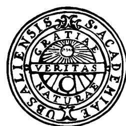

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Soluble amyloid-β aggregates in Alzheimer's disease

HILLEVI ENGLUND

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Abstract

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Soluble oligomeric aggregates of the amyloid-β (Aβ) peptide are suggested to initiate Alzheimer's disease (AD), leading to impaired synapse signalling, widespread neuronal death and loss of cognitive functions. These aggregates seem tightly linked to disease progression, and have therefore gained much attention as potential novel disease markers. In this thesis soluble oligomeric Aβ aggregates in general, and the Aβ protofibril species in particular, have been investigated with the aim to quantify and determine their role in AD pathogenesis.

Sandwich-ELISAs specifically measuring $A\beta_{42}$ peptides are widely used both in AD research and as complements for clinical diagnosis. Here it was demonstrated that presence of soluble Aβ aggregates disturbs such analyses, making it difficult to interpret the results. This discovery was made through analyses of samples from cell- and mouse models carrying the AD causing 'Arctic' APP mutation. When analyzed by ELISA, $A\beta_{42}$ levels were reduced in Arctic samples, in contrast to levels measured by denaturing SDS-PAGE Western blot. The same divergence in $A\beta_{42}$-levels between analyses was observed in CSF samples from Down syndrome infants. The discrepancy between methods was hypothesized to be due to presence of soluble Aβ aggregates leading to impaired ELISA detection caused by epitope masking. This was confirmed by developing a protofibril specific ELISA, by which samples from Arctic cell- and mouse models were demonstrated to have enhanced Aβ protofibril levels.

AD patients have reduced ELISA-measured $A\beta_{42}$-levels in CSF compared to healthy controls. To test if this reduction was due to oligomeric Aβ species present in AD CSF, $A\beta_{42}$-levels were analyzed under both denaturing and non-denaturing conditions. These two measures were combined and an $A\beta_{42}$ oligomer ratio established. Higher ratios were found in AD patients than healthy controls, implying that Aβ oligomers are present in CSF during Alzheimer pathogenesis. The observations from AD patients and young Down syndrome individuals suggest that $A\beta_{42}$ oligomer formation is an early mechanism of AD pathogenesis, which potentially could be used as a biomarker to monitor disease development.

Keywords: Alzheimer's disease, amyloid-beta, protofibrils, ELISA

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List of papers

This thesis is based on the following four papers which are referred to in the text by their roman numerals (I-IV).

I Stenh C, **Englund H**, Lord A, Johansson AS, Almeida C.G, Gellerfors P, Greengard P, Gouras G.K, Lannfelt L and Nilsson L.N. Amyloid-beta oligomers are inefficiently measured by enzyme-linked immunosorbent assay *Ann Neurol 2005;58:147-150*

II **Englund H**, Annerén G, Gustafsson J, Wester U, Wiltfang J, Lannfelt L, Blennow K, Höglund K. Increase in β-amyloid levels in cerebrospinal fluid of children with Down syndrome *Dement Geriatr Cogn Disord. 2007;24(5):369-74*

III **Englund H***, Sehlin D*, Johansson AS, Nilsson L.N.G, Gellerfors P, Paulie S, Lannfelt L, Ekholm Pettersson F. Sensitive ELISA detection of amyloid-β protofibrils in biological samples *J Neurochem. 2007 Oct;103(1):334-45*

IV **Englund H**, Degerman Gunnarsson M, Brundin RM, Hedlund M, Kilander L, Lannfelt L, Ekholm Pettersson F. The reduction of Aβ1-42 in Alzheimer cerebrospinal fluid is partially due to oligomerization *Submitted*

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Abbreviations

Aβ	Amyloid-β
AD	Alzheimer's disease
ADDL	Aβ derived diffusible ligand
AICD	APP intracellular domain
APOE/apoE	Apolipoprotein E (gene/protein)
APP	Amyloid-β precursor protein
Arc	Arctic mutation (APP E693G, Aβ E22G)
αAPPs	Soluble α-secretase cleaved APP fragment
βAPPs	Soluble β-secretase cleaved APP fragment
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CSF	Cerebrospinal fluid
DLB	Lewybody dementia
DS	Down syndrome
ELISA	Enzyme linked immuno-sorbent assay
FTD	Frontotemporal dementia
HAMA	Human anti-mouse activity
HRP	Horse radish peroxidase
IDE	Insulin degrading enzyme
IgG	Immunoglobulin G
LMW-Aβ	Low molecular weight Aβ
LTP	Long term potentiation
mAb	Monoclonal antibody
MCI	Mild cognitive impairment
MMSE	Mini-mental state examination
NFT	Neurofibrillary tangle
PET-PIB	Positron emission tomography with Pittsburgh compound B
PrP	Prion protein
PS	Presenilin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC-HPLC	Size exclusion chromatography on high performance liquid chromatography
SOD1	Superoxide dismutase 1
SPR	Surface plasmon resonance
Swe	Swedish mutation (APP K670N/M671L)
tg	transgenic
wt	wild type

Introduction

Protein misfolding, amyloidoses and neurodegeneration

Proteins are built of amino acid residues connected in chains by peptide bonds and these molecules are essential for almost every biological function. Normal protein interactions depend on a correct three dimensional structure of the protein, achieved during native folding. The primary amino acid sequence of a polypeptide chain contains the information needed for proper folding [1], and correct native folding seems to be the most energetically favorable state. Folding, especially of larger proteins, is facilitated by molecular chaperones, which bind to folding intermediates in the cytoplasm and thereby prevent misfolding [2]. If proteins do become misfolded cellular pathways, such as the ubiquitin-proteasome system, recognize and degrade dysfunctional proteins to prevent them from causing harm [3]. Therefore, correct folding of polypeptide chains and rapid degradation of misfolded ones are crucial mechanisms for maintaining vital cell functions.

Misfolded proteins, which a cell fails to degrade, may start to aggregate due to for example exposure of hydrophobic amino acids. These aggregates can form large insoluble deposits intracellularly and extracellularly, deposits which can progress to amyloid. Amyloid, first described by Rudolf Virchow in 1854, is by definition stained by Congo red dye and is typically composed of long straight unbranched fibrils with a diameter of ~100Å, built up of 4-6 parallel protofilaments having a cross β-sheet structure [4]. Amyloidoses, diseases characterized by amyloid deposition, represent a diverse family of both systemic and tissue-specific diseases. Today more than 25 proteins are known to spontaneously form amyloid and disease in humans [5], but it has been suggested that the ability of proteins to form amyloid fibers is a generic feature, although the propensity to do so varies substantially [6].

Protein deposition and misfolding is a common feature of many neurodegenerative diseases (examples in Table 1), where some of the deposits fulfill the amyloid criteria. A general misfolding mechanism, which shares common pathological features regardless of the primary amino acid sequence, has been suggested (*Figure 1*) [reviewed in 7]. Therefore, molecular studies and experiences with novel therapeutics for one disease could potentially lead to increased understanding of many of the diseases [8].

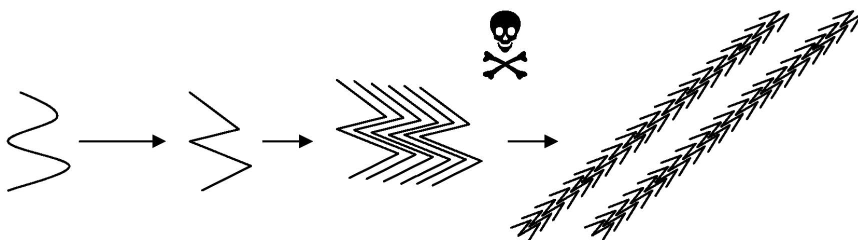

Figure 1. General mechanism suggested for protein misfolding diseases where a natively folded protein becomes misfolded and starts to aggregate into toxic soluble aggregates and insoluble fibrillar deposits.

Table 1. *Neurodegenerative diseases linked to protein misfolding*

Disease	**Neuropathology**	**Proteins**
Alzheimer's disease	Senile plaques, CAA, NFTs	Aβ and Tau
Amyotrophic lateral sclerosis	Intracellular inclusions	SOD1
Creutzfeldt-Jakob's disease	Prion plaques	PrP
Frontotemporal dementia	NFTs, Pick bodies, TDP43-bodies	Tau, TDP-43, ubiquitin
Huntingtons disease	Intracellular inclusions	Huntingtin
Lewy body dementia	Lewy bodies, senile plaques	α-synuclein, Aβ
Parkinson's disease	Lewy bodies	α-synuclein

Alzheimer's disease

Several of these neurodegenerative disorders are dementias as defined by their common feature – the progressive impairment of cognitive functions – though the symptoms differ depending on which brain region is affected. Alzheimer's disease (AD), Vascular dementia, Frontotemporal dementia (FTD) and Lewy body dementia (DLB) are the most common diagnoses. AD is by far the most common, accounting for at least two thirds of all dementia cases and affecting 15 million people worldwide [9].

Neuropathologically, Alzheimer's disease is characterized by a cortical atrophy with widened sulci and enlarged ventricles. Two microscopic lesions, first described by Alois Alzheimer (1864-1915) in 1906, are observed in the cerebral parenchyma: extracellular senile plaques and intracellular neurofibrillary tangles (NFTs) (*Figure 2*) [10]. These neuropathological hallmarks are, together with inflammation, dystrophic neurites, synapse and neuronal loss, activated microglia and astrocytes as well as cerebral amyloid angiopathy (CAA), typically associated with AD neuropathology.

AD primarily affects the temporal and parietal cortex, and the disease progression often starts with neuronal degeneration in the hippocampus and entorhinal cortex [11]. Subsequently, loss of short-term memory is an early symptom. During the course of the disease the cognitive decline continues and symptoms arise depending on which area of the brain is affected. Examples of symptoms are language impairment, problems with spatial abilities and an incapability to manage ordinary activities of daily life.

AD patients are traditionally divided into subgroups depending on age at onset, where an onset before 65 years of age is denoted as early onset AD and after 65 as late onset. Although only a few per cent of all cases have an identified genetic cause (e.g. mutation), approximately 25% of all AD cases are considered to be familial, i.e. when two or more family members are affected [12].

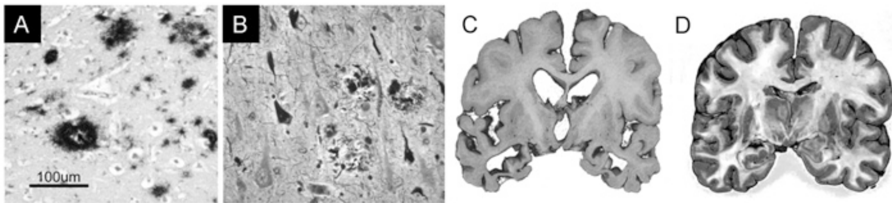

Figure 2. The pathological hallmarks of the Alzheimer's disease brain are extracellular senile plaques immunohistochemically stained for amyloid-β (**A**), and intracellular neurofibrillary tangles visualized by Bielschowsky silver staining (**B**). The widespread nerve cell death is observed macroscopically in AD (**C**) as compared to aged healthy controls (**D**) and seen as hippocampal atrophy, widening of sulci and enlargement of ventricles. Pictures kindly provided by Dr Hannu Kalimo.

Diagnosis and treatment

The definitive AD diagnosis is made by *post mortem* analysis of Aβ plaque load and amount of NFTs in affected brain areas [13, 14]. The clinical diagnosis “probable AD” is made from the medical history and through thorough clinical examination of symptoms according to the DSM-IV [15] and NINCDS-ADRDA [16] criteria (Table 2). In addition, there is a battery of different tests which can be used for evaluation of cognitive status, where Mini-Mental State Examination (MMSE) is widely used for AD patients [17]. The maximal MMSE score is 30 and patients with a result below 26 are considered cognitively impaired [18]. Brain imaging techniques and evaluation of biomarker levels in cerebrospinal fluid (CSF) are utilized as a complement to the clinical examination and to exclude other disorders causing dementia-like symptoms, such as tumors or malnutrition. The accuracy of the clinical AD diagnosis can be up to ~90 % when compared to neuropathologically confirmed AD [19, 20].

AD is often preceded by mild cognitive impairment (MCI), which is classified as a state where an individual has problems with cognitive functions and when there is evidence of memory impairment, but the symptoms do not fulfill the criteria of dementia [21, 22]. MCI can be benign and reversible, remain stable or represent incipient AD. At least one third of MCI develop AD over a few years follow-up [23, 24].

Even though many novel and promising therapeutic strategies for AD are being evaluated, to date there is no cure for AD. Available treatments are one N-methyl D-aspartate (NMDA) receptor antagonist and several acetylcholine esterase inhibitors, which are used as a temporary help to maintain mental functions by modulating neurotransmitter levels, but fail to stop the underlying neurodegenerative processes.

Table 2. *Examples of DSM-IV and NINCDS-ADRDA criteria*

DSM-IV **"Dementia of the Alzheimer type"**	**NINCDS-ADRDA** **"Probable AD"**
Development of cognitive deficits and memory impairment, insidious onset which precedes in a progressive manner	Dementia established by clinical and neuropsychological examination
At least one of: aphasia, apraxia, agnosia or disturbance in executive functioning	Absence of other conditions that could cause dementia symptoms
The cognitive decline cause significant impairment in social or occupational functioning	Progressive impairment of memory and cognitive functions in two or more cognitive areas
Other causes of dementia are excluded	Disease onset between 40 and 90 years of age

Risk factors

Alzheimer's disease is most likely a disease with multifactorial origin where combinations of genetic variances and environmental factors lead to disease development. Getting old is the major risk factor for AD and the number of cases increases markedly with advancing age. The prevalence is 10 % among people aged 70, which increases to 50 % at an age of 85 [25, 26]. Genetic factors are important, as first-degree relatives of AD patients have an increased risk of developing the disease [27]. Several genes have been suggested to increase risk for AD, but except for the APOE gene, results have been difficult to replicate. Polymorphisms of the APOE gene are the strongest identified genetic risk factor for AD, where carriers of the ε4 allele have an increased risk and the ε2 allele seems to reduce the risk [28, 29]. The apoE protein is present in the senile plaques, binds to cholesterol and is suggested to be involved in cholesterol homeostasis [30].

Even though AD cases may be considered familial, most of them do not have an identified genetic cause. There are however rare cases of families

where autosomal dominant inherited point mutations lead to AD. Mutations have been identified in three different genes: the amyloid-β precursor protein (APP), presenilin (PS) 1 and 2 genes, which will be further described below.

Among possible disease causing environmental factors, head trauma, depression, obesity, high cholesterol levels, diabetes and low education have been associated with increased risk of developing AD [31-36]. Conversely, high consumption of fruits, vegetables and omega-3, a moderate coffee intake and a rich social life have been suggested to lower disease risk [37-39].

Molecular mechanisms of AD pathogenesis

It took almost 80 years from the first description of senile plaques until the major component of plaques and CAA was identified. In 1984 Glenner and Wong isolated a peptide, later called amyloid-β (Aβ), from CAA [40] and within a year it was concluded that Aβ was the main component of senile plaques in AD and Down syndrome (DS) brain as well [41].

APP processing

The Aβ peptide is a metabolite of a larger membrane bound precursor protein, APP, which is processed by different secretases into several soluble and membrane bound products. During intracellular transport and at the cell membrane APP can be processed by three secretases: α-, β-, and γ-secretase [reviewed in 42] along two major pathways: the amyloidogenic or the non-amyloidogenic (Figure 3). β- and γ-cleavage leads to Aβ formation, whereas

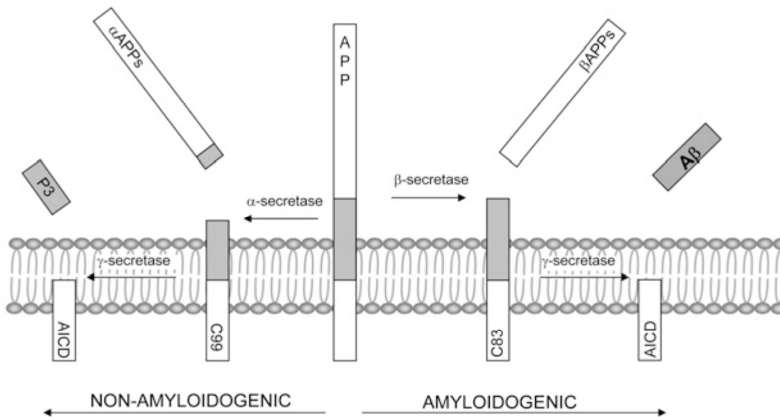

Figure 3. The membrane bound APP molecule can be processed in two ways: by α- and γ-secretase forming non-amyloidogenic end-products, or by β- and γ-secretase resulting in the release of the amyloid-forming Aβ peptide.

α-secretase cleaves between amino acid 16 and 17 in the Aβ sequence and prevent Aβ production. β- and α-cleavage also produce large soluble secreted fragments of APP: αAPPs and βAPPs, which can be found in CSF [43, 44]. The function of APP and its derivates remains elusive, but since APP is located in the cell membrane it has been suggested to be a cell surface receptor [45]. APP knockout mice are viable [46], perhaps due to presence of the homologous APP like proteins (APLPs) [47].

Amyloid-β

The γ-secretase is a protein complex which exerts its activity within the cell membrane and determines the C-terminal truncation of Aβ, where $Aβ_{38}$, $Aβ_{40}$ and $Aβ_{42}$ are the most common truncations [48, 49]. CAA is mainly composed of $Aβ_{40}$, whereas $Aβ_{42}$ is most abundant in parenchymal plaques [50, 51]. $Aβ_{42}$ is also the first species deposited in the brain often as "diffuse plaques", considered by some to be the precursors of mature senile plaques [51, 52]. The non-amyloidogenic pathway is the predominant way for APP-processing, but still the total amount of Aβ produced through the amyloidogenic APP processing over a 70 year lifespan has been estimated at 1-3 kg [53]. Half-life of Aβ in transgenic mouse brain have been estimated to ~2h [54] and Aβ clearance is for example mediated by proteases such as insulin degrading enzyme (IDE) [55] and neprilysin [56].

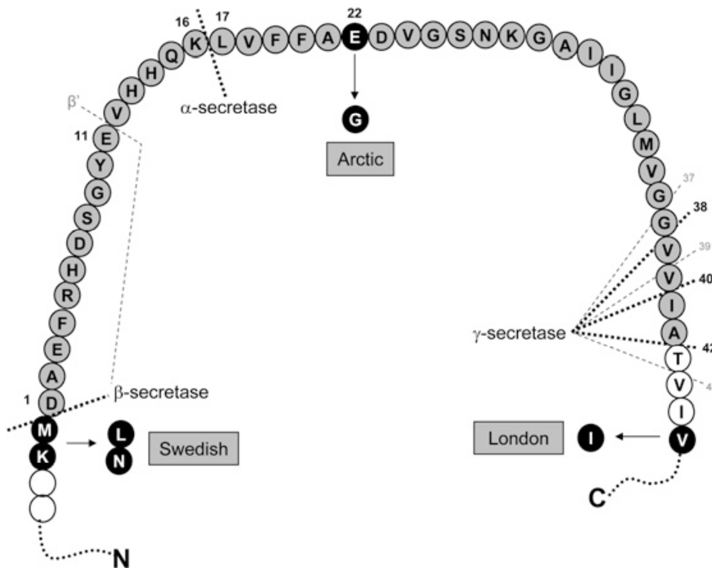

Figure 4. The Aβ peptide sequence flanked by APP mutations which increase amyloidogenic processing. Secretase cleavage sites are marked as dotted lines and examples of familial APP mutations are marked in black.

Although Aβ is an identified part of AD pathology, it seems to have a biological function during non-disease conditions as it is detectable in biological fluids from non-AD persons as well as in non-neuronal cells [57-59]. Interestingly, recent studies report that picomolar concentrations of Aβ actually stimulate synaptic plasticity, suggesting a dual effect of Aβ depending on its concentration [60]. Concentrations of Aβ in the interstitial fluid of the brain have also been reported to increase as the human brain recovers following acute injury, implying a physiological role for Aβ [61].

Aβ aggregation and soluble Aβ aggregates

The Aβ peptide has a high propensity to self-aggregate both *in vivo* and *in vitro*. This propensity has been attributed to its hydrophobic C-terminal and the KLVFF motif in the mid-region of the peptide, and an extended C-terminal further increases the ability to form aggregates [62, 63]. Having been cleaved out of the APP protein, Aβ can be released to the extracellular space or retained intracellularly [64, 65]. Since Aβ plaques are extracellular deposits, the aggregation process of Aβ has been thought to be an extracellular event. Intracellular accumulation of Aβ has however been reported in both human brain [66, 67] and transgenic mice [68, 69], indicating that the aggregation process *in vivo* may start intracellularly.

Aβ aggregation *in vitro* has been studied thoroughly and synthetic Aβ spontaneously forms fibrils which resemble the *in vivo* formed fibrils found in plaques. The Aβ aggregation process is a nucleation dependent process with three main characteristics: a lag phase where a nucleus is formed, a critical concentration below which aggregation will not occur and the potential to be seeded [70]. During seeding, preformed aggregates act as nuclei for the aggregation shortening the lag phase. The process is thought to proceed through several soluble intermediates before the insoluble fibrils are formed (schematically illustrated in *Figure 5*). However, some soluble aggregates – for example globulomers and amyloid derived diffusible ligands (ADDLs) – are described as aggregational end-states rather than intermediates, possibly due to differences in β-sheet arrangement [71].

Several different forms of oligomeric Aβ species have been reported, ranging from 8 kDa dimers to >100 kDa protofibrils (Table 3). Most of the smaller aggregates are described as spherical, whereas the protofibril is an elongated structure. Despite the many different names of these *in vitro* aggregates, they share one common – and important – characteristic: they are all soluble. This, together with their size, set them apart from the large insoluble fibrils which partitions to the pellet during high speed centrifugation. Due to methodological limitations, the aggregation have mainly been studied *in vitro* where concentrations sufficient for detectable aggregation can be obtained. Hence, it is important to remember that some of the described species are still only proven to be a part of the *in vitro* aggregation process,

whereas the details of the *in vivo* process are unclear. However, the recent development of conformation specific antibodies that can specifically detect certain aggregation states have greatly improved the chances of unraveling the processes *in vivo*, and will be further discussed later in this thesis.

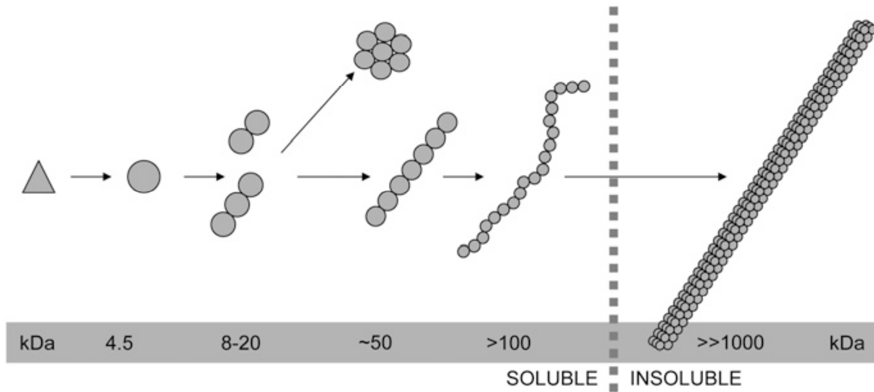

Figure 5. Schematic view of the *in vitro* aggregation process of Aβ. The monomer becomes misfolded and aggregates into low-molecular weight oligomers like dimers, trimers or tetramers. There are several larger soluble aggregates reported, e.g. spherical ADDLs and elongated protofibrils. Some oligomeric species are described as aggregational end-states, whereas others eventually form long, unbranched and insoluble fibrils.

Table 3. *Soluble Aβ aggregates*

Name	Properties	Reference
Dimers – tetramers	8-20 kDa, biologically derived, SDS-stable, LTP-inhibiting, present in human brain and CSF	[72-77]
ADDLs	~10-20mer, >40 kDa, spherical, LTP-inhibiting, present in human brain and CSF, induce tau phosphorylation	[78-82]
Oligomers	15-20mer, >40 kDa, spherical, neurotoxic, present in human brain	[83, 84]
Aβ*56	56 kDa, correlates with memory impairment in tg mice, impairs spatial memory in rats	[85, 86]
Globulomers	~60 kDa, globular, LTP-inhibiting, present in human brain	[87, 88]
Protofibrils	>20mer, >100 kDa, linear with beaded appearance, neurotoxic, impairs LTP, present in cell culture media, tg-mouse brain and human AD brain (unpubl. data), correlates with memory deficits in tg-mice	[89-95]
Aβ spheroids	Synthetic, 10-15 nm in diameter, neurotoxic, induce tau phosphorylation	[96]
Annular aggregates	Synthetic, 150-250 kDa, 7-10 nm in diameter	[97, 98]

The protofibril

In 1997 a semi-stable intermediate termed 'protofibril' was identified during *in vitro* Aβ fibrillogenesis [89, 90]. The Aβ protofibril was described through electron microscopy studies as an elongated structure with a diameter of 4-10 nm and a length of 200 nm. It is toxic to cells *in vitro* [93, 99, 100], impairs LTP [101] and correlates with cognitive deficits prior to plaque onset in young transgenic mice [94]. Other amyloidogenic proteins like α-synuclein, huntingtin, transthyretin and ABri also form protofibrillar structures during aggregation [102-106].

Mutations and duplications

In the early 1990's the first autosomal inherited APP mutations were discovered and today over 20 have been identified. Following the APP mutations two more genes causing early onset familial AD were described: PS1 on chromosome 14 encoding presenilin protein 1, and PS2 on chromosome 1 encoding presenilin protein 2 [reviewed in 107].

The APP gene is located on chromosome 21 [108] and accordingly, Down syndrome (DS) patients are among the youngest to develop AD like pathology and do so almost invariably during their fourth decade of life [109, 110]. Similar to DS patients, duplications of the APP gene [111], and possibly also variants in the promoter sequence increasing APP expression [112], cause AD in a few early onset families.

The Arctic mutation

The intra-Aβ mutation, AβE22G ($A\beta_{Arc}$) or APPE693G (APP_{Arc}), was identified in a Swedish family with hereditary AD [113]. Contrary to other known familial AD-causing mutations, APP_{Arc} does not lead to increased levels of plasma-Aβ, nor does it increase $A\beta_{42}$-formation in the mutation carriers. Instead, decreased $A\beta_{40}$- and $A\beta_{42}$-levels are found in plasma and in cell culture models expressing the Arctic mutation [113, 114]. The biochemical effect of this mutation, where the charged glutamic acid is substituted for an uncharged glycine, is an accelerated formation of protofibrils *in vitro*, for both $A\beta_{40Arc}$ and $A\beta_{42Arc}$ [113, 115]. Human APP with the Arctic mutation has been used to generate transgenic mouse models (tg-ArcSwe) [69], which show increased levels of soluble Aβ aggregates *in vivo* [91, 94].

Toxicity of Aβ

There is a widespread neuronal death and synapse loss in an AD brain. Aβ was reported to be toxic to cultured cells in the late 1980's and it soon became apparent that aggregated, or "aged", Aβ was the most toxic [116]. Much effort has been devoted to find the *most* toxic species, and now focus

is on soluble intermediates of Aβ aggregation as the principle mediators of Aβ toxicity. Accordingly, many of the described soluble oligomeric species have been shown to mediate neurotoxicity in cell cultures [78, 87, 95, 96, 99, 117, 118]. When fractionating Aβ, Ward *et al* demonstrated that protofibrils and fibrils, but not monomers, were toxic to cultured neurons [100]. In addition to direct toxicity studies, different electrophysiological measures have been used to reflect other changes possibly associated with synaptic dysfunction in AD brain. Long term potentiation (LTP) in cultured rat brain slices is linked to synaptic strengthening which is thought to be involved in memory formation, and several of the oligomeric species described impair LTP [72, 74, 80, 101]. Moreover, synapse loss correlates with degree of dementia [119] and soluble Aβ levels correlate with synapse loss in *post mortem* AD brain [120]. Therefore, part of the neurotoxicity is suggested to be caused by soluble Aβ aggregates interfering with synapses. Koffie and colleagues recently demonstrated that oligomeric Aβ surrounds plaques in APP-transgenic mouse brains, and that there is a 60% reduction of excitatory synapses in this 'oligomeric Aβ halo' [121].

As with the Aβ aggregation studies, most toxicity studies are made *in vitro* using cell cultures and synthetic Aβ at non-physiological conditions. In addition, there are differences between toxicity assays and cell lines used, and also difficulties in reproducing results with different production lots of synthetic Aβ, which have made toxicity studies hard to interpret. To circumvent this, some research groups have focused on isolating oligomers from biological samples. For example, small oligomers – dimers and trimers – isolated from cell culture media, human CSF and human brain samples have an LTP-lowering effect [72-76]. Additionally, *in vivo* levels of the soluble Aβ species denoted Aβ*56 correlated with memory impairment in transgenic mice [85].

Several mechanisms of how the Aβ-induced toxicity is mediated have been proposed, such as interference with cell membrane integrity, disturbed ion homeostasis, induction of apoptosis, oxidative stress, mitochondrial dysfunction, interaction with apoE and sequestration of cellular proteins [93, 118, 122-127]. Also, it has been reported that different types of aggregates induce toxicity via different mechanisms [84, 128]. Protein aggregation have been suggested to result in both a gain of toxic function, and a loss of physiological function [129, 130]. For Aβ, aggregation may prevent it from exerting its normal function and thus the toxicity could additionally be due to loss of normal function, rather than gain of toxic function. A loss of normal function has been suggested as a pathogenic mechanism for PS mutation carriers [reviewed by 131, 132]. Of course, these mechanisms are not mutually exclusive and any or all could be involved during AD pathogenesis, simultaneously or at different stages of the disease.

The amyloid cascade hypothesis

Neuropathological findings in AD and DS brain, together with identification of Aβ, genetic mapping of the APP-gene to chromosome 21 and early evidence of mutations causing familial AD resulted in *the amyloid cascade hypothesis* [133]. There it was hypothesized that the insoluble fibrillar Aβ deposits cause dementia. However, histological measurements of Aβ plaque load in post mortem brains have been contradictory with regard to correlating plaque load and degree of dementia [134, 135]. In addition, plaques are also observed in brains of cognitively healthy elderly people [136-138], supporting the main criticism against the hypothesis – that plaque load does not correlate directly with disease severity.

With the identification of several toxic soluble aggregation intermediates, the aggregation prone Arctic mutation, the observation that soluble Aβ levels correlate better with disease progression than plaque load [120, 139-141] and the report that APP transgenic mice could display cognitive deficits prior to plaque onset [142], the amyloid hypothesis was refined, bringing soluble oligomeric Aβ into focus [143] (Figure 6). Converging evidence points at soluble Aβ aggregates as being the initiators of AD pathology. For example, two recent studies both support the hypothesis of Aβ oligomers as disease initiators, one demonstrating the close relationship between oligomeric Aβ and synapse loss [121], and one showing that increased Aβ degradation in mice reduce plaque burden but not oligomeric Aβ levels and the cognitive deficits remained [144]. Similarly, mice with reduced oligomeric levels but intact plaque load had no cognitive impairment [86]. This suggests that therapeutic strategies aiming at reducing Aβ preferably would be directed towards Aβ oligomers.

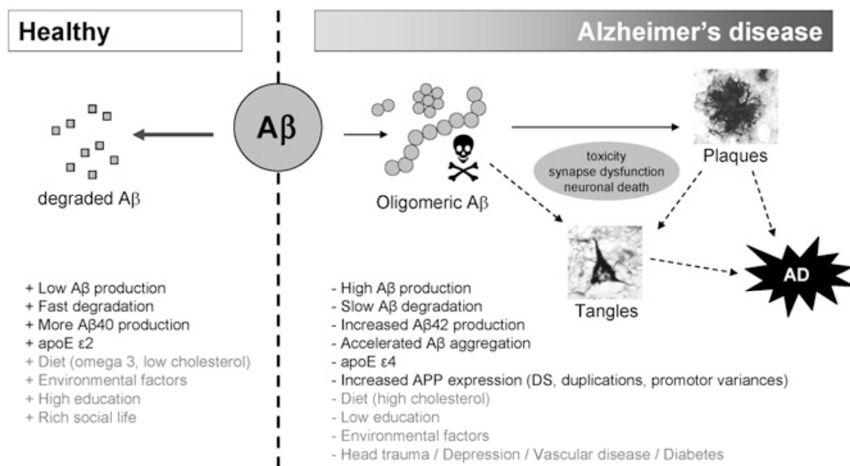

Figure 6. The amyloid cascade hypothesis suggests that an increased Aβ aggregation causes AD. Several factors have been suggested to be both protective and confer enhanced risk for AD by influencing Aβ levels or its aggregation in some way.

The molecular mechanisms of familial AD with identified genetic causes (for examples of mutations, see *Figure 4*) have been suggested to be:

- overproduction of total Aβ as for Swedish mutation carriers [145], APP-duplications and DS patients
- a selective increase in $Aβ_{42}$-levels, as for the PS and London-like APP mutations [146]
- production of mutant Aβ with increased aggregation rate, as for intra-Aβ-sequence mutations like the Arctic mutation [113]

These all lead to increased Aβ concentration and aggregation, which fits well with the amyloid cascade hypothesis. In concordance, the apoE ε4 protein promotes Aβ aggregation *in vitro* whereas ε2 enhances Aβ clearance [147-149]. Nonetheless, the majority of AD cases are patients with no evidence of a genetic cause and for these the Aβ elevating mechanisms leading to a chronic imbalance between Aβ production and clearance remain to be proven. Overproduction of Aβ could be caused by unknown genetic alterations leading to increased APP-expression or by altered enzymatic activity of α-, β- or γ-secretases causing a shift towards amyloidogenic APP-processing. An increase in Aβ concentration could also be caused by impaired clearance, due to reduced activity of Aβ degrading enzymes, or to proteolytic resistance which has been demonstrated for aggregated Aβ [150]. A genetic support of impaired clearance was suggested when a linkage was found between the IDE-region of chromosome 10q and AD [151]. *In vitro* aggregation is influenced by several factors, e.g. fatty acids, pH and metal ions [92, 152], thus environmental and dietary factors could also influence Aβ aggregation and accumulation *in vivo*. There is also a suggestion that the body's ability to produce anti-Aβ antibodies, capable of increasing clearance and attenuating toxicity, could be a protective mechanism [153].

Tau

The second pathological hallmark of AD is the neurofibrillary tangle composed of hyperphosphorylated fibrillar tau. Tangle formation is generally believed to be a secondary event preceded by Aβ amyloid pathology. This is supported by family based genetic studies where mutations in the APP gene lead to AD, whereas mutations in the tau gene lead to FTD, a dementia largely devoid of Aβ amyloid pathology. Consistent with the amyloid cascade hypothesis, Aβ pathology precedes tau tangle formation in a transgenic mouse carrying mutated human APP, PS1 and tau [154, 155]. Links between Aβ and tau have been suggested, e.g. Aβ oligomers induce tau phosphorylation in cell culture models [82].

The tau protein is associated with microtubules, with three or four microtubule binding repeats. Tau facilitates polymerization of tubulin into micro-

tubules and stabilizes them. When tau becomes hyperphosphorylated it has low affinity for microtubules, leading to destabilization of microtubules and impaired axonal transport. The phosphorylated tau can aggregate into paired helical filaments and form intracellular neurofibrillary tangles [reviewed in 156]. Levels of tau in CSF are a general marker of neuronal damage and axonal degeneration and is ~300% higher in AD than in controls [157]. Tau phosphorylated at amino acid 181 can also be quantified in CSF, and seems to reflect the amount of NFTs rather than neurodegeneration *per se* [158].

Present investigations

Aim of the thesis

The aim was to develop novel techniques to measure soluble Aβ aggregates, and to use these methods to quantify such species in samples from Alzheimer's disease, Down syndrome and AD models. This to gain deeper insights into how these species relate to the pathogenesis and development of dementia.

The specific aims were:

- use the aggregation prone Arctic mutation to test if common Aβ analyses are affected by presence of soluble Aβ aggregates in the sample

- to analyze Aβ species in CSF from young DS patients, as a model of very early events in Alzheimer's disease

- to create an Aβ antibody with a conformation dependent epitope and use it to establish a novel Aβ protofibril specific ELISA

- to use cell- and mouse models carrying the Arctic mutation to validate the Aβ protofibril specific assay, since the Arctic mutation is known to facilitate Aβ oligomerization

- to compare soluble oligomeric Aβ content in CSF from patients with Alzheimer's disease, other dementias and healthy controls

Paper I

Aim

The aim of the study was to test if C-terminal specific Aβ ELISAs were affected by the presence of aggregated Aβ species in a sample and if this could potentially lead to misinterpretations of results.

Background

Contradicting observations for the Arctic mutation

Even though β-secretase cleavage was found to be elevated in APP_{ArcSwe} transfected cells [114], Aβ levels measured by C-terminal specific Aβ ELISA were lower in the cell media compared to cells carrying the Swedish mutation alone [113]. Two possible explanations for these contradicting observations were suggested:

- increased β-cleavage without increased extracellular Aβ levels could be due to Aβ being retained within the cell due to e.g. rapid intracellular aggregation
- decreased extracellular Aβ levels as measured by ELISA could be due to a methodological artefact caused by the presence of oligomeric Aβ, since the Arctic mutation had been shown to facilitate Aβ oligomerization *in vitro* [113, 115]

Methodological considerations

Antibody based quantitative immunoassays

Antibodies with affinity for a specific antigen are often utilized for specific detection in various immunoassays. Two antibodies directed against the same antigen enables the establishment of a sandwich ELISA, as first described by Engvall and Perlman in the early 1970s [159]. In a sandwich ELISA antibodies against the antigen of interest are immobilized (coated) on a solid phase. This "capture antibody" is used to capture and retain antigens present in samples. A second antibody, specific for the same antigen, is used to detect the bound antigen-antibody complex. The amount of captured antigen can be measured by covalently binding an enzyme to the detection antibody. Commonly used enzymes include horse-radish peroxidise (HRP) and alkaline phosphatise (ALP) (Figure 7). The enzyme converts its substrate to a coloured product which can be measured spectrophotometrically. Quantification of antigens in samples can be achieved by preparing a standard curve of the antigen of interest diluted to known concentrations plotted against recorded absorbance. To avoid unspecific signals and to increase sensitivity

of the assay, washing steps are performed and detergents that reduce low-affinity protein-protein interactions, e.g. Tween 20, are added in low concentrations to the buffers. Plates are blocked with an excess of an unrelated protein, usually bovine serum albumin (BSA), which may also be included in incubation buffers to minimize unspecific binding. For ELISAs of human samples, especially plasma and serum, it is important to use optimized buffers as sample diluent, to reduce the effect of human anti-mouse activity (HAMA). HAMA is caused by human antibodies with the ability to cross-bind the two ELISA antibodies and thereby produce a false positive signal (*Figure 7*C). Presence of human anti-mouse-IgG-antibodies leading to HAMA is common in plasma and can be avoided by diluting samples in a buffer containing large excess of unrelated IgG antibodies [160, 161].

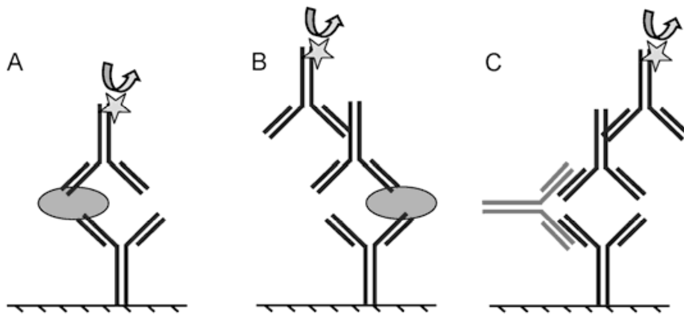

Figure 7. The principle of sandwich ELISA where the immobilized capture antibody binds to antigen from the sample and the detection antibody binds to the captured antigen. The detection antibody can be directly linked to an enzyme (A). Alternatively an additional enzyme-linked antibody, specific for the Fc-part of the detection antibody, is used (B). The HAMA-effect occurs when an antibody in human samples (in grey) cross-binds the ELISA sandwich antibodies, leading to a false positive signal (C).

Sandwich ELISAs are widely used for many kinds of antigens and included in routine diagnostics of several diseases. For AD, the specific ELISA quantification of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides has been instrumental in many aspects of AD research and are today available as commercial kits. The $A\beta_{1-42}$ ELISA is also used clinically. These ELISAs are based on antibodies raised against the neo-epitopes formed when γ-secretase cleaves after amino acid 40 or 42 of the Aβ-sequence. The ELISAs are specific for x-40 or x-42 when the C-terminal capture antibody is combined with a mid-region Aβ-detection antibody. If a detection antibody specific for the N-terminal Aβ neo-epitope is used, the ELISAs measure $A\beta_{1-40}$ and $A\beta_{1-42}$. As APP also contains the Aβ sequence and since soluble APP fragments often greatly exceeds Aβ in biological samples, it is important to select a capture-antibody which lacks affinity for APP and its derivates, to avoid misleading results.

Assay conditions

The cores of amyloid plaques are highly insoluble and formic acid is required to release Aβ from these structures. Ranging from the extremely dense aggregates requiring formic acid down to the water soluble monomer, there are several different pools of Aβ in the brain which can be extracted differently depending on buffers, centrifugation speed and homogenization protocols used. A common definition of 'soluble Aβ' requires the peptide to remain dissolved in a mild physiological buffer, e.g. TBS without detergent, following high speed centrifugation (at least 100 000 xg). Importantly, soluble Aβ may still contain aggregates which are small enough to stay in solution. During analysis of soluble Aβ, detergent may be added to monomerize the peptide.

Aβ measurements in this study

In **paper I** levels of $A\beta_{40}$ and $A\beta_{42}$ in cell media and mouse brain homogenates were measured by ELISA-kits (Covance). These kits use 6E10 (N-terminal Aβ epitope, aa 3-8) as the capture antibody and C-terminal specific ($A\beta_{40}$ or $A\beta_{42}$) as the detection antibodies and have a lower detection limit of about 15 pg/ml. In addition, Aβ and APP were analyzed with SDS-PAGE followed by Western blot, which was adapted to the low Aβ-concentrations found in cell media. This adaptation included a slow SDS-PAGE separation (95 V for 2.5h), post-transfer boiling of nitrocellulose membranes and 2.5 days of incubation with primary antibody, of which the boiling step in particular increased the Aβ signal substantially [162]. 6E10 was used as primary antibody for Western blots as it enables detection of Aβ, APP and αAPPs.

Synthetic Aβ

Synthetic Aβ has been used in **paper I-IV** and since there is large variability between Aβ's aggregation propensity from different manufacturers, different lots and sometimes even different vials within the same lot, the aim has been to use Aβ from a single manufacturer and a single lot for all the experiments in one study. A general protocol was used to prepare synthetic Aβ of different conformations, where the lyophilized Aβ was dissolved in 10 mM NaOH followed by neutralization in PBS. In **paper I** and **paper III,** SEC-HPLC was used to analyze the size distribution of the synthetic Aβ, according to the protocol developed in [115].

Experimental models of Alzheimer's disease

Several *in vitro* and *in vivo* approaches to resemble the human AD pathology have been used to understand the molecular basis of the pathogenesis. Immortalized cell lines are typically transfected with human APP containing one or several familial AD mutations. Similarly, different transgenic mouse

models have been created using human APP or presenilin mutations driven by different promoters [reviewed in 163]. Overexpression of human APP in mice leads to cerebral amyloid plaque pathology, however to resemble tau pathology both mutated APP and tau need to be inserted [155]. The Swedish mutation is often included in cell and mouse models as a signal amplifier since it leads to a tenfold increase in Aβ when compared to wild type (wt) cells [164]. The mutation is located outside the Aβ sequence, and thus the released Aβ has the same sequence as wt Aβ (see *Figure 4*). Inclusion of the Arctic mutation accelerates the pathology leading to intraneuronal Aβ aggregation and amyloid plaques [69] but also to enhanced Aβ protofibril levels (**paper III** and [94]). The Arctic mutation, in combination with the Swedish, was used to increase levels of soluble Aβ aggregates in both cell and mouse models for **paper I** and **paper III**.

Results and discussion

Contradicting results were obtained following the analysis of conditioned cell media and mouse brain homogenates from models expressing APP_{Swe} or APP_{ArcSwe} under different assay conditions. The Arctic mutation resulted in decreased $Aβ_{1-40}$ and $Aβ_{1-42}$ levels when measured under non-denaturing conditions in ELISAs. Under denaturing conditions in SDS-PAGE Western blot contrary results were obtained, as APP_{Swe} samples displayed lower Aβ levels than APP_{ArcSwe} (Fig 1, **paper I**). To explain these opposing findings we hypothesized that the soluble Aβ aggregates present in the Arctic samples, would have less C-terminal epitopes available as this hydrophobic terminus faces into the core of the aggregate [165], thus impairing quantification with an ELISA dependent on free C-terminal epitopes. This theory was supported in a kinetic experiment where aggregation of synthetic $Aβ_{42}$ over time was followed in parallel using SEC, $Aβ_{1-42}$ ELISA and SDS-PAGE Western blot. The $Aβ_{1-42}$ ELISA levels declined with peptide aggregation, but SDS-PAGE Western blot detected levels remained stable during aggregation (fig 2 **paper I**).

Ideally, Western blot studies should have been performed with $Aβ_{40}$ and $Aβ_{42}$ end-specific antibodies, which would have strengthened the comparisons with ELISA data and made it easier to interpret the results. However, due to low Aβ levels in both cell media and brain homogenates from young transgenic mice Western blot analysis was limited to the detection of total-Aβ using the N-terminal 6E10 antibody. In addition, it would have been preferable to use another ELISA-kit not based on 6E10 as the capture antibody, since 6E10's affinity for APP and APP fragments may have disturbed the analysis. However, αAPPs levels were similar in APP_{Swe} and APP_{ArcSwe} cell media, as analyzed by Western blot, why the possible disturbance of αAPPs in the ELISAs should at least have been equal among groups.

An important finding of this study was that results from the extensively used $A\beta_{40}$ and $A\beta_{42}$ ELISAs may be misrepresentative if oligomeric Aβ is present in the sample. There is a consensus that ELISA measured levels of $A\beta_{1-42}$ in CSF are reduced among Alzheimer patients. Whether this phenomenon is linked to the presence of soluble Aβ aggregates in human CSF was further investigated in **paper IV**.

Paper II

Aim

The aim of the study was:
- to investigate how the established markers of AD: $A\beta_{42}$, tau and p-tau change over time in CSF of DS infants
- to test if DS patients display a CSF pattern of Aβ peptides indicative of AD from early childhood

Background

Down syndrome and Alzheimer's disease

DS patients almost invariably develop AD-like pathology during their fourth decade of life. This has been attributed to trisomy of chromosome 21, resulting in an additional copy of the APP-gene and thereby an elevated Aβ-production throughout life. By studying young DS patients, early events in AD pathogenesis can likely be elucidated.

Cerebrospinal fluid

CSF is a clear fluid with a total protein content of ~250 mg/L [166] that surrounds the brain, fills it cavities and drains the interstitial fluid. In total there is about 140 ml of CSF with 25 new ml produced per hour, leading to an exchange of the whole volume 4-5 times a day [reviewed by 167, 168]. 10-12 ml CSF can be collected for analysis by lumbar puncture, and as CSF is in contact with the brain it is thought to reflect biochemical processes in the brain.

Amyloid-β peptides in CSF

Aβ is present in CSF from both AD and non-AD patients and the total Aβ content does not differ significantly between groups [169]. Using mass-spectroscopy or gel based separation followed by Western blot, several different truncated Aβ species have been identified in CSF, where the majority seem to have an intact N-terminus [49, 162, 170, 171]. It was long stated that $A\beta_{40}$ constituted 90% of total Aβ in CSF and $A\beta_{42}$ the remaining 10%, though using urea based SDS-PAGE, Wiltfang *et al* found $A\beta_{38}$ to be the second most abundant Aβ peptide [49]. The same method identified a peptide pattern of $A\beta_{37/38/39/40/42}$ where the relative amounts – but not absolute concentrations – of the peptides ($A\beta_{1\text{-}x}$ to $A\beta_{total}$) were found to be different in AD compared to non-demented disease controls. AD had higher relative values of $A\beta_{38}$ and $A\beta_{40}$ but a lower percentage of $A\beta_{42}$ in CSF, whereas the relative abundance of $A\beta_{37}$ and $A\beta_{39}$ were equal to controls. This suggests that the relationship between C-terminally truncated forms could be important for AD pathogenesis. Comparative studies of Aβ levels in CSF from AD

and healthy controls using C-terminal specific ELISAs, show equal levels of Aβ_{38} and Aβ_{40} [172], whereas Aβ_{42} levels are markedly reduced in AD [173-175]. Low Aβ_{42} levels in itself, or expressed as a ratio against Aβ_{40} [176-178], can be used as a supplement during differential diagnosis of AD and in combination with high tau and p-tau concentrations can predict whether MCI likely represents incipient AD [179]. However, measurement outcome is highly dependent on sample handling and the assay used [180, 181].

Methodological considerations

Longitudinally sampled CSF from DS infants

This study was a substudy of CSF samples from DS children who had participated in a clinical trial with growth hormone treatment. CSF-samples were taken before treatment start (8-10 months of age), during treatment (19-46 months) and after treatment (55-56 months). Some individuals chose not to have lumbar puncture during follow up, which explains the reduction in sample numbers.

Urea-based Western blot

During SDS-PAGE all Aβ species migrate through the gel as a ~4 kDa band and under ‘normal’ analysis conditions the resolution is insufficient to separate different truncated forms. However, by adding urea to the system, a conformational shift is induced resulting in differential migration and separation of various C-terminally truncated Aβ species [49].

xMAP

The bead based xMAP technology enables simultaneous quantification of Aβ_{1-42}, tau and p-tau in the same sample and under native conditions [182], which was favorable in this study where sample volumes were limited. The assay is similar to ELISA, however carboxylated beads are used as the solid phase for immobilization of capture antibodies. Detection is made through fluorescently labelled secondary antibodies, which are quantified through a Luminex device based on flow cytometry-like technology.

Results and discussion

Absolute levels of all Aβ species (37/38/39/40/42) increased from eight months to 4.5 years of age in the DS infants, however during this time no pattern indicative of AD developed as the relative ratio of the peptides remained unchanged. The A$\beta_{42/40}$ ratio, which is decreased in AD patients, remained stable over time in DS CSF. As observed during the studies of ‘Arctic’ samples in **paper I**, there was a discrepancy between results from different Aβ_{1-42} analysis methods. While SDS-PAGE Western blot showed

increased $A\beta_{1-42}$ over time, xMAP $A\beta_{1-42}$ levels instead decreased. One speculative explanation is that this is a methodological artefact caused by aggregation of Aβ, leading to impaired $A\beta_{1-42}$ detection under the native conditions of xMAP analysis, as suggested for ELISA in **paper I**. This would imply that soluble Aβ aggregates form during the early years of life in DS patients, which could represent an early molecular pathology of AD occuring several decades before expected pathology. It has previously been reported that both intracellular $A\beta_{42}$ immunoreactivity and increased levels of soluble Aβ precede plaque pathology in DS brains, and that levels of natively analysed $A\beta_{42}$ decrease between 20 and 60 years of age [183, 184].

The early reduction in xMAP measured $A\beta_{1-42}$ levels in CSF was not accompanied by increasing tau or p-tau levels, contrary to observations from AD and MCI patients. However, AD and MCI probably represent late stages in a pathogenic process initiated years, maybe even decades, before symptoms arise [136, 137].

Paper III

Aim

The aim of the study was:
- to develop a monoclonal antibody with specific affinity for Aβ in its protofibril conformation
- to establish an Aβ protofibril specific sandwich ELISA to enable Aβ protofibril quantification in biological samples

Background

Antibodies

Antibodies, or immunoglobulins, are naturally occurring proteins produced and secreted by B-cells. They are part of the humoral immune system and able to specifically identify and eliminate foreign antigens. Antibodies occur in different isotypes, where immunoglobulin G (IgG) is the most abundant in human blood. Antibodies specific for a certain antigen can be generated either from a single B-cell clone, referred to as monoclonal antibody (mAb), or from a population of B-cells, termed polyclonal antibody (pAb). The latter are made by immunizing a host animal, often a rabbit or goat, and later collecting the serum containing pAbs. The serum can then be affinity purified to enrich the antibodies of interest. In contrast, mAbs are produced by the hybridoma technique where animals, usually mice or rats, are immunized with the desired antigen. The spleen is then removed, B-cells isolated and fused with myeloma cells (Sp2/0-cells for mice [185]). After selection, subcloning and screening an immortalized B-cell-Sp2/0-hybridoma producing antibodies against the antigen of interest can be maintained [186].

Antibodies with conformation dependent epitopes

Amino acids which are a relatively large distance apart in the linear sequence can be in close proximity to each other in the folded structure of the protein, forming novel epitopes. This may also occur during protein aggregation, where the close proximity of amino acids from adjacent monomers can present new surfaces for antibodies to bind. This formation of novel epitopes during aggregation and folding is the principle behind the generation of antibodies with affinity for conformation dependent epitopes. A number of anti-Aβ antibodies with such epitopes have been reported (summarized in Table 4). Interestingly, despite the aggregated Aβ forms being used as antigen, some of the antibodies also recognize aggregates of other amyloidogenic proteins and thus, the existence of a generic amyloid epitope has been suggested [187, 188].

Table 4. *Conformation dependent Aβ antibodies*

Name	mAb	pAb	Aβ epitope: Soluble aggregate	Aβ epitope: Fibrils	Generic amyloid	Ref.
M94, M71		x	ADDLs	x		[189]
WO1 & 2	x			x	x	[188]
A11		x	pre-fibrillar oligomers		x	[83]
M93		x	ADDLs			[79]
8F5	x		Globulomers			[87]
NAB61	x		soluble aggregates	x		[190]
mAb158	x		protofibrils	x		[91]
NU1-NU7	x		ADDLs	(x)		[191]
OC		x	fibrillar oligomers	x	x	[192]

Conformation specific quantification of Aβ

Given the many reports suggesting soluble oligomeric aggregates as the main toxic species in AD, there has been a great interest in specific quantifications of soluble Aβ aggregates. To enable this with ELISA, two main strategies have emerged: either to create oligomer dependent assays by using the same monoclonal anti-Aβ antibody as capture and detection antibody in the sandwich ELISA, or to use conformation specific antibodies against the aggregate of interest. With the first strategy, theoretically, a dimer is the smallest aggregate that will produce a signal as two epitopes need to be available – one for the capture antibody and one for the detection. In general any monoclonal antibody could be used for such an oligomer assay, but for biological samples it is important to use an antibody without affinity for APP or its derivates since their concentration will probably exceed Aβ's and thereby lower the signal (see fig 3 C, **paper III**). To assay for a specific type of oligomer, several groups have developed conformation specific antibodies (Table 4) and applied them in various immunoassays to detect oligomerized Aβ in biological samples. Reported quantifications of soluble Aβ aggregates are summarized in Table 5.

Methodological considerations

Protofibril preparation

Aβ protofibrils used for this study were prepared from synthetic $A\beta_{42}$, with or without the Arctic mutation, which was incubated according to the protocol described in **paper III**. The purity of the preparation was analyzed by HPLC-SEC where protofibrils elute in the void volume on a Sephadex75 column. Fibrillar species were removed from the solution by centrifugation at 17 900 xg for 5 minutes, as described in the initial protofibril reports [90].

Table 5. Quantification of soluble Aβ aggregates in biological samples

Method	Results	Reference
Fluorescence correlation spectroscopy with fluorescent 6E10	Higher signal of aggregated Aβ in AD vs controls	[193]
Oligomer-ELISA based on two 3D6 (Aβ neo-epitope aa 1-6)	Soluble oligomers in tg-mouse brain homogenates	[194]
Dot blot using the oligomer specific pAb A11	Oligomers in AD brain but not in controls	[83]
Sandwich-ELISA with a globulomer specific mAb	Globulomers in AD brain	[87]
Immuno-PCR (BioBarcode) using ADDL pAbs	Higher ADDL-levels in AD vs controls. [ADDL] in CSF in fM-range	[81]
ADDL specific pAbs in sandwich format on SPR	Detection of ADDLs in human CSF and brain	[195]
Sandwich-ELISA using the protofibril selective mAb158	Protofibrils in cell media, mouse and human brain (unpubl. data)	[91, 94]
FRET-based flow cytometry-analysis using a combination of 6E10 and 4G8	Increased levels of Aβ aggregates in AD CSF compared to controls	[196]
Sandwich ELISA with oligomer specific mAb7A1a and anti-$A\beta_{42}$-antibody	Elevated $A\beta_{42}$ oligomer levels in AD brain homogenates compared to controls	[197]
Oligomer-ELISA with two 3D6 or 82E1 (Aβ neo-epitope aa 1-6)	Aβ oligomers in AD and control plasma	[198]

Antibody production

The strategy for creating an antibody with affinity for Aβ in its protofibrillar conformation was to repeatedly immunize mice subcutaneously with protofibrils made from $A\beta_{42Arc}$, to monitor the immune response in serum by measuring antibody titers with ELISA, and then to harvest the spleen and culture antibody-producing tumour cells – hybridomas. During the first screening all hybridomas were tested for anti-Aβ reactivity using direct ELISA where plates were coated with Aβ in different conformations. Aβ positive clones were further analyzed with respect to affinity and conformational selectivity of the antibodies.

Antibody characterization

Previously reported conformation specific antibodies were characterized using dot blot, Western blot or direct ELISA [79, 83, 87, 188, 190, 191], all methods in which the antigen is present in large excess in the coat or on the membrane. A limitation of adhering antigen to the solid phase is the poten-

tial to induce conformational changes resulting in the loss of native epitopes. In addition, the excess of antigen makes these methods less suitable for antibody binding characterization. Instead, an inhibition ELISA was used for affinity and selectivity characterizations in this study. This method allows antibodies and antigen to interact in solution and at low concentrations [199]. The antigen concentration required to inhibit 50% of the antibody's binding to the coat, IC50, can be used as an estimate of the antibody's affinity for the investigated antigen.

Results and discussion

Results from **paper I** together with reports in the literature indicated that a pool of the soluble Aβ fraction is in an aggregated form. Therefore the aim of this study was to develop an antibody specific for Aβ in the protofibril conformation to enable specific quantification of these aggregates in biological samples. After immunization with $A\beta_{42Arc}$ protofibrils, two IgG producing hybridomas (and several IgM) were identified from 1500 screened clones. The mAb158 antibody (IgG2a isotype) was extensively characterized and its approximate IC50-values for different Aβ preparations were estimated (see figure 2 in **paper III** and Table 6). The affinity pattern of mAb158 clearly differed from mAb1C3 (the other IgG obtained) and 6E10, with an at least 200-fold higher affinity for Aβ protofibrils than for LMW-Aβ. Inhibition ELISA is not suitable for insoluble Aβ fibrils, why mAb158's affinity for fibrils was investigated with dot blot (Fig 3, **paper III**). Despite dot blots limitations with regard to quantification, it was obvious that mAb158 had no apparent difference in affinity for Aβ protofibrils and fibrils. Hence, the protofibril epitope recognized by mAb158 seems to be conserved in the fibril structure.

Table 6. *IC50 (nM) for mAb158, mAb1C3 and 6E10*

	Inhibition on LMW-Aβ coat				**Inhibition on Aβ protofibril coat**			
Inhibitory peptide	Aβ PF	AβArc PF	LMW-Aβ	Aβ1-16	Aβ PF	AβArc PF	LMW-Aβ	Aβ1-16
mAb158	4	3	800	1000	5	2.5	1000	5000
mAb1C3	8	5	35	25	6	5	25	25
6E10	9	7	11	8	10	6	10	10

Binding characteristics for the antibodies were also investigated by surface plasmon resonance (SPR) analyses. The antigen, LMW-Aβ or Aβ protofibrils served as covalently linked ligands and the antibodies as analytes that were flowed over the surface at different concentrations. A clear difference could be observed between mAb158 and mAb1C3/6E10 in their LMW-Aβ dissociation phases, confirming the results of the inhibition ELISA (*Figure 8*). Estimating affinity measures from the SPR-experiments were compli-

cated due to the bivalent antibodies and probably both multivalent and heterogeneous antigen.

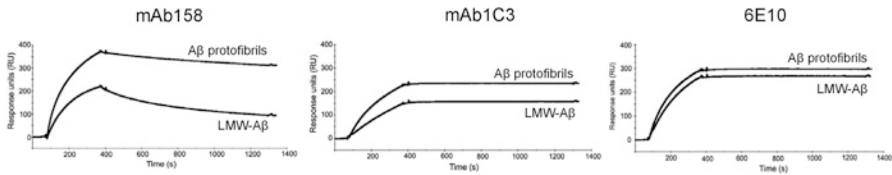

Figure 8. SPR-sensograms for 25 nM of mAb158, mAb1C3 and 6E10 showing their association and dissociation to Aβ protofibrils and LMW-Aβ.

mAb158 exhibits two important characteristics – no affinity for other amyloids and no affinity for native APP or βAPPs (Fig 3, **paper III**). These characteristics made it possible to establish a protofibril specific sandwich-ELISA. Since mAb158 also has affinity for LMW-Aβ, albeit it is lower, the sandwich-ELISA was based on mAb158 as both capture and detection antibody to further ensure the requirement of aggregated Aβ to produce a signal. As seen in figure 4, **paper III**, the ELISA needs a 1000-fold higher concentration of LMW-Aβ than of Aβ protofibrils to produce a comparable signal. This LMW-Aβ preparation was later shown to contain small amounts of larger aggregates, though at concentrations too low to be detected in HPLC-SEC analysis, (Figure 1, **paper III**). The non-aggregating Aβ1-16 fragment was, as expected, not detected in the mAb158 ELISA. Importantly, there was no decrease in the mAb158 ELISA signal following the addition of 500 000-fold molar excess of Aβ1-16 to a 50 pM protofibril sample. However, with a 6E10-6E10 sandwich pair a 1000-fold excess of Aβ1-16 reduced the signal by 50% (Fig 4, **paper III**), which is of importance to consider when using dimer dependent ELISAs for biological samples, e.g. as described in [194, 198]. Thus, the mAb158 ELISA enables specific detection low pM concentrations of protofibrils, without interference from monomers and APP derivates.

To confirm the indication of a high content of soluble Aβ aggregates in cell and mouse models expressing APP_{ArcSwe} observed in **paper I**, the protofibril content in aliquots of the very same cell media samples was measured. These indications were confirmed and cell culture media from APP_{ArcSwe} cells contained more protofibrils than media from APP_{Swe} cells, which in turn differed significantly from untransfected mock cells. The same pattern was observed for TBS-soluble brain homogenates from 10 months old tg-ArcSwe mice (Fig 5, **paper III**). Using this protofibril specific mAb158 ELISA, Aβ protofibrils have now been quantified in several different mouse models, in tg-ArcSwe mice of different ages and in CSF from tg-ArcSwe [94] as well as in human AD brain (unpublished data).

Aggregated Aβ of many shapes and sizes has been proposed as *the* pathogen of AD pathogenesis (Table 3), and it is of course interesting to further

characterize which of these species the mAb158 ELISA detects. To do this, Aβ preparations have been fractionated with gradient ultracentrifugation [modified protocol from 100] prior to ELISA analysis. The ELISA primarily detected soluble aggregates of Aβ in heavier fractions (over ~100 kDa), both in tg-ArcSwe mouse brain homogenates and synthetic Aβ preparations (unpublished data). To further characterize the specificity of the ELISA, ADDLs were prepared according to the published protocol [189], fractionated and analyzed. This preparation contained oligomers of a wide size-range, though only the larger were detected. This is in line with results from the low-denaturing SDS-PAGE where mAb158 mainly detected the high molecular weight Aβ species (figure 2 in **paper III**). It is questionable if *in vitro* generated Aβ protofibrils resemble the structure of *in vivo* formed aggregates. Though, using gradient ultracentrifugation, both *in vitro* and *in vivo* protofibrils accumulated in the same fraction and are therefore at least of similar molecular weight.

To date, the reported levels of soluble Aβ aggregates detected in human CSF have been low – down to fM – requiring highly sensitive assays [81, 193, 196]. The detection limit of the mAb158 ELISA when using streptavidin-HRP, TMB and spectrophotometric detection at 450 nm is ~1pM. Thus, for human CSF analysis increased sensitivity may be needed. This work is on-going and several approaches including luminescent and fluorescent HRP substrates or PCR-based detection such as immuno-PCR and proximity ligation assay [200] are being tested.

Paper IV

Aim

The aim of the study was:
- to develop a method to detect presence of soluble oligomeric Aβ species in biological samples, regardless of their exact conformation
- to test if this method could be used for improved diagnosis of AD

Background

Soluble Aβ aggregates are suggested as the instigators of AD pathogenesis, and thus specifically measuring them would be of obvious benefit when trying to identify novel markers for AD. As discussed in **paper III**, several groups are working on assays for quantification of a specific oligomeric species, e.g. the protofibril or ADDLs. However, it is possible, and perhaps likely, that several of these species co-exist during the progressive AD pathology and thus it would be convenient to measure all soluble aggregates. In an attempt to do this, observations from **paper I** and **paper II** were utilized. Both these studies showed that Aβ, and $Aβ_{42}$ in particular, analyzed under different conditions could give contradictory results. We hypothesized that this effect was caused by epitope masking induced by aggregation, which lead to impaired detection of $Aβ_{42}$ under native conditions. Supporting this theory are reports of reduced availability of C-terminal epitopes for antibody binding in aggregates of $Aβ_{42}$ [201]. Accordingly, cell and mouse samples from models carrying the aggregation-prone Arctic mutation had the lowest ELISA-measured $Aβ_{42}$-values, and were in **paper III** shown to have the highest Aβ protofibril content. As an ELISA-measured reduction in CSF $Aβ_{42}$ is observed among AD patients, it is intriguing to speculate that this reduction is not solely due to $Aβ_{42}$ being depleted from the CSF as it is sequestered to cerebral plaques, since it could also be the result of impaired ELISA-detection caused by the presence of aggregated $Aβ_{42}$ in the CSF.

Methodological considerations

Quantitative SDS-PAGE and Western blot

SDS-PAGE followed by Western blot was used to assay $Aβ_{42}$ under denaturing conditions. Samples were denaturated by boiling for 5 min in sample buffer containing 2% SDS. Even though SDS-stable oligomers have been reported [77], this denaturation was sufficient to dissociate soluble Aβ aggregates as only a ~4 kDa band corresponding to the molecular weight of Aβ monomers was visible on the immunoblot. The intensity of fluorescently labelled secondary antibodies was quantified using the Odyssey system (Westburg). All CSF-samples were run in parallel on separate gels, quanti-

fied against a standard on the respective gel and then the mean value of the two quantifications was used. Samples with means with a coefficient of variance higher than 20% were excluded.

Human CSF samples

CSF samples from patients were collected by lumbar puncture during routine clinical investigation at the Memory Clinic at Uppsala University Hospital and were sent for biochemical analysis of $A\beta_{1\text{-}42}$, tau and p-tau made performed with established ELISAs [202-204]. Since AD is often preceded by MCI, CSF samples from a group of MCI patients were included in addition to the AD samples. Clinical follow-ups of MCI patients reveal that within a couple of years about one third develop AD. However, all nine MCI cases included in **paper IV** later converted to AD, therefore their CSF was expected to reflect an incipient AD pathology. Samples from FTD patients were included to represent a group with ongoing neurodegeneration, but expected to show little Aβ pathology. CSF from cognitively healthy aged individuals served as controls. It is worth remembering that neuropathological AD changes have been reported to be present in one third of cognitively normal 75 year olds [138]. Concerning the high incidence of dementia among the elderly, 5-10% of an elderly control group could convert to AD within 10 years, as discussed by Blennow and Hampel [205]. In addition, a pattern indicative of AD, with low $A\beta_{1\text{-}42}$ in CSF combined with cerebral PET-PIB binding (positron emission tomography using the plaque-binding ligand Pittsburgh compound B) was also observed in elderly individuals with no cognitive impairment [206], indicating that control groups of elderly persons may include individuals with incipient AD.

Results and discussion

An $A\beta_{42}$ oligomer ratio was enabled by combining two $A\beta_{42}$ analyses of the same sample, one made under denaturing conditions and one native analysis. To validate that high $A\beta_{42}$ levels measured with Western blot and low $A\beta_{42}$ levels measured with ELISA were indeed reflective of an aggregated state of Aβ, mouse brain homogenates from tg-ArcSwe were assayed both for their $A\beta_{42}$ oligomer ratio and their protofibril levels using the mAb158 ELISA established in **paper III**. High protofibril levels corresponded to high $A\beta_{42}$ oligomer ratios (figure 2 in **paper IV**), verifying that this ratio is indicative of the presence of soluble Aβ aggregates. The reduced ELISA signal was also observed for $A\beta_{1\text{-}40}$ in **paper I**, and for models expressing the highly aggregating $A\beta_{Arc}$ peptide, it is possible that to a large extent $A\beta_{40Arc}$ also exists in an aggregated form. However, for non-Arctic mutation carriers $A\beta_{42}$ is the aggregation-prone peptide, and thus the ratio was restricted to $A\beta_{42}$ analyses.

The $A\beta_{42}$ oligomer ratio was used to test for the presence of oligomeric Aβ in human CSF samples. As expected, AD and MCI-patients displayed low levels of $A\beta_{42}$ in CSF with ELISA. When $A\beta_{42}$ was analyzed with Western blot there was no such reduction and no significant difference in levels between groups. Accordingly, AD and MCI had higher oligomer ratios than FTD and controls (figure 3, **paper IV**). The results suggest that the reduction of $A\beta_{42}$ in AD and MCI CSF could be due to Aβ aggregation into soluble oligomers, perhaps in combination with the previously suggested mechanism in which CSF Aβ is sequestered with the ongoing Aβ plaque pathology. It is interesting to notice that low ELISA $A\beta_{42}$ in CSF correlate with high cortical PIB-binding [206], which may suggest a correlation between oligomerized Aβ in CSF and cerebral plaque pathology. In a recent study measuring oligomer levels in plasma with an Aβ dimer dependent ELISA, oligomeric and $A\beta_{42}$-levels were closely associated indicating that they are related also in the periphery [198].

To evaluate the diagnostic value of the oligomer ratio, AD and MCI were combined into one group which could be distinguished from the cognitively healthy controls with 91% sensitivity and 89% specificity (*Figure 9A*). A similar pattern was observed for DS infants in figure 2 **paper II**, where opposite results were obtained in the Aβ pattern for xMAP- and Western blot-derived $A\beta_{42}$ levels. Based on these data, the oligomer ratio increased during the first years of life (*Figure 9B*), which may indicate that oligomeric Aβ is formed early in life and could possibly be used to detect preclinical AD.

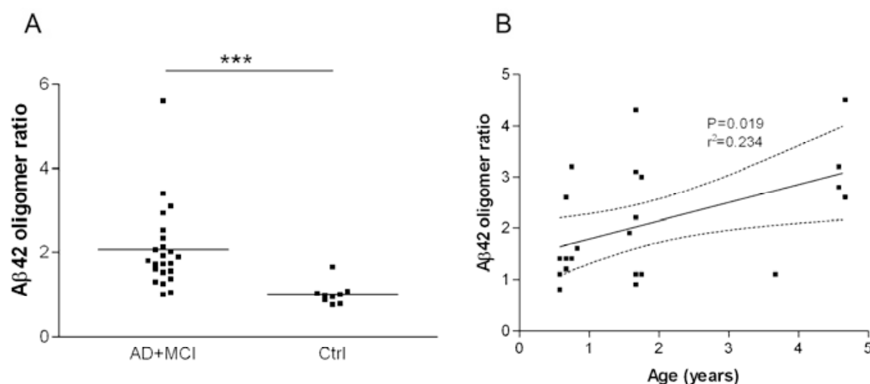

Figure 9. $A\beta_{42}$ oligomer ratio of AD and MCI samples in **paper IV** were combined into one group to calculate sensitivity and specificity. The $A\beta_{42}$ oligomer ratio distinguished AD+MCI from cognitively healthy controls with a sensitivity of 91% and a specificity of 89% (A).The $A\beta_{42}$ oligomer ratio was calculated from the $A\beta_{42}$ Western blot and $A\beta_{42}$ xMAP analyses, made in **paper II**, and a longitudinal increase with age was observed in young Down syndrome patients (B).

To analyze if the oligomer ratio changes with disease progression in AD, longitudinal samples from patients in different clinical and preclinical stages

of the disease are needed. To facilitate such an analysis, a quantitative denaturing method that is less time consuming and is more reliable than Western blot, needs to be developed. The application of ELISA for assaying denatured samples is limited by methodological problems. Generally, antibodies are sensitive to high concentrations of detergent, which may prevent antigen binding, and extensive sample dilution to reduce negative detergent effects is usually not possible due to limited sensitivity of the ELISAs. However, by using a high-sensitivity $A\beta_{1-42}$ kit (Wako Chemicals) in combination with SDS-pretreatment of samples, it was possible to establish an oligomer ratio based on $A\beta_{1-42}$ ELISA analyses made under denaturing and native conditions. Using this method, the signal from synthetic SDS-treated Aβ protofibrils was increased almost 3-fold, and a sub-set of the CSF samples from **paper IV** displayed the same trend as for the original oligomer ratio (Figure 10). This improvement of the $A\beta_{42}$ oligomer ratio will facilitate analysis of large sample sets and will be used to investigate $A\beta_{42}$ oligomer ratios in CSF from controls and AD patients of different stages of the disease.

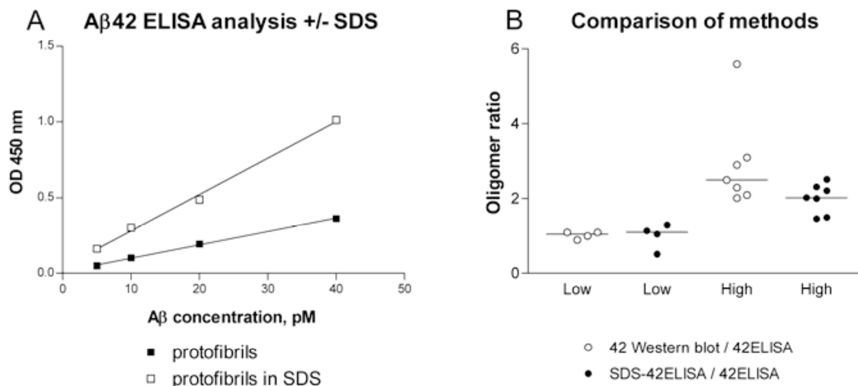

Figure 10. The $A\beta_{42}$ oligomer ratio, presented in **paper IV**, was developed further by using SDS-treatment prior to ELISA analysis instead of Western blot as the denaturing method. This pretreatment led to a ~3-fold increase in signal from synthetic Aβ protofibrils (left). A sub-set of CSF-samples with low or high oligomer ratios in **paper IV** were analyzed with the SDS-ELISA, and similar results were generated with both methods.

Direct measurements of Aβ protofibrils in CSF using the protofibril specific mAb158 ELISA is the next phase of this work, however the assay has not yet been optimized for the expected low protofibril levels. Moreover, as several oligomeric forms of Aβ have been suggested to cause AD, measuring all sizes of soluble aggregates using the $A\beta_{42}$ oligomer ratio in combination with the mAb158 ELISA (which primarily detects aggregates >100 kDa) is a valuable and informative analysis. The drawback of the $A\beta_{42}$ oligomer ratio is that it indicates the presence of oligomers but not necessarily the amount

of them. If larger aggregates cause more extensive epitope masking leading to an increased discrepancy between methods, a high ratio could be obtained both by a few large aggregates or many smaller oligomers.

Following the novel therapeutic strategies to combat AD, which are being evaluated in clinical trials, there is a demand for AD markers both for early diagnosis and to follow disease progression, particularly when monitoring treatment progress. Today, combinations of $A\beta_{42}$, tau and p-tau offers the best diagnostic markers of AD, however they do not change with disease progression since low $A\beta_{42}$ and high tau and p-tau remain stable after disease onset [202, 207]. Other suggested CSF markers of AD which relate to Aβ pathology include decreased αAPPs or increased βAPPs in CSF [43, 44], increased BACE-activity [208] or changes in the ratio of Aβ metabolites [49, 209]. *In vivo* amyloid imaging with PET-PIB distinguishes AD from non-AD at an early stage [210] but do not correlate with memory tests such as MMSE. Structural modelling with magnetic resonance imaging (MRI) and measures of atrophy, currently offer the best opportunities to asses disease progression [reviewed in 211].

Conclusions

Paper I: The Arctic APP mutation increases Aβ levels in cell and mouse models. To observe this increase Aβ has to be assayed under denaturing conditions, since presence of soluble Aβ aggregates impairs quantification during native conditions.

Paper II: Children with Down syndrome have an increasing Aβ concentration in CSF early in life, while tau and p-tau levels remain unchanged. $A\beta_{42}$ analyses of DS CSF made under different conditions are contradictory, possibly due to presence of soluble Aβ aggregates.

Paper III: mAb158 has a conformation dependent epitope, resulting in preferential affinity for Aβ protofibrils. A sandwich ELISA based on mAb158 enables specific quantification of Aβ protofibrils in biological samples. Cell and mouse models bearing the Arctic APP mutation display enhanced Aβ protofibril levels.

Paper IV: Combining native and denaturing $A\beta_{42}$ analyses into an $A\beta_{42}$ oligomer ratio enables measurement of oligomeric Aβ in human CSF samples. AD and MCI patients have elevated $A\beta_{42}$ oligomer ratio compared to cognitively healthy controls.

Future perspectives

Alzheimer's disease is the most common form of dementia affecting 15 million people over the world, a number that is expected to increase dramatically due to demographic changes. The caring costs associated with the US's 5 million AD patients is estimated at $148 billion, which excludes the often extensive care provided by relatives [212]. Today there are no curative drugs for AD and the available treatment can at best offer symptom relief and a minor retardation of the cognitive decline. Thus, there is an immense demand for new therapeutic interventions and with them, also a requirement to identify biomarkers that are indicative of AD and detectable before symptoms appear.

Although sometimes debated, the amyloid cascade hypothesis provides a model for the disease process where the central event is the abnormal release and aggregation of Aβ peptides. Most proposed therapeutic strategies intervene at some level of this hypothesis, either by reducing Aβ production at the point of enzymatic cleavage of APP, or at later stages by inducing neurogenesis to replace dead neurons (therapeutic strategies are schematically summarized in Figure 11). During the last decade huge efforts have been devoted to the development of immunotherapeutic approaches where antibodies against Aβ, either endogenous after active vaccination or recombinant used for passive immunization, are expected to attenuate Aβ toxicity and reduce amyloid burden. The support for this strategy is based on the successful initial report where amyloid burden was both prevented and reduced after active Aβ vaccination in transgenic mice [213]. Following these positive results in animal studies a clinical trial using the same vaccine (fibrillized $Aβ_{42}$) was initiated, but halted during phase II due to meningoenchephalitis in 6% of the patients [214]. At present modified versions of this vaccination protocol are under evaluation, e.g. active vaccinations using Aβ or Aβ derivates, or passive vaccination where antibodies recognizing different epitopes of Aβ, including mAb158 (characterized in **paper III**).

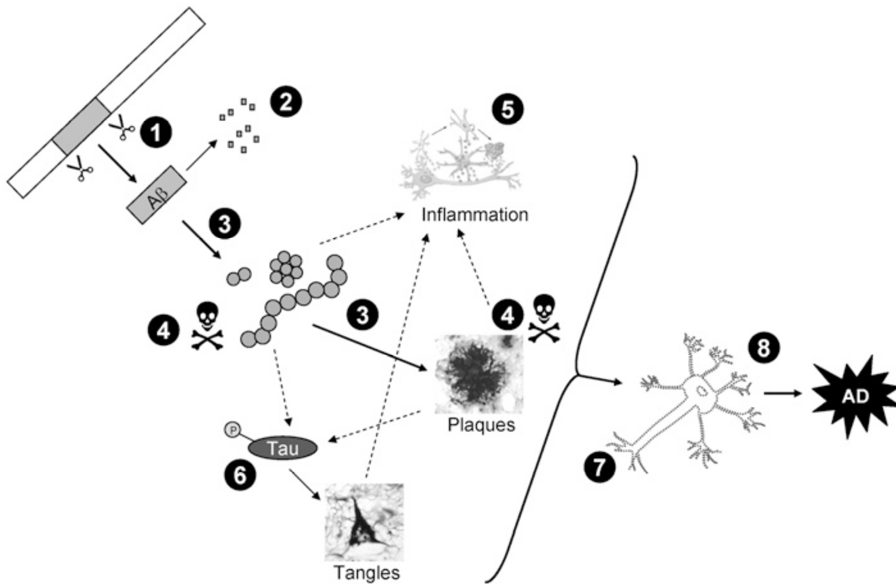

Figure 11. Points of intervention for current strategies of AD treatment: ❶ alter APP-processing by inhibiting β- or γ-secretase and thereby inhibit Aβ formation, ❷ increase Aβ degradation, ❸ inhibit Aβ-aggregation, ❹ neutralize toxic Aβ species, ❺ reduce inflammatory processes, ❻ inhibit tau phosphorylation and thereby reduce tangle formation, ❼ compensate for reduced neurotransmitter levels caused by degenerated synapses, ❽ stem cell therapy to repair neuronal loss.

Today there is a basic understanding of AD pathogenesis and many putative drug targets have been identified, evaluated and utilized in ongoing clinical trials. Therefore a future disease-modifying treatment, or perhaps a combination of therapies, seems possible. Regardless of the therapeutic mechanism, the therapy would preferably be initiated early in the pathogenesis to minimize the synapse loss and neuronal degeneration. This increases the demand for markers capable of detecting pre-symptomatic AD, but also for markers which can report on treatment efficacy. As soluble oligomeric aggregates of Aβ are thought to initiate the pathogenesis, they have of course gained much attention as potential biomarkers and various oligomeric Aβ forms have been found in CSF (summarized in Table 5). Soluble Aβ levels in CSF probably reflect soluble Aβ levels in the brain, as shown in tg-ArcSwe mice with elevated Aβ protofibril levels in both brain and CSF [94]. The soluble Aβ pool is likely most rapidly affected by Aβ directed immunotherapy, as demonstrated with passive immunization using mAb158 (Lord et al, manuscript). Therefore biomarkers reflecting this fraction of Aβ are of great interest for future treatment of AD patients. The general analysis of soluble oligomeric Aβ 'the $A\beta_{42}$ oligomer ratio' described in **paper IV** could provide such a measure, and as this ratio increases in CSF during early childhood in DS patients (**paper II**) it could potentially detect preclinical AD pathology.

AD research has progressed rapidly during the last two decades and the knowledge gained can benefit research also in other amyloidoses and neurodegenerative disorders, as a common mechanism have been suggested for misfolding diseases. Toxic protofibrils of other proteins have been implicated in DLB (α-synuclein), FTD (tau), prion disease (PrP), type II diabetes (IAPP) and familial amyloidotic polyneuropathy (transthyretin) [215-219]. Hence, the $A\beta_{42}$ oligomer ratio described in **paper IV** could also be relevant for these diseases, since epitope masking during aggregation is probably a general phenomenon. In agreement with the decrease of $A\beta_{42}$ in AD CSF, reduced levels of α-synuclein have been reported in CSF among patients suffering from DLB and Parkinson's disease [220], and it would be interesting to assay α-synuclein levels in these samples under both denaturing and non-denaturing conditions. Prefibrillar amyloid aggregates have been reported to share mechanisms of toxicity, like permeabilization of lipid membranes and calcium dysregulation [118, 124, 221]. In addition, some of the conformation specific antibodies described recognize a common amyloid epitope [83, 188], further implying common characteristics among toxic protein aggregates. Accordingly, following the encouraging results from vaccination of transgenic AD mouse models, immunotherapeutic approaches have successfully been tested also in models with α-synuclein and PrP pathology [222, 223].

Svensk sammanfattning

Det som länge kallades *senil demens* och ibland även sågs som en naturlig del av åldrandet har visat sig bestå av grupp av demenssjukdomar där Alzheimers sjukdom är den vanligast förekommande. Vid Alzheimers sjukdom (*eng.* Alzheimer's disease, AD) dör nervcellerna i framförallt tinnings- och hjässloberna och kopplingarna mellan nervcellerna – synapserna – förstörs. Detta leder till en gradvis försämring av hjärnans funktioner i dessa områden, vilket tidigt resulterar i symtom som nedsatt minnesfunktion och problem med rumslig orientering. För närvarande finns ingen botande behandling för AD. De mediciner som finns har endast förmågan att fördröja symtomen under en kortare tid, men stoppar inte processerna som leder till att nervcellerna dör och hjärnan förtvinar.

Forskningen kring AD är intensiv, och flera nya behandlingsstrategier såsom vaccinering har visats vara lovande. Dessa utvärderas just nu i kliniska prövningar världen över. Ett generellt krav för alla potentiellt botande Alzheimerbehandlingar är att de måste startas tidigt, förmodligen redan innan patienten märker av några demenssymtom. Detta så att en alltför utbredd förlust av synapser och nervceller kan undvikas, eftersom döda nervceller är svåra och kanske till och med omöjliga att ersätta. En så pass tidig behandlingsstart innebär att diagnostiken av sjukdomen måste förbättras. För att kunna göra det behöver den molekylära förståelsen kring sjukdomens tidiga processer att öka, vilket skulle kunna leda till att nya och tidiga sjukdomsmarkörer kan identifieras. Eftersom andra demenssjukdomar med likartade symtom, till exempel frontallobsdemens och Lewykroppsdemens, orsakas av andra proteiner är det också viktigt att diagnostiskt kunna särskilja de olika demenstyperna åt, så behandlingen kan riktas mot rätt molekyler.

Trots att det gått hundra år sedan den tyske läkaren Alois Alzheimer för första gången beskrev de sjukliga förändringarna i hjärnan hos en patient med vad som senare kom att kallas Alzheimers sjukdom, kvarstår många frågetecken kring vad som faktiskt orsakar sjukdomen. Det Alois Alzheimer såg: amyloida plack och neurofibrillära nystan (för bilder, se Figur 2 i introduktionen), anses nuförtiden som slutstadier i sjukdomsprocessen. Istället fokuseras forskningen idag i stor utsträckning på förstadier till placken, lösliga aggregat kallade oligomeriserat amyloid-β (Aβ). Dessa former av Aβ är giftiga för nervceller och försämrar nervcellernas förmåga att bilda nya minnen. De anses därför vara de molekyler som startar sjukdomsprocessen i hjärnan.

I denna avhandling har flera aspekter av oligomeriserat Aβ undersökts. Målet har varit att hitta ett sätt att mäta dessa former för att testa om de skulle kunna användas som en framtida markör för Alzheimers sjukdom. I det första delarbetet (**paper I**) undersöktes cell- och djurmodeller vilka bar på den arktiska mutationen. Den arktiska mutationen identifierades i en familj från norra Sverige med ärftlig AD och har i biokemiska studier visat sig leda till påskyndad oligomerisering av Aβ. I denna studie visades att prover från modellsystem med den arktiska mutationen gav felaktigt för låga signaler i en i Alzheimerforskningen vanligt förekommande analysmetod, $A\beta_{42}$-ELISA. Om proverna däremot analyserades under andra förhållanden och med en annan metod, Western blot, syntes inte denna sänkning utan tvärt om en höjning. Sänkningen av signalerna i $A\beta_{42}$-ELISAn föreslogs därför bero på att närvaro av oligomeriserat Aβ störde analysen.

Genen för Aβs ursprungsprotein APP ligger på kromosom 21. Personer med Downs syndrom har en extra kopia av kromosom 21 och har därför en ökad bildning av APP och även förhöjda Aβ-nivåer under sin livstid. Det gör att förändringar i hjärnan, vilka är i princip identiska med de vid Alzheimers sjukdom, uppkommer tidigt (ofta redan i 40-årsåldern) hos personer med Downs syndrom. Mycket tyder på att sjukdomsprocessen som leder till AD har startat långt innan individen märker de första symtomen, men eftersom det kan ta decennier innan personen utveckar symtom är studier av dessa tidiga processer svåra att genomföra. Därför ger prover från unga individer med Downs syndrom en unik möjlighet att kunna studera vad som pågår tidigt i sjukdomsförloppet. I det andra delarbetet (**paper II**) analyserades cerebrospinalvätska (CSF) från barn med Downs syndrom. CSF är en vätska som omger hjärnan och ryggmärgen och genom att mäta nivåerna av olika protein i CSF går det att få en bild av processerna som pågår i hjärnan. I denna studie mättes Aβ i CSF för att se hur Aβ-mönstret ändrades med tiden. Aβ-nivåerna ökade med åldern hos dessa barn, och precis som i **paper I** visade sig resultatet av $A\beta_{42}$-analyserna skilja sig åt beroende på vilken metod som användes. Detta tolkades som att oligomerisering av Aβ sker tidigt i sjukdomsförloppet.

För att specifikt kunna mäta en viss form av oligomeriserat Aβ kallade protofibriller, framställdes i delarbete tre (**paper III**) en antikropp med specifik bindningsförmåga för protofibriller. Antikroppen användes för att sätta upp en analysmetod som mäter mängden protofibriller i biologiska prover. I **paper I** föreslogs det att prover med den arktiska mutationen innehöll en ökad mängd oligomeriserat Aβ, och med hjälp av den nya analysmetoden kunde förhöjda nivåer av protofibriller påvisas i **papper III**. Den här analysmetoden kommer nu att utvärderas med prover från patienter, med målet att kunna använda mätningar av protofibriller som en del i Alzheimerdiagnostiken. Antikroppen som analysmetoden bygger på kommer även att testas som behandling av Alzheimers sjukdom, då den i andra studier visat sig kunna oskadliggöra och minska mängden av de giftiga protofibrillerna.

Observationerna i **paper I-III** tydde alla på att närvaro av oligomeriserat Aβ i ett prov resulterade i en ”falskt” för låg signal i $A\beta_{42}$-ELISAn. För att kunna undersöka närvaron av alla oligomeriserade Aβ-former, och inte enbart protofibrillen som i **paper III**, användes dessa fynd för att etablera en ”oligomerkvot”. Denna kvot användes i **paper IV** för att detektera oligomeriserat Aβ i CSF från AD-patienter och jämnåriga friska kontrollpersoner. Alzheimerpatienter och personer med mild kognitiv störning (MCI), vilket kan utgöra ett förstadium till AD, visade sig ha förhöjda oligomernivåer i CSF i jämförelse med friska kontrollpersoner och individer med frontallobsdemens. Dessa fynd tyder på att närvaron av oligomeriserat Aβ är specifikt för Alzheimers sjukdom, och därför skulle kunna användas som en diagnostisk markör.

Mekanismen där ett protein likt Aβ börjar aggregera och bilda toxiska oligomerer är troligen generell för flera sjukdomar, även om själva proteinet är specifikt för varje sjukdom. Förutom AD finns flera så kallade felveckningssjukdomar där symptomen orsakas av proteinaggregering, till exempel prionsjukdomar, amyotrof lateral skleros (ALS) och Parkinsons sjukdom. Därför är Alzheimerforskningen av generell betydelse då kunskapen delvis kommer att gå att tillämpa på fler sjukdomar. Det skulle till exempel vara möjligt att etablera en oligomerkvot, likt den beskriven i **paper IV**, för lösliga proteinaggregat av till exempel α-synuklein som är inblandat vid Parkinsons sjukdom.

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