Aβ Conformation Dependent Antibodies and Alzheimer's Disease

DAG SEHLIN
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Abstract

Soluble intermediates of the amyloid-β (Aβ) 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 Aβ aggregates have been studied with a particular focus on the Aβ protofibril, which has served as the antigen for developing conformation dependent monoclonal antibodies.

Antibodies generated from mice immunized with Aβ protofibrils were characterized regarding Aβ 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 Aβ protofibrils with a 200-fold higher affinity than to monomeric Aβ without affinity for soluble amyloid-β precursor protein (AβPP) or other amyloidogenic proteins. A sandwich enzyme-linked immunosorbent assay (ELISA) based on mAb158 was used to measure soluble Aβ protofibrils in brain extracts from AβPP-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 Aβ 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 Aβ aggregates have been described and suggested to impair neuron and synapse function. To investigate the soluble Aβ pool, synthetic Aβ and brain extracts from AβPP-transgenic mice and AD patients were ultracentrifuged on a density gradient to separate Aβ by size under native conditions. Four distinct gradient fractions were defined based on the appearance of synthetic Aβ in atomic force microscopy (AFM) and immunoreactivity in our protofibril specific sandwich ELISA. Interestingly, most Aβ from AD patients and AβPP-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.


IV  **Sehlin D**, Englund H, Simu B, Karlsson M, Ingelsson M, Nikolajeff F, Lannfelt L, Pettersson FE. Large toxic aggregates are the major soluble \(\alpha\)\(\beta\) species in AD brain separated with density gradient ultracentrifugation. *Manuscript*

* Shared authorship

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Contents

Introduction...................................................................................................11
  Alzheimer’s disease..................................................................................11
    Clinical picture and neuropathology....................................................12
    AβPP processing and Aβ .................................................................13
    Aβ aggregation and neurotoxicity .......................................................14
  Genetics and risk factors......................................................................17
    The amyloid cascade hypothesis .........................................................18
Diagnosis and therapy ..............................................................................19
  Aβ as a biomarker................................................................................20
  Aβ immunotherapy ..............................................................................21
  Conformation dependent antibodies ....................................................22

Present investigations....................................................................................25
  Aim of the study ....................................................................................25
    Specific aims......................................................................................25
Results and discussion..............................................................................26
  Generation of conformation dependent antibodies ................................26
  Establishment of an Aβ protofibril specific ELISA................................30
  Heterophilic antibody interference in immunoassays..........................32
  Characterization of soluble Aβ aggregates in mice and men............33
Methodological considerations....................................................................36
  Synthetic Aβ ........................................................................................36
  Sample selection and preparation ........................................................37
  Production of monoclonal antibodies ..................................................39
  CDR sequence analysis........................................................................40
  ELISA..................................................................................................40
  Density gradient ultracentrifugation ....................................................44
  Atomic force microscopy ....................................................................46
  Cell toxicity .........................................................................................47

Concluding remarks and future perspectives ................................................48

Sammanfattning på svenska..........................................................................50

Acknowledgements.......................................................................................53

References.....................................................................................................56
## Abbreviations

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

*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 Aβ peptide, but they also contain other components such as apolipoprotein E
(ApoE) [12], proteoglycans [13] and α-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].

**AβPP processing and Aβ**

In 1984, Aβ 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-β precursor protein (AβPP) [22]. AβPP 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 AβPP 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].

![Diagram of AβPP processing](image)

**Figure 2.** Processing of AβPP. The non-amyloidogenic pathway, with α- and γ-secretase cleavage, generates the p3 peptide, whereas the amyloidogenic pathway, with β- and γ-secretase cleavage, generates the Aβ peptide.
APP 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 α-secretase, cleaving APP in the middle of the APP sequence, generating a soluble APP fragment called αAPPs and the C-terminal fragment C83, anchored in the membrane. The non-amyloidogenic peptide p3 is then cleaved out from C83 by γ-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 APP is instead cleaved by β-secretase, also known as beta-site APP cleaving enzyme 1 (BACE1), the soluble APP fragment APPs is formed along with the C-terminal fragment C99, from which Aβ is generated by a subsequent γ-secretase cleavage. This cleavage takes place within the cell membrane and determines the C-terminal amino acid residues of the peptide, with Aβ38, Aβ40 and Aβ42 as the most common forms [29, 30]. Aβ 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]. Aβ is normally cleared by the proteases neprilysin [34] and insulin degrading enzyme [35]. The physiological role of Aβ is unclear, but the Aβ 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 Aβ 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, Aβ metabolism is somehow disturbed and the usual levels of specific Aβ peptides in e.g. CSF is altered. A significant drop in levels of Aβ42 [39], combined with a relative increase in Aβ38 and Aβ40 levels [30] are typical in CSF from an AD patient.

Aβ aggregation and neurotoxicity

Aβ is produced throughout the body, but the formation of extracellular Aβ deposits occurs mainly in the brain. What actually triggers the aggregation and deposition of Aβ 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 Aβ 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 Aβ aggregation and different soluble molecular intermediates in this process. Furthermore, the former assumption that the extracellular Aβ deposits are a result of extracellular aggregation has recently been questioned, with reports of intraneuronal accumulation of Aβ aggregates in both transgenic mouse brain [45-47] and AD brain [48, 49]. Another question is whether Aβ
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 Aβ fibrillization and stabilize Aβ fibrils once formed [50].

As schematically described in figure 3, the Aβ 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]. Aβ 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 Aβ peptide can adopt a β-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 Aβ aggregation. In the fibrillar pathway (low), the monomer goes through a conformational change into a β-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.](image-url)
In off-pathway aggregation, the unstructured Aβ monomer forms soluble oligomers, which are structurally different from the Aβ 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 Aβ species from both pathways (Table 1), some of which may well be similar. Many of these soluble Aβ 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 Aβ 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 Aβ resembles the in vivo reality, where both time scale and molecular environment differ greatly from the test tube.

Table 1. Properties of soluble Aβ aggregates

<table>
<thead>
<tr>
<th>Name</th>
<th>Size</th>
<th>Properties</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimers-tetramers</td>
<td>8-20 kDa</td>
<td>Isolated from cell media, AD brain and CSF, SDS stable, inhibit LTP, induce synapse loss</td>
<td>[67-75]</td>
</tr>
<tr>
<td>Paranuclei</td>
<td>20-30 kDa, 5-6mer, ~5 nm</td>
<td>Generated from Aβ42 in vitro by photochemical cross-linking, neurotoxic</td>
<td>[58, 76]</td>
</tr>
<tr>
<td>ADDLs</td>
<td>&gt;40 kDa</td>
<td>Generated in vitro, do not form fibrils, detected in AD brain and CSF, inhibit LTP</td>
<td>[65, 77-81]</td>
</tr>
<tr>
<td>Aβ oligomers</td>
<td>&gt;40 kDa</td>
<td>Generated in vitro, detected in AD brain, A11-positive, neurotoxic through membrane permeabilization</td>
<td>[52, 82-84]</td>
</tr>
<tr>
<td>Aβ*56</td>
<td>56 kDa</td>
<td>Isolated from tg mice, correlate with memory deficits in tg mice and rats</td>
<td>[85-87]</td>
</tr>
<tr>
<td>Globulomers</td>
<td>~60 kDa</td>
<td>Generated in vitro, do not form fibrils, detected in AD brain, inhibit LTP</td>
<td>[64, 88-90]</td>
</tr>
<tr>
<td>Amylospheroids (ASPDs)</td>
<td>&gt;100 kDa Ø 10-15 nm</td>
<td>Generated in vitro, do not form fibrils, isolated from AD and DLB brain, neurotoxic via presynaptic target</td>
<td>[63, 91]</td>
</tr>
<tr>
<td>Protofibrils</td>
<td>&gt;70 kDa width 6-8 nm</td>
<td>Generated in vitro, detected in tg mouse brain and in AD brain and CSF, neurotoxic, inhibit LTP</td>
<td>[52, 57, 59, 60, 92-100]</td>
</tr>
<tr>
<td>Annular protofibrils</td>
<td>150-250 kDa, Ø 7-10 nm</td>
<td>Generated in vitro, neurotoxic through ion-channel like function</td>
<td>[101-103]</td>
</tr>
</tbody>
</table>

Most of the soluble Aβ 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 Aβ 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 Aβ structures have different mechanisms of action [84, 111] and that several mechanisms may operate
simultaneously. Apart from the direct neurotoxicity, soluble Aβ 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 Aβ [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 ε4 allele of APOE run a higher risk of getting the disease [117], whereas the ε2 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 Aβ, either through altered AβPP processing or through modulation of the Aβ 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 AβPP 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 AβPP and thus Aβ [120]. In addition, families with AβPP 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 AβPP 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 AβPP amino acid sequence, where a lysine and an asparagine residue were substituted for a methionine and a leucin, respectively [124]. For the first time it was shown that an AβPP mutation alters cleavage of AβPP, leading to an increased Aβ production, which speeds up the disease process and causes early onset AD [125, 126]. While the Swedish mutation increases overall production of Aβ by increasing β-secretase cleavage, the London mutation alters γ-secretase cleavage, increasing the production of the more aggregation prone Aβ1-42. In 2001, another AβPP mutation was found in a Swedish family with hereditary AD. This mutation, termed the Arctic mutation, is located at position 22 within the Aβ 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 Aβ protofibrils. In addition, mutations in the presenilins, which are one of the four components of the γ-secretase complex, shift the Aβ42/Aβ40 ratio towards increased production of the more hydrophobic and aggregation prone Aβ42, [128].

Figure 4. The amyloid cascade hypothesis suggests that AD is caused by increased Aβ production, an increased Aβ42/Aβ40 ratio, altered properties of the Aβ peptide or decreased Aβ clearance, leading to aggregation of Aβ, 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 Aβ peptide, a process that is enhanced by mutations in the AβPP or presenilin genes or by an increased gene dose of AβPP, as in DS or AβPP duplications [130]. This hypothesis stated that Aβ fibrillation 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 Aβ aggregation process and its neurotoxic intermediates led to a refined hypothesis, where focus was shifted towards soluble, oligomeric and prefibrillar Aβ [129, 131, 132] (Figure 4). The order of events in the amyloid cascade hypothesis suggests that Aβ 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 Aβ cause early onset AD, while mutations in the tau gene result in FTLD without Aβ pathology [133]. Moreover, enhanced NFT formation in transgenic mice over-expressing both AβPP and tau has been suggested to be influenced by Aβ [134]. In addition, Aβ immunotherapy in such transgenic mice has been reported to reduce Aβ pathology, leading to clearance of tau pathology. The Aβ pathology then reappeared before tau pathology when therapy was terminated, suggesting not only a link between Aβ 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 Aβ 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 Aβ plaque load in affected brain areas. However, the definite AD diagnosis is made by a post mortem analysis of Aβ 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 sown 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 Aβ, by
limiting its production with β- or γ-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].

Aβ as a biomarker
A number of studies have demonstrated a correlation between AD and
decreased Aβ42 levels [39, 141, 142] or a decreased Aβ42/Aβ40 ratio [143-
145] in CSF. Despite the considerable overlap between AD and controls, low
Aβ42 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 Aβ42 indicate the likely progression from MCI to AD
[146]. The low CSF levels of Aβ42 associated with AD have been explained
by absorption of circulating Aβ42 into plaques [39] or by altered Aβ
metabolism in the brain [147]. Low Aβ42 levels could also be the result of
soluble Aβ42 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 Aβ
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 Aβ aggregates.

In 2006, El-Agnaf et al. reported elevated levels of α-synuclein oligomers
in plasma from patients with Parkinson’s disease (PD) [152] and a few years
later, Xia et al. found elevated levels of Aβ 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 Aβ aggregates in AD patients compared to healthy controls. Importantly, the levels of Aβ aggregates also correlated to the patients’ degree of dementia, as measured by their MMSE score. However, the levels of Aβ aggregates in this study were very low, confirming the need for an extremely sensitive assay in order to perform reliable measurements of Aβ aggregates in CSF. In addition, these results need to be confirmed and further studies are required to establish how the level of Aβ aggregates reflect the disease onset and progression.

Aβ immunotherapy

The idea behind Aβ immunotherapy is to take advantage of the body’s immune system to remove the pathogenic Aβ molecules from the brain and thus eliminate the cause of the disease. The first Aβ immunotherapy experiments were performed by Schenk et al. in 1999. Transgenic mice with AD-like pathology were vaccinated with Aβ42 fibrils, resulting in a marked reduction of Aβ pathology [160]. This study was followed by studies showing that Aβ vaccination, besides the beneficial effects on Aβ pathology, could also prevent Aβ 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 Aβ 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 AβPP or Aβ monomers. Moreover, if required, the treatment can be halted without persisting long-term effects.

Treatment of transgenic mice with antibodies binding to the Aβ 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 Aβ pathology and reverse cognitive deficits. Several clinical trials involving passive immunization against Aβ are ongoing [178]. Two different monoclonal antibodies, one directed to the Aβ 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 Aβ 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 Aβ 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 Aβ in the periphery alter the equilibrium of antibody-bound vs -unbound Aβ, which eventually clears Aβ from the brain. The fourth hypothesis is that once bound to the therapeutic antibodies, the toxic effect of Aβ oligomers is neutralized.

Conformation dependent antibodies

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

![Antibody structure](image)

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 Aβ fibrils were described in 2002 [182].

Table 2. Conformation dependent Aβ antibodies

<table>
<thead>
<tr>
<th>name</th>
<th>type</th>
<th>antigen</th>
<th>generic amyloid</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>M93, M94</td>
<td>polyclonal</td>
<td>ADDLs</td>
<td>no</td>
<td>[78]</td>
</tr>
<tr>
<td>WO1, WO2</td>
<td>monoclonal</td>
<td>fibrils</td>
<td>yes</td>
<td>[182]</td>
</tr>
<tr>
<td>A11</td>
<td>polyclonal</td>
<td>pre-fibrillar oligomers</td>
<td>yes</td>
<td>[82]</td>
</tr>
<tr>
<td>M71</td>
<td>polyclonal</td>
<td>ADDLs</td>
<td>no</td>
<td>[81]</td>
</tr>
<tr>
<td>R262, R286</td>
<td>polyclonal</td>
<td>Aβ fibrils</td>
<td>no</td>
<td>[183]</td>
</tr>
<tr>
<td>8F5</td>
<td>monoclonal</td>
<td>Aβ1-42 globulomers</td>
<td>no</td>
<td>[88]</td>
</tr>
<tr>
<td>NAB61</td>
<td>monoclonal</td>
<td>soluble Aβ aggregates and fibrils</td>
<td>no</td>
<td>[176]</td>
</tr>
<tr>
<td>mAb158</td>
<td>monoclonal</td>
<td>Aβ protofibrils and fibrils</td>
<td>no</td>
<td>[100]</td>
</tr>
<tr>
<td>NU1-NU7</td>
<td>monoclonal</td>
<td>ADDLs (and fibrils)</td>
<td>no</td>
<td>[184]</td>
</tr>
<tr>
<td>PFA1, PFA2</td>
<td>monoclonal</td>
<td>Aβ protofibrils and fibrils</td>
<td>no</td>
<td>[185]</td>
</tr>
<tr>
<td>OC</td>
<td>polyclonal</td>
<td>fibrillar oligomers and fibrils</td>
<td>yes</td>
<td>[186]</td>
</tr>
<tr>
<td>7A1, 1G5</td>
<td>monoclonal</td>
<td>Aβ1-42 oligomers</td>
<td>no</td>
<td>[187]</td>
</tr>
<tr>
<td>rpASD1</td>
<td>polyclonal</td>
<td>amylospheroids</td>
<td>no</td>
<td>[91]</td>
</tr>
<tr>
<td>mASD3</td>
<td>monoclonal</td>
<td>amylospheroids</td>
<td>no</td>
<td>[91]</td>
</tr>
<tr>
<td>A-887755</td>
<td>monoclonal</td>
<td>Aβ20-42 globulomers</td>
<td>no</td>
<td>[89]</td>
</tr>
</tbody>
</table>

During the past decade, several different Aβ conformation dependent antibodies, both polyclonal and monoclonal, have been developed and
described (Table 2). While most of these antibodies are Aβ specific, binding to Aβ oligomers or fibrils, some of them have been suggested to bind a generic amyloid epitope [82, 182, 186], possibly targeting the amyloid specific cross-β structure [188]. Aβ 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 Aβ 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 Aβ 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 Aβ 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 Aβ protofibril, for use in immunoassays.

Specific aims

I To characterize the immune response to Aβ protofibrils in mice and develop monoclonal antibodies selectively binding to Aβ protofibrils.

II To characterize Aβ conformation selective antibodies and develop and validate an Aβ protofibril specific immunoassay for quantification of biologically derived soluble Aβ aggregates.

III To investigate the influence of naturally occurring human heterophilic antibodies in quantitative immunoassays of soluble Aβ aggregates.

IV To use density gradient ultracentrifugation for separation and characterization of soluble Aβ species in brain extracts from AD patients and AβPP-transgenic mice.
Results and discussion

As described in the introduction of this thesis, AD is characterized by aggregation of the Aβ 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 Aβ aggregates. Such antibodies can be used for various research purposes as well as for AD immunotherapy and diagnostic immunoassays. Human antibodies binding to Aβ fibrils were first reported already in 1993 [189] and in 2002, the first Aβ fibril selective monoclonal antibodies were developed [182], followed by a few reports of polyclonal antibodies recognizing soluble Aβ oligomers. However, it became evident that developing monoclonal antibodies against soluble Aβ aggregates is a difficult task.

Generation of conformation dependent antibodies

To generate monoclonal antibodies selectively binding to Aβ protofibrils, mice were immunized with protofibrils from synthetic Aβ1-42, 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 AβPP gene, the idea being that the AβPP-KO mice, lacking endogenous expression of Aβ, would produce a stronger immune response to Aβ, as has been shown for the prion protein [197]. Immunized mice were sacrificed to generate antibody producing hybridomas, which were screened for Aβ reactivity with indirect ELISA. Of the 2921 screened hybridomas, 172 produced Aβ positive antibodies but only ten of them had intermediate to high affinity for Aβ protofibrils. These ten antibodies were further characterized with inhibition ELISA (Figure 1, paper I), where the IC50 (antigen concentration required to inhibit half the ELISA signal) was used to estimate the antibody binding affinity [198] for monomeric and protofibrillar Aβ. As displayed in table 3, antibodies were then divided into groups based on isotype, Aβ protofibril affinity and whether they had preferential binding to Aβ protofibrils over monomeric Aβ (i.e. whether the epitope was conformational or linear) as well terminal specificity (N- or C-terminal).

The AβPP-KO mice, as expected, generated a higher proportion of Aβ positive hybridomas compared to the Balb/c mice, but all produced IgM antibodies with low affinity to Aβ. Consequently, despite Balb/c mice producing less than a fifth of the Aβ positive clones, all high and intermediate affinity antibodies came from clones derived from these mice.
This could possibly be explained by the observation that AβPP may play a role in the immune system [199].

Table 3. Monoclonal antibodies generated from Aβ protofibril immunization

<table>
<thead>
<tr>
<th>Clone name</th>
<th>Isotype</th>
<th>Aβ protofibril affinity</th>
<th>Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb1C3</td>
<td>IgG</td>
<td>High (IC50 ~10^{-9} M)</td>
<td>Linear, N-terminal</td>
</tr>
<tr>
<td>mAb158</td>
<td>IgG</td>
<td>High (IC50 ~10^{-9} M)</td>
<td>Conformational, N-terminal</td>
</tr>
<tr>
<td>mAb146</td>
<td>IgM</td>
<td>High (IC50 ~10^{-8} M)</td>
<td>Conformational, N-terminal</td>
</tr>
<tr>
<td>mAb235</td>
<td>IgM</td>
<td>Intermediate (IC50 ~10^{-6} M)</td>
<td>Conformational, N/C-terminal</td>
</tr>
</tbody>
</table>

Interesting to note is that only two of the 172 Aβ positive hybridoma clones produced antibodies of IgG isotype, while all the others produced IgM antibodies. The T-cell epitopes of Aβ, predicted to be located in the middle and the C-terminal regions [200], are hidden in the core of Aβ 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. Aβ devoid of T-cell epitopes induces an IgM response in mice [202] and a strong IgM response to Aβ aggregates has been observed in humans [164, 189] as well as in mice [182], indicating that Aβ 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 Aβ 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 Aβ recognition pattern and heavy-chain CDR sequences was observed between our high affinity pan-Aβ 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 Aβ 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 \( \text{A}\beta \) protofibrils composed of \( \text{A}\beta42\text{wt} \) or \( \text{A}\beta42\text{Arc} \). The protofibrils were compared to unaggregated \( \text{A}\beta40 \), termed low molecular weight-\( \text{A}\beta \) (LMW-\( \text{A}\beta \)), as this preparation may contain dimers, trimers and tetramers [207]. The N-terminal \( \text{A}\beta1-16 \) fragment and the C-terminal \( \text{A}\beta17-40 \) 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-\( \text{A}\beta \). Furthermore, it bound to the N-terminal fragment of \( \text{A}\beta \) with the same affinity as to LMW-\( \text{A}\beta \), 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 \( \text{A}\beta \). 6E10, recognizing a linear epitope at the \( \text{A}\beta \) N-terminus, bound equally well to all \( \text{A}\beta \) 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-\( \text{A}\beta \) and \( \text{A}\beta \) 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-\( \text{A}\beta \) and protofibrils, mAb158 only detected larger aggregates, confirming its conformational selectivity (Figure 2, paper II).

These experiments were all performed with protofibrils prepared from \( \text{A}\beta42 \) and compared to LMW-\( \text{A}\beta40 \) and the C-terminal fragment \( \text{A}\beta17-40 \), 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 \( \text{A}\beta40 \) and \( \text{A}\beta42 \). To investigate this question, \( \text{A}\beta40 \) was aggregated and compared to LMW-\( \text{A}\beta40 \) and \( \text{A}\beta42 \) protofibrils in an inhibition ELISA setting. As displayed in figure 6, mAb158 bound strongly to both \( \text{A}\beta40 \) and \( \text{A}\beta42 \) protofibrils but not to LMW-\( \text{A}\beta40 \). 6E10 bound equally well to all the different preparations. The slightly better binding to \( \text{A}\beta42 \) protofibrils in this experiment is explained by the fact that its protofibril content was around 90% compared to 10-20% for the \( \text{A}\beta40 \) preparation [208]. In addition, mAb158 has been demonstrated to bind to stabilized \( \text{A}\beta40 \) 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_Swe) or a combination of the Swedish and Arctic mutations (AβPP_ArcSwe), 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 Aβ protofibril specific ELISA

The idea to develop an Aβ protofibril specific ELISA comes from the now generally accepted idea that soluble Aβ 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 Aβ 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 Aβ metabolism and aggregation.

Soluble Aβ aggregates are composed of several Aβ 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 Aβ42wt or Aβ42Arc were detected with high specificity compared to LMW-Aβ with a limit of detection (LOD) of approximately 1 pM. Moreover, the addition of a 500 000-fold molar excess of the Aβ1-16 fragment, mimicking the high physiological concentration of soluble AβPP fragments and Aβ monomers, did not affect the Aβ protofibril detection in the mAb158-ELISA. A similar ELISA based on the 6E10 antibody, which binds AβPP with the same affinity as Aβ, showed impaired protofibril detection from a 1000-fold excess of Aβ1-16 (Figure 4, paper II). In addition to Aβ42 protofibrils, the mAb158 ELISA has proven to detect stabilized, β-sheet containing Aβ oligomers and protofibrils with high sensitivity [52].

As synthetic Aβ protofibrils are potentially structurally different from biologically derived soluble Aβ aggregates, it was necessary to test the protofibril ELISA also with biological samples. Conditioned media from transfected AβPP_Swe and AβPP_ArcSwe HEK293-cells were compared to medium from mock cells, without AβPP over-expression. These same samples had had previously been analyzed in a separate study and indirectly proven to contain considerable amounts of aggregated Aβ [148]. The earlier results were confirmed by the protofibril ELISA, where considerably higher protofibril levels were detected in media from the AβPP_ArcSwe transfected cells compared to AβPP_Swe 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 AβPP_Swe and AβPP_ArcSwe transgenic mice, an age at which both plaque and intraneuronal Aβ pathology are present. Brains were homogenized in TBS and centrifuged 100 000 x g to extract the soluble pool of Aβ 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 Aβ 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 Aβ protofibrils in these samples are expected to be significantly lower.

Measuring Aβ 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 Aβ [210]. However, adjusting the centrifugation conditions for the recovery of soluble Aβ from 100 000 x g to 16 000 x g [85], low levels of Aβ 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 FTLD (Figure 7). In this set of samples only 0.25% of the soluble Aβ42 was detected by the protofibril ELISA. However, much of the Aβ in AD brain has been reported to be N-terminally truncated [211] and, as discussed in paper IV, more than 90% of the Aβ42 in our AD samples was N-terminally truncated (Figure 7). This could well explain the low levels detected by the Aβ protofibril specific ELISA, which depends on at least two intact Aβ N-termini for detection. In theory, this problem could be solved by replacing mAb158 with a mid-region Aβ antibody, such as the commercially available 4G8 [206], but this ELISA setup suffers from the AβPP competition discussed above, and failed to achieve the high sensitivity of the mAb158 ELISA, which is necessary for this type of measurements.

Figure 7. Aβ protofibril levels, Aβ42 levels and degree of Aβ42 truncation in human AD and non-AD brain.

CSF from AD patients has been reported to contain low levels of soluble Aβ 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 Aβ aggregates is too low or that their size or conformation is not compatible with the ELISA. mAb158 was generated against the relatively large Aβ protofibril and does not bind to smaller Aβ aggregates (Figure 3D and 4F, paper IV), which explains why the ELISA based on this antibody does not detect small Aβ 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 Aβ and α-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 Aβ protofibril specific ELISA in comparison with the ELISA used by Xia et al. 2009 [153], where the Aβ 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 Aβ-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 Aβ aggregates formed in vivo, AβPPArcSwe transgenic mouse brain homogenates were analyzed in the same set of control experiments. Here, no signals were obtained in the Aβ 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 Aβ protofibril ELISA and in the Aβ 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 Aβ aggregates, could greatly compromise the validity of research and clinical findings reliant on such immunoassays.

Characterization of soluble Aβ aggregates in mice and men

Recent AD research has given soluble Aβ 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 Aβ 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 Aβ 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 Aβ 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 immuno-precipitated from fraction 3, again suggesting the presence of oligomers lacking the structure recognized by mAb158.
Synthetic and biologically derived Aβ aggregates are formed in very different environments. In the brain, Aβ can interact with many other molecules, both during aggregation and as an aggregated protein and the Aβ concentration is in general different \textit{in vitro} and \textit{in vivo}. Moreover, biologically derived Aβ 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 Aβ aggregates from human and transgenic mouse brain extracts show important similarities to synthetic Aβ42 aggregates with regard to their size and distribution in the gradient as well as their binding to conformation dependent antibodies. This implies that soluble Aβ aggregates of large to intermediate size, the most toxic species \textit{in vitro}, are the predominant soluble Aβ species in the brains of AD patients and AβPP-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 Aβ protofibrils and the use of these antibodies in immunoassays. Therefore, a significant part of the work deals with the methodological concerns regarding Aβ interactions with antibodies. In addition, the aggregation prone amyloid-β peptide presents its own methodological complications.

Synthetic Aβ

Many aspects of AD research involve the use of the Aβ peptide in different forms. Studies of Aβ aggregation and structure require large quantities of high purity peptide, consequently recombinant or synthetic Aβ 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 Aβ 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 Aβ quality and aggregation rate vary a lot from different suppliers and even from batch to batch. Moreover, Aβ, especially Aβ42, 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 Aβ peptides with the Arctic E22G intra-Aβ mutation (AβArc), which has a higher rate of aggregation than wild type Aβ (Aβwt). It is therefore important to establish criteria for how to handle the peptide and define the different Aβ preparations used in a study.

In this project, the Aβ protofibril, made from either Aβ42Arc or Aβ42wt, has been used as the antigen for production and characterization of monoclonal antibodies (paper I and II) and for comparison with soluble Aβ aggregates generated in vivo (paper IV). Aβ 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 Aβ eluting in the void of a Superdex 75 column [95]. Non aggregated Aβ40 elutes with a peak corresponding to a mix of Aβ monomers-tetramers, when compared to SEC protein standards (Figure 8A-B). This preparation is therefore termed low molecular weight Aβ (LMW-Aβ). Typical appearance of Aβ protofibrils seen with cryo-TEM is shown in figure 8C.
Sample selection and preparation

While the first study is purely an in vitro study, the other studies deal with biological samples. Soluble Aβ 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 Aβ 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 AβPP processing and Aβ 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 AβPP with the Swedish mutation alone or in combination with the Arctic mutation, to increase Aβ production and/or aggregation. Studies of complex pathological processes, such as extracellular accumulation of Aβ or effects on cognition and memory, require animal models. We have mainly used an AβPP tg mouse model carrying a combination of the Swedish and Arctic mutations (AβPP_ArcSwe) under a Thy-1 promoter, resulting in a marked increase in Aβ production and aggregation in the brain. This leads to early intraneuronal accumulation of Aβ 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 Aβ protofibrils compared to mice with only the Swedish mutation. This result was reiterated [98] when this model was compared to two other AβPP tg mouse models widely used in AD research: tg2576 harboring the Swedish mutation [227] and PSAPP with mutations in both the AβPP gene and the PS-1 gene [228]. An advantage of the AβPP_ArcSwe transgenic model is that, compared to the AβPP_Swe model, its Aβ pathology is more similar to that of AD brain as is the stability of its Aβ deposits [229]. This is a feature of interest when the AβPP_ArcSwe 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-Aβ 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 Aβ 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 Aβ 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 Aβ aggregation proceeds via the addition of small Aβ species to a gradually growing fibrillar structure, samples presumably contain a wide spectrum of heterogeneous intermediates. Consequently, the cut-off point for soluble Aβ aggregates is somewhat arbitrarily defined by the centrifugation conditions. Generally, Aβ 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 Aβ aggregates, but due to the high levels of N-terminal truncation and low content of soluble Aβ 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 Aβ 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 phosphoribosyltransferase (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 Aβ 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 Aβ 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.

![Graph showing serum titers to Aβ protofibrils](image)

*Figure 9. Serum titers to Aβ protofibrils from mice immunized with Aβ 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 Aβ 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 Aβ protofibril reactivity. Mouse serum or hybridoma supernatants were then applied to a
plate coated with Aβ 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 Aβ species in synthetic Aβ 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 Kd by determining the concentration of antigen at IC50 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 Aβ protofibrils or LMW-Aβ, 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-Aβ and Aβ 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 IC50 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.

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

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

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

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).

**Aβ ELISA quantification**

ELISA is widely used for quantification of Aβ, both for research and diagnostic purposes. The most common Aβ species measured with ELISA are Aβ40 and Aβ42 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 Aβ40 or Aβ42 specific antibody in combination with an antibody against the Aβ N-terminus or mid-region. Conformation dependent ELISA quantification of Aβ demands a slightly different approach, either relying on a conformation dependent capture antibody or using the same Aβ antibody (recognizing either a linear or conformational epitope) for both capture and detection.

**Aβ40 and Aβ42 ELISA**

There are two main issues associated with ELISA quantification of Aβ40 and Aβ42. Firstly, total Aβ levels may be underestimated due to incomplete detection of the C-terminus of aggregated Aβ [148, 149]. This phenomenon is described schematically in figure 11A and is a result of the structure of Aβ aggregates, where the hydrophobic C-terminal amino acids are hidden in the core of the Aβ aggregate [201]. Thus, the number of epitopes available to antibodies recognizing the Aβ C-terminus is reduced. This problem can be solved by boiling samples in SDS to denature Aβ 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 Aβ aggregates, where the hidden Aβ C-termini (light shading) are inaccessible to the anti-Aβ40 or anti-Aβ42 coat antibody (A). After monomerizing Aβ aggregates with SDS denaturation, anti-Aβ coat antibodies can easily bind to their epitopes on the C-termini, which enhances Aβ 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 Aβ. This may cause underestimation of Aβ levels if measured with the wrong type of antibodies. An Aβ1-42 ELISA assay, based on an N-terminal and a C-terminal antibody will not be able to detect N-terminally truncated Aβ. In such a case the N-terminal antibody should be replaced by an Aβ mid-region antibody, thus measuring Aβx-42.

Aβ protofibril ELISA
The sandwich ELISA developed for quantification of Aβ 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 AβPP, prevents competition from more abundant molecules that could potentially block the ELISA signal. However, as indicated in paper IV, because mAb158 is Aβ protofibril selective, this ELISA does not detect smaller Aβ oligomers, which may well be present in CSF. Similar ELISA setups, which are not based on conformation selective antibodies, have been reported to bind Aβ 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 Aβ aggregates to be measured prove to be very small.

An issue that has to be considered when trying to quantify Aβ aggregates is their size and concentration. For quantification of Aβ aggregates, we have chosen to express their concentration as Aβ monomer units, as the size of the Aβ 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 Aβ standard protofibrils, we cannot say for sure that the number of conformational epitopes per Aβ
monomer unit is the same. This could be a potential problem if there is high variability in the size of Aβ 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].

![Diagram of forces acting on a particle during ultracentrifugation](image)

*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 Aβ containing sample applied to the top (A). After centrifugation, four fractions were collected (B) with a decreasing percentage of Optiprep (C).](image)

In paper IV, density gradient ultracentrifugation was employed to separate and fractionate Aβ 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 Aβ were applied on top and centrifuged at 384 000 x 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 Aβ aggregates in the gradient was related to their molecular weight.

When performing a study like this, where synthetic Aβ samples are compared to biological samples, a few considerations have to be acknowledged. The Aβ in brain extracts, even with a mild TBS extraction protocol, will contain many different proteins, lipids and polysaccharides. Consequently, this biological Aβ is surrounded by a complex matrix, which
is not present in samples of synthetic Aβ 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 Aβ 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 Aβ 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 nanomolar 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).

![AFM Diagram](image)

*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 Aβ aggregates [60, 65, 110, 239]. In paper IV, we used AFM to visualize fractionated Aβ 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 Aβ 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 Aβ 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 Aβ toxicity [63, 82, 93, 219], we treated PC-12 cells with fractions from our synthetic Aβ42 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 Aβ 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 Aβ peptide, resulting in aggregation and formation of toxic soluble Aβ 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 Aβ aggregates have been targeted with an immunologic approach, by generation of monoclonal antibodies, selectively binding to Aβ protofibrils in a conformation dependent manner. The IgG antibody mAb158 binds to protofibrils with a 200-fold higher affinity than to low molecular weight Aβ, but does not bind soluble AβPP 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 Aβ aggregates are detected in brain extracts from AβPP-transgenic mice and AD patients, without interference from AβPP fragments or monomeric Aβ. The aggregates found in AD and mouse brain are large, soluble Aβ species with an estimated molecular weight of 80-500 kDa. Synthetic aggregates of the same size proved more toxic than low molecular weight Aβ or aggregates larger than 500 kDa.

Targeting and characterizing the pathogenic Aβ 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 Aβ 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 Aβ 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 Aβ irrespective of conformation. In preliminary experiments, this new assay can actually detect minute amounts of Aβ 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 Aβ aggregates can be measured in CSF with a method similar to ours. In this study, the levels of Aβ 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 Aβ 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 Aβ 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 Aβ 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 Aβ 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 Aβ binding sites are currently in different stages of clinical trials. In this regard, the monoclonal antibody generated against Aβ 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 AβPPArcSwe-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.
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 skrumpar 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.


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 Aβ 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 Aβ, 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 Aβ från Alzheimerpatienter och transgena möss. Med hjälp av ultracentrifugerings separerades de lösliga Aβ-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 protofibrillanalysmetod 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 Aβ-aggregaten i våra hjärnprover motsvarade protofibrill i storlek och att aggregat i denna storlek var mest giftiga för celler. Dessa aggregat fick dessutom att fånga upp med vår protofibrillbindande antikropp, vilket visar att de till sin struktur liknar de syntetiska protofibriller vi använt för att skapa antikropparna.

Att utröna vilken typ av lösliga Aβ-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 Aβ-formerna i CSF från Alzheimerpatienter. Detta är viktigt för att en eventuell behandling ska kunna sättas in tidigt och stoppa nervcellsdöden 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 Aβ-formerna i hjärnan kan man således förhoppningsvis förhindra en fortsatt sjukdomsutveckling. Det kan till exempel åstadkommast 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...
All the work was carried out at the Department of Public Health and Caring Sciences at the Rudbeck Laboratory, Uppsala University and at Mabtech AB in Nacka Strand. I am grateful for financial support from the Swedish Research Council, Bertil Hållstens forskningsstiftelse, Hjärnfonden, Stohnes stiftelse, Emma Petterssons testamente and Uddeholms stiftelse.

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Dag Sehlin, Uppsala, November 2010
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