Lysosomal network proteins as biomarkers and therapeutic targets in neurodegenerative disease

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Nothing is constant but change.

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Abstract

The pre-symptomatic stage of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) occurs several decades before the clinical onset. Changes in the lysosomal network, i.e. the autophagosomal, endosomal and lysosomal vesicular system, are among the first alterations observed. There are currently no treatments to slow or cure neurodegenerative diseases, and there is a great need for discovery of treatment targets in cellular pathways where pathology pre-dates the neuronal death. It is also crucial to be able to diagnose neurodegenerative diseases earlier, both to enable early intervention treatment and aid in selecting clinical trial populations before the patient has widespread pathology.

This thesis aims at investigating the potential of lysosomal network proteins as biomarkers and therapeutic targets in neurodegenerative disease.

A targeted search for lysosomal network proteins was performed in cerebrospinal fluid (CSF) from AD patients, and seven proteins: early endosomal antigen 1 (EEA1), lysosomal-associated membrane proteins 1 and 2 (LAMP-1, LAMP-2), lysozyme, microtubule-associated protein 1 light chain 3 (LC3), Rab3 and Rab7, were elevated. The levels of EEA1, LAMP-1, LAMP-2, LC3, lysozyme and Rab3 were also measured in CSF from parkinsonian syndrome patients: PD, clinically diagnosed 4-repeat tauopathy, pathologically confirmed corticobasal degeneration (CBD) and pathologically confirmed progressive supranuclear palsy (PSP) patients. LAMP-1 and LAMP-2 were decreased in PD. LC3 and lysozyme levels were increased in 4-repeat tauopathy patients. EEA1 was decreased and lysozyme increased in PSP, and LAMP-1, LAMP-2, LC3 and lysozyme were increased in CBD. The lysosomal network proteins had different CSF protein profiles in all the parkinsonian syndromes, as well as in AD. It should be emphasized that only a select few of the lysosomal network proteins were observed to be changed, rather than a general change in lysosomal network proteins, which implicates the involvement of these seven proteins in specific pathological processes. The most interesting candidates, LAMP-2 and lysozyme, were selected for further study for their involvement in the pathology of AD.

Lysozyme was found to co-localise with A β plaques in AD patients and overexpression prolonged survival and improved the activity in a *Drosophila* model of AD. Lysozyme was found to alter the aggregation pathway of A β_{1-42} , to counteract the formation of toxic A β species and to protect from A β_{1-42} induced cell toxicity. A β_{1-42} in turn was found to increase the expression of lysozyme in both neuronal and glial cells. These data suggest that lysozyme levels rise in AD as a compensatory response which is protective against A β associated toxicity.

LAMP-2 mRNA and protein were found increased in brain areas relevant for AD pathology and various cellular models showed complex involvement of LAMP-2 in A β related pathology, with extensive crosstalk between LAMP-2 and A β . Exposure to oligomeric A β_{1-42} caused an upregulation of LAMP-2 and in turn, overexpression of LAMP-2 caused a reduction in secreted levels of A β_{1-42} , as well as changing the generation pattern of A β and affecting clearance and secretion of A β_{1-42} . These data indicate that the increased levels of LAMP-2 in AD could be an attempt to regulate A β generation and secretion.

In summary, this thesis reports that utilising lysosomal network proteins as biomarkers and novel therapeutic targets for neurodegenerative diseases holds great promise.

Populärvetenskaplig sammanfattning

Neurodegenerativa sjukdomar som Alzheimers sjukdom och Parkinsons sjukdom uppträder ofta sent i livet och sjukdomarna leder till att hjärnans nervsystem långsamt förtvinar. Det finns inga botemedel mot neurodegenerativa sjukdomar, och det finns ännu ingen möjlighet att ställa en diagnos innan stor skada redan skett i hjärnan.

Biomarkörer är ämnen som avspeglar biologiska processer i kroppen, och som kan ge information om en patients hälsotillstånd. Vid neurologiska sjukdomar är många proteiner förändrade i ryggmärgsvätskan, den vätska som omger hjärnan, vilket beror på sjukdomsprocessen i hjärnan. Många neurodegenerativa sjukdomar kännetecknas av ansamlingar av ihopklumpade proteiner, som senila plack i Alzheimers sjukdom, eller Lewy-kroppar i Parkinsons sjukdom. Nervcellens renhållningssystem, det lysosomala nätverket, har bland annat som roll att bryta ned ihopklumpade proteiner, och studier har visat att detta nedbrytningssystem tidigt förändras i många neurodegenerativa sjukdomar.

Målet för den här avhandlingen var att undersöka och utvärdera om det går att använda proteiner från det lysosomala nätverket för diagnos samt nya behandlingsmetoder mot neurodegenerativa sjukdomar.

Vi visar att nivåerna av några specifika proteiner från det lysosomala nätverket är förändrade i ryggmärgsvätska från patienter med de neurodegenerativa sjukdomarna Alzheimers sjukdom, Parkinsons sjukdom, corticobasal degeneration samt progressiv supranucleär pares. De olika sjukdomarna har olika profiler av dessa proteiner i ryggmärgsvätska, en upptäckt som gör att de skulle kunna användas som tidiga och specifika biomarkörer. Två av dessa proteiner, lysosomal-associated membrane protein 2 (LAMP-2) och lysozym, valdes ut för att vidare studera deras roll vid Alzheimers sjukdom.

Lysozym hade en skyddande effekt i både cell- och djurmodeller av Alzheimers sjukdom, och verkade genom att hindra ihopklumpning av det giftiga protein som finns i senila plack. Dessa resultat visar på att lysozymnivåerna stiger vid Alzheimers sjukdom som en försvarsmekanism. De förhöjda nivåerna av LAMP-2 verkar också vara en försvarsmekanism vid Alzheimers sjukdom, då våra studier visar att LAMP-2 påverkar tillverkningen av det giftiga protein som hittas i de senila placken vid Alzheimers sjukdom. Båda proteinerna visar därmed potential att kunna användas vid utveckling av nya behandlingsmetoder.

Sammanfattningsvis fann vi att proteiner från det lysosomala nätverket kan visa sig mycket användbara vid utveckling av nya diagnos- och behandlingsmetoder för neurodegenerativa sjukdomar.

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List of original publications

This thesis is based on the following original publications, which will be referred to by their Roman numerals.

- I. **Andrea Armstrong**, Niklas Mattsson, Hanna Appelqvist, Camilla Janefjord, Linnea Sandin, Lotta Agholme, Bob Olsson, Samuel Svensson, Kaj Blennow, Henrik Zetterberg, Katarina Kågedal. **Lysosomal Network Proteins as Potential Novel CSF Biomarkers for Alzheimer's Disease.** *Neuromolecular Medicine*, 2014. 16:150–160.
- II. Linda Helmfors/**Andrea Boman**, Livia Civitelli, Sangeeta Nath, Linnea Sandin, Camilla Janefjord, Heather McCann, Henrik Zetterberg, Kaj Blennow, Glenda Halliday, Ann-Christin Brorsson and Katarina Kågedal. **Protective properties of lysozyme on β -amyloid pathology; implications for Alzheimer disease.** *Neurobiology of disease* 2015. 83:122-133.
- III. **Andrea Boman**, Samuel Svensson, Adam Boxer, Julio C. Rojas, William W. Seeley, Anna Karydas, Bruce Miller, Katarina Kågedal, Per Svenningsson. **Distinct lysosomal network protein profiles in parkinsonian syndrome cerebrospinal fluid.** Submitted to *Parkinsonism & Related disorders*.
- IV. **Andrea Boman**, Camilla Janefjord, Glenda Halliday, Henrik Zetterberg, Kaj Blennow, Brett Garner, Paul Saftig, Katarina Kågedal. **The role of LAMP-2 in A β PP processing and A β degradation: implications for Alzheimer's disease.** Manuscript.

Abbreviations

α -synuclein	Alpha synuclein
A β	Amyloid beta
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
APs	Atypical parkinsonian syndromes
BACE1	β -secretase
CBD	Corticobasal degeneration
CBS	Corticobasal syndrome
CMA	Chaperone mediated autophagy
CSF	Cerebrospinal fluid
EEA1	Early endosomal antigen 1
ELISA	Enzyme-Linked Immuno-Sorbent Assay
FRET	Förster resonance energy transfer
GBA	β -glucocerebrosidase
GWAS	Genome-wide association studies
HSC70	Heat shock cognate 71 kDa protein
LAMP-1	Lysosomal-associated membrane protein 1
LAMP-2	Lysosomal-associated membrane protein 2
LC3	Microtubule-associated protein 1 light chain 3
LRRK2	Leucine-rich repeat kinase 2
MCI	Mild cognitive impairment
MSD	Meso Scale Discovery protein assay
NFL	Neurofilament light chain
PD	Parkinson's disease
PSP	Progressive supranuclear palsy
P-tau	Phosphorylated tau ₁₈₁
sAPP α	Soluble amyloid precursor protein α
sAPP β	Soluble amyloid precursor protein β
SD	Standard deviation
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel
SEM	Standard error of mean
TEM	Transmission electron microscopy
TGN	Trans Golgi network
T-tau	Total tau
YKL-40	Chitinase-3-like protein 1

Introduction

Neurodegeneration

Neurodegeneration is the common term for the progressive loss of function, structure or death of neurons. Neurons and glial cells are the main cells found in the nervous system, which includes the brain and spinal cord, where neurons are post-mitotic and normally do not divide or replace themselves when damaged. Common neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), prion disease and Huntington's disease, which are all currently incurable and eventually fatal. Even though the pathology differs between these diseases, there may be common etiology on a subcellular level. Neurodegenerative diseases are often caused by genetic mutations and/or aggregation of misfolded proteins and the greatest risk factor is ageing. However, it is becoming increasingly clear, that the development of the diseases results from a complex interplay between lifestyle, environmental, epigenetic and genetic events (Bossy-Wetzel et al., 2004; Ramanan and Saykin, 2013).

Alzheimer's disease

AD is the most common form of dementia and since ageing is the greatest risk factor and life expectancy is on the rise around the world, the prevalence of dementias is also on the rise. In 2013 there were an estimated 44 million dementia patients worldwide, a majority of these being AD patients, and both the G8 Dementia Summit and the World Health Organisation have identified dementia and AD prevention a major public health priority. It has been predicted that the number of people living with dementia will double every 20 years, reaching 136 million by 2050 (Brookmeyer et al., 2007; International, 2013), causing not only great suffering to the patients and their families, but also enormous health care costs.

Pathology

The clinical manifestations of AD are loss of memory, especially episodic memory, and altered cognitive functions such as language, planning and reasoning, orientation and agnosia. Patients also experience apraxia, and in the later stages, often experience severe motor dysfunction (Blennow et al., 2006). After symptomatic onset, the patient usually only lives for another eight to ten years (Jost and Grossberg, 1995). AD associated neurodegeneration mostly affects the basal forebrain, cortex and hippocampus, causing neuronal death, brain atrophy and ventricular enlargement (Wenk, 2003). These pathological changes start several decades before the symptoms arise (Braak et al., 1999). See Figure 1 for development of the typical brain atrophy and ventricular enlargements during preclinical, mild and severe AD.

Figure 1. Coronal brain sections from patients at three different stages of AD, illustrating the progressive development of typical brain damage in AD. Brain slice images by courtesy of National Institute on Ageing

The neuropathological hallmarks of AD are extracellular plaques, intracellular neurofibrillary tangles and loss of synapses. The plaques and tangles were first described by Dr. Alois Alzheimer in 1907 (Alzheimer et al., 1995). In the 1980's amyloid beta ($A\beta$) and hyperphosphorylated tau were discovered to be the main constituents of the plaques and the tangles respectively (Glennner and Wong, 1984a; Masters et al., 1985; Grundke-Iqbal et al., 1986). Tau tangles have been found to correlate better with the degree of dementia than the $A\beta$ plaques, while synapse loss is the neuropathological event that correlates best with clinical stage in AD (Terry et al., 1991). The pathological lesions of AD are distributed in a characteristic manner with tau pathology starting from the inner regions of the brain and spreading outwards, mainly being observed in the hippocampus and entorhinal cortex. The $A\beta$ pathology does the opposite, being observed primarily in the prefrontal, parietal and temporal cortices (Braak and Braak, 1991). Some AD pathology is seen in the brains of cognitively healthy elderly (Price et al., 2009), although severe pathology is only found in patients showing signs of cognitive decline (Nelson et al., 2009), which is consistent with the long lag phase between the first brain changes and onset of the symptomatic phase of the disease (Jack et al., 2013).

Diagnosis

There is no single test for AD; instead a battery of tests must be performed to rule out other causes of disease. The tests include: complete anamnesis, mental status tests, physical and neurological exam, and a wide array of other tests such as blood tests and brain imaging techniques. New updated guidelines were published in 2011 by the "National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease" regarding clinical diagnosis of dementia due to AD (McKhann et al., 2011), mild cognitive impairment (MCI) due to AD (Albert et al., 2011), preclinical AD (Sperling et al., 2011b) and how to assess AD related brain changes during an autopsy (Hyman et al., 2012). Definite diagnosis is still only possible to confirm post mortem, but these new guidelines have facilitated the ease of diagnosis.

A β

A β is produced via sequential cleavage of its precursor, the amyloid precursor protein (APP) (Goldgaber et al., 1987; Kang et al., 1987), a transmembrane protein which belongs to a family of three similar proteins, APP, APLP1 and APLP2, the last two lacking the A β region. There is functional redundancy between these different proteins, which indicates a key physiological role (Sprecher et al., 1993) and several knock-out combinations of these proteins are postnatally lethal in rodent models (Heber et al., 2000). The exact roles of these proteins are still unclear, but they have been shown to play a role in neurite outgrowth and pruning, neuronal survival, synaptic plasticity, neuronal traffic, signal transduction and cell adhesion (Mattson, 2004; Zheng and Koo, 2006; Zhang et al., 2012; van der Kant and Goldstein, 2015). APP is processed in non-amyloidogenic or amyloidogenic manners, the first leading to cleavage within the A β sequence of APP, the latter generating A β from sequential cleavage of APP (Portelius et al., 2011; Zhang et al., 2011), see Figure 2.

Figure 2. APP can be cleaved in a non-amyloidogenic manner by α -secretase, or in an amyloidogenic manner by β -secretase followed by γ -secretase, resulting in the production of A β .

Amyloidogenic generation of A β is rate-limited by, and starts with β -secretase (BACE1) (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000) cleaving APP at the N-terminal boundary of the A β peptide domain. The resulting membrane bound C-terminal fragment of APP is then further cleaved by a membrane-bound, multimeric protein complex, the γ -secretase complex. This complex is composed of four proteins (Edbauer et al., 2003): presenilin 1 (Sherrington et al., 1995) or presenilin 2 (Rogaev et al., 1995; Finckh et al., 2000), nicastrin (Yu et al., 2000), anterior pharynx-defective 1 and presenilin enhancer 2 (Francis et al., 2002). After cleavage at the γ -secretase site, the A β peptide is released. However, the γ -secretase cleavage is imprecise and generates a variety of A β peptides with varying lengths for example A β_{1-38} , A β_{1-42} with A β_{1-40} being the most abundant form (Matsumura et al., 2014). APP is located on the plasma membrane; however, BACE1 cleavage requires APP internalisation from the cell surface, probably via clathrin-mediated endocytosis (Haass et al., 1995; Lai et al., 1995; Vassar et al., 2014). During internalisation, APP is in contact with a wide variety of protein sorting receptors, and is either targeted for recycling to the plasma membrane or allowed to move into the late endosomes (Bhalla et al., 2012; Seaman, 2012).

BACE1 is found enriched in lipid rafts at the plasma membrane (Riddell et al., 2001; Cordy et al., 2003). Also BACE1 is internalised, and targeted to endosomes via an endosomal retrieval signal (Huse et al., 2000; Pastorino et al., 2002), but it can also recycle to the trans Golgi

network (TGN) (Walter et al., 2001) or the plasma membrane (He et al., 2005; Wahle et al., 2005). BACE1 has an acidic pH optimum of pH 4.5 (Vassar et al., 1999), which is similar to the pH 4.5–5.5 that is found in late endosomes (Sorkin and Von Zastrow, 2002); however, BACE1 activity is not exclusively restricted to endosomes.

The location for γ -secretase activity is less clear, but many studies indicate that a complete, proteolytically active, γ -secretase complex, with all subunits cannot be found in endoplasmic reticulum, Golgi, or post-Golgi transport vesicles but rather at the plasma membrane and in the network of endosomes, lysosomes and autophagosomes (Pasternak et al., 2004; Kaether et al., 2006; Nixon, 2007; Dries and Yu, 2008). The relative γ -secretase amount of cleavage that takes place at these locations is unclear and may differ between cell types. It has been shown that the γ -secretase cleavage, for example with respect to which A β fragments are produced, differs in endosomes and the plasma membrane (Fukumori et al., 2006). This probably depends upon the pH or lipid composition of the compartment, but it is clear that altered subcellular localisation of γ -secretase has an impact upon AD pathology. Several A β isoforms are observed in AD, with A β_{1-42} , A β_{1-43} and the N-terminally truncated isoforms A β_{3-42} and A β_{11-42} being neurotoxic, whilst C-terminal truncation of A β generating A β_{1-41} , A β_{1-40} and below are non-toxic (Jonson et al., 2015). The A β_{1-42} to A β_{1-40} ratio increases during AD, A β_{1-42} is highly prone to aggregation and is the main constituent of plaques in the AD brain (Portelius et al., 2010). During A β aggregation, monomers first self-associate to form oligomers, then prefibrillar species, fibrils and finally insoluble plaques. A β aggregation is characterised by a lag phase in which seeds are formed, and a logarithmic phase in which the seeds elongate into fibrils that catalyse further fibrillisation, which in turn accelerates the aggregation process (Wogulis et al., 2005). A β oligomers are thought to be the most toxic aggregation form but the exact mechanism of A β oligomer formation and toxicity is still unknown (Benilova et al., 2012; Kaye and Lasagna-Reeves, 2013).

Tau

Tau is a microtubule-associated protein and is in neurons mainly located in the axons (Binder et al., 1985) and is thought to regulate stability and assembly of the microtubules (Weingarten et al., 1975; Cleveland et al., 1977b; Bre and Karsenti, 1990). The binding strength to the microtubule asserts the stabilising effect, and the binding strength is regulated by splicing and phosphorylation of tau (Cleveland et al., 1977a). Tau is encoded by one gene, but alternative splicing of its three exons gives rise to six different isoforms that differ by way of having three or alternatively four microtubule binding repeats (3R or 4R) and zero to two amino terminal inserts (Goedert et al., 1989). In a healthy adult brain there is a 50:50 ratio of 3R and 4R tau (D'Souza and Schellenberg, 2005), but this ratio can be altered in neurodegenerative tauopathies (Liu and Gong, 2008). The tau protein has 79 phosphorylation sites, which can be phosphorylated by a wide array of kinases (Crespo-Biel et al., 2012). In neurodegeneration, tau is hyper-phosphorylated both at regular epitopes as well as on novel epitopes, some being AD-specific (Yu et al., 2009). Hyper-phosphorylation causes tau to detach from the microtubule and form neurofibrillary tangles (Bramblett et al., 1993; Alonso et al., 1994; Binder et al., 2005), which appear in many different types of neurodegenerative diseases.

The amyloid cascade hypothesis and beyond

The causative events behind AD are still debated, but the most influential theories today relate to the toxic effects of A β and tau. It is believed, based on results in both cellular and animal models, that A β pathology lies upstream of tau (Busciglio et al., 1995; Gotz et al., 2001; Oddo et al., 2003; Choi et al., 2014). However, there is definitively crosstalk between A β and tau, since tau is crucial for conveying the A β toxicity as shown in both cellular and animal tau knockout models exposed to A β (Rapoport et al., 2002; King et al., 2006; Roberson et al., 2007).

The dominating theory regarding the cause of AD is described in the amyloid cascade hypothesis (Selkoe, 1991; Hardy and Higgins, 1992). This hypothesis has been modified over the years, and in the current form states that an increase in toxic A β species, caused by either an increase in A β production and/or a decrease in A β clearance (Mawuenyega et al., 2010), triggers tau pathology, and leads to synaptic failure and neuronal death (Hardy and Selkoe, 2002; Karran et al., 2011). Many scientific findings support this hypothesis. Multiple A β isoforms accumulate in AD brains (Portelius et al., 2010). Naturally occurring genetic mutations in human APP and APP processing genes cause an increased ratio of A β ₁₋₄₂ to A β ₁₋₄₀ which leads to AD. A naturally occurring APP mutation that decreases A β production has been found to be protective against AD (Jonsson et al., 2012). Individuals with Down's syndrome who naturally overproduce A β due to trisomy of chromosome 21 where APP is situated, suffer from early-onset AD like dementia (Glennner and Wong, 1984b). In various experimental settings A β has been found to be toxic for synapses, and neurons (Geula et al., 1998; Lesne et al., 2006), and to activate microglia to cause damage (Del Bo et al., 1995; Griffin et al., 2006). However, the exact mechanism by which A β would cause AD in humans is still unclear, and there are also many scientific findings which do not support the amyloid cascade hypothesis. There is a low correlation between A β load and neurodegeneration or cognitive stage (Blessed et al., 1968; Braak and Braak, 1991). High plaque loads are commonly found in elderly people without cognitive decline (Savva et al., 2009). This together with the last years repeatedly failed clinical trials of A β targeted therapies, has raised the question of the value of the amyloid cascade hypothesis (Selkoe, 2011; Herrup, 2015).

Most likely, AD, like many other diseases, is the end stage of a disease process that is unique for each individual. It involves A β and tau pathology, as well one or many of the following factors: hypoxia, oxidative stress, failure of mitochondrial bioenergetics, neuroinflammation, impaired autophagy and alteration of blood-brain barrier permeability (Raz et al., 2015).

Lifestyle risk versus protective factors

As mentioned above, ageing is the greatest risk factor for developing AD. Around 1% of 65 year old individuals suffer from AD, and the incidence rate rises exponentially, doubling every 5.5 years (Ziegler-Graham et al., 2008), reaching 25% for people aged 85 and above (Ferri et al., 2005). There are several lifestyle-related modifiable risk- and protective factors for AD. Being multilingual, having achieved a higher educational level, having a mentally-engaging occupation and partaking in regular social and physical activities seem to be protective, whilst for example head trauma and cardiovascular risk factors (such as smoking and type 2 diabetes) seem to be detrimental (Blennow et al., 2006; Di Marco et al., 2014). A recent meta

study of over 16 000 research articles found statistical significance for several types of interventions in lifestyle factors, such as education level, body weight and drinking habits, that may decrease new incidence of AD (Xu et al., 2015). However, it is becoming increasingly clear that focus should not be on modifying one single risk factor but rather that a multifaceted approach addressing several lifestyle factors is needed for successful AD prevention (Sindi et al., 2015).

Genetic risk versus protective factors

Familial inherited AD is often caused by mutations in the genes *PSEN1* (St George-Hyslop et al., 1992; Van Broeckhoven et al., 1992; Sherrington et al., 1995), *PSEN2* (Sherrington et al., 1996) and *APP* (St George-Hyslop et al., 1987; Goate et al., 1991) encoding proteins that are involved in A β production (Lambert and Amouyel, 2011). Recently a protective mutation in *APP* was found that lowers A β production and thereby the risk for AD (Jonsson et al., 2012). 99% of all cases of AD are not inherited, yet genetic predisposition also plays an important role in the pathology of sporadic AD, with a heritability estimate of 60-80% (Gatz et al., 2006). Apolipoprotein E (ApoE) plays a role in lipid transport, immunoregulation, and in neuronal growth and regeneration. Three isoforms exist, ApoE2, ApoE3 and ApoE4, which differ at two sites of the amino acid sequence. *Apolipoprotein E4* is a strong genetic risk factor for both inherited and sporadic AD, but its effect is not sufficient on its own to cause disease (Corder et al., 1993; Saunders et al., 1993; Farrer et al., 1997). ApoE binds to A β and has an effect on A β clearance, whereas ApoE4 is thought to be less efficient than the other isoforms (Deane et al., 2008). ApoE4 heterozygotes have a tripled risk for AD and homozygotes a 15-fold elevated risk with a dose-dependent effect upon age of onset (Corder et al., 1993; Saunders et al., 1993). The ApoE2 allele is thought to have a protective effect and delays age of onset (Corder et al., 1994; Farrer et al., 1997). Only 20–25% of the general population carry one or more alleles coding for ApoE4, but 40–65% of AD patients are carriers, and ApoE4 is thought to cause a third of the estimated AD heritability (Lambert et al., 2013).

Genome-wide association studies (GWAS) have identified other AD risk genes that could be linked with the A β cascade and/or tau pathology. Twenty-one genes have been proven to convey AD risk, and novel candidates appear for each new study. The AD risk associated genes cluster within a few pathways: cholesterol and lipid metabolism, immune system and inflammatory response and vesicle cycling. Some of the risk associated genes appear in more than one pathway (Van Cauwenberghe et al., 2015), see Figure 3.

Cholesterol and Lipid Metabolism	Immunity and Inflammation	Vesicular Transport
APOE4	APOE4	APOE4
CLU	CR1	SORL1
ABCA7	ABCA7	BIN1
	EPHA1	PICALM
	INPP5D	EPHA1
	MEF2C	CD2AP
	CD33	FERMT2
		CASS4
		FERMT2
		CASS4

Figure 3. The three main pathways for AD risk associated genes, as identified via GWAS.

Current treatment strategies

The AD pathology affects primarily the cholinergic and the glutamatergic neurons (Davies and Maloney, 1976; Whitehouse et al., 1982). Cholinergic neurons use acetylcholine for signal transduction and there are several different cholinesterase inhibitors available for AD symptom management. These inhibitors delay the degradation of acetylcholine in the synaptic cleft, and therefore signaling is extended, which helps boost the signaling of the few remaining cholinergic neurons. Glutamate is a major neurotransmitter in the brain and crucial in the formation of new memories through the mechanism of long-term potentiation (Francis, 2003). Abnormally high glutamate concentrations are found in AD brains, which can overstimulate NMDA receptors, which in the long run leads to neuronal death (Greenamyre and Young, 1989; Meldrum, 1990; Penney et al., 1990). Another AD symptom management drug is therefore an NMDA “re-setter” (Chohan and Iqbal, 2006).

It should be noted that neither of these drugs cure AD, but they can ameliorate the symptoms of neurodegeneration, before the neuronal death is too severe.

Future treatment strategies and clinical trials

Epidemiological studies have identified various interventions that reduce the risk of AD, but none have been successful in clinical trials so far. The completed and ongoing intervention trials as described by type of intervention are (Andrieu et al., 2015):

- Drug treatments: AD specific pharmacological interventions, non-steroidal anti-inflammatory drugs, antihypertensive treatment, hormone replacement therapy and other compounds: Ginkgo biloba etc.
- Nutritional supplements: Vitamin B, omega-3 fatty acids, flavanol and vitamin E.
- Cognitive activity or training.
- Physical exercise.
- Multi-domain interventions.

Apart from intervention approaches, disease modifying therapies are also being tested, and several completed and ongoing trials are targeting A β , tau or neuronal dysfunction and death.

Anti-amyloid strategies:

- Decrease A β production: β -secretase inhibition, γ -secretase inhibition/modulation, α -secretase enhancement.

- Decrease A β aggregation: decrease metal ion mediated fibrillisation, decrease oligomer formation via reduction of A β monomer, decrease plaques via blocking β -sheet formation.
- Increase A β degradation: Insulin-degrading enzyme activation, neprilysin activation, increase A β clearance.
- A β vaccinations: “active” vaccination with truncated A β peptide, passive immunisation with monoclonal antibody against A β epitope, passive immunisation with antibody against specific conformational forms of A β .

Tau focused strategies:

- Decrease tau and neurofibrillary tangle formation: prevent tau hyperphosphorylation, decrease tau aggregation, stabilise microtubules.
- Active and passive immunisation against tau.

Neuroprotection and/or neuroregeneration strategies:

- Anti-oxidants and other agents to preserve metabolic and/or mitochondrial function.
- Anti-apoptotic agents.
- Decrease inflammatory damage.
- Nerve growth factor enhancement.
- Stem-cell replacement.

Unfortunately, over the last years a long list of failed clinical trials has raised concerns. Three main hypotheses have been used to explain these failures: 1) The wrong mechanisms were targeted, 2) The treatments do not engage the targets in patients, 3) Treatment was administered too late in the disease progression (Sperling et al., 2011a). Current ongoing trials address the need to start treatment at an earlier stage of the disease as well as targeting several targets at once. There is still a need to explore the pathogenesis in the early phase of AD to be able to find novel diagnostic markers as well as drug targets.

Parkinsonian syndromes

Parkinsonian syndromes are neurodegenerative diseases mainly affecting the motor system. Parkinsonian syndrome patients display bradykinesia and one or more of the following signs; resting tremor, rigidity and altered postural reflexes. PD makes up about 80% of parkinsonian cases. The other 20% can be symptomatic, for example vascular PD caused by multiple small strokes, drug-related PD, or caused by other neurodegenerative diseases, so called atypical parkinsonian syndromes (APSs) (Mark, 2001).

Parkinson's disease

PD is a neurodegenerative disease of the motor system in the brain and affects 1-2% of people over 65 years. Most cases are sporadic but over ten genes have been identified that can cause PD, such as the gene that encodes alpha-synuclein (α -synuclein) (Klein and Westenberger, 2012). Symptoms affecting thinking and behaviors such as depression, sleep disturbances and mood changes, arise long before the motor problems (Schrag, 2004). When the motor symptoms appear, they include bradykinesia, shaking, rigidity and difficulties walking. In later stages of the disease dementia appears (Xia and Mao, 2012). The pathology is caused by the death of dopamine-generating cells in the substantia nigra in the midbrain and by the

accumulation of α -synuclein into Lewy bodies inside neurons (Braak et al., 2004; Lees et al., 2009). The Lewy body locations are often related to expression of symptoms in the patient (Braak and Del Tredici, 2008), but little is known about what causes the pathologic processes.

Atypical parkinsonian syndromes

APs such as corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP) is a small and heterogeneous group of diseases that share the bradykinesia and rigidity with PD, but display additional features (Mark, 2001). PD is a synucleinopathy, whilst both CBD and PSP are tauopathies displaying intracytoplasmic aggregates of tau in both neurons and glial cells (Arai et al., 2001).

Corticobasal degeneration

Due to the large number of misdiagnosed patients, the term corticobasal syndrome (CBS) is generally used for patients with a clinical diagnosis of CBD, whilst CBD refers to patients with the diagnosis confirmed via postmortem autopsy. Initial symptoms of CBD are similar to PD with bradykinesia, rigidity and altered postural reflexes which usually are presented only on one side of the body. As the disease progresses both sides will be affected (Wenning et al., 1998; Litvan et al., 1999). Other symptoms include cognitive impairment, hesitant and halting speech, apraxia, decline in spatial function, dysphagia, abnormal muscle postures, myoclonus and loss of discriminative sensation involving one or more parts of the body (Pillon et al., 1995; Boeve et al., 1999). CBD is caused by a gradual deterioration of neurons in the cerebral cortex and the basal ganglia and an accumulation of tau in both neurons and glial cells. The hallmarks of CBD are tau-positive cell processes throughout the gray and white matter, “ballooned neurons”, “astrocytic plaques” and “coiled bodies” in astrocytes and oligodendrocytes in both the gray and the white matter. (Arai et al., 2001; Forman et al., 2002). Several subtypes of CBD with distinct clinical phenomena exist, which further complicates accurate diagnosis (Armstrong et al., 2013).

Progressive supranuclear palsy

The pattern of signs and symptoms for PSP can be quite different from person to person, but the most common first symptom is altered postural reflexes, causing falls, stiffness and difficulty walking (Steele et al., 1964; Litvan et al., 1999; Williams et al., 2005). Other common early symptoms include changes in personality such as apathy, forgetfulness, irritability and mood swings (Pillon et al., 1995; Aarsland et al., 2001). Individuals affected by PSP have trouble voluntarily shifting their gaze downwards, often have trouble controlling their eyelids and have problems maintaining eye contact. The eye problems are usually the first clues that PSP is the correct diagnosis. Speech often becomes slurred and swallowing solid foods or liquids can be difficult (Steele et al., 1964). PSP is caused by a gradual deterioration of neurons in the brainstem including the substantia nigra and a hallmark of PSP is accumulation of tau in both neurons and glial cells. Both tau neurofibrillary tangles and neuropil threads are found in PSP, giving rise to the hallmark “tufted astrocytes” and the “coiled bodies” in oligodendrocytes of the white matter (Litvan et al., 1996; Arai et al., 2001). Several subtypes of

PSP with distinct clinical phenotypes exist, which further complicates accurate diagnosis (Williams and Lees, 2009).

Parkinsonian syndrome diagnosis and treatment

There is currently no treatment to slow or stop the neurodegeneration in PD, but L-DOPA or dopamine agonists can be used for dopamine replacement. As the dopaminergic neurons die, the drug effect is lost. The side effects of the drug include dyskinesia or involuntary movements and exaggerated reward seeking (Dunnett and Bjorklund, 1999). There are currently no available biomarkers for PD, with diagnosis relying solely on clinical diagnosis guidelines (Hughes et al., 1992).

The APSs generally have a worse prognosis than PD, patients rarely respond to dopaminergic medication and therefore palliative care is the only treatment option. For PSP, the most common complications are choking, pneumonia, head injury and fractures caused by falls, so care must be focused on managing these complications, and ensuring good nutrition. If attended properly, most PSP patients can live a decade or more after the first symptoms appear (Muller et al., 2001; Lang, 2005). For CBD, the myoclonus can be managed with drugs, and physical and speech therapy can help with managing other symptoms. However, despite proper attention, most patients live only six to eight years after the first symptoms occur, and the cause of death is often pneumonia (Muller et al., 2001; Lang, 2005). No biomarkers are currently available, with diagnosis relying solely on clinical diagnosis guidelines for both PSP (Litvan et al., 1996) and CBD (Armstrong et al., 2013).

Cerebrospinal fluid biomarkers

Cerebrospinal fluid

Cerebrospinal fluid (CSF) is the clear body fluid inside and around the brain and in the spine. It is mainly produced by glial cells of the choroid plexus located in the brain ventricles, but about a third comes from the linings of the ventricular and subarachnoid spaces. There are multiple functions for CSF, including: maintaining neutral buoyancy so the weight of the brain does not damage neurons and cut off blood supplies to the brain, regulating cerebral blood flow, giving protection against impact, maintaining chemical stability and clearing waste products. CSF is reabsorbed into the bloodstream, allowing for homeostatic regulation of neuroendocrine factors, as well as flushing waste products from the interstitial fluid during sleep (Iliff et al., 2012). The areas around the brain and spinal cord can contain only 135-150 ml, but CSF is produced at a rate of 500 ml per day; therefore there is a full turnover 3-4 times per day when CSF is drained mainly into the blood, leading to a rapid flux of molecules into the CSF, and making it a relatively accessible “window to the brain”. CSF can be sampled by a trained clinician using a lumbar puncture, which is a simple and minimally invasive procedure, if performed correctly and which rarely cause complications, with headache being the most common complaint (Andreasen et al., 2001). In spite of the low risk of the procedure, patient fear of a lumbar puncture has limited the worldwide use of CSF based diagnostics.

Biomarkers

The definition of a biomarker is an objectively measurable indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic (Biomarkers Definitions Working, 2001). Even though the term is recent, biomarkers have been used for research and diagnosis for a long time. A classic example is body temperature being a biomarker for fever. Biomarkers can be anything from molecules, genes and, gene products to complex organ functions, which can be assayed or imaged using a variety of techniques. This thesis focuses on protein biomarkers in CSF. Apart from genomic and proteomic platform approaches to finding novel biomarkers, also metabolomics, lipidomics, glycomics and secretomics approaches are used to identify novel biomarkers in CSF.

Several aspects must be taken into consideration when investigating the qualities of a novel candidate biomarker, namely its sensitivity, specificity, robustness, accuracy and the reproducibility of its measurement. The end use of a biomarker can also differ, varying from estimating disease risk (prognostic biomarker), evaluating disease stage (staging biomarker), help with clinical diagnosis (diagnostic biomarker) and monitoring therapeutic success (theragnostic biomarker) (Blennow et al., 2010).

Unfortunately, multicenter studies have shown widely differing cut-off values and considerable variability in concentrations and diagnostic performance when comparing the same biomarkers in CSF. This variation could be caused by several factors such as a variation in CSF sampling procedures, CSF blood contamination, CSF handling temperature after lumbar puncture, type of test tubes for sample collection, freezing and thawing procedures and length of storage (Mollenhauer et al., 2010; Mattsson et al., 2011).

Established biomarkers for Alzheimer's disease

Three proteins in CSF have been established internationally as biomarkers for AD: $A\beta_{1-42}$, total tau (T-tau) and phosphorylated tau₁₈₁ (P-tau). These three biomarkers have been incorporated into the new diagnostic criteria for AD from the International Working Group and the US National Institute on Aging Alzheimer's Association (Dubois et al., 2014).

Several groups have worked in consort to standardise the measurements as well as validate the markers in large cohorts. Apart from using the three markers together for identifying AD patients (Blennow and Hampel, 2003; Bloudek et al., 2011), the ratio of total T-tau or P-tau to $A\beta_{1-42}$ has been shown to be predictive of cognitive decline in cognitively normal subjects (Fagan et al., 2007; Li et al., 2007) as well as for individuals with MCI or very mild dementia (Hansson et al., 2006; Snider et al., 2009; Landau et al., 2010; Buchhave et al., 2012).

$A\beta_{1-42}$ is decreased in the CSF of AD patients (Motter et al., 1995). The decrease is considered to be caused by retention of $A\beta_{1-42}$ in plaques (Strozyk et al., 2003), although other explanations could be lowered $A\beta_{1-42}$ production due to neuronal death or reduced synaptic activity (Cirrito et al., 2008). Decreased $A\beta_{1-42}$ in CSF is not exclusive for AD; other neurodegenerative dementias, such as dementia with Lewy bodies (Mollenhauer et al., 2005) and multiple sclerosis with dementia (Mori et al., 2011), can show a decrease in CSF $A\beta_{1-42}$. Decreased $A\beta_{1-42}$ is not even exclusive to neurodegenerative dementia, as it has also been reported for HIV-associated neurological diseases (Clifford et al., 2009).

Tau is highly expressed in the central nervous system, mostly in neurons, but also at low levels in astrocytes and oligodendrocytes. In many neurodegenerative diseases, tau becomes hyperphosphorylated and is then unable to stabilise the microtubules properly. Tau is highly expressed in cortical axons, and is therefore increased in cortical diseases such as AD (Blennow and Hampel, 2003) and Creutzfeldt-Jacob disease (Otto et al., 1997; Blennow et al., 2005). P-tau correlates to the number of tau tangles in AD (Buerger et al., 2006) but not in other diseases with increased T-tau such as frontotemporal lobe dementia (Buerger et al., 2002) and Creutzfeldt-Jacob disease (Riemenschneider et al., 2003).

Biomarkers for parkinsonian syndromes

There are currently no validated biomarkers for PD, and the levels of the biomarkers $A\beta_{1-42}$, T-tau and P-tau in the CSF from PD patients are similar to that of controls (Parnetti et al., 2011). The search for PD specific biomarkers has been focusing on α -synuclein, and α -synuclein concentrations in CSF has been studied by several groups with conflicting results (Mollenhauer et al., 2008; Schulz-Schaeffer, 2010; Parnetti et al., 2013). The levels of α -synuclein seem to be decreased in PD but no cut off levels have been established. Therefore, patients with synucleinopathy cannot be distinguished from healthy individuals. Focus has therefore been put on determining the diagnostic accuracy of different combinations of CSF α -synuclein, T-tau and P-tau ratios (Mollenhauer et al., 2011). It has been suggested that low T-tau to α -synuclein, or low P-tau to α -synuclein ratios can discriminate PD from Lewy body dementia, AD and frontotemporal dementia (Kasuga et al., 2010).

CBD and PSP also lack any validated biomarkers. In spite of PSP and CBD being tauopathies, the results from studies measuring T-tau and P-tau in CSF are inconclusive (Urakami et al., 1999; Urakami et al., 2001; Noguchi et al., 2005; Borroni et al., 2009), and some studies indicate that it could be more useful to analyse levels of tau fragments and tau phosphorylation (Guillozet-Bongaarts et al., 2007; Portelius et al., 2008; Borroni et al., 2009).

There are also no validated biomarkers to differentiate PD versus APSs. CSF levels of neurofilament light chain (NFL) have been found to be elevated in several APSs among them PSP and CBD, and statistical models might be used to differentiate PD from these diseases (Hall et al., 2012; Sako et al., 2015). However, NFL is also increased in many other neurodegenerative diseases and does not seem to be effective for distinguishing between different forms of APS (Magdalinou et al., 2014).

There is also promising data showing that other CSF proteins, including $A\beta_{1-42}$, and neurofilament heavy chain, could differentiate patients with PSP from healthy control individuals, or those with other parkinsonian syndromes (Holmberg et al., 1998; Noguchi et al., 2005; Brettschneider et al., 2006), but further studies are needed.

APS specific biomarker studies have apart from α -synuclein looked at markers for inflammation: monocyte chemoattractant protein-1 and chitinase-3-like protein 1 (YKL-40) as well as $A\beta$ related markers: soluble amyloid precursor protein α and β (sAPP α and β) and $A\beta_{1-42}$ (Magdalinou et al., 2014). A problem with biomarker studies in CSF for PSP and CBD is the difficulty obtaining a differential diagnosis in the initial stages of the disease. Hence, studies performed on patients diagnosed only clinically, could unknowingly be investigating another disease. A recent study used a panel approach, where a combination of the nine

biomarkers mentioned above could potentially differentiate PD patients from APSs patients. This study was performed on CBD and PSP patients, with 14% and 18% pathologically confirmed participants respectively (Magdalinou et al., 2015).

Novel biomarker approaches

It is becoming increasingly clear that the key to curing neurodegenerative disease is to be able to diagnose it earlier and thereby enable treatment to begin prior to the patient suffering widespread pathology and neuronal loss.

Blood is a biological fluid which is easy to obtain through very simple, minimally invasive and cheap procedures, which are readily available at any general practitioner. It has been suggested that blood-based markers could serve as the first analysis step in a multi-stage assessment, where any bio-marker positive patient could then be referred for more advanced analyses. The search for blood-based biomarkers of neurodegeneration has not yet led to any clinical applications, and the field is struggling with challenges with reproducibility and standardisation, however, global initiatives are currently being put in place to combat these issues (Lista et al., 2015).

CSF $A\beta_{1-42}$, T-tau and P-tau will most likely continue to be the primary CSF biomarkers for diagnosis of AD. However, novel markers are needed for AD prognosis, clinical staging, therapeutic monitoring and identification of individuals at increased risk of rapid cognitive decline. Since many neurodegenerative conditions present with AD-like clinical symptoms, making a differential diagnosis difficult, and many patients have mixed pathologies, which further complicates diagnosis, there is a great need for validated biomarkers for non-AD neurodegenerative disease (Sancesario and Bernardini, 2015). CSF biomarker studies were previously hampered by limited access to samples from participants with long enough clinical follow-up times. Fortunately, several large collections have been built up, and large, longitudinal AD biomarker studies are ongoing, such as the: Alzheimer's Disease Neuroimaging Initiative, Australian Imaging Biomarkers and Lifestyle Study of Ageing and the Dominantly Inherited Alzheimer Network (Fagan and Perrin, 2012). Novel biomarkers must be validated in large, well-characterised research cohorts, and standardisation must be achieved in and between different research laboratories in order to maximise reproducibility as well as permit meta-studies (Mattsson et al., 2011). For example, the report of diurnal variations in CSF $A\beta$ levels (Bateman et al., 2007), illustrates the importance of sample collection and processing procedures to be standardised at every step in the process.

Many studies are now focused on combinations of biomarkers rather than finding a single biomarker for a disease. Promising novel upcoming protein biomarkers are other forms of $A\beta$ and tau, and various proteins involved in APP processing, synapse loss, neurodegeneration, neuroinflammation, complement system, oxidative stress and apolipoproteins. There are also several novel approaches, such as studying metabolomes, DNA and RNA oxidation and miRNAs in CSF (Fagan and Perrin, 2012). Another interesting aspect since personalised medicine is on the rise is that changes in analyte levels over time within the patient has been shown to be a more reliable measure than baseline measures alone (de Leon et al., 2007).

None the less, the etiology of the different neurodegenerative diseases is largely unknown and there is a huge need for exploration of cellular pathways underlying the pathogenesis in the

early phases of disease, to find promising novel biomarkers and drug targets. One cellular system that is changed early on in multiple neurodegenerative diseases is the lysosomal network.

The lysosomal network

Cellular degradation

There are two main degradation systems in the cell, the autophagic-lysosomal pathway for long lived proteins and organelles, and the ubiquitin-proteasomal pathway for short-lived proteins. These two systems crosstalk and can, to some degree, compensate for loss of function for each other (Knecht et al., 2009). The organelles called lysosomes degrade material for later recycling of building blocks within the cell, and the organelles endosomes and autophagosomes bring material for degradation from outside and inside the cells, respectively. The endosomal, lysosomal and autophagosomal vesicles are in constant flux, with extensive crosstalk and fusion events occurring at a fast rate. This interrelated system of vesicles is in this thesis referred to as the lysosomal network, see Figure 4.

Figure 4. Schematic overview of the lysosomal network, consisting of autophagosomes, endosomes and lysosomes.

Endosomes

Endosomes are vesicles of 100-500 nm in diameter. They become enlarged and drop in pH (from pH 6.0-6.5 to 4.5-5.5) as the vesicle matures from an early to late endosome. The endosome is the major sorting compartment of the cellular endomembrane system, sorting material based upon location tags from the three pathways: TGN to and from endosomes, plasma membrane to and from early endosomes, and from late endosomes to lysosomes. At the plasma membrane, a common internalisation method is receptor-mediated endocytosis via clathrin-coated vesicles (Andersson, 2012). Once endocytic vesicles have uncoated, they fuse with early endosomes. Recycling back to the plasma membrane proceeds via recycling endosomes, and material not tagged for recycling is degraded when early endosomes mature into late endosomes before fusing with lysosomes (Doherty and McMahon, 2009).

Autophagosomes

Autophagosomes are vesicles of 150-900 nm in diameter. Autophagosome formation takes about five to ten minutes in mammals (Mizushima et al., 2001). The formation of this double membrane vesicle is tightly regulated. A lot of progress has been made in elucidating the regulation of the formation of the autophagosome, but the process is still not fully understood. The macromolecules and organelles enclosed in the vesicle after the formation of the autophagosome are rapidly brought to lysosomes for vesicle fusion and then degradation. This process is called macroautophagy, and is in this thesis referred to as autophagy, although alternative forms of autophagy exist. Alternative forms of autophagy are chaperone-mediated autophagy (CMA) and microautophagy, which take place in the lysosomes. Autophagy is part of normal cell growth and development wherein the protein mTOR plays an important regulatory role. Autophagy has an important role in the degradation of damaged organelles, cell membranes and proteins, as well as the release of nutrients during starvation and the destruction of some types of bacteria within the cell (Eskelinen and Saftig, 2009; Shibutani and Yoshimori, 2014).

Lysosomes

Lysosomes are vesicles of 100-1200 nm in diameter and have substantially lower pH than the surrounding cytosol (pH 4.8 versus 7.2). Lysosomes are the cells' waste disposal system containing a battery of acid hydrolases such as lipases, amylases, proteases and nucleases, for the breakdown of waste materials and cellular debris. Apart from digesting macromolecules delivered via endocytosis and autophagy, the organelle can also phagocytose cytosolic material via invagination, protrusion, and/or septation of the lysosomal limiting membrane, a process called microautophagy (Luzio et al., 2007). Material can also be taken into the lysosomes for degradation via CMA, whereby proteins that have the KFERQ consensus peptide sequence are recognised by the binding of a heat shock cognate 71 kDa protein (hsc70)-containing chaperone complex. The complex then moves to the lysosomes for degradation, the protein is unfolded and internalised via LAMP-2 located in the lysosomal membrane (Cuervo and Wong, 2014).

The importance of the lysosomal compartment for cellular health and survival is highlighted by the occurrence of inherited metabolic lysosomal storage diseases such as Danon, Gaucher or Niemann-Pick type C1 disease. In these lysosomal storage diseases, mutations affecting lysosomal function cause abnormal accumulation of undegraded materials inside the lysosomes. The symptoms of the diseases vary, depending on the particular disorder, and can be mild to severe, including developmental delay, movement disorders, seizures, dementia, deafness and blindness. There are no cures for lysosomal storage diseases and treatments provide mostly symptomatic relief (Platt et al., 2012).

The lysosomal network in neurons

Neurons are post-mitotic cells making them more vulnerable to accumulation of toxic components than dividing cells. Neurons also have a high metabolic rate and highly specialised structures, such as axons, dendrites and synapses, all performing specific functions (Lee, 2012; Son et al., 2012).

Inhibition of autophagy is known to cause neurodegeneration in mature neurons (Boland and Nixon, 2006; Hara et al., 2006), and autophagy is induced in several diseases to protect cells against neuronal cell death by removing toxic components (Todde et al., 2009).

The lysosomal network is a sensitive system, where any imbalance in autophagic flux can lead to cell death, either from an increase in autophagosome formation and/or a decrease in degradation. Autophagy is involved in several physiological processes such as normal development, cellular homeostasis, life span expansion, tumor suppression and immunity, as well as in stress associated conditions (Lee, 2012). Autophagy, CMA and lysosomal protein degradation have also been shown to decline with age (Cuervo and Dice, 2000a; Martinez-Vicente et al., 2005). The multitudes of intracellular processes that are dependent upon the lysosomal network have led to the proposal that their failure with age is key in the pathogenesis of several age-related pathologies. Impaired autophagy has been detected in many human diseases, including cancer, cardiovascular disease and neurodegenerative disease (Son et al., 2012).

The lysosomal network in Alzheimer's disease

A β is generated in the lysosomal network, most of the produced A β is excreted, but the intracellular A β is mostly found in autophagosomes and in lysosomes (Lee, 2012).

Lysosomes are also one of the primary compartments for A β and tau degradation (Kenessey et al., 1997; Mueller-Steiner et al., 2006). A β_{1-42} being more resistant to degradation than A β_{1-40} (Ling et al., 2009) and phosphorylated tau being harder to degrade than unphosphorylated tau (Mercken et al., 1995). With age, elevated oxidative stress results in the accumulation of damaged proteins leading to an even greater load on the lysosomal degradation system that is already declining due to age. Accumulation of lysosomes in dystrophic neurites of AD patients was already described in the 1960's (Suzuki and Terry, 1967), and upregulation and/or dysfunction of the lysosomal network in AD brain was later confirmed in the 1990's (Cataldo and Nixon, 1990; Cataldo et al., 1995). The upregulation of the lysosomal network occurs early in the disease process, when the levels of oligomeric A β are rising, but no plaques have formed (Nixon and Cataldo, 2006). Exposing neurons to A β leads to accumulation of A β in lysosomes and an AD like axonal destruction (Ditaranto et al., 2001; Chafekar et al., 2008; Lee et al., 2011). A β accumulation in lysosomes has also been observed in animal models of AD (Langui et al., 2004). A β aggregation is exacerbated by low pH, so the acidic pH in the lysosomes promotes the aggregation and build-up of A β and other undegraded material, which can cause lysosomal leakage (Ditaranto et al., 2001). Furthermore, autophagosomes containing undegraded material, accumulate in AD neurons, indicating that the fusion with or transport to the lysosomes can be dysfunctional (Nixon et al., 2005).

The lysosomal network in parkinsonian syndromes

Even though genetic variants account for only a small proportion of patients, they give insights into the underlying pathophysiology of PD. The loci coding for β -glucocerebrosidase (GBA), α -synuclein, parkin, pink1, DJ-1, leucine-rich repeat kinase 2 (LRRK2) and VPS35 have all been implicated in the pathology of PD, and they can all affect degradation in the lysosomal network. Mitochondrial dysfunction and α -synuclein aggregation play major pathogenic roles in PD (Dauer and Przedborski, 2003; Vila et al., 2008; Hattingen et al., 2009). Lysosomal degradation is the main degradation pathway for α -synuclein as well as being the only mechanism for turnover of dysfunctional mitochondria, via a specialised macroautophagy process called mitophagy (Chu et al., 2007; Mizushima, 2007; Vogiatzi et al., 2008). Parkin and PINK1, whose mutations cause an autosomal recessive form of PD, play important regulatory roles in mitophagy (Dagda et al., 2009; Vives-Bauza et al., 2010), and one of the roles of DJ-1s is to maintain mitochondrial homeostasis (Ottolini et al., 2013). LRRK2 also associates with mitochondria where it interacts with parkin (Smith et al., 2005). VPS35 is a component of the retromer cargo-recognition complex critical for endosome-TGN trafficking (Vilarino-Guell et al., 2011).

The expression levels of the CMA proteins LAMP-2A and hsc70 are reduced in the substantia nigra and amygdala of PD brains compared with age-matched AD and control samples, and α -synuclein inclusions in these areas contain autophagy-related proteins. Decreasing LAMP-2A levels in dopaminergic cell lines reduced the CMA activity and increased the half-life of α -synuclein (Alvarez-Erviti et al., 2010). LAMP-2A is reduced in association with increased α -synuclein dysregulation of CMA and this occurs before substantial α -synuclein aggregation in PD (Murphy et al., 2015). CMA is therefore considered to be the main mechanism by which α -synuclein gets into the lysosomes, but macroautophagy can compensate for loss of CMA function (Cuervo et al., 2004).

The degradative capacity of lysosomes is impaired in PD. In experimental models of PD, a mitochondrial-driven oxidative attack causes toxic leakage from lysosomes and a buildup of autophagosomes, which cannot fuse with the dysfunctional lysosomes (Dehay et al., 2010).

Mutations in the gene encoding the lysosomal protein GBA are involved in the pathogenesis of PD as well as the lysosomal storage disease Gaucher disease. Mutations in the GBA gene reduce or eliminate the GBA activity and sphingolipids can build up inside cells, especially inside macrophages and monocytes (Chen and Wang, 2008). It is suggested that mutations in the GBA gene impairs the function of lysosomes and thereby contributes to the faulty breakdown of toxic substances in nerve cells. A recent study also showed lower GBA activity in PD patients without GBA mutations suggesting a more complex involvement of GBA in the pathogenesis of PD (Alcalay et al., 2015).

Little is known about the underlying pathology of CBD and PSP, although a recent GWAS study identified new susceptibility loci, and a PSP risk was the *STX6* gene, encoding a SNARE-class protein that regulates vesicle membrane fusion, which could potentially indicate lysosomal network involvement in PSP (Kouri et al., 2015).

LAMP-2 in neurodegeneration

LAMP-2 is a lysosomal membrane protein with three isoforms LAMP-2A, 2B and 2C, which differ in their transmembrane domain as well as the cytosolic tail. The isoforms are differently expressed in various tissues but all three isoforms are expressed in the brain (Eskelinen et al., 2005; Murphy et al., 2015). Through different mechanisms LAMP-2 has functions in lysosome motility and integrity, CMA, cholesterol metabolism and autophagosome-lysosome docking (Eskelinen et al., 2005; Eskelinen, 2006). Mutations in the LAMP-2 gene cause the congenital Danon's disease, with heart and skeletal muscle weakening and intellectual disabilities, leading to pre-mature death (D'Souza R et al., 2014). LAMP-2A plays a role in neurodegenerative movement disorders where alterations in CMA are part of the pathology, and decreased levels of LAMP-2A have been reported in PD as well as in models of Huntington's disease (Koga et al., 2011; Cuervo and Wong, 2014).

Lysozyme in neurodegeneration

Lysozyme is both a lysosomal luminal, and secreted protein that plays a part in the innate immune system (Ganz, 2004). How much lysozyme that is kept intracellularly and how much that is secreted is dependent upon cell type and external stimuli. It is secreted by epithelial cells, macrophages, astrocytes and microglia, and it has been estimated that for example monocytes secrete two thirds of its produced lysozyme (Lemansky and Hasilik, 2001). Lysozyme is a glycoside hydrolase and possesses bacteriolytic (Fleming, 1922), anti-oxidant and anti-inflammatory properties (Ogundele, 1998; Liu et al., 2006; Lee et al., 2009). Lysozyme is active over a broad pH range (pH 3 to 8) so it can hydrolyze substrates both within and outside cells (Banerjee et al., 1973). Lysozyme has been reported to be increased in CSF during inflammation (Hällgren et al., 1982), and was recently shown to inhibit A β ₁₋₄₀ (Luo et al., 2013, 2014) and A β ₁₇₋₄₂ (Das et al., 2014) aggregation. Moreover, in a recent large microarray investigation of more than 12,500 genes in five Alzheimer mouse models, lysozyme was one of the key immune genes identified to be overexpressed (Matarin et al., 2015).

The lysosomal network for diagnosis and treatment of neurodegeneration

Prior research points to the lysosomal system as a major actor in many neurodegenerative diseases, and the changes observed pre-dates clinical symptoms by many decades. Therefore, it is of greatest interest to investigate the possibility of using proteins from the lysosomal network both as diagnostic markers as well as evaluate their potential as therapeutic targets, which will be the focus of this thesis.

Aims of the thesis

The general aim of this thesis was to investigate lysosomal network proteins in neurodegenerative disease in the aspect of their potential as sensitive and specific biomarkers and therapeutic targets. The aim also included to investigate the involvement of the lysosomal network proteins lysozyme and LAMP-2 in AD pathology, and to evaluate their potential as future therapeutic targets. The more specific aims of the four papers included in this thesis are listed below.

Paper I

The aim of paper I was to investigate whether the early pathological changes in the lysosomal network observed in AD patients' brains are mirrored in their CSF. A targeted search for thirty-four endosomal, autophagosomal and lysosomal proteins was performed.

Paper II

The aim of paper II was to investigate the lysosomal and innate immunity protein lysozyme and its role in AD pathology. Lysozyme, which previously was reported to prevent the aggregation of $A\beta_{1-40}$ in vitro, was to be investigated in; in vitro assays, cellular models, a novel *Drosophila* model and in human brain, CSF and serum. Lysozyme levels and localisation in AD patients and *Drosophila* were determined, as well as its interactions with $A\beta_{1-42}$, and its effect on $A\beta_{1-42}$ aggregation and toxicity.

Paper III

The aim of paper III was to investigate whether levels of lysosomal network proteins are changed in parkinsonian syndromes, since faulty protein degradation plays an important role in many neurodegenerative disorders. CSF from PD, CBD and PSP patients was to be investigated for changes in levels of endosomal, autophagosomal and lysosomal proteins; to determine if any difference could be detected between the diseases.

Paper IV

The aim of paper IV was to investigate the mechanistic involvement of the lysosomal protein LAMP-2 in AD pathology. Through different mechanisms LAMP-2 has crucial functions in autophagy and degradation. The effect of $A\beta$ on the expression and secretion of LAMP-2 and vice versa, was also investigated, as well as LAMP-2s effect on changes to generation and cellular processing of $A\beta$.

Methods

The following section describes the experimental procedures used in papers I-IV, on which the results in this thesis are based. The methodologies are summarised here, and detailed descriptions of the experimental procedures are provided in the respective paper.

Ethics

Human de-identified and archived CSF samples were obtained from the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden, according to regulations from the Swedish Central Ethical Board.

Brain tissues were obtained from the Sydney Brain Bank at Neuroscience Research Australia and the New South Wales Tissue Resource Centre at the University of Sydney. Tissue collection procedures were approved by the University of New South Wales Human Research Ethics Committee, ethical permission HE10/327.

Investigations of the patients and collection and analysis of human CSF samples obtained from the Karolinska Hospital, were approved by the ethics committee, and informed consent was obtained from the subjects. Ethical permit numbers: Karolinska Hospital, Per Svenningsson/Stockholms Läns landsting, ethical permission 2011/500-31/1 and 2012/2224-32/4.

Investigations of the patients, collection and analysis of human CSF samples and brain autopsy diagnosis confirmation procedures for CSF samples obtained from the University of California San Francisco, were approved by the ethics committee and informed consent was obtained from the subjects. Ethical permit numbers: University of California San Francisco, Adam Boxer ethical permission 10-04442 and Bruce Miller ethical permission 10-03946.

Models used in the studies

Human postmortem brain

To study how protein expression is affected in AD, human postmortem tissue from hippocampus, frontal cortex, temporal cortex and cerebellum was used. Since human brain tissue is of great value for studying disease mechanisms, but does not give information about progressive mechanisms, brains from different disease stages were analysed. However, differences between sample handling and postmortem times can differ substantially and thereby affect protein expressions. Tissues received from the Sydney Brain Bank were selected based on their pathological characterisation with AD according to established neuropathological criteria (Montine et al., 2012).

Clinical diagnosis of PSP and CBS/CBD is based on physical manifestations of the disease and is hampered by the fact that the symptoms overlap with many other common neurodegenerative diseases; therefore, diagnosis can only be confirmed postmortem. PSP and CBD patients, from the University of California, San Francisco Memory and Aging Center were clinically diagnosed using, for PSP, the National Institute of Neurological Disorders and Stroke-Society for Progressive Supranuclear Palsy criteria (Litvan et al., 1996), and for CBD, according to the Armstrong criteria (Armstrong et al., 2013). Both patient groups were pathologically confirmed using standard procedure (Gallyas, 1971).

The LAMP-2 RNA levels in hippocampus were extracted from the microarray dataset “Alzheimer’s disease at various stages of severity”, code GDS810 / 221866 at the NCBI database: <http://www.ncbi.nlm.nih.gov/sites/GDSbrowser> (Blalock et al., 2004).

Human CSF and serum

The studies were performed on the following sample sets:

- Biochemically diagnosed high-T-tau CSF samples (n=5)
- Biochemically diagnosed AD CSF samples (n=10)
- Biochemically diagnosed AD CSF and serum samples (n=10)
- Biochemically and clinically diagnosed AD CSF samples (n=25)
- Clinically diagnosed PD CSF samples (n = 18)
- Clinically diagnosed 4-repeat tauopathy CSF samples, CBS (n=6) and PSP (n=5)
- Clinically diagnosed and pathologically confirmed CSF samples, PSP (n=8) and CBD (n=7)

A significant methodological problem with CSF analysis is the need for matched healthy control groups that are medically and psychiatrically normal. The patients in this thesis were age and gender matched to control individuals. The control individuals, for the parkinsonian syndrome samples set sought medical advice for benign neurological conditions, such as tension headache and unclassified sensory disturbances. The control individuals for the AD and high-T-tau samples, sought medical advice due to minor cognitive or neurological symptoms, but had normal levels of the CSF AD biomarkers P-tau_{181p}, T-tau and A β ₁₋₄₂. The participants underwent lumbar puncture in the L3-4 or L4-5 areas, the CSF was collected in polypropylene tubes, centrifuged, frozen, later thawed, aliquoted and restored at -80 °C pending analysis.

Drosophila melanogaster

The advantages of *Drosophila* as a model are plenty, including short generation time, large numbers of offspring, easy and cost effective to propagate and a well-studied genome. Many molecular mechanisms such as autophagy are highly conserved between species; however, care should be taken when drawing conclusions about human disease from a *Drosophila* model, since it does not capture the complete pathogenesis.

The Gal4/UAS system was used for CNS-specific expression of transgenes, and the following strains were used: C155-gal4 flies carrying UAS-containing genes encoding human wild type lysozyme (Kumita et al., 2012), A β ₁₋₄₂ (Crowther et al., 2005) and a novel strain encoding both lysozyme and A β ₁₋₄₂ were generated for the study. The *Drosophila* longevity assay only indicates whether the fly is alive or not, and is therefore best run in parallel with a locomotor assay to also assess the health of the flies. This is done indirectly by measuring their velocity, negative geotacticity as well as angle of movement, which all change with increasing age and ill-health. The iFly software (Jahn et al., 2011), recording the locomotor activities in 3D, gives a richer picture of the disease process than earlier locomotor assay recording formats.

Cells

The cell lines used in this thesis were human neuroglioma (H₄APP_{swc} cells), human neuroblastoma (SH-SY5Y) cells and LAMP-2 overexpressing human neuroblastoma (LAMP-2 SH-SY5Y) cells. The SH-SY5Y cell line can be differentiated into neuronal-like cells upon retinoic acid treatment that stops cell proliferation and induces extension of neuritic processes. Human LAMP-2A, LAMP-2B, LAMP-2C inserted into the pCINeo vector as described elsewhere (Schneede et al., 2011), were stably transfected using the Lipofectamine LTX reagent (Life Technologies, Karlsbad, CA, USA). Conventional immortalised cell lines are an excellent tool used to study molecular and cellular processes in a simple and controlled environment and do not require ethical permission. Although they are usually poor representations of cells found in intact tissues, they are a good substitute to primary neuronal cultures since they do not require the sacrificing of animals.

Visualisation and quantitative measurement of proteins

Enzyme-Linked Immuno-Sorbent Assay (ELISA)

The ELISAs in this thesis were sandwich assays, where a capture antibody in the bottom of the wells binds a specific antigen in the sample. Detection is then performed via a biotinylated detection antibody which binds to a streptavidin-enzyme complex (for example horseradish peroxidase) that reacts with a substrate to develop colour, measured as changed absorbance using a spectrometer. The colour intensity is a measure of the antigen concentration in the sample. Sandwich ELISAs are excellent for crude samples, reducing non-specific binding to the assay wells, and the assay can be quantitative if a standard curve is used; however, the assay usually requires large volumes of sample. The following ELISAs were used: Levels of A β ₁₋₄₂, T-tau and P-tau in CSF were measured using the INNOTEST® ELISA (Innogenetics, Ghent, Belgium). Insoluble A β ₁₋₄₂ levels in brain preparations were measured using the Beta Amyloid x-42 ELISA kit (Covance, Princeton, NJ, USA). Levels of aggregated forms of A β without detection of monomers were determined using the previously described ELISA (Holtta et al., 2013). All analyses were performed by certified laboratory technicians blinded to the specifics of the clinical sample.

Meso Scale Discovery protein assay (MSD)

The MSD technique is similar to the ELISA technique but uses electrochemiluminescence for detection. The advantages of the MSD assay over normal ELISA is that it is not temperature sensitive, and gives a higher signal to background ratio. Each assay comes with standards, which makes MSD a quantitative technique; however, there is a risk for cross-reaction for the multiplex assays. The lysozyme, sAPP and A β ₁₋₄₂ assays were used (MSD, Rockville, MD, USA).

Western blotting

In Western blot, denatured proteins are separated according to size on a sodium dodecyl sulfate (SDS) polyacrylamide gel (PAGE), transferred to a membrane, non-specific sites are blocked, and then antibodies directed against the protein of interest are applied, followed by secondary horseradish peroxidase-conjugated antibodies. A substrate that becomes

luminescent when cleaved by the peroxidase is used to visualise the protein and the light is captured by a photosensitive film. The gray black signal correlates to amount of specific protein in the sample. Determination of total protein concentration in a sample prior to Western blotting can be performed via the DCTM Protein Assay (Bio-Rad, California, CA, USA), or a housekeeping protein can be used as reference on the blot. Densitometric analysis is used to quantify protein concentrations. The advantages of Western blotting are the small sample volumes needed, that several proteins can be analysed on the same blot in the same sample, and that different cleavage products can be identified. However, the assay is only semi-quantitative, and inconvenient when analysing large numbers of samples.

Flow cytometric visualisation of fluorescently labelled protein uptake

Flow cytometry is a laser-based technology where cells are suspended in a stream of fluid that passes through a laser beam and detected by an electronic detection apparatus fitted with fluorescence filters. It allows simultaneous multi-parametric analysis of up to thousands of particles per second. Choosing optimal voltage thresholds and the issue of bleeding of one fluorescent signal into other channels are commonly encountered problems with flow cytometry. In this study, only one fluorophore was used, and a 1:1 mixture of empty and fluorophore filled cells was used to establish the optimal voltage and light scatter settings of the cytometer, minimising the risk of false positive results.

Immunohistochemistry and immunocytochemistry

These techniques can be used for investigating protein expression levels, as well as protein localisation and co-localisation. Brain tissue, fly heads or cells are first fixed, then permeabilised, blocked and labeled with specific antibodies, followed by fluorescently labeled secondary antibodies and examined using a confocal fluorescence microscope. Methodological considerations are the dependency on specificity of the primary antibody, and its compatibility with the secondary antibody, and the risk of the fixative agent altering the epitope of interest. The advantage of the method is the possibility to study endogenous proteins in their natural subcellular locations.

Microscopic visualisation of proteins and organelles

Confocal microscopy is commonly used to study cellular processes since it enables visualisation along one single focal plane, and therefore protein and organelle interactions can be investigated. However, care must be taken when attributing significance to co-localisation since signals could originate from adjacent focal planes.

Transmission electron microscopy (TEM) is used to image cellular structures with higher magnification. To increase the resolution, TEM, uses a beam of electrons rather than photons, which is used in a regular microscope, to increase the resolution.

Intermolecular interaction between two proteins can be studied by Förster resonance energy transfer (FRET) using steady-state fluorescence spectroscopy. Fluorescence from a labelled donor protein excited at its correct wavelength will, if in close proximity to a fluorescently labelled acceptor protein, transfer energy, causing a different colour emission from the

acceptor. One of the disadvantages of this technique is the possibility that the fluorescent tag affects protein function, and localisation, which is avoided by the use of smaller sized tags.

A β preparation and characterisation

The A β species and aggregation state that is considered most toxic is strongly debated, current consensus point to A β_{1-42} oligomers. Both oligomeric and fibrillary preparations of recombinant A β_{1-42} were used in these studies, prepared using well-established protocols (Stine et al., 2003). Multiple factors can disturb the A β aggregation process, which is why a variety of methods have been used to ascertain the aggregation state of the end product in this study, Western blot, size-exclusion chromatography and electron microscopy. Aggregation of A β was assessed via the widely used ThT assay that measures the fluorescence emitted from ThT upon binding to fibrillised proteins. For treatment of cells with oligomeric A β , the selected concentration was 1 μ M, which is roughly 500 times the concentration neurons are thought to be exposed to in the brain (Cleary et al., 2005).

Statistical Analysis

The mean value and standard deviation (SD) were calculated for all data, with the exception of the *Drosophila* assays where it is customary to use standard error of mean (SEM). The nonparametric Mann–Whitney U test was used to test for significant differences between two groups, and One-way ANOVA, followed by either Dunnett's or Tukey's post hoc tests was used to test for significant differences between multiple groups. Correlation analysis to measure dependence between two quantities was performed using the Spearman's rank correlation coefficient. For *Drosophila* longevity statistics, Kaplan-Meier survival curves and log-rank (Mantel-Cox) statistical analyses were performed. Statistical significance was defined for p-values of less or equal than 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****). All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA USA).

Results

Below is a brief description of the backgrounds, aims and study settings as well as the findings in the papers included in this thesis. For more detail, see the full papers at the end of the thesis.

Paper I

Background

The pre-symptomatic stage of AD occurs several decades before the clinical onset, highlighting the need for validated biomarkers that reflect this early period. Changes in the lysosomal network, i.e., the endosomal, lysosomal and autophagy systems, are among the first alterations observed in an AD brain.

Aim and study settings

The aim of study I was to investigate whether lysosomal network proteins could be detected in CSF, and whether any differences could be detected in AD patients compared to controls. A targeted search for lysosomal network proteins was performed in CSF from AD patients versus controls. Any significant results were confirmed with a second validation cohort of CSF samples, and further validated with a non-AD neurodegeneration cohort.

Main results

Thirty-four proteins were investigated, and six of them, EEA1, LAMP-1, LAMP-2, LC3, Rab3 and Rab7, were significantly increased in the CSF from AD patients compared with neurological controls. In patients with non-AD neurodegeneration, EEA1 but none of the other six proteins was found to correlate with increased general neuronal damage, as assessed by T-tau levels. LAMP-2 was found to positively correlate with P-tau levels in AD patients.

Paper II

Background

Data from preclinical and clinical studies show that immune system-mediated actions contribute to and drive AD pathogenesis. The lysosomal protein lysozyme is a major player in the innate immune system. It has recently been identified to be overexpressed in mouse models of AD, and has recently been shown to prevent A β ₁₋₄₀ and A β ₁₇₋₄₂ aggregation *in vitro*.

Aim and study settings

The aim of study II was to address whether the lysosomal protein lysozyme has a function in AD pathology.

A multi-faceted study was performed, including assessment of: 1) The lysozyme levels in brain tissue, CSF and serum samples from AD patients, 2) The protective effect of lysozyme on A β ₁₋₄₂ toxicity using cell and *Drosophila* models of AD, 3) The *in vitro* binding properties of lysozyme with A β ₁₋₄₂ and the impact of lysozyme on the A β ₁₋₄₂ aggregation process and 4) The effect of A β ₁₋₄₂ on lysozyme expression.

Main results

- 1) Lysozyme was increased in CSF of AD patients and co-localised with A β plaques in AD post-mortem brains.
- 2) In the *Drosophila* model, neuronal co-expression of lysozyme and A β reduced the formation of soluble and insoluble A β species, prolonged survival and improved the activity of the transgenic flies. In the cell model lysozyme protected against A β mediated toxicity.
- 3) The *in vitro* studies revealed that lysozyme associates with A β_{1-42} and alters its aggregation pathway to counteract the formation of toxic A β species.
- 4) A β_{1-42} was found to trigger lysozyme expression in both neuronal and glial cells.

Paper III

Background

Clinical diagnosis of parkinsonian syndromes such as PD, CBD and PSP is hampered by overlapping symptomatology and lack of biomarkers for diagnosis. And thus a definitive diagnosis is only possible post-mortem. Faulty protein degradation plays an important role in many neurodegenerative disorders, and since lysosomal network proteins were identified in paper I as potential novel biomarkers for AD, the same proteins could potentially also play an important role in the pathogenesis of parkinsonian syndromes.

Aim and study settings

The aim of study III was to investigate whether levels of lysosomal network proteins are changed in parkinsonian syndromes, as well as their potential for differential diagnosis.

A targeted search for six lysosomal network proteins was performed in CSF from three separate cohorts, each versus appropriate control samples: 1) PD patients, 2) clinically diagnosed 4-repeat tauopathy patients 3) pathologically confirmed PSP and CBD patients.

Main results

The lysosomal network proteins EEA1, LAMP-1, LAMP-2, LC3, lysozyme and Rab 3 had different CSF protein levels and profiles in the parkinsonian syndromes, a difference further accentuated when pathologically confirmed CBD and PSP patients were tested.

- 1) LAMP-1 and LAMP-2 were significantly decreased in PD.
- 2) LC3 and lysozyme levels were significantly increased in 4-repeat tauopathy patients.
- 3) EEA1 was decreased and lysozyme increased in PSP; and LAMP-1, LAMP-2, LC3 and lysozyme were increased in CBD.

Paper IV

Background

In paper I, dysfunction in the lysosomal network was found to be mirrored in the CSF from AD patients where a specific subset of lysosomal network proteins, among them LAMP-2, were found at elevated levels. Through different mechanisms LAMP-2 has functions in lysosome motility and integrity, CMA, cholesterol metabolism and autophagosome-lysosome docking. Mutations in the LAMP-2 gene cause a congenital disease with mental disabilities

and LAMP-2 plays a role in neurodegenerative movement disorders where alterations in CMA are part of the pathology, such as PD and Huntington's disease.

Aim and study settings

The aim of study IV was to investigate the mechanistic involvement of the lysosomal protein LAMP-2 in AD pathology.

A multi-faceted study was performed, including assessment of: 1) LAMP-2 levels in brain tissue and CSF samples from AD patients, 2) The effect of toxic $A\beta_{1-42}$ exposure on LAMP-2 expression, secretion and subcellular localisation in neuroblastoma cells 3) The effect of LAMP-2 overexpression on APP processing and $A\beta$ generation, uptake of and response to exposure of toxic $A\beta_{1-42}$ in neuroblastoma cells.

Main results

- 1) Hippocampus and frontal cortex, but not temporal cortex or cerebellum in AD cases had increased mRNA and protein expression of LAMP-2, and the increased LAMP-2 levels in hippocampus correlated with increased levels of $A\beta_{1-42}$.
- 2) Oligomeric $A\beta_{1-42}$ caused an upregulation of intracellular LAMP-2 in neuroblastoma cells, but did not trigger the release of LAMP-2 into the extracellular milieu. More lysosomes were observed but the subcellular localization did not seem affected.
- 3) Overexpression of LAMP-2 in neuroblastoma cells caused a reduction in secreted levels of $A\beta_{1-42}$ and changed the generation pattern of $A\beta$. LAMP-2 overexpression did not change the cellular uptake of $A\beta_{1-42}$, but caused a delayed clearance and secretion of $A\beta_{1-42}$.

Discussion

Involvement of lysosomal network proteins in neurodegenerative disease

In paper I, II and III it was found that proteins from the lysosomal network and other degradation pathways could be detected in human CSF, both luminal proteins such as cathepsin D and transmembrane proteins such as LAMP-2. All proteins displayed the correct size via Western blot analysis, indicating that it was full length proteins that were detected in the CSF, which is surprising when it comes to membrane bound proteins, such as Niemann-Pick type C1 protein with thirteen transmembrane regions (Davies and Ioannou, 2000). Whether the membrane bound proteins were released into the CSF attached to membranes from intact exosome vesicles or membrane slivers or even attached to complete organelles leaking out from disintegrating neurons remain to be determined. What is known is that the tight junctions of the choroid plexus can release vesicles from the brain up to the size of 100 nm (Harrington et al., 2009), and in neurodegeneration and old age, the blood-brain barrier becomes even leakier, with the deterioration starting in the hippocampal area (Montagne et al., 2015).

Out of several candidate proteins, selected for likelihood of involvement in AD pathology, seven of them, EEA1, LAMP-1, LAMP-2, LC3, lysozyme, Rab3 and Rab7, were significantly increased in AD patients, some found at almost three-fold elevated levels compared to controls (paper I). Interesting to note was that proteins involved in proteasomal degradation the pre-lysosomal stages of CMA were not changed in AD CSF. The upregulation of endosomal genes and proteins are early pathological events in the AD brain (Bronfman et al., 2007; Ginsberg et al., 2010; Jiang et al., 2010). This is followed by an upregulation of lysosomal genes and proteins and increased lysosomal biogenesis, however, in AD, this protective response is eventually overwhelmed (Nixon et al., 2005; Yu et al., 2005; Boland and Nixon, 2006).

The seven proteins that were increased came from all vesicular compartments in the lysosomal network; endosomes, lysosomes and autophagosomes. It should be emphasised that it was only a select few lysosomal network proteins that were observed to be increased; it was not a general increase in all lysosomal proteins, which hints to an involvement of these seven proteins in specific pathological mechanisms. When these proteins were measured in CSF from patients with non-AD neurodegenerations, i.e. patients with high T-tau, but normal P-tau and $A\beta_{1-42}$, only EEA1 was increased which infers that the other proteins have the potential to be increased due to AD specific pathology. Why these seven particular proteins were selectively increased in AD requires more study. The most interesting candidates, LAMP-2 and lysozyme, were selected and their roles in AD pathology were investigated in paper II and IV.

LAMP-2 was chosen for its crucial role in lysosomal function and for its involvement in CMA, that has been found impaired in many movement neurodegenerative disorders (Koga et al., 2011; Cuervo and Wong, 2014). After correlation studies with the classical AD biomarkers T-tau, P-tau and $A\beta_{1-42}$, LAMP-2 was found to positively correlate with P-Tau,

which compared to T-tau and A β_{1-42} , is more specific for AD since it is not increased in many tauopathy neurodegenerative diseases (Riemenschneider et al., 2003; Buerger et al., 2006; Kapaki et al., 2007).

Due to the strong implication of inflammation in AD (Wyss-Coray and Rogers, 2012), another candidate that caught our attention was the lysosomal protein lysozyme, that is also an innate immunity protein. Lysozyme has been found overexpressed in mouse models of AD (Matarin et al., 2015), and to inhibit A β_{1-40} (Luo et al., 2013, 2014) and A β_{17-42} (Das et al., 2014) aggregation. Lysozyme was also found to positively correlate with P-Tau (unpublished data).

In paper III, the seven lysosomal network proteins that were significantly increased in AD were tested in CSF from parkinsonian syndrome patients, and then another picture emerged. LAMP-1 and LAMP-2 were significantly decreased in PD, LC3 and lysozyme levels were significantly increased in 4-repeat tauopathy patients, EEA1 was decreased and lysozyme increased in PSP, and LAMP-1, LAMP-2, LC3 and lysozyme were increased in CBD, see table 1.

	AD	PD	4repeat tauopathy	CBD	PSP
EEA-1	↑				↓
LAMP-1	↑	↓		↑	
LAMP-2	↑	↓		↑	
LC3	↑		↑	↑	
Lysozyme	↑		↑		↑
Rab3	↑				
Rab7	↑				

Table 1. The different patterns of lysosomal protein levels in the neurodegenerative diseases analysed in paper I-IV.

Following up on the pathological mechanisms for the selective increase of these proteins in these parkinsonian syndromes is outside the scope of this thesis, but it could be speculated that the decrease of LAMP-2 in PD CSF corresponds to the reported lower LAMP-2 levels in PD brains and the impaired CMA activity in PD (Cuervo and Wong, 2014; Murphy et al., 2015). LAMP-1 has been reported to be unchanged in substantia nigra pars compacta or amygdala in the PD brain (Murphy et al., 2014). However, in this thesis LAMP-1 was found to be reduced in PD CSF, an explanation for these conflicting data could potentially be found in the complex overlap of LAMP-1 and LAMP-2 function (Eskelinen, 2006) or the data might indicate that other areas in the brain may also be affected, and have lower levels of LAMP-1. Too little is known about lysosomal network involvement in CBD and PSP to draw any conclusion about pathological mechanisms but it could be speculated that the lysosomal network is also involved early on in these neurodegenerative diseases.

The potential of using lysosomal network proteins for early diagnosis

The upregulation of the lysosomal network occurs early in the AD disease process, at the time where the levels of oligomeric A β are rising, but no plaques have yet formed (Nixon and Cataldo, 2006). According to the current model of the temporal appearance of the major AD biomarkers, changes in A β levels are the first to appear (Jack et al., 2013). Changes in the lysosomal network would thereby have the potential to precede the classical biomarkers by many years. Unfortunately, this was outside the scope of this thesis, but if the changes in the lysosomal network could be detected in MCI and pre-MCI patients, they would hold great promise for being early markers for AD; maybe even early enough to appear at a stage before irreversible brain damage has occurred. As such they would have great potential for use in early diagnosis as well as enrichment for clinical trials. Samples from longitudinal studies following patients for decades ending in clear defined diagnoses would be needed to be able to determine the feasibility of these potential novel early biomarkers.

In the PD brain, motor symptoms appear first when 80% of dopaminergic neurons have degenerated (Riederer and Wuketich, 1976; Tissingh et al., 1998a; Tissingh et al., 1998b); however, defective lysosomal network activity can be detected early in models of PD (Martinez-Vicente et al., 2008; Alvarez-Erviti et al., 2010; Dehay et al., 2010). Even though little is known of the timing of the appearance of the lysosomal network aberrations in PD, most likely as with AD, they appear years, if not decades before symptoms arise, and it could be speculated that the same hold true in the case of CBD and PSP. Samples from longitudinal studies following patients for decades would be needed to be able to further study the lysosomal network involvement in these parkinsonian syndromes, and their feasibility as potential novel early biomarkers.

The potential of using lysosomal network proteins for differential diagnosis

In all four diseases studied; AD, CBD, PD, and PSP; a select few proteins were increased, rather than a general increase in all proteins. Also, different patterns of the lysosomal network proteins emerged between the diseases studied, which hints to an involvement of these proteins in specific pathological mechanisms that differ between the diseases. It is clear that different parts of the lysosomal network dysfunction in the different diseases. The transport of autophagosomes and their maturation to lysosomes is suggested to be impaired in AD, whilst dysfunctional CMA and mitophagy seem to be the main problem in PD (Son et al., 2012), whilst little is known about lysosomal network involvement in CBD and PSP. Therefore lysosomal network proteins have the potential to be used to aid in differential diagnosis, but extensive further studies are needed to verify the preliminary data presented in this thesis.

The potential of using lysosomal network proteins as therapeutic targets

To map out physiological processes on a molecular level would not only help explain disease pathogenesis, but would also give rise to opportunities to develop new therapeutics. The importance of the lysosomal network in normal cellular function in complex organisms is highlighted by the many diseases where the system is in imbalance. Apart from

neurodegenerative diseases, dysfunction has also been reported in cancer, autoimmune disorders and diabetes for example (Moffitt et al., 2010).

Targeting the lysosomal system could be done using many different approaches, increasing exocytosis, increasing autophagy and increasing lysosomal degradation. However, the importance of the lysosomal network in cell function also heeds caution, since tampering could lead to unexpected and unwanted side effects in other cellular processes. Promoting lysosomal exocytosis, as a means to get rid of excess undegraded material, has been shown experimentally (Medina et al., 2011), however, this approach has raised the concern that potentially toxic material is just moved to the extracellular space to exert its detrimental effect there instead, making it a non valid treatment option (Schultz et al., 2011).

Promoting lysosomal degradation is another tempting target, but even here caution is needed. The lysosomal degradation of LAMP-2 has been shown to be a dynamic process that regulates the activity of the CMA pathway (Cuervo and Dice, 2000b), illustrating that general interference with lysosomal degradation rate is too non-specific as a target.

Induction of autophagy has been reported successful in neurodegenerative diseases such as PD and Huntington's disease (Harris and Rubinsztein, 2012), but in neurodegenerative disease where defective lysosomal degradation has been found to be the cause, it would hypothetically be detrimental to induce autophagy. For example, in Niemann-Pick type C1 disease, where defective lysosomal proteolysis seems to be the problem (Elrick et al., 2012). The complexity of choosing stimulation of autophagy as a target in neurodegeneration is further highlighted by conflicting data from the AD field where results from animal models shows induction of autophagy to be beneficial in young animals, but detrimental in older animals (Ling and Salvaterra, 2011; Majumder et al., 2011).

It is clear that since different parts of the lysosomal network are defective in different neurodegenerative diseases, no single approach could be used. Rather, disease mechanism specific target must be identified for each disease. A clear illustration of how specific proteins in the lysosomal network could be targeted in neurodegenerative disease comes from a cell based study pinpointing vATPase as the protein responsible for aberrations in lysosomal acidification and proteolysis malfunction. This however, was demonstrated in a model of inherited AD caused by presenilin 1 mutations (Lee et al., 2015), whereas most AD cases are sporadic with fully functional presenilin 1.

The lysosomal network proteins included in this thesis, LAMP-1, LAMP-2, LC3, lysozyme, Rab3 and Rab7 could all potentially be suitable as targets in neurodegenerative disease. Therapeutic strategies targeting endocytosis could therefore focus on the feasibility of either EEA1, Rab3 or Rab7 as therapeutic target. Strategies targeting autophagic flux could focus on LC3, and strategies targeting the lysosomal compartment could focus on either LAMP-1, LAMP-2 or lysozyme as therapeutic targets. See Figure 5 for the lysosomal network locations of these proteins.

EEA1 localises exclusively to early endosomes and has an important role in endosomal trafficking and sorting, by bringing early and late endosomes physically closer for fusion and delivery of cargo (Barysch et al., 2009; Mishra et al., 2010). Rab proteins are G proteins involved in regulating membrane traffic, including vesicle formation, movement, and

membrane fusion, through which cell surface proteins are trafficked from the Golgi apparatus to the plasma membrane and recycled. Rab7 functions as a key regulator in endo-lysosomal trafficking, and Rab3 is a regulator of secretory and synaptic vesicles (Stenmark and Olkkonen, 2001). LC3 or other ATG8 family proteins are required for the formation of

Figure 5. The lysosomal network locations for the proteins EEA1, LAMP-1, LAMP-2, LC3, lysozyme, Rab3 and Rab7. The proteins have been depicted in their primary location, but can due to vesicular flux also appear in other compartments.

autophagosomal membranes, and the mammalian LC3 isoforms' participation in autophagy/mitophagy is under strict regulation (Klionsky and Schulman, 2014; Stolz et al., 2014). LAMP-1 together with LAMP-2 comprise 50% of all lysosomal membrane proteins and are thought to be responsible in part for maintaining lysosomal integrity, pH and catabolism (Eskelinen, 2006). The expression of LAMP-1 and LAMP-2 is linked, and they are thought to share similar functions. A LAMP-1 knock out will lead to increased expression of LAMP-2 and the resulting phenotype is similar to wild type, which makes it difficult to determine the precise function of LAMP-1 (Andrejewski et al., 1999). Following up on the potential of EEA1, LAMP-1, LC3, Rab3 and Rab7 as therapeutic target was unfortunately outside the scope of this thesis.

Lysozyme as a potential therapeutic target

In paper II, lysozyme was identified as a potentially interesting therapeutic target in AD pathology. Lysozyme was found to be increased in the CSF and co-localised with A β plaques in AD patients, and rescued from A β toxicity in both cellular and animal models. The mode

of action seems to be through that lysozyme associates with $A\beta_{1-42}$ and alters its aggregation to counteract the formation of toxic $A\beta$ species. Induction of lysozyme expression could be a treatment option since overexpressing it in *Drosophila* caused no adverse effects, and lysozyme related pathology only appears in humans with mutations that affects its folding properties (Dumoulin et al., 2006).

LAMP-2 as a potential therapeutic target

In paper I, III and IV, LAMP-2 was identified as a potentially interesting therapeutic target, in both PD and AD, where LAMP-2 was found to be increased in CSF from AD patients and decreased in CSF from PD patients. LAMP-2 mRNA and protein levels were found to be elevated in brain areas relevant for AD pathology and various cellular models showed complex involvement of LAMP-2 in $A\beta$ related pathology, with extensive crosstalk between LAMP-2 and $A\beta$. Oligomeric $A\beta_{1-42}$ caused an upregulation of LAMP-2, and in turn, overexpression of LAMP-2 caused a reduction in secreted levels of $A\beta_{1-42}$, altered the generation pattern of $A\beta$ and affected clearance and secretion of $A\beta_{1-42}$.

The lysosomal network in both neurons and glial cells is involved in degradation of endocytosed $A\beta$ (Majumdar et al., 2011; Li et al., 2012), and a recent publication has highlighted the intricacies and importance of LAMP-2 in lysosomal degradation, with different systems compensating for LAMP-2 deficiency (Furuta et al., 2015). We observed that the increased LAMP-2 levels in the neuroblastoma cells was accompanied by more lysosomes in the perinuclear area, indicating a ramping up of the lysosomal compartment, most likely to deal with the increased load of $A\beta$ for degradation. These data indicate that the increased levels of LAMP-2 in AD could be an attempt to regulate $A\beta$ generation and secretion. Since all the three isoforms of LAMP-2 have identical luminal domains but differ in transmembrane and cytoplasmic domains (Eskelinen et al., 2005) their effect on $A\beta_{1-42}$ is most likely conveyed before the protein to be degraded has entered the lysosome, alternatively if no direct interaction occurs between LAMP-2 and $A\beta_{1-42}$, through an extra-lysosomal interaction.

The isoform LAMP-2A seems particularly interesting as a PD target, due to the malfunction of CMA pathways in this disease (Cuervo and Wong, 2014). Also in AD LAMP-2 clearly seems to have a direct link with the $A\beta$ pathology, although which isoform/s and the mechanism by which this is regulated is still unclear.

Concluding remarks and future directions

Utilising the lysosomal network proteins for biomarkers and novel therapeutic targets for neurodegenerative diseases holds great promise. The possibility of discovering new treatment targets in cellular pathways where pathology pre-dates much of the neuronal death observed in neurodegeneration brings new hope in the field where many current clinical trials have failed, and there is yet no product on the market to halt or counteract the neurodegeneration. The possibility of discovering biomarkers that could aid in early and specific diagnosis of various neurodegenerative diseases would not only benefit patients, but also aid in selecting appropriate study populations for preventative and intervention studies.

However, the findings of these studies are preliminary. Validating novel biomarkers requires a concerted effort and any novel therapeutic targets must be investigated in detail to eliminate risk of any unwanted side effects. Extensive further studies are required to be able to deduce the potential of targeting the dysfunctional lysosomal network in the process of neurodegeneration as an intervention therapy.

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