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Amyloid- β Protofibrils in Alzheimer's Disease

Focus on Antibodies, Inflammation and Astrocytes

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Abstract

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Soluble amyloid-beta ($A\beta$) aggregates, including $A\beta$ protofibrils, play a central role in Alzheimer's disease (AD) and constitute a potential diagnostic biomarker and a therapeutic target. $A\beta$ protofibrils promote synapse dysfunction and neurodegeneration, but the mechanisms behind these effects remain unclear. The aim of this thesis was to increase the knowledge of $A\beta$ protofibrils in AD pathology.

When measuring low abundant antigens, such as soluble $A\beta$ aggregates, in plasma and CSF by immunoassays, there is a possibility of interference by heterophilic antibodies (HA). In paper I, we show that HA generate false positive signals, by cross-binding the assay antibodies, when plasma and CSF from AD patients and healthy controls were analyzed for soluble $A\beta$ aggregates, using sandwich ELISAs.

Natural anti- $A\beta$ antibodies exist in AD patients and healthy individuals. Circulating $A\beta$ and anti- $A\beta$ antibodies may form immune complexes, masking epitopes on the anti- $A\beta$ antibody, which makes the anti- $A\beta$ antibody concentration difficult to measure. In paper II, the ELISpot technique enabled us to successfully measure B cell production of anti- $A\beta$ antibodies. Our results show that anti- $A\beta$ protofibril antibody production is present in both AD patients and healthy individuals, but is significantly higher in AD patients, indicating that the immune system attempt to eliminate the toxic $A\beta$ species.

Insufficient lysosomal degradation is proposed to cause sporadic AD. In paper III, we used a co-culture system of astrocytes, neurons and oligodendrocytes, to clarify the role of astrocytes in $A\beta$ protofibril clearance. Astrocytes are the most prominent glial cell type in the brain, but their role in AD remains elusive. We found that astrocytes effectively engulf, but inefficiently degrade $A\beta$ protofibrils. This result in a high intracellular load of toxic, partly N-terminally truncated $A\beta$ and lysosomal dysfunction. Moreover, we found that secretion of microvesicles, containing N-terminally truncated $A\beta$, induce neuronal apoptosis. In paper IV, we show that treatment with the protofibril selective antibody mAb158 lead to enhanced $A\beta$ clearance and thereby prevent $A\beta$ neurotoxicity.

Taken together, this thesis contributes with important knowledge on the role of $A\beta$ protofibrils in AD pathogenesis and technical aspects that should be considered when measuring $A\beta$ in human tissues.

Keywords: Alzheimer's Disease, Amyloid-beta, Protofibrils, ELISA, ELISpot, Astrocyte, Monoclonal Antibody, Immunofluorescence

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List of Papers

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

- I Sehlin D*, Söllvander S*, Paulie S, Brundin R, Ingelsson M, Lannfelt L, Pettersson FE, Englund H. (2010) Interference from heterophilic antibodies in amyloid- β oligomer ELISAs. *J Alzheimers Dis.* 21:1295-301.
- II Söllvander S, Ekholm-Pettersson F, Brundin R-M, Westman G, Kilander L, Paulie S, Lannfelt L, Sehlin D. (2015) Increased number of plasma B cells producing autoantibodies against A β 42 protofibril in Alzheimer's disease. *J Alzheimers Dis.* 48: 63-72
- III Söllvander S, Nikitidou E, Brolin R, Sehlin D, Lannfelt L, Erlandsson A. Accumulation of oligomeric amyloid-beta in astrocytes result in giant astrocytic endosomes and microvesicle-induced apoptosis of neurons. *Submitted manuscript*
- IV Söllvander S, Nikitidou E, Sehlin D, Söderberg L, Lannfelt L, Erlandsson A. Treatment with mAb158 dramatically increases degradation of A β protofibrils in astrocytes. *Manuscript*

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Abbreviations

A β	Amyloid- β
A β PP	A β precursor protein
AD	Alzheimer's disease
ADDLs	Amyloid- β derived diffusible ligands
APH-1	Anterior pharynx defective-1
ApoE	Apolipoprotein E
Arc	Arctic mutation (A β PP E693G, A β E22G)
ChE	Cholinesterase
CSF	Cerebrospinal fluid
DAPI	4',6-diamino-2-phenylindole
ELISA	Enzyme linked immune-sorbent assay
ELISpot	Enzyme-Linked ImmunoSpot
E14	Embryonal day 14
F(ab')	Fragment antigen-binding
GFAP	Glial fibrillary acid protein
GLAST,	Glutamate-aspartate transporter
GLT-1	Glutamate transporter-1
HA	Heterophilic antibodies
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
LAMP-1	Lysosomal membrane-associated protein 1
LAMP-2	Lysosomal membrane-associated protein 2
LMW-A β	Low molecular weight
mAb	Monoclonal antibody
MMSE	Mini-mental State Examination
MCI	Mild cognitive impairment
NMDA	N-methyl-D-aspartate
PBMC	Peripheral blood mononuclear cells
PEN-2	Presenilin enhancer-2
PS1	Presenilin 1
PS2	Presenilin 2
SDS	Sodium dodecyl sulfate
Swe	Swedish mutation (A β PP K670N/M671L)
TEM	Transmission electron microscopy

TNF- α
tg
TUNEL

Tumor necrosis factor- α
Transgenic
Terminal deoxynucleotidyl transferase dUTP
nick labeling

Introduction

Alzheimer's disease

In 2010, the prevalence of people with dementia was approximately 35 million worldwide. This number is predicted to almost double every 20 years if there are no changes in prevention strategies or mortality number [1]. Alzheimer's disease (AD) constitutes 60-80% of all dementia cases and is thereby the most common dementia disorder followed by vascular dementia, Lewy body dementia and Frontotemporal dementia [2]. AD was first described 1906 by the German physician Alois Alzheimer after the investigation of a 50 years old patient named Auguste Dieter. Auguste suffered from memory loss and disorientation and she had problems with writing and reading. When examining Auguste's brain after her death, various abnormalities including senile plaques and neurofibrillary tangles were found and reported for the first time [3]. These abnormalities, amyloid plaques containing fibrillar amyloid-beta ($A\beta$) peptides and neurofibrillary tangles, containing hyperphosphorylated tau protein, are still the neuropathological hallmarks of AD (Figure 1) [4]. Apart from plaque and tangles, a loss of neuronal cells is seen in the brain of AD patients and there are also degeneration of axons and dendrites, especially in the limbic structures (e.g. hippocampus and amygdala) of the brain.

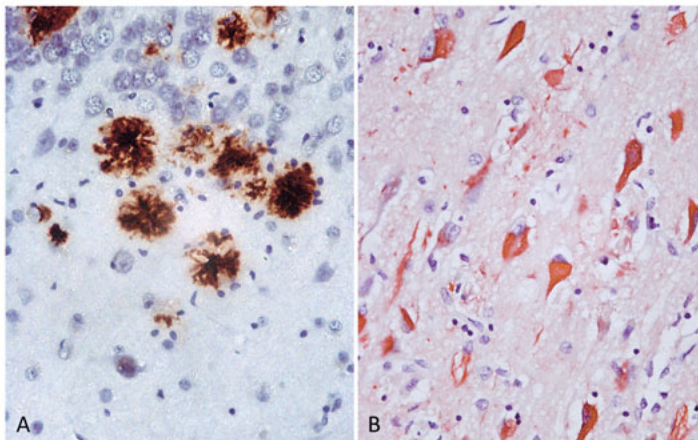


Figure 1. Neuropathological hallmarks of AD. Amyloid plaques (A) and neurofibrillary tangles (B) are lesions frequently observed in sections from AD brains. Pictures kindly provided by Paul O'Callaghan.

The neuronal degeneration and dysfunction noticed in AD brains give rise to the symptomatic picture of the disease [5, 6]. The first signs of AD are mild memory deficits, primarily affecting short term memory, leading to difficulties remembering recent events of everyday life, e.g. recalling names of familiar persons and objects and remembering recent conversations. Subsequently, AD gradually affects multiple cognitive and behavioral processes and ultimately progress into severe dementia, where the patient is unaware of time and place, has a changed personality and has problems identifying even closely related family members. Advanced AD is also accompanied by deficits in the motor system, leading to symptoms such as slow movements and hampered motor coordination. Death of AD patients occurs on average 9 years after diagnosis, due to secondary conditions, such as pneumonia or other common infections [7].

Tau

Tau is a microtubule-associated protein regulating microtubule assembly and stability by associating and dissociating from microtubules via dephosphorylation and phosphorylation, respectively. Pathologically, tau forms neurofibrillary tangles within neurons by aggregation of hyperphosphorylated tau. Pathologic aggregated tau causes malfunction of cell transport and mitochondrial functions and is hence proposed to act as an essential mediator of neurotoxicity [8]. In AD, the formation of tangles is proposed to be triggered by A β , while tau is not believed to have any altering effect on A β [9-11].

A β precursor protein (A β PP)

The accumulation and aggregation of A β peptides is a main event in AD. The A β peptide is produced by processing the A β precursor protein (A β PP) [12]. A β PP, encoded by the A β PP gene on chromosome 21 [13], is a heterogeneous, ubiquitously expressed type I integral membrane protein, consisting of a large extracellular region, a single transmembrane part and a small cytosolic tail [14]. Alternative splicing of A β PP yields several isoforms, where the three major isoforms are 695, 751 and 770 residues long. The 695 isoform is widely expressed by neuronal cells, while the other isoforms are primarily apparent in the periphery, but are occasionally found in the brain [15, 16]. The function of A β PP is only partly understood. It has been suggested to play a role as a cell surface receptor [17] and to be important for neuronal function, including synapse formation, plasticity, neurite outgrowth and neuronal migration, as well as brain development and learning and memory [18-24].

A β PP processing

Processing of A β PP occurs in two pathways, the non-amyloidogenic pathway and the A β producing amyloidogenic pathway (Figure 2). A β PP processing involves enzymatic activities of α -secretase [25], β -secretase [26-28] and γ -secretase [29]. First, A β PP is proteolytically cleaved by either α -secretase, in the non-amyloidogenic pathway or β -secretase, in the amyloidogenic pathway, releasing A β PP derivatives into the lumen of the cell or to the extracellular matrix. α -secretase, mediated by the ADAM family of proteases, sheds off a large soluble ectodomain fragment, α -A β PPs, leaving a 83 residues long C-terminal fragment in the membrane. In neurons, ADAM10 is the most likely candidate for α -secretase activity [30]. The membrane-bound aspartyl-protease BACE1 has been identified as the β -secretase enzyme. To produce A β , BACE1 cleaves A β PP at the N-terminal, before position 1 in the A β domain, releasing β -A β PPs and retaining a 99 residues long C-terminal fragment in the membrane. After α -secretase or β -secretase cleavage the remaining transmembrane fragment is processed stepwise by γ -secretase, consisting of an aspartyl protease multiprotein complex, constituting presenilin (PS), nicastrin, anterior pharynx defective-1 (APH-1) and presenilin enhancer-2 (PEN-2) [29, 31] of which PS is the catalytic component [32]. γ -secretase releases P3 or A β peptide [33].

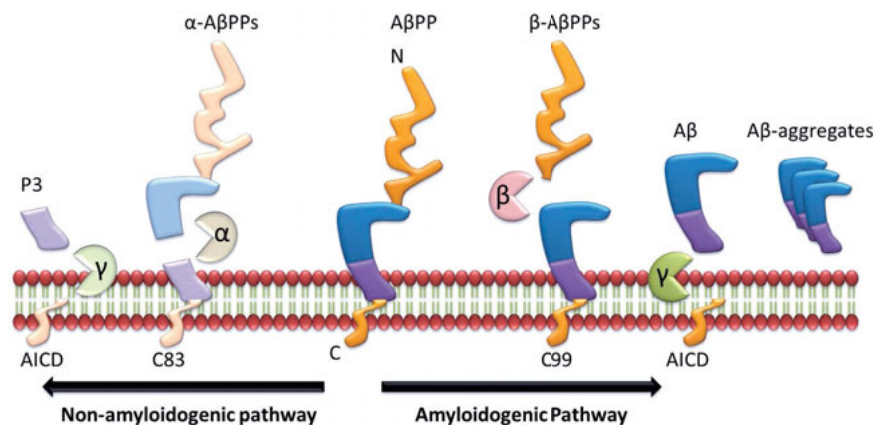


Figure 2. A β PP processing. A β PP can be processed in two different pathways. In the amyloidogenic pathway, A β PP is first cleaved by β -secretase with subsequent cleavage by γ -secretase. This leads to formation of A β peptides that are prone to aggregate and give rise to amyloid plaques. Initial cleavage by α -secretase results in processing through the non-amyloidogenic pathway.

A β aggregation

γ -secretase cleaves the C-terminal at different sites in a sequential manner, generating A β peptides of various chain lengths [34-36]. Predominantly, the 4 kDa A β_{40} is produced along with a minor portion of A β_{42} . In addition, other A β -variants ranging from 38-43 amino acids have been observed [37, 38]. Since A β_{42} contains two additional hydrophobic amino acids, this peptide is more prone to aggregate than A β_{40} . The accumulation of A β causes formation of a wide range of soluble A β aggregates, ranging from small oligomers e.g. A β dimers, trimers and tetramers [39-43], A β^* 56 [44, 45], A β derived diffusible ligands (ADDLs) [46, 47] and globulomers [48, 49], to larger oligomers [50, 51], including oligomer paranuclei [52], protofibrils [53-56] and annual protofibrils [57]. Protofibrils are described as curvilinear, short flexible fibrils generally 4-10 nm in diameter and with a length of up to 200 nm [54, 58, 59]. The end stage in the A β aggregation process is insoluble amyloid fibrils depositing into fibrillar amyloid plaques [60]. Amyloid fibrils are highly stable and have a cross- β structure, containing parallel β -sheets with strands perpendicular to the fibril axis [61]. Before the fibril formation, A β aggregates are only transiently formed. This makes it difficult to separate these forms from each other and to characterize their conformation. There is no consistent classification system for soluble A β aggregates and the literature is therefore unclear. Soluble A β assemblies that are distinct from A β protofibrils are commonly referred to as A β oligomers.

A lot of the knowledge about the A β peptide is based on studies of synthetic or modified A β peptides *in vitro* and the biological relevance of these results can be questioned. It has been hypothesized that A β monomers can assemble and aggregate along different pathways e.g. the on-pathway, ending up as amyloid fibrils and the alternative, off-pathway, ending up as soluble end-stage A β oligomers, e.g. ADDLs and globulomers (Figure 3). The on-pathway acts through a nucleus-dependent fibrillization process. During a lag phase, a nucleus is established from which fibrils can grow under thermodynamically favorable conditions. A β monomers have been suggested to start the process by forming a hairpin structure, nucleating monomer folding into dimers, trimers and oligomers, which act as building blocks for protofibrils, maturing into fibrils [62, 63]. A β oligomers in the on-pathway contain intramolecular bonds and antiparallel β -sheets [61].

The off-pathway includes A β oligomers possessing spherical, irregular secondary structures, distinct from the ordered protofibril and fibrillar conformations. These off-pathway oligomers will not aggregate further unless they have made a thermodynamically favorable, conformational change [48, 63-65].

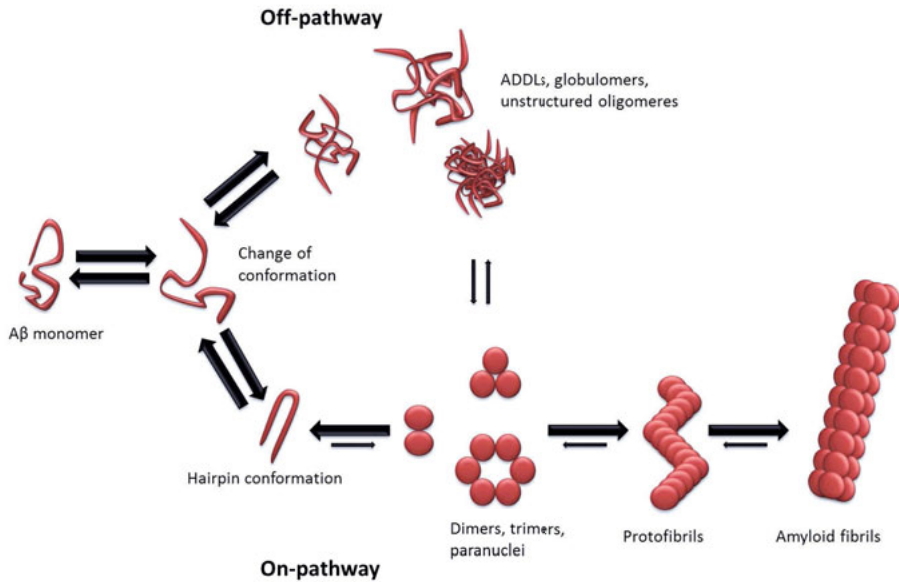


Figure 3. A β aggregation pathways. Schematic illustration of A β monomers aggregating into fibrils via the off-pathway and the on-pathway. In the off-pathway, A β aggregates into spherical, irregular secondary structures and will not aggregate further into fibrils, if not going through a conformational change first. The on-pathway follows a nucleus-dependent fibrillization process. A β monomers folds into a hairpin structure from which dimers, trimers and oligomers are formed. These forms act as building blocks for protofibrils, which elongate and eventually become insoluble fibrils.

Genetics and risk factors

AD exists both as late onset sporadic AD and early onset familial AD, with only a small percentage of the patients classified as autosomal dominant familial AD [2]. Down's syndrome patients, known to have an extra copy of chromosome 21, develop amyloid pathology and dementia around 45 years of age. The AD pathology is a direct consequence of A β PP overexpression and thus increased A β levels [66, 67]. Early onset AD has also been reported in families with inherited A β PP duplications [68]. Further, mutations in the A β PP gene on chromosome 21 have been found to be associated to familial AD [69-72]. A β PP gene mutations are located at, or close to, the A β cleavage sites or within the A β peptide (Figure 4). Mutations near the β -secretase cleavage sites, e.g. the Swedish mutation, leads to increased total A β production [73], while mutations near the γ -secretase cleavage sites, e.g. London mutation, is associated with an increased A β_{42} /A β_{40} ratio [74]. The Arctic mutation and mutations within the A β sequence promotes A β protofibrillar and A β fibrillar formation, due to changed peptide properties [58]. A

recently found A β PP mutation, A673T, is intriguing as it was shown to be protective against AD [75]. Familial AD is also associated with mutations in the genes regulating proteolytic processing of A β ; Presenilin 1 (PS1) and Presenilin 2 (PS2), that similar to the mutations close to the γ -cleavage site, increase the A β_{42} /A β_{40} ratio [76]. Worth mentioning is that no mutations in the tau gene have been shown to induce A β pathology [77].

Although the majority of AD patients are diagnosed with late onset, sporadic AD, much less is known about the cause of this type of the disease and whether A β has an equally high importance in the sporadic form is not yet completely investigated. Genetic and environmental risk factors are regarded to be involved in sporadic AD and studies have shown that the familial heritability of AD is high [78-80]. Sporadic AD is age-dependent, with the prevalence increasing exponentially with age. Roughly, 0.5-1% of individuals between 60-64 years of age and 10-30% of the individuals above 85 years of age are diagnosed with AD [81]. Beside age, other proposed risk factors associated with sporadic AD include head trauma, cardiovascular diseases, gender and educational level [82-85].

There is only one well established “risk-gene” associated with sporadic AD; the apolipoprotein E (APOE) gene [86, 87]. A β binds to ApoE and ApoE is present in the brain, in cerebrospinal fluid (CSF) and in amyloid plaques. ApoE has been suggested to be involved in A β PP membrane insertion and processing, A β aggregation and in clearance of A β [88]. ApoE consists of three major isoforms, ϵ 2, ϵ 3 and ϵ 4 and of these the ϵ 4 allele is associated with an increased risk for AD while ApoE ϵ 2 seems to be protective [86, 89]. In addition, there are a number of genes associated with low to moderate risk for increased susceptibility of AD. These genes are found to have a role in lipid metabolism, immune response or endosomal/lysosomal function [90].

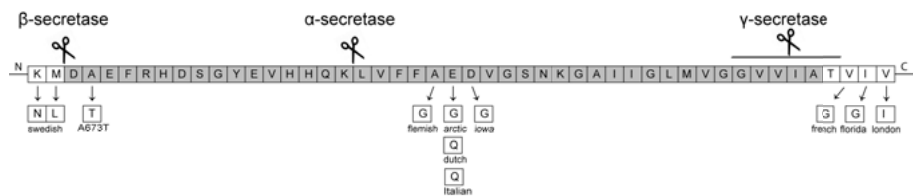


Figure 4. A β PP mutations. Autosomal dominant mutations in the A β PP gene are associated with AD. The mutations are located at, or close to, the A β cleavage sites or within the A β peptide sequence. The mutations lead to increased total A β production, increased A β_{42} /A β_{40} ratio or enhanced A β protofibrillar and A β fibrillar formation. Interestingly, the A673T A β PP mutation has been shown to be protective against AD.

A β toxicity

It is well established that A β plays an important pathologic role in AD. However, which A β species that cause most neurological damage is debated. The neurodegeneration can be a direct neurotoxic effect or an indirect effect of A β , which e.g. reduce mitochondrial activity, prevent synaptic transmission and axonal transport, disrupt membranes or generate oxidative stress [91].

Fibrillar A β was for a long time considered to be the neurotoxic species [92-94]. However, the amount of amyloid plaques in the brain does not correlate well with the clinical symptomatology of AD [95, 96]. Instead, the pathologic picture correlates much better with the concentration of soluble aggregated A β species [46, 56, 95-100]. Moreover, oligomeric species of A β have more impact on the viability of neurons, compared to both monomers and fibrils [101-103]. Thus, soluble A β oligomers and protofibrils are believed to be the major neurotoxic species and A β plaques are assumed to exist as reservoirs for A β , rather than being toxic themselves [100].

The amyloid hypothesis

The *amyloid hypothesis* (Figure 5) was generated in early 1990s by Hardy and Higgins, based on the discovery that A β in amyloid plaques and A β PP/PS mutations result in A β pathology [104]. The *amyloid hypothesis* states that accumulation of A β is the trigger for a cascade of events culminating in AD. A β aggregation is believed to destroy synapses, induce neuroinflammation, form neurofibrillary tangles and ultimately cause neuronal cell death. Particularly, brain regions involved in learning and memory, e.g. hippocampus are affected. Increased amyloid burden and loss of synapses and neurons cause progressive dementia. The *amyloid hypothesis* has not changed much since first reported. However, the focus has shifted from A β plaques as the main toxic entity to soluble aggregates of A β since these A β intermediates have been found to be neurotoxic and correlate with cognitive decline [105-107].

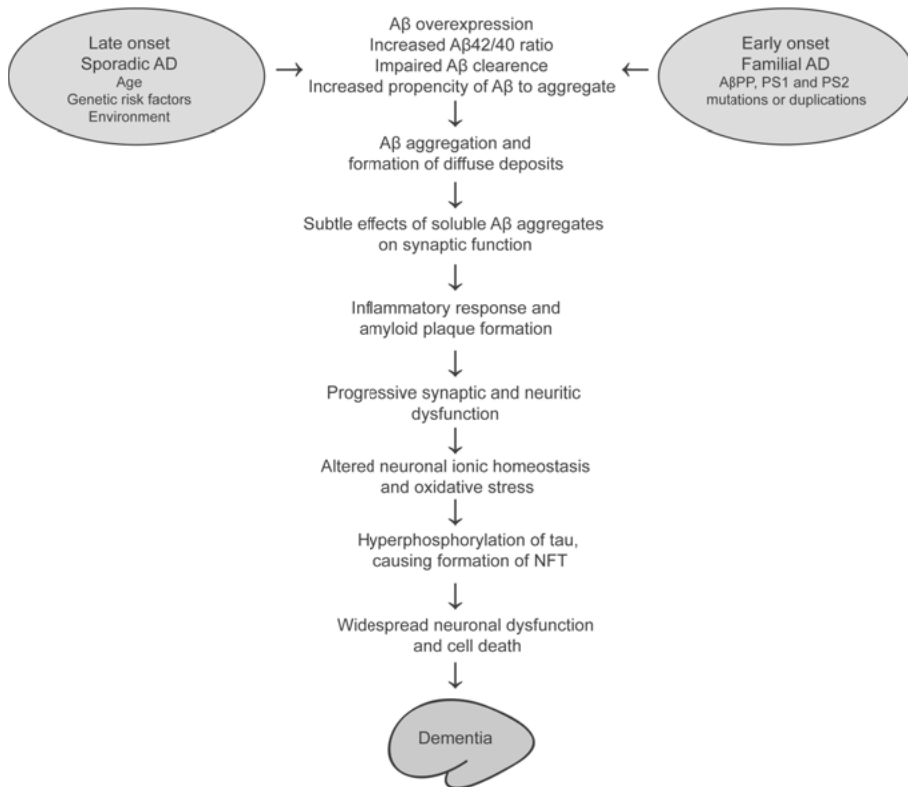


Figure 5. The amyloid cascade hypothesis suggests that A β accumulation due to A β overexpression, increased A β_{42} /A β_{40} ratio, altered A β propensities or impaired A β clearance results in A β aggregation. In turn, A β aggregates initiate a cascade of events including formation of neurofibrillary tangles, dysfunction of synapses, induced neuroinflammation and neuronal cell death, culminating in cognitive decline and AD [107].

A β clearance and degradation

Defect A β clearance may contribute to pathological accumulations of cerebral A β in late onset sporadic AD [108]. A β are cleared from the brain by various mechanisms including degradation by proteases [109-111], clearance by blood brain barrier interstitial fluid bulk flow, cerebrospinal fluid absorption [112-117] and endocytosis by cells [118-123]. Endocytosis can be divided into phagocytosis and pinocytosis. Phagocytosis is devoted to specialized cells, such as microglia in the brain, and is highly regulated process involving specific cell surface receptors. Pinocytosis occurs in all cells and can be categorized into four mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated and clathrin/caveolae-independent endocytosis. Pinocytosis is known to regulate hormone-mediated signal

transduction, immune surveillance, antigen presentation and homeostasis [124].

In addition to microglia, other cell populations possess phagocytic features in the CSN e.g. astrocytes, oligodendrocytes and neurons [125, 126]. In fact, astrocytes have been reported to engulf dead cells both *in vitro* and *in vivo*, but then store, rather than degrade the ingested material due to poor acidification of the astrocytic lysosomes [127]. *In vitro*, both microglia and astrocytes are able to phagocytose soluble A β . Whether this also occurs *in vivo* has been questioned, but A β deposits have been demonstrated in both cell types in the AD brain [125, 128-131]. The pathway of endocytosis seems to be decided by the aggregation state of A β . In microglia, soluble A β are mainly taken up by pinocytosis and fibrillary A β by phagocytosis [132]. Internalization of A β by astrocytes is poorly understood, but macropinocytotic uptake and receptor-mediated phagocytosis have been suggested [133].

Following engulfment, the degradation occurs through the endosomal-lysosomal pathway [134] (Figure 6), in which the formation of early endosomes is the initial step. Early endosomes are characterized by the presence of Rab4 and Rab5 GTPases. Rab4 and Rab5 have a role in sorting molecules from the early endosome to various destinations in the cell, including Rab7 and Rab9 positive late endosomes and subsequently the lysosomes. Except for Rab GTPases, lysosomal membrane-associated protein 1 and 2 (LAMP-1 and LAMP-2) are two well characterized proteins present from early endosomes to mature lysosomes. The lysosome is the main component, degrading waste material in form of macromolecules, organelles and protein aggregates. During the endosomal/lysosomal pathway, both the membrane composition and endosomal luminal milieu changes. Most importantly, the proton pump V-ATPase changes the pH from around 6.2 in early endosomes to 5.5 and 5.0 in late endosomes and lysosomes respectively. The acidification controls receptor-ligand uncoupling, lysosome enzyme activity and transport [135].

Endosomal changes in the brain are observed early in AD with increased transcription levels of endosomal genes e.g. PICALM (encoding a clathrin assembly protein) and EEA1 (encoding EEA-1 protein localized in early endosomes) and upregulation of proteins important in the endosomal-lysosomal pathway e.g. Rab4, Rab5, Rab7 and LAMP-1 [136-140]. Of particular interest is the observation that endosomal/lysosomal related dysfunctions in AD occurs before the appearance of A β and tau pathology [141, 142].

Microvesicles

Membrane vesicles of various sizes, e.g. exosomes and larger microvesicles are generated and secreted from cells into the extracellular matrix. Their

functions include removal of unnecessary proteins from the cell and involvement in cell-cell communication [143]. Microvesicles, with a diameter of 100-1000 nm, are formed by outward budding and pinch off the plasma membrane [144]. Exosomes, with a diameter of 30-100 nm, are instead formed by invagination of the late endosome membrane, forming small vesicles that accumulate in the luminal space of the endosome, now called a multivesicular body [145, 146]. If not transferred to the lysosome for degradation, the multivesicular body fuses with the plasma membrane, by a calcium-dependent mechanism and release the exosomes into the extracellular space [147] (Figure 6). It has been proposed that A β is accumulated in multivesicular bodies and then released by exosomes into the extracellular environment [148].

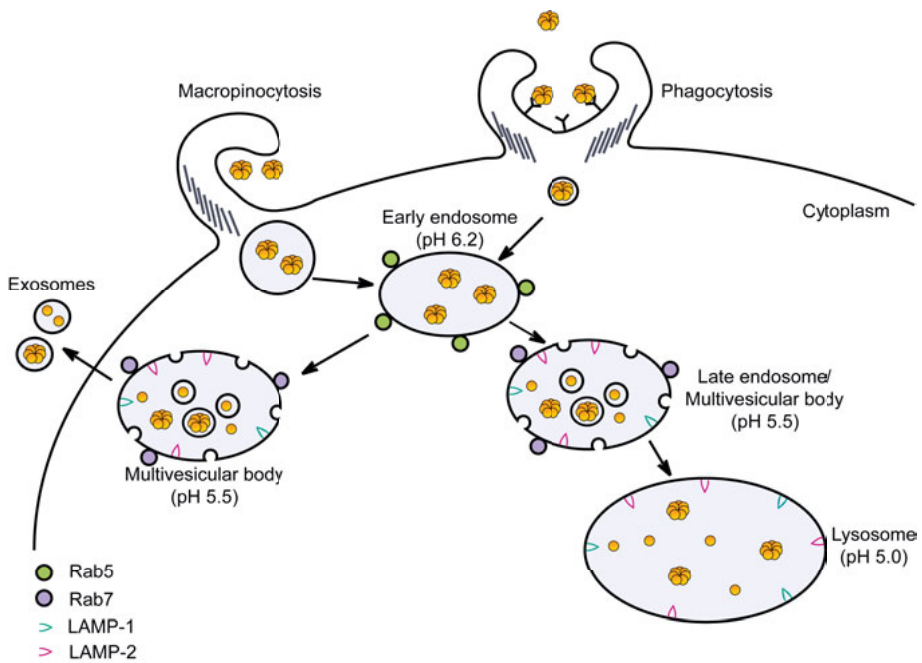


Figure 6. Extracellular material can be taken up by endocytosis through various mechanisms including macropinocytosis and receptor mediated phagocytosis. After internalization, the material is transported and degraded through the endosomal/lysosomal pathway. In this pathway, the pH will subsequently drop causing activation of degrading enzymes. Several proteins are required for the endosomal/lysosomal pathway to work properly, including Rab5, Rab 7 and LAMP-1 and LAMP-2. Exosomes, derived from the late endosome, may release material into the extracellular space.

AD diagnostics and therapy

At present, there is no curative treatment for AD. The current therapies provide symptomatic benefits without altering the underlying disease process. The two therapeutic strategies used today are cholinesterase (ChE) inhibitors, for mild to moderate AD and an N-methyl-D-aspartate (NMDA) antagonist, for moderate to severe AD. ChE inhibitors prevent breakdown of acetylcholine and have only a modest effect in AD since the inhibitor only prolongs the half-life of the produced neurotransmitter. However, it does not inhibit the ongoing loss of neurons, contributing to the cognitive decline [149]. Stimulation of NMDA receptors by the principal brain excitatory neurotransmitter glutamate is important for learning and memory functions [150], but the excessive levels noticed in AD can instead evolve in excitotoxicity, followed by neurodegeneration. An uncompetitive NMDA receptor antagonist is used as a treatment to impede this outcome [151].

The diagnosis of AD is based on the medical history of the patient, imaging analysis of the brain, physical and neurological examination and cognitive assessment. A definitive diagnosis can however only be made by post-mortem autopsy of the brain. Blood and serological tests are performed to exclude other causes for the symptoms. Analysis of CSF may be used as diagnostic criteria for AD [152], where a combination of low levels of A β ₄₂ and high levels of tau and phospho-tau in CSF are associated with AD [153, 154]. Even though these tests are useful, they are not suitable to follow disease progression.

Novel AD biomarkers are needed that better reflect the disease progression and mirror drug responses. As soluble A β oligomers and protofibrils have been implied to be causatives for AD, they are believed to be good biomarker candidates. Highly sensitive assays for A β oligomers are needed to quantify the very low levels of soluble A β oligomers in CSF. Fukumoto et al. (2010) reported increased levels of high molecular weight A β oligomers in CSF from patients with AD and mild cognitive impairment (MCI), compared to control subjects. The levels also correlated to the disease state and the most severe cases had higher levels of high molecular weight A β oligomers [155]. Since the study by Fukumoto was published, several studies have reported the existence of soluble A β aggregates in CSF [155-162], but their diagnostic potential is still questionable.

A β immunotherapy

Schenk et al. [163] reported in year 1999 that immunization with synthetic, pre-aggregated A β ₄₂ reduced the plaque burden and the extent and progression of AD pathology in an AD mouse model over-expressing mutant A β PP. Since then, active and passive immunization has been the main target for

developing a future treatment for AD. Active immunization, using synthetic intact A β ₄₂ or conjugated A β ₄₂ fragments, stimulates the host immune system to recognize and induce the defense against A β . AD patients, responding to the treatment, were found to have a slower rate of decline of cognitive functions. These patients were also found to produce high levels of antibodies against A β [164]. Unfortunately, active immunization also generated cases of meningoencephalitis, probably due to an inflammatory response caused by activated T cells [165, 166]. Therefore the phase II study was halted in 2002 [166].

Treating AD mice with antibodies directed towards A β resulted in enhanced clearance and/or prevention of deposition of A β plaques, resulting in reduced amyloid burden [167-169]. Cognitive function was improved in the mice, but also unwanted adverse effects in form of vascular amyloid and small hemorrhages were noticed [170]. To avoid adverse effects, antibodies have been redesigned to minimize interactions with effector proteins, such as complement proteins and Fc γ receptors [171]. Several clinical trials using monoclonal antibodies against A β peptide are ongoing. Bapineuzimab (targeting the N-terminal of A β) from Pfizer/Janssen and Solanezumab (targeting the central domain of A β) from Eli Lilly have just ended their evaluation as immunotherapeutics for AD in two large phase III clinical trials. Unfortunately, both failed to show any evidence of clinical benefits and this led to termination of the Bapineuzimab clinical trial [172, 173]. In the Solanezumab trial, positive effects on cognitive measures were detected in mild AD patients, indicating that treatment has to start early to give an effect [172]. Currently, antibodies against soluble A β oligomers or protofibrils are studied as AD immunotherapeutic drugs. One of these is the BAN2401, from Bio-Arctic Neuroscience AB and Eisai, targeting A β protofibrils. BAN2401 is presently in phase 2b clinical trials [174].

AD and inflammation

The importance of the inflammatory system in AD is well documented and supported by both cellular and genetic studies. Genes for immune receptors; TREM2 and CD33 have been found to be associated with AD [175-177]. The amyloid cascade hypothesis states that the immune system is activated following A β accumulation. However, recent studies suggest that an early involvement of immune system exists in AD and it has been suggested that inflammation exacerbates, precedes or causes AD [178-180]. In brains of AD patients there is an ongoing neuroinflammation, manifested by a large number of activated microglia, reactive astrocytes and inflammation markers (e.g. cytokines, chemokines, complement proteins and reactive oxygen species), surrounding the amyloid plaques [181-183]. Microglia is considered to be the macrophages of the brain. It is assumed that A β triggers an innate

response by binding several cell-surface receptors including CD36, TLR4 and TLR6 on microglia, thereby inducing production of pro-inflammatory cytokines, e.g. IL-1 β and TNF, and chemokines [184-186]. During AD progression, microglia will keep a highly responsible primed phenotype that rapidly switches to a pro-inflammatory phenotype upon A β exposure, neurodegeneration and ischemic changes. Other cellular changes contributing to neuroinflammation in AD includes astrogliosis, increased levels of complement factors by oligodendrocytes, reduced inhibitor expression by neurons and production of immune molecules and regulating vascular inflammation by endothelial cells [187].

Except for inflammation in the brain, alterations of the immune system on a systemic level have been found in AD patients and have been proposed to contribute to the pathogenesis of AD. It has even been suggested that the systemic response can induce the progression of AD [188, 189]. The cytokine expression in AD patients are suggested to demonstrate higher levels of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6), compared to control subjects [190, 191]. The immune system of offspring with parental history of AD has responsiveness towards inflammation already at middle-age, indicating that the immune response in AD patients may be altered [192].

Natural anti-A β antibodies

Naturally occurring antibodies against A β have been found in the periphery in both AD and non-demented individuals [193]. The function of anti-A β antibodies is not clearly understood, but they have been implied to inhibit fibrillization and affect clearance of A β [194-196]. Thus, infusion of natural antibodies from healthy individuals, hopefully including natural A β -autoantibodies, is currently studied in AD. Positive effects on mouse behavior and AD cognition [194, 197, 198] have been reported but no positive treatment effects have been seen in clinical AD trials [199, 200]. In AD patients, plasma concentrations of anti-A β antibodies have demonstrated contradicting results with increased, decreased or equal levels, compared to control subjects. This might depend on the degree of existing antibody-antigen complexes and antigen specificity [201-205]. Recently, it has been proposed that natural anti-A β antibodies mainly show affinity towards oligomeric species of A β [194]. This would suggest that the immune system reacts specifically towards toxic A β oligomers and not necessarily towards the native, monomeric forms of A β .

Astrocytes in AD

The existence of neuroglia was first reported by Rudolf Virchow, year 1846. The cells were considered to be the supporting substance between neuronal parts in the brain [206, 207]. During the years that followed, the astroglia, named after its star-like appearance, was described. Although the number of astrocytes varies depending on brain area, it is estimated that 20-40% of the total mammalian brain cell number consists of astrocytes [208]. Astrocytes can be divided into two groups, based on morphology and anatomic location, *fibrous* and *protoplasmic* astrocytes. The *fibrous* astrocytes have numerous long fiber-like processes and are spread out within the white matter. *Protoplasmic* astrocytes have a bushy appearance with several stem branches, giving rise to innumerable thin processes [209].

Astrocytes are highly organized and spread throughout the whole brain, in a non-overlapping manner, coupled with each other through gap junctions. Astrocytes surround neuronal somata and dendrites, and provide fine ensheathment of synapses [210, 211]. The role of astrocytes in CNS is multifunctional, including guidance of axons and neuroblast migration during development, formation and function of synapses, synaptic pruning [212-216], regulation of CNS blood flow [217], maintenance of fluid, ion, pH and transmitter homeostasis [218-220], regulation of the blood brain barrier [221] and metabolic support for neurons [222]. Glial fibrillary acid protein (GFAP) is a widely used astrocytic marker, present in terminally differentiated astrocytes. GFAP is upregulated in reactive astrocytes, but the expression in *protoplasmic* and early *fibrous* astrocytes is less apparent [223]. Moreover, GFAP is expressed by cells of radial glia origin in CNS and by other cells in peripheral organs [224, 225]. Other markers used to identify astrocytes include S100- β , glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1), but these are not exclusively expressed by astrocytes. All markers vary in expression and location, demonstrating that there are specialized populations of astrocytes. It would be valuable to find markers that represent distinct astrocytic subpopulations [226, 227].

Astrocytes are also implemented in inflammation [228]. Reactive astrogliosis is a pathological hallmark of injured and diseased CNS. Upon astrogliosis, the astrocytes respond by increased gene expression of a number of structural proteins including GFAP, vimentin and nestin. Moreover, the astrocytes go through morphological changes, such as hypertrophy, resulting in enlarged cell body and increased number of processes. This is crucial for the formation of an astrocyte scar around tissue lesions [229, 230]. Astrocytes importance in inflammation is further supported by their expression of toll like receptors, scavenger receptors and complement receptors and their secretion of cytokines and chemokines [231-235]. In AD, reactive astrogliosis are prominent before onset of disease and in mouse models, they have even been reported to precede amyloid plaques [236-238]. Further, in astrocytic cultures, reactive astrogliosis can be induced by amyloid plaques isolated from AD patients [239].

Aims

The overall aim of this thesis was to increase the knowledge regarding A β protofibrils in AD pathology.

Specific aims

- I To investigate if heterophilic antibody (HA) interference is a common problem when using sandwich ELISAs for measuring oligomeric and protofibrillar A β in plasma and CSF from Arctic mutation carriers, sporadic AD patients and control individuals.
- II To investigate anti-A β antibody production on a cellular level by measuring the amount of anti-A β antibody producing B cells, instead of the plasma level of anti-A β antibodies. This may more accurately represent the situation in AD patients and control subjects.
- III To clarify the role of astrocytes in A β protofibril clearance by studying uptake, degradation and toxicity of A β protofibrils in a co-culture system of neurons, astrocytes and oligodendrocytes.
- IV To investigate if degradation of A β ₄₂ protofibrils by astrocytes can be enhanced by treatment with the protofibril selective antibody mAb158.

Methods

Human samples

Human plasma, CSF (**Paper I**) and whole blood (**Paper II**) from diagnosed AD patients or non-AD patients were provided by the Memory Disorder Unit, Uppsala University Hospital. Clinical AD diagnosis was determined by clinical and neuropsychological examination including neuroimaging and Mini-mental State Examination (MMSE). All study participants were included after obtaining a written informed consent.

In **Paper I**, we aimed to measure A β levels in plasma and CSF, with or without anti-heterophilic antibody (HA) treatment. In the AD patient group, we included plasma and CSF from sporadic AD and MCI and in the non-AD group we included plasma and CSF from healthy individuals and patients with Frontotemporal degeneration. Frontotemporal degeneration is a heterogeneous neuropathological disorder, both clinically and pathologically, without A β pathology [240]. We also included AD patients who were carriers/non-carriers of the Arctic mutation in **Paper I**. Arctic mutation carriers were specifically interesting to study due to their increased levels of A β protofibrils in the brain which also may be reflected in the periphery [58, 241]. Whole blood from age-matched AD patients and healthy volunteers were collected in **Paper II** (Table 1) to isolate peripheral blood mononuclear cells (PBMCs) for subsequent measurement of anti-A β producing B cells.

Table 1. Age matched AD patients and healthy individuals included in paper III.

Age (years)	AD patients		Healthy individuals	
	♂	♀	♂	♀
82-91	11	3	6	7
72-81	13	14	13	14
62-71	4	5	2	5
48-61	-	-	2	1

Model systems

Both *in vitro* and *in vivo* models are frequently used in AD research. Model systems are important for understanding specific mechanisms essential for the disease and to test new diagnostic and therapeutic strategies. Since all

model systems have both advantages and disadvantages, it is important to carefully select a suitable system for the studies of interest. Due to the anatomical similarity with the human brain, the possibility to use genetically modified strains and the existence of numerous behavioral tests to examine neural dysfunction, mice are extensively used as AD animal models [242]. Cell culture models are a more direct way to study the role of specific cellular or molecular processes in a disease, e.g. neuronal and glial response upon A β exposure. Moreover, cell cultures are easy to reproduce and enable reduced animal numbers to be used in research. Both *in vitro* and *in vivo* models carrying the familial inherited genetic mutations have been important for AD research, but they do not mirror the complete complexity of this heterogeneous disease.

Animal model

Non-transgenic mice and tg-ArcSwe mice, characterized by increased levels of soluble A β protofibrils, intraneuronal A β accumulation and accelerated senile plaque pathology [243-245], were used in **Paper I**. Both monoclonal antibody mAb158 ELISA and mAb82E1 ELISA are based on antibodies derived from mice and therefore, mice samples are not affected by HA in these two immunoassays. As a positive control in **Paper I**, we used saline perfused brain homogenates from frontal cortex, known to contain A β pathology [246]. The homogenates were investigated to assure that the A β signal remained in A β protofibril ELISA after anti-HA treatment and depletion of antibodies by protein G. To obtain brain homogenates, mice were intracardially perfused with saline solution to rinse traces of blood derivative. Brains were homogenized by using a tissue grinder with teflon pestle. To avoid degradation of proteins, complete protease inhibitor cocktail was used. To remove cell debris and to obtain a preparation of TBS-soluble extracellular and cytosolic proteins, including a wide range soluble A β , the homogenates were centrifuged at 100 000 $g \times 1$ h [95-97, 247, 248].

To compare if *in vitro* data from co-cultures reflected the pattern *in vivo* in **Paper III** and **IV**, immunohistochemistry was performed on brain slices from tg-ArcSwe mice. A saline perfusion step was followed by fixation of the brain in 4% phosphate-buffered formaldehyde to avoid autolysis. Brains were frozen and cryo-sectioned sagittally to a thickness of 14 μ m and then permeabilized and blocked in with 0.3% Triton X-100 in PBS, containing 5% normal goat serum.

Cell culture models

In **Paper III** and **IV** we used differentiated neural stem cell cultures. In order to grow these cells, cerebral cortex was dissected from C57/BL6 mice embryos, 14 days of age (E14). The cells were dissociated and cultured as

free floating neurospheres in cell culture media supplemented with bFGF and EGF. The mitogens allowed the cells to maintain their stem cell potential and promoted cell proliferation. The neural stem cells were allowed to differentiate for 7 days in mitogen free medium, to a mixed culture of astrocytes, neurons and oligodendrocytes (Figure 7). As we sought to follow the role of astrocytes in $A\beta_{42}$ protofibril clearance in the absence of microglia, which originate from a different progenitor, this cell culture system was ideal [127].

In **Paper III**, we aimed to study the toxic ability and the $A\beta$ content of microvesicles (including both larger vesicles and exosomes) derived from conditioned medium harvested day 6 and 12 following $A\beta_{42}$ protofibril removal. To study the neurotoxic effect, conditioned media were administered to pure E14 cortical neurons. Following dissection, neurons were directly seeded on poly-L-ornithine and laminin coated cover slips and cultured in neurobasal medium for 12 days prior to experiment.



Figure 7. Representative images of mixed neural cell cultures. Neural stem cells were allowed to differentiate to a mixed culture of astrocytes (GFAP, A), oligodendrocytes (CNPase, B) and neurons (β III-tubuline, C).

Microvesicle isolation

Microvesicles were isolated by repeated centrifugation steps. Conditioned medium were centrifuged for 5 min at 300 x g to remove any cell remnants, 10 min at 2000 x g to remove apoptotic bodies and 1.5 h ultracentrifugation at 135 000 x g at 4°C to isolate large microvesicles and exosomes. There should not exist any excess $A\beta$ aggregates in the media contaminating the microvesicle isolation as we extensively washed and transferred the cells to new wells after the 24 h of $A\beta_{42}$ protofibril exposure.

Synthetic A β

Throughout this thesis, synthetic A β has been used. The quality of A β and its propensity to aggregate vary between manufacturers and even between lot-to-lot. In order to minimize variations in A β we aimed to use the same lot in all experiments throughout each study [249, 250]. A β has, due to its hydrophobicity, a propensity to stick to glass and plastic surfaces in tubes, plates, pipette tips and columns. This may be avoided by addition of small amount of detergents.

When setting up experiments for production of A β of various forms, non-aggregated synthetic A β are used as starting material. Many laboratories use HFIP for monomerization of A β . However, when dissolving A β in acids, A β pass through the pI (5.1), which may cause formation of oligomers/aggregates [56]. Instead, we and others routinely use sodium hydroxide to dissolve A β , bringing A β to a high pH (pH \approx 10.5-11), followed by neutralisation with PBS, avoiding A β to pass its pI. Pretreatment with sodium hydroxide produces peptide solutions with higher yields of low molecular weight A β and lower levels of pre-existent aggregates [251-253].

In **Paper I-IV**, we have worked with monomeric and/or protofibrillar A β . Importantly, A β exists as a mixture of monomers and low-order oligomers in quasiequilibrium rather than solely as monomers [51, 55, 254, 255], and therefore low molecular weight A β are actually a more correct term to use [54]. Protofibrillar A β is defined by us as aggregates larger than 75 kDa, eluting in the void volume on a size exclusion Superdex 75 column. The biotinylation (**Paper II**) or the HiLyte™ Fluor 555-label (**Paper IV**) of A β protofibrils did not disturb the protofibril conformation.

Enzyme-linked immunosorbent assay (ELISA)

The Enzyme-linked immunosorbent assay (ELISA) is used to analyze the concentration of a specific antigen or antibody in a sample [256]. The method is used in **Paper I-IV**. ELISA is an immunological technique depending on the interaction between antigens and antibodies. In our studies, the sandwich ELISA setup has been used to identify and calculate concentration of A β monomers, oligomers and protofibrils in human body fluids, mice homogenates, cell culture medium, cell lysates and microvesicle preparations. Moreover, mAb158 ELISA has been used to characterize A β ₄₂ protofibrils after biotinylation and HiLyte™ Fluor 555-labeling. In the first step of a sandwich ELISA, antibodies are immobilized on a microplate surface. Next, the surface is blocked and the samples are added and only antigens recognized by the antibody will be captured, while non-specific proteins in the samples will be removed by washing. A secondary antibody, linked with a detection enzyme e.g. horseradish peroxidase (HRP), is then added, followed

by the addition of a substrate that leads to a detectable signal in the presence of antigen.

A β ₄₀ and A β ₄₂ ELISA

Various in house A β ₄₀ and A β ₄₂ ELISA setups, in numerous different combinations, were used in this thesis. The aggregation state of A β is important to consider when measuring synthetic or biological A β . The hydrophobic C-terminal is often hidden in the core of aggregates leading to underestimation of A β levels in C-terminus dependent ELISA assays [257, 258]. To circumvent this problem samples containing A β can be boiled in SDS to dissolve the aggregates and unmask the C-terminus. It is important to dilute samples after SDS treatment prior to the ELISA in order to avoid interference from SDS. N-terminal truncation is yet another factor that can lead to underestimation in samples. To measure N-terminally truncated A β in cell culture or *in vivo* [259, 260], an antibody binding the middle of A β -sequence, together with an A β _{40/42} antibody is to be preferred.

ELISA measuring soluble A β aggregates

In the mAb158 ELISA, the same monoclonal antibody is used both for capture and detection. Thus, more than one epitope have to be present to be detected by the ELISA (Figure 8). The mAb158 ELISA does not detect monomeric A β or low molecular weight A β oligomers. Only larger A β protofibrils are measured with this method [244]. In order to detect smaller aggregates, the mAb82E1 ELISA was employed. This ELISA is designed in a similar setup as the mAb158 ELISA, but uses the A β N-terminal specific antibody mAb82E1 as capture and detection antibody. Compared to mAb158, mAb82E1 is a non-conformational selective antibody recognizing all forms of A β . Therefore, theoretically, the mAb82E1 ELISA is able to detect aggregates from small dimers up to large A β aggregates [161]. To describe measured levels of A β oligomers or protofibrils, we express the concentration in monomer concentration units. The size and the number of conformational epitopes per A β monomer unit may differ between biological samples and synthetic A β used as a standard in our ELISAs. This may be a problem if A β aggregates in biological samples have high variability in size.

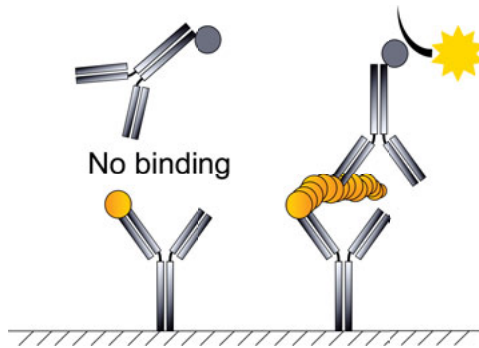


Figure 8. mAb158 ELISA. The A β protofibril specific, mAb158 ELISA uses the same antibody (mAb158) for both capture and detection. Thus, to achieve a signal in the ELISA, more than one epitope are needed.

HA interference in sandwich ELISAs

The aim of **Paper I** was to investigate if HA interference was a problem when analyzing plasma and CSF from AD patients and controls, by mAb158 ELISA and mAb82E1 ELISA. HAs, including human anti-mouse antibodies (HAMA), are polyreactive antibodies recognizing antibodies from other species occurring as a result of exposure to animals or animal products during life. HA can cross-bind assay antibodies, thus generating false positive signals in ELISAs [261] (Figure 9). To avoid HA interference, the samples in **Paper I** were subjected to a commercial neutralizing HA buffer containing a large excess of unrelated antibodies. The samples were only diluted 1:4-1:6 due to the expected low levels of A β oligomers and protofibrils. Therefore, in ELISA setups, where HA interference has not been considered, low dilution of samples could generate false positive signals. In CSF, antibody concentrations are low, allowing nearly complete depletion of HA using protein G.

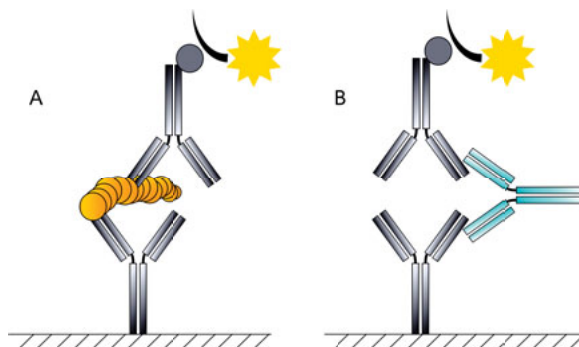


Figure 9. Principle of HA interference. In a sandwich ELISA, an immobilized capture antibody specifically binds its antigen. A second specific binding to the antigen occurs by an enzyme labelled detection antibody (A). False positive signals may be generated by HA due to crossbinding of assay antibodies in the sandwich ELISA (B).

PBMC preparation

PBMCs, containing lymphocytes, monocytes, macrophages and dendritic cells, were separated from blood by Ficoll-Paque separation technique. Ficoll-Paque is a density gradient medium and upon centrifugation, blood cells will migrate differently in the media. In short, blood diluted in PBS was carefully layered over Ficoll Paque-PLUS. After centrifugation, the following layers were distinct: plasma, PBMCs, Ficoll Paque, granulocytes and erythrocytes. The cloudy layer, consisting of PBMCs was carefully collected and washed. The cells were then counted and resuspended in freezing medium for storage in liquid nitrogen. Before use, PBMCs were carefully thawed.

Flow cytometry

In flow cytometry, suspended particles or cells from 0.2-150 μm in size are carried by a fluid stream to a laser intercept. By appropriately positioned lenses in the intercept, the particles or cells will be registered by scattering the laser light and, if tagged by any fluorescent molecule, they will fluoresce. The scattered and fluorescent light will be collected by detectors converting the received optical signals to electronic signals. In **Paper II**, flow cytometry was used to determine B cell numbers in AD patients and healthy controls. Before analysis of flow cytometry, PBMCs were stained for 1 h with the fluorescently labeled pan B cell surface marker anti CD19 [262].

B cell ELISpot

Antibodies secreted from cells can be measured on a single-cell level using the immunoassay enzyme-linked immunospot (ELISpot). The advantages using ELISpot when measuring natural anti-A β antibodies (**Paper II**) is that the assay has a high sensitivity and is less affected by protease activity and A β /anti-A β antibody complex binding. We used B cell ELISpot to investigate if B cells producing A β_{40} monomers or A β_{42} protofibrils could be detected and if they differed in AD patients compared to healthy controls. PBMCs, prestimulated with R848 and recombinant human IL-2 to efficiently activate B cells, were cultured in human-anti-IgG coated wells specific for ELISpot assay and secreted antibodies were captured. Anti-A β antibodies were detected by biotinylated A β and Streptavidin-ALP. Development occurred by filtered BCIP until spots were evident. Each spot correspond to an individual antibody-secreting cell (Figure 10). An alternative way to measure anti-A β antibodies by ELISpot is to first immobilize A β in the well and then add PBMCs. However, this approach was less suitable as A β conformation may be affected by surface binding [263]. Moreover, it may often

cause increased background and lower sensitivity and higher antigen concentrations are needed. Although A β ELISpot is of great advantage when measuring A β antibodies produced by B cells, both low and high binding intensity antibodies will be detected. Thus, the assay cannot provide a measurement of the affinity of the detected antibodies.

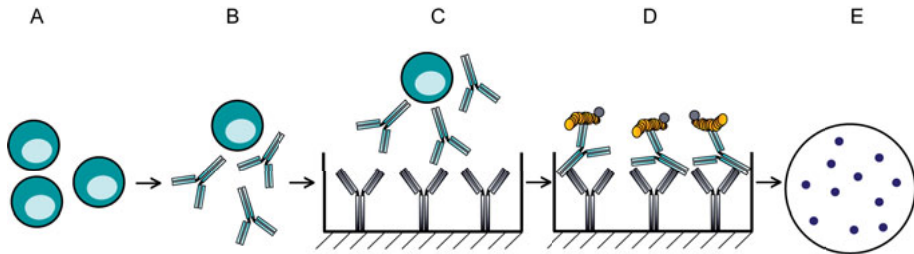


Figure 10. To initiate the antibody production from B cells, PBMCs were stimulated with R848 and hIL-2 (A-B). PBMCs were added to ELISpot wells, coated with anti-IgG capture antibodies (C). The captured anti-A β antibodies, secreted from the B cells were recognized by biotinylated A β (D) and visualized by Streptavidin-ALP. The dots in the ELISpot wells represent individual B cells secreting anti-A β antibodies (E).

Immunofluorescence and other staining techniques

Immunofluorescence is a common technique based on antigen-antibody interaction. Antibodies, chemically conjugated with a fluorophore, bind directly or indirectly to a specific antigen of interest in e.g. cultured cells and tissue samples. In direct immunofluorescence, the primary antibody, detecting the antigen of interest, is labeled a fluorophore and can be visualized in fluorescence or confocal microscopy. In **Paper III** and **IV** we have applied indirect immunofluorescence. Here, the primary antibody is unlabeled and the secondary antibody, recognizing the constant part of the primary antibody, is carrying a fluorophore, amplifying the sensitivity. By using fluorophores of different colors, multiple antigens can be detected in the same sample.

To visualize the individual cell types in the primary cell culture model used in **Paper III** and **IV** we have used well-known markers. For astrocytes, antibodies recognizing the intermediate filament protein GFAP were used. Importantly, it has been reported that as little as 15% of astrocytic area can be observed by GFAP, leading to underestimation of astrocytes size [210]. Neurons and oligodendrocytes were recognized by microtubule component, β III-tubulin and CNPase antibodies, respectively. Rab5 was stained to visualize early endosomes, Rab7 visualizing late endosomes and LAMP-1 and LAMP-2 to identify endosomal/lysosomal compartments.

A β , N-terminally labeled with HiLyte™ Fluor 555-label was used in some experiments in **Paper III** and **IV** to follow A β clearance and degrada-

tion in the neuronal and glial co-cultures. Vectashield mounting medium containing 4',6-diamino-2-phenylindole (DAPI) was used to stain cell nuclei in **Paper III** and **IV**. DAPI is extensively used in fluorescence microscopy and binds to A-T rich regions in DNA. To label apoptotic cells in **Paper IV**, Terminal deoxynucleotidyl transferase dUTP nick labeling (TUNEL) assay was used. TUNEL identifies free 3'-hydroxyl termini generated by DNA fragmentation. Degraded DNA may also be displayed by necrotic cells [264]. Therefore, TUNEL should not be considered a specific marker for apoptosis.

The LysoTracker® Red DND-99 dye accumulates and labels all vesicles with low internal pH within the cells and is thus not lysosome-specific. LysoTracker consists of a fluorophore linked to a weak base that is only fluorescent at acidic pH. LysoTracker was used in **Paper III** to study whether A β was present in acidic vesicles.

Microscopic techniques

Results in **Paper III** and **IV** are mainly based on imaging techniques to study mechanisms of A β protofibril clearance in primary cell cultures. By using fluorescence microscopy we studied uptake and accumulation of A β and co-localization and expression of enzymes in the endosome/lysosome pathway. To verify that A β ₄₂ protofibrils were localized intracellularly, confocal microscopy was used, providing a three-dimensional image. This technique allows the specimen to be imaged one “point” at a time and the images can then be joined to a z-stack to get the three-dimensional image. In short, out-of-focus light from above and below a focus point are eliminated as a small pinhole aperture allows only light emitted from the desired focal point to pass through. In time-lapse, image sequences are captured with regular intervals and viewed at a greater speed to give an accelerated view of dynamic process. Time-lapse microscopy was used to image the cell culture over time, to follow A β accumulation and other cellular processes.

In order to visualize isolated microvesicles secreted by the cell culture we used transmission electron microscope (TEM). TEM uses electrons instead of light to produce an image. As electrons have a much lower wavelength than light they provide resolution in the low nanometer range e.g. small structures in the cells can be detected. In TEM, a beam of electrons are focused on a single element of a studied ultra-thin specimen. The electrons interact with the specimen and only electrons going through the sample will convert into light when meeting a phosphor screen. When electrons are absorbed or scattered on their way through the specimen, dark areas are formed and when more electrons pass through they will form lighter areas resulting in an image.

Present investigations

Detection of A β oligomers and protofibrils in human body fluids using sandwich ELISAs. Is the signal true?

One purpose of this thesis was to evaluate A β protofibrils as a CSF biomarker. At first glance, our data looked promising and we found positive signals in the protofibril specific mAb158 ELISA when measuring human CSF. However, when performing control experiments, we found HA interference causing false positive signals in the ELISA. Further, we noticed that interference of HA was often neglected among researchers in the AD field, when measuring A β oligomer and protofibril levels in CSF using immunoassays. Therefore, in **Paper I**, we aimed to study the extent of HA interference when measuring A β oligomers and protofibrils in human plasma and CSF.

Human plasma and CSF from Arctic mutation carriers, sporadic AD patients and control individuals was examined with the mAb158 ELISA to investigate if A β protofibrils could be detected. In both plasma and CSF, the mAb158 ELISA generated A β protofibril signals. In plasma AD patients had significantly higher A β protofibril levels than control individuals. Similar results were obtained also with the mAb82E1 ELISA. All signals in the mAb158 ELISA and the majority of signals in the mAb82E1 ELISA in plasma samples, were reduced to below the limit of detection when diluted in anti-HA plasma diluent instead of the standard ELISA buffer (Figure 11 A-C, **Paper I**, Table 1 & Figure 2 A-C). Most CSF samples generating signal in the mAb158 ELISA lost their signal upon protein G depletion. In some CSF samples, positive signals remained but were greatly reduced (Figure 11 D, **Paper I**, Table 1 & Figure 2 D). This can be explained by the presence of HAs of other isotypes than IgG since protein G favor binding to IgG and binds other isotypes to a lesser extent. To verify the results from protein G depletion in CSF, anti-HA sample diluent was also tested in a few samples with high signals in the standard buffer, and also in these samples the signal disappeared after treatment (**Paper I**, figure 3 B).

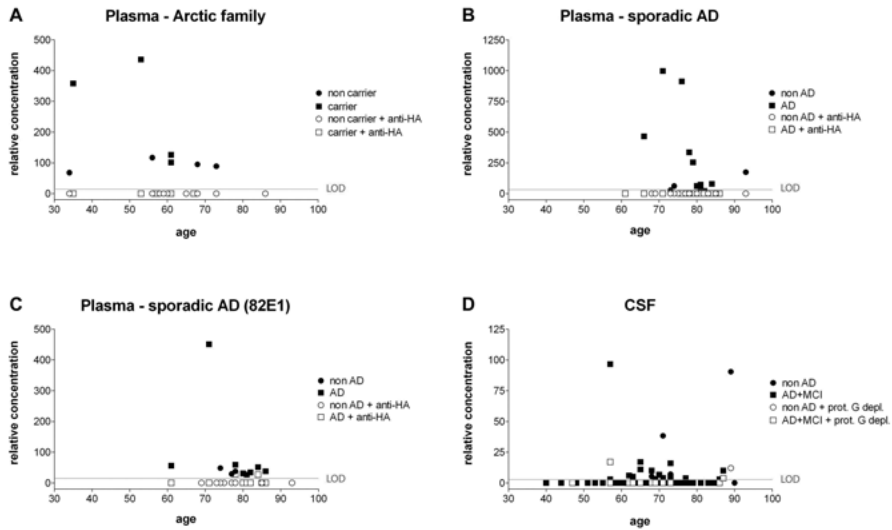


Figure 11. HA interference in oligomer/protofibril ELISAs. Plasma (A-C) and CSF (D) from AD patients or control individuals were analyzed by the mAb158 ELISA (A,B & D) or the mAb82E1 ELISA (C). Positive signals obtained in A β protofibril and oligomer ELISAs were greatly reduced when treating the samples with anti-HA treatment.

The results from our studies indicated that plasma and CSF samples from both AD and control subjects contained HA in sufficient levels to interfere with A β sandwich ELISAs. In a selection of positive HA samples, the capture, the detection, or both capture and detection antibodies of the mAb158 ELISA were exchanged for irrelevant mouse antibodies. Without anti-HA treatment the samples still gave positive signal (**Paper I**, Figure 3 A-B), while the signal dropped below limit of detection with anti-HA treatment (**Paper I**, Figure 3 A). This verifies the notion that the signals were in fact unrelated to A β , and that HA interfering with the ELISA caused false positive results. Thus, it is of importance to consider the possibility of false positive results due to HA interference when carrying out immunoassay based studies of soluble A β aggregates.

To ensure that A β protofibrils from *in vivo* samples were correctly measured by mAb158 ELISA, brain homogenates from A β PP transgenic mice, containing substantial amounts of A β protofibrils, were analyzed [244]. A β signals were received in the standard mAb158 setup even after anti-HA treatment, but were lost when exchanging antibodies in the ELISA to irrelevant antibodies. Non-transgenic mice did not obtain any signals in the mAb158 ELISA (**Paper I**, Figure 3 C).

Although we did not find any true signals of soluble A β oligomers or protofibrils in **Paper I**, these A β species may still be present in CSF and plasma. In fact, by combining A β_{42} concentrations determined by a denaturing and a non-denaturing method into an A β_{42} oligomer ratio, we have indirectly

detected the presence of A β oligomers in human CSF from AD patients [258]. Numerous studies have been able to measure soluble A β oligomers in human CSF [155-162]. To our knowledge, HA have not been considered in most of these studies. To ensure a true A β signal and to avoid distrust, these studies should perform and present further characterization of the results by e.g. perform serial dilutions of samples to confirm the linearity of the signal, perform immunodepletion to confirm their A β specificity, excluding HAs as signals, and recovery of signal from samples spiked with an oligomer standard. One study measuring A β oligomers in CSF is worth to mention. Fukumoto et. al (2010) measured elevated levels of soluble A β oligomers in AD CSF compared to control CSF, by using fragment antigen-binding F(ab') fragments of anti-A β antibodies as detection instead of whole antibodies, thus minimizing the risk of HA interference [155].

Anti-A β antibodies in healthy controls and AD patients

Since A β oligomers in CSF are difficult to measure by traditional ELISA we sought to investigate another approach. Instead of measuring protofibrils directly we measured the anti-A β antibody response from B cells to see if they differed between AD patients and healthy controls. If we were able to measure anti-A β antibody producing B cells in humans correctly, this could be an important diagnostic marker. Anti-A β antibodies are also interesting as many of research groups are evaluating anti-A β antibodies as a therapeutic approach for AD. Thus, it is important to know the effect of these antibodies *in vivo*, since they can play a role in the natural defense against AD. A β protofibrils are toxic and may induce an antibody response from B cells. Numerous studies have demonstrated that humans are able to synthesize antibodies against A β . However, it has not been established if these antibodies are important for A β clearance and acts protective against AD [265], or if they exist in a higher degree in AD patients as a result of the disease process [201].

ELISA has been widely used to measure anti-A β antibodies in human serum and plasma [266]. In serum/plasma, A β and anti-A β antibodies can form a complex with each other making it troublesome to measure anti-A β antibody concentration by conventional methods. The problem of A β /anti-A β antibodies complex binding can be circumvented by dissociation of A β from the anti-A β antibody at low pH. After dissociation, serum from AD patients has been shown to have higher anti-A β antibody levels than controls [266]. In **Paper II** we used an alternative method, instead of measuring the anti-A β antibodies concentration directly; the number of B cells producing anti-A β antibodies of IgG subclass was analyzed with the ELISpot technique. The number of anti-A β antibody producing B cells were measured in a large set of age matched AD patients and healthy controls. Samples that were unable

to produce a general IgG response upon stimulation were excluded from the study as were samples with low PBMC numbers. Although no differences in total B cell number were seen, AD patients had a higher number of IgG producing B cells in their blood compared to healthy controls (**Paper II**, Figure 2 A-B). In AD, the immune response is generally active [267] and this could lead to a higher activation of B cells in AD patients compared to healthy controls.

Both the number of B cells producing anti-A β_{40} monomer antibodies and anti-A β_{42} protofibril antibodies were studied. Interestingly, B cells were significantly more prone to produce antibodies recognizing anti-A β_{42} protofibrils than A β_{40} monomers (Figure 12 A & **Paper II**, Figure 3 A). We speculate that this could be a result of the immune system responding to the increased A β_{42} protofibril load as an attempt to remove the toxic A β_{42} protofibrils. A β_{40} monomers are the native form of A β and have probably physiological functions. There was low or no existing numbers of B cells recognizing A β_{40} monomers and they did not differ between AD patients and healthy controls (Figure 12 B & **Paper II**, Figure 3 B). This result was predicted since the immune system should not react towards self-proteins [268-272]. Interestingly, AD patients had a higher degree of anti-A β_{42} protofibril antibodies compared to healthy individuals (Figure 12 C & **Paper II**, Figure 3 C). This difference was even greater in AD patients with ApoE $\epsilon 4$ genotype. In this study, we found no correlation between B cell producing anti-A β_{42} protofibril antibodies and the mini mental status in the AD group (**Paper II**, Figure 4).

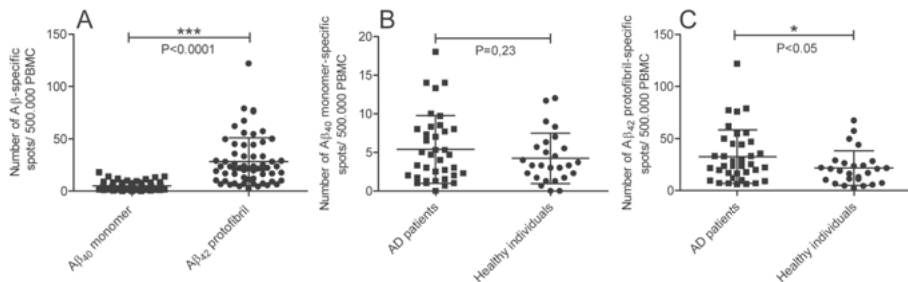


Figure 12. B cells mainly produced anti-A β antibodies towards A β_{42} protofibrils and not A β_{40} monomers (A). No difference was seen in the level of anti-A β_{40} monomer antibody producing B cells when comparing AD patients with healthy controls (B). However, AD patients had a higher level of anti-A β_{42} protofibril producing B cells compared to healthy controls (C).

The existing overlap of the number of anti-A β_{42} protofibril antibody producing cells in AD patients and healthy controls and the lack of correlation between anti-A β_{42} protofibril antibody producing cells and mini mental status makes this measure less suitable as a biomarker for AD. However, anti-A β_{42}

protofibril antibodies role as therapeutics should be further evaluated, since they seem to be a part of the natural defense against A β ₄₂ protofibrils.

The role of astrocytes in A β clearance

Being the most numerous cell type in the brain, astrocytes are important for many functions, but their role in AD is poorly understood. It has been suggested that insufficient lysosomal degradation is a main cause of sporadic AD [273, 274]. Ineffective degradation of A β may lead to spreading of AD pathology, due to A β secretion from the phagocytic cells [275]. Many studies have been focusing on the role of microglia in A β clearance, but the importance of astrocytes in A β clearance needs to be further investigated. It has been reported that monomeric A β is engulfed and degraded by astrocytes [120-122]. However, in these studies it is unclear whether A β was degraded or transported to the lysosomes for storage. Our research group has recently shown that astrocytes effectively engulf whole dead cells, but store, rather than degrade the ingested material [276]. This may be the case for A β as well, since astrocytes with high A β load have been found in AD brain [128].

In **Paper III**, co-cultures of astrocytes (70%), neurons (25%) and oligodendrocytes (5%) were exposed to A β ₄₂ protofibrils for 24 h to study uptake, degradation, secretion and toxic effects of A β . A β ₄₂ protofibrils, labeled with fluorescent HiLyte™ Fluor 555 were efficiently taken up by astrocytes and oligodendrocytes (**Paper III**, Figure 1 A & C, respectively). A β were often found to co-localize with DAPI stained condensed nuclei intracellularly (Figure 13 & **Paper III**, Figure 1 D). To verify that it was A β and not the 555-labeling agent that was detected after the engulfment, unlabeled A β ₄₂ protofibrils were immunostained using A β antibodies binding to the N-terminus of A β , A β protofibrils or A β ₄₂. All A β antibodies detected intracellular A β deposits, verifying a specific A β signal (**Paper III**, Additional file 2 A-D).

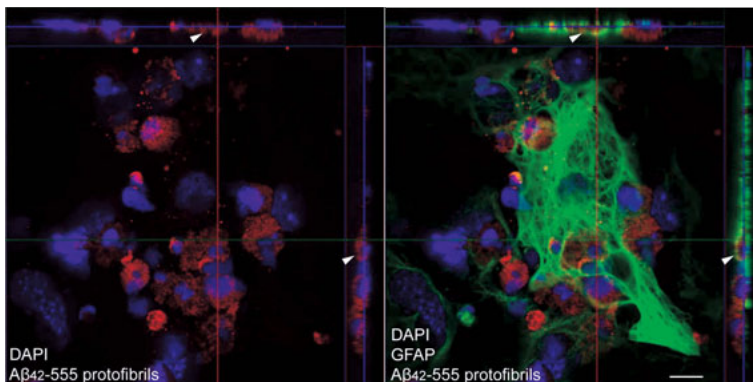


Figure 13. Confocal image of astrocyte containing A β inclusions. Scale bar: 10 μ m.

Interestingly, in the brains of tg-ArcSwe mice we found A β , often co-localized with condensed nuclei of dead cells, ingested by reactive astrocytes (**Paper III**, Figure 2 E). This confirms that our *in vitro* data from the cell culture studies reflected cellular processes of A β pathology *in vivo*. In neurons, stained with β III-tubulin, A β ₄₂ protofibril levels were often below the detection limit (**Paper III**, Figure 1 B) suggesting that A β ₄₂ protofibrils were not taken up by neurons or that they were successfully degraded. When studying the cells using immunostaining and time-lapse over the course of 12 days after A β removal, we found that both astrocytes and oligodendrocytes degraded A β extremely slow, leading to intracellular accumulation of A β protofibrils (**Paper III**, Figure 3 A, B & D-G). A β _{1-x}, A β _{x-42} and A β protofibril ELISAs were used to measure A β levels in both the soluble and the insoluble fraction of the cell lysate. The insoluble fraction was analyzed in order to determine if accumulated A β were packed into insoluble aggregates that were pelleted during the centrifugation. The ELISAs demonstrated that a larger part of the accumulated A β was N-terminally truncated (**Paper III**, Figure 5).

Immunostainings using the lysosome specific LAMP-1 antibody showed that A β were slowly transported to lysosomal compartments within astrocytes (**Paper III**, Figure 4 A). Moreover, immunostainings of 14-month-old tg-ArcSwe mice brains, using antibodies against GFAP and A β were performed. The results demonstrated that astrocytes, as expected, were tightly localized around A β plaques and A β co-localized, in the outermost layer of the A β plaque, with both GFAP and LAMP-2 (**Paper III**, Figure 4 C-D). These data indicates that A β may be situated in lysosomal compartments inside the glial cells that tightly surround the plaque. Degradation does not always occur following arrival to the lysosome. In fact we found that A β ₄₂ containing lysosomes did not stain with the LysoTracker, indicating that A β ₄₂ stored in the glial cells were situated in immature lysosomes (**Paper III**, Figure 4 B)

Time-lapse recordings showed formation of enlarged, dynamic vacuoles, ~50 μ m in diameter, in the astrocytes after 2-3 days of A β ₄₂ protofibril exposure (Figure 14 & **Paper III**, Figure 6 A-B). The vacuoles were probably induced by the high A β load since no vacuoles were formed in corresponding control cultures. Immunostainings using the early endosomal marker Rab5 and the late endosomal marker Rab7 identified the vacuoles, suggesting them to be enlarged early endosomes (**Paper III**, Figure 6 C).

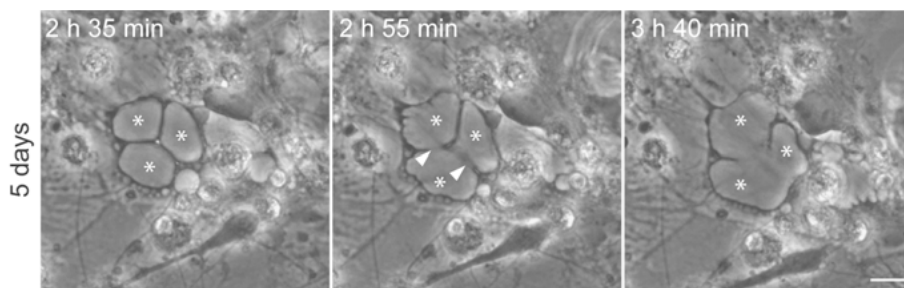


Figure 14. Enlarged, dynamic early endosomes (*), ~50 μm in diameter, appeared inside the astrocytes, 2-3 days after $\text{A}\beta_{42}$ protofibril exposure. The images show 3 enlarged endosomes that fuses (white arrows) to one larger endosome, 5 days after $\text{A}\beta_{42}$ protofibril exposure. Scale bar: 10 μm .

$\text{A}\beta$ exposure had no toxic effect on astrocytes and oligodendrocytes, but neurons were negatively affected in the long term, indicating that the cell death is due to a secondary mechanism (**Paper III**, Figure 7). We speculated that the severe lysosome failure, induced by accumulation of $\text{A}\beta$ in astrocytes, could lead to secretion of toxic agents into the extracellular environment, causing neurotoxicity. To identify the possible mechanism for the secondary toxicity, we investigated if microvesicles secreted by $\text{A}\beta_{42}$ protofibril treated co-cultures induce apoptosis of cortical neurons.

There was a significant increase in apoptosis in neural cultures treated with microvesicles from $\text{A}\beta_{42}$ protofibril exposed co-cultures, compared to microvesicles from untreated co-cultures. Hence, microvesicles from $\text{A}\beta$ exposed cell cultures induced neurotoxicity. Further, we demonstrated by $\text{A}\beta_{1-x}$ and $\text{A}\beta_{x-42}$ ELISA, that the microvesicles contained $\text{A}\beta$ which were mainly truncated in the N-terminal (**Paper III**, Figure 8). Compared to full length $\text{A}\beta$, N-terminally truncated $\text{A}\beta$ is known to be more resistant to degradation, more aggregation prone and more toxic [277].

mAb158 enhance clearance of $\text{A}\beta$ by astrocytes

Currently, different anti- $\text{A}\beta$ antibodies, including the humanized version of the $\text{A}\beta$ protofibril selective mAb158, BAN2401, are evaluated in clinical trials for AD. mAb158 has previously been shown to inhibit fibrillogenesis and $\text{A}\beta$ toxicity *in vitro* and to lower $\text{A}\beta$ protofibril levels, prevent $\text{A}\beta$ deposition and promote clearance of $\text{A}\beta$ in tg-ArcSwe mice [278, 279]. However, the cellular mechanisms of mAb158 and other therapeutically $\text{A}\beta$ antibodies remain to be elucidated. $\text{Fc}\gamma$ -receptor mediated phagocytosis of $\text{A}\beta$ [165, 167, 169, 280], disaggregation of $\text{A}\beta$ aggregates [281], neutralization of $\text{A}\beta$ toxicity and sequestration of $\text{A}\beta$ monomers [282] are suggested pathways for antibody mediated $\text{A}\beta$ clearance pathways.

In **Paper IV**, we aimed to investigate whether mAb158 was able to enhance the degradation of A β through Fc γ receptor phagocytosis. Differentiated neural stem cell cultures (composed of neurons and glia) were used in order to compare clearance of A β_{42} protofibrils when treating with mAb158 or not. The study showed that mAb158 was able to reduce A β accumulation already after 24 h in astrocytes, if added to cell cultures at the same time as the A β_{42} protofibrils (Figure 15 A-B & **Paper IV**, Figure 1 A-B). However, mAb158 addition, 3 days after A β_{42} protofibril exposure, had no A β lowering effect (Figure 15 C, **Paper IV**, Figure 1 C). Twelve days after A β_{42} protofibril + mAb158 treatment, the A β load in astrocytes had been further reduced (**Paper IV**, Figure 1 D-F). To actively follow the course of events after adding A β_{42} protofibrils and mAb158 to the cell cultures, time-lapse recordings were performed. The recordings visualized co-localization of low A β and mAb158 levels within astrocytes (**Paper IV**, Figure 2 A-B). The A β /mAb158 co-localization was further confirmed by confocal microscopy (**Paper IV**, Figure 2 C). The reduced A β accumulation in astrocytes in the presence of mAb158 might be a result of A β internalization through Fc γ receptor mediated phagocytosis. Possibly, the Fc γ receptor pathway is more efficient to degrade A β_{42} protofibrils than e.g. macropinocytosis and phagocytosis by other receptors. Alternatively, mAb158 inhibits uptake of A β . However, we did not detect any larger A β /mAb158 complexes in the media during the time-lapse experiments. Moreover, when analyzing the cell culture media by A β_{1-x} ELISA after 24 h A β_{42} protofibril exposure, with or without mAb158 treatment, no significant differences were observed in A β levels (**Paper IV**, Figure 6).

As noticed in **Paper III**, there was a longitudinal decrease of neuronal viability after A β exposure. Interestingly, A β_{42} protofibril induced neurotoxicity was eliminated when co-administered with mAb158, but not when mAb158 was administered 3 days after A β_{42} protofibril exposure (Figure 15 D & **Paper IV**, Figure 3). Thus, whether mAb158 inhibits the A β uptake by astrocytes or if they enhance degradation of A β in astrocytes, the neurotoxicity is reduced by mAb158. This is important as neurodegeneration is a central process in the pathology of AD.

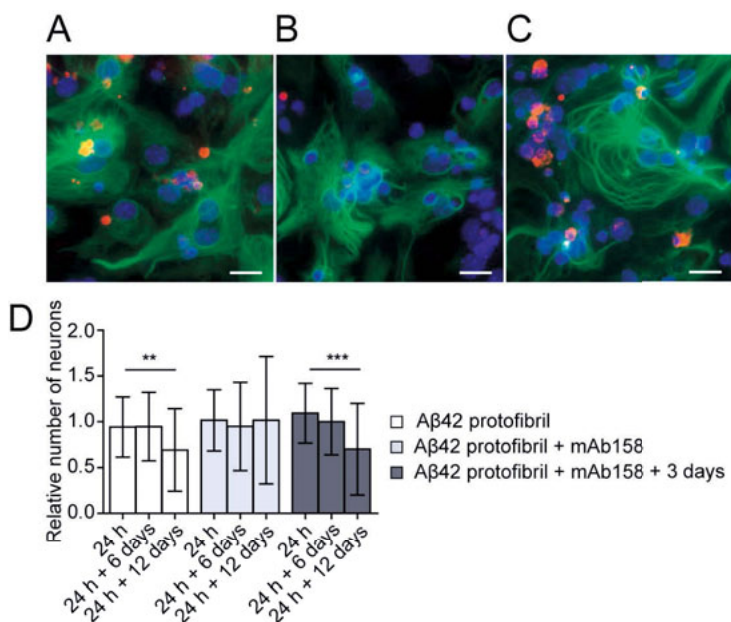


Figure 15. mAb158 reduces A β_{42} protofibril accumulation in astrocytes. Astrocytes accumulate A β after A β_{42} -555 protofibril exposure (A). mAb158 added together (B), but not after A β_{42} -555 protofibrils (C), markedly reduced A β accumulation. The secondary, neurotoxic effect of A β_{42} protofibrils were prevented if co-administered with mAb158 (D). Scale bars: 20 μ m.

The glycosylation site is crucial for the ability of IgG to bind to the Fc γ receptor. Fc γ receptor binding may be impaired by using the N297D antibody. N297D has the same CDR sequence as mAb158 but is mutated at the glycosylation site, i.e. it binds equally well to A β protofibrils, but does not have the same glycosylation pattern. Compared to A β_{42} protofibril exposed cell cultures, a reduction in astrocytic A β accumulation was seen when treating cell cultures with A β_{42} protofibrils and N297D (**Paper IV**, Figure 4 A & C). Possibly, N297D still binds to high affinity Fc γ receptors, thereby enabling degradation of A β . Alternatively, the degradation of A β may occur through the recently described intracellular TRIM21 Fc receptor. The high affinity receptor TRIM21 can mediate immune responses by interacting with antibodies through a domain in the Fc region, independent of glycosylation [283]. Since TRIM21 interacts with a different domain in the Fc region of the antibody than Fc γ receptor, recombinant mAb158 containing the N297D mutation could still bind. A β levels, both A β_{1-x} and A β_{x-42} , were reduced by treatment with mAb158, recombinant mAb158 and N297D, compared to cell cultures exposed to A β_{42} protofibrils only (**Paper IV**, Figure 5), supporting the results from the immunostainings and time-lapse experiments.

The importance of scavenger receptors for A β_{42} protofibril clearance by astrocytes was investigated by pre-treating cells with the general scavenger

receptor blocker fucoidan. When analyzing the cell lysate, samples treated with fucoidan consistently had lower levels of both $A\beta_{1-x}$ and $A\beta_{x-42}$, compared to corresponding treatment without fucoidan (**Paper IV**, Supplementary Table 1). The effect could depend on antibody guidance of $A\beta_{42}$ protofibrils through an alternative pathway (e.g. $Fc\gamma$ receptor or TRIM21 dependent pathway) that might have a higher degradation rate than the scavenger receptor pathway. The decrease of $A\beta$ levels can also be due to reduced uptake by astrocytes as scavenger receptors are inhibited.

Taken together, our data indicate that $A\beta$ clearance can be enhanced by mAb158, but the mechanisms behind this most likely involves more than one pathway. Therefore, further studies are needed in order to clarify these processes.

Further studies

To elucidate the mechanisms involved in the decreased accumulation of $A\beta$ in astrocytes after antibody treatment, we will perform further experiments. One possible explanation for our results may be that mAb158 prevent $A\beta$ from entering the astrocytes, thus keeping $A\beta$ in the cell culture media. In time-lapse recordings, the background signals were low, without increased amount of complexes floating in the medium. Further, we did not find any difference in media concentrations between $A\beta_{42}$ protofibrils with or without mAb158 and N297D (**Paper IV**, Figure 6). Our hypothesis is that astrocytes engulf $A\beta$ with equal rate in the absence and presence of antibodies, but more detailed analysis of medium will be performed in future studies.

The antigen-binding site of the antibody is called the $F(ab')$ fragment, which is composed of one constant and one variable domain of the heavy and light chains. If cleaving an antibody by pepsin enzyme below the hinge region, the antibody will be divided into an $F(ab')_2$ fragment and an pFc' fragment. It would be interesting if we could treat cultures with only the $F(ab')_2$ fragment of mAb158 to study the effect on $A\beta$ accumulation in astrocytes. If we still see decreased $A\beta$ accumulation by $F(ab')_2$, this may suggest a $Fc\gamma$ receptor independent mechanism, since the Fc part is lacking in the $F(ab')_2$ fragment. We will also study the involvement of the TRIM21 pathway on $A\beta$ degradation in astrocytes.

Finally, *in vivo* studies of tg-ArcSwe mice will be performed. We have brain material from tg-ArcSwe mice intraperitoneally injected once weekly for 4 weeks with mAb158, recombinant mAb158, N297D or PBS. This material will be used to study if $A\beta$ inclusions in astrocytes are reduced by mAb158 treatment.

Conclusion

The molecular and cellular mechanism of soluble A β aggregates is in focus for many research teams, and various soluble A β aggregates are investigated as both diagnostic markers and therapeutic target for AD. Great efforts have been made to investigate soluble A β aggregates in plasma and CSF as reliable biomarkers for AD. However, this has proven to be challenging due to the low concentrations of these A β species in CSF and plasma. We did not find any A β aggregates in CSF in the work of this thesis, but instead we proved the importance of heterophilic antibody (HA) interference when measuring A β aggregates in plasma and CSF by immunoassays. Results from the study described in **Paper I** have led to some awareness regarding HA. Still, recent articles have measured A β oligomers in AD, without specifying if HA has been concerned [155-161]. There is a lot of focus on understanding mechanisms of action of antibodies when targeting A β *in vivo* and to investigate their role as AD therapeutics. Natural anti-A β antibodies in the human body have been identified, but discrepancies remain regarding the levels, and whether they differ between AD patients and control individuals. In **Paper II**, we managed to indirectly measure natural anti-A β antibodies by ELISpot. We found that B cells in humans mainly produce antibodies against the toxic A β protofibrils but not against A β monomers. Interestingly, AD patients had higher numbers of anti-A β antibody producing B cells than healthy controls. Due to an overlap between the numbers of B cells producing anti-A β antibodies in AD patients and healthy controls and a lack of correlation between anti-A β antibody producing B cells and mental status in AD patients, the diagnostic potential for anti-A β antibody producing B cells are low. Yet, our results indicates that a natural defense mechanism is ongoing in the body to eliminate A β protofibrils, implicating that anti-A β protofibril antibodies used in therapeutics could be effective during right circumstances.

In **Paper III**, we found that astrocytes effectively engulf, but inefficiently degrade A β protofibrils, resulting in a high intracellular load of toxic, partly N-terminally truncated A β and severe lysosomal dysfunctions. Moreover, we propose that the phagocytic astrocytes induce apoptosis of neurons by secreting microvesicles containing N-terminally truncated A β . Hence, astrocytes can play an important role in the progression of AD and may constitute a important therapeutic target. BAN2401, the humanized version of mAb158 is presently evaluated in phase 2b clinical trials. mAb158 targets A β protofi-

brils and has been shown to reduce A β levels in mouse studies [278], but the mechanisms of the antibody in reducing protofibrils are still to be clarified. In **Paper IV** we found that mAb158 reduced accumulation of A β within astrocytes. Further studies are needed to elucidate the exact mechanism behind this effect. From our current data, we hypothesize that mAb158 manages to induce a more efficient degradation of A β in astrocytes, either through Fc γ receptor mediated phagocytosis or through an intracellular pathway, possibly including the recently described receptor TRIM21.

In conclusion, the results from our *in vitro* and *in vivo* data included in this thesis, increase the knowledge of both A β protofibrils and A β antibodies on a biological level. Our results may be beneficial when developing both new diagnostics and therapeutics for AD and for improved understanding of AD pathology.

Sammanfattning på svenska

Alzheimers sjukdom är den vanligaste orsaken till demens och medför stort lidande för patienter och anhöriga. Vid Alzheimers sjukdom försämras minnet och andra kognitiva förmågor p.g.a. att nervceller i hjärnan förtvinar och inte förmår att kommunicera med varandra. Alzheimers sjukdom är en åldersrelaterad sjukdom och risken för att drabbas ökar exponentiellt efter 65 års ålder. I vissa sällsynta fall av ärftlig Alzheimers sjukdom så kan man dock drabbas betydligt tidigare i livet. Riskfaktorer som kan associeras med Alzheimers sjukdom är bland annat kön, utbildningsnivå och ärftlighet. De tidigaste symptomen vid Alzheimers sjukdom yttrar sig oftast som minnes-svårigheter och sämre förmåga att utföra dagliga sysslor. I ett senare skede får den drabbade en utbredd glömska, förändrad personlighet och svårigheter att orientera sig i tid och rum. Ofta drabbas även rörelseapparaten, så att rörelserna blir långsamma och svårkoordinerade.

En viktig aktör i sjukdomsförloppet vid Alzheimers sjukdom är proteinet ”amyloid prekursor protein”, vilket klyvs och frisätter peptiden amyloid-beta ($A\beta$). $A\beta$ bildas naturligt i kroppens alla celler och för höga nivåer av $A\beta$ leder till Alzheimers sjukdom. På grund av sin vattenavstötande natur har $A\beta$ en benägenhet att klumpa ihop sig och bilda både lösliga och olösliga aggregat som deponeras i amyloida plack. Flera studier har visat att det är främst de lösliga $A\beta$ -formerna som skadar hjärnan vid sjukdomen. I slutet på 1990-talet hittades den ”Arktiska mutationen” ($A\beta$ PP E22G) i en svensk familj. Den arktiska $A\beta$ -peptiden ansamlas och bildar större former av lösliga aggregat, så kallade protofibriller, snabbare än $A\beta$ utan mutationen. Detta fynd gav kliniska indicier för att just protofibriller orsakar sjukdomen.

De ”bromsmediciner” som existerar idag mildrar symptomen hos vissa patienter under något år, men behandlingen angriper inte den bakomliggande sjukdomsorsaken, utan nedbrytningen av nervceller i hjärnan fortskrider. Forskningen kring nya behandlingsstrategier vid Alzheimers sjukdom är intensiv och nya, lovande terapier, däribland immunterapi, utvärderas just nu i kliniska prövningar världen över. För att dessa nya behandlingar skall kunna utvärderas i ett tidigt skede och på ett optimalt sätt krävs det nya, bättre markörer för sjukdomen. De biomarkörer som finns idag, sänkta nivåer av $A\beta$ 42 och förhöjda nivåer av tau och fosfo-tau i ryggmargsvätska (CSF), är förhållandevis bra på att urskilja sjukdomen tidigt, men de är inte optimala för att kunna följa sjukdomsförloppet.

Idag pågår det flera studier där lösliga A β -aggregat analyseras som potentiella diagnostiska markörer. Det är därför viktigt att känna igen olika metodiska problem som kan uppstå vid dessa mätningar. Sandwich enzyme-linked immunosorbent assay (ELISA), utnyttjar antikroppars förmåga att känna igen olika typer av antigen, i vårt fall A β -protofibriller. Ett problem som kan uppstå vid immunologiska mätningar av antigen, t.ex. A β aggregat, som finns i låga nivåer i human plasma och CSF, är påverkan av heterofila antikroppar. Människor som exponerats för djur och/eller produkter från djur kan producera heterofila antikroppar i plasma och CSF. Heterofila antikroppar som då känner igen antikroppar från andra arter korsbinder antikropparna i metoden och kan därmed skapa falskt positiva resultat. I många forskningsfält så är man noga med att ta hänsyn till heterofila antikroppar, men inom forskningsfältet för Alzheimers sjukdom har flera studier påvisat A β utan att de tagit hänsyn till influens av heterofila antikroppar. I **Paper I** visade vi att de positiva signaler som erhöles i sandwich ELISA berodde på förekomst av heterofila antikroppar och inte av A β oligomerer och protofibriller i plasma och CSF. Förekomsten av lösliga A β aggregat kan fortfarande vara riktig, och har uppmäts, i både plasma och CSF, men eftersom nivåerna är väldigt låga krävs det känsliga och specifika metoder för att kunna mäta dessa korrekt.

Autoantikroppar mot A β har också varit under utredning som biomarkörer för Alzheimers sjukdom. Dessutom utvärderas de som behandling för Alzheimers sjukdom. I plasma kan det bildas komplex av A β och dess autoantikroppar. Detta leder till underestimering av A β -autoantikroppsnivåerna när mätningar görs med konventionella immunologiska metoder. Som en alternativ metod studerade vi i **Paper II** förekomst av A β -autoantikroppar med hjälp av den så kallade ELISpot-metoden. Med ELISpot lyckades vi räkna antalet B celler som producerar autoantikroppar mot A β i stället för att, som tidigare, direkt försöka mäta nivån av A β -autoantikroppar i blodet. Detta gav en mer sanningsenlig bild av nivåerna och det visade sig att autoantikropparna reagerar med A β -protofibriller och inte mot A β -monomerer. Dessutom fann vi, trots stort överlapp, att Alzheimerpatienter hade ett högre antal B celler som producerade autoantikroppar mot A β -protofibriller, jämfört med friska kontrollindivider. Vi fann ingen korrelation mellan antalet B celler som producerade autoantikroppar mot A β -protofibriller och minnessvårigheter hos Alzheimerpatienter. Detta talar emot A β -autoantikroppars roll som biomarkörer. Våra resultat visar att ett naturligt antikroppssvar var riktat mot de toxiska A β -protofibrillerna. Denna kunskap kan vara viktig för utvecklingen av nya behandlingar för Alzheimers sjukdom.

Immunsystemet har en viktig roll vid AD och runt amyloida plack i Alzheimer-hjärnan finner man ett stort antal immunceller i form av aktiverade microglia och reaktiva astrocyter. Man har sett att astrocyter kan ta upp A β men området är relativt utforskat och i avhandlingens två avslutande studier har vi fokuserat på astrocyters roll i Alzheimers sjukdom. I **Paper III** har vi

studerat huruvida astrocyter tar upp, bryter ner och utsöndrar de toxiska A β -protofibrillerna. Våra resultat pekar på att astrocyter snabbt tar upp A β -protofibriller men att deras förmåga att bryta ner A β inte är tillräckligt effektiv. Detta leder till att A β -aggregat, delvis nedrutna och toxiska, lagras i astrocyterna. Detta skapar fel i astrocytens nedbrytningskedja och utsöndring av blåsor, innehållandes A β , är giftiga för nervcellerna. I **Paper IV** visar vi att mAb158, en antikropp som är riktad mot A β protofibriller, kan förhindra ansamlingen av A β -protofibriller i astrocyter och även utsläpp av toxiska A β -former. Som en fortsättning på denna studie kommer vi att titta på olika mekanismer som kan vara inblandade i upptaget av A β och även vilka processer som kan leda till ökad nedbrytning av A β -protofibriller inuti astrocyterna.

Sammanfattningsvis har avhandlingen gett ökad kunskap om A β -protofibrillers och A β -antikroppars biologiska roll. Våra resultat kan bidra till utveckling av nya diagnostiska metoder och läkemedel för Alzheimers sjukdom och till en djupare förståelse patologin vid Alzheimers sjukdom.

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Am I finished?! Really!? Wohoo!! Finally! Well, before I start celebrating, I would like to take some time and thank everybody who has made this thesis possible.

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