Pathogenic variants in a dozen genes have been demonstrated, of which the *C9orf72*, *GRN*, and *MAPT* genes are implicated in more than half of familial cases. A point mutation in the *CHMP2B* gene (c.532-1G > C) has been identified as a cause of autosomal dominant dementia in a Danish family with behavioral variant FTD.¹ The *CHMP2B* protein is a core component of the heteromeric 'endosomal sorting complex required for transport III' (ESCRT-III). This protein complex is involved in biogenesis of endocytic multivesicular bodies (MVBs), membrane repair, and regulation of autophagy.^{2,3} Most well-described is the role of ESCRT-III in the endo-lysosomal sorting of ubiquitinated cargo: The protein complex binds transiently to endosomal surfaces and is responsible for the scission of membranes budding into the MVB compartment during the formation of intraluminal vesicles. The mutation renders *CHMP2B* and thereby the ESCRT-III unable to dissociate from its binding partners and disassemble from endosomal membranes, where the protein accumulates.^{4,5} The result is defective trafficking of endosomes and impaired fusion of endosomes and autophagosomes with lysosomes.^{3,6} Correspondingly, enlarged endosomes have been observed in *CHMP2B*-FTD patient brain tissue and fibroblasts, as well as animal and cell models expressing the *CHMP2B* mutation.^{3,4,6–9} It has not been fully established whether the organelle malformation itself or mechanisms downstream of it are driving neurodegeneration.^{7,8} However, the mutations that cause FTD converge on defects in the autophagy-lysosomal and ubiquitin-proteasomal systems.¹⁰ Disturbed proteostasis particularly affects neurons, presumably owing to their specialized morphology and post-mitotic longevity. A distinct neuronal histopathology has been described in post-mortem tissue from *CHMP2B*-FTD patients, namely the presence of cytoplasmic

ubiquitin and p62 inclusions (FTLD-UPS), in the absence of tau, TDP-43 or FUS deposits.^{11,12} This hallmark has been recapitulated in three independent, transgenic mouse models expressing mutant *CHMP2B*.^{13–16} Interestingly, reactive gliosis was present in all *CHMP2B*-mice, and one study showed that the endo-lysosomal defects are present in both microglia and neurons,^{14,17} indicating involvement of the innate immune system – another common feature of FTD subtypes.

At present, there are no disease-modifying treatment options and no disease-specific biomarkers for FTD that can inform clinicians about expected disease onset, rate of progression, timing of potential treatment, or monitoring of the response to pharmacological intervention. Collection of cerebrospinal fluid (CSF) is essential for establishing such biomarkers. Peptides that reflect brain pathology can reach the CSF as a consequence of neurodegeneration, through secretion of soluble proteins or secretory vesicles, or from the blood across the blood-brain barrier (BBB).¹⁸ We have recently shown that a marker of axonal degeneration, neurofilament light (NfL), starts to increase in CSF and serum several years before debut of clinical *CHMP2B*-FTD.^{19,20} Genetic disorders provide ideal settings for investigating this preclinical stage, and the Danish *CHMP2B*-FTD family offers a genetically homogeneous cohort for investigating the underlying pathology in a familial FTD subtype. The *CHMP2B* animal and cell models have shown that endo-lysosomal components such as certain cathepsins, LAMPs and Rab proteins are affected by *CHMP2B* mutation,^{4,7,8,14,21} but the mediators of proteostasis have not yet been assessed in CSF from *CHMP2B*-FTD patients. To this end, we quantified a panel of CSF peptides in patients and controls. The targeted mass spectrometry panel was identified in an explorative proteomics study as tryptic peptides of proteins associated with endocytosis, lysosomal function, and the ubiquitin-proteasome system, that could be reliably quantified using solid-phase extraction and parallel reaction monitoring mass spectrometry.²³

2 | METHODS

2.1 | Participants

This cross-sectional study included 31 members of the Danish *CHMP2B*-FTD family: 11 symptomatic and 7 presymptomatic *CHMP2B* mutation carriers and 13 non-carrier controls. Patients were diagnosed at the Danish Dementia Research Centre, Rigshospitalet, Denmark, using international consensus criteria for behavioral variant FTD.²⁴ Members of the *CHMP2B*-FTD family are continuously offered pre-clinical genetic testing and genetic counseling. If consented to, serum and CSF are obtained from each family member for research purposes, without disclosing the genetic status, if so desired by the individual. Family members who do not carry the *CHMP2B* mutation and have no neurological or cognitive deficits on examination serve as the control group. Addenbrooke's Cognitive Examination (ACE) was employed as a measure of cognitive disease burden. The study was approved by the Ethics Committee of the Capital Region of Denmark (H-1-2012-041).

2.2 | CSF acquisition

Lumbar punctures were performed according to standard protocol. CSF samples were analyzed in clinical routine at the Department of Clinical Biochemistry, Rigshospitalet. Samples intended for PRM-MS were centrifuged at 2000 g for 10 min, and aliquots of 250 μ L were immediately stored at -80°C in the Danish Dementia Biobank, pending biochemical assay at the Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, the Sahlgrenska Academy, University of Gothenburg.

2.3 | Pre-analysis sample preparation

The methods have previously been described in detail, see Brinkmalm et al.,²² Sjödin et al.,²³ and Fernström et al.²⁵ In short, 100 μ L CSF was mixed with 25 μ L internal standard (IS) containing heavy isotope-labeled peptides (C-term $^{13}\text{C}/^{15}\text{N}$ -labeled K and L, and cysteine carbamidomethylation) corresponding to all peptides included in the analysis (JPT Peptide Technologies and Thermo Fisher Scientific) as well as uniformly ^{13}C -labeled ubiquitin (Silantes). The samples were reduced and alkylated by the addition of 25 μ L 30 mM 1,4-dithiothreitol (in 50 mM NH_4HCO_3 ; shaking incubation at $+60^{\circ}\text{C}$ for 30 min and then 30 min at room temperature) and 25 μ L 70 mM iodoacetamide (in 50 mM NH_4HCO_3 ; shaking at room temperature in the dark for 30 min), respectively. Digestion was performed by adding 25 μ L 0.08 $\mu\text{g}/\mu\text{L}$ sequencing grade modified trypsin (Promega Co.) diluted in 50 mM NH_4HCO_3 followed by shaking incubation for 18 h at $+37^{\circ}\text{C}$ which was ended by the addition of 25 μ L 10% trifluoroacetic acid. SPE was performed using standard procedure and Oasis HLB 96-well μ Elution Plates (2 mg sorbent and 30 μm particle size; Waters Co.). The plate was conditioned ($2 \times 300 \mu\text{L}$ methanol) and equilibrated ($2 \times 300 \mu\text{L}$ H_2O). The samples were loaded followed by

RESEARCH IN CONTEXT

- 1. Systematic review:** PubMed searches were conducted to compare data from biomarker, animal, and cell model studies on impaired proteostasis in neurodegenerative diseases, with focus on frontotemporal dementia (FTD). Knowledge is lacking regarding which components of the endo-lysosomal and ubiquitin-proteasomal systems are affected, and whether such proteins might be utilized as biomarkers.
- 2. Interpretation:** This is the first study to assess CSF levels of proteostatic markers in a genetically homogenous FTD cohort. From mass spectrometry data, we identified six significantly different protein levels when comparing *CHMP2B* mutation carriers with family controls. Particularly interesting was the finding of lower CSF ubiquitin in *CHMP2B*-FTD patients, challenging the view that ubiquitin levels solely reflect neurodegeneration.
- 3. Future directions:** The proposed markers should be included in upcoming studies on fluid biomarkers in familial FTD to confirm the alterations. For certain peptides, for example, ubiquitin, quantification of full-length protein in CSF from *CHMP2B*-FTD patients is relevant.

a wash ($2 \times 300 \mu\text{L}$ H_2O) and were finally eluted ($2 \times 100 \mu\text{L}$ methanol). The samples were dried by vacuum centrifugation and frozen at -80°C pending analysis.

2.4 | PRM-MS

The target panel of interest included 47 peptides derived from 20 proteins. Table S1 shows all peptide measurements and protein estimates. Frozen and dried samples were dissolved, and injected and separated using a Dionex UltiMate 3000 standard-LC system (Thermo Fisher Scientific) in reversed phase on a Hypersil GOLD HPLC C18 column (length 200 mm; inner diameter 2.1 mm; particle size 1.9 μm ; Thermo Fisher Scientific). Analysis was performed on a hybrid Q Exactive mass spectrometer (Thermo Fisher Scientific) using electrospray ionization and a HESI-II ionization probe (Thermo Fisher Scientific). The instrument operated in positive ionization mode and data acquisition was performed using a scheduled PRM method with a retention time isolation window of 2 min, an isolation window of m/z 3, a resolution setting of 70k, an automatic gain control target of 1×10^6 and a maximum injection time of 300 ms.

Peak detection and area integration were performed using Skyline v3.6,²⁶ targeting $[\text{M} + \text{H}]^{1+}$ y-ions from a data-independent acquisition method with a fixed isolation window of m/z 3 and an orbitrap analyzer resolution setting of 70k at m/z 200. The ratio of the sum of the product ion areas of tryptic to isotope-labeled peptide was used for quantification.

TABLE 1 Patient characteristics.

	Presymptomatic <i>CHMP2B</i> mutation carriers	Symptomatic <i>CHMP2B</i> mutation carriers	Healthy family members	<i>p</i> -value
No. of participants	7	11	13	0.405
Sex, females/males	4/3	3/8	5/8	0.511
Mean age, years (range)	52.0 (32.7–68.1)	62.9 (53.6–73.3)	60.5 (38.1–71.1)	0.060
Mean ACE score (range)	92 (88–95)	74.9 (60–89)	93.3 (87–100)	<0.001

2.5 | Immunoprecipitation and PRM-MS

SNAP-25/synaptotagmin-1 were quantified with an in-house assay consisting of enrichment with immunoprecipitation (mouse monoclonal antibodies clone 41.1 (Synaptic Systems) and SMI81 (Biosite)) followed by quantitation with liquid chromatography/tandem mass spectrometry (LC-MS/MS, see section above). The instrument was set to acquire scheduled pairs or triplets of fragmentation scans (PRM scans) in profile mode, allowing simultaneous detection of the CSF peptide and the corresponding IS. LC-MS/MS raw files acquired with Xcalibur software version 2.2 SP1.48 (Thermo Fisher Scientific) were imported into Pinpoint software version 1.3.0 (Thermo Fisher Scientific), and peak areas of the CSF and IS peptides were generated. CSF levels of SNAP-25tot, and synaptotagmin-1 were calculated by multiplying the ratio of the LC-MS peak areas with the concentration of the corresponding IS.

2.6 | Statistics

Statistical analyses were carried out using R statistical software, version 1.2.1335. Normal distribution of peptide values was checked with QQ-plots, histograms, and Shapiro-Wilk normality tests. Non-normally distributed data were logarithmically transformed. Comparisons of mean protein or peptide levels in the clinical groups were calculated with ANOVA and post-hoc comparisons were conducted using Tukey test with single-step adjustment as implemented in the *multcomp* toolbox.²⁷ Since there was a tendency towards significantly different ages in the clinical groups, age was included as a covariate in these comparisons. Correlations between continuous variables were assessed using the Spearman's Rank correlation coefficient. Descriptive statistics were carried out with Fisher's exact test or one-way ANOVA with post-hoc Tukey test. *p*-values below 0.05 were considered significant.

3 | RESULTS

3.1 | Patient characteristics

Participants' demographics and ACE scores are presented in Table 1. Female-to-male ratios did not significantly differ between groups. Mean age differed between symptomatic and presymptomatic patients by 10.8 years (*p* = 0.055, Tukey test). As expected, ACE scores were

significantly lower in symptomatic participants compared with both presymptomatic and healthy participants.

3.2 | CSF protein concentrations

Comparing *CHMP2B* mutation carriers with controls revealed significantly increased protein levels of complement C9, lysozyme C (lysozyme), and transcobalamin II (TCN2) in mutations carriers, and significantly lower levels of ubiquitin, amyloid precursor protein (APP) and cathepsin B (CTSB). No significant differences were detected for AP2B1, CTSD, CTSF, CTSL, CTSZ, DPP7, GM2A, HEXB, LAMP1, LAMP2, FUCA1, TPP1, SYT1, or SNAP25 (Table S2). The six protein estimates significantly associated with *CHMP2B* mutation were based on the levels of 14 peptides in total. Peptide pairs and triplets originating from the same protein were highly correlated, except the combination of the pro-peptide CTSB₅₈₋₇₁ and the light chain peptide CTSB₈₀₋₈₇ (*R*_s = 0.38, *p* = 0.062), which were both highly correlated with the heavy chain peptide, CTSB₂₁₀₋₂₂₀ (*R*_s = 0.73–0.77, *p* < 0.0001). When dividing the *CHMP2B* mutation carriers into symptomatic and presymptomatic participants, the levels of 10 of the 14 peptides were significantly different among the three clinical groups (Figure 1, Table S2).

We have recently found an increased level of NfL in CSF from both symptomatic and presymptomatic *CHMP2B* mutation carriers, as well as an increased CSF/serum albumin ratio in symptomatic *CHMP2B*-FTD patients^{19,20} – the former being a well-established marker of axonal degeneration, the latter reflecting potential compromise of BBB integrity. In the current study, none of the 10 peptides were significantly correlated with CSF NfL levels in *CHMP2B* mutation carriers (Table S2). The complement C9, lysozyme, and TCN2 peptides correlated significantly with the CSF/serum albumin ratios, as shown in Figure 2. The complement C9 peptides and one of two lysozyme peptides were significantly correlated with age (Table S2). Notably, CSF/serum albumin ratios did not correlate with age in the mutation carrier group (*R*_s = 0.11, *p* = 0.670). No correlations between peptide levels and ACE-scores were observed in mutation carriers (Table S2).

4 | DISCUSSION

Increasing evidence implicates proteostatic dysfunction as an early event in the development of neurodegenerative diseases, including

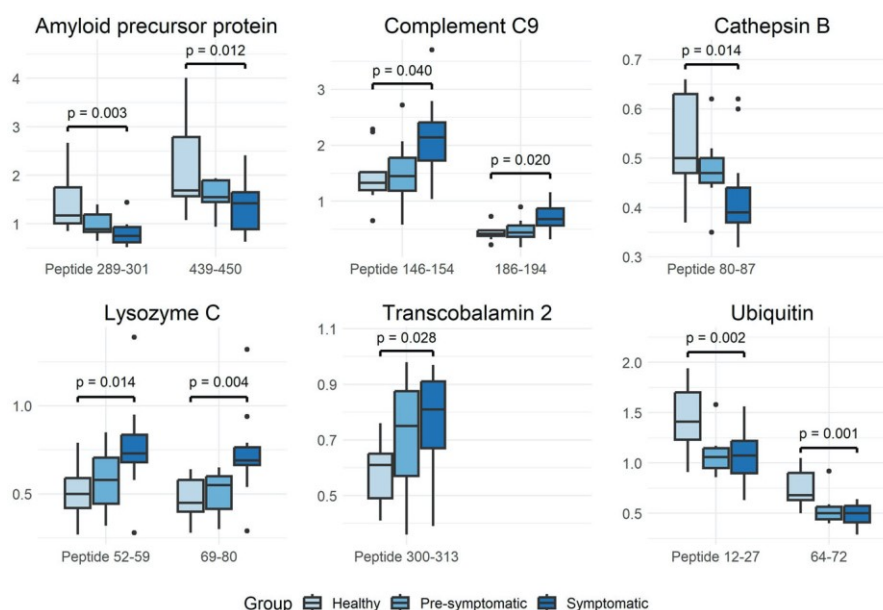


FIGURE 1 Significantly different CSF peptide levels between the three clinical groups: Symptomatic *CHMP2B* mutation carriers, presymptomatic carriers, and healthy family controls. Y-axis values represent a ratio between tryptic peptide and added internal standard. Calculations were performed using ANOVA with post-hoc Tukey multiple comparisons of means test.

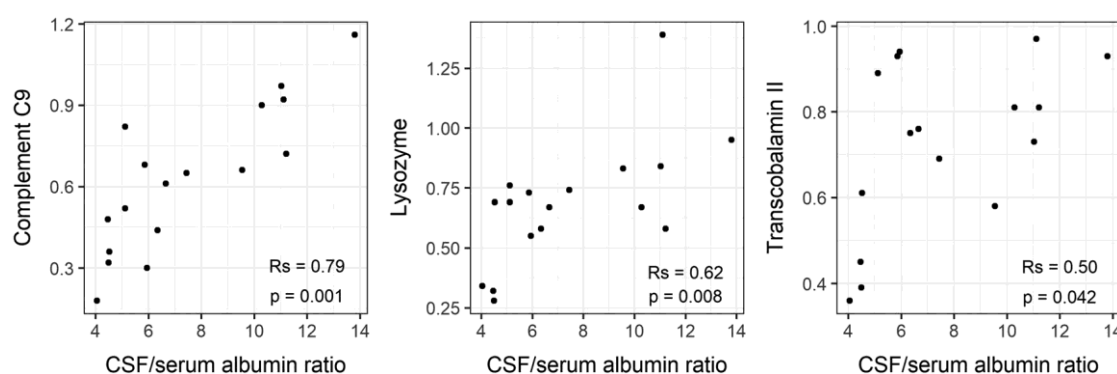


FIGURE 2 Correlations between CSF/serum albumin ratios and complement C9 (peptide 186–194), lysozyme (peptide 52–59), and transcobalamin II (peptide 300–313) in *CHMP2B* mutation carriers. Y-axis values represent a ratio between tryptic peptide and added internal standard. Calculations were performed using Spearman's correlation. R_s , Spearman's rank correlation coefficient.

FTD and *CHMP2B*-FTD specifically. To explore potential mediators of impaired proteostasis in *CHMP2B*-FTD, this study investigated CSF levels of a panel of peptides functionally involved in endo-lysosomal or ubiquitin-proteasome function. A total of 47 peptides originating from 20 proteins were analyzed and their concentrations compared among 31 members of the Danish *CHMP2B*-FTD family. PRM-MS revealed significantly higher levels of complement C9, lysozyme, and TCN2 in *CHMP2B* mutation carriers compared with controls, and significantly lower levels of ubiquitin, APP and CTSB (Figure 1).

Neuronal inclusions containing ubiquitin and the ubiquitin-binding autophagy receptor p62 remain the only immunohistochemical hallmarks of *CHMP2B*-FTD postmortem brain tissue.^{11,12} How the cerebral deposition of ubiquitin is reflected in CSF, has not been investigated. Here, both presymptomatic carriers and symptomatic *CHMP2B*-

FTD patients had significantly lower ubiquitin levels than controls. Two studies have previously included measurements of CSF ubiquitin in FTD patients.^{28,29} Both found the concentration of ubiquitin to be at the same level as healthy controls. Patients with AD and Creutzfeldt–Jakob disease (CJD) showed significantly increased levels of CSF ubiquitin, demonstrated across several studies.^{29–34} Elevated CSF ubiquitin in AD and particularly in CJD has been linked to the extent of neuroaxonal damage.^{28–31} This is supported by correlations between CSF ubiquitin and established markers of neurodegeneration, that is, NfL and total-tau,^{28,29,35} as well as observed rise in CSF ubiquitin following traumatic brain injury.³⁶ However, seemingly unaltered CSF levels in other neurodegenerative disorders such as PD indicate that ubiquitin is not exclusively a biomarker of nonspecific neurodegeneration,^{29,33,35,37} but instead suggests some degree

of association with disease mechanisms or affected brain region. Of note, the studies that have analyzed CSF ubiquitin levels in behavioral type FTD did not distinguish between genetic subtypes, which may differ in terms of ubiquitin dynamics, as it might be the case with NFL.²⁰

A possible explanation for the lower CSF ubiquitin levels in *CHMP2B* mutation carriers lies in the impairment of the ESCRT machinery, responsible for internalizing ubiquitinated proteins into MVBs, where they are sorted for either lysosomal degradation with accompanying release of ubiquitin or trafficking back to the plasma membrane. We speculate that the accumulated MVBs observed in *CHMP2B* models are maintaining an intracellular reservoir of ubiquitin, not reaching the CSF. One might expect that such neuronal ubiquitin deposits would increase CSF levels as neurodegeneration progresses, similar to levels of NFL in *CHMP2B*-FTD.¹⁹ In this regard, an apparent spatial mismatch between the frontotemporal cortical neurodegeneration and the neuronal ubiquitin inclusions – which are predominantly found in the hippocampus¹¹ – might partly explain the seemingly non-parallel courses of CSF ubiquitin and NFL in *CHMP2B*-FTD. Another contributing possibility is compromised ESCRT-dependent formation of exosomes, potentially leading to fewer ubiquitinated proteins being transported to the extracellular space. However, silencing one of the direct binding partners of *CHMP2B*, VPS4B, in HeLa cells has previously been shown to increase overall exosome secretion, not reduce it.³⁸ If validated, decreased CSF ubiquitin levels may distinguish *CHMP2B*-FTD or FTD collectively from other neurodegenerative disease entities, as it has only been found significantly reduced in one other neurodegenerative disease cohort.²³

CSF levels of complement C9 and lysozyme were increased in *CHMP2B*-FTD patients compared with controls. The complement proteins constitute part of the innate immune system and are produced, in the CNS, by neurons and glial cells.³⁹ They mediate proinflammatory stimulation and chemoattraction between these cell types, as well as clearance of pathogens and damaged cells via the membrane attack complex (MAC), and tagging of synapses for phagocytosis by microglia.³⁹ Lui et al. have demonstrated that lysosomal defects and complement activation in microglia can lead to neurodegeneration through excessive synaptic pruning in a mouse model of FTD caused by *GRN* mutation.⁴⁰ Astrocytes generated from induced pluripotent stem cells from *CHMP2B*-FTD patients have been shown to be more reactive and display increased production of complement C3, which was confirmed in the brains of mice expressing mutant *CHMP2B*.⁴¹ Complement C9, the terminal constituent of the complement cascade and major part of the cytolytic MAC, has not previously been investigated in CSF or in vitro models in FTD, but its elevation in CSF indicates involvement of innate immune function. This is supported by increased levels of CSF lysozyme – a glycan cleaving polypeptide produced by microglia.⁴² Findings of higher CSF lysozyme in AD patients than controls have been inconsistent,^{22,23,43,44} although a protective effect of the protein has been proposed based on rescue experiments in *Drosophila* models.^{43,44} To our knowledge, the role or quantities of lysozyme in FTD have not previously been studied. Importantly, both complement C9 and lysozyme were associated with the CSF/serum

albumin ratio (Figure 2).²⁰ Correlations between soluble MAC components and BBB dysfunction is known from patients with traumatic brain injury,⁴⁵ and the positive correlation between complement C9 and age has previously been observed in AD.⁴⁶ Earlier studies have reported increased levels of CSF lysozyme as part of an inflammatory reaction to a variety of CNS pathologies.⁴⁷ Passage of lysozyme from blood through an impaired BBB as well as local production by microglia within the CNS, have been suggested as the underlying mechanisms. Thus, it is plausible that complement C9 and lysozyme emanate from serum and contribute to the measured CSF amounts in *CHMP2B*-FTD patients. In relation to this, we have previously observed a systemic immune response in *CHMP2B*-FTD patients.⁴⁸ Innate immune activation is also supported by results from transgenic *CHMP2B Drosophila* models^{49–51} and *CHMP2B* mouse models, which have all exhibited reactive gliosis.^{13–16} One such mouse model revealed that accumulation of endo-lysosomal byproducts was present in microglia as well as neurons.¹⁴ A follow-up study showed an early microglial activation in the transgenic mice, progressing into overt microgliosis with a proinflammatory phenotype, neuronal loss, and behavioral changes.¹⁷ In addition, the authors detected increased levels of lysosomal cathepsin D and LAMP-1,¹⁴ which has also been reported in *Grn* knockout mice and patients with *GRN*-FTD.⁵²

In the present study, CSF concentrations of these lysosomal proteins were unchanged, whereas CTSB was significantly reduced in *CHMP2B*-FTD patients. Several studies have demonstrated increased CSF levels of CTSB in AD, traumatic brain injury, inflammatory neurological diseases, and aging.^{23,53,54} Lysosomal leakage of CTSB with ensuing unchecked proteolytic activity in the cytosol has been suggested as the deleterious mechanism.^{53,54} Since ESCRT-III is involved in endo-lysosomal membrane repair, one might expect *CHMP2B* mutation to increase the concentration of CTSB in CSF, rather than reduce it in *CHMP2B*-FTD patients, which we observe. The potential reduction in CTSB requires further exploration.

In this study, the quantity of APP was significantly lower in CSF from *CHMP2B*-FTD patients compared with controls. The transmembrane APP protein functions as a cell surface receptor on neurons, involved in neurite growth and synaptogenesis.⁵⁵ It is cleaved by secretases into smaller fragments relating to the amyloidogenic or non-amyloidogenic pathways.⁵⁵ In AD, the CSF concentrations of soluble APP did not differ between patients and controls in a recent meta-analysis,⁵⁶ neither did the levels of the major isoforms of APP in studies using targeted proteomics.^{22,23} In FTD, albeit less well studied, findings point to lower levels of soluble APP in patients with different types of FTLD.^{57–60} Inside neurons, APP is ubiquitinated and targeted to the intraluminal vesicles of MVBs through interaction with the ESCRT machinery.⁶¹ The internalization of APP and delivery to lysosomes has been shown to parallel that of the epidermal growth factor receptor (EGFR),⁶¹ the degradation of which is delayed in *CHMP2B* mutation models^{3,62} – supposedly due to entrapment of EGFR in mutant *CHMP2B* positive compartments.³ Thus, as described for ubiquitin, APP may be retained in the MVB compartment, reducing the proportion of APP available for cleavage into soluble forms at the plasma membrane, lowering the CSF concentration in *CHMP2B*-FTD patients.

Lastly, TCN2 was significantly elevated in the patient group. It is responsible for transporting vitamin B12 into tissues via its receptor (CD320), where it is degraded in lysosomes, while dissociated B12 is exported into the cytosol.⁶³ It is unknown whether the ESCRT system is involved in this process, but ubiquitination of CD320 does not appear to occur.⁶³ A strong correlation between TCN2 levels in plasma and CSF has been demonstrated elsewhere,⁶⁴ and it is likely that the bulk of TCN2 in CSF originates from serum. In line with this, TCN2 was significantly associated with the CSF/albumin ratio in our study.

Research into mediators of disrupted proteostasis has been limited in FTD, particularly for genetic subtypes. This study has screened a panel of potential biomarkers associated with endo-lysosomal function in *CHMP2B*-FTD. Of note, the proteins investigated are multifunctional and hence significantly different CSF levels do not necessarily equate to significant differences in cellular proteostasis. Although the number of statistical comparisons has been attempted to be kept at a minimum, the rarity of the disorder means that the size of our cohort is modest in relation to the number of analytes tested, increasing the risk of false positives. However, the cohort is pathogenetically homogenous, which is necessary for disease entities with such diverse genetic backgrounds as FTD. The findings require validation in an independent cohort and for certain peptides, for example, ubiquitin, further quantification of the full-length protein in CSF from *CHMP2B*-FTD patients will be relevant. The ubiquitin peptides generated by our enzymatic digestion approach can be derived from both free and conjugated forms of the protein – thus, we cannot assess if intact, free ubiquitin is decreased in *CHMP2B*-FTD. In AD, however, previous studies have shown that both free and conjugated ubiquitin levels are increased.⁶⁵ Furthermore, a recent study employing the same technique as herein could confirm that the levels of these particular ubiquitin peptides are increased in CSF from AD patients.²³ Taken together, the data support different concentrations of CSF ubiquitin in AD, FTD, and healthy controls. The lack of correlation between the studied peptides and CSF NfL suggests that the altered protein levels in mutation carriers are not secondary to axonal neurodegeneration, as expressed by CSF NfL levels, but perhaps represent another part of the disease mechanism.

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CONFLICT OF INTEREST STATEMENT

K.B. has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, BioArctic, Biogen, and JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Ono Pharma, Pharmatrophix, Prothena, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper. H.Z. has served at scientific advisory boards and/or as a consultant for Abbvie, Alektor, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Passage Bio, Pinteon Therapeutics, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectricron, Fujirebio, Alzecure, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). The remaining authors (A.T., S.S., A.H.S., P.E., P.R., C.S.M., E.E.H., T.T.N., A.B., and J.E.N.) have no conflicts of interest to declare. Author disclosures are available in the [supporting information](#).

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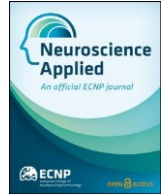
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Review

Cerebrospinal fluid ubiquitin as a biomarker for neurodegenerative diseases: A systematic review

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ABSTRACT

Ubiquitin plays a vital role in neuronal proteostasis, as a major but often overlooked component of neurotoxic protein aggregates across neurodegenerative diseases. Although neuropathological changes can be present for years before clinical onset, early and accurate diagnosis remains an immense challenge in this disease category. The level of ubiquitin in cerebrospinal fluid (CSF) has been assessed as a biomarker for several disease entities. This systematic review compares current findings and evaluates the potential of CSF ubiquitin as a fluid biomarker. A systematic literature search identified studies comparing CSF ubiquitin levels between a control group and patients with one of the following diseases: Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia (FTD), Lewy body dementia (DLB), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). All included studies were reviewed systematically by two independent authors. 171 studies were identified. A total of 17 studies met the eligibility criteria and were included. Nine out of 13 studies found a significant increase of CSF ubiquitin in AD patients compared with control groups. Correlations between CSF ubiquitin and other established biomarkers were demonstrated in seven studies. A single study was included for both HD and DLB respectively, each showing significantly higher CSF ubiquitin in patients compared to controls. In patients with PD, FTD or ALS, CSF ubiquitin levels were generally equal to those of the control groups, with two studies showing significantly decreased concentrations in a PD and an FTD cohort. Presently, the available body of research is insufficient to assess whether CSF ubiquitin could contribute to the clinical setting, alongside established markers of neurodegeneration. The correlation of elevated CSF ubiquitin with AD is well-founded, whilst validation of reduced or unchanged levels in the other neurodegenerative diseases will determine the usefulness of the biomarker in clinical practice.

1. Introduction

Neurodegenerative diseases are characterized by progressive deterioration of the nervous system, with gradual and selective loss of particularly vulnerable populations of neurons. These processes eventually manifest as motor deficits and cognitive decline (Schmidt et al., 2021). The degenerative mechanisms often develop decades before the onset of clinical features (Dugger and Dickson, 2017). However, the current lack of sensitive and specific biomarkers continues to make early and accurate diagnosis an immense challenge.

The accumulation of misfolded and aggregated proteins is a neuropathological hallmark of most neurodegenerative diseases, implying an

underlying severe impairment of cellular proteostasis and protein degradation (Ciechanover and Kwon, 2015; Ross and Poirier, 2004). Neurons are predisposed to the harmful effects of these protein deposits as well as damaged organelles, due to their longevity and inability to undergo cell division. In eukaryotes, the two major pathways of protein degradation are the ubiquitin-proteasome system (UPS) and autophagy-lysosomal pathway (ALP). Progressive breakdown and perturbation of these systems, crucial for neuronal survival and functioning, results in accumulation of harmful protein aggregates and is a hallmark of aging as well as the onset of neurodegenerative disease (Alves-Rodrigues et al., 1998; López-Otín et al., 2013; Schmidt et al., 2021; Watanabe et al., 2020).

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1.1. Ubiquitin and protein degradation in neurodegenerative diseases

The highly conserved 76 amino acid protein, ubiquitin, exists in abundance in all eukaryotic cells and plays a key role in both of the main cellular mechanisms for protein turnover, the UPS and the ALP. Ubiquitin is conjugated to substrate proteins through C-terminal glycine residue linkages. Ubiquitination occurs via the sequential actions of the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. The fate of each modified protein is determined by the nature of the mono- or polyubiquitin linkage, with the majority being directed to proteasomal degradation and elimination through the UPS. Degradation-dependent and degradation-independent ubiquitin-signaling has been shown to play a major role in neuronal survival through both the UPS and ALP, as well as to be involved in membrane trafficking, DNA repair, transcription regulation, cell signaling, neuroinflammation and cell death control (Bard et al., 2018; McClellan et al., 2019; Schmidt et al., 2021; Watanabe et al., 2020). The functions rely on the availability of free ubiquitin and ubiquitin conjugates in intracellular pools (Park and Ryu, 2014). These reservoirs are maintained by the actions of deubiquitinating enzymes (DUBs) and *de novo* synthesis of monomeric ubiquitin. In neurodegenerative diseases, polyubiquitination of aggregating neurotoxic proteins have been shown to sequester ubiquitin and UPS components and deplete the pool of free ubiquitin, with detrimental effects on the ubiquitin-mediated processes (Boland et al., 2018; Dantuma and Bott, 2014). As this mechanism appears to be a shared disease feature, interventions aimed at reinforcing the proteolytic systems by increasing the recognition or handling of neurotoxic proteins offer wide-ranging therapeutic potential (Boland et al., 2018; Dantuma and Bott, 2014).

Whether misfolding and aggregation of specific proteins or degeneration of the proteolytic systems should be considered the principal neurodegenerative driving mechanism remains undetermined (Deger et al., 2015). However, the involvement of both mechanisms is supported by mutations in genes coding for either aggregating proteins or UPS components leading to the development of neurodegenerative disease (Schmidt et al., 2021). Moreover, a positive feedback loop has been shown to exist, where increased aggregation of toxic proteins further aggravates the impairment of proteasomal activity (Bence et al., 2001).

1.2. Ubiquitination of proteopathic aggregates

Modification of neurotoxic aggregates by ubiquitin and associated enzymes has been demonstrated in several neurodegenerative diseases (Table 1): In Parkinson's disease (PD) and Lewy body dementia (DLB), accumulations of α -synuclein constitute the major component of pathological deposits (synucleinopathies), either as cytoplasmic spherical

filamentous inclusions in the midbrain or cortex (Lewy bodies) or thread-like dystrophic processes in presynaptic terminals of neurons (Lewy neurites). Immunoreactivity for ubiquitin can often be found as a halo around the α -synuclein core of the aggregates, as they are targeted for degradation by the UPS (Boland et al., 2018; Spillantini et al., 1998). Interestingly, some studies have found that unmodified α -synuclein is degraded by the proteasome in a ubiquitin-independent manner, suggesting that ubiquitination of the accumulating protein is an aberrant process reflecting loss of proteolytic control by a comprised UPS (Tofaris et al., 2003).

Diseases characterized by neuronal and glial accumulations of misfolded and insoluble tau protein include Alzheimer's disease (AD) and the FTLT-tau subtype of frontotemporal dementia (FTD). In these tauopathies, tau oligomers undergo ubiquitination to enhance proteasomal degradation as well as capture by autophagy receptors such as p62 (Boland et al., 2018; Li et al., 2021). The degradation signaling has previously been attributed to K48 and K63 polyubiquitin linkages, respectively, but emerging evidence also implicates K6, K11, K27, K29 and mono-ubiquitination in the processes, while K33 remains the least studied linkage type (Li et al., 2021; Tracz and Bialek, 2021). Recent evidence has suggested that tau ubiquitination may contribute to neuropathology by promoting its aggregation and mislocalization to dendrites. However, as ubiquitination promotes the clearance of soluble tau oligomers, which exhibit high seeding activity and toxicity compared to late-stage neurofibrillary tangles, the ubiquitination is most likely non-toxic in itself (Li et al., 2021).

TAR DNA-binding protein 43 (TDP-43) aggregates are a pathological hallmark of sporadic and genetic, tau-negative amyotrophic lateral sclerosis (ALS) and FTD (FTLD-TDP), and are frequently associated with ubiquitin and ubiquitin-associated proteins. Soluble TDP-43 is primarily degraded by the UPS, while aggregated conformations of TDP-43 are eliminated via autophagy (Scotter et al., 2014). The pathophysiological role of TDP-43 ubiquitination has been a long-standing question: Interactions between TDP-43 and specific E2 and E3 enzymes can either counteract toxic protein aggregation or appear to aggravate it, particularly in combination with other post-translational modifications such as phosphorylation and truncation (Tran and Lee, 2022). Aberrant effects of ubiquitination may also depend on the pathological TDP-43 species that are available as substrates, including misfolded, oligomeric, truncated and aggregated protein, or insoluble inclusions. Moreover, unchecked polyubiquitination of TDP-43 has been shown to sequester essential UPS components and deplete the intracellular pool of free ubiquitin (Farrawell et al., 2020). The same authors have presented similar findings in cell models of ALS with pathological aggregations of FUS or SOD1. In fact, this is a shared feature in neurodegenerative proteinopathies, which contributes to disruption of protein homeostasis and impedes the additional functions of ubiquitin.

Beta-amyloid pathology in AD is increasingly recognized as an intracellular as well as extracellular phenomenon. The toxicity of intraneuronal, soluble β -amyloid 1–42 (A β 42) has previously been overshadowed by the considerably more abundant extracellular plaques, but are now recognized as a mediator of synaptic deterioration and prognosticator of cognitive decline (Gouras et al., 2010; Takahashi et al., 2017). The well-documented presence of ubiquitin in extracellular plaques in AD patients suggests that harmful A β 42 are released from neurons, either intended for clearance as an active protective mechanism, or passively due to neurodegeneration. Conversely, neuronal uptake of A β 42 forms is likely implicated in the propagation of pathology, akin to prion transmission (Jucker and Walker, 2013). The significance of such extracellular ubiquitination is not established, but it has been shown to potentially delay the breakdown of A β 42 by the protease insulin-degrading enzyme (IDE), as well as modulate immune responses (Bellia et al., 2019; Majetschak et al., 2011; Takahashi et al., 2017).

Table 1
Neurodegenerative diseases and their characteristic protein aggregates.

Disease	Primary protein aggregates	Ubiquitin component
Alzheimer's disease	Beta-amyloid	Present (de Vrij et al., 2004; Mori et al., 1987; Perry et al., 1987)
Amyotrophic lateral sclerosis	SOD1 TDP-43 FUS	Present (Mizusawa et al., 1989; Neumann et al., 2006)
Frontotemporal dementia	TDP-43 Tau FUS	Present (Neumann et al., 2006; Seelaar et al., 2010)
Huntington's disease	Huntingtin with polyglutamine expansion	Present (Fernández-Nogales et al., 2014; Kalchman et al., 1996)
Lewy body dementia	α -synuclein	Present (Goedert et al., 2001)
Parkinson's disease	α -synuclein	Present (Galloway et al., 1988; Goedert et al., 2001)

1.3. CSF-ubiquitin as a biomarker in neurodegenerative diseases

Ubiquitin is thought to be released into the cerebrospinal fluid (CSF) either passively due to neurodegeneration or by means of active secretion into the extracellular space. Although an invasive method of analysis, CSF sampling provides a specific and sensitive analytic tool, reflecting central nervous system conditions. However, the studies of CSF biomarkers in general, and CSF ubiquitin in particular are very heterogeneous and an overall assessment of the literature including gaps in knowledge could guide future studies and clinical decisions regarding the relevance of using CSF ubiquitin.

A novel biomarker in CSF may provide an additional tool for early diagnosis of neurodegenerative disease and differential diagnosis among them, or for assessment of disease progression and monitoring of response to medical intervention. Thus, the purpose of this systematic review was to review available literature on the potential clinical application of CSF ubiquitin in the diseases: AD, PD, DLB, ALS, FTD, and Huntington's disease (HD). The diseases being chosen based on representing a relatively homogenous patient group regarding average age of onset as well as an insidious disease progression. The diseases share common features of pathology involving harmful protein aggregates, as well as representing a universal challenge concerning accurate and early diagnosis (Table 1). Furthermore, biomarkers that reflect the effects of emerging disease-modifying drugs in clinical trials, irrespective of the specific aggregation pathology, are necessary to elucidate treatment response or even guide the initiation of treatment.

2. Material and methods

2.1. Eligibility criteria

Studies selected for the systematic review were original, full-text articles published in English, investigating the CSF levels of ubiquitin in AD, ALS, FTD, HD, DLB or PD. Studies had to include CSF samples from at least one of the mentioned diseases as well as a control group. Controls should either be healthy individuals or patients with other neurodegenerative or non-neurodegenerative, neurological diseases. Each study had to include at a total of least 10 participants.

2.2. Search strategy and literature selection

Six separate systematic literature searches were performed in English using the PubMed MeSH database to identify all relevant articles. Searches were performed on April 1, 2023. The search strings are listed below. After completing the literature searches, both title and abstracts were screened for relevance according to the eligibility criteria. Searches were performed with the filters "humans" and "English" applied. The following MeSH searches were carried out.

- Ubiquitin AND (Alzheimer disease OR AD) AND (Cerebrospinal fluid OR CSF)
- Ubiquitin AND (Parkinson disease OR PD) AND (Cerebrospinal fluid OR CSF)
- Ubiquitin AND (Frontotemporal dementia OR FTD) AND (Cerebrospinal fluid OR CSF)
- Ubiquitin AND (Lewy body disease OR DLB OR Lewy body dementia) AND (Cerebrospinal fluid OR CSF)
- Ubiquitin AND (Huntington's disease OR HD OR Huntington Chorea) AND (Cerebrospinal fluid OR CSF)
- Ubiquitin AND (Amyotrophic lateral sclerosis OR ALS) AND (Cerebrospinal fluid OR CSF)

Screening of title and abstracts for inclusion of studies was done by two authors.

2.3. Data extraction and study quality

Complete data extraction of the selected studies was performed by two independent authors using a standard data extraction form that included: Year of publication and first author; investigated diseases and types of control groups; study design and purpose; diagnostic criteria; inclusion and exclusion criteria; sex and age distribution for both disease and control groups; methods including CSF handling and analysis; blinding of analysts; and results of statistical analyses.

3. Results

3.1. Literature selection

Table 2 presents a flowchart of the literature search and selection process, and an overview of the included studies. The six separate searches in the PubMed database yielded 171 entries, of which 138 were excluded based on title and abstract because they were either reviews, did not include CSF sampling, or were otherwise irrelevant. An additional 8 results were excluded due to being duplicates. The remaining 25 studies were evaluated carefully, extracting 17 studies that met the eligibility criteria and were included in the qualitative analysis. Eight of these studies included multiple diseases and were not excluded, but only counted once in the total number of studies. See Table 2.

3.2. Preanalytical variables

Sampling of CSF and processing of samples varied among the included studies. Only two studies reported time of CSF sample collection during the day – in the morning hours before noon (Chalbot et al., 2011; Constantinescu et al., 2010), with fourteen not including any information on timing, and one study performing the CSF sampling post-mortem (Kudo et al., 1994). Fourteen studies stated that CSF samples were kept at temperatures of minus 70–80 °C, two studies reported storage temperatures of minus 20–30 °C (Heywood et al., 2015; Manaka et al., 1992), and one did not inform about storage (Blennow et al., 1994). In seven studies the CSF samples were stored in polypropylene tubes, whereas one study used silicone coated tubes (Blennow et al., 1994) and the remaining nine did not report anything on the matter.

3.3. Analytical variables

Nine studies analyzed CSF ubiquitin levels with different mass spectrometry techniques and the remaining eight used either enzyme-linked immunosorbent assay (ELISA) (Blennow et al., 1994; Chalbot et al., 2011; Kandimalla et al., 2011, 2014), competitive ELISA (cELISA) (Iqbal et al., 2005) competitive enzyme-linked immunoflow assay (CELIFA) (Kudo et al., 1994) radioimmunoassay (RIA) (Manaka et al., 1992) or Western Blot (Mendonça et al., 2011). For mass spectrometry approaches, three studies used liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Abu-Rumeileh et al., 2020; Heywood et al., 2015; Oeckl et al., 2014), two used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (Constantinescu et al., 2010; Vinther-Jensen et al., 2015), one used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Davidsson et al., 2002) and three used parallel reaction monitoring-mass spectrometry (PRM-MS) (Sjödén et al., 2017, 2019; Toft et al., 2023). Two studies employed a PRM-MS technique that quantified the CSF levels of two proteotypic ubiquitin peptides (Sjödén et al., 2019; Toft et al., 2023), whilst the other studies measured the intact ubiquitin protein, in either its free or conjugated form.

Table 2

Flowchart of literature search and selection process.

Disease	# identified in PubMed search	# excluded based on title and abstract	# assessed with eligibility criteria ^a	# excluded based on eligibility criteria ^a	# of studies included in this review ^a	Studies included ^a
AD	55	40	15	2	13	Abu-Rumeileh et al., (2020) Blennow et al., (1994) Chalbot et al., (2011) Davidsson et al., (2002) Heywood et al., (2015) Iqbal et al., (2005) Kandimalla et al., (2011) Kandimalla et al., (2014) Kudo et al., (1994) Manaka et al., (1992) Oeckl et al., (2014) Sjödén et al., (2017) Sjödén et al., (2019)
ALS	17	15	2	0	2	Mendonça et al., (2011) Oeckl et al., (2014)
FTD	12	7	5	2	3	Abu-Rumeileh et al., (2020) Oeckl et al., (2014) Toft et al., (2023)
HD	3	2	1	0	1	Vinther-Jensen et al., (2015)
DLB	5	3	2	1	1	Heywood et al., (2015)
PD	79	71	8	3	5	Constantinescu et al., (2010) Heywood et al., (2015) Oeckl et al., (2014) Sjödén et al., (2017) Sjödén et al., (2019)

^a Note that studies may appear multiple times in these columns if they include more than one of the disease groups examined in this review.

3.4. Patient data

The clinical workup of patients and controls differed between studies. All studies provided information on which diagnostic criteria had been used to diagnose the participating patients. A total of six studies reported having performed diagnostic imaging – either CT, MRI, or undefined brain imaging – on all included patients, except patients belonging to a pilot study cohort in one study (Sjödén et al., 2019). Thirteen studies included patients with a diagnosis of AD, of which six measured core CSF biomarkers for AD, namely t-tau, p-tau, or Aβ42, as a part of the clinical workup (Abu-Rumeileh et al., 2020; Heywood et al., 2015; Kandimalla et al., 2011, 2014; Sjödén et al., 2017, 2019). The 17 studies involved a total of 2366 participants, encompassing 841 AD patients, 30 with ALS, 63 with FTD, 94 with HD, 27 with DLB and 124 with PD. The control groups amounted to 1187 participants, comprising 449 healthy controls, 316 non-neurodegenerative or neurological controls, 191 patients with a different type of dementia or cognitive impairment than the one of interest in the study and 231 patients with other neurodegenerative or neuroinflammatory diseases than the ones included in our study. Of the 17 studies included, two stated having matched patient and control groups for age and sex (Abu-Rumeileh et al., 2020; Constantinescu et al., 2010), and two additional studies had matched only for age (Heywood et al., 2015; Kandimalla et al., 2014).

3.5. CSF ubiquitin in patients and controls

CSF sampling information, analytical methods, patient characteristics and results from the reviewed studies are shown in Table 3.

3.5.1. Alzheimer's disease

Thirteen studies evaluated CSF ubiquitin levels in AD patients against controls. A total of five studies used ELISA, with three of the

studies detecting increased levels of ubiquitin in AD patients, reaching statistical significance (Blennow et al., 1994; Kandimalla et al., 2011, 2014). The two additional studies using ELISA also detected increased levels of ubiquitin in AD compared to controls, but either did not reach statistical significance (Chalbot et al., 2011) or no *p*-value was provided (Iqbal et al., 2005; Chalbot et al., 2011 assessed the discriminative power of CSF ubiquitin between AD patients divided into age groups. Levels of ubiquitin were higher in AD patients over 75 years of age than in patients aged 45–75 years, but the difference was not statistically significant (Chalbot et al., 2011). Six studies used different kinds of mass spectrometry techniques of which five reported statistically significant, increased levels of ubiquitin in the AD groups (Abu-Rumeileh et al., 2020; Heywood et al., 2015; Oeckl et al., 2014; Sjödén et al., 2017, 2019). In contrast, one study only detected ubiquitin in the control group and not in the samples from the AD patients (Davidsson et al., 2002). Two additional studies using RIA and CELIFA detected increased ubiquitin levels, but no *p*-value was calculated (Manaka et al., 1992) or the results for the AD group were determined to be statistically significant only when compared to the healthy controls (HC) and not the neurological controls (NC) (Kudo et al., 1994).

3.5.2. Parkinson's disease

CSF ubiquitin concentrations in patients diagnosed with PD were analyzed in five studies (Constantinescu et al., 2010; Heywood et al., 2015; Oeckl et al., 2014; Sjödén et al., 2017, 2019). The studies all applied mass spectrometry, with only two studies presenting findings reaching statistical significance (Constantinescu et al., 2010; Sjödén et al., 2019). Constantinescu et al. used SELDI-TOF-MS and reported similar levels in the PD group compared to HC, as well as a significantly decreased level of ubiquitin in the PD group compared to progressive supranuclear palsy (PSP) controls. In the study by Sjödén et al. from 2019, the use of a PRM-MS technique revealed significantly decreased

Table 3

Characteristics and findings of CSF ubiquitin studies.

Study	CSF handling	CSF analysis	Cohort size (sex [F/M] and age mean/median [years])	Study findings
Manaka et al., (1992)	Timing: n.a. Storage: −20 °C Tubes: n.a.	RIA	AD: n = 7 (sex n. a. age n.a.) CJD: n = 4 (sex n. a. age n.a.) MID: n = 10 (sex n.a. age n.a.) HC: n = 18 (sex n. a. age n.a.)	↑CSF Ub, AD vs HC, $p = \text{n.a.}$ ↑CSF Ub, AD vs MID, $p = \text{n.a.}$ ↑CSF Ub, AD vs CJD, $p = \text{n.a.}$
Blennow et al., (1994)	Timing: n.a. Storage: n.a. Tubes: Silicone	ELISA	AD: n = 29 (20/9, 71.7) VaD: n = 14 (5/9, 70.1) HC: n = 13 (2/9, 65.2)	↑CSF Ub, AD vs HC, $p < 0.0001$ ↑CSF Ub, AD vs VaD, $p < 0.0001$
Kudo et al., (1994)	Timing: Post mort. Storage: 70–80 °C Tubes: n.a.	CELIFA	AD: n = 17 (12/5, 78.0) NC: n = 17 (10/7, 81) HC: n = 12 (3/9, 73)	↑CSF Ub, AD vs HC, $p < 0.02$ ↑CSF Ub, AD vs NC, $p = \text{n.s.}$
Davidsson et al., (2002)	Timing: n.a. Storage: 70–80 °C Tubes: n.a.	MALDI-TOF-MS	AD: n = 15 (9/6, 77.2) HC: n = 12 (10/2, 67.3)	AD: CSF Ub not detected HC: CSF Ub not detected
Iqbal et al., (2005)	Timing: n.a. Storage: 70–80 °C Tubes: n.a.	cELISA	AD: n = 334 (221/113, 74.3) NAD: n = 115 (65/50, 61.4) AD/L: n = 19 (7/11, 75.2)	↑CSF Ub, AD vs HC, $p = \text{n.a.}$ ↑CSF Ub, AD vs AD/L, $p = \text{n.a.}$
Constantinescu et al., 2010	Timing: Morning Storage: 70–80 °C Tubes: PP	SELDI-TOF-MS	PD: n = 56 (15/41, 56) MSA: n = 42 (25/17, 65) PSP: n = 39 (18/21, 68) CBD: n = 9 (5/4, 71) HC: n = 24 (12/12, 70)	→CSF Ub, PD vs HC, $p = \text{n.s.}$ ↓CSF Ub, PD vs PSP, $p = 0.002$ ↓CSF Ub, PD vs MSA, $p = \text{n.s.}$ ↓CSF Ub, PD vs CBD, $p = \text{n.s.}$
Kandimalla et al., (2011)	Timing: n.a. Storage: 70–80 °C Tubes: PP	ELISA	AD: n = 51 (sex n. a., 62.9) NAD: n = 69 (sex n.a., 56.3) NC: n = 79 (sex n. a., 52.3) HC: n = 49 (sex n. a., 62.2)	↑CSF Ub, AD vs NAD, $p < 0.005$ ↑CSF Ub, AD vs NC, $p < 0.005$ ↑CSF Ub, AD vs HC, $p < 0.005$
Mendonça et al., (2011)	Timing: n.a. Storage: 70–80 °C Tubes: n.a.	Western blot	ALS: n = 10 (12/8, 30–67) HC: n = 6 (sex n. a., age n.a.)	ALS: CSF Ub not detected HC: CSF Ub not detected
Chalbot et al., (2011)	Timing: Morning Storage: 70–80 °C Tubes: PP	ELISA	AD _{45–75} : n = 54 (25/29, 69.3) AD _{>75} : n = 85 (24/61, 80.4) NC _{45–75} : n = 19 (6/13, 59.5) HC _{45–75} : n = 21 (9/12, 59.4)	↑CSF Ub, AD _{45–75} vs HC, $p = \text{n.s.}$ ↑CSF Ub, AD _{45–75} vs NC, $p = \text{n.s.}$ ↑CSF Ub, AD _{>75} vs HC, $p = \text{n.s.}$ ↑CSF Ub, AD _{>75} vs NC, $p = \text{n.s.}$ ↑CSF Ub, AD _{>75} vs AD _{45–75} , $p = \text{n.s.}$
Oeckl et al., (2014)	Timing: n.a. Storage: n.a.	LC-MS/MS	AD: n = 20 (11/9, 70.0) ALS: n = 20 (6/	↑CSF Ub, AD vs HC, $p < 0.05$ ↑CSF Ub, AD vs

Table 3 (continued)

Study	CSF handling	CSF analysis	Cohort size (sex [F/M] and age mean/median [years])	Study findings
	70–80 °C Tubes: n.a.		14, 57.5) PD: n = 25 (8/17, 70.0) bvFTD: n = 15 (7/8, 60.0) HC: n = 28 (18/10, 63.5) CJD: n = 19 (11/8, 67.0) PSP: n = 17 (6/11, 64.0) PPA: n = 6 (3/3, 72.0)	FTLD _{total} , $p < 0.01$ →CSF Ub, ALS vs HC, $p = \text{n.s.}$ →CSF Ub, PD vs HC, $p = \text{n.s.}$ →CSF Ub, FTL _{total} vs HC, $p = \text{n.s.}$ Corr. (all): Ub vs age: $r = 0.27$ $p = 0.0008$ Corr. (all): Ub vs p-tau: $r = 0.15$ $p = 0.13$ Corr. (bvFTD, AD, CJD, PPA, PSP, HC): Ub vs t-tau, $r = 0.89$, $p < 0.0001$ Ub vs p-tau, $r = 0.15$, $p = \text{n.s.}$ Ub vs Aβ42, $r = -0.37$, $p < 0.0001$ Corr. (AD, HC): Ub vs p-tau, $r = 0.53$, $p = 0.0001$ Corr. (bvFTD, AD, PPA, PSP, HC): Ub vs Q-alb, $r = 0.18$, $p = 0.10$ ↑CSF Ub, AD vs HC, $p < 0.0001$ ↑CSF Ub, AD vs NAD, $p < 0.0001$ ↑CSF Ub, AD vs NC, $p < 0.0001$ Corr. (all): Ub vs MMSE ranges, $p < 0.0001$ Corr. (all): Ub vs Aβ42, $r = -0.76$, $p < 0.0001$ Corr. (all): Ub vs t-tau, $r = 0.80$, $p < 0.0001$
Kandimalla et al., (2014)	Timing: n.a. Storage: 70–80 °C Tubes: n.a.	ELISA	AD: n = 44 (18/26, 61.9) NAD: n = 64 (15/49, 57.2) NC: n = 71 (19/52, 55.0) HC: n = 44 (14/30, 60.8)	↑CSF Ub, AD vs HC, $p < 0.0001$ ↑CSF Ub, AD vs NAD, $p < 0.0001$ ↑CSF Ub, AD vs NC, $p < 0.0001$ Corr. (all): Ub vs MMSE ranges, $p < 0.0001$ Corr. (all): Ub vs Aβ42, $r = -0.76$, $p < 0.0001$ Corr. (all): Ub vs t-tau, $r = 0.80$, $p < 0.0001$
Heywood et al., (2015)	Timing: n.a. Storage: −30 °C Tubes: PP	MRM-LC-MS/MS	AD: n = 16 (10/6, 78.1) PD: n = 7 (2/5, 68.1) DLB: n = 17 (7/10, 72.1) HC: n = 15 (10/5, 61)	↑CSF Ub, AD vs HC, $p < 0.0015$ ↑CSF Ub, DLB vs HC, $p < 0.011$ ↑CSF Ub, PD vs HC, $p = \text{n.s.}$ Corr. (AD): Ub vs Aβ42, $r = 0.25$, $p = \text{n.s.}$ Corr. (AD): Ub vs p-tau, $r = 0.47$, $p = 0.004$ Corr. (AD): Ub vs t-tau, $r = 0.15$, $p = \text{n.s.}$ Corr. (DLB): Ub vs Aβ42, $r = 0.002$, $p = \text{n.s.}$ Corr. (DLB): Ub vs p-tau, $r = 0.61$, $p = 0.0003$ Corr. (DLB): Ub vs t-tau, $r = 0.31$, $p = 0.03$ Corr. (HC): Ub vs

(continued on next page)

Table 3 (continued)

Study	CSF handling	CSF analysis	Cohort size (sex [F/M] and age mean/median [years])	Study findings
				$A\beta_{42}$, $r = 0.0004$, $p = \text{n.s.}$ Corr. (HC): Ub vs p-tau, $r = 0.54$, $p = 0.01$ Corr. (HC): Ub vs t-tau, $r = 0.84$, $p < 0.0001$ $\uparrow$ CSF Ub, HD_{manifest} vs HC, $p = 0.007$ (age-adjusted $p = 0.011$) $\uparrow$ CSF Ub, $HD_{\text{pre-manifest}}$ vs HC, $p = \text{n.s.}$ Corr. (HD _{manifest}): Ub vs UHDRS-TFC, $p = 0.001$ Corr. (all gene-expansion carriers): Ub vs CAP-scare, $p = 0.019$ 1) $\uparrow$ CSF Ub, AD vs HC, $p < 0.001$ 1) Corr. (HC): Ub vs t-tau, $p < 0.006$ 1) Corr. (HC): Ub vs p-tau, $p < 0.002$ 1) Corr. (HC): Ub vs $A\beta_{42}$, $p = \text{n.s.}$ 1) Corr. (AD): Ub vs t-tau/p-tau/ $A\beta_{42}$, $p = \text{n.s.}$ 2) $\uparrow$ CSF Ub, AD vs HC, $p < 0.001$ 2) Corr. (HC): Ub vs t-tau/p-tau, $p < 0.001$ 2) Corr. (HC): Ub vs $A\beta_{42}$, $p = \text{n.s.}$ 2) Corr. (AD): Ub vs t-tau/p-tau/ $A\beta_{42}$, $p = \text{n.s.}$ 3) $\uparrow$ CSF Ub, AD vs HC, $p < 0.003$ 3) Corr. (HC): Ub vs t-tau/p-tau/ $A\beta_{42}$, $p < 0.001$ 3) Corr. (AD): Ub vs t-tau/p-tau, $p < 0.001$ 3) Corr. (AD): Ub vs $A\beta_{42}$, $p = \text{n.s.}$ 4) $\downarrow$ CSF Ub, PD vs HC, $p < \text{n.s.}$ 4) $\downarrow$ CSF Ub, PD vs PSP, $p = \text{n.s.}$ 4) Corr. (HC): Ub vs t-tau, $p < 0.001$ 4) Corr. (HC): Ub vs p-tau, $p = 0.002$ 4) Corr. (HC): Ub vs $A\beta_{42}$, $p = \text{n.s.}$ 4) Corr. (PD): Ub vs t-tau/p-tau, p
Vinther-Jensen et al., (2015)	Timing: n.a. Storage: 70-80 °C Tubes: PP	SELDI-TOF-MS	HD_{manifest} : n = 55 (20/29, 50) $HD_{\text{pre-manifest}}$: n = 39 (13/19, 36) HC: n = 27 (11/13, 42)	
Sjödin et al., (2017)	Timing: n.a. Storage: 70-80 °C Tubes: n.a.	SPE-PRM-MS	Cohort 1) AD: n = 9 (4/5, 75) HC: n = 15 (6/9, 71) Cohort 2) AD: n = 14 (8/6, 75) HC: n = 15 (4/11, 74) Cohort 3) AD: n = 37 (24/13, 73) HC: n = 45 (50/15, 75) Cohort 4) PD: n = 15 (7/8, 69) PSP: n = 11 (6/5, 71) HC: n = 11 (5/6, 69)	

Table 3 (continued)

Study	CSF handling	CSF analysis	Cohort size (sex [F/M] and age mean/median [years])	Study findings
				< 0.001 4) Corr. (PD): Ub vs $A\beta_{42}$, $p = \text{n.s.}$ 1) $\uparrow$ CSF Ub, AD vs HC, $p \leq 0.05$ 2) $\downarrow$ CSF Ub, PD vs pro-AD, $p \leq 0.05$ 2) $\downarrow$ CSF Ub, PD vs pro-AD, $p \leq 0.05$ 2) $\rightarrow$ CSF Ub, AD vs MCI, $p = \text{n.s.}$ 2) $\rightarrow$ CSF Ub, PD vs MCI, $p = \text{n.s.}$ 2) Corr. (PD): Ub vs p-tau, $p < 0.0001$ 2) Corr. (PD): Ub vs t-tau, $p = \text{n.s.}$ 3) $\downarrow$ CSF Ub, PD vs HC, $p \leq 0.05$ 3) $\downarrow$ CSF Ub, PD vs AD, $p < 0.001$ 3) $\rightarrow$ CSF Ub, AD vs HC, $p = \text{n.s.}$ 3) Corr. (AD): Ub vs p-tau, $p < 0.0001$ 3) Corr. (AD): Ub vs t-tau, $p = \text{n.s.}$ 3) Corr. (HC): Ub vs t-tau/p-tau, $p < 0.0001$ $\uparrow$ CSF Ub, AD vs HC, $p = 0.003$ $\rightarrow$ CSF Ub, FTD vs HC, $p = \text{n.s.}$ $\uparrow$ CSF Ub, AD vs FTD, $p = 0.002$ $\downarrow$ CSF Ub, AD vs prion, $p < 0.001$ $\downarrow$ CSF Ub, FTD vs prion, $p < 0.001$ Corr. (AD, FTD): Ub vs t-tau, $p < 0.001$ Corr. (AD): Ub vs NfL, $r = 0.43$ $p = 0.0007$ Corr. (FTD): Ub vs NfL, $p = \text{n.s.}$ $\downarrow$ CSF Ub, FTD _{symptomatic} vs HC, $p = 0.002$ $\downarrow$ CSF Ub, FTD _{presymptomatic} vs HC, $p = \text{n.s.}$ $\rightarrow$ CSF Ub, FTD _{symptomatic} vs FTD _{pre-symptomatic} , $p = \text{n.s.}$ Corr. (FTD): Ub vs NfL, $p = \text{n.s.}$
Sjödin et al., (2019)	Timing: n.a. Storage: 70-80 °C Tubes: PP	PRM-MS	Cohort 1) AD: n = 19 (9/10, 76) HC: n = 24 (8/16, 72) Cohort 2) AD: n = 6 (2/4, 70) pro-AD: n = 10 (7/3, 66) PD: n = 10 (5/5, 60) MCI: n = 15 (6/9, 67) Cohort 3) AD: n = 36 (23/13, 73) PD: n = 11 (6/5, 69) HC: n = 44 (30/14, 75)	
Abu-Rumeileh et al., (2020)	Timing: n.a. Storage: 70-80 °C Tubes: PP	LC-MS/MS	AD: n = 38 (14/24, 68.9) FTD: n = 30 (17/13, 66.5) Prion: n = 84 (39/45, 67.6) HC: n = 28 (13/15, 64.7)	
Toft et al., (2023)	Timing: n.a. Storage: 70-80 °C Tubes: n.a.	PRM-MS	FTD _{symptomatic} : n = 11 (3/8, 62.9) FTD _{presymptomatic} : n = 7 (4/3, 52.0) HC: n = 13 (5/8, 60.5)	

AD/L: Alzheimer's disease with Lewy Type Dementia; bvFTD: behavioral variant Frontotemporal Dementia; CBD: Corticobasal Degeneration; CELISA: competitive enzyme-linked immunoflow assay; cELISA: competitive Enzyme-linked Immunosorbent assay; Corr: correlation; ELISA: enzyme-linked Immunosorbent assay; F: females; HC: healthy Controls; LC-MS/MS: Liquid chromatography – mass spectrometry; M: males; MALDI-TOF-MS: matrix-assisted laser desorption-ionization mass spectrometry; MCI: mild Cognitive Impairment;

MID: multiple Infarction Dementia; MRM-LC-MS/MS: multiple reaction monitoring-liquid chromatography mass spectrometry; MSA: multiple System Atrophy; n.a.: not available; n.s.: non-significant; NAD: non-Alzheimer's disease dementia; NC: neurological Controls; NfL: CSF neurofilament light; PP: polypropylene; Prion: prion disease; PRM-MS: parallel reaction monitoring-mass spectrometry; PPA: primary progressive aphasia; pro-AD: prodromal Alzheimer's dementia; RIA: radioimmunoassay; SELDI-TOF-MS: surface enhanced laser desorption/ionization mass spectrometry; SPE-PRM-MS: solid-phase extraction-parallel reaction monitoring mass spectrometry; Ub: CSF ubiquitin; VaD: vascular Dementia.

levels of ubiquitin in the PD group when compared to HC.

3.5.3. *Lewy body dementia*

One study examined levels of CSF ubiquitin in DLB patients compared with healthy controls, finding significantly increased levels, employing a LC-MS/MS technique (Heywood et al., 2015).

3.5.4. *Frontotemporal dementia*

Three studies evaluated the concentration of CSF ubiquitin in FTD. Two studies using LC-MS/MS found equal levels in patients and HC (Abu-Rumeileh et al., 2020; Oeckl et al., 2014), whilst one study performing PRM-MS observed significantly lower CSF ubiquitin levels in FTD patients (Toft et al., 2023). Regarding FTD subtypes, Abu-Rumeileh et al. included 13 patients with familial FTD (9 with C9orf72 mutation and 4 with GRN mutation) and 17 patients with corticobasal degeneration (CBD) or PSP. The study by Oeckl et al. had 15 behavioral variant FTD (bvFTD) patients, 17 PSP patients and 6 with primary progressive aphasia (PPA). Toft et al. included 18 participants with bvFTD due to CHMP2B mutation.

3.5.5. *Huntington's disease*

CSF ubiquitin in patients with Huntington's disease was quantified in one study using a SELDI-TOF-MS technique. The study demonstrated increased levels of ubiquitin in both the manifest and pre-manifest HD groups when compared to gene-expansion negative family controls, but with only the HD-manifest patients reaching statistical significance (Vinther-Jensen et al., 2015).

3.5.6. *Amyotrophic lateral sclerosis*

Two studies measured CSF ubiquitin in ALS patients. Using western blot, ubiquitin was not detected in CSF from either the ALS or healthy control group (Mendonça et al., 2011). Oeckl et al. did not detect any statistically significant differences in ubiquitin levels between ALS patients and controls using LC-MS/MS (Oeckl et al., 2014).

3.6. *Comparative diagnostic value of CSF ubiquitin in different neurodegenerative diseases*

To discriminate between the different diseases, three studies included analyses assessing the comparative diagnostic value of CSF ubiquitin. Discrimination between AD and FTD was investigated in two studies, both discovering a significantly higher level of ubiquitin in AD patients: Oeckl et al. performed ROC analysis for the classification, reporting an AUC of 0.776, a sensitivity of 53 % and a specificity of 100 %, using a cut-off level of 32.1 ng/mL ubiquitin (Oeckl et al., 2014). Abu-Rumeileh et al. reported an AUC of 0.880 with 87 % sensitivity and 80 % specificity at a cut-off value of 45.7 ng/mL (Abu-Rumeileh et al., 2020). Sjödin et al. showed a significant decrease of CSF ubiquitin in PD patients compared with AD and prodromal AD patients (Sjödin et al., 2019), whilst a previous study did not observe any difference between AD and PD (Oeckl et al., 2014). CSF ubiquitin levels were markedly higher in Creutzfeldt-Jakob disease (CJD) than AD in two out of two studies (Abu-Rumeileh et al., 2020; Manaka et al., 1992), with one calculating an AUC of 0.848 with 77 % sensitivity and 90 % specificity, at a 68.3 ng/mL cut-off value (Abu-Rumeileh et al., 2020). No

considerable differences in CSF ubiquitin were found between AD patients and patients with either ALS (Oeckl et al., 2014), DLB (Iqbal et al., 2005), vascular dementia (Blennow et al., 1994; Manaka et al., 1992), or MCI (Sjödin et al., 2019). Constantinescu et al. observed elevated CSF ubiquitin in PSP patients compared to PD, but this could not be reproduced in a subsequent study (Sjödin et al., 2017).

3.7. *Correlations of ubiquitin with established neurodegenerative biomarkers*

Correlations with t-tau, p-tau, Aβ42 or NfL were calculated in seven of the studies (Table 3). When including both AD, FTD, CJD, PPA and PSP patients, as well as healthy controls, Oeckl et al. demonstrated a strong positive correlation for CSF ubiquitin with t-tau, and a weak negative correlation with Aβ42. No association with p-tau was found for the entire cohort, but a moderate correlation was significant when including only AD patients and controls in this calculation (Oeckl et al., 2014). Across the additional five studies, significant correlation between CSF ubiquitin and t-tau was found in AD patients (Abu-Rumeileh et al., 2020; Kandimalla et al., 2014; Sjödin et al., 2017), FTD, PD and controls (Heywood et al., 2015; Kandimalla et al., 2011; Sjödin et al., 2017, 2019). Correlation with p-tau was also shown in AD (Heywood et al., 2015; Sjödin et al., 2017, 2019), DLB (Heywood et al., 2015), PD (Sjödin et al., 2017) and controls (Heywood et al., 2015; Sjödin et al., 2017, 2019). Three studies besides Oeckl. et al. included measurements of Aβ42 (Heywood et al., 2015; Kandimalla et al., 2014; Sjödin et al., 2017). Two did not find ubiquitin significantly correlated with Aβ42 in AD, DLB, PD, or controls, while one study demonstrated significant correlation in AD patients and controls combined (Kandimalla et al., 2014). One study tested for and found a positive correlation between ubiquitin and NfL in AD (Abu-Rumeileh et al., 2020), and two studies found no significant correlation between the two in FTD patients (Abu-Rumeileh et al., 2020; Toft et al., 2023).

3.8. *CSF ubiquitin as a disease severity marker*

Two studies observed correlations between the levels of CSF ubiquitin and different disease severity markers. When patients in the AD group were categorized by intervals based on their MMSE score, one study showed a significant difference in the mean levels of ubiquitin, with higher levels of ubiquitin correlating with worsening of MMSE-score (Kandimalla et al., 2014). Another study, including patients who were carriers of the HD gene premanifest and manifest of disease, found correlations with CAG-Age Product (CAP) score as well as Unified HD Rating Scale - Total Functional Capacity (UHRS-TFC) scores reaching statistical significance. Levels of ubiquitin in the manifest gene expansion carriers were found to increase with increasing CAP scores and decreasing UHRS-TFC scores (Vinther-Jensen et al., 2015).

4. Discussion

Ubiquitin plays a key role in maintaining neuronal functioning and survival, as well as being a major but often overlooked component of pathological protein aggregates associated with neurodegenerative diseases (Watanabe et al., 2020). The purpose of this study was to review the available literature assessing CSF ubiquitin as a potential biomarker for diseases sharing common neuropathology involving, among other features, abnormal protein aggregation.

Patients with AD were the most abundantly represented patient group in the different studies included. When compared to control cohorts a significant increase in CSF levels was found in nine out of the 13 studies involving AD patients. Higher CSF ubiquitin levels could distinguish AD from FTD in two studies (Abu-Rumeileh et al., 2020; Oeckl et al., 2014), and from PD in one study (Sjödin et al., 2019). Despite limitations of the included studies, the results for the AD patient groups suggest a justifiable association between increased CSF ubiquitin

and AD pathology. For the additional disease entities included in this review – ALS, FTD, HD, DLB and PD – similar conclusions cannot be drawn as available research is too sparse. Regarding ALS, the studies did not show any differences in CSF ubiquitin levels (Mendonça et al., 2011; Oeckl et al., 2014). For both HD and DLB, one study was available and showed a significantly increased ubiquitin concentration in comparison to controls (Heywood et al., 2015; Vinther-Jensen et al., 2015). In FTD and PD, respectively, one of three and one of five studies found significantly altered CSF levels in patients compared with healthy controls, with patients having lower ubiquitin levels than controls (Sjödin et al., 2019; Toft et al., 2023).

A definition of diagnostic criteria applied to each disease group was included in all studies. However, the studies were often limited by the lack of definite inclusion and exclusion criteria for both patients and controls, as well as limited documentation of selection and definition of control groups. Common to all studies was relatively small sample sizes, with nine of the studies having below 20 participants in the disease groups. Only four studies had matched patient and control cohorts for age (Abu-Rumeileh et al., 2020; Constantinescu et al., 2010; Heywood et al., 2015; Kandimalla et al., 2014). The activities of the two major proteolytic systems in the cell, the ALP and UPS, decline with age, suggesting a collapse of cell proteostasis being one of the principal hallmarks of aging as well as of neurodegenerative diseases, with a possible effect on baseline levels of CSF ubiquitin (López-Otín et al., 2013; Rubinsztein et al., 2011; Schmidt et al., 2021; Tomaru et al., 2012). Of the reviewed studies, nine included a correlation between CSF ubiquitin concentrations and age: Three demonstrated a positive correlation (Iqbal et al., 2005; Oeckl et al., 2014; Vinther-Jensen et al., 2015) and the remainder presented insignificant results. Based on available literature, it is indeterminable whether CSF ubiquitin levels remain unchanged or tend to slightly increase with age. Future studies should include measurements of CSF ubiquitin over time, as the temporal profile of CSF ubiquitin is likely to be associated with disease stage, but cannot be established with the cross-sectional data presented in the studies included here. Beyond establishing whether age-associated changes exist, longitudinal assessments are required to ascertain the extent of fluctuation; evaluate the potential as an early indicator of developing pathology; and explore the likelihood of an initial “sequestration-phase” followed by a subsequent “release-phase” with advancing neurodegeneration.

The different studies reviewed here showed great heterogeneity in study protocol and execution including analytical and pre-analytical variables. Methods of analysis were mainly divided between immunoassays and mass spectrometry techniques. Immunoassays traditionally show high sensitivity through immuno-selectivity offered by the specific antibody-antigen complexes, but limitations include selectivity only being as good as that offered by the antibody in use. Anti-ubiquitin antibodies exhibit differences in affinity for conjugated versus non-conjugated ubiquitin. Mass spectrometry techniques show high sensitivity as well as specificity but produce complex results requiring trained staff for accurate interpretations (Cross and Hornshaw, 2016). When conducting research on biomarkers, one of the many challenges is lack of reproducibility across studies and laboratories. Preanalytical errors still account for 60–70 % of all problems in relation to laboratory diagnostics (Lippi et al., 2011). Chalbot et al. and Constantinescu et al. were the only two studies that reported the timing of CSF sampling, and generally information on CSF sample collection, handling, transport, and storage was lacking or varied between the included studies (Chalbot et al., 2011; Constantinescu et al., 2010). Confounders in relation to CSF sampling of ubiquitin include the potential influence of diurnal variation and volume of sample fluid aspirated from each subject (Janssens et al., 2019). Several studies have suggested the presence of a physiological circadian rhythm of CSF protein levels, implying this could also apply to other biomarkers such as ubiquitin (Bateman et al., 2007; Bjerke et al., 2010). Further studies should aim for a standardized approach to study design and execution, including pre-analytical as well as analytical variables,

producing results suitable for comparison.

In this review, the included neurodegenerative diseases exhibited different patterns of change in CSF ubiquitin: Levels were found to be elevated in AD patients as opposed to reduced amounts in PD and FTD populations. Furthermore, although only one study involved patients with DLB, the increased ubiquitin concentrations in this population stand contrast with the results for the PD group, as the diseases share the same pathological hallmark of misfolded alpha-synuclein inclusions. Regarding the increased levels in AD, a possible explanation may lie in the pathological protein aggregates comprising extracellular plaques of β -amyloid as well as hyperphosphorylated tau inclusions, termed neurofibrillary tangles. When these tangles mature, the neuron eventually dies and leaves behind “ghost” tangles in the interstitial space (DeTure and Dickson, 2019; Dugger and Dickson, 2017). Hypothetically, these types of ubiquitinated aggregates outside of the cell could constitute a source of CSF-ubiquitin, following ubiquitin-release via e.g., extracellular DUBs, interactions with glia or other mechanisms. The synucleinopathies are characterized by the presence of Lewy bodies and Lewy neurites within neurons and have not been associated with the same burden of extracellular pathology. This, however, mainly pertains to PD, given that the majority of DLB cases present with some degree of Alzheimer-type neuropathology (Koga et al., 2021; Walker et al., 2019), which might contribute to the elevated levels observed in DLB patients. While inclusions are primarily localized in the brainstem and limbic regions in PD, Lewy body pathology extends to involve the neocortex in DLB (Koga et al., 2021; Walker et al., 2019). Greater propagation of pathology could potentially result in an increased exposure of ubiquitinated proteins to the extracellular space, and ultimately the CSF, in DLB compared with PD. Furthermore, recent studies have reported heightened levels of NfL in DLB in comparison to PD (Ashton et al., 2021; Gaetani et al., 2019). The more extensive axonal degeneration in DLB may lead to an increased release of ubiquitin. In addition to DLB, CSF ubiquitin was found to be reduced in a cohort of patients diagnosed with FTD caused by *CHMP2B* mutation. A hallmark protein aggregation has not yet been identified in this genetic subtype of FTD, only accumulations of ubiquitin- and p62-positive intraneuronal inclusions (FTLD-UPS) (Holm et al., 2009). The mutation leads to buildup of deformed multivesicular bodies containing ubiquitinated proteins (Ugbode and West RJH, 2021), possibly causing additional intracellular entrapment of ubiquitin. Another potential cause of reduced CSF ubiquitin could involve impaired release of extracellular vesicles harboring ubiquitinated proteins, a process theoretically impacted by *CHMP2B* mutation.

As previously mentioned, the sequestration of ubiquitin and UPS constituents by polyubiquitinated protein aggregates, followed by depletion of cellular ubiquitin reservoirs, appears to be common features of neurodegenerative diseases, potentially impairing both UPS and ALP activity. Whether there is a compensatory upregulation of free ubiquitin varies between studies and may depend on the specific disease model investigated (Farrawell et al., 2020). A related missing link is the extent to which intracellular ubiquitin levels reflect the quantities that ultimately translocate to the interstitial space. These processes require further elucidation in future studies.

Under normal circumstances the concentration of ubiquitin in blood is three-to fivefold higher than CSF levels (Majetschak et al., 2011). For ubiquitin to be used as a CSF biomarker, it is relevant to examine whether elevated levels simply reflect emanation from serum. To this end, four studies included here reported a well-established measure of blood-brain barrier integrity, namely the CSF/serum albumin ratio (Blennow et al., 1994; Chalbot et al., 2011; Oeckl et al., 2014; Toft et al., 2023). None of the studies found the ubiquitin levels in patients to correlate with the albumin quotient, indicating that CSF ubiquitin is intrathecally derived, and probably diffuses from the brain and ISF to the CSF through the ependymal cells lining the ventricles.

Increased expression levels of CSF ubiquitin corresponded with disease progression and severity scales in two studies (Kandimalla et al.,

2014; Vinther-Jensen et al., 2015), indicating that CSF ubiquitin might reflect the extent of ongoing neurodegeneration. In patients with prion disease, a class of infectious and neurodegenerative diseases caused by the propagation of pathological scrapie isoform of the prion protein (PRPsc), the disease course is typically characterized by rapidly progressing neurodegeneration and dementia. Several studies have observed substantially increased levels of CSF ubiquitin in CJD patients, discriminating the subjects from both healthy controls as well as patients with AD and other comparable diseases (Abu-Rumeileh et al., 2020; Oeckl et al., 2014; Steinacker et al., 2010). The association with neurodegeneration is supported by correlations of CSF ubiquitin with neurofilament light (NfL) and t-tau (Abu-Rumeileh et al., 2020; Oeckl et al., 2014; Sjödin et al., 2017, 2019), as well as increased CSF ubiquitin following traumatic brain injury or brain ischemia (Kurimura et al., 1997; Majetschak et al., 2005).

Two studies evaluated the performance of CSF ubiquitin in discriminating between AD and FTD patients (Abu-Rumeileh et al., 2020; Oeckl et al., 2014). The studies reported sensitivities of 53–87 % and specificities of 80–100 %. While the values are not unlike what was recently presented for A β 42 in a systematic review (Kokkinou et al., 2021), the authors reported a lower diagnostic performance of CSF ubiquitin compared with the neurodegenerative markers NfL and t-tau (Abu-Rumeileh et al., 2020). Some studies argue that CSF ubiquitin can be considered a specific biomarker for AD, due to correlations with A β 42, tau, and the APOE ϵ 4 allele (Kandimalla et al., 2014). However, correlation with AD markers does not point to specificity, but only for association with AD pathology – just as ubiquitin has been shown associated with the protein aggregates in the other diseases included in this review. Nevertheless, other findings do suggest some degree of connection with disease pathomechanisms or affected brain region: Studies that include a variety of neurodegenerative diseases do not observe generally elevated levels of CSF ubiquitin, but only in certain diseases, i.e. AD and CJD (Abu-Rumeileh et al., 2020; Oeckl et al., 2014); in CJD, the CSF concentration is reported to differ significantly between molecular subtypes of the disease (Abu-Rumeileh et al., 2020); and in other diseases with increased NfL or t-tau, such as ALS, FTD and PD, CSF ubiquitin levels have been found unaltered or even significantly decreased, as described.

Collectively, whether CSF ubiquitin levels exclusively reflect neurodegeneration or if association with certain types of neurodegenerative pathomechanisms exists, remains to be fully elucidated. This is relevant in terms of implementation in the clinical setting, where other biomarkers of neurodegeneration such as NfL are already being used, both from serum and CSF samples, to assess the presence or absence of neurodegeneration (Willemse et al., 2021). NfL is a sensitive and unspecific marker of neuroaxonal damage and is exclusively present in neurons. Ubiquitin, however, participates in various cellular processes in all eukaryotic cells, and elevated levels may not necessarily relate to neurodegenerative diseases. The concentrations of NfL in plasma closely align with those in CSF (Blennow et al., 1994; Khalil et al., 2018). In contrast, a recent study assessed the plasma levels of ubiquitin for correlation with mild cognitive impairment (MCI) and mild dementia in a cohort of 230 elderly but did not observe any significant differences related to cognitive functioning as measured by MMSE (McFarlane et al., 2023). Whereas Single Molecular Array is considered the gold standard for measuring NfL, accurately quantifying ubiquitin is challenging due to the high homology between ubiquitin species, such as free ubiquitin, conjugated monomeric and conjugated polymeric ubiquitin (Kandimalla et al., 2014; Oeckl et al., 2014; Steinacker et al., 2010; Wang et al., 1991). This is reflected in the diversity of analytical methods used in the included studies, as well as the observation that the reference range for CSF ubiquitin in healthy controls varies by 10-fold between studies (Blennow et al., 1994; Kandimalla et al., 2011).

Alternative strategies to exploit the biomarker potential of ubiquitin and UPS function might be required: While using serum ubiquitin as a measure of neurodegenerative disease burden might be challenging

(McFarlane et al., 2023) – perhaps due to other sources than CNS-derived ubiquitin – markers such as the CSF/serum ubiquitin ratio and the DUB UCHL1 have shown promising results, and may outperform CSF ubiquitin as biomarkers (Blennow et al., 1994; Oeckl et al., 2020; Öhrfelt et al., 2016). Given the ongoing preclinical research directed at therapeutically modulating UPS activity, the ubiquitination state of specific substrates or broader ubiquitination profiles – rather than soluble amounts ubiquitin or UPS components – could potentially serve as future predictive biomarkers, or contribute prognostic information about the disease stage based on the levels of (dys)ubiquitination.

The present review exhibits a number of limitations. Most important is the constraint imposed by the number of exploitable studies that met the pre-defined criteria, resulting in an increased emphasis on CSF ubiquitin in AD. This skewness could be explained by prevalence of AD and the clinical practice of performing lumbar punctures in the diagnostic evaluation of AD in specialized centers. In turn, this practice is encouraged by the availability of CSF biomarkers of amyloid and tau pathology, whereas lumbar puncture for the diagnosis other neurodegenerative diseases is used more sparingly. Hence, CSF available for research investigations would primarily be derived from patients with AD. Furthermore, it is essential to acknowledge the potential influence of publication bias on the results of this review. Studies reporting negative findings are less likely to be published and would therefore not appear in our searches. The heterogeneous analytical and sampling methodologies described in the studies, alongside potential confounding factors such as disease stage, medication usage or co-morbidity, may impede the generalizability of the results as well as the attribution of CSF ubiquitin changes to the disease alone. The studies included in this review are all cross-sectional in design and thus, the correlations between CSF ubiquitin and other clinical or biochemical measures such as CSF A β 42, p-tau or t-tau may yield valuable insights into the disease mechanisms, but cannot establish causal relationships. Lastly, in order for correlations between biomarkers to be comparable between studies, it is necessary to make calculations on individual disease groups, and not combine several groups or include all participants, which was often the case in the reviewed studies.

To facilitate the clinical utilization of CSF ubiquitin, or any other CSF biomarker, several practical aspects must be considered: CSF collection is an invasive procedure typically performed in specialized clinics, and the sampling and storage of CSF must be carefully standardized (Campo et al., 2012; Simonsen et al., 2013). Additionally, it is essential to harmonize the interpretation of results obtained from different analytical modalities to ensure their clinical applicability. Neurodegenerative diseases characterized by “late onset” are particularly complex and multifactorial. Patients with such conditions often present with a multitude of comorbidities and concurrently prescribed medications. These patient-specific variables warrant in-depth investigation to ascertain whether alterations in CSF ubiquitin levels genuinely reflect the underlying neurodegenerative disease.

Research into CSF-ubiquitin aligns with the broader objective of comprehending the biological underpinnings of neurodegenerative diseases, particularly with trends focusing on shared driving mechanisms, rather than disease-specific protein aggregation – the importance of which is emphasized by the high prevalence of patients with multiple concurrent proteinopathies (Karanth et al., 2020). Despite the extensive research on ubiquitin over decades, the available evidence supporting the utility of CSF ubiquitin as a biomarker in neurodegenerative diseases is sparse. Given its direct involvement in protein degradation and its dysregulation, the marker may offer mechanistic insights, perhaps in combination with other biomarkers. This requires that the differing levels in PD, FTD and ALS can be verified. If CSF ubiquitin levels solely represent a surrogate measure of neurodegeneration, it is difficult to imagine a diagnostic value of the biomarker and an unmet need for it in the clinical practice. Other already well-established biomarkers are more easily measured and show greater fold changes between patients and controls.

5. Conclusion

Ubiquitination is common in neurotoxic protein aggregates, and CSF ubiquitin levels has been evaluated as a biomarker in several neurodegenerative diseases. Studies vary in methodology due to the lack of standard measurement. In AD studies, elevated CSF ubiquitin is near-consistent. So is correlation with tau, while A β 42 results lacked reproducibility. In the few studies available, HD and DLB patients showed increased CSF ubiquitin, while levels in ALS, FTD and PD have been similar to or below those of the controls. If the latter is validated, CSF ubiquitin may contribute more disease-specific information than all-cause neurodegeneration, which would increase the potential for reaching the clinical setting. This is otherwise unlikely, as more readily applicable neurodegenerative biomarkers have been implemented.

Data availability

The data supporting this systematic review are from previously published studies, which have been cited in the manuscript.

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Declaration of interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Anja H. Simonsen reports financial support was provided by Absalonfonden. Steen G. Hasselbalch reports financial support was provided by the Danish Ministry of Health and the Danish Health Foundation.

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Protocol S1. Differentiation of iPSCs to induced microglia

Modified from Haenseler et al. ¹¹⁵ and van Wilgenburg et al. ¹¹⁶

Day 1

- Prepare 24-well AggreWell Microwell Plate (StemCell Technologies) with Anti-Adherence Rinsing Solution (StemCell Technologies)
- Prepare Embroid Body Differentiation Medium (EBdiff medium)
 - 50 ng/mL BMP4 (Miltenyi Biotec), 50 ng/mL VEGF (Peprotech), 20 ng/mL SCF (Peprotech), and 1 % RevitaCell in mTeSR Plus Medium (StemCell Technologies)
- Harvest two confluent 6-wells of iPSCs with Accutase (StemCell Technologies) (incubate for 7 min at 37 °C)
- Wash off all cells with 1 mL mTeSR1 Plus (StemCell Technologies) and transfer to a 2 mL tube
- Centrifuge for 5 min at 1200 x g
- Remove supernatant and resuspend in 1 mL mTeSR Plus (combine the two 2 mL tubes in 1 tube) and count cells
- Transfer 4 million cells to a new 2 mL tube and spin down (5 min at 1200 x g)
- Aspirate rinsing solution from AggreWell and wash two times with PBS^{-/-}
- Remove supernatant and resuspend the 4 million cells in 2 mL EBdiff and transfer to AggreWell
- Centrifuge Aggrewell 1 min at 100 x g and incubate at 37 °C

Day 2-3

- Carefully remove 1 mL of EBdiff medium and add 1 mL of EBdiff slowly down the side of the well
- Repeat to get 75 % medium change

Day 4

- Prepare 4 mL preheated EBdiff per Aggrewell
- Rinse one 6-well plate with 1 mL Anti-Adherence Rinsing Solution (leave in at least 5 min) and wash two times with PBS^{-/-}

- Place an inverted 40 μ m cell strainer on a 50 mL falcon
- Wash the cell strainer once with PBS^{-/-}
- Dislodge emboid bodies (EBs) using a P1000 pipette and transfer contents to the inverted cell strainer
- Wash EBs off the cell strainer into a 6-well using the new 4 mL EBdiff
- Incubate at 37 °C for 2-3 days and change medium daily

Day 7

- Coat 10 cm dish or T75 flask with growth factor reduced Matrigel (Corning)
- Prepare 15 mL Microglia Factory Medium per factory
 - 1X Glutamax (ThermoFisher Scientific), 50 μ M β -mercaptoethanol (ThermoFisher Scientific), 100 ng/ml M-CSF (Genscript), 25 ng/ml IL-3 (Genscript) in X-VIVO15 medium (Lonza) with 0.5 % penicillin/streptomycin (ThermoFisher Scientific)
- Remove Matrigel (ThermoFisher Scientific) and add 10 mL Microglia Factory Medium to the coated 10 cm dish
- Transfer EBs from 6-well to 15 mL falcon using a 5 mL serological pipette
- Carefully remove as much EB medium as possible from the 15 mL falcon
- Use a 5 mL serological pipette to wash EBs gently with the remaining 5 mL Microglia Factory Medium and transfer all EBs to the dish
- Incubate at 37 °C and leave untouched for a week

Day 14

- Add 10 mL Microglia Factory Medium to 10-dish/T75
- Continue to feed twice a week with 5 mL medium

3-6 weeks after factory setup

- Prepare wanted format with 50 μ g/mL fibronectin coating
- Prepare required volumes of Microglia Factory Medium and Microglia Differentiation Medium (MGdiff)

- 1X Glutamax, 1X N-2 Supplement (ThermoFisher Scientific), 50 μ M β -mercaptoethanol, 10 ng/mL GM-CSF (PeproTech), 100 ng/ml IL-34 (PeproTech), 5 ng/ml TGF- β (PeproTech) in Advanced DMEM/F-12 medium (ThermoFisher Scientific) with 0.5 % penicillin/streptomycin
- Gently harvest supernatant of factories (max 90 %) containing precursors and pass through a cell strainer into a 50 mL falcon
- Centrifuge cells at 400 x g for 5 min
- Discard supernatant (pour out in one motion), resuspend in 1 mL MGdiff medium, and count cells
- Plate 100.000 cells per cm^2 in the wanted format and incubate at 37 °C
- Continue to harvest factories weekly or biweekly

Full medium change (MGdiff medium) every 3-4 days

- 14 days after plating, microglia-like cells can be used for experiments

Protocol S2. Differentiation of iPSCs to induced neurons

Modified from Chen et al.¹¹⁸

Day 1

- Coat the wanted number of 6-wells with 5 % laminin 521N in PBS^{+/+} (depending on the experiment)
- Harvest a confluent 6-well of *NGN2*-transfected iPSCs with EDTA (incubate for 7 min at 37 °C)
- Resuspend cells in 1 mL mTeSR Plus, add 250-350 mL to laminin-coated 6-wells, and incubate at 37 °C

Day 3-4

- 6-wells of > 90 % confluent iPSCs are added 5 µg/ml doxycycline for 4 hours to induce *NGN2* expression
- 60 mm plates are coated with laminin
- Prepare Neuronal differentiation medium-1 (Ndiff-1)
 - 1 % Glutamax, 1 % B27 Supplement (ThermoFisher Scientific), 1 % N-2 Supplement, 1 % Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS-A) (ThermoFisher Scientific), 10 µM SB431542 (StemCell Technologies), 100 nM LDN-193189 (Miltenyi Biotech), 2 µg/mL doxycyclin, and 1X ROCK (Y-27632) inhibitor in 1:1 Neurobasal medium and Advanced DMEM-F12 medium with 1 % penicillin/streptomycin
- Harvest cells with Accutase (incubate for 7 min at 37 °C) and centrifuge at 500 x g for 2 minutes
- Resuspend cells in 500 µL Ndiff-1 and count cells
- Plate 1.65 million cells in one laminin-coated 60 mm dish in Ndiff-1 medium

Day 5

- Full medium change (Ndiff-1 without ROCK inhibitor)

Day 7-8

- Coat wanted plate format with poly-L-ornithine and incubate for 1.5 hours at 37 °C

- Wash plates 3 times with PBS^{-/-} and air-dry for 15 minutes
- Coat with laminin and incubate at 37 °C for 2 hours
- Wash cells once with PBS^{-/-} and detach with (room temperate) Accutase for 2 minutes
- Prepare Neuronal differentiation medium-2 (Ndiff-2)
 - 1 % B27 Supplement, 1 % N-2 Supplement, 200 μ M L-ascorbic acid (Sigma), 10 ng/mL BDNF (StemCell Technologies), and 10 ng/mL GDNF (StemCell Technologies), 200 μ M dibutyryl-cAMP (StemCell Technologies) in BrainPhys medium (StemCell Technologies) with 0.5 % penicillin/streptomycin
- Remove Accutase and resuspend cells in 1 mL Ndiff-2
- Centrifuge at 500 x g for 2 minutes
- Remove supernatant and resuspend cells in 600 μ L Ndiff-2 with 2 μ g/mL doxycycline and count cells
- Plate 500.000 cells in a 12-well plate or 75.000 cells in a Lab-Tek II 8-well Chamber Slide System

Day 9-10 and 12

- 50 % medium change (Ndiff-2 medium with 2 μ g/mL doxycycline)
- Optional: Top-up with laminin (1 μ g per 12-well) on day 10

Day 12-25

- 50 % medium changes every 3-4 days (Ndiff-2 medium without doxycycline)

Day 25-60

- 50 % medium changes once a week (Ndiff-2 medium without GDNF, cAMP, or doxycycline)

Table S1. Enrichment analyses

Comparison of enriched pathways and biological processes in symptomatic and presymptomatic carriers of the *CHMP2B*^{homo} variant versus healthy controls. The top 10 up- and downregulated pathways identified via three enrichment analyses are listed, along with proteins from the untargeted CSF proteomics dataset implicated in each pathway. The presented data met a 0.05 significance threshold with *p*-values adjusted for multiple testing (Benjamini-Hochberg correction).

Symptomatic carriers vs controls - All significantly altered proteins (n = 669)		
Pathways	Proteins	Adjusted <i>p</i> -values
KEGG 2021 Human		
Complement and coagulation cascades	CFD;CPB2;ITGAM;SERPINA1;CFH;SERPINC1;PROS1;CFI;F13A1;C4BPA;PLG;C4BPB;C8B;C8A;KNG1;SERPINA5;C4B;C3;VTN;C5;C8G;C6;C7;C9;FGB;FGA;SERPIND1;F12;FGG;SERPINF2;F11;PLAUR;F2;F9;CFHR2;CFHR4;SERPING1;CFHR5;MASP2;F13B;CD46;KLKB1;CFB	2.149e-38
Cell adhesion molecules	CNTNAP2;ITGAM;NLGN2;ICAM2;NRXN3;CD99L2;GLG1;PTPRF;ICAM1;CDH5;ALCAM;CDH1;NRCAM;NCAM1;NEO1;MPZL1;NTNG1;NTNG2;CADM3;CADM1;NEGR1;L1CAM;NFASC;SELL;HLA-DPB1;ESAM;NECTIN1	7.134e-11
Axon guidance	EPHB6;EPHA5;NTNG1;EPHA4;NTNG2;EPHA7;SEMA7A;UNC5A;SEMA4D;CAMK2A;UNC5D;EFNA5;L1CAM;EFNB2;PPP3CA;SHH;PLXNA2;SLIT1;NEO1;CAMK2G;NGEF;PLXNA4;WNT4	0.000004
Glycosaminoglycan biosynthesis	NDST2;EXT1;NDST1;EXTL2;CHST7;XYLT1;B3GNT2;CHST1;B3GALT6;HS6ST1;HS6ST3	0.00007
Staphylococcus aureus infection	CFD;ITGAM;CFH;FGG;CFI;PLG;DEFA1;ICAM1;C4B;C3;C5;HLA-DPB1;MASP2;CFB	0.00017
Coronavirus disease	CFD;IFNAR2;FCB;FGA;FGG;F13A1;C8B;F2;C8A;C4B;C3;C5;C6;C8G;C7;C9;F13B;MASP2;IL6S	0.00051
	T;CFB;HBEGF;IFNAR1	
Cholesterol metabolism	CETP;LRP1;APOH;APOC1;APOA2;APOA1;APOA4;LPA;APOB	0.00134
Mannose type O-glycan biosynthesis	FKRP;B4GAT1;B3GAT2;CHST10;LARGE1;POMGNT1	0.00274
WikiPathway 2023 Human		
Complement System WP2806	CFD;CFH;PROS1;CFI;ICAM2;F13A1;C4BPA;PLG;CFP;C8A;ICAM1;CPN1;C3;VTN;C5;C6;C7;C9;FGB;FGA;PRNP;CD93;F12;FGG;ADIPOQ;F11;PLAUR;APOA1;CFHR2;SELL;CFHR4;ALB;SERPING1;MASP2;PTX3;CALR;CD46;KLKB1;CFB	5.552e-30
Complement And Coagulation Cascades WP558	CFD;CPB2;SERPINA1;CFH;SERPINC1;PROS1;CFI;PLG;KNG1;SERPINA5;C4B;C3;C8G;C6;C7;C9;FGB;SERPIND1;F12;SERPINF2;PLAUR;F2;F9;SERPING1;MASP2;F13B;CD46;KLKB1;CFB	2.544e-25
Complement System In Neuronal Development And Plasticity WP5090	CFD;PATJ;FCN3;ITGAM;CFH;PROS1;CFI;C4BPA;C4BPB;CFP;C8B;C8A;CX3CL1;C4B;C3;VTN;C5;C6;C8G;C7;C9;MBP;DLGAP5;CAP1;SERPING1;CYCS;MASP2;CD46;CFB	1.112e-16
Complement Activation WP545	CFD;C8B;CFP;C8A;C3;C4B;C5;C6;C8G;C7;C9;MASP2;CFB	5.840e-12
Network Map Of SARS CoV 2 Signaling Pathway WP5115	ITIH4;ITIH3;CFH;CFI;SERPINA10;CFP;C8A;SERPINA5;CPN1;APOM;APOH;APOD;LBP;APO L1;FGB;HSPA8;FGA;GSN;IL1R2;IGFBP3;FGG;APOA2;APOA1;GP1BA;TGFB2;TF;LRG1;ALB;APOC1;SAA1;HRG;CFB	1.866e-10

GO Biological Process 2023

Axon Guidance (GO:0007411)	EPHB6;NOTCH2;DRAXIN;SEMA7A;EPHA10;LRTM2;NRXN3;EFNA5;EFNB2;SHH;ALCAM;RELN;PLXNA2;SLIT1;NRCAM;NCAM1;NEO1;PLXNA4;EPHA5;EPHA4;EPHA7;UNC5A;SEMA4D;BDNF;L1CAM;VEGFA;NELL2;NELL1;GAP43;NFASC	3.763e-12
Negative Regulation Of Peptidase Activity (GO:0010466)	SERPINA3;SERPINA1;LRP1;SERPIND1;SERPINA10;SERPINF2;SPINK5;SERPINB9;PARK7;SERPINA6;SERPINA7;SERPINA4;KNG1;SERPINA5;VTN;SPOCK2;SERPING1;SPOCK1;TIMP1;SERPIN1	3.763e-12
Negative Regulation Of Blood Coagulation (GO:0030195)	FGB;FGA;CPB2;F12;F11;FCG;SERPINF2;PDGFB;PLG;GP1BA;F2;KNG1;VTN;APOH;KLKB1	5.527e-12
Cell-Cell Adhesion Via Plasma-Membrane Adhesion Molecules (GO:0098742)	ITGAM;PTPRS;CDH9;CELSR2;CDH8;PTPRF;ICAM1;CDH5;IGSF21;ALCAM;CDH1;SLITRK1;CDH2;HMCN1;NTNG1;CADM3;CADM1;PCDHGC3;UNC5D;L1CAM;TGFB2;PTPRD;SELL;CDH10;IL1RAPL1;CDH12;CDH13;ADGRL1;ESAM;CDH18;NECTIN1	9.972e-12
Negative Regulation Of Endopeptidase Activity (GO:0010951)	SERPINA3;SERPINA1;SERPIND1;SERPINA10;SERPINF2;SERPINB9;SERPINA6;SERPINA7;MT3;SERPINA4;KNG1;SERPINA5;VTN;SPOCK2;SERPING1;SPOCK1;TIMP1;SERPIN1;RECK;LTF	1.209e-11
Axonogenesis (GO:0007409)	EPHB6;NOTCH2;APP;DRAXIN;SEMA7A;EPHA10;LRTM2;NRXN3;EFNA5;EFNB2;SHH;RELN;SLITRK1;SLITRK4;SLIT1;SLITRK5;NRCAM;NEO1;EPHA5;NTNG1;EPHA4;NTNG2;EPHA7;SEMA4D;BDNF;APLP2;APLP1;L1CAM;NELL2;SHTN1;NELL1;NFASC	1.330e-11
Positive Regulation Of Cell Junction Assembly (GO:1901890)	CNTNAP2;COL16A1;NLGN2;CLSTN2;MYOC;CLSTN3;BDNF;CLSTN1;PTPRJ;IL1RAP;THY1;VEGFA;PTPRD;NELL1;SLITRK1;SLITRK4;IL1RAPL1;SLITRK5;S100A10	2.203e-11
Nervous System Development (GO:0007399)	NOTCH2;APP;IGSF8;NLGN2;ADAM22;STMN3;EFNA5;NRGN;SHH;RELN;PTPRZ1;CDH1;SLITRK1;SPOCK2;ADAM23;SPOCK1;NRCAM;NPTX1;PCDH1;CDK5RAP2;GSN;MOG;BDNF;GSS;APLP2;MAMDC2;APLP1;SPINK5;LSAMP;CRIM1;L1CAM;GFRA3;GFRA2;VEGFA;OLFM1;NELL1;NFASC;ADGRB2;ADGRB1;PPT1;CSPG5;SERPIN1;LY6H;MDGA2;NBL1	5.108e-9
Neuron Projection Guidance (GO:0097485)	EPHB6;EPHA5;NOTCH2;EPHA4;DRAXIN;EPHA7;SEMA7A;EPHA10;SEMA4D;BDNF;LRTM2;NRXN3;EFNA5;L1CAM;EFNB2;NELL2;NELL1;SHH;NFASC;RELN;SLIT1;NRCAM;NEO1	5.546e-9

Symptomatic carriers vs controls - Upregulated proteins (n = 326)

Pathways	Proteins	Adjusted p-values
KEGG 2021 Human		
Complement and coagulation cascades	CFD;CPB2;ITGAM;SERPINA1;CFH;SERPINC1;PROS1;CFI;F13A1;C4BP A;PLG;C4BPB;C8B;C8A;KNG1;SERPINA5;C4B;C3;VTN;C5;C8G;C6;C7;C9;FCG;FGA;SERPIND1;F12;FCG;SERPINF2;F11;PLAUR;F2;F9;CFHR2;CFHR4;SERPING1;CFHR5;MASP2;F13B;KLKB1;CFB	1.838e-50
Staphylococcus aureus infection	CFD;ITGAM;CFH;FCG;CFI;PLG;DEF A1;ICAM1;C4B;C3;C5;HLA-DPB1;MASP2;CFB	4.165e-8
Coronavirus disease	CFD;IFNAR2;FCB;FGA;FCG;F13A1;C8B;F2;C8A;C4B;C3;C5;C6;C8G;C7;C9;F13B;MASP2;CFB	5.908e-7
Cholesterol metabolism	CETP;APOH;APOC1;APOA2;APOA1;APOA4;LPA;APOB	0.00006
Amoebiasis	ITGAM;C8G;LAMA2;IL1R2;C9;HSPB1;SERPINB9;C8B;C8A;VCL	0.00025

WikiPathway 2023 Human

Complement System WP2806	CFD;CFH;PROS1;CFI;ICAM2;F13A1;C4BPA;PLG;CFP;C8A;ICAM1;CPN1;C3;VTN;C5;C6;C7;C9;FCB;FGA;CD93;F12;FGG;ADIPOQ;F11;PLAUR;APOA1;CFHR2;SELL;CFHR4;ALB;SERPING1;MASP2;PTX3;KLKB1;CFB	1.621e-37
Complement And Coagulation Cascades WP558	CFD;CPB2;SERPINA1;CFH;SERPINC1;PROS1;CFI;PLG;KNG1;SERPINA5;C4B;C3;C8G;C6;C7;C9;FCB;SERPIND1;F12;SERPINF2;PLAUR;F2;F9;SERPING1;MASP2;F13B;KLKB1;CFB	7.892e-33
Complement System In Neuronal Development And Plasticity WP5090	CFD;PATJ;FCN3;ITGAM;CFH;PROS1;CFI;C4BPA;C4BPB;CFP;C8B;C8A;C4B;C3;VTN;C5;C6;C8G;C7;C9;MBP;DLGAP5;CAPI1;SERPING1;MASP2;CFB	1.541e-21
Network Map Of SARS CoV 2 Signaling Pathway WP5115	ITIH4;ITIH3;CFH;CFI;SERPINA10;CFP;C8A;SERPINA5;CPN1;APOM;APOH;APOD;LBP;APO L1;FCB;FGA;GSN;IL1R2;IGFBP3;FGG;APOA2;APOA1;GPIBA;TGFB2;TF;LRG1;ALB;APOC 1;SAA1;HRG;CFB	1.861e-18
Complement Activation WP545	CFD;C8B;CFP;C8A;C3;C4B;C5;C6;C8G;C7;C9;MASP2;CFB	2.783e-16
Blood Clotting Cascade WP272	FCB;FGA;F9;F12;F11;FGG;SERPINF2;PLG;F13B;F2;KLKB1	5.701e-13
COVID 19 Thrombosis And Anticoagulation WP4927	FCB;FGA;FGG;F13A1;PLG;F13B;F2	1.278e-11
Folate Metabolism WP176	FCB;SERPINA3;FGA;FGG;SAA4;ALB;SAA1;APOA1;PLG;F2;APOB;ICAM1	3.607e-8
Selenium Micronutrient Network WP15	FCB;SERPINA3;FGA;FGG;SAA4;ALB;SAA1;APOA1;PLG;F2;APOB;ICAM1	5.180e-7
Kinin Kallikrein Pathway WP5089	F12;SERPING1;KLKB1;KNG1;CPN	7.118e-7
GO Biological Process 2023		
Negative Regulation Of Blood Coagulation (GO:0030195)	FCB;FGA;CPB2;F12;F11;FGG;SERPINF2;PLG;GPIBA;F2;KNG1;VTN;APOH;KLKB1	1.431e-14
Negative Regulation Of Endopeptidase Activity (GO:0010951)	SERPINA3;SERPINA1;SERPIND1;SERPINA10;SERPINF2;SERPINB9;SERPINA6;SERPINA7;S ERPINA4;KNG1;SERPINA5;VTN;SERPING1;TIMP1;RECK;LTF	6.198e-12
Zymogen Activation (GO:0031638)	FCB;FGA;F9;APOH;F12;CD5L;C1RL;F11;FGG;KLKB1;HGFAC	3.313e-8
Proteolysis (GO:0006508)	FCN3;CPB2;CFH;TMPRSS5;DHH;FBXO2;F12;PLG;PEPD;PGC;CPN1;DPP4;PSMA6;SHH;ADA MTS13;F9;ADAMTS1;ANPEP;PAPPA;LPA;KLKB1;HABP2;HGFAC;TRABD2A	1.983e-7
Extracellular Matrix Organization (GO:0030198)	VIT;COL28A1;COL16A1;GSN;MIA;LUM;SERPINF2;SPINK5;PLG;NPNT;ADAMTS13;ADAMTS 1;ADAMTS14;HMCN1;TGFB1;LCP1;RECK	7.76675e-7
High-Density Lipoprotein Particle Remodeling (GO:0034375)	CETP;APOM;APOC1;APOA2;APOA1;APOA4	1.07234e-5
Positive Regulation Of Immune System Process (GO:0002684)	C6;C8G;C7;C9;C8B;CFP;C8A	2.25683e-5
Homotypic Cell-Cell Adhesion (GO:0034109)	FCB;FGA;FGG;HSPB1;MYH9;GPIBA;CDHR5;VCL	7.20569e-5
Antibacterial Humoral Response (GO:0019731)	FCB;IGHM;FGA;SLPI;DEFA1;IGKV3-20;JCHAIN;LTF	1.06795e-4

Symptomatic carriers vs controls - Downregulated proteins (n = 343)			
Pathways	Proteins	Adjusted p-values	
KEGG 2021 Human			
Cell adhesion molecules	NTNG1, NTNG2, CNTNAP2, CADM3, NLGN2, CADM1, NEGR1, NRXN3, CD99L2, L1CAM, GLG1, PTPRF, NFASC, ALCAM, NRCAM, ESAM, NCAM1, NEO1, NECTIN1	2.100e-9	
Axon guidance	EPHB6, EPHA5, NTNG1, EPHA4, NTNG2, EPHA7, SEMA7A, UNC5A, SEMA4D, CAMK2A, UNC5D, EFNA5, L1CAM, EFN2B, PPP3CA, PLXNA2, SLIT1, NEO1, CAMK2G, PLXNA4	5.343e-9	
Glycosaminoglycan biosynthesis	NDST2, EXT1, EXTL2, XYLT1, B3GNT2, CHST1, HS6ST1, HS6ST3	0.00022	
Mannose type O-glycan biosynthesis	FKRP, B4GAT1, CHST10, LARGE1, POMGNT1	0.00202	
Other types of O-glycan biosynthesis	LFNG, GALNT7, C1GALT1C1, ST6GAL2, GALNT16, XXYL1T1	0.00622	
WikiPathway 2023 Human			
Splicing Factor NOVA Regulated Synaptic Proteins WP4148	NTNG1, CADM3, CADM1, CHL1, APLP2, CLSTN1, EFNA5, AGRN, CAMK2G, NEO1	5.935e-7	
Spinal Cord Injury WP2431	RTN4R, EFN2B, EPHA4, PPP3CA, GAP43, PTPRZ1, BDNF, NCAN, PLXNA2, SLIT1, XYLT1, PDYN	0.00017	
Glycosaminoglycan Synthesis In Fibroblasts WP5395	NDST2, EXT1, EXTL2, XYLT1, CHST1, HS6ST1, HS6ST3	0.00063	
Glycolysis And Gluconeogenesis WP534	LDHB, TPI1, PKM, GOT1, MDH1, GOT2, ALDOA	0.00090	
Hippo Signaling Regulation Pathways WP4540	PRKAR1B, CDH10, CDH22, CDH12, CDH13, CDH9, FGFR3, CDH8, CDH18	0.00323	
Clear Cell Renal Cell Carcinoma Pathways WP4018	LDHB, TPI1, PKM, MDH1, PDGFB, CDH13, ALDOA, VEGFA	0.00596	
Synaptic Vesicle Pathway WP2267	NSF, SYT1, AP2A1, PARK7, AP2B1, CPLX1	0.00870	
GO Biological Process 2023			
Axon Guidance (GO:0007411)	EPHB6, NOTCH2, DRAXIN, SEMA7A, EPHA10, LRTM2, NRXN3, EFNA5, EFN2B, ALCAM, RELN, PLXNA2, SLIT1, NRCAM, NCAM1, NEO1, PLXNA4, EPHA5, EPHA4, EPHA7, UNC5A, SEMA4D, BDNF, L1CAM, VEGFA, NELL2, NELL1, GAP43, NFASC	3.785e-19	
Axonogenesis (GO:0007409)	EPHB6, NOTCH2, APP, DRAXIN, SEMA7A, EPHA10, LRTM2, NRXN3, EFNA5, EFN2B, RELN, SLIT, RK1, SLITRK4, SLIT1, SLITRK5, NRCAM, NEO1, EPHA5, NTNG1, EPHA4, NTNG2, EPHA7, SEMA4D, BDNF, APLP2, APLP1, L1CAM, NELL2, NELL1, NFASC	1.419e-17	
Neuron Projection Guidance (GO:0097485)	EPHB6, EPHA5, NOTCH2, EPHA4, DRAXIN, EPHA7, SEMA7A, EPHA10, SEMA4D, BDNF, LRTM2, NRXN3, EFNA5, L1CAM, EFN2B, NELL2, NELL1, NFASC, RELN, SLIT1, NRCAM, NEO1	1.367e-13	
Cell-Cell Adhesion Via Plasma-Membrane Adhesion Molecules (GO:0098742)	NTNG1, CADM3, PTPRS, CADM1, PCDHGC3, UNC5D, L1CAM, CDH9, CELSR2, CDH8, PTPRF, PTPRD, IG-SF21, ALCAM, SLITRK1, CDH10, CDH22, IL1RAPL1, CDH12, CDH13, ADGRL1, ESAM, CDH18, NECTIN1	1.255e-12	

Nervous System Development (GO:0007399)	NOTCH2,APP,IGSF8,NLGN2,ADAM22,STMN3,EFNA5,NRGN,RELN,PTPRZ1,SLITRK1,SPOC K2,ADAM23,SPOCK1,NRCAM,NPTX1,PCDH1,MOG,BDNF,GSS,APLP2,APLP1,LSAMP,CRIM 1,LI1CAM,GFRA2,VEGFA,OLFM1,NELL1,NFASC,ADGRB2,ADGRB1,SERPINI1,LY6H,MDGA2 ;NBL1	1.959e-12
Positive Regulation Of Cell Junction Assembly (GO:1901890)	CNTNAP2,NLGN2,CLSTN2,CLSTN3,BDNF,CLSTN1,THY1,VEGFA,PTPRD,NELL1,SLITRK1, SLITRK4,IL1RAPL1,SLITRK5	5.236e-10
Positive Regulation Of Synapse Assembly (GO:0051965)	PTPRD,NLGN2,CLSTN2,SEMA4D,CLSTN3,SLITRK1,BDNF,SLITRK4,CLSTN1,IL1RAPL1,SLI TRK5	8.494e-10
Regulation Of Neuron Projection Development (GO:0010975)	RTN4R,NTNG1,EPHA4,NTNG2,NDRG4,PTPRS,BDNF,NTRK3,SEZ6,RTN4RL2,MT3,CX3CL1, EFNB2,RELN,RTN4RL1,IL1RAPL1,SPOCK1,SERPINI1,CAMK2C	4.206e-8
Neuron Projection Morphogenesis (GO:0048812)	NTNG1,APP,EPHA4,NTNG2,CNTNAP2,NRN1,BDNF,APLP2,APLP1,CELSR2,ALCAM,SLITR K1,SLITRK4,ADGRB1,SLITRK5,NRCAM,NBL1	1.272e-7
Regulation Of Synapse Assembly (GO:0051963)	PTPRD,NLGN2,CLSTN2,CLSTN3,SLITRK1,BDNF,SLITRK4,CLSTN1,IL1RAPL1,SLITR K5	1.641e-7

Presymptomatic carriers vs controls - All significantly altered proteins (n = 197)

Pathways	Proteins	Adjusted p-values
KEGG 2021 Human		
Complement and coagulation cascades	CPB2,VWF,C1R,SERPINC1,F11,PLG,CD46,KLKB1,KNK1	0.00003
Cholesterol metabolism	CETP,SORT1,APOC1,APOA2,APOA1,LRP2	0.00079
Lysosome	ABCA2,SORT1,SMPD1,PPT1,GNS,SUMF1	0.04509
WikiPathway 2023 Human		
Complement System WP2806	F11,ALB,APOA1,ADM,PLG,LRP2,CD46,CFP,KLKB1,ELANE,CPN1	6.198e-7
Complement And Coagulation Cascades WP558	ITIH4,C1R,SERPINA10,FN1,APOA2,APOA1,CFP,CPN1,APOM,ALB,APOC1,HRG,APOL1	6.505e-7
Network Map Of SARS CoV 2 Signaling Pathway WP5115	KLKB1,KNK1,CPN1	0.00002
Kimin Kallikrein Pathway WP5089	VWF,F11,PLG,KLKB1	0.00204
Blood Clotting Cascade WP272	CETP,APOA2,APOA1	0.00296
Lipid Particles Composition WP3601	CETP,APOC1,APOA2,APOA1	0.00456
Statin Inhibition Of Cholesterol Production WP430	CETP,APOA2,APOA1	0.00651
Familial Hyperlipidemia Type 3/5/2 WP5110/ WP5112/ WP5109	CETP,APOA2,APOA1	0.00798
GO Biological Process 2023		
HDL Particle Remodeling (GO:0034375)	CETP,APOM,APOC1,APOA2,APOA1	0.00034

Proteolysis (GO:0006508)	GCT5;CPB2;CPB1;ERAP2;FBXO2;PLG;PCSK6;PEPD;CNDP1;CPN1;ADAMTS4;SHH;ADAMT S2;KLKB1;ELANE	0.00072
Regulation Of Cholesterol Transport (GO:0032374)	ABCA2;CETP;APOC1;APOA2;APOA1	0.00094
Axonogenesis (GO:0007409)		
Extracellular Matrix Organization (GO:0030198)	NOTCH2;EFNA1;LG11;SHTN1;SHH;DSCAM;EPHA10;SEMA4D;S100A6;UNC5C;FGFR2	0.00094
Negative Regulation Of Blood Coagulation (GO:0030195)	VIT;COL28A1;ADAMTS4;ADAMTS2;ECM2;SMOC1;SPINK5;PLG;GAS6;ELANE	0.00239
Regulation Of Cholesterol Efflux (GO:0010874)	CPB2;F11;PLG;KLKB1;KNG1	0.00239
Reverse Cholesterol Transport (GO:0043691)	CETP;ABCA2;SHH;PONI;APOA1	0.00264
Axon Guidance (GO:0007411)	CETP;APOM;APOA2;APOA1	0.00264
Nervous System Development (GO:0007399)	NOTCH2;EFNA1;LG11;SHH;DSCAM;EPHA10;SEMA4D;UNC5C;NCAM1	0.00268
	NOTCH2;LG11;IGSF8;TENM1;MOG;DSCAM;SPINK5;FN1;EGFR;SHH;ERBB3;SMPD1;PPT1;P CDH1;NBL1	0.00377

Presymptomatic carriers vs controls - Upregulated proteins (n = 93)

Pathways	Proteins	Adjusted p-values
KEGG 2021 Human		
Complement and coagulation cascades	PB2;SERPINC1;F11;PLG;KLKB1;KNG1	0.000299
Cholesterol metabolism	CETP;APOC1;APOA2;APOA1	0.00458
WikiPathway 2023 Human		
Network Map Of SARS CoV 2 Signaling Pathway WP5115	ITIH4;APOM;SERPINA10;ALB;APOC1;APOA2;APOA1;CFP;APOL1;HRG;CPN1	8.604e-7
Complement System WP2806	F11;ALB;APOA1;PLG;CFP;KLKB1;ELANE;CPN1	0.000001
Kinin Kallikrein Pathway WP5089	KLKB1;KNG1;CPN1	0.00017
Complement And Coagulation Cascades WP558	CPB2;SERPINC1;PLG;KLKB1;KNG1	0.00028
Statin Inhibition Of Cholesterol Production WP430	CETP;APOC1;APOA2;APOA	0.00029
Lipid Particles Composition WP3601	CETP;APOA2;APOA1	0.00029
Familial Hyperlipidemia Type 3 /5/2 WP5110/ WP5112/ WP5109	CETP;APOA2;APOA1	0.00059
GO Biological Process 2023		
High-Density Lipoprotein Particle Remodeling (GO:0034375)	CETP;APOM;APOC1;APOA2;APOA1	0.000004
Negative Regulation Of Blood Coagulation (GO:0030195)	CPB2;F11;PLG;KLKB1;KNG1	0.00009
Reverse Cholesterol Transport (GO:0043691)	CETP;APOM;APOC1;APOA2;PONI;APOA1;SHH	0.00018

Negative Regulation Of Peptidase Activity (GO:0010466)	SERPINA10;SPINK5;SERPINA6;SERPINA4;KNG1	0.0007789
Presymptomatic carriers vs controls - Downregulated proteins (n = 104)		
Pathways	Proteins	Adjusted p-values
KEGG 2021 Human		
Cell adhesion molecules	MAG;VCAN;ESAM;NCAM1;F11R;JAM3	0.01377
Tight junction	MYL6;MSN;MYH11;F11R;JAM3	0.01377
WikiPathway 2023 Human		
Complement And Coagulation Cascades WP558	LMAN1;VWF;C1R;CD46	0.03382
Glial Cell Differentiation WP2276	MAG;MSN	0.03382
Burn Wound Healing WP5055	ACHE;TAGLN;S100A6;FGFR2	0.03382
EGFR Tyrosine Kinase Inhibitor Resistance WP4806	ERBB3;GAS6;IL6R;FGFR2	0.04088
GO Biological Process 2023		
Smooth Muscle Contraction (GO:0006939)	MYH11;SLCSA1;MYLK	0.02607
Axonogenesis (GO:0007409)	NOTCH2;EFNA1;EPHA10;SEMA4D;S100A6;UNC5C;FGFR2	0.02607
Regulation Of Osteoblast Differentiation (GO:0045667)	SEMA4D;BAMBI;SMOC1;IL6R;FGFR2	0.02607
Axon Guidance (GO:0007411)	NOTCH2;EFNA1;EPHA10;SEMA4D;UNC5C;NCAM1	0.03091
Gland Morphogenesis (GO:0022612)	MSN;FGFR2	0.03996
Hypotonic Response (GO:0006971)	SLC12A5;MYLK	0.03996
Positive Regulation Of Fibroblast Proliferation (GO:0048146)	FN1;S100A6;GAS6	0.03996
Maintenance Of Blood-Brain Barrier (GO:0035633)	ESAM;F11R;JAM3	0.03996
Neuron Projection Guidance (GO:0097485)	NOTCH2;EFNA1;EPHA10;SEMA4D;UNC5C	0.03996

Table S2. Germ layer differentiation

Cell culture medium utilized for inducing differentiation of iPSC into ectoderm, endoderm, and mesoderm

Ectoderm	Endoderm and mesoderm
DMEM/F12 medium	Neurobasal medium and DMEM/F12 medium (1:1)
Fetal bovine serum (20 %)	Glutamax (1 %)
Glutamax (1 %)	B27 supplement (1 %)
Non-essential amino acids (1 %)	N2 supplement (1 %)
Penicillin/Streptomycin (1 %)	Penicillin/Streptomycin (1 %)

Table S3. Antibodies and primers

Primary antibodies used for immunofluorescence staining and western blot

Primary antibody target	Company	Catalog no.
AIM	Abcam	Ab45408
CD36	Santa Cruz Biotechnology	Sc-7309
CHMP2B	ThermoFisher Scientific	PA5-70058
FOXP1	Life Technologies	PA5-26794
IBA1	Wako	019-19741
MAP2	Novus Biologicals	NB-300-213
NANOG	Peptotech	500-P236
NeuN	Merck/Millipore	MAB377
OCT3/4	Santa Cruz Biotechnology	sc-8628
P2RY12	Sigma/Merck	HPA014518
SSEA-3	BioLegend	330302
SSEA-4	BioLegend	330401
SMA	DAKO	M0851
SOX17	R&D Systems	AF1924
Synapsin1	Synaptic Systems	106001
TMEM119	Sigma/Merck	HPA051870
TUBB3	Merck/Millipore	MAB1637
TRA-1-60	BioLegend	330602
TRA-1-81	BioLegend	330702

Vinculin	Sigma	V9131
VGLUT1	Invitrogen	48-2400

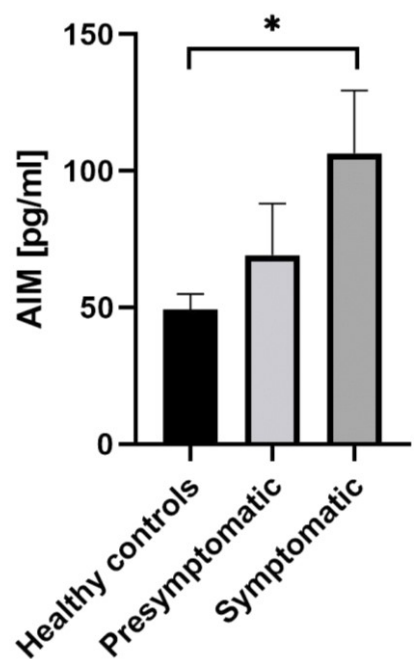
Secondary antibodies used for immunofluorescence staining and western blot

Secondary antibody target	Company	Catalog no.
Anti-chicken IgG	Life Technologies	A11041
Anti-goat IgG	Sigma	SAB4600032-250UL
Anti-mouse IRDye 800CW	Li-Cor	926-32210
Anti-mouse IgG	Sigma	SAB4600388-125UL
Anti-rabbit IRDye 800CW	Li-Cor	926-32211
Anti-rabbit IgG	Sigma	SAB4600044-250UL
Anti-rat IgG	Sigma	SAB4600037-250UL

Primers used for qPCR

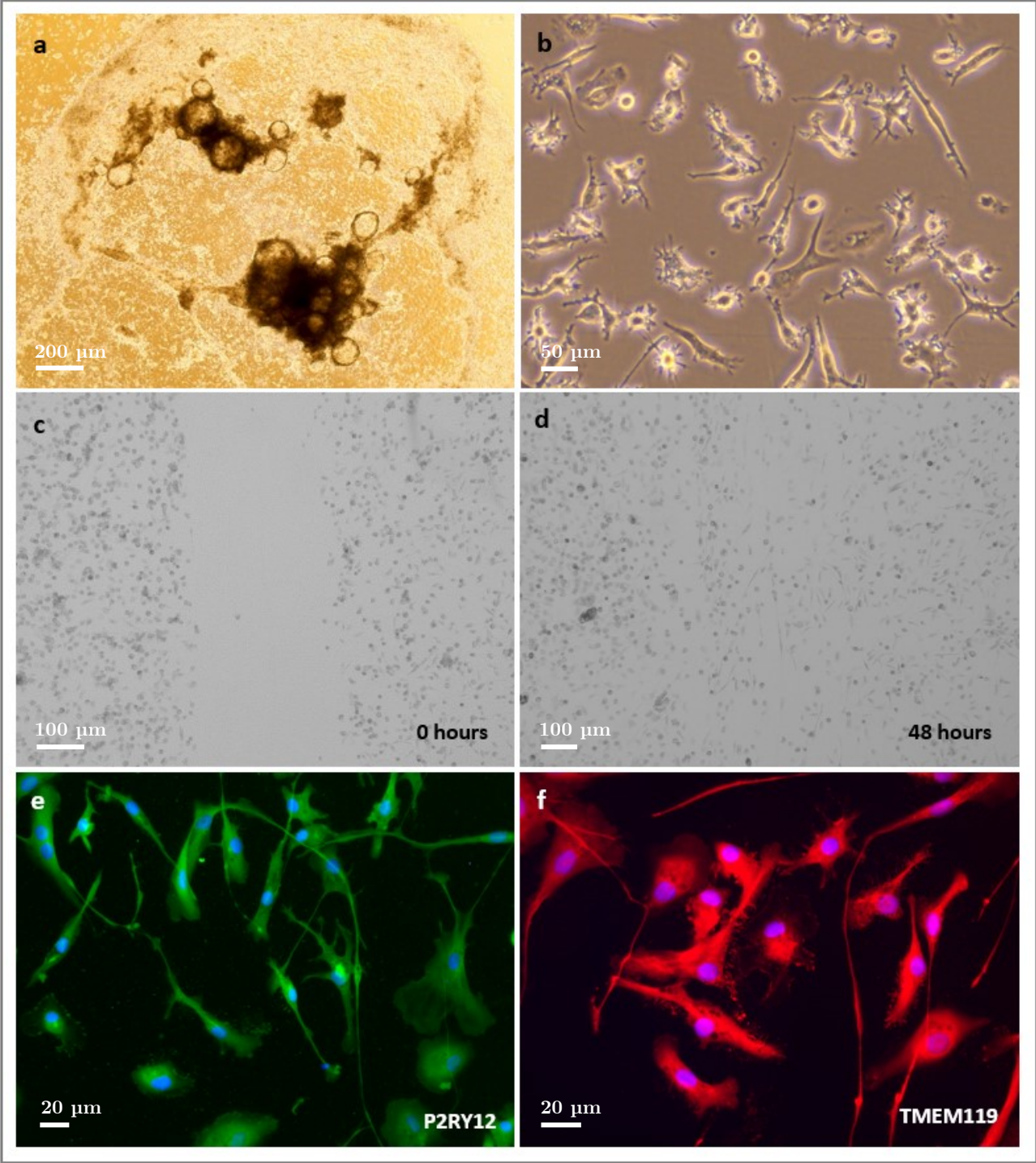
Primers targets	Sequence	Company
CD45 (PTPRC) Forward	5'-CAGTTCATGCAGCTAGCAAGTGG-3'	Sigma
CD45 (PTPRC) Reverse	5'-CCACTGTTGTCTTATCAGACGAGGAA-3'	Sigma
CX3CR1 Forward	5'-CAACTCTCCTGGACTGCCTTGA-3'	Sigma
CX3CR1 Reverse	5'-CAGGTTGGCAGTAGCATGAATGA-3'	Sigma
P2RY12 Forward	5'-CAGGATGGTGGTGACCCAAA-3'	Sigma
P2RY12 Reverse	5'-GCGCTTTGCTTTAACGAGTTCTG-3'	Sigma

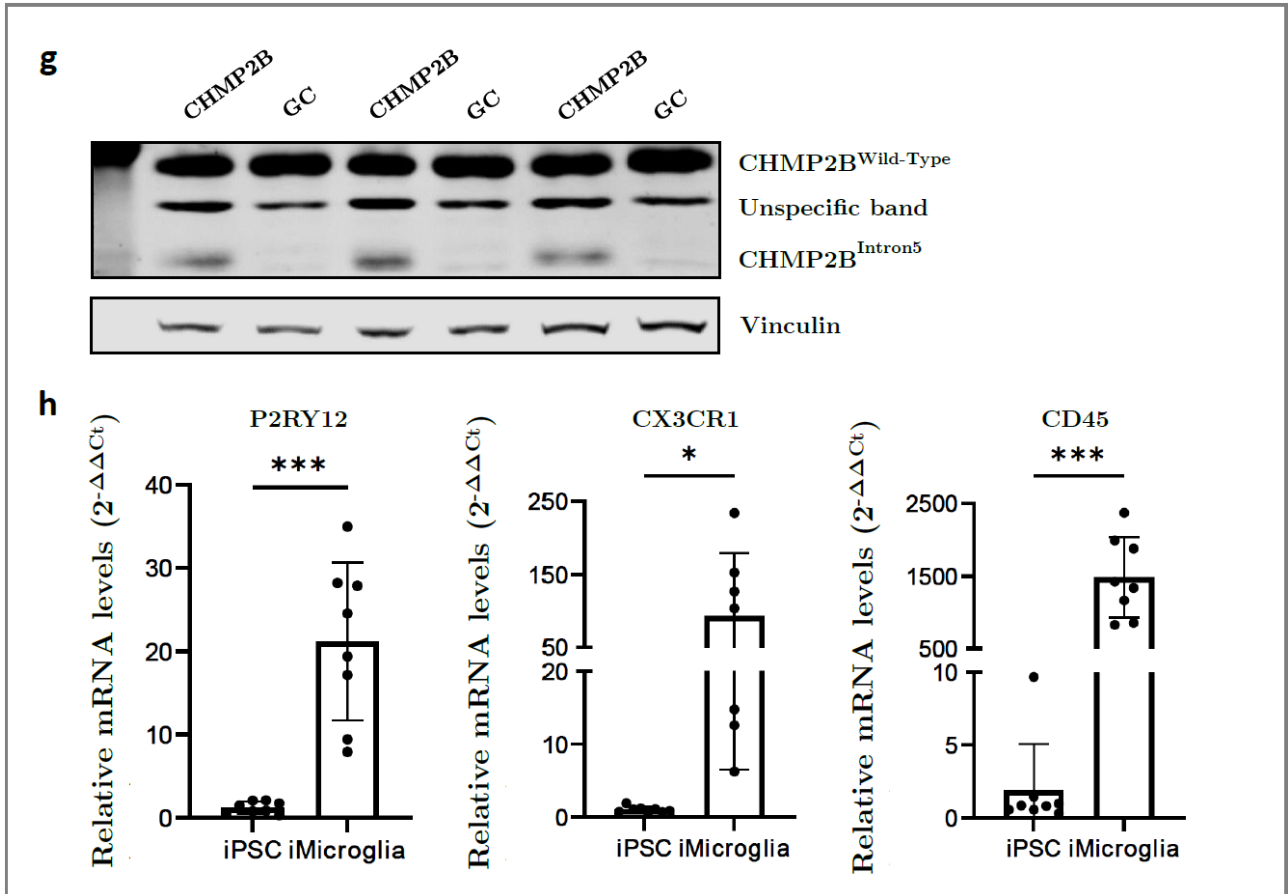
Figure S1. Cerebrospinal fluid AIM levels in the clinical groups



ELISA-based quantification of CSF AIM levels. Each bar in the plot represents the mean AIM concentration in CSF samples from 16 non-carrier family members (Healthy controls), 12 presymptomatic carriers of the *CHMP2B* variant (Presymptomatic), and 8 symptomatic carriers (Symptomatic). Error bars represent SEM. Group means were compared using the Kruskal-Wallis Test, with a significance level of 0.05.

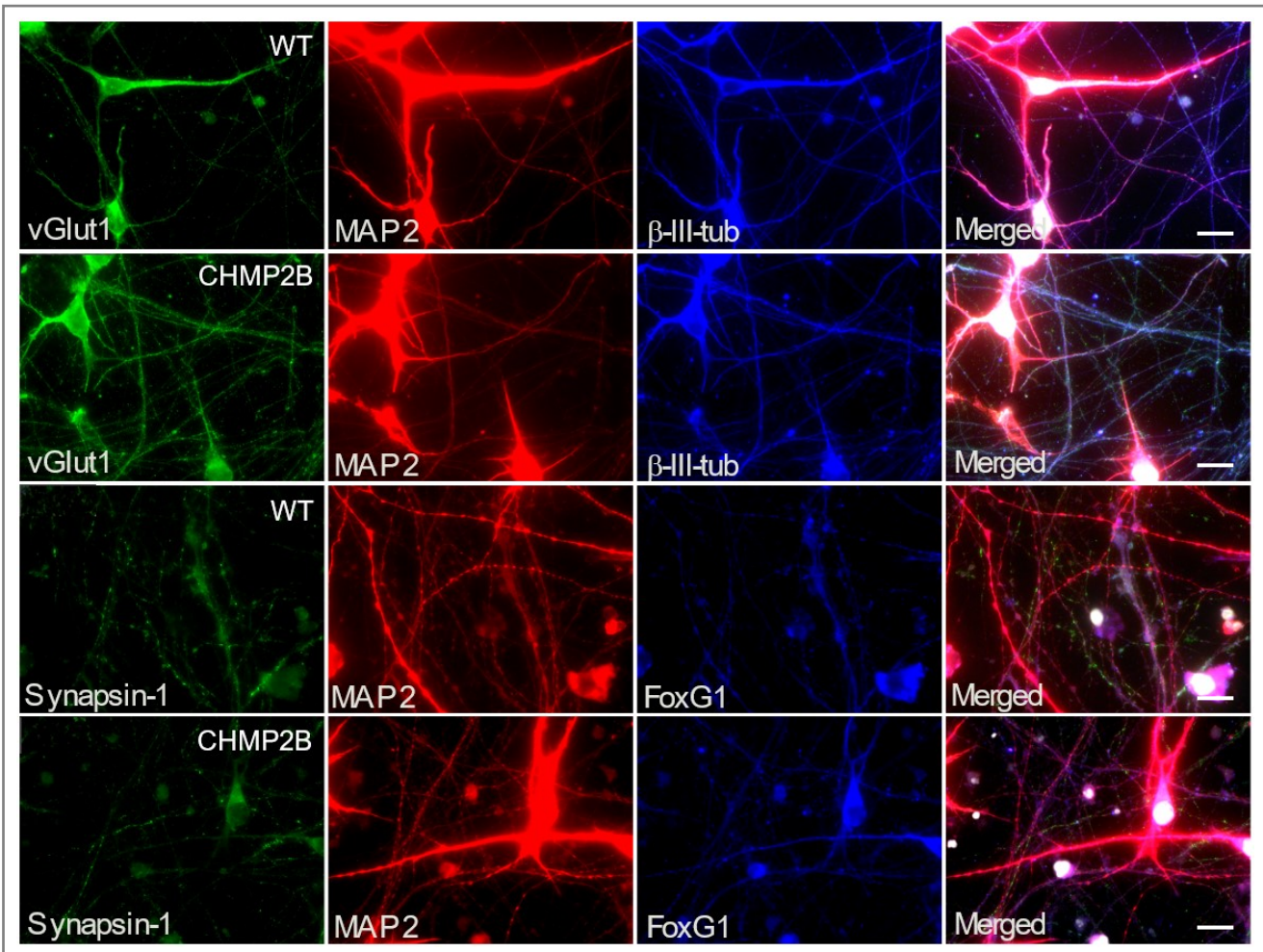
Figure S2. Characterization of induced microglia





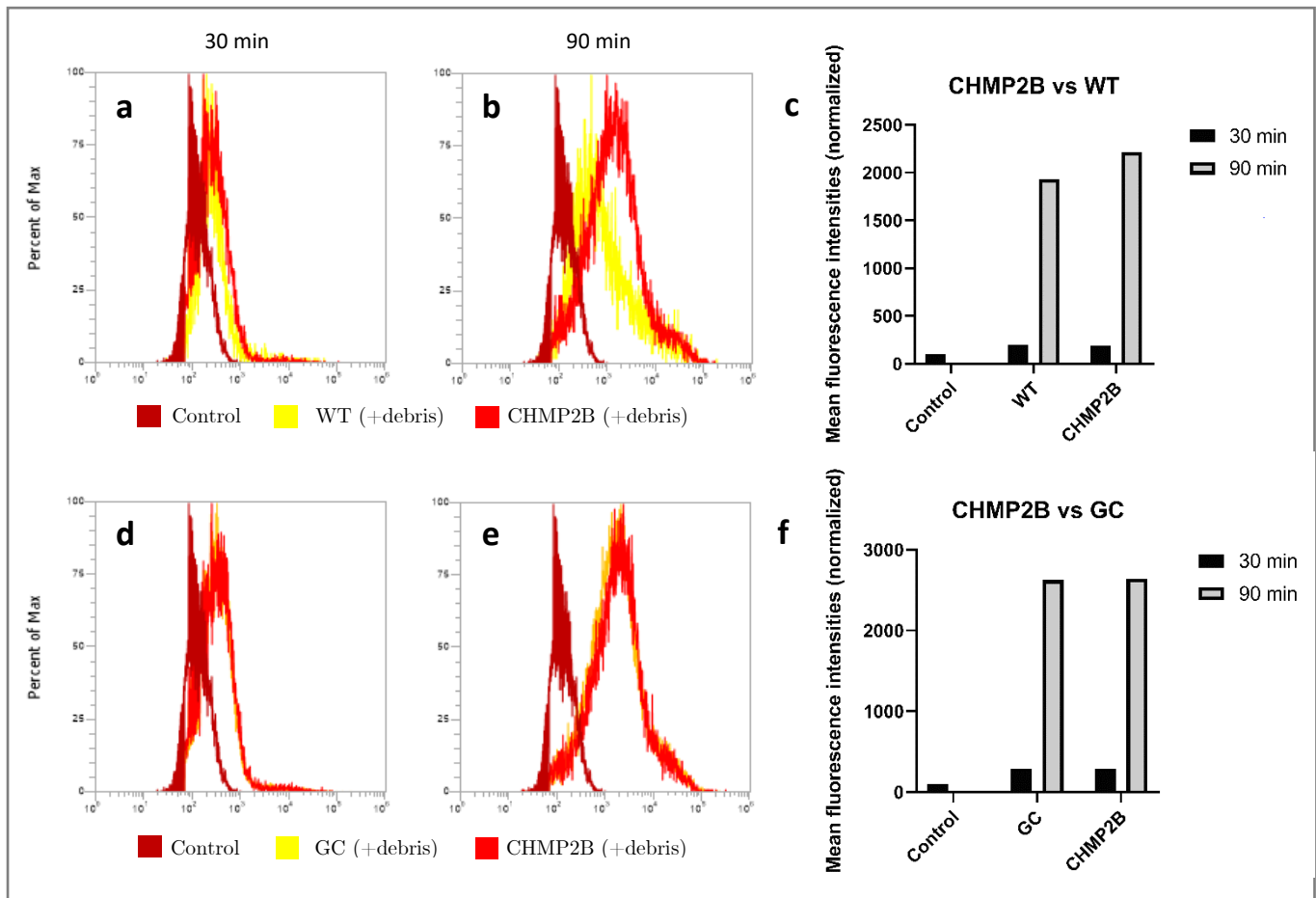
Characterization of iPSC-derived microglia. (a) Light microscopy image (4X) of a 6-week-old “microglia factory” with cystic, yolk-like structures and surrounding stromal tissue. The factories produce microglia-like precursors that either float in the cell culture medium or attach around the factory. Two weeks after replating of the precursors (b), induced microglia are ramified, migrating, and filled with numerous clear vesicles, indicative of active pinocytosis (20X light microscopy). (c, d) Scratch-wound assay to test the non-directed, surveillance motility of the cells. Following the scratch induced with a pipette tip, microglia repopulate the scratched area within 36-48 hours (4X light microscopy). (e, f) The protocol yielded pure populations of microglia positive for commonly used microglial markers like the calcium-binding protein IBA1, the purinergic receptor P2RY12, and the transmembrane protein TMEM119 (40X IF). (g) Induced microglia carrying the *CHMP2B* variant (CHMP2B) present with an additional band around 22 kDa, which is not present in gene-corrected microglia (GC), representing truncated CHMP2B protein. (h) mRNA expression of markers P2RY12, CX3CR1, and CD45 was elevated in induced microglia (iMicroglia) compared with undifferentiated iPSCs ($2^{-\Delta\Delta C_t}$). Each data point represents an individual cell line with or without the *CHMP2B* variant.

Figure S3. Characterization of induced neurons



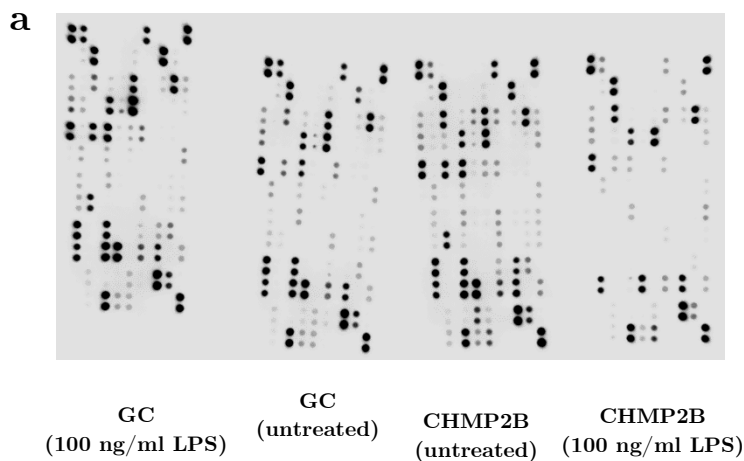
Immunofluorescence staining of induced neurons. iPSC-derived neurons with the *CHMP2B* variant (CHMP2B) and wild-type controls (WT) stained with neuronal markers β -tubulin III (β -III-tub, blue, upper panels) and MAP2 (red), forebrain patterning marker FOXG1 (blue, lower panels), and neuronal maturations markers VGLUT1 (green, upper panels) and Synapsin 1 (green, lower panels). Scale bar, 20 μ m.

Figure S4. Phagocytosis of neuronal debris

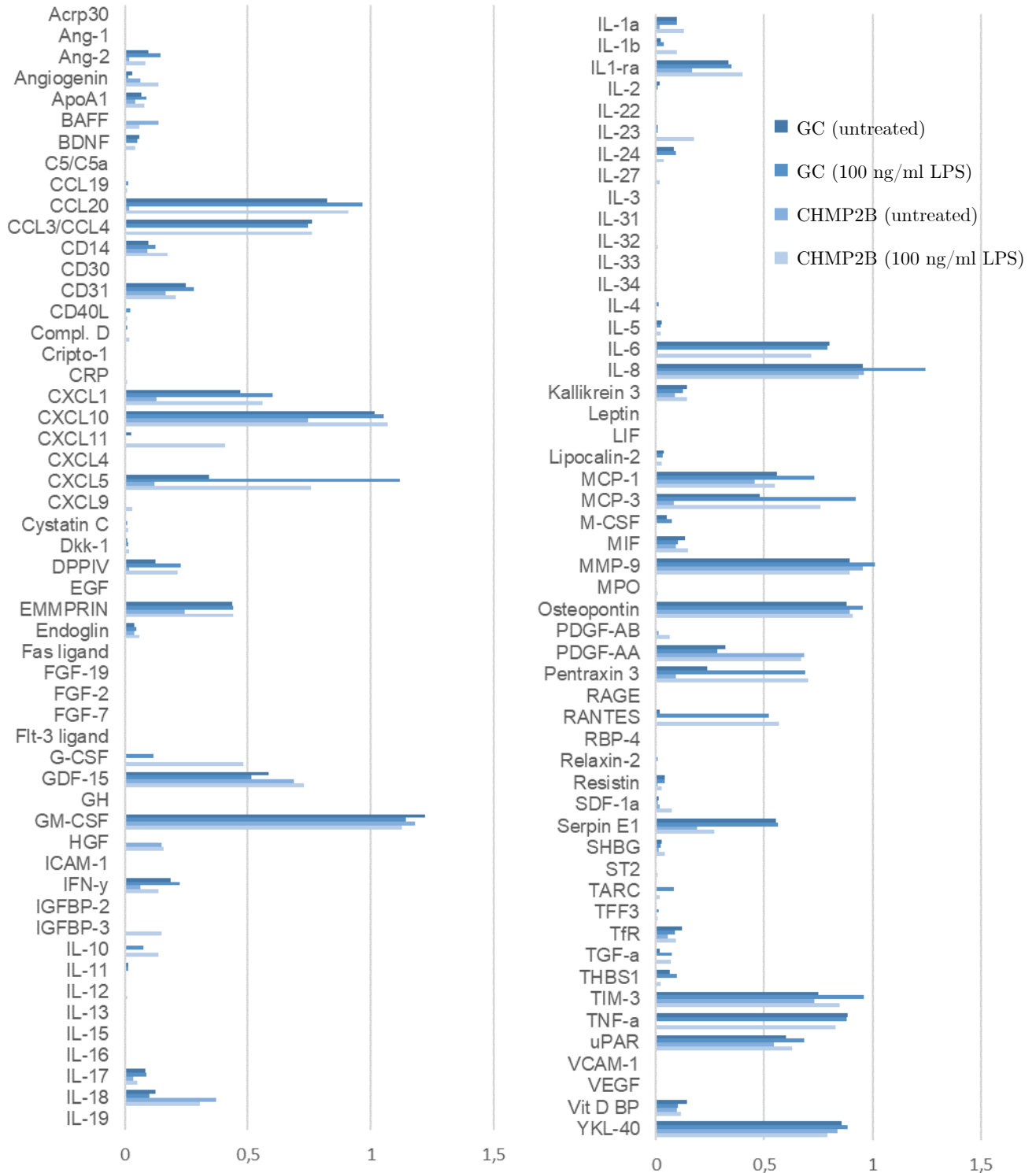


Phagocytosis of fluorescence-labeled neuronal debris by microglia. UV-mediated apoptosis was induced in fluorescently labeled iPSC-derived neurons, which were added to microglia generated from iPSCs from the same patient, for 30 or 90 minutes. In (a-c), stimulated microglia carrying the *CHMP2B* variant (CHMP2B) were compared with stimulated wild-type microglia (WT) and an unstimulated microglial line (Control). After 90 minutes, *CHMP2B* microglia had taken up more apoptotic neurons than the wild-type lines (14.8 % higher mean fluorescence). However, when compared with gene-corrected microglia (GC) (d-f), no apparent differences were observed ($n = 1$).

Figure S5. Cytokines in microglia medium

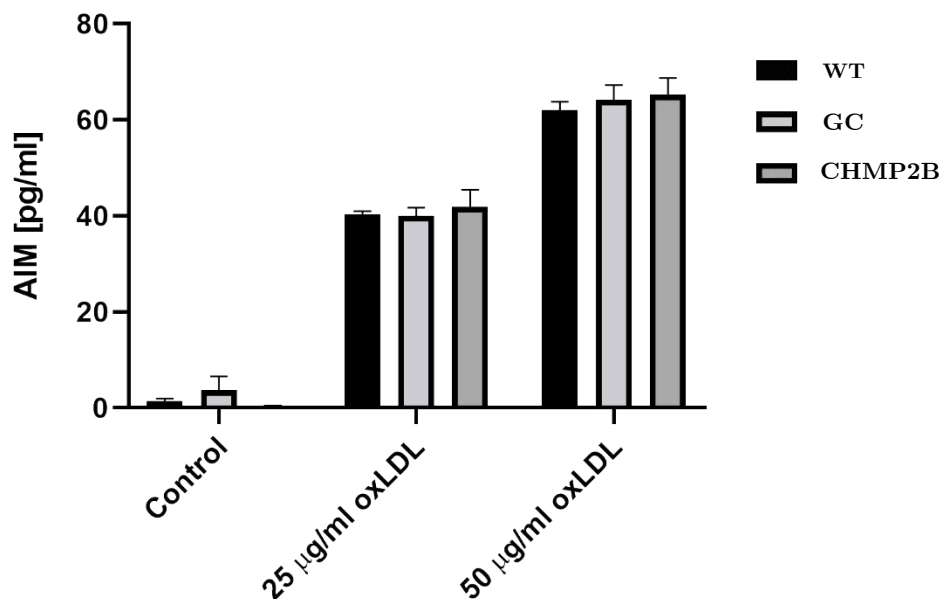


b

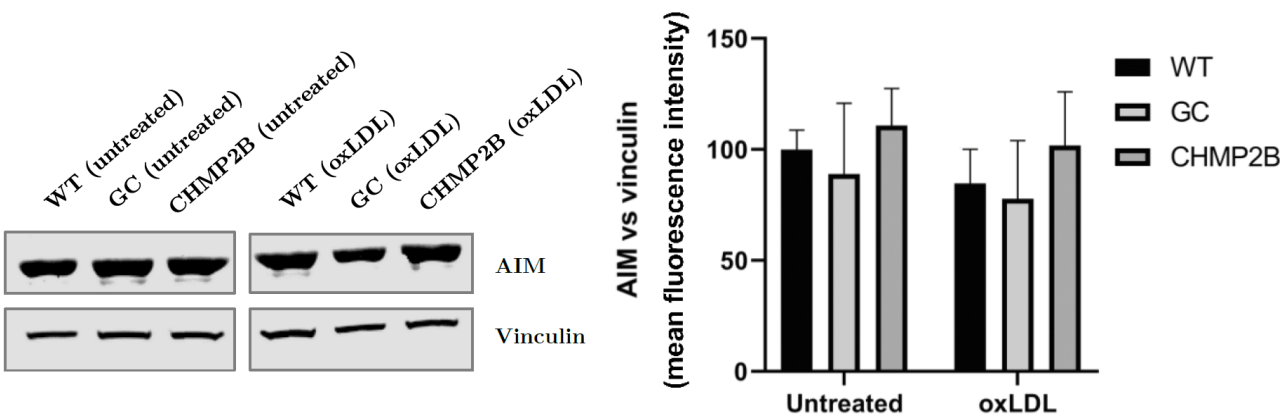


Cytokine screening. (a) Media from induced microglia carrying the *CHMP2B* variant (*CHMP2B^{Intron5}*) and gene-corrected controls (GC) were applied to membranes with capture antibodies for 105 cytokines in duplicates. Each pair of dots represents one cytokine. (b) A few cytokines were exclusively produced by *CHMP2B* microglia (HGF, BAFF) or produced in higher quantities (IL-18, IL-23, PDGF-AA). Levels of inflammatory cytokines such as TNF- α , IFN- γ , IL-1, IL-6, and IL-17 were lower in *CHMP2B* microglia when compared with gene-corrected controls, but LPS treatment increased their secretion.

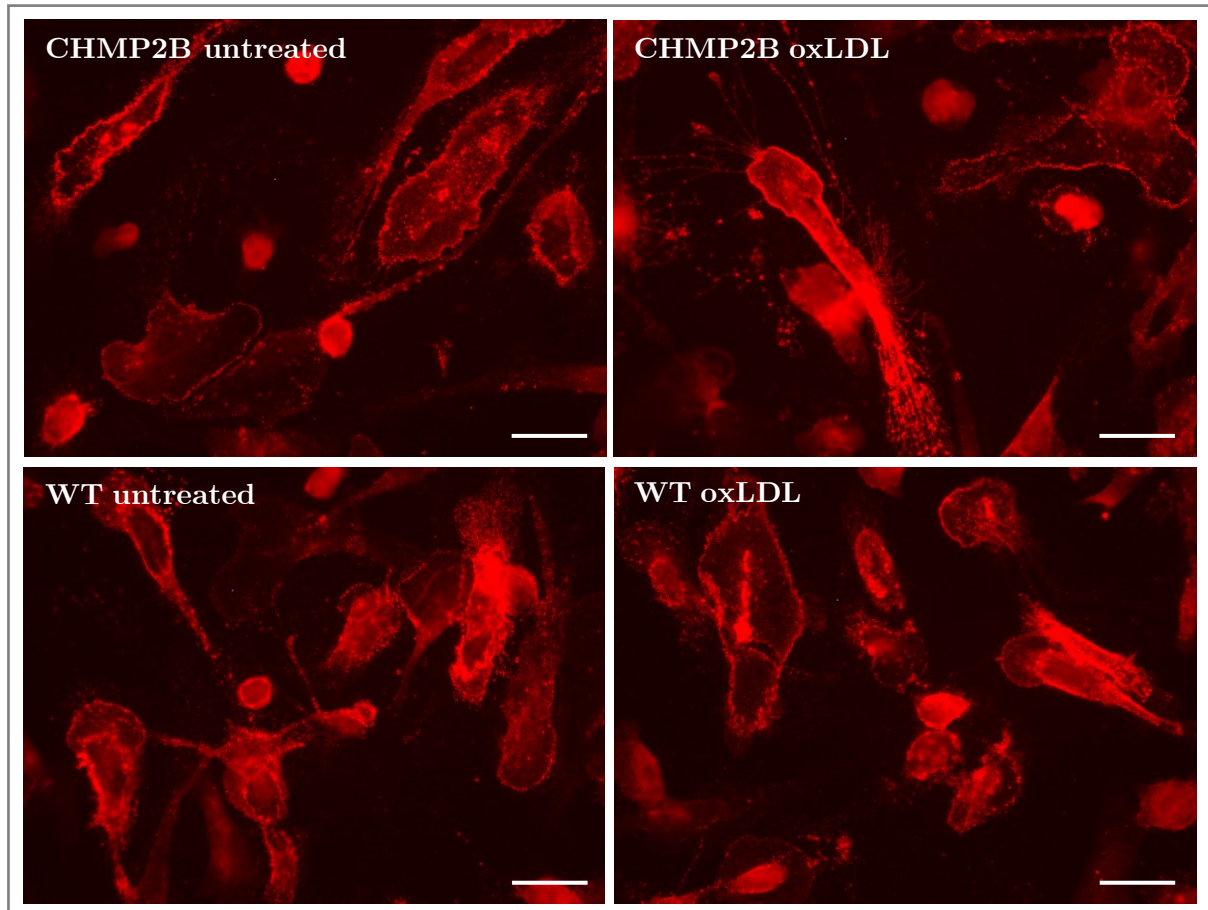
Figure S6. Extracellular and intracellular AIM and CD36



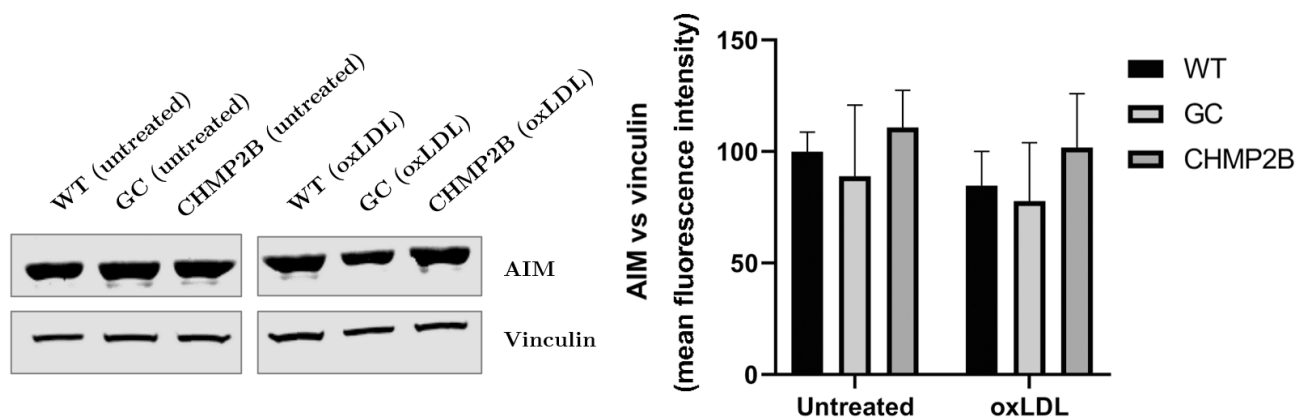
ELISA-based quantification of extracellular AIM levels in the medium of induced microglia. Each bar in the plot represents the mean AIM concentration of three different microglial lines ($n = 3$), which are either wild-type control lines (WT), gene-corrected control lines (GC), or cell lines carrying the pathogenic *CHMP2B* variant (CHMP2B). The cells were stimulated with 25 or 50 µg/mL human oxidized low-density lipoprotein (oxLDL) for 4 hours prior to media collection, leading to dose-dependent increases in AIM, without significant differences between the cell lines using Kruskal-Wallis Test. Error bars represent SEM.



Western blot analysis of AIM in induced microglia. Each bar in the plot represents the mean fluorescence band intensity normalized to the house-keeping protein vinculin ($n = 2$) of wild-type microglia (WT), gene-corrected microglia (GC), or *CHMP2B* microglia (CHMP2B) with or without treatment with 50 µg/mL oxLDL. oxLDL did not affect overall AIM levels, and no apparent differences between the cell lines were observed. Error bars represent SEM.

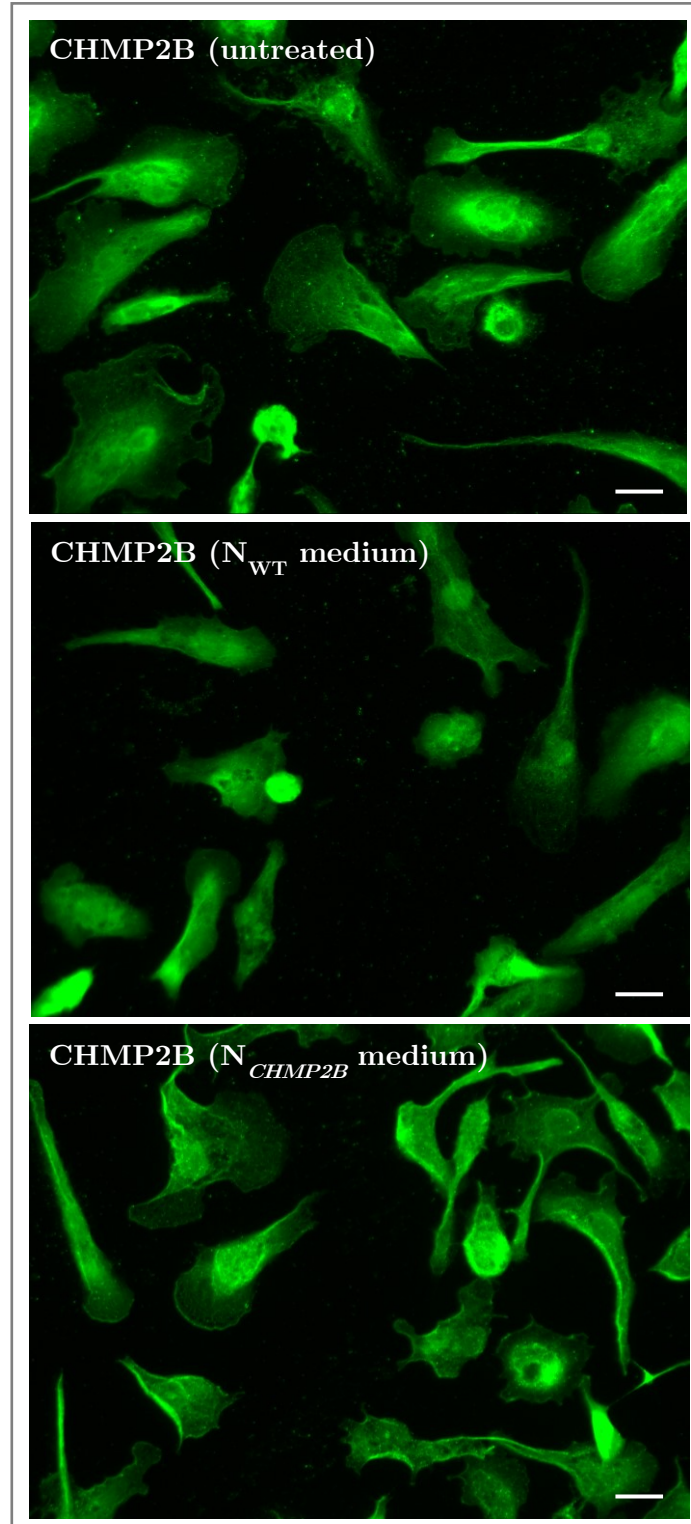


Immunofluorescence staining for CD36 in induced microglia. iPSC-derived microglia with the *CHMP2B* variant (CHMP2B) and wild-type controls (WT) stained for the binding receptor of AIM, CD36, with or without treatment with 50 $\mu\text{g}/\text{mL}$ oxLDL for 4 hours. The CD36 receptor is present at the plasma membrane and in intracellular vesicular structures without apparent differences between the cell lines. Similar staining of induced neurons did not reveal any observable expression of the receptor (not shown). Scale bar, 20 μm .



Western blot analysis of CD36 in induced microglia. Each bar in the plot represents the mean fluorescence band intensity from two samples of wild-type microglia (WT), gene-corrected microglia (GC), or *CHMP2B* microglia (CHMP2B), normalized to vinculin, with untreated WT cells as reference. Treatment with 50 $\mu\text{g}/\text{mL}$ oxLDL did not affect overall AIM levels, and no apparent differences between the cell lines were observed. Error bars represent SEM of $n = 2$.

Figure S7. Immunofluorescence staining for AIM



Immunofluorescence staining for AIM in induced microglia carrying the *CHMP2B* variant. iPSC-derived *CHMP2B* microglia (CHMP2B) untreated or treated for 4 hours with conditioned medium from either induced wild-type neurons (N_{WT} medium) or induced neurons harboring the *CHMP2B* variant (N_{CHMP2B} medium). Based on visual inspection, stimulation with conditioned medium from *CHMP2B* neurons appears to increase the staining intensity associated with the plasma membrane in the *CHMP2B* microglia. Scale bar, 20 μ m.

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