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# A $\beta$ Conformation Dependent Antibodies and Alzheimer's Disease

DAG SEHLIN



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### **Abstract**

Sehlin, D. 2010. A $\beta$  Conformation Dependent Antibodies and Alzheimer's Disease. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 623. 69 pp. Uppsala. ISBN 978-91-554-7949-7.

Soluble intermediates of the amyloid- $\beta$  (A $\beta$ ) aggregation process are suggested to play a central role in the pathogenesis of Alzheimer's disease (AD) by causing synaptic dysfunction and neuronal loss. In this thesis, soluble A $\beta$  aggregates have been studied with a particular focus on the A $\beta$  protofibril, which has served as the antigen for developing conformation dependent monoclonal antibodies.

Antibodies generated from mice immunized with A $\beta$  protofibrils were characterized regarding A $\beta$  binding properties and the amino acid sequences of their antigen binding sites. A conformation dependent IgG antibody, mAb158, was further characterized and found to bind to A $\beta$  protofibrils with a 200-fold higher affinity than to monomeric A $\beta$  without affinity for soluble amyloid- $\beta$  precursor protein (A $\beta$ PP) or other amyloidogenic proteins. A sandwich enzyme-linked immunosorbent assay (ELISA) based on mAb158 was used to measure soluble A $\beta$  protofibrils in brain extracts from A $\beta$ PP-transgenic mice. Low levels of protofibrils could also be detected in human AD brain. However, positive signals generated from measurements in AD and control CSF samples were attributed to interference from heterophilic antibodies (HA), generating false positive signals by cross-binding the assay antibodies; consequently, a study on HA interference in A $\beta$  oligomer ELISAs was initiated. A large set of plasma and CSF samples from AD and non-AD subjects were analyzed with and without measures taken to block HA interference, revealing that virtually all signals above the assay limit of detection were false and generated by HA interference.

Many types of soluble A $\beta$  aggregates have been described and suggested to impair neuron and synapse function. To investigate the soluble A $\beta$  pool, synthetic A $\beta$  and brain extracts from A $\beta$ PP-transgenic mice and AD patients were ultracentrifuged on a density gradient to separate A $\beta$  by size under native conditions. Four distinct gradient fractions were defined based on the appearance of synthetic A $\beta$  in atomic force microscopy (AFM) and immunoreactivity in our protofibril specific sandwich ELISA. Interestingly, most A $\beta$  from AD patients and A $\beta$ PP-transgenic mice separated in the same fraction as toxic synthetic protofibrils.

*Keywords:* Alzheimer's disease, amyloid-beta, protofibrils, conformation, monoclonal antibody, ELISA

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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**, Hedlund M, Lord A, Englund H, Gellerfors P, Paulie S, Lannfelt L, Pettersson FE. Heavy-chain complementarity-determining regions determine conformation selectivity of anti-A $\beta$  antibodies. *Neurodegener Dis*, 2010. [Epub ahead of print]
- II **Sehlin D\***, Englund H\*, Johansson A-S, Nilsson L, Gellerfors P, Paulie S, Lannfelt L, Pettersson FE. Sensitive ELISA detection of amyloid- $\beta$  protofibrils in biological samples. *J Neurochem*, 2007; 103: 334-45
- III **Sehlin D\***, Söllvander S\*, Paulie S, Brundin RM, Ingelsson M, Lannfelt L, Pettersson FE, Englund H. Interference from heterophilic antibodies in A $\beta$  oligomer ELISAs. *J Alzheimers Dis*, 2010; 21: 1295-1301
- IV **Sehlin D**, Englund H, Simu B, Karlsson M, Ingelsson M, Nikolajeff F, Lannfelt L, Pettersson FE. Large toxic aggregates are the major soluble A $\beta$  species in AD brain separated with density gradient ultracentrifugation. *Manuscript*

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# Abbreviations

A $\beta$	Amyloid- $\beta$
AD	Alzheimer's disease
ADDLs	Amyloid- $\beta$ derived diffusible ligands
AFM	Atomic force microscopy
ApoE	Apolipoprotein E
A $\beta$ PP	Amyloid- $\beta$ precursor protein
Arc	Arctic mutation (A $\beta$ PP E693G, A $\beta$ E22G)
CDR	Complementarity determining region
CSF	Cerebrospinal fluid
DS	Down's syndrome
ELISA	Enzyme linked immuno-sorbent assay
FTD	Frontotemporal dementia
HA	Heterophilic antibodies
IAPP	Islet amyloid polypeptide
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LMW-A $\beta$	Low molecular weight A $\beta$
LTP	Long term potentiation
LOD	Limit of detection
mAb	Monoclonal antibody
MCI	Mild cognitive impairment
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFT	Neurofibrillary tangle
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
Swe	Swedish mutation (A $\beta$ PP K670N/M671L)
TEM	Transmission electron microscopy
tg	Transgenic
TTR	Transthyretin
wt	Wild type



# Introduction

## Alzheimer's disease

Alzheimer's disease (AD) was first described in 1906 by the German physician Alois Alzheimer [1], although its symptoms had been known since the ancient Greeks. One of Dr. Alzheimer's patients, Auguste D, died at the age of 55, after years of suffering from severe memory problems and confusion. When Alzheimer performed a brain autopsy he discovered dense deposits around neurons and bundles of twisted fibers inside them. These pathological structures, today termed amyloid plaques and neurofibrillary tangles (NFTs), still constitute the two major hallmarks of AD and the basis for a definite diagnosis. For a long time it was generally accepted that AD was a rare condition affecting people below 65 years of age, but in the late 1960s it was discovered that elderly suffering from "senile dementia" had in fact the same pathology and a relationship between age, cognitive decline and AD was established [2-4]. Still, not until the 1980s was it understood that AD is a disease affecting a significant portion of elderly people and the disease started to become acknowledged for its serious impact on a society with a gradually aging population. Today, 35 million people are estimated to suffer from AD, a figure expected to double within the next 20 years, and the current yearly cost for care of these patients exceeds the annual revenue of the world's two largest companies [5, 6].

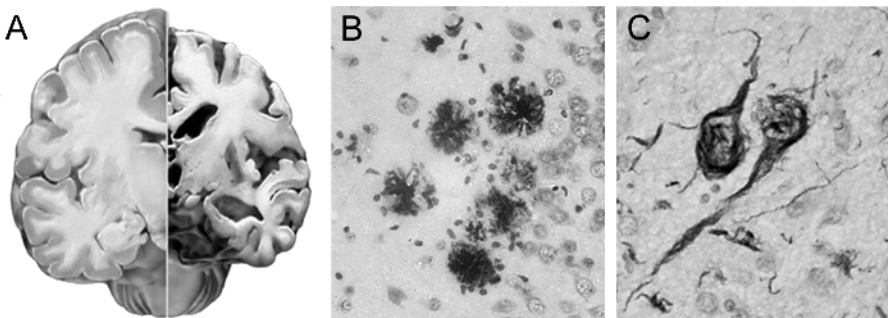
AD is one in a group of neurodegenerative diseases where proteins aggregate and form insoluble intra- or extracellular amyloid deposits in the brain. Amyloid, which is spontaneously formed *in vivo* by more than 25 different proteins [7], is defined by birefringent Congo red staining, an ultrastructure of long, unbranched fibrils with a 10 nm diameter and a characteristic cross- $\beta$  sheet X-ray diffraction pattern [8]. As reviewed by Ross [9], recent research has established that many neurodegenerative diseases, often affecting different regions of the nervous system with distinct clinical manifestations, share common cellular and molecular pathogenic mechanisms, driven by misfolding and aggregation of proteins. While amyloid- $\beta$  (A $\beta$ ) and tau are the proteins associated with aggregation and neurotoxicity in AD, tau pathology alone is seen as NFTs and Pick bodies in frontotemporal lobar degeneration (FTLD),  $\alpha$ -synuclein forms Lewy bodies in Parkinson's disease (PD) and dementia with Lewy bodies (DLB), intracellular inclusions of the protein huntingtin are seen in Huntington's

disease and aggregated superoxide dismutase-1 (SOD-1) is involved in the pathology of amyotrophic lateral sclerosis (ALS). The prion protein (PrP) is the key protein in a group of amyloid diseases including Creutzfeldt-Jakob disease (CJD) and kuru. Diseases caused by the prion protein are so far the only amyloid diseases proven to be transmissible in humans, although this mechanism has been suggested in other disorders as well [10].

## Clinical picture and neuropathology

AD generally presents with a gradual loss of short-term memory, often in combination with difficulties in remembering words. The initial symptoms are usually misattributed to normal ageing and dementia is not suspected until the problems start to interfere with social or professional activities. A progressive decline in cognitive functions, such as problems with memory acquisition and retention, disorientation and impaired language skills are often accompanied by emotional changes such as depression, anxiety and apathy [11]. The gradual neurodegeneration of the AD brain eventually causes a complete inability of the AD patient to lead an independent life and death usually occurs 5-15 years after disease onset, often from secondary infections, such as pneumonia or urinary tract infections.

On the macroscopic level, AD neuropathology is characterized by a reduced brain volume with enlarged sulci and ventricles, caused by massive atrophy, most evident in the temporal and parietal cortex and in the hippocampus (Figure 1A). Microscopically, the two histopathological hallmarks of AD, amyloid plaques and NFTs, appear as dense structures in the brain parenchyma (Figure 1B and C).



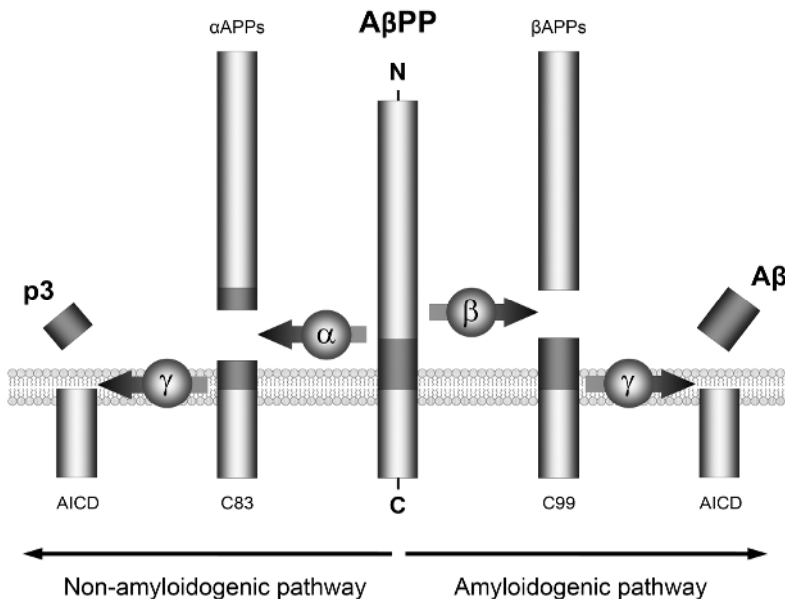
*Figure 1.* A normal brain hemisphere (left) is compared to an AD brain hemisphere (right) with enlarged ventricles and massive atrophy in the hippocampus and the temporal lobe (A) © 2007 Stacey Janis, Alzheimer's Association. All rights reserved. The two neuropathological hallmarks of AD are extracellular amyloid plaques (B) and intracellular neurofibrillary tangles (C) Pictures kindly provided by Paul O'Callaghan.

Plaques are extracellular deposits, primarily composed of fibrils of the A $\beta$  peptide, but they also contain other components such as apolipoprotein E

(ApoE) [12], proteoglycans [13] and  $\alpha$ -synuclein [14]. Plaques are divided in two categories; neuritic plaques have a dense congophilic amyloid core and are surrounded by dystrophic neurites, activated microglia and reactive astrocytes, whereas diffuse plaques lack the amyloid core and the surrounding glial cells [15]. NFTs are deposits of the tau protein [16]. Tau is a microtubule-associated protein mainly concentrated to the axons of the neurons [17], where it stabilizes the microtubules and is involved in axonal transport [18]. In AD, tau becomes hyperphosphorylated, leading to release from microtubules, resulting in aggregation and deposition in neurons [19].

## A $\beta$ PP processing and A $\beta$

In 1984, A $\beta$  was purified and sequenced from amyloid in AD brain vessels [20]. It was later established that this protein is also the main component of amyloid plaques [21] and originates from the cell-surface receptor-like amyloid- $\beta$  precursor protein (A $\beta$ PP) [22]. A $\beta$ PP is a transmembrane protein, which through alternative splicing occurs in three main isoforms, consisting of 770, 751 or 695 amino acid residues, the shortest one being the predominant variant in neurons [23]. The physiological role of A $\beta$ PP is not fully understood but it has been suggested to function as a receptor [24] and to be involved in neurite outgrowth [25], neuronal migration and brain development [26].



*Figure 2.* Processing of A $\beta$ PP. The non-amyloidogenic pathway, with  $\alpha$ - and  $\gamma$ -secretase cleavage, generates the p3 peptide, whereas the amyloidogenic pathway, with  $\beta$ - and  $\gamma$ -secretase cleavage, generates the A $\beta$  peptide.

A $\beta$ PP is proteolytically cleaved through two distinct pathways – the non-amyloidogenic and the amyloidogenic pathway (Figure 2) (reviewed in [27]). Non-amyloidogenic cleavage is initiated by  $\alpha$ -secretase, cleaving A $\beta$ PP in the middle of the A $\beta$  sequence, generating a soluble A $\beta$ PP fragment called  $\alpha$ APPs and the C-terminal fragment C83, anchored in the membrane. The non-amyloidogenic peptide p3 is then cleaved out from C83 by  $\gamma$ -secretase, a multiprotein complex composed of presenilin (PS) 1 or 2, nicastrin, presenilin enhancer 2 (PEN-2) and anterior pharynx defective 1 (Aph-1) [28]. If A $\beta$ PP is instead cleaved by  $\beta$ -secretase, also known as beta-site A $\beta$ PP cleaving enzyme 1 (BACE1), the soluble A $\beta$ PP fragment  $\beta$ APPs is formed along with the C-terminal fragment C99, from which A $\beta$  is generated by a subsequent  $\gamma$ -secretase cleavage. This cleavage takes place within the cell membrane and determines the C-terminal amino acid residues of the peptide, with A $\beta$ 38, A $\beta$ 40 and A $\beta$ 42 as the most common forms [29, 30]. A $\beta$  is produced during normal metabolism [31, 32] and has a rapid turnover with a half-life estimated as two hours in a transgenic mouse model [33]. A $\beta$  is normally cleared by the proteases neprilysin [34] and insulin degrading enzyme [35]. The physiological role of A $\beta$  is unclear, but the A $\beta$  concentration in the interstitial fluid has been reported to increase with neuronal activity after brain injury [36] and to vary with the sleep-wake cycle [37]. In addition, picomolar concentrations of A $\beta$  have been shown to actually stimulate synaptic plasticity [38], in opposition to its detrimental effects at higher concentrations, suggesting a role in regulation of neuronal activity. In AD, A $\beta$  metabolism is somehow disturbed and the usual levels of specific A $\beta$  peptides in e.g. CSF is altered. A significant drop in levels of A $\beta$ 42 [39], combined with a relative increase in A $\beta$ 38 and A $\beta$ 40 levels [30] are typical in CSF from an AD patient.

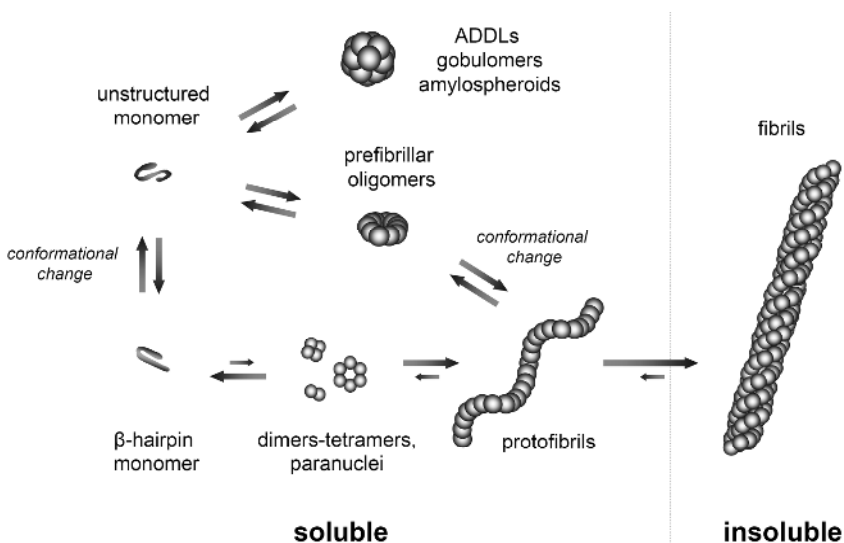
## A $\beta$ aggregation and neurotoxicity

A $\beta$  is produced throughout the body, but the formation of extracellular A $\beta$  deposits occurs mainly in the brain. What actually triggers the aggregation and deposition of A $\beta$  is largely unknown, but probably there is an imbalance between clearance and production of the peptide. Though a pathological hallmark of AD, the burden of amyloid plaques in AD brain does not correlate with degree of dementia [40]. Instead, studies have shown that the level of soluble A $\beta$  in the brain, preceding fibril formation and deposition, is a better correlate of disease progression [41-43] and synaptic loss [44]. Based on this insight, considerable effort has been invested in studies of A $\beta$  aggregation and different soluble molecular intermediates in this process. Furthermore, the former assumption that the extracellular A $\beta$  deposits are a result of extracellular aggregation has recently been questioned, with reports of intraneuronal accumulation of A $\beta$  aggregates in both transgenic mouse brain [45-47] and AD brain [48, 49]. Another question is whether A $\beta$

aggregation is dependent on interactions with other molecules, as suggested by the presence of multiple components in the amyloid plaques. For example, proteoglycans have been reported to enhance A $\beta$  fibrillization and stabilize A $\beta$  fibrils once formed [50].

As schematically described in figure 3, the A $\beta$  peptide is suggested to aggregate through the fibrillar pathway, ending up as amyloid fibrils, or off-pathway, resulting in soluble end-stage oligomers [51, 52]. A $\beta$  is a natively unstructured protein and its inherent propensity to self-assemble is driven by the mid-region KLVFF motif [53] and by the hydrophobic C-terminus, where the longer peptide variants are more prone to aggregate [54]. Based on the properties of these regions, the A $\beta$  peptide can adopt a  $\beta$ -hairpin structure, where the C-terminus is folded over the mid-region and stabilized by hydrogen bonds and salt bridges [55, 56]. The peptide can then aggregate into small species such as dimers [57] or paranuclei [58], serving as building blocks for protofibrils [59, 60], which elongate into filaments that eventually become insoluble amyloid fibrils [61]. This is a nucleation-dependent process with three main characteristics [62]:

1. A lag phase, where a nucleus is formed, from which the fibrils can grow
2. A critical concentration, below which no aggregation will occur
3. The ability to be seeded by the addition of a nucleus



*Figure 3.* Schematic picture of A $\beta$  aggregation. In the fibrillar pathway (low), the monomer goes through a conformational change into a  $\beta$ -hairpin structure, forming paranuclei during the lag phase. In the elongation phase, paranuclei assemble into soluble protofibrils, which eventually become insoluble fibrils. The unstructured monomer can also aggregate off-pathway, forming end-stage oligomers, often with a globular shape, that do not aggregate further (top). Prefibrillar oligomers (middle) may undergo a conformational change and join the fibrillar pathway.

In off-pathway aggregation, the unstructured A $\beta$  monomer forms soluble oligomers, which are structurally different from the A $\beta$  species along the fibrillar pathway, and do not aggregate further [63-65] or do so only after a conformational change [66]. Several reports, mostly from *in vitro* experiments, have described different A $\beta$  species from both pathways (Table 1), some of which may well be similar. Many of these soluble A $\beta$  aggregates have been detected in transgenic mouse or AD brain extracts or in CSF from AD patients. This detection is often dependent on antibodies selective for a certain A $\beta$  conformation and indeed, this reveals something about the structure of the aggregates. However, it is important to point out that it is difficult to say to what extent the *in vitro* aggregation of A $\beta$  resembles the *in vivo* reality, where both time scale and molecular environment differ greatly from the test tube.

Table 1. *Properties of soluble A $\beta$  aggregates*

Name	Size	Properties	Ref
Dimers-tetramers	8-20 kDa	Isolated from cell media, AD brain and CSF, SDS stable, inhibit LTP, induce synapse loss	[67-75]
Paranuclei	20-30 kDa, 5-6mer, ~5 nm	Generated from A $\beta$ 42 <i>in vitro</i> by photochemical cross-linking, neurotoxic	[58, 76]
ADDLs	>40 kDa 10-20mer	Generated <i>in vitro</i> , do not form fibrils, detected in AD brain and CSF, inhibit LTP	[65, 77-81]
A $\beta$ oligomers	>40 kDa 10-20mer	Generated <i>in vitro</i> , detected in AD brain, A11-positive, neurotoxic through membrane permeabilization	[52, 82-84]
A $\beta$ *56	56 kDa 12mer	Isolated from tg mice, correlate with memory deficits in tg mice and rats	[85-87]
Globulomers	~60 kDa 12mer	Generated <i>in vitro</i> , do not form fibrils, detected in AD brain, inhibit LTP	[64, 88-90]
Amylospheroids (ASPDs)	>100 kDa Ø 10-15 nm	Generated <i>in vitro</i> , do not form fibrils, isolated from AD and DLB brain, neurotoxic via presynaptic target	[63, 91]
Protofibrils	>70 kDa width 6-8 nm	Generated <i>in vitro</i> , detected in tg mouse brain and in AD brain and CSF, neurotoxic, inhibit LTP	[52, 57, 59, 60, 92-100]
Annular protofibrils	150-250 kDa, Ø 7-10 nm	Generated <i>in vitro</i> , neurotoxic through ion-channel like function	[101-103]

Most of the soluble A $\beta$  aggregates presented in table 1 have been shown to mediate neurotoxicity, which is usually measured as their capacity to reduce the viability of neurons or neuron-like cells in culture. There are several theories about the mechanisms behind A $\beta$  neurotoxicity e.g. oxidative stress, mitochondrial dysfunction, interference with membrane permeability, formation of ion-channel like pores, disturbed ion homeostasis, induction of apoptosis or the nucleation dependent polymerization itself [83, 94, 104-110]. It has been proposed that different A $\beta$  structures have different mechanisms of action [84, 111] and that several mechanisms may operate

simultaneously. Apart from the direct neurotoxicity, soluble A $\beta$  aggregates impair synaptic activity and plasticity *in vivo* [69, 112] and *in vitro* [65, 79, 88, 113], often assessed by their ability to inhibit long term potentiation (LTP), a synaptic process linked to memory formation. Synapse loss in AD correlates with degree of dementia [40] as well as with levels of soluble A $\beta$  [44], and the mechanism behind it has been suggested to be linked to a reduced number of surface AMPA and NMDA receptors [73, 114].

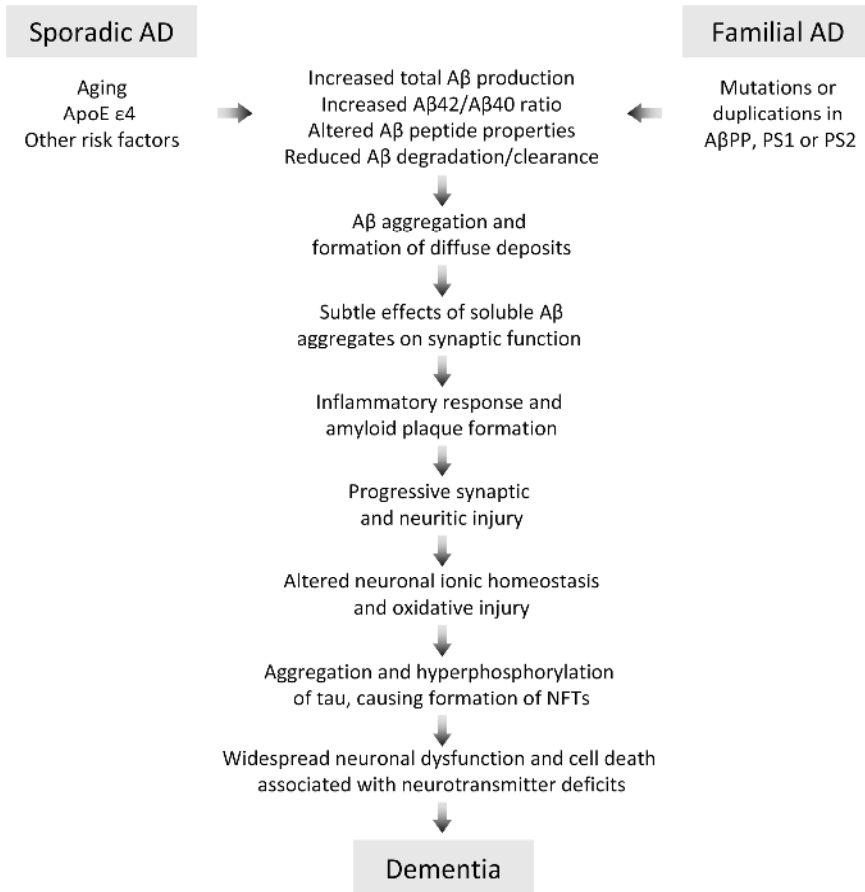
## Genetics and risk factors

AD has probably many causes and both environmental factors and genetic predisposition are important, but the main risk factor for AD is age, with an exponential increase in prevalence from 1.5% at the age of 65 to 25% over the age of 85 [115]. There is often a familial history in AD and first-degree relatives of an AD patient run a greater risk of getting the disease. Twin studies have shown that the heritability of AD is as high as 79% [116], but the only well established genetic risk factor for late onset AD, with an age of onset after 65, is that of the apolipoprotein E (APOE) gene. Carriers of the  $\epsilon$ 4 allele of APOE run a higher risk of getting the disease [117], whereas the  $\epsilon$ 2 allele is protective [118]. The ApoE protein is involved in cholesterol homeostasis [119] and can be found in amyloid plaques in AD brain.

Though rare, there are a number of known mutations affecting A $\beta$ , either through altered A $\beta$ PP processing or through modulation of the A $\beta$  peptide, causing early onset Alzheimer's disease with a dominant inheritance pattern. Furthermore, people with Down's syndrome (DS) have an extra copy of chromosome 21, on which A $\beta$ PP is located, and show Alzheimer-like symptoms and pathology around 45 years of age, with plaques apparent in their thirties, due to elevated levels of A $\beta$ PP and thus A $\beta$  [120]. In addition, families with A $\beta$ PP locus duplications, leading to early onset AD by the same mechanism, have been reported [121, 122].

In 1991 came the first report of a mutation in A $\beta$ PP causing early onset familial AD – the London mutation [123], but the mechanism behind it was not known. The year after, patients from a Swedish family with hereditary, early onset AD were reported to have a double mutation at position 670 and 671 in the A $\beta$ PP amino acid sequence, where a lysine and an asparagine residue were substituted for a methionine and a leucine, respectively [124]. For the first time it was shown that an A $\beta$ PP mutation alters cleavage of A $\beta$ PP, leading to an increased A $\beta$  production, which speeds up the disease process and causes early onset AD [125, 126]. While the Swedish mutation increases overall production of A $\beta$  by increasing  $\beta$ -secretase cleavage, the London mutation alters  $\gamma$ -secretase cleavage, increasing the production of the more aggregation prone A $\beta$ 1-42. In 2001, another A $\beta$ PP mutation was found in a Swedish family with hereditary AD. This mutation, termed the Arctic mutation, is located at position 22 within the A $\beta$  amino acid sequence, where

a glutamic acid is substituted for a glycine [127]. This substitution changes the conformation or flexibility of the peptide, resulting in a faster rate of aggregation and a stabilization of A $\beta$  protofibrils. In addition, mutations in the presenilins, which are one of the four components of the  $\gamma$ -secretase complex, shift the A $\beta$ 42/A $\beta$ 40 ratio towards increased production of the more hydrophobic and aggregation prone A $\beta$ 42, [128].



*Figure 4.* The amyloid cascade hypothesis suggests that AD is caused by increased A $\beta$  production, an increased A $\beta$ 42/A $\beta$ 40 ratio, altered properties of the A $\beta$  peptide or decreased A $\beta$  clearance, leading to aggregation of A $\beta$ , causing synaptic and neuritic injury, amyloid deposition, altered ionic homeostasis and oxidative injury followed by formation of NFTs and eventually leading to neurodegeneration and dementia [129].

## The amyloid cascade hypothesis

In 1991, Hardy and Higgins formulated the *amyloid cascade hypothesis*, based on the observations that amyloid plaques are formed by aggregation of

the A $\beta$  peptide, a process that is enhanced by mutations in the A $\beta$ PP or presenilin genes or by an increased gene dose of A $\beta$ PP, as in DS or A $\beta$ PP duplications [130]. This hypothesis stated that A $\beta$  fibrillization and deposition as plaques is the key event in AD and that tau pathology, neurodegeneration and inflammation are direct consequences of that. However, the poor correlation between plaque load and cognitive decline in combination with increased knowledge about the A $\beta$  aggregation process and its neurotoxic intermediates led to a refined hypothesis, where focus was shifted towards soluble, oligomeric and prefibrillar A $\beta$  [129, 131, 132] (Figure 4). The order of events in the amyloid cascade hypothesis suggests that A $\beta$  aggregation and its effects on synapses and neurons precedes tau aggregation and deposition as NFTs and many studies support this theory. Mutations resulting in increased formation, aggregation and deposition of A $\beta$  cause early onset AD, while mutations in the tau gene result in FTL D without A $\beta$  pathology [133]. Moreover, enhanced NFT formation in transgenic mice over-expressing both A $\beta$ PP and tau has been suggested to be influenced by A $\beta$  [134]. In addition, A $\beta$  immunotherapy in such transgenic mice has been reported to reduce A $\beta$  pathology, leading to clearance of tau pathology. The A $\beta$  pathology then reappeared before tau pathology when therapy was terminated, suggesting not only a link between A $\beta$  and tau, but also that NFT formation is a downstream event in the cascade [135].

## Diagnosis and therapy

The current diagnosis of AD is generally based on the medical history of the patient in combination with a set of neuropsychiatric tests for evaluation of the patient's cognitive functions. The mini-mental state examination (MMSE) is the most common test, evaluating the patients' memory, orientation, language, attention and calculation. In clinics with the required technical resources, a more thorough investigation can be made, involving assessment of CSF A $\beta$  and tau levels as well as brain imaging with computer tomography (CT) or magnetic resonance imaging (MRI). These techniques are used to visualize the atrophy in different brain regions. In rare cases, positron emission tomography (PET) is used to measure the brain activity or A $\beta$  plaque load in affected brain areas. However, the definite AD diagnosis is made by a *post mortem* analysis of A $\beta$  plaque load and amount of NFTs in the brain [136]. With the improvement of imaging techniques and biomarker assays that are currently under development, a more accurate diagnosis with a better biochemical support of the clinical assessment will hopefully soon be a reality.

Today AD patients can be given treatment which will, in some cases, help to maintain their cognitive functions for a limited period of time. A number of acetylcholine esterase inhibitors, maintaining the levels of the neuro-

transmitter acetylcholine in the brain, are currently used for treatment of patients with mild to moderate AD and beneficial effects are observed for some patients [137]. Antagonists of the N-methyl D-aspartate (NMDA) receptor are believed to reduce glutamate induced neurotoxicity and have been shown to give brief beneficial effects on patients in a more advanced stage of AD [138]. However, there is still no curative therapy for AD, although a number of different strategies are currently being tested [139]. Many of them are aimed at altering the aggregation or metabolism of A $\beta$ , by limiting its production with  $\beta$ - or  $\gamma$ -secretase inhibitors or by increasing its clearance, for example with immunotherapy. Other approaches are directed towards the tau pathology or involve anti-inflammatory drugs, which have been reported to reduce the risk of AD [140].

### A $\beta$ as a biomarker

A number of studies have demonstrated a correlation between AD and decreased A $\beta$ 42 levels [39, 141, 142] or a decreased A $\beta$ 42/A $\beta$ 40 ratio [143-145] in CSF. Despite the considerable overlap between AD and controls, low A $\beta$ 42 levels can be used as a support for a clinical diagnosis of AD and to distinguish the disease from other neurological disorders with similar symptoms. In addition, if combined with high levels of tau and phospho-tau, low CSF levels of A $\beta$ 42 indicate the likely progression from MCI to AD [146]. The low CSF levels of A $\beta$ 42 associated with AD have been explained by absorption of circulating A $\beta$ 42 into plaques [39] or by altered A $\beta$  metabolism in the brain [147]. Low A $\beta$ 42 levels could also be the result of soluble A $\beta$ 42 aggregates present in the CSF of AD patients that are incompletely measured because of their hidden C-termini [148, 149]. The latter theory is supported by several reports of elevated levels of soluble A $\beta$  aggregates detected in CSF from AD patients [77, 99, 150, 151]. However, most of these studies were conducted with technically complicated methods and the results have thus far not been reproduced [77, 150, 151]. Therefore, there is still a need for robust and simple methods with the ability to deliver consistent measurements of soluble A $\beta$  aggregates.

In 2006, El-Agnaf et al. reported elevated levels of  $\alpha$ -synuclein oligomers in plasma from patients with Parkinson's disease (PD) [152] and a few years later, Xia et al. found elevated levels of A $\beta$  oligomers in AD plasma samples [153]. These observations were made with similar ELISA assays, utilizing one antibody for both capture and detection, as previously described [97, 154-158]. However, results presented in this thesis suggest that these results may largely have been based on false positive signals caused by interference from heterophilic antibodies (HA) [159]. In a study from 2010 by Fukumoto et al. with a similar approach, HA interference was addressed through the use of antibody fragments instead of whole antibody molecules, possibly solving that problem [99]. This study showed significantly higher CSF levels

of high molecular weight A $\beta$  aggregates in AD patients compared to healthy controls. Importantly, the levels of A $\beta$  aggregates also correlated to the patients' degree of dementia, as measured by their MMSE score. However, the levels of A $\beta$  aggregates in this study were very low, confirming the need for an extremely sensitive assay in order to perform reliable measurements of A $\beta$  aggregates in CSF. In addition, these results need to be confirmed and further studies are required to establish how the level of A $\beta$  aggregates reflect the disease onset and progression.

## A $\beta$ immunotherapy

The idea behind A $\beta$  immunotherapy is to take advantage of the body's immune system to remove the pathogenic A $\beta$  molecules from the brain and thus eliminate the cause of the disease. The first A $\beta$  immunotherapy experiments were performed by Schenk et al. in 1999. Transgenic mice with AD-like pathology were vaccinated with A $\beta$ 42 fibrils, resulting in a marked reduction of A $\beta$  pathology [160]. This study was followed by studies showing that A $\beta$  vaccination, besides the beneficial effects on A $\beta$  pathology, could also prevent A $\beta$  induced memory deficits in transgenic mice [161, 162]. Encouraged by these promising results, Elan Pharmaceuticals initiated a clinical vaccination trial where AD patients were vaccinated with A $\beta$  fibrils. A positive immune response was observed in 53% of the subjects [163] and there were reports of plaque clearance and a slowed disease progression [164-167]. However, phase II trials were halted due to side effects caused by T-cell mediated, inflammatory induced meningoencephalitis in 6% of the patients [168-170].

After this drawback, the idea of using passive vaccination against AD has been promoted, for several reasons. A potentially harmful, T-cell mediated immune response, as discussed above, could be avoided with this approach. In addition, the immune system in elderly people is weakened and active vaccination may not induce an effective humoral immune response to the antigen. In contrast, passive administration of antibodies allows the determination of an exact antibody dose and the target can be determined based on the binding properties of the antibody, allowing for example the exclusion of binding to A $\beta$ PP or A $\beta$  monomers. Moreover, if required, the treatment can be halted without persisting long-term effects.

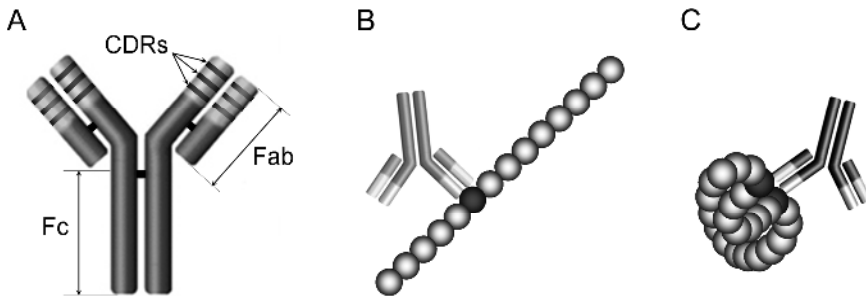
Treatment of transgenic mice with antibodies binding to the A $\beta$  N-terminus [171], mid-region [156, 172, 173] or C-terminus [174, 175], as well as with conformation dependent antibodies [89, 176, 177] have been successful and reported to reduce A $\beta$  pathology and reverse cognitive deficits. Several clinical trials involving passive immunization against A $\beta$  are ongoing [178]. Two different monoclonal antibodies, one directed to the A $\beta$  N-terminus and one to the mid-region, as well as pooled IgG antibodies from healthy blood donors, called IVIg, are currently in phase III clinical

trials. So far, however, only modest improvements on cognition have been recorded, possibly because the antibody treatment is given late in the disease process.

The mechanism behind the antibody mediated A $\beta$  clearance is still not fully understood. However, four main hypotheses, not necessarily excluding each other, are discussed by Citron [139]. One theory suggests a direct resolution of A $\beta$  aggregates by antibodies. A second is that antibodies bound to plaques attract microglial cells, which clear the deposits by phagocytosis. The third hypothesis is a peripheral sink mechanism, where antibodies binding A $\beta$  in the periphery alter the equilibrium of antibody-bound vs -unbound A $\beta$ , which eventually clears A $\beta$  from the brain. The fourth hypothesis is that once bound to the therapeutic antibodies, the toxic effect of A $\beta$  oligomers is neutralized.

### Conformation dependent antibodies

As discussed above, soluble A $\beta$  aggregates are a possible biomarker for early diagnosis of AD and a promising target for AD immunotherapy. A strategy to directly target the pathogenic A $\beta$  species is to develop antibodies binding selectively to A $\beta$  aggregates in a conformation dependent manner.



*Figure 5.* The antibody structure; the assembled pairs of heavy and light chains give rise to the Fc and Fab regions. The hypervariable CDRs are marked with arrows (A). A linear epitope is illustrated as a dark region on a linear sequence (B), whereas a conformation dependent epitope is made up of two dark regions forming a neoepitope in the folded protein (C).

Antibodies, or immunoglobulins (Ig), are part of the humoral immune system and are produced and secreted by B-cells as five different isotypes, the most common of which is IgG. Antibodies are designed to specifically bind to foreign antigens and, assisted by other components of the immune system, eliminate them. Upon maturation of the B-cell, three antibody gene segments, called the V, D and J segment, are rearranged to determine the structure and specificity of the antibody and ensure the large variety of antibodies present in the immune system. The antibody consists of two

heavy and two light chains, linked together with disulfide bonds, forming the two major functional parts of the antibody – the Fab (fragment, antigen binding) region and the Fc (fragment, crystallizable) region (Figure 5A). The Fab region has a constant and a variable domain, the latter incorporating three hypervariable complementarity-determining regions (CDRs), which make up the three dimensional structure of the antigen binding site.

Many of the antigens recognized by antibodies are proteins and the part of the antigen recognized by the antibody is called an epitope. An epitope can be linear, composed of 5-10 consecutive amino acid residues (Figure 5B). It can also be conformation dependent and then composed of amino acid residues that may originate from different regions of the amino acid sequence. These will then come in proximity upon protein folding or conformational change (Figure 5C). Antibodies used in the laboratory can be monoclonal (mAb), i.e. produced by one single clone of B-cells and hence identical, or polyclonal (pAb), as in the body, where many different B-cell clones produce different antibodies to the same antigen.

In the field of amyloid research, polyclonal conformation dependent antibodies were raised against the amyloidogenic protein CAR as early as 1973 by Linke et al. [179]. After reports of conformation specific monoclonal antibodies against the prion protein (PrP) [180] and transthyretin (TTR) [181], the first monoclonal antibodies selectively binding to A $\beta$  fibrils were described in 2002 [182].

Table 2. *Conformation dependent A $\beta$  antibodies*

<b>name</b>	<b>type</b>	<b>antigen</b>	<b>generic amyloid</b>	<b>ref.</b>
M93, M94	polyclonal	ADDLs	no	[78]
WO1, WO2	monoclonal	fibrils	yes	[182]
A11	polyclonal	pre-fibrillar oligomers	yes	[82]
M71	polyclonal	ADDLs	no	[81]
R262, R286	polyclonal	A $\beta$ fibrils	no	[183]
8F5	monoclonal	A $\beta$ <sub>1-42</sub> globulomers	no	[88]
NAB61	monoclonal	soluble A $\beta$ aggregates and fibrils	no	[176]
mAb158	monoclonal	A $\beta$ protofibrils and fibrils	no	[100]
NU1-NU7	monoclonal	ADDLs (and fibrils)	no	[184]
PFA1, PFA2	monoclonal	A $\beta$ protofibrils and fibrils	no	[185]
OC	polyclonal	fibrillar oligomers and fibrils	yes	[186]
7A1, 1G5	monoclonal	A $\beta$ <sub>1-42</sub> oligomers	no	[187]
rpASD1	polyclonal	amylospheroids	no	[91]
mASD3	monoclonal	amylospheroids	no	[91]
A-887755	monoclonal	A $\beta$ <sub>20-42</sub> globulomers	no	[89]

During the past decade, several different A $\beta$  conformation dependent antibodies, both polyclonal and monoclonal, have been developed and

described (Table 2). While most of these antibodies are A $\beta$  specific, binding to A $\beta$  oligomers or fibrils, some of them have been suggested to bind a generic amyloid epitope [82, 182, 186], possibly targeting the amyloid specific cross- $\beta$  structure [188]. A $\beta$  conformation dependent antibodies have also been isolated from AD patients [189] and reported to occur among IgG antibodies isolated from human serum [190] and plasma [191]. Furthermore, various forms of A $\beta$  conformation dependent antibody fragments have been generated by phage display [192-194].

Some of the antibodies in table 2 have been used for specific detection of soluble A $\beta$  aggregates in tissue from transgenic mice [100, 195, 196] and AD patients [77, 81, 82, 89, 91]. In addition, a few of these antibodies have been used for passive immunization of transgenic mice, with promising results, such as normalization of synaptic deficits [89] and positive effects on learning and memory [176, 177]. However, it remains to be proven what can be accomplished in AD patients, passively immunized with A $\beta$  conformation dependent antibodies. This question is currently being addressed in ongoing clinical trials involving humanized conformation dependent antibodies.

# Present investigations

## Aim of the study

The general aim of this study was to generate and characterize monoclonal antibodies with high affinity and specificity for the pathogenic A $\beta$  protofibril, for use in immunoassays.

## Specific aims

- I To characterize the immune response to A $\beta$  protofibrils in mice and develop monoclonal antibodies selectively binding to A $\beta$  protofibrils.
- II To characterize A $\beta$  conformation selective antibodies and develop and validate an A $\beta$  protofibril specific immunoassay for quantification of biologically derived soluble A $\beta$  aggregates.
- III To investigate the influence of naturally occurring human heterophilic antibodies in quantitative immunoassays of soluble A $\beta$  aggregates.
- IV To use density gradient ultracentrifugation for separation and characterization of soluble A $\beta$  species in brain extracts from AD patients and A $\beta$ PP-transgenic mice.

## Results and discussion

As described in the introduction of this thesis, AD is characterized by aggregation of the A $\beta$  peptide via soluble intermediates into large insoluble fibrils, which deposit as amyloid plaques in the AD brain. The research of the past decade has implicated the intermediates in this process as the main pathogen in AD. Based on this premise emerged the idea to generate conformation dependent antibodies, providing the ability to directly target these soluble A $\beta$  aggregates. Such antibodies can be used for various research purposes as well as for AD immunotherapy and diagnostic immunoassays. Human antibodies binding to A $\beta$  fibrils were first reported already in 1993 [189] and in 2002, the first A $\beta$  fibril selective monoclonal antibodies were developed [182], followed by a few reports of polyclonal antibodies recognizing soluble A $\beta$  oligomers. However, it became evident that developing monoclonal antibodies against soluble A $\beta$  aggregates is a difficult task.

### Generation of conformation dependent antibodies

To generate monoclonal antibodies selectively binding to A $\beta$  protofibrils, mice were immunized with protofibrils from synthetic A $\beta$ 1-42, both with and without the Arctic mutation [95]. Two different strains of mice were immunized with the antigen: Balb/c mice, widely used for mAb production, and C57BL/6J mice with a knocked out A $\beta$ PP gene, the idea being that the A $\beta$ PP-KO mice, lacking endogenous expression of A $\beta$ , would produce a stronger immune response to A $\beta$ , as has been shown for the prion protein [197]. Immunized mice were sacrificed to generate antibody producing hybridomas, which were screened for A $\beta$  reactivity with indirect ELISA. Of the 2921 screened hybridomas, 172 produced A $\beta$  positive antibodies but only ten of them had intermediate to high affinity for A $\beta$  protofibrils. These ten antibodies were further characterized with inhibition ELISA (Figure 1, **paper I**), where the IC<sub>50</sub> (antigen concentration required to inhibit half the ELISA signal) was used to estimate the antibody binding affinity [198] for monomeric and protofibrillar A $\beta$ . As displayed in table 3, antibodies were then divided into groups based on isotype, A $\beta$  protofibril affinity and whether they had preferential binding to A $\beta$  protofibrils over monomeric A $\beta$  (i.e. whether the epitope was conformational or linear) as well terminal specificity (N- or C-terminal).

The A $\beta$ PP-KO mice, as expected, generated a higher proportion of A $\beta$  positive hybridomas compared to the Balb/c mice, but all produced IgM antibodies with low affinity to A $\beta$ . Consequently, despite Balb/c mice producing less than a fifth of the A $\beta$  positive clones, all high and intermediate affinity antibodies came from clones derived from these mice.

This could possibly be explained by the observation that A $\beta$ PP may play a role in the immune system [199].

Table 3. Monoclonal antibodies generated from A $\beta$  protofibril immunization

Clone name	Isotype	A $\beta$ protofibril affinity	Epitope
mAb1C3	IgG	High (IC <sub>50</sub> $\sim 10^{-9}$ M)	Linear, N-terminal
mAb158	IgG	High (IC <sub>50</sub> $\sim 10^{-9}$ M)	Conformational, N-terminal
mAb146	IgM	High (IC <sub>50</sub> $\sim 10^{-8}$ M)	Conformational, N-terminal
mAb235	IgM	Intermediate (IC <sub>50</sub> $\sim 10^{-6}$ M)	Conformational, N/C-terminal

Interesting to note is that only two of the 172 A $\beta$  positive hybridoma clones produced antibodies of IgG isotype, while all the others produced IgM antibodies. The T-cell epitopes of A $\beta$ , predicted to be located in the middle and the C-terminal regions [200], are hidden in the core of A $\beta$  aggregates such as protofibrils and fibrils [201] and may therefore be less accessible for presentation on the surface of antigen-presenting cells – a requirement for specific T-cell stimulation. A $\beta$  devoid of T-cell epitopes induces an IgM response in mice [202] and a strong IgM response to A $\beta$  aggregates has been observed in humans [164, 189] as well as in mice [182], indicating that A $\beta$  aggregates have a reduced ability to generate a specific T-cell response, which is required to induce production of IgG antibodies. Furthermore, other antigens of a repetitive nature have been shown to induce a T-cell-independent immune response resulting in IgM production [203]. These are factors that may underlie the difficulties in obtaining protofibril selective monoclonal antibodies with a switched isotype, something which has also been observed by others [176].

### CDR analysis

A few high and intermediate affinity antibodies were selected for amino acid sequence analysis of the antigen binding CDRs. CDR sequence similarities between the antibodies in table 3 were then calculated for each individual CDR and compared to the binding properties of the antibodies (Figure 3, **paper I**), revealing a relationship between degree of sequence similarity and type of A $\beta$  binding pattern. There was a high degree of sequence similarity between the heavy-chain CDR1 and CDR2 of the high affinity, conformation dependent antibodies (mAb158 and mAb146), which suggests that these regions are important for recognition of a conformational vs. a linear epitope. A corresponding similarity in the A $\beta$  recognition pattern and heavy-chain CDR sequences was observed between our high affinity pan-A $\beta$  binding IgG antibody, mAb1C3 and the antibody 12A11 [204]. The light-chain CDRs, on the other hand, were very similar for all high affinity antibodies, implying that these regions are important for A $\beta$  binding in general. This is supported

by the resemblance of the light-chain CDRs in our antibodies and other antibodies with published sequences [185, 204, 205].

### **Characterization of IgG antibodies**

Antibodies of IgG isotype are usually preferred in immunoassays and immunotherapy, which is why we decided to further characterize our two IgG clones, mAb1C3 and mAb158. The commercially available, N-terminal specific antibody 6E10 [206] was used as a reference, since it is widely used in various immunoassays. Again, inhibition ELISA was used to determine the antibodies' binding affinity for A $\beta$  protofibrils composed of A $\beta$ 42wt or A $\beta$ 42Arc. The protofibrils were compared to unaggregated A $\beta$ 40, termed low molecular weight-A $\beta$  (LMW-A $\beta$ ), as this preparation may contain dimers, trimers and tetramers [207]. The N-terminal A $\beta$ 1-16 fragment and the C-terminal A $\beta$ 17-40 fragment were included in the analysis to give an indication of epitope locations. As seen in figure 2, **paper II**, mAb158 had a 200-fold higher affinity for protofibrils compared to LMW-A $\beta$ . Furthermore, it bound to the N-terminal fragment of A $\beta$  with the same affinity as to LMW-A $\beta$ , whereas it had no measurable affinity for the C-terminal fragment, indicating that mAb158 binds to an epitope mainly involving amino acid residues from the N-terminus of A $\beta$ . 6E10, recognizing a linear epitope at the A $\beta$  N-terminus, bound equally well to all A $\beta$  preparations except for the C-terminal fragment, as did mAb1C3, though with a slightly higher affinity for protofibrils.

To further analyze the conformation selectivity of mAb158, LMW-A $\beta$  and A $\beta$  protofibrils were separated by SDS-PAGE. Samples were mixed with SDS but without heating, to preserve high-molecular weight aggregates. Following transfer, membranes were immunoblotted with the three different antibodies. While 6E10 and mAb1C3 readily detected both LMW-A $\beta$  and protofibrils, mAb158 only detected larger aggregates, confirming its conformational selectivity (Figure 2, **paper II**).

These experiments were all performed with protofibrils prepared from A $\beta$ 42 and compared to LMW-A $\beta$ 40 and the C-terminal fragment A $\beta$ 17-40, raising the question whether the difference in affinity for these different species may in fact be a difference in affinity for the C-terminus of A $\beta$ 40 and A $\beta$ 42. To investigate this question, A $\beta$ 40 was aggregated and compared to LMW-A $\beta$ 40 and A $\beta$ 42 protofibrils in an inhibition ELISA setting. As displayed in figure 6, mAb158 bound strongly to both A $\beta$ 40 and A $\beta$ 42 protofibrils but not to LMW-A $\beta$ 40. 6E10 bound equally well to all the different preparations. The slightly better binding to A $\beta$ 42 protofibrils in this experiment is explained by the fact that its protofibril content was around 90% compared to 10-20% for the A $\beta$ 40 preparation [208]. In addition, mAb158 has been demonstrated to bind to stabilized A $\beta$ 40 oligomers [52].

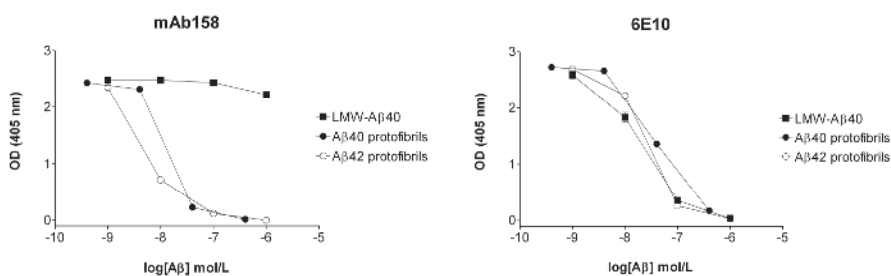


Figure 6. Inhibition ELISA, comparing the binding of mAb158 and 6E10 to LMW-Aβ40 and to protofibrils made of Aβ40 or Aβ42. mAb158 bound slightly better to Aβ42 protofibrils than to Aβ40 protofibrils, due to incomplete aggregation of Aβ40.

An important issue with Aβ antibodies is whether they bind to naturally occurring AβPP or soluble fragments thereof. Antibodies recognizing linear epitopes within the Aβ sequence typically do. This feature may be useful for certain applications, but when used in immunoassays for detection of Aβ aggregates it is not desirable, since the aggregates to be detected in e.g. CSF are expected to be present in extremely low concentrations [77] compared to the nanomolar concentrations of soluble AβPP fragments [209]. To address this question, mAb158 and 6E10 were used for immunoprecipitation analysis of brain homogenate from transgenic mice, over-expressing human AβPP with the Swedish mutation (AβPP<sub>Swe</sub>) or a combination of the Swedish and Arctic mutations (AβPP<sub>ArcSwe</sub>), which cause increased Aβ production and aggregation respectively. Conditioned media from human embryonic kidney-293 (HEK-293) cells transfected with the same AβPP variants as the mice were also analyzed. Non-transgenic mice and mock cells served as negative controls and synthetic Aβ protofibrils as positive control. 6E10 precipitated soluble AβPP from both transgenic mouse samples and medium from transfected cells, whereas mAb158 pulled down only the protofibrils serving as a positive control (Figure 3, **paper II**).

A few antibodies previously reported to bind Aβ fibrils [182] or oligomers [82] have also been shown to bind aggregates from other amyloidogenic molecules such as α-synuclein, islet amyloid polypeptide (IAPP) and TTR, suggesting a common epitope amongst amyloid aggregates. Such antibodies reveal interesting characteristics of amyloidogenic proteins but may be of limited use in immunoassays or immunotherapy, where the target is normally only one protein. As displayed in figure 3, **paper II**, mAb158 does not bind fibrils of medin, IAPP or α-synuclein, suggesting that it does not recognize a generic amyloid epitope, but a conformation dependent epitope specific for Aβ.

In summary, mAb158 is a monoclonal, conformation dependent, Aβ protofibril selective IgG antibody that does not bind to either AβPP or other amyloid assemblies, which are several of the characteristics required of a suitable candidate for Aβ protofibril specific immunoassays.

## Establishment of an A $\beta$ protofibril specific ELISA

The idea to develop an A $\beta$  protofibril specific ELISA comes from the now generally accepted idea that soluble A $\beta$  aggregates are responsible for neurotoxicity as well as synapse malfunction and loss, leading to massive degeneration of the AD brain. An assay measuring this elusive A $\beta$  species could possibly be used for early diagnosis, to facilitate early intervention with future therapies, or to evaluate the outcome of the many upcoming therapeutic strategies targeting A $\beta$  metabolism and aggregation.

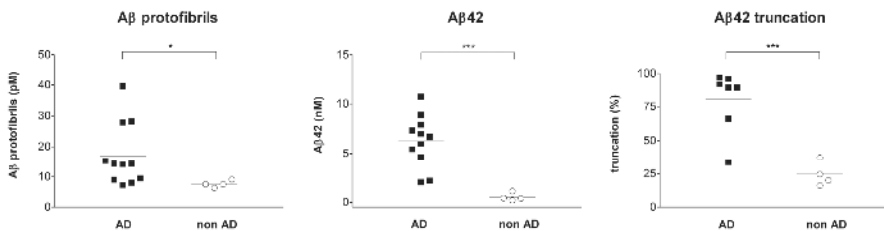
Soluble A $\beta$  aggregates are composed of several A $\beta$  units, which suggests there is a high probability that several identical epitopes, conformational or linear, are exposed on their surface. This property of the target molecule has the advantage that the same antibody can be used for both capture and detection [152-158], ensuring that monomers will not be detected. With a mAb158-mAb158 sandwich ELISA setup, protofibrils made of A $\beta$ 42wt or A $\beta$ 42Arc were detected with high specificity compared to LMW-A $\beta$  with a limit of detection (LOD) of approximately 1 pM. Moreover, the addition of a 500 000-fold molar excess of the A $\beta$ 1-16 fragment, mimicking the high physiological concentration of soluble A $\beta$ PP fragments and A $\beta$  monomers, did not affect the A $\beta$  protofibril detection in the mAb158-ELISA. A similar ELISA based on the 6E10 antibody, which binds A $\beta$ PP with the same affinity as A $\beta$ , showed impaired protofibril detection from a 1000-fold excess of A $\beta$ 1-16 (Figure 4, **paper II**). In addition to A $\beta$ 42 protofibrils, the mAb158 ELISA has proven to detect stabilized,  $\beta$ -sheet containing A $\beta$  oligomers and protofibrils with high sensitivity [52].

As synthetic A $\beta$  protofibrils are potentially structurally different from biologically derived soluble A $\beta$  aggregates, it was necessary to test the protofibril ELISA also with biological samples. Conditioned media from transfected A $\beta$ PP<sub>Swe</sub> and A $\beta$ PP<sub>ArcSwe</sub> HEK293-cells were compared to medium from mock cells, without A $\beta$ PP over-expression. These same samples had previously been analyzed in a separate study and indirectly proven to contain considerable amounts of aggregated A $\beta$  [148]. The earlier results were confirmed by the protofibril ELISA, where considerably higher protofibril levels were detected in media from the A $\beta$ PP<sub>ArcSwe</sub> transfected cells compared to A $\beta$ PP<sub>Swe</sub> transfected cells (Figure 5B, **paper II**).

Though cell cultures are often biologically relevant, they do not provide the full complexity of the human brain. To better mimic the molecular situation in AD, brains were taken from 10 month old A $\beta$ PP<sub>Swe</sub> and A $\beta$ PP<sub>ArcSwe</sub> transgenic mice, an age at which both plaque and intraneuronal A $\beta$  pathology are present. Brains were homogenized in TBS and centrifuged 100 000 x g to extract the soluble pool of A $\beta$  and this extract was analyzed with the ELISA and compared to brains of non-transgenic mice. Interestingly, we saw the exact same pattern as for cell media (Figure 5C, **paper II**). These findings confirmed the existence of A $\beta$  protofibrils *in vivo*,

suggesting that they may well exist and potentially be measured also in brain homogenates and CSF from AD patients, even if the concentrations of A $\beta$  protofibrils in these samples are expected to be significantly lower.

Measuring A $\beta$  protofibrils in human tissue and body fluids proved to be a challenging task. Brain homogenate prepared in the same way as the mouse brains gave signals close to the LOD of the ELISA. This was somewhat surprising, as AD brain has been reported to contain substantial amounts of soluble A $\beta$  [210]. However, adjusting the centrifugation conditions for the recovery of soluble A $\beta$  from 100 000 x g to 16 000 x g [85], low levels of A $\beta$  protofibrils could indeed be detected in most AD brains but not in brains from control subjects or patients with frontotemporal dementia (FTD), a subcategory of FTL (Figure 7). In this set of samples only 0.25% of the soluble A $\beta$ 42 was detected by the protofibril ELISA. However, much of the A $\beta$  in AD brain has been reported to be N-terminally truncated [211] and, as discussed in **paper IV**, more than 90% of the A $\beta$ 42 in our AD samples was N-terminally truncated (Figure 7). This could well explain the low levels detected by the A $\beta$  protofibril specific ELISA, which depends on at least two intact A $\beta$  N-termini for detection. In theory, this problem could be solved by replacing mAb158 with a mid-region A $\beta$  antibody, such as the commercially available 4G8 [206], but this ELISA setup suffers from the A $\beta$ PP competition discussed above, and failed to achieve the high sensitivity of the mAb158 ELISA, which is necessary for this type of measurements.



*Figure 7.* A $\beta$  protofibril levels, A $\beta$ 42 levels and degree of A $\beta$ 42 truncation in human AD and non-AD brain.

CSF from AD patients has been reported to contain low levels of soluble A $\beta$  aggregates, measured by direct [77, 99, 150, 151] and indirect [149] methods. However, when assessed with our protofibril specific ELISA, no detectable signals were generated, suggesting either that the concentration of soluble A $\beta$  aggregates is too low or that their size or conformation is not compatible with the ELISA. mAb158 was generated against the relatively large A $\beta$  protofibril and does not bind to smaller A $\beta$  aggregates (Figure 3D and 4F, **paper IV**), which explains why the ELISA based on this antibody does not detect small A $\beta$  aggregates (Figure 2, **paper IV**) and implies that the antibody specificity rather than the ELISA setup is the size limiting factor.

## Heterophilic antibody interference in immunoassays

Sandwich immunoassays, typically sandwich ELISAs, where one antibody captures and a second detects the target molecules, have proven invaluable for specific and sensitive detection of a target molecule in human body fluids, such as serum, plasma and CSF. The simplicity, sensitivity and reproducibility of this type of assay have made it a standard procedure in most labs and many companies have specialized in immunoassay production. However, any sandwich immunoassay used to analyze human samples is likely to be susceptible to heterophilic antibody (HA) interference, especially if the capture and detection antibodies are from the same species [212-214]. HA are naturally occurring, polyreactive antibodies binding to antibodies from foreign species, often with a low affinity and specificity. The term HA sometimes include human anti-animal antibodies (HAAA), particularly human anti-mouse antibodies (HAMA), which arise as a result of exposure to animals or animal products [215]. HA may interfere with immunoassays by cross-binding the capture and detection antibody to create a false positive signal. Due to the low affinity of most HA, interference can be decreased by sample dilution, but if the concentration of the analyte is low, a solution to the problem is to remove HA prior to analysis [216] or to add a large excess of irrelevant antibodies to which HA can bind [217, 218]. In AD and PD research, immunoassays involving multiple antibodies have been used to measure the elusive soluble oligomers in human body fluids and higher levels of A $\beta$  and  $\alpha$ -synuclein oligomers have been reported in AD and PD patients respectively [77, 99, 151-153]. However, the possibility of HA interference is not mentioned in any of these reports, raising doubts regarding the credibility of the results. In one of the studies though, Fab fragments were used instead of intact antibodies [99], a strategy likely to minimize the risk of false positives as most heterophilic antibodies are directed to the Fc part.

In **paper III**, we aimed to investigate the effect of HA interference on our A $\beta$  protofibril specific ELISA in comparison with the ELISA used by Xia et al. 2009 [153], where the A $\beta$  N-terminal specific antibody 82E1 served as both capture and detection antibody. A large set of plasma and CSF samples were therefore selected for analysis with and without measures taken to counteract HA interference. Plasma samples were analyzed following dilution in ordinary ELISA incubation buffer and in commercially available buffers designed to neutralize HA, typically with a large excess of unrelated antibodies. CSF samples, containing substantially lower amounts of antibodies than plasma, were instead depleted of IgG with protein G. The general outcome of these analyses was a near complete reduction of positive ELISA signals in all samples (Figure 2, **paper III**). This suggests that positive ELISA signals obtained with both ELISAs were in fact produced by HA mediated cross-binding of the capture and detection antibodies. This

conclusion was further supported by a number of control experiments presented in figure 3, **paper III**. When substituting the capture or detection mAb158 antibody, or both, with A $\beta$ -irrelevant mouse derived antibodies, positive signals remained in the subset of plasma and CSF samples that generated a signal in the original ELISA setup. These signals were similarly abolished when samples were diluted in HA neutralizing ELISA buffer or IgG depleted with protein G. Furthermore, to prove that our anti-HA treatment did not affect true ELISA signals generated by A $\beta$  aggregates formed *in vivo*, A $\beta$ PP<sub>ArcSwe</sub> transgenic mouse brain homogenates were analyzed in the same set of control experiments. Here, no signals were obtained in the A $\beta$  irrelevant ELISA setup and the normal ELISA analysis was unaffected by all forms of anti-HA treatment.

In the experiments above, HA interference is convincingly, albeit indirectly proven. However, as a proof of concept and a controlled replication of HA interference, a goat-anti-mouse IgG antibody was tested in the A $\beta$  protofibril ELISA and in the A $\beta$  irrelevant ELISA, with the expectation that it would cause interference by cross-binding the assay antibodies. The expected signals were seen in both ELISA setups and disappeared upon dilution of the anti-mouse-antibody in anti-HA buffer.

Immunoassays often serve as the primary platform in which clinical drug candidates and biomarkers are monitored. Therefore, a failure to evaluate and compensate for HA interference, not least in the analysis of soluble A $\beta$  aggregates, could greatly compromise the validity of research and clinical findings reliant on such immunoassays.

## Characterization of soluble A $\beta$ aggregates in mice and men

Recent AD research has given soluble A $\beta$  aggregates a central role in the pathogenesis of the disease. However, there is an ongoing debate regarding the identity of the soluble aggregates responsible for synapse loss and neurotoxicity associated with AD. As discussed in the introduction, all kinds of soluble A $\beta$  aggregates ranging from dimers up to large protofibrillar species have been suggested and shown to induce detrimental effects in different models, both *in vitro* and *in vivo*. However, these molecules have generally been generated or isolated by methods that could modify their molecular size or structure. We therefore sought to characterize synthetic and biologically derived soluble A $\beta$  aggregates as natively as possible. This was done with density gradient ultracentrifugation, a method that separates molecules based on their size, in the absence of detergents, denaturing agents or solid matrices.

Thus, samples of synthetic A $\beta$  and brain extracts from transgenic and non-transgenic mice as well as from AD and non-AD subjects were centrifuged in a density gradient. The gradient was then collected in four fractions, containing molecules of different sizes, with the largest molecules

in fraction 1 and the smallest in fraction 4. ELISA analysis revealed that synthetic, freshly dissolved A $\beta$ 40 was mainly found in fraction 3 and 4 and that no A $\beta$  protofibrils could be detected in these fractions. A $\beta$ 42, incubated at high concentration for 30 minutes, was more evenly distributed throughout the density gradient and could be measured in all four fractions, though the highest concentration was found in fraction 2. This fraction also proved to contain the highest level of A $\beta$  aggregates detected with our A $\beta$  protofibril ELISA. As for the A $\beta$ 40 preparation, no protofibrillar A $\beta$ 42 was detected in fraction 3 or 4 (Figure 2, **paper IV**), suggesting that our protofibril ELISA cannot detect smaller A $\beta$  oligomers, as observed by others with a similar method [99]. The size of the molecules in the different fractions of ultracentrifuged synthetic A $\beta$ 42 was estimated based on atomic force microscopy (AFM) analysis (Table 1, **paper IV**). The four fractions of the A $\beta$ 42 preparation were then tested for their effect on PC12 cell viability in an MTT assay, revealing the two middle fractions as the most toxic (Figure 2, **paper IV**). This is in line with previous reports about toxicity of A $\beta$  aggregates [63, 65, 92, 93, 219] and the notion that among small A $\beta$  oligomers, cell toxicity increases with size [220].

With this well characterized synthetic A $\beta$  serving as a kind of comparative standard, the TBS soluble pool of brain extracts from transgenic mice and human AD and non-AD subjects were fractionated and analyzed. Mouse brains contained eight-fold more soluble A $\beta$ 40 than A $\beta$ 42 and most of it ended up in fraction 2, where it could readily be measured with the A $\beta$  protofibril ELISA (Figure 3, **paper IV**). The low amount of A $\beta$  in fraction 3 was, as for synthetic A $\beta$ , not detectable with the protofibril ELISA. Furthermore, soluble A $\beta$  in AD brain was mainly found in fraction 2 but here, unlike in mice, most of the A $\beta$  was A $\beta$ 42, though some of the AD cases had high A $\beta$ 40 levels (Figure 4, **paper IV**). Very low A $\beta$  levels were detected in non-AD brains. Despite the high A $\beta$ 42 levels in fraction 2 from AD brain, nothing was detected with the protofibril ELISA, probably due to the high level of A $\beta$ 42 N-terminal truncation. In a different approach, fraction 2 and 3 from human brain extracts were immunoprecipitated with the protofibril selective mAb158 and analyzed with high sensitive A $\beta$ 1-40 and A $\beta$ 1-42 ELISAs. This method, requiring only one intact N-terminus (instead of at least two for the ELISA), proved sensitive enough to reveal that, despite the relatively equal distribution of A $\beta$ 42 and A $\beta$ 40 in the input material, the mAb158 precipitate from fraction 2 of the AD brains contained almost exclusively A $\beta$ 42. The failure to recover A $\beta$ 40 from this fraction may be due to differences in the conformation of A $\beta$ 40 and A $\beta$ 42 aggregates; alternatively, non-aggregated A $\beta$ 40 may have separated in this fraction due to a native interaction with larger carrier proteins. No A $\beta$  was immunoprecipitated from fraction 3, again suggesting the presence of oligomers lacking the structure recognized by mAb158.

Synthetic and biologically derived A $\beta$  aggregates are formed in very different environments. In the brain, A $\beta$  can interact with many other molecules, both during aggregation and as an aggregated protein and the A $\beta$  concentration is in general different *in vitro* and *in vivo*. Moreover, biologically derived A $\beta$  aggregates are suspended in a complex matrix and may consequently not separate as synthetic aggregates in density gradient ultracentrifugation. Nevertheless, these results suggest that soluble A $\beta$  aggregates from human and transgenic mouse brain extracts show important similarities to synthetic A $\beta$ 42 aggregates with regard to their size and distribution in the gradient as well as their binding to conformation dependent antibodies. This implies that soluble A $\beta$  aggregates of large to intermediate size, the most toxic species *in vitro*, are the predominant soluble A $\beta$  species in the brains of AD patients and A $\beta$ PP-tg mice.

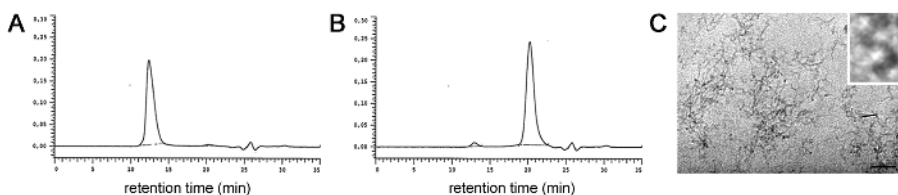
## Methodological considerations

A major part of this work, at least when measured in time and effort, has been focused on generation and characterization of conformation dependent monoclonal antibodies recognizing A $\beta$  protofibrils and the use of these antibodies in immunoassays. Therefore, a significant part of the work deals with the methodological concerns regarding A $\beta$  interactions with antibodies. In addition, the aggregation prone amyloid- $\beta$  peptide presents its own methodological complications.

## Synthetic A $\beta$

Many aspects of AD research involve the use of the A $\beta$  peptide in different forms. Studies of A $\beta$  aggregation and structure require large quantities of high purity peptide, consequently recombinant or synthetic A $\beta$  is often used. In many studies, the solvent 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) is used to dissolve the peptide and dissociate any preformed aggregates in the peptide preparation to get control over the peptide aggregation process. We have used 10 mM sodium hydroxide to dissolve the lyophilized A $\beta$  peptide, which has a pI of 5.1, in order to minimize isoelectric precipitation [92]. Previous studies [221] as well as our own experience have shown that synthetic A $\beta$  quality and aggregation rate vary a lot from different suppliers and even from batch to batch. Moreover, A $\beta$ , especially A $\beta$ 42, is highly hydrophobic, leading to aggregation kinetics that are often difficult to control – it also tends to bind to tubes, plates and chromatography columns and the addition of small amounts of detergents have proven necessary to reduce this effect. This problem is even more prominent for A $\beta$  peptides with the Arctic E22G intra-A $\beta$  mutation (A $\beta$ Arc), which has a higher rate of aggregation than wild type A $\beta$  (A $\beta$ wt). It is therefore important to establish criteria for how to handle the peptide and define the different A $\beta$  preparations used in a study.

In this project, the A $\beta$  protofibril, made from either A $\beta$ 42Arc or A $\beta$ 42wt, has been used as the antigen for production and characterization of monoclonal antibodies (**paper I and II**) and for comparison with soluble A $\beta$  aggregates generated *in vivo* (**paper IV**). A $\beta$  protofibrils are defined by a molecular weight greater than 70 kDa and a curvilinear morphology in transmission electron microscopy (TEM) [59, 60, 95, 222]. Their size is determined with size exclusion chromatography as a population of A $\beta$  eluting in the void of a Superdex 75 column [95]. Non aggregated A $\beta$ 40 elutes with a peak corresponding to a mix of A $\beta$  monomers-tetramers, when compared to SEC protein standards (Figure 8A-B). This preparation is therefore termed low molecular weight A $\beta$  (LMW-A $\beta$ ). Typical appearance of A $\beta$  protofibrils seen with cryo-TEM is shown in figure 8C.



**Figure 8.** A $\beta$  protofibrils (A) and LMW-A $\beta$  (B) separated with size exclusion chromatography. A $\beta$  protofibrils, enlarged in the upper right corner, visualized with cryo-TEM. The size of the scale bar is 200 nm. (C) (From figure 2, **paper II**).

## Sample selection and preparation

While the first study is purely an *in vitro* study, the other studies deal with biological samples. Soluble A $\beta$  aggregates were measured and characterized in homogenized brain tissue from transgenic mice and human brains in both **paper II and IV** and in **paper III** plasma and CSF samples from almost 150 subjects were used to investigate the effect of HA interference in A $\beta$  oligomer ELISAs.

## Animal and cell models

Most types of medical research involving complex molecular disease mechanisms rely on various model systems. In the case of AD, events such as A $\beta$ PP processing and A $\beta$  secretion and aggregation can be studied in cell models [100, 223-225]. These studies, carried out in our lab, were performed on either transiently transfected HEK-293 cells or stably transfected SHSY-5Y cells, all expressing human A $\beta$ PP with the Swedish mutation alone or in combination with the Arctic mutation, to increase A $\beta$  production and/or aggregation. Studies of complex pathological processes, such as extracellular accumulation of A $\beta$  or effects on cognition and memory, require animal models. We have mainly used an A $\beta$ PP tg mouse model carrying a combination of the Swedish and Arctic mutations (A $\beta$ PP<sub>ArcSwe</sub>) under a Thy-1 promoter, resulting in a marked increase in A $\beta$  production and aggregation in the brain. This leads to early intraneuronal accumulation of A $\beta$  and an onset of plaque pathology from five to six months [46], which results in spatial learning deficits [98, 226]. Moreover, in **paper II**, we showed that these mice had higher brain levels of soluble A $\beta$  protofibrils compared to mice with only the Swedish mutation. This result was reiterated [98] when this model was compared to two other A $\beta$ PP tg mouse models widely used in AD research: tg2576 harboring the Swedish mutation [227] and PSAPP with mutations in both the A $\beta$ PP gene and the PS-1 gene [228]. An advantage of the A $\beta$ PP<sub>ArcSwe</sub> transgenic model is that, compared to the A $\beta$ PP<sub>Swe</sub> model, its A $\beta$  pathology is more similar to that of AD brain as is the stability of its A $\beta$  deposits [229]. This is a feature of interest when the A $\beta$ PP<sub>ArcSwe</sub> mouse model is compared to AD brain in **paper IV**.

## Human samples

Human CSF and plasma samples, used in **paper III**, were collected during clinical investigation at the Memory Clinic at Uppsala University Hospital. Samples were selected based on the patients' diagnoses, with one group of AD and MCI patients and one group of non-AD subjects. A follow-up of the MCI patients revealed that they all converted to AD within a few years and were thus considered to represent a group of patients with early AD neuropathology. The non-AD group consisted of healthy control subjects and patients with FTD, a non-A $\beta$  related form of dementia. Plasma samples were selected from members of the Arctic family [127], both mutation carriers and non-carriers, as we considered that samples from Arctic mutation carriers could potentially contain measurable quantities of soluble A $\beta$  aggregates. In addition, plasma was collected from sporadic AD cases and age matched healthy controls.

In **paper IV**, brain tissue samples, provided by Uppsala Biobank were taken from temporal cortex of seven AD cases, three FTD cases and one control subject in order to study biologically derived soluble A $\beta$  aggregates. The clinical diagnosis of these patients was made at the Memory Clinic at Uppsala University Hospital and later confirmed by a *post mortem* neuropathological diagnosis.

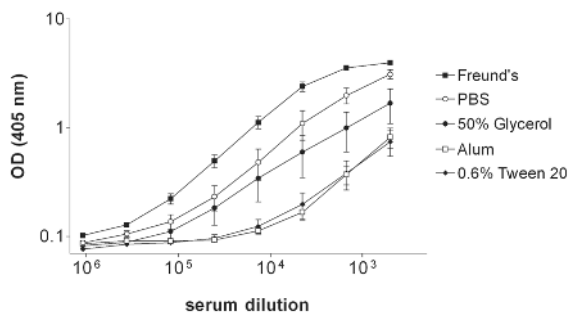
## Tissue preparation

Before performing immunological analyses of mouse tissue, the mouse is perfused with a saline solution to eliminate antibodies and other blood molecules that may interact with assay antibodies. Brains from transgenic mice as well as from AD, FTD and control patients were homogenized in a tissue grinder with a teflon pestle in a tris buffered saline (TBS) solution. A protease inhibitor cocktail was added to prevent proteases from degrading proteins and thereby impairing the analysis. The homogenate was then centrifuged at 100 000 x g to remove cell debris and obtain a preparation of soluble molecules. However, since A $\beta$  aggregation proceeds via the addition of small A $\beta$  species to a gradually growing fibrillar structure, samples presumably contain a wide spectrum of heterogeneous intermediates. Consequently, the cut-off point for soluble A $\beta$  aggregates is somewhat arbitrarily defined by the centrifugation conditions. Generally, A $\beta$  found in the supernatant after a one hour centrifugation at 100 000 x g is considered soluble [42-44, 210, 230], but other definitions exist [85]. For transgenic mouse brain, this protocol is suitable for detection and quantification of soluble A $\beta$  aggregates, but due to the high levels of N-terminal truncation and low content of soluble A $\beta$  aggregates (**paper IV**) human AD brain required a modified protocol. Human brain samples were therefore centrifuged for one hour at 16 000 x g after homogenization to increase the amount of measurable soluble A $\beta$  aggregates.

## Production of monoclonal antibodies

For production of antibodies, an animal's immune response to a foreign antigen is exploited. When an animal is immunized with an antigen, this triggers a cascade of events that eventually leads to elimination of the foreign antigen. A key event in this cascade is the production of antibodies by mature B-cells, called plasma cells. Serum taken from an immunized animal contains many different antibodies to the same antigen. These are produced by different clones of plasma cells and the serum is therefore called polyclonal. To produce a monoclonal antibody, B-cells are isolated from the spleen, and sometimes also from lymph nodes, of an immunized animal and fused with an immortalized myeloma cell line. The fusion is facilitated by permeabilization of the cell membranes with polyethylene glycol (PEG). After fusion, non fused B-cells die naturally and non fused myeloma cells, lacking the B-cell hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) gene, die in the presence of hypoxanthine-aminopterin-thymidine (HAT) medium, which blocks nucleotide synthesis. Fused cells, with an intact HGPRT gene survive thanks to their ability to produce nucleotides by the salvage pathway [231, 232]. Single fusion cell cultures are prepared and clones producing antibodies with the desired properties are selected e.g. with ELISA.

In this study, mice were immunized with A $\beta$  protofibrils for production of monoclonal antibodies. To enhance the immune response, five different adjuvants were tested; protofibrils were mixed 1:1 with PBS, Freund's complete adjuvant (FCA), alum, 0.6% Tween or 50% glycerol. Freund's adjuvant gave the highest serum titers (Figure 9). After fusion, hybridomas producing antibodies reactive to A $\beta$  protofibrils in an indirect ELISA were selected and subcloned to assure monoclonality. Cell culture media, containing the monoclonal antibodies, were collected and further analyzed with inhibition ELISA.



*Figure 9.* Serum titers to A $\beta$  protofibrils from mice immunized with A $\beta$  protofibrils with different adjuvants. Freund's complete and incomplete adjuvant gave the highest titers, followed by PBS, 50% glycerol, alum and 0.6% Tween 20.

The selected hybridomas, producing monoclonal antibodies of interest, were cultivated in bulk to obtain a large amount of cell medium containing the monoclonal antibody. IgG antibodies were then purified on a protein G-sepharose column. However, purification of the IgM antibodies was more problematic. Normally, IgM antibodies are purified with protein L, but in this case several of the antibodies precipitated at low concentrations. Attempts were made to purify the IgM antibodies with SEC, but maintaining them in cell medium containing bovine serum proteins proved to be the best way to store these antibodies.

## CDR sequence analysis

The CDR sequences of a selection of the antibodies generated by immunization with A $\beta$  protofibrils were analyzed. DNA was extracted from selected hybridoma clones and the amino acid sequences of the antibodies' heavy and light chain variable regions were determined. The antibody CDRs were defined as the smallest unique amino acid sequence within a specific area of the antibody framework region, after comparison between several antibodies. When CDRs were of different length, the CDR sequences were matched so that the highest possible number of matching amino acids was obtained. All CDR sequences of four of our antibodies were then compared and the result was expressed as per cent sequence similarity for each CDR (Figure 3, **paper I**). The CDR sequences of our antibodies were also compared to CDRs of five antibodies produced and characterized by others [185, 204, 205]

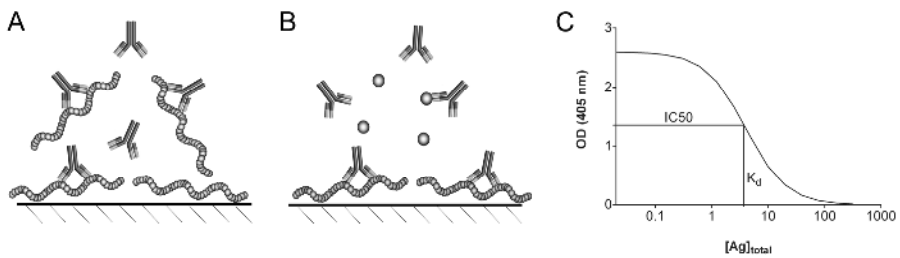
## ELISA

ELISA [233] is the standard immunoassay for quantitative detection of a specific antigen or antibody, as it is a sensitive and simple method with low variability, requiring only standard laboratory equipment. There are numerous variants of the method, but they share a common dependence on a specific interaction between an antibody and an antigen, where either the antigen or antibody has been immobilized on a solid phase. Binding can be revealed in several ways, typically involving the use of a secondary enzyme conjugated reagent such as an anti-Ig antibody or, as often the case in sandwich ELISA, streptavidin. Finally, a substrate for the enzyme is added and this is converted to a chromogenic or fluorogenic signal with an intensity proportional to the underlying antibody-antigen interaction. The two most commonly used ELISA enzymes are alkaline phosphatase (ALP) and horseradish peroxidase (HRP). In the present study several different applications of the ELISA method were employed. Indirect ELISA was used for measuring serum titers and screening of hybridomas for A $\beta$  protofibril reactivity. Mouse serum or hybridoma supernatants were then applied to a

plate coated with A $\beta$  monomers, protofibrils or fibrils and antibody reactivity was detected with an ALP-coupled anti-mouse IgG/IgM-antibody (**paper I and II**). Inhibition ELISA was used for more detailed characterization of antibody-antigen interactions (**paper I and II**) and sandwich ELISA was used for quantification of different A $\beta$  species in synthetic A $\beta$  preparations as well as in different biological samples (**paper II, III and IV**).

### Inhibition ELISA and antibody affinity

Inhibition ELISA is a variant of the indirect or direct ELISA, but here the antigen is first incubated with the antibody in solution. This approach has the advantage of eliminating the difference in epitope availability caused by differences in antigen size and ability to bind to the solid phase. In addition, since lower antigen concentrations are used in solution compared to the solid phase, it allows for a more accurate differentiation of high affinity binding interactions.



*Figure 10.* Principle of inhibition ELISA with the same antigen in coat and solution (A) or with different antigens in coat and solution (B). Approximation of  $K_d$  by determining the concentration of antigen at IC<sub>50</sub> in the inhibition ELISA graph (C).

For a more sensitive characterization of the antibodies in **paper I and II**, an inhibition ELISA protocol was developed, where the antibody was incubated with the antigen, in this case A $\beta$  protofibrils or LMW-A $\beta$ , in solution for 1 h. The antibody-antigen solution was then allowed to react with the immobilized antigen for 10 min. The analyses were performed on both LMW-A $\beta$  and A $\beta$  protofibril coated plates to ensure that the choice of coating antigen had no effect on the result (Figure 10A-B). The short incubation time was employed to make sure that only a small fraction of the free antibody in the antibody-antigen solution bound to the immobilized antigen, preserving the equilibrium of the pre-incubation solution. To control that the equilibrium was not significantly affected, the antibody-antigen solution was placed on a second antigen-coated plate and analyzed again, with the same outcome. The IC<sub>50</sub> is the antigen concentration required to inhibit half the signal generated by the antibody's binding to the antigen-coated plate. This concentration was then used as an estimate of the

antibody's affinity for this particular antigen (Figure 10C), based on the theory presented below [198].

An antibody's dissociation constant,  $K_d$ , is a measure of the antibodies affinity for its antigen and can be expressed as in equation 1.

$$K_d = [\text{Ab}] [\text{Ag}] / [\text{Ab}\cdot\text{Ag}] \quad (1)$$

Where  $[\text{Ab}]$  and  $[\text{Ag}]$  are the concentrations of antibody and antigen in solution, respectively and  $[\text{Ab}\cdot\text{Ag}]$  is the concentration of antibody-antigen bound in complex. If the total amount of antibody,  $[\text{Ab}]_{\text{total}} \ll K_d$ , which is the case in the inhibition ELISA, it follows from equation 1 that at IC50, when half  $[\text{Ab}]_{\text{total}}$  is in complex with the antigen, i.e.  $[\text{Ab}] = [\text{Ab}\cdot\text{Ag}]$ , the concentration of antigen in solution,  $[\text{Ag}]$ , is approximately equal to the total concentration of antigen,  $[\text{Ag}]_{\text{total}}$ , and hence to  $K_d$  [198]:

$$K_d = [\text{Ab}] [\text{Ag}] / [\text{Ab}\cdot\text{Ag}] = [\text{Ag}] \approx [\text{Ag}]_{\text{total}} \quad (2)$$

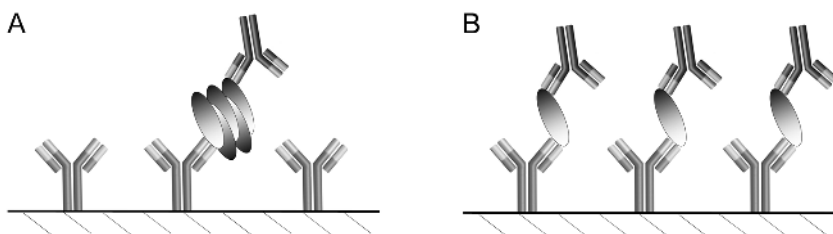
This means that  $K_d$  can be approximately determined from an inhibition ELISA diagram as the concentration of antigen required to inhibit half the ELISA signal (Figure 10C).

### **A $\beta$ ELISA quantification**

ELISA is widely used for quantification of A $\beta$ , both for research and diagnostic purposes. The most common A $\beta$  species measured with ELISA are A $\beta$ 40 and A $\beta$ 42 and sandwich ELISA kits of varying sensitivity and reliability are available from a number of different suppliers. These ELISAs are typically composed of a C-terminal A $\beta$ 40 or A $\beta$ 42 specific antibody in combination with an antibody against the A $\beta$  N-terminus or mid-region. Conformation dependent ELISA quantification of A $\beta$  demands a slightly different approach, either relying on a conformation dependent capture antibody or using the same A $\beta$  antibody (recognizing either a linear or conformational epitope) for both capture and detection.

#### *A $\beta$ 40 and A $\beta$ 42 ELISA*

There are two main issues associated with ELISA quantification of A $\beta$ 40 and A $\beta$ 42. Firstly, total A $\beta$  levels may be underestimated due to incomplete detection of the C-terminus of aggregated A $\beta$  [148, 149]. This phenomenon is described schematically in figure 11A and is a result of the structure of A $\beta$  aggregates, where the hydrophobic C-terminal amino acids are hidden in the core of the A $\beta$  aggregate [201]. Thus, the number of epitopes available to antibodies recognizing the A $\beta$  C-terminus is reduced. This problem can be solved by boiling samples in SDS to denature A $\beta$  aggregates, making C-termini available (Figure 11B). In this procedure, sample dilution is necessary after denaturation to make sure the SDS concentration is low enough not to disturb the antibody-antigen interactions.



*Figure 11.* Impaired ELISA detection of A $\beta$  aggregates, where the hidden A $\beta$  C-termini (light shading) are inaccessible to the anti-A $\beta$ 40 or anti-A $\beta$ 42 coat antibody (A). After monomerizing A $\beta$  aggregates with SDS denaturation, anti-A $\beta$  coat antibodies can easily bind to their epitopes on the C-termini, which enhances A $\beta$  detection (B).

Secondly, as discussed in **paper IV**, some biological samples used in AD research, especially AD brain, contain considerable amounts of N-terminally truncated A $\beta$ . This may cause underestimation of A $\beta$  levels if measured with the wrong type of antibodies. An A $\beta$ 1-42 ELISA assay, based on an N-terminal and a C-terminal antibody will not be able to detect N-terminally truncated A $\beta$ . In such a case the N-terminal antibody should be replaced by an A $\beta$  mid-region antibody, thus measuring A $\beta$ x-42.

### *A $\beta$ protofibril ELISA*

The sandwich ELISA developed for quantification of A $\beta$  protofibrils, described in **paper II**, is based on the protofibril selective antibody mAb158 (**paper I** and **II**). Since the same antibody is used for both capture and detection, this setup ensures that no monomers are detected, as a monomer does not have two identical epitopes. Furthermore, the conformation selectivity of mAb158, combined with its lack of affinity for A $\beta$ PP, prevents competition from more abundant molecules that could potentially block the ELISA signal. However, as indicated in **paper IV**, because mAb158 is A $\beta$  protofibril selective, this ELISA does not detect smaller A $\beta$  oligomers, which may well be present in CSF. Similar ELISA setups, which are not based on conformation selective antibodies, have been reported to bind A $\beta$  dimers [153], which is the smallest unit that could theoretically be detected in this type of ELISA. This approach could be useful if the biologically derived A $\beta$  aggregates to be measured prove to be very small.

An issue that has to be considered when trying to quantify A $\beta$  aggregates is their size and concentration. For quantification of A $\beta$  aggregates, we have chosen to express their concentration as A $\beta$  monomer units, as the size of the A $\beta$  protofibrils used to standardize the assay is not precisely defined. However, as we don't know whether the sizes of biologically derived aggregates are comparable to our synthetic A $\beta$  standard protofibrils, we cannot say for sure that the number of conformational epitopes per A $\beta$

monomer unit is the same. This could be a potential problem if there is high variability in the size of A $\beta$  aggregates *in vivo*.

## Density gradient ultracentrifugation

Ultracentrifugation is a complex method that can be used in different forms for separation of cells, cellular components or macromolecules [234]. In ultracentrifugation, a sample is centrifuged at high speed for a specific time period in a viscous medium and several different forces act on the particles in a sample. This is illustrated in figure 12, where  $F_s$  is the sedimentation or gravitational force,  $F_f$  is the frictional force and  $F_b$  is buoyancy or the “floating” force [235].

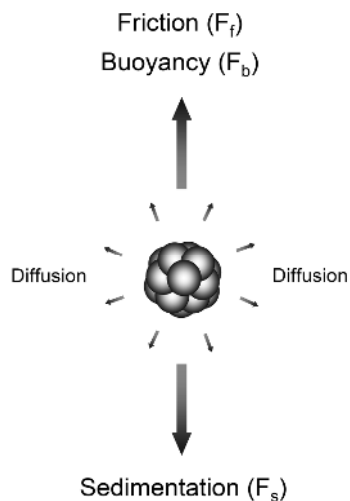
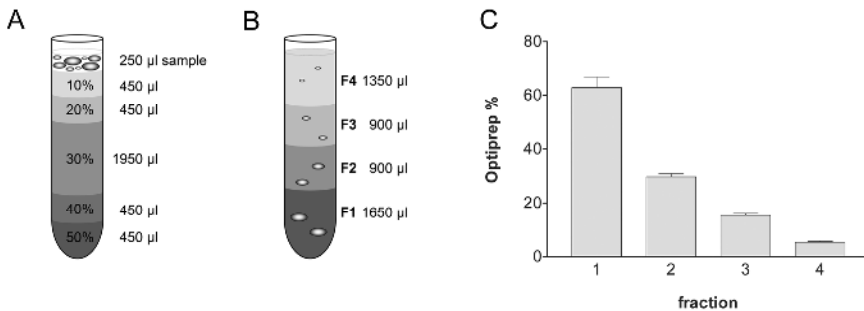


Figure 12. Forces acting on a particle during ultracentrifugation. Sedimentation ( $F_s$ ) is directed away from the axis of the rotor, whereas the frictional ( $F_f$ ) and buoyant ( $F_b$ ) forces have the opposite direction. Diffusion is multidirectional.

Sedimentation is proportional to the mass and acceleration of the particle. This is the force driving particles away from the centre of the centrifuge rotor, whereas friction, depending on the shape and size of the particle, and buoyancy, determined by the density (mass/volume) of the particle, act in the opposite direction. Hence, large and dense molecules generally travel faster and further down the centrifugation tube. For separation of a mixed sample, with particles (e.g. proteins) of different sizes, the sample can be centrifuged in a density gradient, typically made of sucrose of different percentage. This gradient can either be poured in layers (e.g. 10%, 20%, 30%) or as a continuum of increasing concentration, with the greatest density at the bottom. An additional variable important for separation of particles in this setting is time. If centrifuged to equilibrium, the particles will stop moving

in the gradient when the buoyant force is equal to the sedimentation force, i.e. when the density of the particle is equal to the density of the medium at that particular position in the gradient [236]. If, on the other hand, the centrifugation is stopped before reaching equilibrium, particles will separate based on their molecular weight. Here, all three forces act on the particle and the sedimentation velocity, which is dependent on the size, shape and density of the particle (normally corresponding to its molecular weight), determines the distance travelled in the gradient [236]. Finally, result can be affected by diffusion, which increases with increased particle concentration and decreases with increased friction. This is dependent on the shape of the particle and is proportional to its radius [235].



*Figure 13.* Optiprep density gradient before centrifugation: steps ranging from 10% to 50% with 250 µl of A $\beta$  containing sample applied to the top (A). After centrifugation, four fractions were collected (B) with a decreasing percentage of Optiprep (C).

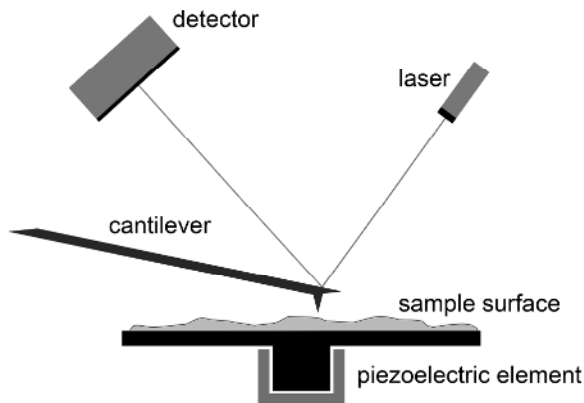
In **paper IV**, density gradient ultracentrifugation was employed to separate and fractionate A $\beta$  aggregates based on their different sizes. We used Optiprep, also known as iodixanol, which unlike other iodinated gradient media (such as sucrose) is iso-osmotic in aqueous solutions and naturally forms gradients within three hours [237]. The gradient was poured stepwise, with increasing concentrations of optiprep (figure 13A). Samples containing A $\beta$  were applied on top and centrifuged at 384 000  $\times$  g for 3 h at 4°C in a near-vertical rotor. Four fractions were collected, with a volume and optiprep density as displayed in figure 13B-C; the largest molecules separated in fraction 1 and the smallest in fraction 4. The centrifugation time and speed employed in this study did not bring the system to equilibrium and we assumed that the distance travelled by the A $\beta$  aggregates in the gradient was related to their molecular weight.

When performing a study like this, where synthetic A $\beta$  samples are compared to biological samples, a few considerations have to be acknowledged. The A $\beta$  in brain extracts, even with a mild TBS extraction protocol, will contain many different proteins, lipids and polysaccharides. Consequently, this biological A $\beta$  is surrounded by a complex matrix, which

is not present in samples of synthetic A $\beta$  dissolved in PBS. This difference may influence the way proteins travel through the gradient, as the friction could be altered by the surrounding molecules. Moreover, as this is a completely native method, any preformed complexes between A $\beta$  and other molecules will remain intact unless dissociated by the forces of centrifugation. This could lead to an overestimation of the molecular size of the A $\beta$  aggregates. Finally, the four fractions of each ultracentrifuged sample were collected dropwise through a hole pierced in the bottom of the tube. One risk associated with this collection method relates to the fact that the separation solution flows faster down the centre of the tube than at the sides; therefore, later fractions may be contaminated with remnants from earlier fractions lagging behind on the tube's inner wall.

## Atomic force microscopy

Atomic force microscopy is a powerful technique for imaging structures with nanometer resolution. The sample is applied to a surface which is connected to a piezoelectric element moving the sample surface in the x-y planes. The sample structure is then probed by an oscillating cantilever with a sharp tip at the end. As the tip follows the topography of the sample, various intermolecular forces keep it at nanometer distances above the surface. The cantilever movements over the surface are then recorded by a photodetector, measuring the deflection of a laser aimed at the end of the cantilever [238] (figure 14).



*Figure 14.* Principle of AFM. A cantilever with a sharp tip at the end moves over the sample surface. The cantilever movements are recorded by a laser and a photodetector, visualizing the structure of the surface.

AFM is a method that has been frequently used to analyze the size and shape of A $\beta$  aggregates [60, 65, 110, 239]. In **paper IV**, we used AFM to visualize fractionated A $\beta$  aggregates and analyze their structure and size.

With the particular instrument setting we used, there was no possibility to analyze the molecules in solution. Instead, samples were applied to a mica surface, air dried and then rinsed with water to remove salt crystals, which can otherwise disturb the analysis. With this non-physiological treatment, there is a risk that the A $\beta$  molecules may lose their native structure. Indeed, the appearance of some of the aggregates studied, e.g. protofibrils, looked more round than expected from previous TEM experiments. Moreover, the AFM technique allows only a minute fraction of the total sample surface to be scanned at a time, requiring multiple sections of the sample to be scanned to ensure selected images are representative for the sample. AFM is more sensitive and has a higher resolution than TEM, used to visualize the protofibrils in **paper II**, but TEM has the advantage that a larger sample surface can be analyzed in a given scan.

## Cell toxicity

Numerous reports state that A $\beta$  aggregates are neurotoxic and a common way to examine their toxicity is to assess their effect on the viability of PC-12 cells, a cell line derived from a rat adrenal pheochromocytoma, which can be differentiated to produce neuron-like morphology when stimulated with nerve growth factor [240]. The viability of the cells can then be assessed with the MTT assay, which is based on an intracellular reduction of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into a purple formazan product, which is exocytosed and form insoluble crystals at the cell surface. The formazan crystals are solubilized and measured spectrophotometrically at 570 nm, reflecting the cell viability [241]. As this assay is frequently used to measure A $\beta$  toxicity [63, 82, 93, 219], we treated PC-12 cells with fractions from our synthetic A $\beta$ 42 samples in **paper IV** and assessed their effect on the viability of the cells. Though claimed to be non toxic, the gradient medium Optiprep, turned out to affect the MTT assay results, requiring normalization of all fractions, not only with regard to A $\beta$  concentration, but also to Optiprep density.

# Concluding remarks and future perspectives

According to the amyloid cascade hypothesis, AD is initiated by an imbalance between production and clearance of the A $\beta$  peptide, resulting in aggregation and formation of toxic soluble A $\beta$  aggregates, culminating in degeneration of the AD brain. This hypothesis is not yet fully proven but is considered the most probable explanation for the pathogenic course of the disease.

In this thesis, the pathogenic A $\beta$  aggregates have been targeted with an immunologic approach, by generation of monoclonal antibodies, selectively binding to A $\beta$  protofibrils in a conformation dependent manner. The IgG antibody mAb158 binds to protofibrils with a 200-fold higher affinity than to low molecular weight A $\beta$ , but does not bind soluble A $\beta$ PP or other forms of amyloid. This selective and conformation dependent binding to protofibrils correlates to a specific CDR sequence in the antibody's variable antigen binding domain.

A protofibril specific ELISA, with mAb158 as both capture and detection antibody, measures synthetic protofibrils down to low picomolar concentrations and soluble A $\beta$  aggregates are detected in brain extracts from A $\beta$ PP-transgenic mice and AD patients, without interference from A $\beta$ PP fragments or monomeric A $\beta$ . The aggregates found in AD and mouse brain are large, soluble A $\beta$  species with an estimated molecular weight of 80-500 kDa. Synthetic aggregates of the same size proved more toxic than low molecular weight A $\beta$  or aggregates larger than 500 kDa.

Targeting and characterizing the pathogenic A $\beta$  species in samples derived from AD patients is potentially important for the development of reliable diagnostic markers of the disease. However, mAb158 ELISA detection of soluble A $\beta$  aggregates in CSF has been problematic, either due to low concentrations, or possibly because these aggregates are of different size and/or conformation. As shown in **paper IV**, the mAb158 ELISA does not detect smaller oligomers, which may lack the epitope required for mAb158 detection. This could explain why no A $\beta$  aggregates have been detected in CSF with this method. Therefore, besides testing different strategies to make the assay more sensitive, an alternative assay setup is currently being evaluated, based on an antibody which binds to A $\beta$  irrespective of conformation. In preliminary experiments, this new assay can actually detect minute amounts of A $\beta$  aggregates in CSF, but further analyses are needed to verify these results and their diagnostic relevance.

Interesting results have recently been published, stating that low levels of large soluble A $\beta$  aggregates can be measured in CSF with a method similar to ours. In this study, the levels of A $\beta$  aggregates were found to be significantly higher in AD patients compared to controls [99] and, importantly, the high levels also correlated with progression of the disease.

Thus, soluble A $\beta$  aggregates are indeed a promising AD biomarker and in combination with present AD biomarkers, it could help us make AD diagnoses more accurate and contribute to a better understanding of disease mechanisms. This will require further development of A $\beta$  immunoassays and ideally the initiation of longitudinal studies, in which age as well as disease onset and progression can be compared with CSF levels of A $\beta$  aggregates. In addition, as proven in **paper III**, interference from heterophilic antibodies can seriously affect immunoassay results and as a general phenomenon must always be considered when measuring minute amounts of an analyte in human body fluids.

Identifying the neurotoxic soluble A $\beta$  species would provide a specific target for intervention in the AD pathogenesis. Among the most promising and extensively modelled strategies for curing AD is immunotherapy, first tried as active vaccination and later modified in favour of a passive immunization approach. Antibodies with different properties and A $\beta$  binding sites are currently in different stages of clinical trials. In this regard, the monoclonal antibody generated against A $\beta$  protofibrils, described in this thesis, was not developed solely for diagnostic assays. A project running in parallel with the present studies, partly academic and partly managed by Bioarctic Neuroscience AB, is evaluating mAb158 as a candidate for immunotherapy. The first studies showed that passive immunization of A $\beta$ PP<sub>ArcSwe</sub>-transgenic mice with mAb158 reduced protofibril levels, but did not alter plaque pathology if administered after plaque onset. When tested in a preventive setting, with immunization before development of plaques, amyloid deposition was greatly reduced and protofibril levels decreased [177]. These promising results encouraged the company to proceed with humanization of the antibody, which is now called BAN2401. In the summer of 2010 a phase I clinical trial was initiated in the USA to evaluate if BAN2401 is suitable for AD therapy.

# Sammanfattning på svenska

De flesta har hört talas om Alzheimers sjukdom, men vad är egentligen Alzheimers sjukdom och vad beror den på? Vad är det som händer i hjärnan och som gör att den långsamt skrumpnar ihop och slutar fungera som den ska? Hur kan man komma åt de skadliga processerna i hjärnan och stoppa dem? Och hur vet man att man har fått Alzheimers sjukdom?

Alzheimers sjukdom är en progressiv, neurodegenerativ sjukdom som ibland felaktigt blandas ihop med normalt åldrande och som inom loppet av 5-15 år leder till döden, ofta till följd av sekundära infektioner, till exempel lunginflammation. I Sverige talas det om så många som 150 000 drabbade och antalet drabbade ökar med den åldrande befolkningen världen över. Dessvärre finns idag inget botemedel mot Alzheimers sjukdom, utan endast lindrande behandling, vars verkan upphör efter något års medicinering.

Det kan vara svårt att säkert ställa diagnosen Alzheimers sjukdom då den måste särskiljas från andra typer av demenssjukdom, som kan ha helt andra molekylära orsaker. Patienter söker ofta hjälp för att de glömmet saker, har problem att orientera sig eller inte hittar ord. Det är framförallt deras sjukdomshistoria, tillsammans med ett antal tester där man undersöker språk, minne och spatial förmåga, som ligger till grund för diagnosen. Utöver detta kan man undersöka hjärnan med olika röntgenmetoder samt göra ett antal analyser på ryggmärgsvätska (*eng. cerebrospinal fluid – CSF*), vilket kan ge viss vägledning, men det finns ännu inga säkra biokemiska markörer för sjukdomen. Först vid en hjärnobduktion kan man säkert fastställa att det är Alzheimers sjukdom, då man med mikroskop kan urskilja två speciella strukturer som är karaktäristiska för sjukdomen: amyloida plack, vars huvudsakliga beståndsdel är fibriller av proteinet  $\beta$ -amyloid ( $A\beta$ ) samt neurofibrillära nystan, som består av proteinet *tau*. Man trodde tidigare att det var framförallt de olösliga  $A\beta$ -fibrillerna som skadade nervcellerna, men senare tids forskning har flyttat fokus till lösliga förstadier till dessa fibriller – oligomerer och protofibriller.

Den här avhandlingen beskriver ett antal studier med det övergripande målet att ta fram antikroppar som selektivt kan binda till de skadliga protofibrillerna. Antikropparna är sedan tänkta att användas för att utveckla nya diagnostiska analysmetoder samt för behandling av Alzheimers sjukdom.

I de första två studierna beskrivs utvecklingen av antikroppar som känner igen och binder till protofibriller på grundval av deras proteinkonformation. Antikropparna karaktäriserades med avseende på deras förmåga att binda till

olika former av A $\beta$  och en känslig analysmetod skapades med utgångspunkt från en av antikropparna. Denna metod användes sedan för att mäta mängden protofibriller i hjärnextrakt från s.k. transgena möss, vars gener modifierats så att de uttrycker en muterad form av A $\beta$ , som aggregerar och bildar plack i hjärnan, liknande dem man ser hos Alzheimerpatienter. Tanken är att denna analysmetod skall användas för att mäta protofibriller i CSF-prover från Alzheimerpatienter och friska kontroller för att se om de skiljer sig åt. Tidigare studier har dock visat att koncentrationen av protofibriller vid sådana mätningar förväntas vara extremt låg, varför mätmetoden behöver vara extremt känslig, något som visade sig försvåra studierna.

Ett annat problem som kan uppstå när man använder antikroppar i den här typen av immunologiska analysmetoder är att s.k. heterofila antikroppar, som finns i både blodplasma och CSF hos en stor del av befolkningen, kan korsbinda de antikroppar man använder sig av och därmed skapa felaktigt positiva signaler (se figur 1 i **studie III**). Detta är ett känt fenomen inom andra forskningsfält, men inom Alzheimerfältet har ingen tidigare fokuserat på problemet, trots att immunologiska mätmetoder ofta används. I den tredje studien undersökte vi därför hur vår mätmetod påverkades av heterofila antikroppar i plasma och CSF och jämförde med en liknande metod, som tidigare rapporterats kunna mäta en mindre variant av protofibriller, s.k. oligomerer. Det visade sig att båda metoderna var känsliga för påverkan från heterofila antikroppar och falska signaler uppträdde således i hela 25% av proverna.

Den fjärde och sista studien ägnades åt att mer i detalj studera de lösliga formerna av naturligt förekommande A $\beta$  från Alzheimerpatienter och transgena möss. Med hjälp av ultracentrifugering separerades de lösliga A $\beta$ -aggregaten med avseende på deras storlek och delades in i fyra fraktioner med de största molekylerna i fraktion ett och de minsta i fraktion fyra. Fraktionerna analyserades därefter med vår egenutvecklade protofibrill-analysmetod samt med en extremt känslig typ av mikroskopi där man kan urskilja enstaka molekyler (atomkraftsmikroskopi). Sedan mättes vilken påverkan de olika fraktionerna hade på levande celler. Dessa analyser avslöjade att merparten av A $\beta$ -aggregaten i våra hjärnprover motsvarade protofibriller i storlek och att aggregat i denna storlek var mest giftiga för celler. Dessa aggregat gick dessutom att fånga upp med vår protofibrill-bindande antikropp, vilket visar att de till sin struktur liknar de syntetiska protofibriller vi använt för att skapa antikropparna.

Att utröna vilken typ av lösliga A $\beta$ -aggregat som ansamlas i hjärnan vid Alzheimers sjukdom är viktigt framförallt av två skäl. För det första vill man så tidigt som möjligt kunna diagnostisera sjukdomen och detta kan förhoppningsvis uppnås genom att mäta halten av de skadliga A $\beta$ -formerna i CSF från Alzheimerpatienter. Detta är viktigt för att en eventuell behandling ska kunna sättas in tidigt och stoppa nervcellsödnen innan den blivit alltför

utbredd. För det andra vill man kunna behandla sjukdomen och detta underlättas naturligtvis av att man vet vad som orsakar skadan. Om man lyckas minska mängden av de skadliga A $\beta$ -formerna i hjärnan kan man således förhoppningsvis förhindra en fortsatt sjukdomsutveckling. Det kan till exempel åstadkommas genom att de skadliga proteinerna binds upp av antikroppar och sedan bryts ned av celler i närheten.

De resultat som framkommit i de här studierna styrker uppfattningen att protofibriller är skadliga och ger oss dessutom ett redskap för att mäta dem i hjärnvävnad från Alzheimer-patienter vilket är viktigt för fortsatt forskning och utveckling av diagnostiska metoder. Därutöver har vår antikropp, genom ett projekt drivet av Bioarctic Neuroscience AB, vidareutvecklats till en mänsklig variant av den musantikropp vi tagit fram. Sedan sommaren 2010 ingår denna antikropp, under namnet BAN2401, i en klinisk studie där den testas som ett potentiellt framtida läkemedel mot Alzheimers sjukdom.

# Acknowledgements

I understand you skipped the first part of the thesis and popped right into this section. No problem, you can read it later, or just the short version:



It all started right after the summer of 2002. I came to the lab and met this professor, **Lasse**, going on and on about some protofibrils that no one had ever seen, but he was quite convincing and I started my work. After a year, practically guided by **Charlotte**, I was sent to **Staffan** at Mabtech in Nacka Strand to develop antibodies against these magic protofibrils, at this stage actually isolated by **Ann-Sofi** on her HPLC machine. It was a beautiful time. I thrived in the friendly atmosphere and the extraordinary working environment – I had a beautiful sea view from my lab bench. The Antibody came to us just before Christmas in 2004 and there was much rejoicing.

**Mimmi** worked on with antibody production at Rudbeck. **Hille**, **Frida** and I characterized The Antibody and developed the ELISA, eventually resulting in **paper I** and **II**. Here, my first son, Hugo, was born and my work gradually moved to Uppsala. **Anna** and **Lars**, assisted by the people at **BioArctic**, started the first immunization study with The Antibody and I missed the analysis stage, as I decided to go for a long parental leave.

Back at work, I had a hard time understanding what I was supposed to do, but **Hille** was nice and I inherited the Ultracentrifugation project, skilfully handled by **Barbro**. In about three years, she centrifuged, fractionated and aliquoted samples, resulting in more than four hundred million tubes, stored in the freezer until analysis. No wonder **paper IV** is not published yet.

Apart from generating The Antibody, the overall aim was to establish an assay able to detect protofibrils in CSF from Alzheimer patients. If you had read the thesis, you would know that we haven't accomplished that, despite years of hard work and numerous failures. The most remarkable one was that when tiny signals, finally squeezed out from the ELISA, turned out to be caused by heterophilic antibody interference – signals were false! But this debacle actually led to the publication of **paper III**, written with our new team member **Sofia**, just after my second parental leave, this time with Emil.

To sum this up, I'm not entirely convinced I have ever seen any actual protofibrils, but something in Alzheimer brains is in fact recognized by The Antibody and, who knows? This something might be protofibrils...

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Dag Sehlin, Uppsala, November 2010

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