Evaluation of the Endo-Lysosomal System and the Ubiquitin-Proteasome System in Neurodegenerative Diseases

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ABSTRACT

Neurodegeneration is the process of neuronal cell loss where the symptoms will reflect the regions affected. Neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD) and frontotemporal dementia (FTD) are all characterised by an accumulation of protein aggregates suggesting impaired production or turnover of these proteins. Hence, dysfunctional proteostasis is implicated in neurodegenerative disorders. In AD, there is a decreased turnover of endocytic and autophagic vesicles and an accumulation of endo-lysosomal proteins and ubiquitin in brain tissue. Lysosomal dysfunction has been indicated in PD by the link of disease risk and genetic alterations associated with lysosomal storage disorders as well as by decreased expression of lysosomal proteins in disease afflicted regions. Disease causing mutations and genetic risk factors in FTD suggest altered function of the autophagic and endo-lysosomal system to be involved in the pathogenesis.

The aim of this thesis was to examine dysfunctional proteostasis in neurodegenerative diseases by developing assays to monitor proteins from the autophagic and endo-lysosomal system and the ubiquitin-proteasome system in human cerebrospinal fluid (CSF). Proteins from the endo-lysosomal system and the ubiquitin-proteasome system have been identified and quantified in CSF using mass spectrometry (MS)-based proteomics. Principally, three methods have been developed; 1) lysosomal membrane protein LAMP2 was purified from CSF by immunoprecipitation followed by tryptic digestion and quantification by liquid chromatography (LC) and parallel reaction monitoring MS (PRM-MS); 2) full length ubiquitin was isolated from CSF by solid-phase extraction (SPE) followed by quantification by LC PRM-MS; and 3) finally, a panel of endo-lysosomal proteins, *e.g.*, LAMP2, and ubiquitin, were analysed using tryptic digestion, peptide isolation by SPE and quantification by LC PRM-MS. CSF samples from cohorts including subjects with AD, PD, clinical FTD subtypes and FTD mutation carriers, as well as controls, were analysed with the developed assays.

In AD the CSF levels of several endo-lysosomal proteins, including LAMP2, were elevated compared to controls. CSF ubiquitin was also found to be elevated in AD compared to controls. In contrast, CSF levels of endo-lysosomal proteins and ubiquitin in PD were found to be decreased. Investigation in clinical subtypes of FTD and mutation carriers showed limited alterations in the CSF levels of endo-lysosomal proteins, suggesting dysfunctional proteostasis not to be readily detected in CSF in FTD. Our results showing altered CSF levels of proteins involved in proteostasis in AD and PD might indicate pathological alterations in the autophagic and endo-lysosomal system and the ubiquitin-proteasome system. Although further studies are needed, CSF ubiquitin in AD and endo-lysosomal proteins and ubiquitin in PD might serve as potential biomarkers in these disorders.

Keywords: Alzheimer's disease, Parkinson's disease, frontotemporal dementia, dysfunctional proteostasis, cerebrospinal fluid, mass spectrometry

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SAMMANFATTNING PÅ SVENSKA

I neurodegenerativa sjukdomar sker en fortlöpande nervcellsdöd. Symptomen speglar de regioner i hjärnan som drabbas vid varje specifik sjukdom. Neurodegenerativa sjukdomar inkluderar bland annat Alzheimers sjukdom, Parkinsons sjukdom och frontallobsdemens. Gemensamt för neurodegenerativa sjukdomar är förekomsten av ansamlingar i hjärnan av specifika proteiner. Detta tyder på en ökad produktion eller minskad nedbrytning av dessa proteiner. Nervceller har en begränsad förmåga att förnya sig och kräver därför ett effektivt system för nedbrytning som upprätthåller miljön i cellen under en individs livstid. Nedbrytning sker primärt genom två system. Via endocytos eller autofagi levereras proteiner till lysosomen för nedbrytning. Alternativt märks proteiner av ubiquitin för att brytas ner av proteasomen. Tidiga förändringar i den lysosomal nedbrytningsvägen i nervceller har påvisats i Alzheimers sjukdom och det finns även en ökad mängd ubiquitinmärkta proteinansamlingar i hjärnan. Ärftliga riskfaktorer och minskade nivåer av lysosomal proteiner i sjukdomsdrabbade regioner i hjärnan tyder på en central roll för lysosomal funktion i Parkinsons sjukdom. Orsaken till frontallobsdemens är inte sällan ärftlig och de gener som är sjukdomsorsakande tyder på förändrad proteinnedbrytningsförmåga.

Att kunna identifiera och skilja sjukdomar åt i ett tidigt skede är viktigt för att kunna utveckla effektiva behandlingar. I den här avhandlingen har vi utvecklat metoder för att mäta nivåerna i ryggvätska av proteiner med en funktion i nedbrytningssystemen, för att se om dessa skiljer sig åt mellan prover från individer med Alzheimers sjukdom, Parkinsons sjukdom och frontallobsdemens, samt friska kontrollpersoner. Biomarkörer produceras av kroppen och speglar ett sjukdomstillstånd eller en biologisk process. För Alzheimers sjukdom finns väl validerade biomarkörer i ryggvätska, men för Parkinsons sjukdom och frontallobsdemens finns ännu inga kliniskt användbara biomarkörer. Avhandlingens resultat tyder på att nivåerna av flera lysosomala proteiner är förhöjda i ryggvätska vid Alzheimers sjukdom, bland annat det lysosomala membranproteinet LAMP2. I motsats visas tydligt sänkta nivåer av lysosomala proteiner i ryggvätska vid Parkinsons sjukdom. Även nivåerna av ubiquitin i ryggvätska är höjda vid Alzheimers sjukdom och sänkta vid Parkinsons sjukdom. Vid frontallobsdemens uppmättes inga tydliga skillnader i nivåerna av proteiner i ryggvätska. Sammantaget indikerar fynden att ubiquitin i ryggvätska kan vara en potentiell biomarkör vid Alzheimers sjukdom och att lysosomala proteiner och ubiquitin kan vara potentiella biomarkörer vid Parkinsons sjukdom. Fortsatta studier med de biokemiska metoder vi har utvecklat krävs för att fastställa om dessa fynd går att använda för diagnos/prognos av neurodegenerativa sjukdomar i klinik.

LIST OF PAPERS

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

I. **Sjödin S**, Öhrfelt A, Brinkmalm G, Zetterberg H, Blennow K, and Brinkmalm A. *Targeting LAMP2 in human cerebrospinal fluid with a combination of immunopurification and high resolution parallel reaction monitoring mass spectrometry*.
Clinical Proteomics 2016, 13:4.

II. **Sjödin S**, Hansson O, Öhrfelt A, Brinkmalm G, Zetterberg H, Brinkmalm A, and Blennow K. *Mass Spectrometric Analysis of Cerebrospinal Fluid Ubiquitin in Alzheimer's Disease and Parkinsonian Disorders*.
Proteomics Clinical Applications 2017, 11:11-12.

III. **Sjödin S**, Brinkmalm G, Öhrfelt A, Parnetti L, Paciotti S, Hansson O, Hardy J, Blennow K, Zetterberg H, and Brinkmalm A. *CSF Levels of Endo-Lysosomal Proteins and Ubiquitin in Alzheimer's and Parkinson's Disease*.
Manuscript.

IV. **Sjödin S**, Woollacott I, Brinkmalm G, Foiani M, Heller C, Lashley T, Öhrfelt A, Blennow K, Brinkmalm A, Rohrer J, and Zetterberg H. *Cerebrospinal Fluid Levels of Lysosomal Proteins and Ubiquitin in Clinical and Familial Subtypes of Frontotemporal Dementia*.
Manuscript.

Related papers not included in this thesis.

Sjödin S, Andersson KK, Mercken M, Zetterberg H, Borghys H, Blennow K, and Portelius E. *APLP1 as a cerebrospinal fluid biomarker for gamma-secretase modulator treatment*.
Alzheimer's Research and Therapy 2015, 7:77.

Brinkmalm G, **Sjödin S**, Simonsen AH, Hasselbalch SG, Zetterberg H, Brinkmalm A, and Blennow K. *A Parallel Reaction Monitoring Mass Spectrometric Method for Analysis of Potential CSF Biomarkers for Alzheimer's Disease*.
Proteomics Clinical Applications 2018, 12:1.

CONTENT

ABBREVIATIONS

18F-FDG	2-[18F]-fluoro-2-deoxy-D-glucose
Aβ	Amyloid β
$A\beta_{1-42}$	42 amino acid-long amyloid β
aa	Amino acids
AD	Alzheimer's disease
AP2	AP-2 complex subunit beta
APP	Amyloid precursor protein/amyloid beta A4 protein
bvFTD	Behavioural variant frontotemporal dementia
C9	Complement component C9
C9ORF72	Chromosome 9 open reading frame 72
CatB	Cathepsin B
CatD	Cathepsin D
CatF	Cathepsin F
CatL1	Cathepsin L/L1
CatZ	Cathepsin Z
CHMP2B	Charged multivesicular body protein 2B
CMA	Chaperone-mediated autophagy
CSF	Cerebrospinal fluid
DC	Direct current
DPP2	Dipeptidyl peptidase 2
EE	Early endosome
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport

ESI	Electrospray ionisation
FTD	Frontotemporal dementia
FTDC	Frontotemporal Dementia Criteria Consortium
FTLD	Frontotemporal lobar degeneration
GM2A	Ganglioside GM2 activator
GRN	Progranulin
HEXB	Beta-hexosaminidase subunit beta
HLB	Hydrophilic-lipophilic balance
Hsc70	Heat shock-cognate protein of 70 kDa
IP	Immunoprecipitation
IWG	International working group
IWG-2	International working group 2
LAMP1	Lysosome-associated membrane protein 1
LAMP2	Lysosome-associated membrane protein 2
LC	Liquid chromatography
LC3	Microtubule-associated proteins 1A/1B light chain 3
LE	Late endosome
LRRK2	Leucine-rich repeat serine/threonine-protein kinase 2
lvPPA	Logopenic variant primary progressive aphasia
LysC	Lysozyme C
m/z	Mass to charge ratio
MAPT	Microtubule-associated protein tau
MCI	Mild cognitive impairment
MCI-AD	Mild cognitive impairment due to Alzheimer's disease

MRI	Magnetic resonance imaging
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
nfvPPA	Nonfluent variant primary progressive aphasia
NIA-AA	National Institute on Aging-Alzheimer's Association
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
NINDS	National Institute of Neurological Disorders and Stroke
P-tau	Phosphorylated tau
P-tau$_{181}$	Tau phosphorylated at Thr 181
PAGE	Polyacrylamide gel electrophoresis
PD	Parkinson's disease
PET	Positron emission tomography
PICALM	Phosphatidylinositol-binding clathrin assembly protein
PINK1	PTEN-induced putative kinase protein 1
PPA	Primary progressive aphasia
PRM	Parallel reaction monitoring
PRM-MS	Parallel reaction monitoring mass spectrometry
PSP	Progressive supranuclear palsy
QqQ	Triple quadrupole
RF	Radiofrequency
sMCI	Mild cognitive impairment remaining stable
SPE	Solid-phase extraction
SRM	Selected reaction monitoring

svPPA	Semantic variant primary progressive aphasia
T-ALF	Tissue alpha-L-fucosidase
T-tau	Total tau
TCO2	Transcobalamin-2
TDP-43	Transactive response DNA-binding protein of 43 kDa
TMEM106B	Transmembrane protein 106B
TPP1	Tripeptidyl-peptidase 1
Ub	Ubiquitin
VPS35	Vacuolar protein sorting-associated protein 35
WB	Western blotting

1 INTRODUCTION

1.1 Neurodegenerative Diseases

Neurodegeneration is the progressive loss of neurons resulting in a number of potential afflictions, presenting with for example dementia syndrome or motor neuron deficits, depending on regional involvement in the brain. Examples of neurodegenerative disorders are Alzheimer's disease (AD) [1], Parkinson's diseases (PD) [2] and frontotemporal dementia (FTD) [3].

The worldwide prevalence of dementia in 2015 was estimated to be 46.8 million, to double every 20 years and reach 131.5 million in 2050 [4]. The worldwide cost of dementia in 2018 is appreciated to reach US$ 1 trillion [4]. Although absolute numbers are increasing there seem to be a decrease in dementia incidence [5].

1.1.1 Alzheimer's Disease

AD is the most common cause of dementia, representing 60-80% of all cases [6]. After 65 years of age the incidence doubles every fifth year [7] and the approximate prevalence is 5% in the population 60 years of age and older [8]. Alois Alzheimer, in a paper published in 1907, first described a patient with impaired episodic memory, disorientation and dysphasia [9]. Neuropathological investigation revealed symmetric atrophy of the brain and depositions of neurofibrils and extracellular miliary foci, so called plaques [9].

AD is a progressive disease with neurodegeneration early affecting the medial temporal lobe, including the *hippocampus* and entorhinal cortex [10, 11], and early synaptic pathology [12]. The pattern of neurodegeneration translates symptomatically and presents with an impaired episodic memory, aphasia, apraxia and/or agnosia [13].

To date there is no available treatment for AD. The identification of the primary component of plaques, the amyloid β (Aβ) peptide [14, 15] found to originate from the amyloid precursor protein (APP) [16-18], lead to the formulation of the "*amyloid cascade hypothesis*", stating Aβ to be the instigator and driver of the disease [19]. The principle focus of drug development has been to target the production or facilitate the removal [20-22] of the potentially toxic Aβ peptides [23-25]. Symptomatic treatments exist and include acetylcholinesterase inhibitors and a N-methyl-D-aspartate receptor antagonist [26].

1.1.1.1 Pathology

Neuropathological characterisation in AD reveals amyloid plaques and neurofibrillary tangles containing aggregated Aβ [14, 15] and hyperphosphorylated and truncated tau protein [27-29], respectively. APP is processed in an amyloidogenic pathway by β-secretase [30] and subsequently γ-secretase [31, 32], generating a range of Aβ peptides including an aggregation-prone 42 amino acid-long variant ($A\beta_{1-42}$). APP has been suggested to be involved in cell adhesion, neurogenesis and neurite outgrowth [33]. Physiological functions of Aβ peptides remain largely unknown, however have been suggested to have neuroprotective properties and at low concentrations enhance long term potentiation [34]. Tau binds to and stabilises microtubules, an interaction regulated by phosphorylation [35]. Hyperphosphorylation and truncation of tau cause its release from microtubules, which destabilises axons and enables aggregation of tau into neurofibrillar tangles [35].

When examining suspected AD neuropathologically, the presence, distribution and frequency of amyloid plaques and neurofibrillary tangles are determined [36-39]. Appearance of neurofibrillary tangles follows a pattern concurring with developing symptomology by progression from subcortical to cortical regions [40]. In the opposite direction amyloid plaques appear from cortical to subcortical regions [37]. Pathology is also found in cognitively healthy individuals, indicating a preclinical phase of the disease [41]. Pathologic onset may precede symptomatic onset by decades.

1.1.1.2 Diagnosis

The diagnostic criteria presented in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) provided a framework describing probable AD with the requirement of dementia, progressive impairment of memory and cognitive function and absence of other disease, systemic or in the brain, causing the symptoms [13]. A specificity and sensitivity of 80% and 70%, respectively, have been shown for the 1984 criteria [42], but this varies between clinics [43]. Revised criteria were later proposed by the National Institute on Aging-Alzheimer's Association (NIA-AA) [44]. A new definition of dementia was presented, and is required in combination with an insidious onset, progressive development, an amnestic and/or a nonamnestic presentation for the diagnosis of probable AD [44]. According to these criteria definite AD can only be confirmed by histopathologic evidence.

In 2007, the international working group (IWG) presented diagnostic criteria for probable AD to be used for research purposes which included the supportive use of biomarkers; brain imaging and biochemical measurements in cerebrospinal fluid

(CSF) [45]. Further refinement has been made in the IWG-2 criteria [46], now requiring one of the following three biomarkers to be positive; 1) a low concentration of CSF $A\beta_{1-42}$ and high total tau (T-tau) or phosphorylated tau (P-tau); 2) positron emission tomography (PET) showing increased retention of an amyloid radioligand; or 3) an autosomal dominant mutation. Biomarkers have also been included in the criteria presented by NIA-AA where they have a supportive role or are suggested to be used for research purposes [41, 44, 47].

A treatment would be expected to be most efficient in the early stages of disease, in the preclinical and prodromal phases of disease. For drug development and research purposes efforts to define the early stages of disease have been made where the preclinical phase requires biomarkers indicating amyloid pathology and neurodegeneration [41, 46]. The prodromal phase, including mild cognitive impairment (MCI) [48], is similarly supported by biomarkers [47].

1.1.1.3 Heritability and Risk Factors

Although sporadic AD accounts for most cases, there are also autosomal dominant mutations causing early onset familial AD. These mutations are found in genes encoding proteins associated with Aβ pathology, including, *APP* [49], and the γ-secretase subunit-encoding presenilin 1 (*PSEN1*) and 2 (*PSEN2*) genes [50-52]. However, mutations in the *APP* and *PSEN* genes are found in less than 1% of cases [53].

The most prominent risk factor for developing AD, apart from aging, is the apolipoprotein E-encoding ε4 allele of the *APOE* gene [54], which in a gene dose-dependent manner increases the risk and decreases age of onset. Around 15% of the general Swedish population are ε4 allele carriers [55]. Interestingly, the *APOE* ε2 allele has been found to be protective [56]. The most common allele is ε3 (55-60% of the Swedish population are homozygous ε3 carriers [55]) which is neutral in terms of AD risk [56]. An increased risk has also been associated with polymorphisms in the *CR1* (complement receptor type 1), *CLU* (clusterin), and *PICALM* (phosphatidylinositol-binding clathrin assembly protein) genes [8]. Lifestyle risk factors include comorbidities associated with a sedentary lifestyle, including cardiovascular disease and type 2 diabetes [8]. Protective are factors associated with an active physical and social life, as well as educational level, providing a cognitive reserve delaying symptomatic disease onset [8].

1.1.1.4 Biomarkers

CSF has proven a useful biological fluid for identifying biomarkers in AD. CSF is produced by the choroid plexus in the ventricles of the brain, as well as from the brain interstitial fluid, and flows into the subarachnoid space surrounding the spinal cord and brain [57]. Thus, the central nervous system is bathed in CSF. An adult

produce approximately 600 mL CSF per day having a total volume of 150 mL at any given moment. Reabsorption of CSF occurs through arachnoid villi draining into venous sinuses [57]. Production of CSF is facilitated in a regulated manner at the blood-CSF barrier, the choroid plexus, where an epithelial cell layer, connected by tight junctions, maintains an osmolarity gradient [57]. CSF stands in contact with the brain *parenchyma* by ependymal cells in the ventricles and at the pial-glial membrane of the brain allowing for regulation of the contents of the interstitial fluid [57]. CSF is accessible through lumbar puncture which is a standardised procedure. There is a risk of post-lumbar puncture headache, however it is not very frequent [58].

The AD CSF core biomarkers are $A\beta_{1-42}$, T-tau and P-tau [59]. The level of $A\beta_{1-42}$ in CSF correlates inversely with accumulating plaque load in the brain [60, 61]. CSF T-tau level reflects neuronal and axonal degeneration [62, 63] and is associated with disease intensity and progression rate [64-66]. P-tau levels in CSF correlate with tangle load in the brain [67]. Numerous studies have shown lower concentrations of CSF $A\beta_{1-42}$, and higher concentrations of T-tau and P-tau in AD compared to controls and in MCI due to AD (MCI-AD) compared to those with MCI remaining stable (sMCI) [68]. Using CSF $A\beta_{1-42}$, T-tau and P-tau have shown a sensitivity and specificity of 95% and 87%, respectively, for discriminating MCI-AD from sMCI [69].

To distinguish AD from other neurodegenerative disorders or vascular disease, structural imaging could be useful. Magnetic resonance imaging (MRI) shows atrophy of the entorhinal cortex and *hippocampus* in the preclinical and prodromal phases of AD [10, 11, 70]. Although absolute hippocampal volume is lower in AD compared to controls, hippocampal volume decrease with aging similarly in both groups [71]. Retention of the radioligand 2-[18F]-fluoro-2-deoxy-D-glucose (18F-FDG) in the brain is due to decreased tissue metabolism and can be visualized by PET [72]. In AD, increased 18F-FDG retention is seen in the posterior cingulate, parietotemporal and prefrontal association cortices [73, 74]. In preclinical AD, 18F-FDG retention is seen in the *hippocampus* and progress to the cortices with disease development [75]. Furthermore, accumulating Aβ in the brain in AD can be shown using PET and an Aβ binding radioligand, Pittsburgh compound B [76]. Tau radioligands are under development, however poses problems as tangles exists intracellularly and fibrillar tau adopts complex conformations [77]. A tau ligand, ^{18}F-AV-1451, binding tau deposits, has however been shown to associate with disease stage, neurodegeneration and cognitive decline in AD [78].

1.1.2 Parkinson's Disease

In 1817, PD was described by James Parkinson, however then referred to as shaking palsy [79]. Shaking palsy was described as a slow progressing disease with tremor

and fatigue at rest, bent forward posture, a propensity to pass into running from walking, however with spared cognition [79]. PD mainly affects dopamine-producing ("dopaminergic") neurons in *substantia nigra*, which produces the classical motor symptoms [2]. Additionally there are non-motor symptoms, including for example olfactory dysfunction, cognitive impairment and rapid eye movement sleep behaviour disorder [2]. Non-motor symptoms can present in a prodromal stage of disease [80].

PD is the second most common neurodegenerative disease with a prevalence of 1% in the population 60 years of age and older [81]. The approximate lifetime risk is 2% for men and 1% for women [82] and mean age of onset is 70 years [83]. A number of other diseases commonly present with Parkinson-like motor symptoms and are collectively referred to as parkinsonian disorders. Progressive supranuclear palsy (PSP), of which there are several disease subtypes [84], is a parkinsonian disorder presenting with, for example, supranuclear gaze palsy, slowing of vertical saccades, postural instability, falls and poor responsiveness to levodopa [85].

1.1.2.1 Pathology

The main pathological finding in PD are intraneuronal Lewy bodies containing an aggregated form of α-synuclein [86]. Lewy bodies are also the principle pathological component of dementia with Lewy bodies [87-89] and multiple system atrophy [88]. In PD, Lewy body pathology appears in six suggested stages from the brain stem, through the basal ganglia and *substantia nigra*, through the mesocortex and into the cortex [90]. Neuronal loss occur in the brainstem, midbrain, including the ventrolateral region of *substantia nigra pars compacta*, basal forebrain, *amygdala* and *hypothalamus* [2].

PSP includes a pathological heterogeneous spectrum of subtypes [84]. However, PSP is a tauopathy with primarily four repeat tau forming neurofibrillary tangles, oligodendral coiled bodies and tufted astrocytes [91]. Pathological involvement includes subcortical regions; *globus pallidus*, subthalamic nucleus, *substantia nigra*, *locus coeruleus* and the dentate nucleus of the *cerebellum* [91]. In subjects with cognitive impairment there is cortical tau pathology [92]. Atrophy is noticeable in the midbrain and mild in the frontal cortex [91].

1.1.2.2 Diagnosis

The National Institute of Neurological Disorders and Stroke (NINDS) diagnostic criteria for PD include staging in possible, probable and definite PD [93]. Possible PD display two of the following symptoms; bradykinesia, rest tremor, rigidity and/or asymmetric onset. Bradykinesia or rest tremor is needed. Additionally, there must not be any features suggestive of alternative diagnoses and there is a substantial response to levodopa. Probable PD includes three of the following symptoms; bradykinesia,

rest tremor, rigidity and/or asymmetric onset [93]. Additionally, symptoms have to be present for three years with no feature suggestive of alternative diagnoses and there is a substantial response to levodopa. A diagnosis of definite PD requires the possible PD criteria to be met in addition to histopathological confirmation [93]. The NINDS diagnostic criteria for possible PD perform with a positive predictive value and sensitivity of 93% and 87%, respectively [94]. Comparably, the criteria for probable PD show a positive predictive value of 92% and a sensitivity of 72% [94].

1.1.2.3 Heritability and Risk Factors

In PD, approximately 14% report a family history of disease [83]. Less than 10% of familial cases are caused by monogenic mutations [83]. Autosomal dominant late-onset PD is caused by mutations in for example *SCNA* (α-synuclein) [95], *LRRK2* (leucine-rich repeat serine/threonine-protein kinase 2) [96, 97] and *VPS35* (vacuolar protein sorting-associated protein 35) [98]. Recessive inherited early-onset PD is caused by mutations in *PRKN* (Parkin) [99] and *PINK1* (PTEN-induced putative kinase protein 1) [100, 101], *PARK7* (DJ-1) [102] and *ATP13A2* (cation-transporting ATPase 13A2) [103]. Genetic predisposition in PD is linked to mutations in the *GBA* gene coding for the lysosomal enzyme, β-glucocerebrosidase [104, 105]. Heterozygous carriers of *GBA* mutations have a five-fold increased risk of developing PD [104, 105]. *GBA* mutations cause Gaucher's disease, a lysosomal storage disorder [106]. Additional polymorphisms, for example in genes *SMPD1* (sphingomyelin phosphodiesterase) and *CTSD* (cathepsin D, CatD) associated with lysosomal storage disorders, have been linked to the risk of developing PD [107]. Exposure factors contributing to an increased risk of PD are certain pesticides, dairy products and traumatic brain injury, whereas protective factors include smoking, caffeine and physical activity [82].

1.1.2.4 Biomarkers

There are no biomarkers used in the diagnosis of PD. Biomarkers aiding diagnosis of prodromal PD would be valuable in accurate identification of subjects and development of treatments [108]. Investigations of CSF α-synuclein levels have shown modest decrease in PD compared to controls and AD in most studies [109-111]. The CSF level of DJ-1 has been suggested to be elevated [112] or decreased [111] in CSF in PD compared to controls. Furthermore combining CSF tau and DJ-1 might aid in differentiation between PD and the parkinsonian disorder, multiple system atrophy [113]. Indeed, combinations of CSF biomarkers including AD CSF core biomarkers, neurofilament light chain and α-synuclein have shown potential in differentiating PD from parkinsonian and dementia disorders [110, 114]. Furthermore, being a PD risk factor [104, 105], the activity of lysosomal β-glucocerebrosidase has been investigated in CSF and show lower activity in PD compared to control groups [115-117]. Additional lysosomal enzymes, CatD [117,

118] and β-hexosaminidase [115-118], have shown CSF activities with conflicting results.

There are imaging techniques to investigate dopamine terminal dysfunction due to degeneration of dopaminergic neurons in the *substantia nigra* using radioligands and PET or single photon emission computed tomography [2]. However such techniques are not able to separate PD from other disorders with degeneration of the *substantia nigra* [2].

1.1.3 Frontotemporal Dementia

FTD is a syndrome including a spectrum of clinical presentations. FTD results from frontotemporal lobar degeneration (FTLD), which displays a pathological complex pattern of regional neurodegeneration [119] and molecular neuropathology [120]. FTLD research emerged from the initial pathologic and symptomatic descriptions by Arnold Pick in the late 19th century [3]. Clinically, FTD can be divided into behavioural or language type presentations. Behavioural variant FTD (bvFTD) [121], representing 57% of FTD cases [122], presents with for example disinhibition, loss of empathy and executive impairment [121]. There are three primary subgroups of FTD with language impairment, primary progressive aphasia (PPA) [123]; logopenic variant (lvPPA), nonfluent variant (nfvPPA) and semantic variant PPA (svPPA) [124].

FTD is the third most common type of dementia after dementia due to AD and vascular disease in individuals younger than 65 years of age [125]. In FTD, age at onset typically occur in the sixth decade of life [122, 126-129]. The prevalence and incidence ranges between 15 and 22, and 3 and 4 per 100 000 individuals, respectively [122].

1.1.3.1 Pathology

In FTLD, molecular pathology is classified according to the nature of accumulating protein inclusions [120]. Forty percent of FTLD cases are classified as FTLD-tau [3, 120] with inclusions of hyperphosphorylated tau [130]. FTLD-tau is a pathologic feature of for example Pick's disease, PSP and corticobasal degeneration [131]. In FTLD-TDP there are cytoplasmic inclusions and dystrophic neurites positive for transactive response DNA-binding protein of 43 kDa (TDP-43) [132, 133], ubiquitin [132, 133] and p62 [134]. Additionally there are four subtypes of FTLD-TDP; A, B, C and D [135]. Type A show dystrophic neurites and cytoplasmic inclusions primarily in top cortical layers [135]. Type B display moderate numbers of cytoplasmic inclusions and low numbers of dystrophic neurites in all cortical layers [135] and in addition cytoplasmic inclusions in lower motor neurons [120]. In type C, in superficial cortical layers, there are long dystrophic neurites and few cytoplasmic inclusions [135]. Finally, type D shows short dystrophic neurites and intranuclear

inclusions in all cortical layers [135]. In FTLD-FET, representing 15% of all FTLD [120], there are inclusions of fused in sarcoma protein [136], Ewing's sarcoma protein [137] and TATA-binding protein-associated factor 15 [137]. In individuals with *CHMP2B* (charged multivesicular body protein 2B) mutations [79], there is FTLD-UPS pathology [138]. FTLD-UPS pathology is characterized by cytoplasmic tau- and TDP-43-negative, and ubiquitin- and p62-positive inclusions, frequent in hippocampal neurons and less frequent in frontal and temporal cortical neurons [138].

1.1.3.2 Diagnosis

Criteria for behavioural type FTD diagnosis was presented by the Lund and Manchester groups in 1994 [139]. In 1998, there was an update on the criteria for behavioural type FTD which further incorporated criteria for language impairments, progressive nonfluent aphasia and semantic dementia with aphasia and agnosia, due to FTLD [140].

The International Behavioural Variant FTD Criteria Consortium (FTDC) presented revised criteria for bvFTD incorporating possible bvFTD, probable bvFTD and bvFTD with definite FTLD pathology [121]. A bvFTD diagnosis requires a progressive decline in behaviour and/or cognition. In short, in possible bvFTD three of the following symptoms need to present; disinhibition, apathy, loss of empathy, compulsive behaviour, hyperorality and/or executive deficits. In addition to the symptoms required for possible bvFTD, in probable bvFTD there is a significant functional decline and for example MRI showing frontal and/or anterior temporal lobar atrophy. In bvFTD with definite FTLD pathology there is additionally known disease causing mutations or histopathological evidence.

The criteria for the three primary variants of PPA (lvPPA, nfvPPA and svPPA) were refined by Gorno-Tempini *et al*. [124]. In short, lvPPA shows word retrieval and word repetition impairment, nfvPPA displays impaired language production, and svPPA presents with impaired word comprehension. These subtypes are supported by for example MRI showing atrophy of the left posterior perisylvian or parietal lobe in lvPPA, left posterior frontoinsular in nfvPPA, and anterior temporal lobe in svPPA. Similar to bvFTD, definite pathology is concluded by known disease causing mutations or histopathological evidence.

1.1.3.3 Heritability and Risk Factors

FTD is associated with a large genetic component where a family history exists in 40% of cases [126-128] and a familial cause in more than 10% of cases [126, 127]. Familial FTD is most frequently [127, 141, 142] caused by a hexanucleotide expansion of the *C9ORF72* (chromosome 9 open reading frame 72) gene [143, 144], *GRN* (progranulin) mutations [145, 146] and *MAPT* (microtubule-associated protein

tau, or simply tau) mutations [147]. Less frequent familial causes are for example mutations in the genes *VCP* (encoding valosin-containing protein) [148] and *CHMP2B* [79]. Additionally, genetic alterations in *TMEM106B* (encoding transmembrane protein 106B) has been identified as a risk factor for FTLD [149]. Possible non-genetic risk factors include head trauma and thyroid disease [122].

1.1.3.4 Biomarkers

There are no fluid biomarkers used in FTD diagnosis to date. However, imaging by MRI showing atrophy and hypoperfusion or decreased tissue metabolism by PET, are supportive [121, 124]. CSF $A\beta_{1-42}$ is lower, and T-tau and tau phosphorylated at Thr 181 ($P\text{-}tau_{181}$) are higher in AD compared to FTD [150]. However, the CSF level of the shorter $A\beta_{1-38}$ has been indicated to be decreased in FTD compared to controls and AD [151, 152]. On the contrary, CSF level of neurofilament light chain is higher in FTD compared to controls and AD [153, 154]. CSF level of neurofilament light chain is associated with disease severity [154, 155]. Additionally the CSF level of neurofilament light chain has been shown to be elevated in *GRN* mutation carriers compared to *C9ORF72* and *MAPT* carriers [156]. Furthermore, the ratio of CSF $P\text{-}tau_{181}$ to T-tau levels is lower in subjects with FTLD-TDP pathology compared to FTLD-tau [157, 158]. However, studies comparing potential CSF biomarkers in clinical subtypes of FTD are limited [159]. Recently, neurofilament light concentration in blood has emerged as a promising biomarker for the intensity of the neurodegenerative process in FTD, irrespective of the underlying molecular cause [160].

1.2 Proteostasis in Health and Disease

Proteostasis is primarily maintained by the degradation of proteins and organelles by the autophagic and endo-lysosomal system [161, 162] and the ubiquitin-proteasome system [163] (Figure 1). These systems are fundamentally important in neurons which are post-mitotic cells, requiring lifelong environmental maintenance, and in addition promote neuronal development, plasticity, survival and synaptic function [162, 164-166]. The autophagic and endo-lysosomal system and the ubiquitin-proteasome system are not simply separate entities as substrates and components are shared and regulatory components of one system are degraded by the other and vice versa [167]. Additionally there are compensatory mechanisms where autophagy offer protection following proteasomal inhibition [168, 169], however autophagic inhibition disrupts proteasomal degradation [170].

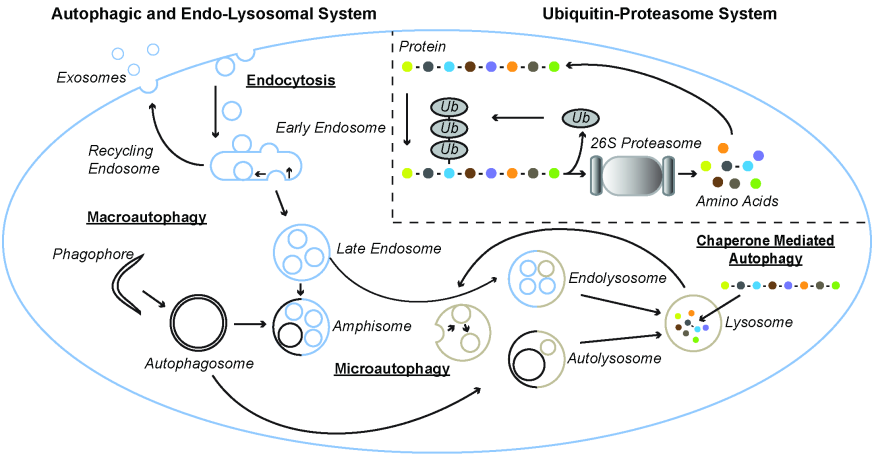

Figure 1. Overview of the primary systems in proteostasis. In the autophagic and endo-lysosomal system substrates are delivered for degradation by the lysosome through endocytosis or autophagy. In the ubiquitin-proteasome system substrates are labeled by ubiquitin and targeted for degradation by the 26S proteasome.

1.2.1 The Autophagic and Endo-Lysosomal System

The autophagic and endo-lysosomal system governs the engulfment of extra- and intracellular substrates through endocytosis and autophagy, respectively, for the delivery to and degradation by the lysosome. The system is an intricate vesicle system with continuous vesicle maturation and vesicle fusions with the purpose to introduce the substrates to the lysosomal lumen as well as to maintain and propagate the vesicle population.

1.2.1.1 Lysosome

The lysosome was first described by Christian de Duve in 1955 [171]. The lysosome is an organelle enclosed by a phospholipid bilayer with an acidic environment of pH 4.5-5 [172]. The lysosomal acidity is maintained by a v-ATPase proton pump and the degradative ability of the lysosome is conducted by more than 60 hydrolases, digesting proteins, peptides, lipids, glycosides *etc*. [172]. Digested components are actively transported out of the lysosome by membrane proteins [173]. Fifty percent of the membrane proteins are constituted by highly glycosylated proteins, lysosome-associated membrane protein 1 and 2 (LAMP1 and LAMP2) and lysosomal integral membrane protein 1 and 2, which forms an intraluminal glycocalyx protecting the membrane and membrane proteins from digestion [173]. Lysosomal biogenesis and maintenance is dependent on a continuous process of endosomal maturation, fusion of late endosomes (LE) and lysosome, and trans-Golgi network delivery of lysosomal proteins [174]. Lysosomes may fuse with the plasma membrane, which is a secretory path for conventional lysosomes triggered by increased cytosolic Ca^{2+}, providing membrane for plasma membrane repair [175]. Additionally, in specialised cells such

as melanocytes and mast cells, secretory lysosomes secrete melanin and histamine, respectively [176].

1.2.1.2 Endocytosis

Endocytosis covers numerous routes of entry into the cell [177]. In macrophages and neutrophils, phagocytosis is one such specialized route. Ubiquitous routes of endocytosis include *e.g.*, clathrin-mediated endocytosis, caveolae-mediated endocytosis and micropinocytosis. Primary endocytic vesicles formed through endocytosis fuse with the early endosome (EE) (Figure 1), a morphologically heterogeneous tubular and vacuolar structure [161]. From here cargo can be directed for recycling to the plasma membrane through recycling endosomes [178] or towards degradation by sorting into maturing LE. The maturation and formation of LE from EE, involves the conversion and exchange of the Rab GTPases, Rab5 to Rab7, which promote an accumulation phosphatidylinositol 3,5-bisphosphate and recruitment of the necessary fusion machinery [161]. LEs are also referred to as multi-vesicular bodies, due to having numerous intraluminal vesicles. These intraluminal vesicles are formed in the EE and LE by the endosomal sorting complex required for transport (ESCRT) machinery, consisting of four complexes (ESCRT-0, -I, -II and -III), directed by ubiquitinated membrane proteins and phosphatidylinositol 3-phosphate [179]. The membrane and contents of the intraluminal vesicles becomes readily degradable by the lysosomal hydrolases. Lysosomal hydrolases and membrane proteins are delivered to the endocytic pathway through mannose-6-phosphate receptor dependent or independent routes allowing for maintenance of the lysosomal population and lysosomal biogenesis [174]. LE either fuse or mature into lysosomes [180] or fuse with the plasma membrane and expel the intraluminal vesicles as exosomes [181].

1.2.1.3 Autophagy

Self-eating or autophagy is the process of facilitating digestion of cytosolic components. There are three types of autophagy; macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy.

Macroautophagy is the process where a double membrane vesicle is formed around the substrates to be degraded. Macroautophagy and the formation of the autophagosome occurs in four stages; induction, nucleation, elongation and fusion [182]. The induction phase is initiated by a protein complex regulated and activated by for example nutritional status and starvation [182]. This is followed by the recruitment of the nucleation complex, which is a phosphatidylinositol 3-kinase complex, producing phosphatidylinositol 3-phosphate [182]. Next, elongation and fusion is driven by the activity of two ubiquitin-like conjugating systems, which conjugate phosphatidylethanolamine and microtubule-associated proteins 1A/1B light chain 3 (LC3) [183]. After completion, the autophagosome fuse with either the

endosome to form an intermediate amphisome, or directly with the lysosome to form an autolysosome (Figure 1). Autophagy is often considered a route for *in bulk* digestion of cytosolic contents, however autophagy can be selective as exemplified by reticulophagy (endoplasmic reticulum) [184], mitophagy (mitochondria) [185], lipophagy (lipid droplets) [186], or aggrephagy (protein aggregates) [187]. In aggrephagy p62 recruits ubiquitinated protein aggregates and interact with LC3 [188, 189]. Similarly, dysfunctional mitochondria are targeted for mitophagy [190, 191].

CMA is the process where proteins containing a Lys-Phe-Glu-Arg-Gln motif, or chemically equivalent, are targeted for degradation [192]. The motif is recognized by the chaperone protein heat shock-cognate protein of 70 kDa (hsc70) which recruits the target to the lysosomal transmembrane protein LAMP2. Upon target binding LAMP2 multimerise [193] and the substrate is translocated into the lysosomal lumen. LAMP2 exists in three isoforms; A, B and C [194], of which LAMP2A is responsible in CMA [192]. LAMP2 constitute the rate limiting step of CMA [195], which has been shown to decrease with normal ageing [196]. CMA is upregulated in response to starvation and inhibition of macroautophagy or the proteasome [192].

Similar to CMA, microautophagy occurs at the lysosomal membrane which bud inwards and form vesicles to be degraded [197]. The process has been better characterised in yeast compared to mammalian cells [197].

1.2.2 The Ubiquitin-Proteasome System

The ubiquitin-proteasome system is an evolutionary conserved pathway for protein degradation where substrates are labelled by ubiquitin and targeted for destruction by the 26S proteasome [198, 199]. Ubiquitin [200] was first isolated from bovine thymus by Goldstein *et al*. [201]. Independently, a polypeptide was isolated, a component identified to be involved in ATP-dependent proteolysis [202, 203], which was later confirmed to be ubiquitin [204, 205]. The 26S proteasome is a protein complex consisting of a cylindrical 20S core particle, with protease activity producing short peptides, and a gating 19S subunit, containing ubiquitin receptor and deubiquitination activity [206]. However, there is also 20S and 26S proteasome ubiquitin independent degradation [207].

Ubiquitin is conjugated to protein substrates [208, 209] through the action of E1-activating enzymes [210], E2-conjugating enzymes and E3 ligases [211]. The process requires ATP and produces a thiolester intermediated [210] forming an iso-peptide bond between the N-terminal Gly of ubiquitin and Lys in substrate proteins [208]. The E3 enzyme is primarily responsible for selecting the substrate to which ubiquitin will be covalently attached [212]. The human genome encodes two ubiquitin E1-activating enzymes, approximately 40 E2-conjugating enzymes and more than 600

E3 ligases [213]. Additionally, ubiquitination is reversible and ubiquitin can be removed from substrates by deubiquitinases [214].

Ubiquitin is a post-translational modification existing as mono- or polyubiquitin chains [215]. Apart from targeting substrates for the proteasome, ubiquitin is also involved in for example regulating endocytosis and degradation of membrane receptors [216], and regulating transcription [217].

1.2.3 Proteostasis in Neurodegeneration

Dysfunctional proteostasis is a pathological feature of most neurodegenerative diseases [218-220]. Elimination of key components in autophagy [221, 222] and the proteasome [223] results in neurodegeneration and accumulation of ubiquitin-positive protein inclusions. The importance of functional proteostasis in neurons is exemplified in lysosomal storage disorders frequently presenting with neurological complications [224]. In lysosomal storage disorders, accumulation of substrates occurs primarily due to dysfunctional transport or lysosomal degradation exemplified by increased retention of cholesterol and glycosphingolipids in Niemann-Pick type C disease and decreased degradation of GM2 gangliosides in Sandhoff disease [225].

A common feature of neurodegenerative disorders is the accumulation of protein aggregates [226], suggesting protein production or turnover to be impaired. Protein aggregates inhibit the proteasome [227], including tau [228] and Aβ [229-231]. CMA has been implicated in the degradation but is also inhibited by proteins associated with a number of neurodegenerative diseases including tau [232], α-synuclein [233-235], LRRK2 [236] and ubiquitin carboxyl-terminal hydrolase isozyme L1 [237]. Macroautophagy, or aggrephagy [187], is involved in the degradation of protein aggregates [188, 189], huntingtin [188] and tau [228, 238]. In healthy neurons autophagosomes are infrequently observed, suggesting a rapid turnover and fusion with lysosomes [239]. However, in AD there is a pathological accumulation of intraneuronal autophagic vacuoles, indicating impaired turnover [239]. Thus an important route for maintaining proteostasis is impaired.

The proteolytic machinery display a decline in function with normal aging [240], having implications in age associated neurodegenerative diseases. Life span is affected by proteasomal activity [241] and autophagy [242, 243], and can be extended by upregulation of these systems [241-243]. Adding to this, caloric restriction, which activates autophagy, extends the life span in mice [244, 245]. With aging, in post-mitotic cells, there is an accumulation of undegradable lipofuscin in lysosomes [246]. The lipofuscin laden population of lysosomes might not be able to effectively engage in conventional degradation [246]. The rate of CMA also decreases with normal aging [196], possibly due to altered lysosomal membrane composition [247]. Intervention to induce or activate the proteolytic machinery in

neurodegenerative disorders might however be problematic depending on whether induction or turnover is failing. Collectively, maintaining proteostasis is fundamental in health and disease of the central nervous system.

1.2.3.1 Proteostasis in AD

In sporadic AD, there is an intraneuronal enlargement of early endosomes [248, 249], suggested to occur at a preclinical stage of disease [248], as well as an accumulation of pre-lysosomal autophagic vesicles [239]. These alterations are accompanied by an increased deposition of lysosomal hydrolases (*e.g.*, cathepsin B (CatB) and CatD) [250-252] and expression of regulators of endosomal vesicle trafficking and maturation (*e.g.*, Rab4, Rab5 and Rab7) [253-255]. APP processing to Aβ has been shown to occur following endocytosis [256]. After endocytosis, APP is sorted into intraluminal vesicles of LEs and is subsequently degraded [257, 258], accomplished in part by CatD [259-261]. Missorting of APP might enhance the amyloidogenic pathway [257, 258]. β-secretase localises to early and recycling endosomes [262, 263] and co-localise with APP in endocytic vesicles after neuronal stimulation [263]. Endocytic recycling of β-secretase replenishes the pool of plasma membrane β-secretase necessary for continuous Aβ production [264]. γ-secretase exists in the lysosomal membrane [265] where it cleaves APP [266].

In neurons, after formation of autophagosomes and endocytic vesicles in distal neurites, these vesicles are transported in a retrograde manner to fuse with lysosomes at the perikaryon. However, in AD there is a disruption of this transport and vesicles accumulate within neurites [239, 267-270]. These vesicles do not have the degradative ability of the lysosome; however contain the necessary components for Aβ production [267, 268]. Neurites with accumulating vesicles might thus provide potent sites for Aβ production [271-273] and can be found in association with plaques [267, 268].

Also the ubiquitin-proteasome system is affected in AD. The proteasomal activity is reduced in AD within several regions of the brain associated with pathology [228, 274]. There is an inhibitory effect on the proteasome of protein aggregates [227], tau [228] and Aβ [229-231]. Furthermore, in the cortical regions there is an increased deposition of ubiquitin [275, 276]. Interestingly, in AD an ubiquitin variant with a 19 amino acid-long C-terminal extension has been found and is caused by a dinucleotide deletion in the transcript [277]. The extended ubiquitin variant is ubiquitinated for degradation, however in this state inhibit proteasomal function [278].

Through genome-wide association studies two genes involved in endocytosis [279, 280], *BIN1* (myc box-dependent-interacting protein 1) and *PICALM*, have been found to confer an increased risk for developing AD [281, 282]. Such findings further indicate endocytosis to be involved in the pathological processes of AD.

1.2.3.2 Proteostasis in PD

In PD, there is an increased number of autophagic vacuoles in neurons of the *substantia nigra* [283]. In inducible models of disease there is also an increase of autophagic vacuoles and accompanied decrease of lysosomes, supported by increased levels of LC3 and decreased LAMP1 [284]. Similarly, in the *substantia nigra* of PD subjects the protein levels of LAMP1 [284], LAMP2 and hsc70 [285] are lower and LC3 higher [284, 285] compared to controls. Furthermore the amount of CatD, LAMP1 [286] and LAMP2 [287] has been shown to decrease in neurons with accumulating amounts of α-synuclein. Collectively, these alterations indicate that there is an impairment of vesicle turnover and autophagic flux.

Lysosomal function in neurons is important as indicated by pathology in the central nervous system in lysosomal storage disorders [224]. There is a link between genetic alterations associated with lysosomal storage disorders and the risk of developing PD [107]. Such a link is genetic alterations in the *GBA* gene, contributing an increased risk of developing PD [104, 105]. β-glucocerebrosidase degrade glucosylceramide and glucosylsphingosine, and β-glucocerebrosidase deficiency cause Gaucher's disease [106]. The amount of β-glucocerebrosidase as well as activity is decreased in the *substantia nigra* of subjects with PD, both in *GBA* gene mutation carriers [288] and non-carriers [288, 289]. Furthermore, in PD, accumulation of α-synuclein is associated with decreased levels and activity of β-glucocerebrosidase [290]. Indeed α-synuclein has been indicated to inhibit β-glucocerebrosidase activity, causing accumulation glucosylceramide [291]. Glucosylceramide in turn has been shown to stabilise oligomeric α-synuclein species [291].

CMA is also implicated in PD and is involved in the degradation of α-synuclein [233, 234]. However overexpression of wild type α-synuclein [235], mutant α-synuclein [235, 292] or dopamine-modified α-synuclein [293] inhibit CMA. Inside the lysosome, CatB, CatD and cathepsin L/L1 (CatL1) are involved in the degradation of α-synuclein [294].

Additional involvement of proteostasis in PD is implicated by the monogenic disease causing mutations. For example LRRK2 [96, 97] regulate autophagy [295], Parkin [99] and PINK1 [100, 101] orchestrate mitophagy [190, 296, 297], and VPS35 is involved in endocytic trafficking [98].

1.2.3.3 Proteostasis in FTD

The pathological subtypes of FTLD share in common an accumulation of protein aggregates indicating dysfunctional proteostasis to be a pathological feature of disease [120]. Additional support is given by a large genetic component in disease [127] involving genes associated with the autophagic and endo-lysosomal system.

Autosomal dominant inheritance of FTD results from *GRN* mutations [145, 146] and GRN deficiency cause neuronal ceroid lipofuscinosis, a lysosomal storage disorder [298]. In a cohort, 8% of FTLD cases were found to be carriers of *GRN* mutations [127]. *GRN* is associated with lysosomal gene expression, biogenesis and size [299]. In *GRN* mutation carriers there is an increased expression of lysosomal associated proteins CatD, LAMP1 and LAMP2 and TMEM106B in the frontal cortex [300]. Additionally, in GRN deficient mice there is an increased expression of proteins CatB [301], CatD [300], CatL1, dipeptidyl peptidase 2 (DPP2), beta-hexosaminidase subunit beta (HEXB) [301], LAMP1 [300-302], TMEM106B [300] and tripeptidyl-peptidase 1 (TPP1) [301].

TMEM106B, a transmembrane protein localising to the LE and lysosome [303-305], has been identified as a risk factor for FTD [149], and is involved in lysosomal trafficking [306, 307]. The expression of TMEM106B is increased in *GRN* mutations carriers [300, 303]. In opposite to GRN deficient mice, TMEM106B deficiency cause a decrease in the level of lysosomal proteins including CatB, DPP2 and LAMP1 [301]. *TMEM106B* overexpressing cells display enlarged lysosomes [303, 305] with poor acidification [303] and reduced lysosomal degradation [305]. Also *TMEM106B* knock-out impair lysosomal acidification [301]. In turn lysosomal alkalisation increases the expression of TMEM106B [303, 304] and GRN [303, 304, 308]. In mice, the effects of GRN deficiency is in part reverted by knockout of *TMEM106B* [301]. GRN and TMEM106B indicate a central role of lysosomal function in FTLD.

Furthermore, involvement of autophagy and endosomal maturation and trafficking in FTD is implicated by the functions of additional proteins with genes harbouring disease causing mutations. C9ORF72 [143, 144] is involved in regulating autophagy [309-315]; CHMP2B [316] is a component of ESCRT-III [317] and mutations affect endosome-lysosome fusion [318]; and p62 [319] binds polyubiquitin-labelled protein aggregates [320] and facilitates aggrephagy [188].

2 AIM

2.1 General Aim

The aim is to examine the involvement of dysfunctional proteostasis in neurodegenerative diseases by developing novel assays for proteins involved in the autophagic and endo-lysosomal system and the ubiquitin-proteasome system as tools to study this pathological process in human cerebrospinal fluid.

2.2 Specific Aims

1. Examine lysosomal alterations in Alzheimer's disease by targeting the lysosomal membrane protein LAMP2 in cerebrospinal fluid as a potential surrogate marker for lysosomal status.
2. Examine alterations in the ubiquitin-proteasome system in neurodegenerative diseases by quantification of ubiquitin in cerebrospinal fluid.
3. Target a panel of endo-lysosomal proteins and ubiquitin in cerebrospinal fluid to examine alterations in the endo-lysosomal system the ubiquitin-proteasome system in neurodegenerative diseases

3 MATERIALS AND METHODS

3.1 Subjects and Sample Collection

3.1.1 Subjects

Subjects and samples have been recruited and collected, after providing written informed consent, in accordance with approvals given by regional ethical committees. Two principle groups of subjects have been included; biochemically or clinically characterised subjects.

Samples in two biochemically characterized cohorts (cohorts 1 and 2, see Table 1) have been collected after clinical routine analysis at the Clinical Neurochemistry Laboratory, Mölndal, Sweden. These samples have been used as pilot materials for method validation. Subjects have been defined as AD or controls by their CSF AD core biomarker profile [59], based on the CSF levels of; $A\beta_{1\text{-}42}$, T-tau and P-tau_{181}. The cohorts fulfil the IWG-2 biomarker criterion [46], having a low level of $A\beta_{1\text{-}42}$, and high level of T-tau and/or P-tau_{181}. The cut-off levels used has been; $A\beta_{1\text{-}42}$ ≤550 ng/L, T-tau ≥400 ng/L, and P-tau_{181} ≥80 ng/L. These cut-off levels are in line with previously defined levels [69, 321].

Clinically characterised subjects included a subpopulation of the Swedish BioFINDER study (www.biofinder.se) recruited at Skåne University Hospital, Sweden. Cohorts 3 and 4 (Table 1) from the Swedish BioFINDER study included cognitively healthy controls and subjects diagnosed with AD dementia according to the NINCDS-ADRDA criteria [13], PD according to the NINDS diagnostic criteria [93], and PSP according to the NINDS and Society for PSP International Workshop criteria [85]. Cohort 5 (Table 1) included participants recruited at the Center of Memory Disturbances of the University of Perugia, Italy. Subjects where diagnosed with AD according to the NIA-AA criteria [44, 47] and PD according to the NINDS diagnostic criteria [93]. Subjects where diagnosed as MCI according to the Petersen's criteria [48]. Of these subjects some developed AD (MCI-AD) and some remained stable (sMCI) over a follow up period. Cohort 6 (Table 1) included subjects with FTD disease subtypes, and controls being cognitively normal or with subjective complaints and where recruited from the Specialist Cognitive Disorders Service at the National Hospital for Neurology and Neurosurgery or from University College London FTD cohort studies, UK. The FTD disease subtypes included subjects diagnosed with bvFTD according to the FTDC criteria [121], and subjects with lvPPA, nfvPPA and svPPA according to the criteria devised by Gorno-Tempini *et al*. [124]. Additionally, the FTD subjects in Cohort 6 had been genotyped and included a

number of subjects with familial FTD, carrying disease causing mutations in *GRN* (N = 3), *MAPT* (N = 4) or hexanucleotide expansion in *C9ORF72* (N = 3).

All clinically characterised subjects included in Papers II-IV have been assessed by cognitive testing, psychiatric and neurological assessments in addition to brain imaging, by experts in neurodegenerative disorders. Furthermore all subjects with AD and MCI-AD fulfilled the IWG-2 biomarker criterion as described above. Controls and participants with sMCI had no more than one abnormal CSF AD core biomarker. No biomarker criterion was applied to participants with PD, PSP or FTD disease subtypes.

Table 1. Cohorts included in Papers I-IV.

Cohort	Paper I	Paper II	Paper III	Paper IV
1[a]	Controls (N = 14) AD (N = 14)	Controls (N = 15) AD (N = 9)	Controls (N = 10) AD (N = 7)	
2[a]		Controls (N = 15) AD (N = 14)	Controls (N = 14) AD (N = 12)	
3[b]		Controls (N = 45) AD (N = 37)	Controls (N = 44) AD (N = 36)	
4[b]		Controls (N = 11) PD (N = 15) PSP (N = 11)	PD (N = 11)	
5[b]			sMCI (N = 15) MCI-AD (N = 10) AD (N = 6) PD (N = 10)	
6[b]				Controls (N = 20) lvPPA (N = 15) bvFTD (N = 20) nfvPPA (N = 16) svPPA (N = 12)

[a]The cohort includes subjects biochemically characterised and selected based on their CSF AD core biomarker profile.

[b]The cohort includes clinically characterised participants.

3.1.2 CSF

In Papers I-IV CSF was collected in a standardised manner [322, 323]. Twelve mL of CSF was collected via lumbar puncture through the L3/L4 or L4/L5 interspace into polypropylene tubes. CSF was then centrifuged at 2000 g for 10 minutes at room temperature (Paper III, Study 3) [322] or +4 °C [323]. The supernatant was aliquoted and stored at −80 °C pending analysis.

3.2 Mass Spectrometry-Based Proteomics

In a cell, the proteome consists of the expressed proteins and their post-translational modifications. Proteins regulate cellular processes and functions by altering protein quantities, protein-protein interactions, and post-translational modifications. Exploring the proteome can inform us about the status of a cell and, potentially, in extension the status of an organism. Mass spectrometry (MS)-based proteomics offer a powerful approach to investigate the proteome [324]. Shortly, MS-based proteomics includes the following steps; sample preparation, sample separation and detection using a mass spectrometer.

MS-based proteomics has been the principal methodology adapted to answer the questions devised in Papers I through IV. A combination of explorative and targeted proteomics has been used to govern protein and peptide identification and quantification.

3.2.1 Sample Preparation

Sample preparation is prerequisite to be able to detect and quantify proteins in complex biologic matrices. In plasma the range of concentrations for low to high abundant proteins exceeds ten magnitudes. Under such conditions reducing the complexity of the sample can improve sensitivity 1 000-fold [325]. Alternatively, the physiochemical properties of the target protein or peptide can be exploited for selective enrichment. Although sensitivity is significantly improved, with each step of sample preparation there is a loss of the analyte as well as a trade-off in throughput and tentatively also repeatability.

3.2.1.1 Immunoprecipitation

Immunoprecipitation (IP) is used to reduce sample complexity by enriching the target protein or peptide using antibodies. An antibody is conjugated to a stationary phase, for example a column [326], well plate [327] or magnetic beads [328]. The sample is incubated with the antibody-complex followed by washing and elution. Combining selective enrichment using IP with detection by MS allows for the investigation of the full complexity of the enriched protein and can be used for studying protein-protein interactions.

In Paper I, LAMP2 was enriched from CSF using IP by conjugating a monoclonal anti-LAMP2 antibody (Abcam plc., Cambridge, UK) to magnetic beads with anti-mouse IgG antibodies (Dynabeads M280, Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA).

3.2.1.2 Solid-Phase Extraction

In practice solid-phase extraction (SPE) works as liquid chromatography (LC) and uses a stationary and liquid phase. There are stationary phases in silica or polymeric materials employing reversed phase, ion exchange or mixed mode extraction. In reversed phase the analyte is extracted according to hydrophobicity by being loaded onto the stationary phase using a polar liquid and then eluted using a non-polar liquid. In ion exchange, cation or anion, the extraction is based on net polarity. The affinity of the protein/peptide to the stationary phase is contested by charge competition and/or changing the pH. Mixed mode offer combinations of the above, mixing reversed phase with ion exchange. SPE has limited ability to selectively enrich an analyte, but is extensively used for removing salts and detergents, as well as for concentrating the sample.

In Papers II-IV Oasis hydrophilic-lipophilic balance (HLB, 96-well μElution Plate; 2mg sorbent and 30 μm particle size; Waters Co., Milford, MA, USA) has been used with minor modifications to the manufacturer's generic protocol. In a reversed phase manner samples have been loaded in a non-polar liquid and eluted using methanol. The HLB material carries, as the name implies, both hydro- and lipophilic characteristics.

3.2.1.3 Protease Digestion

Protein digestion using sequence specific proteases is employed in MS based proteomics to facilitate instrumental and data analysis [324]. Trypsin cleaves C-terminally of Lys and Arg generating peptides with masses suitable for MS analysis [329]. Having a C-terminal basic amino acid, tryptic peptides fragment into full y-ion series with identity informative high mass y-ions. Tryptic digestion have been applied prior to explorative and targeted proteomics in Papers I, III and IV.

3.2.2 Liquid Chromatography

To enable analysis of complex samples in conjunction with mass spectrometry, separation in a second dimension is typically employed. LC is such a separation approach where a mixture of molecules is separated according to physiochemical properties using a stationary and liquid phase. The stationary phase is found in a column, with different dimensions depending on application, packed with for example porous silica beads or monolithic material. In reversed phase chromatography there is a lining of non-polar molecules on the porous material, for example covalently attached carbon chains of differing length (C4, C8, C18 *etc.*). A non-polar stationary phase enables separation of molecules by polarity, by contesting the analytes affinity to the stationary phase when increasing the non-polar concentration of the liquid phase, commonly an organic fluid such as acetonitrile or methanol.

High-performance liquid chromatography is a conventional technique for liquid chromatography in proteomics operating at flow rates ranging from nano- to millilitres per minute at high pressures from tenths to several hundred bars. When reducing the particle size of the columns used in conventional high-performance liquid chromatography to less than 2 μm this will be referred to as ultra-performance chromatography [330]. Reducing the particle size has proven beneficial for throughput, resolution and sensitivity [330].

3.2.3 Mass Spectrometry

Using MS the mass to charge ratio (*m/z*) of charged particles is measured. The mass of a molecule is determined by its elemental composition. Thus MS provides the mean of identification. A mass spectrometer is a molecular scale consisting of three principal components; 1) an ion source, 2) one or more mass analysers, and 3) at least one detector. Most modern mass spectrometers also include some device for selecting and fragmenting ions to perform tandem mass spectrometry (MS/MS). The ion source produces ions in gas phase required for detection and introduction into the high vacuum of the typical mass analyser. The most commonly used ion sources in biological MS are electrospray ionisation (ESI) [331, 332] and matrix-assisted laser desorption ionisation [333-335]. The mass analyser provides the mean of separating ions by *m/z* and is exemplified by an orbitrap [336], time of flight [337] or a quadrupole [338]. The most common detector in a mass spectrometer is an electron multiplier. The orbitrap, in addition to being a mass analyser, also functions as a detector [336].

MS is a diversified tool in proteomics useful for both explorative hypothesis generating and targeted hypothesis driven experiments. MS has the potential of reflecting the full complexity of the proteome, including alternative splicing, post translational modification (*e.g.*, glycosylation and phosphorylation) and protein degradation.

3.2.3.1 Electrospray Ionisation

The principle of MS is to determine *m/z* of ions in gas phase. Thus, the means of transforming molecules in solid or liquid state to gas phase is needed. ESI [339] provides such means and was applied in conjunction with mass spectrometers [331, 332]. A key feature of ESI in proteomics is that ESI provide the possibility to analyse large biomolecules; peptides, polypeptides and proteins [332, 340]. Depending on utilised mass analyser, limiting the mass range, this is enabled by the generation of a large range of charge states. ESI is a soft ionisation method which makes it possible to detect post-translational modifications and protein-protein interactions.

In ESI, the liquid is emitted from a needle into a strong electric field (typically 1-5 kV potential applied over a few millimetres) at atmospheric pressure. The surface

tension of the emitted liquid is opposed by the electrostatic attraction at the other end of the field, forming an elliptic droplet at equilibrium. At a specific voltage threshold, the Taylor cone voltage, the equilibrium is disrupted and a Taylor cone is formed from which tip charged droplets spray [341]. Evaporation shrinks droplets and when the repulsive force between charges in the droplet exceeds the surface tension, the Rayleigh limit, smaller droplets are produced through Coulomb explosion. Ions in gas phase are formed by continuous evaporation and explosion of the droplets [342]. How ions are formed during the process has not been conclusively determined [343].

ESI is most often used in conjunction with LC at different flow rates depending on application. Low flow rates, less than 1 µL/min, offer high sensitivity and a large dynamic range, by producing small droplets increasing the ionisation efficiency [342] and limiting charge competition [344], and is suitable for explorative proteomics or when there is only small amounts of sample. Operating at flow rates above 1 µL/min, offer robustness and high throughput typically required in analytical or clinical assays. Higher flow rates require the assistance of a drying gas, typically nitrogen, and heating to aid evaporation.

3.2.3.2 Quadrupole Mass Filter

The quadrupole mass filter was first described in 1953 [338]. It operates with four opposing rods, forming pairs. The pairs exert a combined radiofrequency (RF) potential and direct current (DC) potential of opposing sign. At a given RF, RF potential and DC potential, only ions of a certain narrow *m/z* range will be able to pass through the quadrupole filter. The ratio between the RF and DC potentials determines the range of *m/z* able to pass. Ions pass through the quadrupole filter with a trajectory governed by attraction and repulsion by the alternating potential. Ions with an unstable trajectory, with an *m/z* too low or high, will not be able to travel through. Scanning the ratio of RF potential to DC potential enables acquisition over large ranges of *m/z*, however at the cost of sensitivity.

A linear arrangement of quadrupoles in a triple quadrupole (QqQ) mass spectrometer [345] enables MS/MS by having two quadrupole mass filters for isolation of precursor and product ions, respectively. These two quadrupoles are separated by a collision cell; a quadrupole, hexapole, or octapole, where fragmentation induced by a neutral gas, collision induced dissociation, is facilitated. An electron multiplier is often used as a detector in QqQ mass spectrometers. The narrow isolation and fast cycle times of the quadrupole enable selective and sensitive measurements and a broad dynamic range [346]. However, resolving power and mass accuracy is a limitation. Quadrupole mass filters are frequently combined with high resolution mass analysers, for example time of flight [347] and orbitrap [348] analysers.

3.2.3.3 Orbitrap Mass Analyser

The orbitrap [336] is a mass analyser providing orbital trapping of ions in an electrostatic field. The orbitrap consist of a symmetrical outer, barrel shaped electrode, and an inner spindle-like electrode. After injection of ions perpendicular to and at an offset to the axis of the electrodes the ions' attraction to the centre electrode acts as a centripetal force, forcing the ions into a circular motion around the inner electrode. Independent of initial energy the ions also oscillates in the axial direction at a frequency corresponding to *m/z*. The frequency of axial oscillation (ω) is described by equation 1 where z = charge, m = mass and k = force constant.

$$\omega = \sqrt{z/m \times \quad k} \qquad \text{(1, reference [336])}$$

The outer electrode is split into two, separated by an insulating material. This enables detection of an image current produced by the oscillating ions. The image current is amplified and *m/z* calculated from the frequency using Fourier transformation. Resolving power increases proportionally with time spent scanning but, decreases with increasing *m/z*. With today's commercial instruments a resolving power of 1 000 000 at *m/z* 200, can be achieved. Accurate measurements below 3 ppm can readily be achieved [349] or even sub-ppm using internal calibration [350]. Combining a quadrupole mass filter and an orbitrap analyser in hybrid instruments brings together narrow isolation, high resolving power and accurate measurements.

3.2.3.4 Explorative Proteomics

Protein and peptide identification is a key feature of MS enabled by for example peptide mass fingerprinting, peptide sequence tags or MS/MS. Two principle approaches can be used to identify proteins and investigate the proteome; bottom-up or top-down proteomics, analysing protease digested or full length proteins and endogenous peptides, respectively. When optimized, a single explorative data-dependent bottom-up analysis have the potential of identifying thousands of proteins and tenth of thousands peptides, covering a major portion of the proteome [351]. However, repeatability and reproducibility in data-dependent MS is limited [352-354]. To deduce the identity, product ion mass spectra from MS/MS are matched against databases using algorithms such as SEQUEST [355] or Mascot [356]. Searching vast numbers of spectra and assigning them to the correct peptides generate false positive results. A decoy database will allow for controlling the false discovery rate which reduce the number of false positives and increase the number of true positives [357, 358].

In Papers I and III, explorative proteomics employing nano-flow LC in combination with data-dependent bottom-up analysis on a hybrid and tribrid mass spectrometer (both from Thermo Fisher Scientific Inc.), respectively, have been used to identify proteins and peptides for quantification.

3.2.3.5 Quantitative Mass Spectrometry

A number of approaches exist for the quantification of proteins and peptides using MS. These include large scale explorative data-dependent methods using for example stable isotope labelling by amino acids in cell culture [359] or tandem mass tagging [360]. In addition there are label free approaches using spectral counting or signal intensities [361]. Data-dependent assays are valuable in relative quantification, generating large amounts of information useful for formulating hypotheses. However, they typically lack the throughput and repeatability required in a clinical setting. In targeted MS a limited number of proteins are targeted in a hypothesis driven manner using data-independent methods such as selected ion monitoring, selected reaction monitoring (SRM) [346] or parallel reaction monitoring (PRM) [362].

Stable isotope-labelled proteins or peptides are suitable internal standards for quantification in targeted MS. These are for example peptides with amino acids (aa) enriched with ^{13}C and ^{15}N. Absolute quantification (AQUA) peptides are such standards [363]. The isotope-labelled peptide will be chemically equivalent to its endogenous counterpart and will thus co-elute in chromatography and ionise similarly. The only difference will be a shift in mass. When analysing tryptic peptides using C-terminal $^{13}C/^{15}N$ isotope-labelled Lys or Arg, a complete series of labelled y-ions is generated. Additionally, for Lys and Arg there is a mass difference of 8 and 10 Da, respectively, aiding distinction of the isotopic envelopes of isotope-labelled and endogenous peptide. C-terminal $^{13}C/^{15}N$ Lys- or Arg-labelled crude tryptic peptides (Thermo Fisher Scientific Inc. and JPT Peptide Technologies GmbH, Berlin, Germany) were used for quantitative analyses in Papers I, III and IV. Uniformly labelled ^{13}C or ^{15}N ubiquitin (Silantes, GmbH, München, Germany) was used in Papers II-IV.

3.2.3.6 SRM

The QqQ mass spectrometer [345] enabled the development of a targeted quantitative method called SRM [346]. In SRM, a selected precursor ion and typically two to four product ions are isolated sequentially in the first and third quadrupole, respectively. A pair of product and precursor ions is referred to as a transition. Monitoring only selected transitions using narrow isolation, typically unit resolution (*m/z* 0.7), is crucial for sensitivity.

SRM has proven potent in biomarker research, screening large numbers of candidate biomarkers in a complex biological matrix, for example plasma, in a single analysis [364, 365]. Depending on sample complexity, proteins in the range of four to five orders of magnitude can be quantified and the methodology can have a lower limit of detection at low attomole level [366].

3.2.3.7 PRM

PRM is a mass spectrometric method where several product ions of a selected precursor ion are monitored simultaneously, or in parallel [362]. PRM is most often performed on hybrid mass spectrometers combining quadrupole mass filters with high resolution mass analysers such as time of flight [367] or orbitrap [348]. A key benefit of using these types of hybrid instruments is the selectivity provided by a narrow isolation of precursor ions in the quadrupole and high resolution enabling distinction between ions of similar *m/z*, reducing interferences [368]. Having these characteristics PRM contests conventional SRM in dynamic range and linearity when analysing complex samples [362].

An inherent feature of PRM is that it provides with each measurement the means to identify the analyte, reducing the need of a *priori* knowledge in method development. Also, PRM enables to a large degree post-acquisition processing since no product ions have to be chosen beforehand. This allows for diagnostics and selection of those product ions most suitable for quantification, for example product ions consistently detected from sample to sample and free of interferences. In the analysis of complex samples, PRM has shown a dynamic range over three orders of magnitude [362].

In Papers I-IV parallel reaction monitoring mass spectrometry (PRM-MS) was used to target the proteins of relevance involved in the endo-lysosomal system and the ubiquitin-proteasome system. These proteins are shown in Table 2. In Paper I, tryptic peptides from LAMP2 were targeted by scheduled isolation of ions using an isolation window of *m/z* 8. Thus, both tryptic and stable isotope-labelled peptides were isolated simultaneously. Additionally, alternating survey and MS/MS scans were acquired. In Papers II-IV a narrower isolation window was employed isolating either the tryptic or stable isotope-labelled peptide. Additionally, only MS/MS scans were acquired.

Table 2. Proteins targeted by PRM-MS in Papers I-IV.

Protein Name	UniProtKB Accession	Abbreviation	Paper
AP-2 complex subunit beta	P63010	AP2	III, IV
Amyloid precursor protein/amyloid beta A4 protein	P05067	APP	III, IV
Complement component C9	P02748	C9	III, IV
Cathepsin B	P07858	CatB	III, IV
Cathepsin D	P07339	CatD	III, IV
Cathepsin F	Q9UBX1	CatF	III, IV
Cathepsin L1	P07711	CatL1	III, IV
Cathepsin Z	Q9UBR2	CatZ	III, IV
Dipeptidyl peptidase 2	Q9UHL4	DPP2	III, IV
Ganglioside GM2 activator	P17900	GM2A	III, IV
Beta-hexosaminidase subunit beta	P07686	HEXB	III, IV
Lysosome-associated membrane glycoprotein 1	P11279	LAMP1	III, IV
Lysosome-associated membrane glycoprotein 2	P13473	LAMP2	I, III, IV
Lysozyme C	P61626	LysC	III, IV
Tissue alpha-L-fucosidase	P04066	T-ALF	III, IV
Transcobalamin-2	P20062	TCO2	III, IV
Tripeptidyl-peptidase 1	O14773	TPP1	III, IV
Ubiquitin	P0CG48	Ub	II-IV

3.3 PAGE and Western Blotting

In polyacrylamide gel electrophoresis (PAGE), proteins are often subjected to denaturation using an anionic detergent (*e.g.*, sodium dodecyl sulphate) which uniformly charges the protein. The protein is then forced to migrate according to size through a polyacrylamide mesh by applying a current. For selective detection using Western blotting (WB) [369] the proteins in the gel is transferred by the aid of a current to a solid membrane, often polyvinylidene fluoride or nitrocellulose, which is then blocked from unspecific protein absorption. Detection can then be facilitated by using only a primary antibody binding the protein of interest. For increased sensitivity the detection is enhanced by adding a secondary antibody binding the primary as well as by using a streptavidin-biotin system. Chemiluminescence is a common type of detection using antibody or biotin/streptavidin-conjugated

horseradish peroxidase to which substrates are added to produce excited luminol molecules. Alternatively, fluorescence as the means of detection is facilitated by employing fluorophore-conjugated antibodies.

PAGE and WB were employed in Paper I and II. In Paper I, successful IP of LAMP2 was confirmed by PAGE and WB. In Paper II, the elution profile of ubiquitin from the Oasis HLB SPE material (Waters Co.) was investigated by separating eluted samples by PAGE and detecting ubiquitin using WB. PAGE was performed similarly to the original method described by Laemmli [370].

3.4 ELISA

Enzyme-linked immunosorbent assay (ELISA) is a frequently used immunoassay technique for quantification of analytes in clinical and research settings. In short, using sandwich ELISA, the samples are diluted and added to pre-coated blocked wells containing a capture antibody. A second selective antibody which could be biotin-conjugated is added to sandwich the analyte. Then, typically horseradish peroxidase conjugated with streptavidin and substrate are added to facilitate colorimetric detection.

Through Papers I-IV sandwich ELISAs have been used for the determination of the levels of CSF $A\beta_{1\text{-}42}$, T-tau and P-tau_{181}. These procedures have been done by skilled technicians using commercially available sandwich ELISAs, namely INNOTEST β-AMYLOID(1-42), hTAU Ag and PHOSPHO-TAU(181P) (Fujirebio Europe, Ghent, Belgium). ELISA has been performed according to the manufacturer's instructions.

3.5 Statistical Analyses

Statistical analyses have been performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) and JMP Pro (SAS Institute Inc., Cary, NC, USA). Graphs have been created using GraphPad Prism. Normal distribution has been assessed using combinations of boxplots and histograms showing the sample distribution. In addition, Shapiro-Wilk test has been used to quantitatively investigate the distribution. The low number of the participants in the cohorts and the non-Gaussian distribution of the data have facilitated the decision to use non-parametric analyses. Groups of two were compared using Mann-Whitney U-test (GraphPad Prism) or the equivalent Wilcoxon 2-sample rank sum test (JMP Pro). Three or more groups were compared using Kruskal-Wallis test with Dunn's test for multiple comparisons. Comparisons generating p-values ≤ 0.05 were considered to show statistically significant differences. Spearman's rank correlation was used for association analyses. The correlation coefficient, Spearman's ρ, was used at a level of ≥ 0.65

(Paper II) or 0.80 (Papers III and IV) to indicate a correlation in combination with p-values ≤0.01 (Papers I-IV) to indicate a slope significantly different from 0.

4 RESULTS AND DISCUSSION

4.1 CSF LAMP2 Level in AD

LAMP2 is an abundant component of the lysosomal membrane [173] and a rate limiting component of CMA [192]. The importance of LAMP2 in the autophagic and endo-lysosomal system is further indicated by the accumulation of autophagic vacuoles in LAMP2 deficiency [371] and cardiomyopathy in Danon's disease [372]. Indeed, LAMP2 has been suggested to have a role in autolysosome formation [373].

In AD, there is an accumulation of autophagic vacuoles in dystrophic neurites [239] and an enlargement of endosomal vesicles [248, 249] at a preclinical stage of disease [248]. Similar alterations, with enlarged endosomes and accumulating autophagic vesicles, are seen when inducing endocytosis by overexpressing Rab5 [374], when disrupting axonal vesicle transport [270] or impairing lysosomal proteolytic activity [270, 375]. LAMP2 has been shown at increased levels in the *hippocampus* [268] and entorhinal region [376] of AD mice disease models [268, 376]. LAMP2 might serve as a surrogate marker for lysosomal status.

LAMP2 was measured in CSF by combining purification by IP, tryptic digestion and quantification by PRM-MS (Paper I). LAMP2 consists of 410-411 aa (depending on isoform) and is a 45 kDa highly glycosylated protein (UniProtKB accession P13473). LAMP2 was identified by WB as a band of approximately 80 kDa. In addition, six LAMP2 peptides (aa 46-53, 133-144, 145-152, 153-161, 281-289 and 334-351) were identified using tryptic digestion and bottom up explorative MS. Collectively, our findings in Paper I suggest that precipitated LAMP2 from CSF is most likely a large part of the full length protein. Applying a different approach for sample preparation, LAMP2 in CSF was subjected to tryptic digestion, purification by SPE and quantification by PRM-MS (Paper III). Here, an additional LAMP2 peptide, aa 261-273, was identified following bottom up explorative MS. In Paper III it is unclear which form or forms of LAMP2 that are measured.

The three LAMP2 peptides (aa 133-144, 145-152 and 153-161) quantified in Paper I were found to correlate to a high degree. Typically, the four LAMP2 peptides monitored in Paper III (aa 133-144, 145-152, 153-161 and 281-289) also correlated, however to different extent depending on cohort. There were no significant correlations between the three LAMP2 peptides analysed in both Papers I and III, neither in AD nor in controls (Figure 2). Probably lack of associations reflects that the two assays, to an extent, monitor peptides from different protein forms.

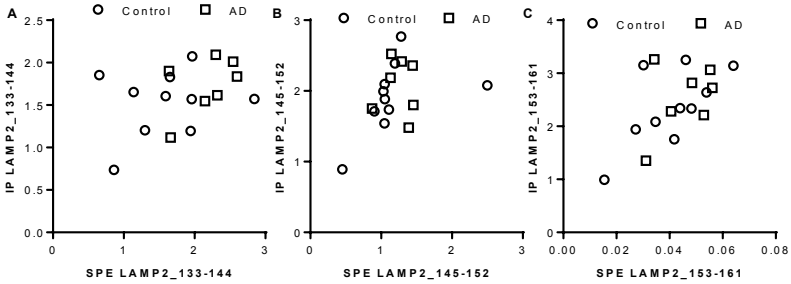

Figure 2. Association between LAMP2 CSF levels monitored in Papers I and III. Tryptic LAMP2 peptides were monitored using PRM-MS following IP or SPE in Papers I and III, respectively. There were no correlations between the LAMP2 peptides (A) aa 133-144, (B) 145-152, or (C) 153-161, neither in AD (N = 7) nor in controls (10). Shown is the ratio between tryptic and stable isotope-labelled peptide following IP and SPE, respectively. Associations were investigated using Spearman's rank correlation, with $\rho \geq 0.8$ and $p \leq 0.01$ indicating a correlation.

In Paper I, LAMP2 was found at increased level in CSF in subjects with an AD CSF core biomarker profile compared to subjects with a control profile (Figure 3A-C). Paper III included simultaneous quantification of a panel of endo-lysosomal proteins and ubiquitin in CSF by PRM-MS and similarly showed an increased CSF LAMP2 level in AD (Figure 3D-G). Replication in a second independent biochemically characterised cohort revealed no significant difference in CSF LAMP2 level in AD compared to controls (Paper III). Neither did investigation in clinically characterised cohorts show significantly different CSF levels of LAMP2 in AD nor MCI-AD compared to sMCI, or in AD compared to controls (Paper III). Previous investigations of the CSF level of LAMP2 are limited but have shown increased LAMP2 in AD compared to controls in biochemically characterised subjects [377], similarly to our present findings.

In Paper I, there was no association between LAMP2 peptides and AD CSF core biomarkers; $A\beta_{1\text{-}42}$, T-tau and P-tau_{181}. However, CSF LAMP2 peptides correlated with CSF P-tau_{181} levels in MCI-AD, AD and PD (Paper III). Armstrong *et al*. [377] have also found an association between CSF LAMP2 and P-tau_{181} in biochemical AD. P-tau is the major component of tangles [27-29] and CSF P-tau reflects the amount of tangles in the brain [67].

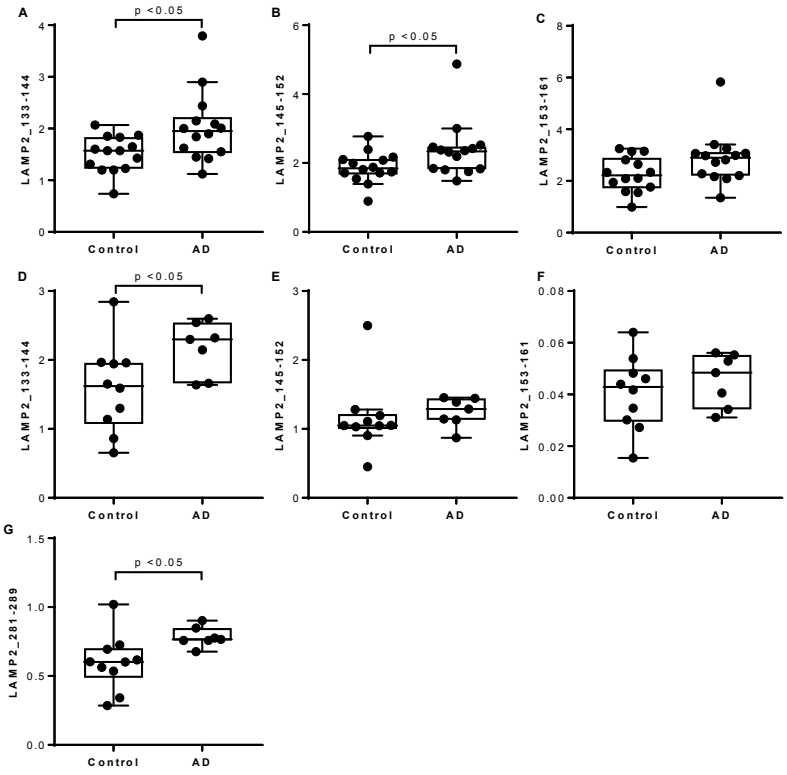

Figure 3. LAMP2 CSF level in AD. Combining IP, tryptic digestion and PRM-MS the CSF levels of LAMP2 peptides (A) aa 133-144 and (B) 145-152 were found to be significantly elevated in subjects with an AD CSF core biomarker profile (N = 14) compared to controls (N = 14). LAMP2 peptide (C) aa 153-161 was seemingly elevated in AD compared to controls. (D-G) Following tryptic digestion of CSF and SPE, LAMP2 peptides aa 133-144, 145-152, 153-161 and 281-289 were analysed by PRM-MS. The levels of tryptic peptides (D) aa 133-144 and (G) 281-289 were significantly elevated in biochemical AD (N = 7) compared to controls (N = 10). Shown is the ratio between tryptic and stable isotope-labelled peptide. (A-C) Groups were compared using Mann-Whitney U-test. (D-G) Groups were compared using Wilcoxon 2-sample rank sum test. Modified figure from Paper I (reference [378]).

4.2 CSF Ubiquitin Level in Neurodegenerative Diseases

Ubiquitin targets substrates for degradation by the proteasome [163] and serves a multifaceted role as a post-translational modification [213, 215]. Impairing autophagy [221, 222] or the ubiquitin-proteasome system [223] results in neurodegeneration and accumulation of ubiquitin positive protein inclusions [221-223]. Protein aggregates, positive for ubiquitin are targeted for autophagy, or aggrephagy, by p62 [188]. Indeed, protein inclusions associated with neurodegenerative diseases are labelled with ubiquitin including plaques [379, 380], tangles [379-381] and Lewy bodies [379, 382].

The proteasomal activity decreases with aging [240] and protein aggregates [227] including aggregates of tau [228] and Aβ [229-231], inhibits the proteasome. In AD the activity of the proteasome is decreased in regions of the brain associated with disease [274]. Additionally, in AD, there is an increased cortical level of ubiquitin [275, 276]. Presently, CSF ubiquitin has been targeted to investigate potential pathological alterations in the ubiquitin-proteasome system in neurodegenerative diseases.

Full length ubiquitin was isolated from CSF using SPE followed by quantification by PRM-MS (Paper II). Additionally two ubiquitin peptides (aa 12-27 and 64-72) were monitored by PRM-MS following tryptic digestion of CSF and SPE (Paper III). Control and AD subjects of cohorts 1-3 (Table 1) were pooled groupwise and associations were found between ubiquitin peptides, aa 12-27 and 64-72, respectively, and full length ubiquitin in control, AD and PD subjects (Figure 4). Tryptic ubiquitin peptides (aa 12-27 and 64-72) likely originate from full length ubiquitin also measured in Paper II. Beside free available ubiquitin, additional potential sources of these peptides are ubiquitinated proteins and polyubiquitin chains. Furthermore, the monitored peptides originate from ubiquitin species that are devoid of branching ubiquitin chains at Lys amino acid position 11, 27 and 63. Similarly, both the CSF level of full length ubiquitin and tryptic ubiquitin peptides, aa 12-27 and 64-72, were monitored in clinical FTD subtypes (Paper IV). Correlations were identified between CSF full length ubiquitin, and peptides aa 12-27 and 64-72, in control, lvPPA and nfvPPA subjects (Figure 5). No correlations were identified in bvFTD or svPPA disease subgroups (Figure 5).

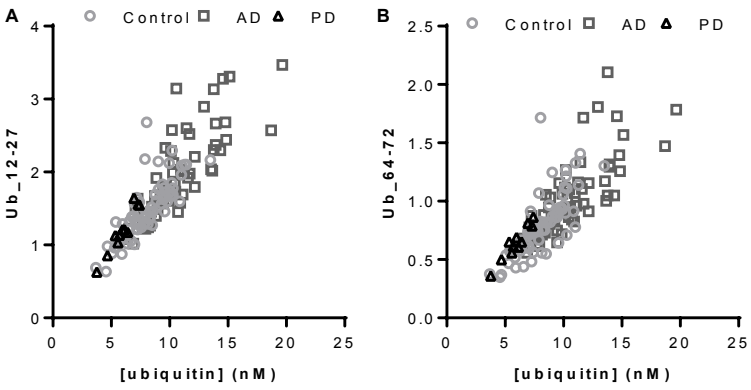

Figure 4. Association between CSF levels of ubiquitin monitored in Papers II and III. Full length ubiquitin and tryptic ubiquitin peptides were monitored in Papers II and III, respectively, in Control (N = 68), AD (N = 55) and PD (N = 11) subjects. (A) There were correlations between ubiquitin peptide aa 12-27 and full length ubiquitin in controls, AD and PD. (B) There were also correlations between ubiquitin peptide aa 64-72 and full length ubiquitin in Control, AD and PD subjects. Shown is the ubiquitin concentration (nM) and the ratio between tryptic and stable isotope-labelled peptide (Ub_12-27 or Ub_64-72). Associations were investigated using Spearman's rank correlation, with $\rho \geq 0.8$ and $p \leq 0.01$ indicating a correlation.

In Paper II, investigation of two independent cohorts consisting of subjects with an AD CSF core biomarker or a control biomarker profile, showed significantly increased levels in CSF of full length ubiquitin in AD subjects compared to controls (Figure 6A and B). A clinically characterised cohort confirmed a significantly elevated CSF level of full length ubiquitin in AD compared to cognitively healthy controls (Figure 6C). Tryptic ubiquitin peptides (aa 12-27 and 64-72) were monitored by PRM-MS following digestion of CSF and SPE (Paper III). The CSF ubiquitin levels were increased in AD compared to controls in two independent biochemically characterised cohorts (Figure 7A-D). Two independent clinically characterised cohorts showed increased, however not significantly elevated, CSF levels of ubiquitin in MCI-AD or AD compared to sMCI, and in AD compared to controls (Figure 7E-H). The CSF level of ubiquitin has previously been shown to be elevated in AD compared to controls [383-386] and in MCI-AD compared to sMCI [387].

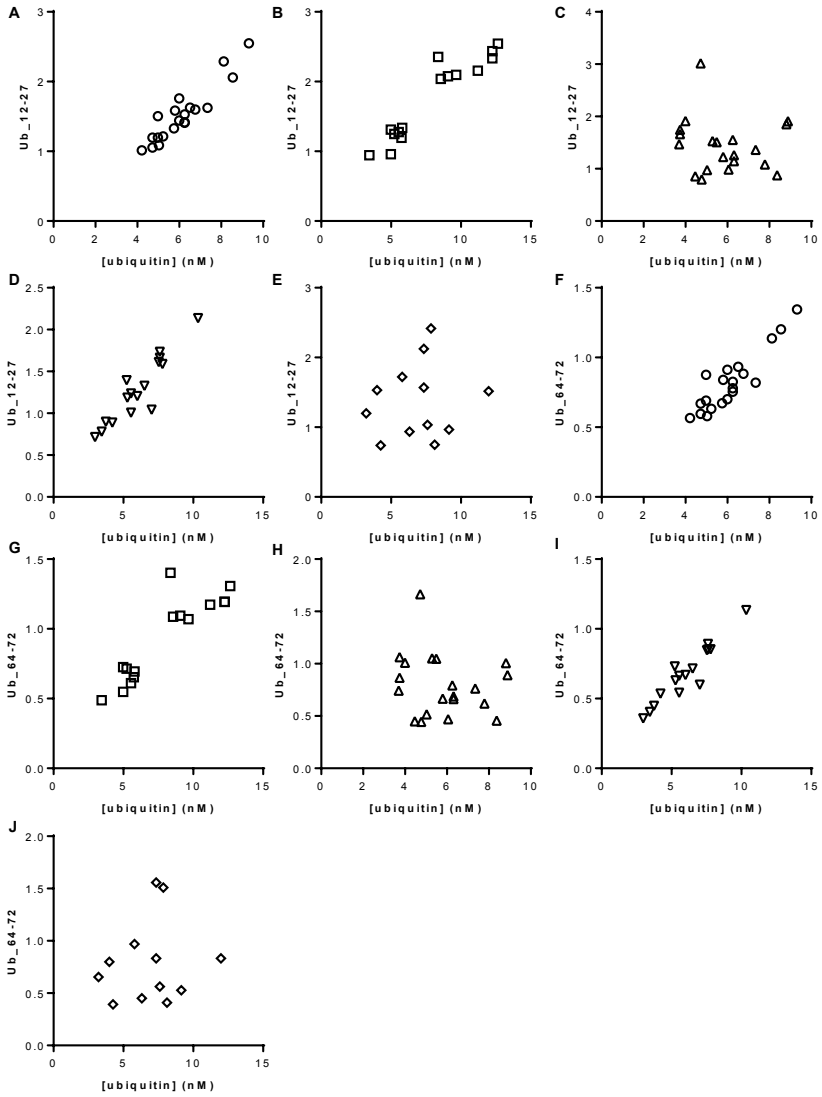

Figure 5. Associations between CSF full length ubiquitin and tryptic peptides in FTD. There were correlations between the levels of CSF full length ubiquitin and ubiquitin peptide aa 12-27 in (A) Control (N = 20), (B) lvPPA (N = 15) and (D) nfvPPA (N = 16) subjects. There were no correlations in subjects with (C) bvFTD (N = 16) or (E) svPPA (N = 12). Similarly there were correlations between full length ubiquitin and ubiquitin peptide aa 64-72 in (F) Control, (G) lvPPA and (I) nfvPPA subjects. There were no correlations in (H) bvFTD or (J) svPPA subjects. Shown is the ubiquitin concentration (nM) and the ratio between tryptic and stable isotope-labelled peptide (Ub_12-27 or Ub_64-72). Associations were investigated using Spearman's rank correlation, with $\rho \geq 0.8$ and $p \leq 0.01$ indicating a correlation.

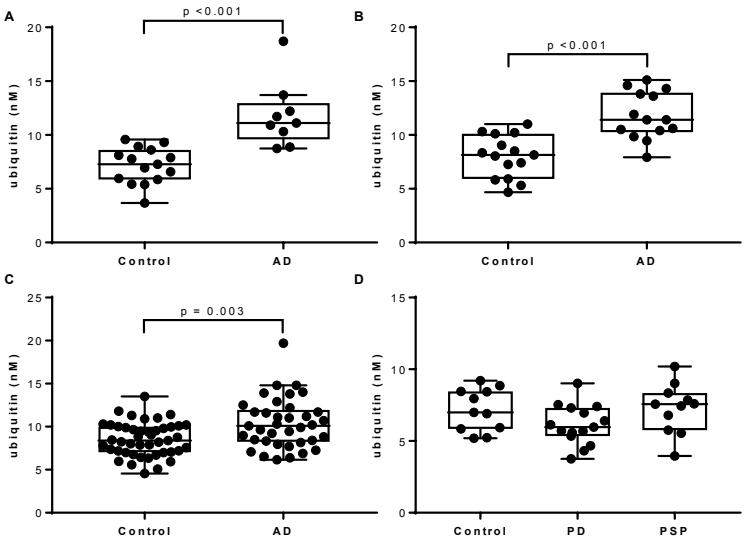

Figure 6. Full length ubiquitin CSF concentrations in neurodegenerative diseases. (A) The concentration of CSF ubiquitin was found to be elevated in subjects with an AD CSF core biomarker profile compared to controls (AD, N = 9; Control, N = 15). (B) Similarly, in an independent cohort with biochemical AD (N = 14) and controls (N = 15), the concentration was elevated in AD. (C) There was a significantly increased concentration of CSF ubiquitin in clinically characterised AD subjects (N = 37) compared to controls (N = 45). (D) There were no significant differences between clinically characterised PD (N = 15), PSP (N = 11) or control (N = 11) subjects. Groups were compared using Mann-Whitney U-test or Kruskal-Wallis test with Dunn's test for multiple comparisons. Modified figure from Paper II (reference [388]).

The CSF level of full length ubiquitin was also investigated in subjects clinically characterised with PD and PSP (Paper II). The CSF level of full length ubiquitin was seemingly lower in PD compared to PSP and controls, however not statistically significant different (Figure 6D). In PSP the concentration of ubiquitin in CSF has been indicated to be unaltered compared to controls [385], and conflictingly elevated compared to controls and PD [389]. Compared to the high level of ubiquitin labelling of tangles in AD, subcortical and brainstem tangles in PSP show a low degree of ubiquitin [390, 391]. The CSF level of ubiquitin in PD was further explored by monitoring tryptic ubiquitin peptides (aa 12-27 and 64-72; Paper III). In a clinically characterised cohort the CSF level was found to be significantly decreased in PD compared to MCI-AD (Figure 7E-F). Replication in an additional clinical cohort showed the CSF level to be decreased in PD compared to AD and controls (Figure 7G-H). Lewy bodies are labelled by ubiquitin [379, 382] and conditional knock-out of 26S proteasomal activity in mice causes nigrostriatal degeneration and

accumulation of ubiquitin positive protein inclusions [223]. The CSF ubiquitin concentration in PD has been suggested to be unchanged [385, 386, 389]. The CSF level of α-synuclein has been shown to be decreased in PD in multiple studies [109-111]. Perhaps for the same reason, yet to be defined, ubiquitin exists at lower CSF levels in PD.

Furthermore, the CSF levels of full length ubiquitin and tryptic peptides (aa 12-27 and 64-72) were monitored in clinical FTD subtypes (Paper IV). There were no differences in the CSF levels of full length ubiquitin or ubiquitin peptides between groups compared; controls, lvPPA, bvFTD, nfvPPA and svPPA. Similarly, a previous investigation showed no difference in CSF ubiquitin concentration between subjects with PPA, and bvFTD, and controls [385]. Also we found no difference in the CSF full length ubiquitin or tryptic ubiquitin peptide levels in subjects with FTD carrying disease causing mutations in *GRN*, *MAPT* or a hexanucleotide expansion in *C9ORF72*. However the number of mutation carriers analysed was low. Ubiquitin positive inclusions exists in FTLD-tau (*e.g.*, Pick's disease [392] and PSP [390, 391]), FTLD-TDP [133], FTLD-FET [136], and in FTLD-UPS [138] pathological subtypes. Future investigation of CSF ubiquitin in known pathological subtypes would be of interest.

Associations were identified between CSF full length ubiquitin and T-tau and P-tau$_{181}$ in controls, AD and PD (Paper II). In PSP there was only a correlation between ubiquitin and P-tau$_{181}$ (Paper II). Correlations were also identified between ubiquitin peptides and P-tau$_{181}$ in controls, AD and PD, and between ubiquitin peptides and T-tau in controls (Paper III). In Paper IV, full length ubiquitin correlated with P-tau$_{181}$ in the lvPPA group. Additionally, both ubiquitin peptides correlated with age in the svPPA group. The CSF P-tau level reflects the tangle load in the brain [67] and tau ubiquitination has been identified [393, 394]. Furthermore, T-tau has been suggested to reflect severity of neurodegeneration [64-66]. Creutzfeldt-Jakob disease is an example of an aggressive neurodegenerative disorder with substantially elevated CSF T-tau [395] and ubiquitin [385, 396].

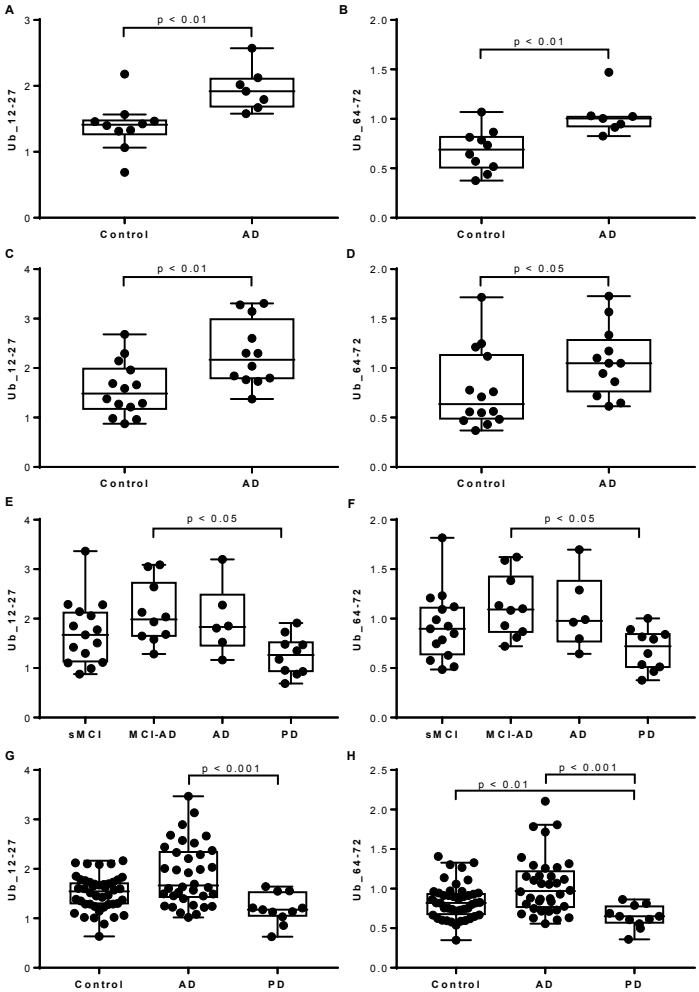

Figure 7. Ubiquitin CSF levels in neurodegenerative diseases. The levels of CSF tryptic ubiquitin peptides (A) aa 12-27 and (B) 64-72 were elevated in subjects with an AD CSF core biomarker profile compared to controls (AD, N = 7; Control, N = 10). Similarly in an independent biochemically characterised cohort the ubiquitin peptides (C) aa 12-27 and (D) 64-72 were elevated in AD (N = 12) compared to controls (N = 14). Investigation in a clinically characterised cohort however showed decreased levels of peptides (E) aa 12-27 and (F) 64-72 in PD (N = 10) compared to MCI-AD (N = 10). sMCI, N = 15; and AD, N = 6. Replication in a clinically characterised cohort showed decreased level of (G) aa 12-27 in PD (N = 11) compared to AD (N = 36), and decreased level of (H) aa 64-72 in PD compared to AD and controls (N = 44). Shown is the ratio between tryptic and stable isotope-labelled peptide (Ub_12-27 or Ub_64-72). Groups were compared using Wilcoxon 2-sample rank sum test or Kruskal-Wallis test with Dunn's test for multiple comparisons.

4.3 Targeting Endo-Lysosomal Proteins and Ubiquitin in Neurodegenerative Diseases

Developing our experience with targeting single analytes in Papers I and II, multiple analytes, including endo-lysosomal proteins and ubiquitin, were analysed in Papers III and IV. Simultaneous quantification of a panel of proteins would allow for a more comprehensive investigation of alterations in proteostasis in neurodegenerative diseases. A bottom up explorative MS screening of CSF (Paper III) resulted in the identification and selection of the proteins shown in Table 2 for quantification by PRM-MS.

The identified proteins included are primarily involved in the functions of the lysosome. No protein with an apparent association with the function or regulation of autophagy was identified. The proteins selected for quantification included lysosomal hydrolases. These were lysosomal proteases; CatB [397], CatD [398], cathepsin F (CatF) [397], CatL1 [397], cathepsin Z (CatZ) [397], DPP2 [399], and TPP1 [400]. Also the fucosidase, tissue alpha-L-fucosidase (T-ALF) was targeted [401] as well as proteins involved in degradation of GM2 gangliosides, ganglioside GM2 activator (GM2A) and HEXB [402]. Lysozyme C (LysC) was also targeted which is a glycoside hydrolase secreted into biological fluids where it degrades the glycosides in the cell wall of primarily gram-positive bacteria [403]. In addition, lysosomal membrane proteins LAMP1 and LAMP2 [173], and transcobalamin-2 (TCO2), delivering cobalamin (vitamin B12) to the lysosome [404], were targeted. Endocytosis was represented by targeting AP-2 complex subunit beta (AP2) which is fundamental in clathrin-mediated endocytosis [405]. From the ubiquitin-proteasome system, ubiquitin [163] was selected for quantification. Additionally proteins not explicitly involved in proteostasis were targeted as control proteins, including complement component C9 (C9), a component of the innate immunity membrane attack complex [406] and APP [16]. In total the PRM-MS panel method developed explore the CSF levels of 50 tryptic peptides from 18 proteins. A summary of significantly altered CSF protein levels in neurodegenerative diseases identified in Papers I-IV is shown in Table 3. Similarly, proteins monitored and their functional association or localisation in the autophagic and endo-lysosomal system or the ubiquitin-proteasome system is shown in Figure 8.

Table 3. Summary of altered CSF protein levels in disease groups.

Paper	Group	Change	Protein
I	AD[a] Vs. Control[a]	Up	LAMP2
II	AD[a,b] Vs. Control[a,b]	Up	Ubiquitin
III	AD[a] Vs. Control[a]	Up	AP2, CatB, GM2A, LAMP1, LAMP2, ubiquitin
	PD[b] Vs. MCI-AD[b]	Down	AP2, CatB, CatF, HEXB, LAMP1, LAMP2, ubiquitin
	PD[b] Vs. AD[b]	Down	AP2, C9, CatB, CatF, GM2A, TCO2, ubiquitin
	PD[b] Vs. Control[b]	Down	AP2, CatF, GM2A, ubiquitin
IV	bvFTD[b] Vs. lvPPA[b]	Down	APP, CatZ
	nfvPPA[b] Vs. bvFTD[b]	Down	TCO2
	MAPT[b] Vs. C9ORF72[b]	Down	DPP2
	MAPT[b] Vs. GRN[b]	Down	CatF

[a]Cohort with biochemically characterised participants.

[b]Cohort with clinically characterised participants.

The levels of CSF panel proteins were explored in two independent cohorts with subjects designated as AD or controls, based on the AD CSF core biomarker profile (Paper III). Investigation of the first cohort showed significantly elevated CSF levels of AP2, CatB, GM2A and LAMP1 in AD compared to controls (Figure 9A-G). CSF LAMP2 and ubiquitin were also elevated in AD (Figure 3D-G, and Figure 7A and B, respectively). The second cohort similarly revealed significantly elevated CSF levels of AP2 and GM2A in AD compared to controls (Figure 9H-J). CSF ubiquitin was also elevated in AD (Figure 7C and D). As previously described in AD there are morphological alterations of endocytic vesicles [248, 249] and an accumulation of autophagic vacuoles [239] accompanied by an increased expression of lysosomal hydrolases, for example CatB and CatD [250-252], and regulators of the endocytic machinery [253-255]. A previous investigation [377], similarly to our present findings, showed increased CSF levels of endocytic proteins early endosome antigen 1, Rab3 and Rab7; lysosomal proteins CatL1, LAMP1 and LAMP2; and autophagic protein LC3 in AD compared to controls [377]. Additional investigations have shown CSF LAMP1 and GM2A to be increased in AD compared to controls [386] and CatB to be increased in plasma, however not in CSF [407]. To our knowledge, CSF AP2 has not previously been investigated in AD. A subunit of adaptor protein 2, AP-2 complex subunit alpha-1, was shown to be less abundant in AD temporal cortex compared to controls [408]. Herein the beta subunit was investigated (AP2). Additionally, Musunuri *et al*. [408] also showed lower expression of clathrin heavy chain 1 (involved in clathrin-mediated endocytosis [405]) in AD temporal cortex compared to controls. As discussed in the section above, CSF ubiquitin has previously been shown to be elevated in AD compared to controls [383-386].

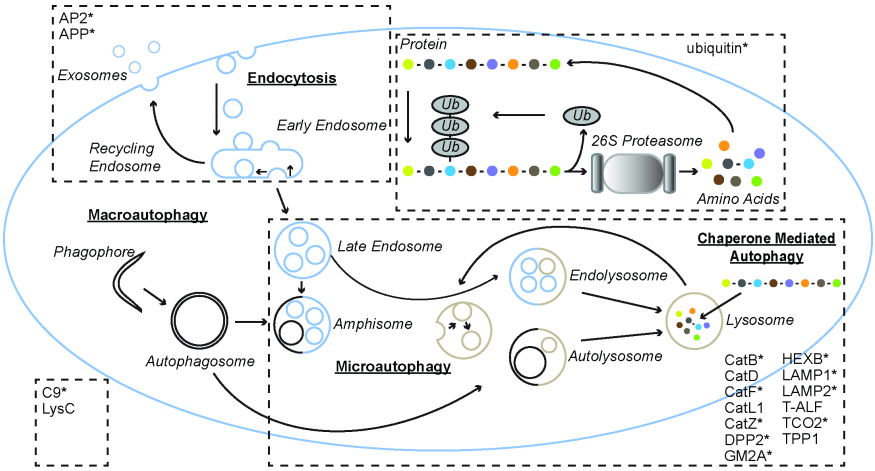

Figure 8. Proteins in CSF investigated in Papers I-IV. Shown are the autophagic and endo-lysosomal system and the ubiquitin-proteasome system. The proteins investigated in CSF and their functional association or localisation within these systems are indicated. Proteins labelled with a star have been identified at altered CSF levels in neurodegenerative diseases.

The levels of CSF endo-lysosomal proteins and ubiquitin were further analysed in clinically characterised cohorts (Paper III). The CSF levels of AP2, CatB, CatF, HEXB, LAMP1, LAMP2 (Figure 10) and ubiquitin (Figure 7E and F) were significantly decreased in PD compared to MCI-AD. There were no significantly different CSF protein levels in MCI-AD or AD compared to sMCI. The second investigation revealed significantly decreased levels in CSF of AP2, C9, CatB, CatF, GM2A and TCO2 in PD compared to AD (Figure 11). AP2, CatF and GM2A were significantly decreased in PD compared to controls (Figure 11). The level of CSF ubiquitin was decreased in PD compared to control and AD (Figure 7G and H). There were no significantly different levels of CSF proteins when comparing AD subjects with cognitively healthy controls.

Similar to AD [239], in PD there is an accumulation of autophagic vacuoles [283]. The level of LAMP2, and hsc70, has been shown to be reduced in the *substantia nigra*, *amygdala* [285] and anterior cingulate cortex [287] in PD. Interestingly α-synuclein is a potential CMA substrate [233-235]. CMA is governed by LAMP2 and hsc70 [192]. Furthermore, lysosomal alterations seem to be associated with accumulating α-synuclein indicated by changes in the neuronal level of LAMP1 [286], LAMP2 [287, 290], CatD [286, 290], cathepsin A [290] and β-glucocerebrosidase [290]. Lysosomal dysfunction has been increasingly implicated in PD by association of genetic alterations contributing an increased risk of disease [104, 105, 107].

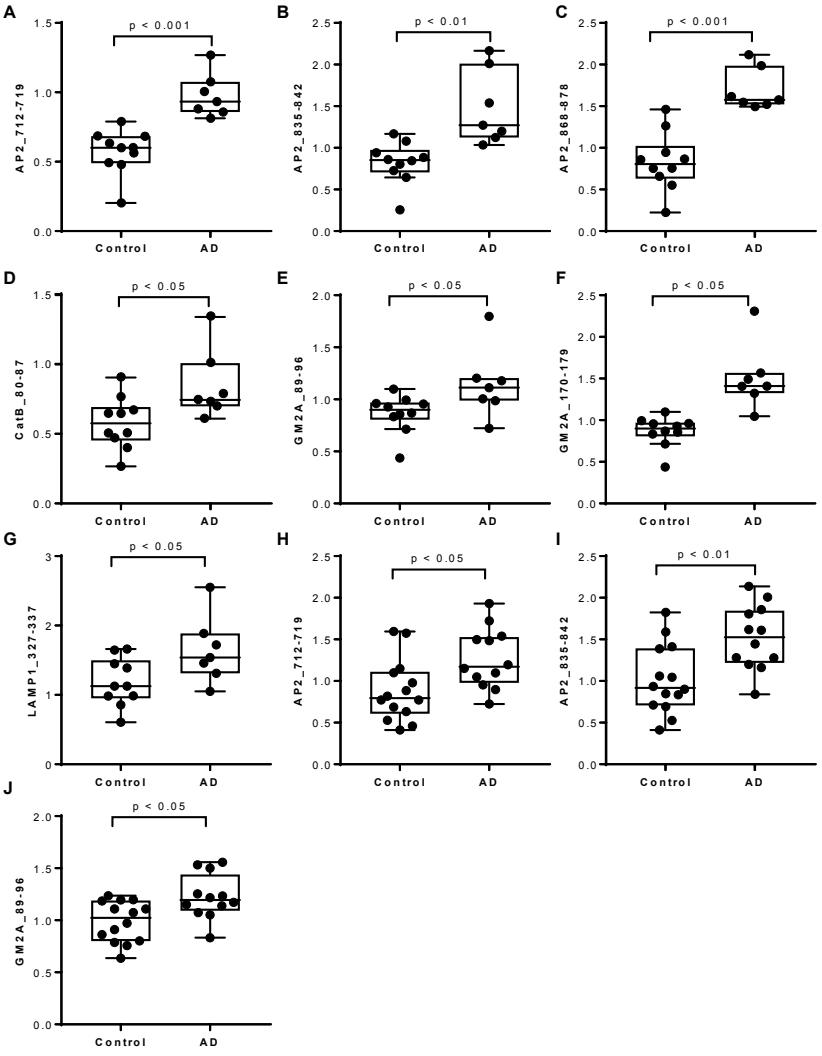

Figure 9. CSF levels of endo-lysosomal proteins in biochemical cohorts. The levels of tryptic peptides from (A-C) AP2, (D) CatB, (E and F) GM2A and (G) LAMP1 in CSF were found to be significantly elevated in AD (N = 7) compared to controls (N = 10). Replication in an independent cohort showed significantly elevated levels of tryptic peptides from (H and I) AP2 and (J) GM2A in AD (N = 12) compared to controls (N = 14). Shown is the ratio between tryptic and stable isotope-labelled peptide. Groups were compared using Wilcoxon 2-sample rank sum test.

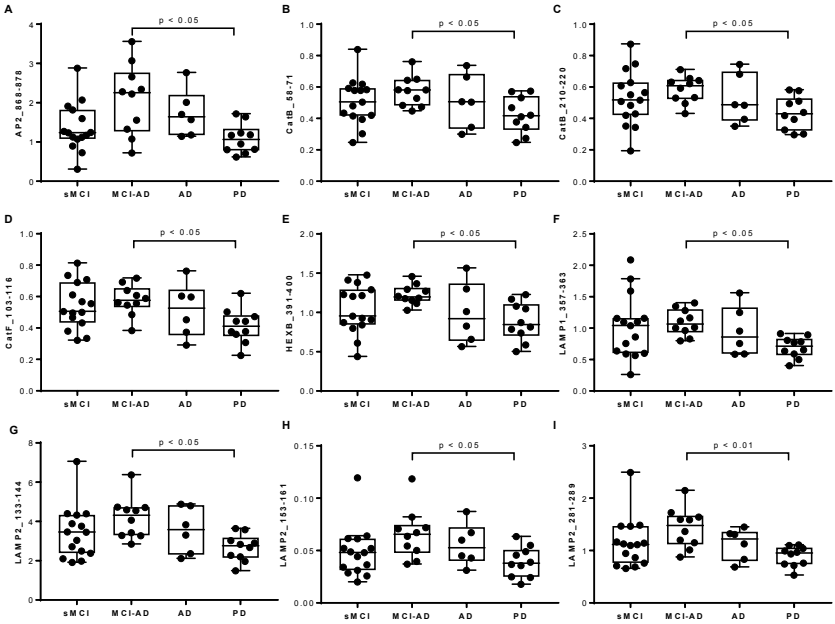

Figure 10. CSF levels of endo-lysosomal proteins in a clinical cohort. The levels of tryptic peptides from (A) AP2, (B and C) CatB, (D) CatF, (E) HEXB, (F) LAMP1 and (G-I) LAMP2 in CSF were found to be significantly decreased in PD (N = 10) compared to MCI-AD (N = 10). AD, N = 6; and sMCI, N = 15. Shown is the ratio between tryptic and stable isotope-labelled peptide. Groups were compared using Kruskal-Wallis test with Dunn's test for multiple comparisons.

To date there is no biomarker in use for diagnosis in PD. CSF α-synuclein levels have in most previous studies been found to be modestly decreased in PD compared to controls [109-111]. A combination of biomarkers might hold the greatest diagnostic value when differentiating parkinsonian disorders [110, 114]. The enzymatic activities and protein levels of a number of lysosomal proteins in CSF have previously been investigated. β-glucocerebrosidase has shown reduced activity in CSF in PD [115-117] and to be further decreased in *GBA* gene mutation carriers [117]. CatD has shown decreased [117] or unchanged [118] activities in PD. Similarly CSF β-hexosaminidase activities have shown conflicting results [115-118]. The level of CSF LAMP2 has been indicated to be decreased in PD [409] whereas LAMP1 has been indicated to be decreased [409] or unaltered [386]. CSF GM2A levels have been shown to be increased in dementia with Lewy bodies but unaltered in PD compared to controls [386]. Finally, the CSF ubiquitin concentration has been indicated to be unaltered in PD compared to controls [385, 386, 389].

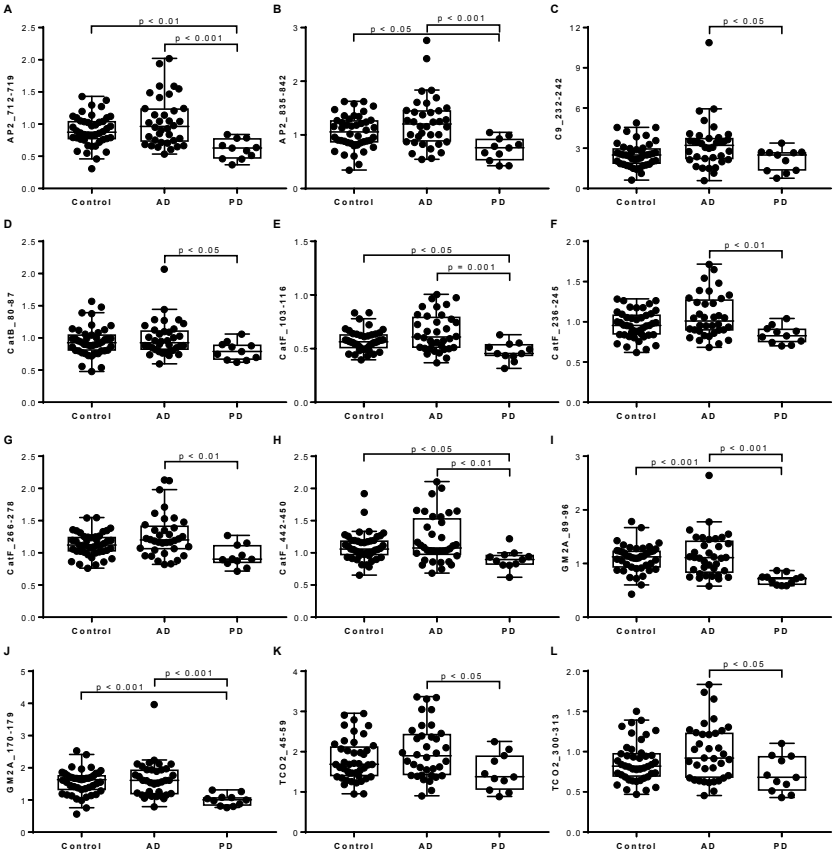

Figure 11. CSF levels of endo-lysosomal proteins in a second clinical cohort. The levels of tryptic peptides from (A and B) AP2, (C) C9, (D) CatB, (E-H) CatF, (I and J) GM2A and (K and L) TCO2 in CSF were found to be significantly decreased in PD (N = 11) compared to AD (N = 36). Additionally, peptide levels from proteins (A and B) AP2, (E and H) CatF and (I and J) GM2A were significantly decreased in PD (N = 11) compared to controls (N = 44). Shown is the ratio between tryptic and stable isotope-labelled peptide. Groups were compared using Kruskal-Wallis test with Dunn's test for multiple comparisons.

Further investigation of the levels of endo-lysosomal proteins in CSF was performed in subjects with clinical FTD subtypes (Paper IV). Significantly decreased levels in CSF of APP and CatZ were identified in bvFTD compared to lvPPA (Figure 12). AD pathology has been shown to exist in lvPPA [410, 411] and increased levels of soluble APP fragments in CSF have been shown in AD compared to FTD [151, 412], which might explain the difference in APP CSF level between bvFTD and lvPPA. A significant decreased level of CSF TCO2 was found in nfvPPA compared to bvFTD (Figure 12). There were no significant differences in CSF protein levels in FTD

subtypes compared to controls. In mutation carriers, CSF CatF and DPP2 levels were found to be lower in *MAPT* compared to *GRN* and *C9ORF72* carriers, respectively (Figure 13). However, only a small number of mutation carriers were analysed. FTLD is characterised by accumulation of protein inclusions [120]. In FTD there is a large genetic component [126-128]. Genetic alterations are found in genes encoding proteins involved in autophagy and endo-lysosomal function, including p62 [188, 319, 320], CHMP2B [316-318], TMEM106B [149, 301, 303-307], progranulin [145, 146, 298-302] and C9ORF72 [143, 144, 309-315]. Thus, altered proteostasis is implicated in disease. For example in *GRN* mutation carriers there is an increased expression of lysosomal proteins [300]. However, altered proteostasis in FTD seem not to be readily detectable in CSF targeting our panel of endo-lysosomal proteins and ubiquitin.

In Paper III, associations were identified between CSF AP2 peptides and P-tau$_{181}$ in controls, MCI-AD, AD and PD, between an APP peptide and P-tau$_{181}$ in AD, between LAMP2 peptides and P-tau$_{181}$ in MCI-AD, AD and PD, and between AP2 peptides and T-tau in controls. There was a correlation between a C9 peptide in AD and a T-ALF peptide in PD and Aβ_{1-42}. Also a LAMP1 peptide in controls and a GM2A peptide in MCI-AD correlated with age. In Paper IV CSF AP2 peptides correlated with P-tau$_{181}$ in lvPPA, nfvPPA and svPPA. An APP peptide in lvPPA and a CatB peptide in svPPA also correlated with P-tau$_{181}$. Also an AP2 peptide in lvPPA and nfvPPA, respectively, and an APP peptide in lvPPA correlated with T-tau. Furthermore, when identifying differences between groups using the panel method, not all proteins analysed were increased or decreased, suggesting there is no general decrease or increase in CSF protein levels in the diseases (PD or AD).

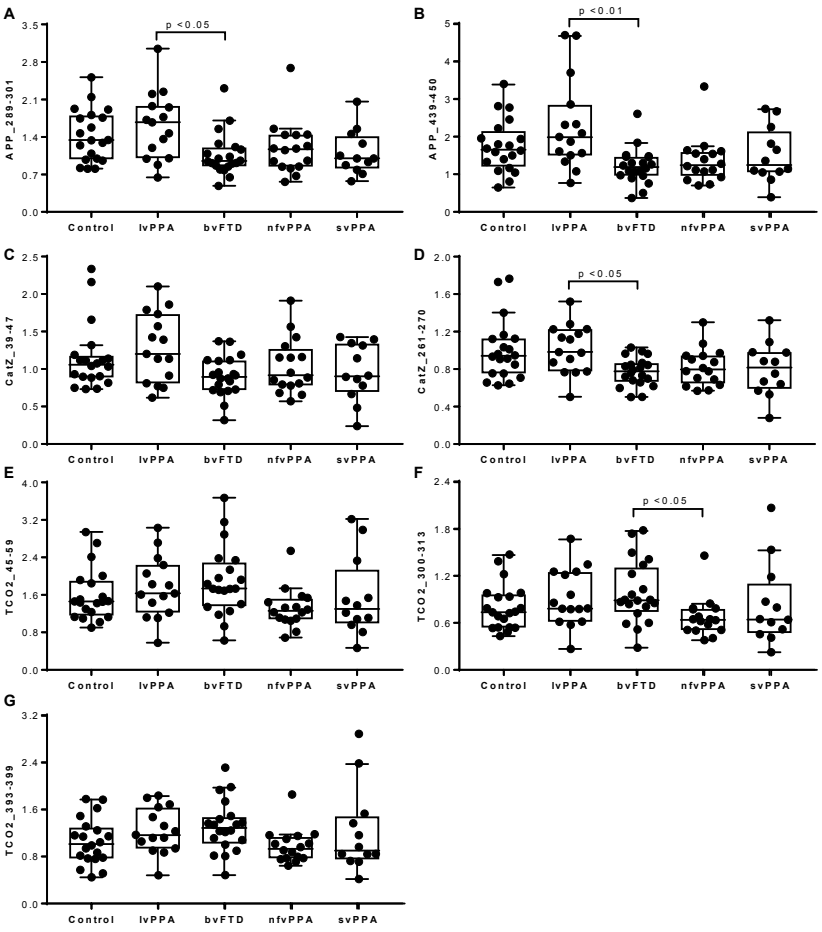

Figure 12. CSF levels of endo-lysosomal proteins in clinical FTD subtypes. (A and B) The levels of tryptic peptides from APP in CSF were found to be decreased in bvFTD (N = 20) compared to lvPPA (N = 15). (C and D) Of the tryptic CatZ peptides aa 39-47 and 261-270, (D) aa 261-270 was significantly decreased in bvFTD compared to lvPPA. (E-G) Of the tryptic TCO2 peptides aa 45-59, 300-313 and 393-399, (F) 300-313 was significantly decreased in nfvPPA (N = 16) compared to bvFTD. Controls, N = 20; and svPPA, N = 12. Shown is the ratio between tryptic and stable isotope-labelled peptide. Groups were compared using Kruskal-Wallis test with Dunn's test for multiple comparisons.

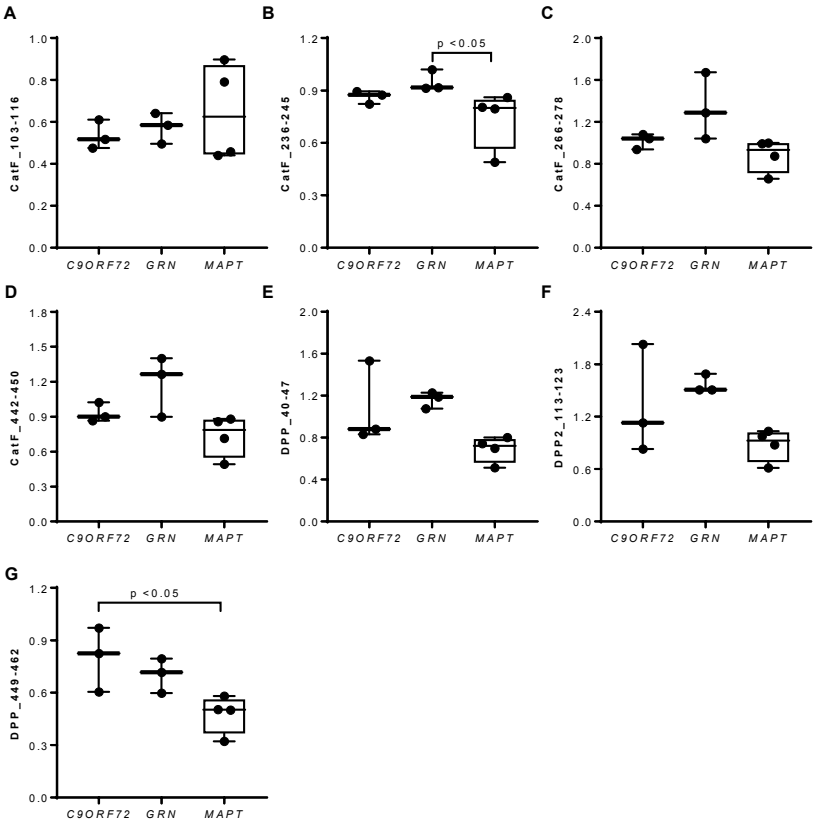

Figure 13. CSF levels of endo-lysosomal proteins in FTD mutation carriers. (A) The CSF level of tryptic CatF peptide, aa 103-116, was not different between MAPT (N = 4), GRN (N = 3) and C9ORF72 (N = 3) mutation carriers. (B) The CSF level of CatF peptide, aa 236-245, was significantly lower in MAPT compared to GRN carriers, and seemingly lower compared to C9ORF72. CatF peptides (C) aa 266-278 and (D) 442-450 were lower in MAPT compared to C9ORF72 and GRN carriers. The CSF levels of DPP2 peptides (E) aa 40-47 and (F) 113-123 were lower in MAPT compared to C9ORF72 and GRN carriers. (G) The CSF level of DPP2 peptide, aa 449-462, was significantly lower in MAPT compared to C9ORF72 carriers, and seemingly lower compared to GRN. Shown is the ratio between tryptic and stable isotope-labelled peptide. Groups were compared using Kruskal-Wallis test with Dunn's test for multiple comparisons.

5 CONCLUSION AND FUTURE PERSPECTIVES

This thesis has examined dysfunctional proteostasis in neurodegenerative diseases by developing assays to monitor proteins from the endo-lysosomal system and the ubiquitin-proteasome system as tools to study this pathological feature in CSF.

The lysosomal membrane protein LAMP2 was monitored as a potential indicator of lysosomal status in AD. Selective purification of LAMP2 in CSF using IP showed a larger fragment or full length LAMP2 to exist in CSF. Tryptic peptide levels from this form of CSF LAMP2 were identified to be increased in AD compared to controls. Similarly, LAMP2 digested in CSF and isolated by SPE showed increased CSF levels in AD, however, the CSF levels of LAMP2 in clinical AD subjects were not significantly elevated. Our results thus indicate LAMP2 to reflect pathological alterations in AD, possibly impaired turnover of endocytic and autophagic vesicles. However, CSF LAMP2 likely has limited value as a potential biomarker in AD.

Next, ubiquitin was targeted in CSF to investigate the status of the ubiquitin-proteasome system in neurodegenerative diseases. The CSF concentration of ubiquitin was elevated in AD compared to controls as shown by two assays targeting full length ubiquitin or tryptic ubiquitin peptides, respectively. Possibly, elevated levels in CSF reflect increased amounts of ubiquitin in the central nervous system which act in an attempt to rid accumulating proteins or protein aggregates by the proteasome or through aggrephagy, respectively. Interestingly, we found lower concentrations of ubiquitin in PD compared to AD and controls. Additionally the CSF levels of ubiquitin were monitored in clinical FTD subtypes however no differences were detected. In FTLD, there is an accumulation of ubiquitin-labelled protein inclusions in the brain, however such alterations seem not to be reflected by the concentration of ubiquitin in CSF. Seemingly, CSF ubiquitin reflects alterations in ubiquitin homeostasis in AD and PD, interestingly in opposite directions, and might thus serve as a potential biomarker in these two disorders.

Further exploration of alterations in the autophagic and endo-lysosomal system and the ubiquitin-proteasome system was pursued by developing a PRM-MS panel method monitoring the CSF concentrations of multiple proteins. In addition to LAMP2 and ubiquitin, the CSF concentrations of several lysosomal proteins and the endocytic protein, AP2, were elevated in AD compared to controls. However in participants with clinical AD the concentrations were not significantly elevated. Alterations in the CSF level of endo-lysosomal proteins might indicate pathological alterations in AD; however the proteins may not be potent as potential biomarkers. Monitoring CSF protein levels in PD showed reduced levels of lysosomal proteins.

Reduced levels were identified in two independent clinical cohorts. Lysosomal dysfunction is largely implicated in PD by the increased risk of disease having genetic alterations associated with lysosomal storage disorders and decreased levels of lysosomal proteins in disease afflicted regions. Ubiquitin and endocytic protein AP2 CSF levels were also altered in PD indicating detectable pathological alterations in the proteolytic machinery beyond the lysosome. Investigation in clinical FTD subtypes did not clearly show possible pathological alterations to be reflected by the CSF levels of endo-lysosomal proteins in CSF. There was no difference in FTD subtypes compared to controls. Exploration in FTD disease mutation carriers also suggested limited ability of endo-lysosomal proteins in CSF to reflect pathology. *GRN* mutation carriers likely have an altered lysosomal function.

Now, using the methods at hand, exploration is warranted in additional cohorts consisting of subjects with PD including carriers of risk associated genetic alterations. As indicated in this thesis, alterations of CSF levels of endo-lysosomal proteins are most prominent in PD. Further investigation is needed in larger cohorts exploring combinations of endo-lysosomal proteins and ubiquitin to determine added differentiating value in the diagnosis of parkinsonian disorders. Longitudinal studies are also warranted to investigate when during disease, alterations in CSF are detectable. A treatment strategy would be expected to most effective in an early stage of disease, before too much irreversible damage has occurred. Similarly, CSF ubiquitin should be pursued in longitudinal studies in participants with AD. Additionally, further investigation of larger cohorts of familial FTD, with *GRN, MAPT and TMEM106B* mutations and *C9ORF72* expansion, and sporadic cases with known FTLD subtype, is needed to monitor if lysosomal dysfunction is reflected in the levels of endo-lysosomal proteins in CSF.

In the future, we hope that biomarker panels like the one we have developed will be useful to facilitate the development of disease-modifying treatments against neurodegenerative diseases. Reduced CSF concentrations of lysosomal proteins in PD may reflect lysosomal dysfunction in the disease and the lysosomal biomarker panel could potentially be used both as an inclusion criterion and to determine if treatments aimed at correcting lysosomal dysfunction in the disease engage their target and are given at sufficiently high doses.

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