Targeted Aβ proteomics –
A tool to study the pathogenesis of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disorder of the central nervous system. Diagnosis and monitoring of sporadic AD has long depended on clinical examination of individuals with end-stage disease. The accumulation of amyloid-β (Aβ) peptides in specific brain regions is believed to represent the earliest event in the pathogenesis of the disease and there is developing consensus for the use of cerebrospinal fluid (CSF) Aβ as a core biomarker for the mild cognitive impairment stage of AD.

Aβ has been the subject of extensive research aimed at identifying markers for the disrupted balance between the production and clearance of the peptide. Many studies on Aβ in plasma, cell media, and CSF have been based on immunoassays such as enzyme-linked immunosorbent assays where specific antibodies are used to discriminate between for example the 40- and 42-amino acid long Aβ peptides (Aβ1-40 and Aβ1-42, respectively). The aim of this thesis was to develop a targeted Aβ proteomic approach using immunoprecipitation (IP) and mass spectrometry (MS).

To study Aβ in CSF, a highly specific IP was developed and combined with MS. Using various Aβ specific antibodies with different epitopes, more than 20 Aβ isoforms have so far been identified and verified. Furthermore, a relative abundance pattern including Aβ1-16, and Aβ1-42 in CSF, distinguished sporadic AD patients from non-demented control subjects with a high degree of accuracy in two independent studies.

The IP-MS method was automated and further optimized which improved the speed of sample preparation and thus sample capacity. By adding isotopically labelled internal standards, variations in the IP and the MS desorption/ionization processes were diminished. This increased the possibility of using the method in AD diagnostics and of estimating the concentration of the Aβ isoforms present in CSF.

To address from which processing pathways the shorter isoforms arise, for example Aβ1-15/16/17, a cell model accurately reflecting the Aβ isoform pattern in CSF was developed. The optimized and automated IP-MS method was used to determine changes in the Aβ isoform pattern induced by α-, β-, and γ-secretase inhibitor treatment. All isoforms longer than and including Aβ1-17 were γ-secretase dependent, whereas shorter isoforms were γ-secretase independent. These shorter isoforms, including Aβ1-15, were reduced by treatment with α- and β-secretase inhibitors, suggesting the existence of a third and previously unknown APP processing pathway.

The described APP processing pathway was further investigated by exploring the effects of γ-secretase inhibition on the Aβ isoform pattern in brain and CSF from transgenic mice. As in the cell model, all fragments longer than and including Aβ1-17 decreased upon γ-secretase inhibition, whereas the shorter isoforms, e.g. Aβ1-15, increased. These data, together with the cell model data, strongly suggest that Aβ1-15 and Aβ1-16 may be generated through a third metabolic pathway by concerted β- and α-secretase cleavage of APP.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Alzheimers sjukdom är den vanligaste orsaken till demens och den karaktäriseras bland annat av så kallade senila plack i hjärnan. Huvudkomponenten i senila plack är amyloid-β (Aβ) peptider och speciellt en 42 aminosyror lång Aβ peptid (Aβ1-42). Man tror idag att utfällningen av Aβ i specifika delar av hjärnan är den tidigaste händelsen i sjukdomsförloppet.

Likvor (cerebrospinalvätska) används idag för diagnostisering av många olika sjukdomar, då den är i direkt kontakt med hjärnan. Likvorns molekylära sammansättning avspeglar troligtvis de biokemiska processerna som sker i hjärnan. Man har bland annat visat att nivån av Aβ1-42 är lägre i likvor vid Alzheimers sjukdom jämfört med friska personer. Målet med denna avhandling var att utveckla en metod, som är baserad på antikroppar och masspektrometri (MS) för att identifiera och studera nya former av Aβ i likvor.

Genom att utnyttja olika antikroppar för att rena och koncentrera Aβ från likvor och MS för detektion, har i dagsläget fler än 20 olika varianter av Aβ identifierats. Förekomsten av fyra olika Aβ-peptider i likvor visade sig kunna särskilja Alzheimerspatienter från friska kontroller med en riktighet på 86 %. Vidare identifierades en ny potentiell markör för Alzheimers sjukdom. I två oberoende studier framkom att nivåerna av en Aβ-peptid bestående av 16 aminosyror (Aβ1-16) var högre hos patienter med Alzheimers sjukdom jämfört med friska kontrollpersoner.

Aβ1-42 bildas från ett större protein som heter amyloidprekursorprotein (APP) genom samverkan av två olika enzymer som heter β- och γ-sekretas. För att utreda hur till exempel Aβ1-16 bildas användes celler som hade behandlats med preparat som hämmar β- och γ-sekretas. Vidare hämmades även α-sekretas, som är ett enzym som klyver mitt i Aβ sekvensen och motverkar bildandet av Aβ1-42. På detta sätt identifierades en tredje och tidigare okänd APP-processväg, där först β- och sedan α-sekretas klyver APP, vilket leder till frisättning av Aβ1-16. Denna processväg är oberoende av γ-sekretas och verifierades även i en studie av γ-sekretashämmade möss. Vidare presenteras även data som visar att Aβ1-16 är en positiv och mycket känslig biomarkör för γ-sekretashämning, vilket kan få betydelse i framtida behandlingsstudier.

Sammanfattningsvis visar denna avhandling att Aβ1-16 potentiellt kan användas som en markör för klinisk diagnostisering vid Alzheimers sjukdom samt att Aβ och dess olika varianter i likvor eventuellt kan användas för att följa läkemedelsbehandlingar.

LIST OF PAPERS

This thesis is based on the following papers:

I. **Portelius, E**; Westman-Brinkmalm, A; Zetterberg, H; Blennow, K. Determination of β-amyloid peptide signatures in cerebrospinal fluid using immunoprecipitation-mass spectrometry. Journal of proteome research. 5, 1010-1016, 2006

II. **Portelius, E**; Zetterberg, H; Andreasson, U; Brinkmalm, G; Andreasen, N; Wallin, A; Westman-Brinkmalm, A; Blennow, K. An Alzheimer's disease-specific β-amyloid fragment signature in cerebrospinal fluid. Neuroscience letters. 409, 215-219, 2006

III. **Portelius, E**; Tran, A; Andreasson, U; Persson, R; Brinkmalm, G; Zetterberg, H; Blennow, K; Westman-Brinkmalm, A. Characterization of amyloid β peptides in cerebrospinal fluid by an automated immunoprecipitation procedure followed by mass spectrometry. Journal of proteome research. 6, 4433-4439, 2007

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V. **Portelius, E**; Zhang, B; Gustavsson, M; Brinkmalm, G; Westman-Brinkmalm, A; Zetterberg, H; Lee, V; Trojanowski, J; Blennow, K. Effects of γ-secretase inhibition on the amyloid β isoform pattern in a mouse model of Alzheimer's disease. Submitted

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Portelius, E; Brinkmalm, G; Tran, A; Andreasson, U; Zetterberg, H; Westman-Brinkmalm, A; Blennow, K; Öhrfelt, A. Identification of novel N-terminal fragments of amyloid precursor protein in cerebrospinal fluid. Experimental neurology. In press, 2009

Review publications

Andreasson, U; **Portelius, E**; Andersson, ME; Blennow, K; Zetterberg, H. Aspects of β-amyloid as a biomarker for Alzheimer´s disease. Biomarkers in medicine. 1, 59-78, 2007

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CONTENTS

ABBREVIATIONS

Aβ	Amyloid-β
ACE	Angiotensin-converting enzyme
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease family
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BACE	β-site APP-cleaving enzyme
BBB	Blood-brain barrier
CID	Collision-induced dissociation
CSF	Cerebrospinal fluid
ECD	Electron capture dissociation
ECE	Endothelin-converting enzyme
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FAD	Familial Alzheimer's disease
FTICR	Fourier transform ion cyclotron resonance
IDE	Insulin-degrading enzyme
IP	Immunoprecipitation
IRMPD	Infrared multiphoton dissociation
LC	Liquid chromatography
LRP	Low-density lipoprotein receptor-related protein
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Mass selection/mass separation
NEP	Neprilysin
PET	Positron emission tomography
PIB	Pittsburgh Compound-B
PS	Presenilin
SAD	Sporadic Alzheimer's disease
Tg	Transgenic
TOF	Time-of-flight

INTRODUCTION

ALZHEIMER'S DISEASE

Alzheimer's disease (AD), first described by Alois Alzheimer in 1906,[1] is the main cause of dementia in the ageing population, accounting for 50-60% of approximately 20-30 million dementia cases around the world.[2] It is a slowly progressive, neurodegenerative disorder of the central nervous system characterized by advancing episodic memory impairment and general cognitive symptoms such as deteriorating judgement, decision-making and orientation. Neuropathologically AD is characterized by the abnormal accumulation of intraneuronal neurofibrillary tangles and extracellular senile plaques in specific brain regions together with massive neuronal and synaptic degeneration (*Figure 1*).

Of all AD cases the majority have the sporadic form (SAD), of unknown cause, while a minority (accounting for less than 0.1%) have the familial form (FAD) caused by autosomal dominant mutations with an onset before the age of 65 years.[3] The first identified mutation causing FAD was a missense mutation in the amyloid precursor protein (*APP*) gene on chromosome 21 (V717I).[4] While investigating other FAD families, several additional *APP* mutations were found most of which were located within or immediately flanking the amyloid-β (Aβ) region of APP.[5] These mutations in the *APP* gene have been shown to increase Aβ production, although by different mechanisms. To date, more than 20 different missense mutations have been identified in *APP*.[6]

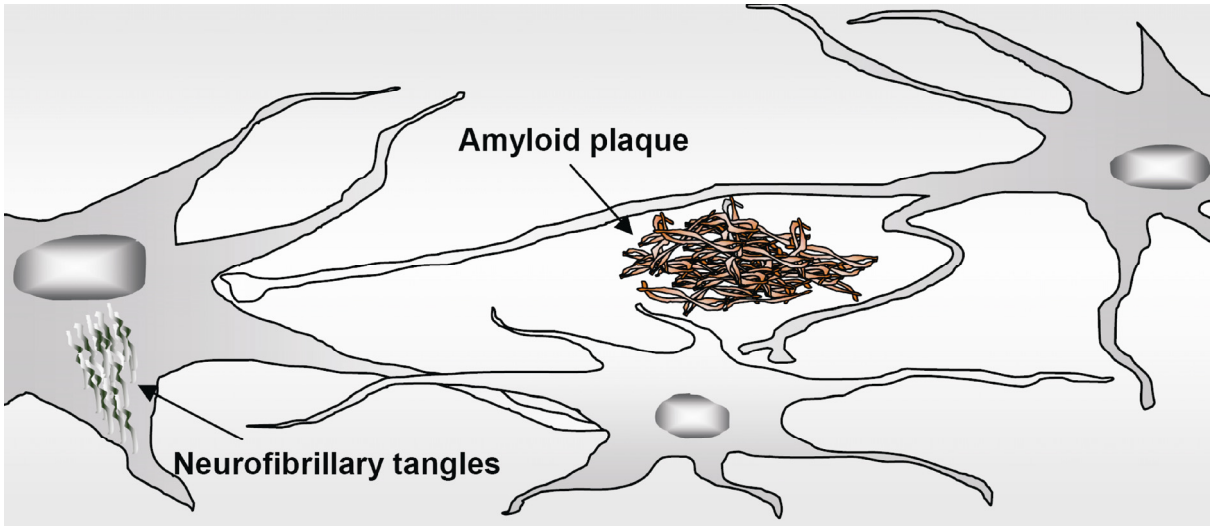

Figure 1. Intraneuronal neurofibrillary tangles and extracellular senile plaques.

Down's syndrome cases that possess an extra *APP* gene have an increased Aβ production and may develop AD dementia symptoms early in life through over-expression of APP.[7] Recently, genomic duplications in the *APP* locus were reported in

families displaying early onset AD in the absence of trisomy 21 suggesting that increased expression of APP can give rise to AD pathology.[8]

Yet, these mutations in the *APP* gene explain only a few familial cases. Most FAD cases are caused by mutations in the highly homologous genes of the presenilin enzymes involved in the production of Aβ, presenilin 1 (*PS1)* located on chromosome 14 and presenilin 2 (*PS2)* on chromosome 1, which appear to accelerate Aβ plaque formation.[9, 10] It has been shown that these mutations increase the Aβ1-42/Aβ1-40 ratio in plasma of affected individuals, in transfected cells, and in transgenic animals.[11-14] To date more than 150 AD-promoting PS mutations have been identified.[15]

There is a link between AD and the apolipoprotein E (*APOE*) ε4 allele, calculated to account for most of the genetic risk for late-onset disease, operating mainly by modifying the age of onset.[16-18] Although it is not fully understood how *APOE* ε4 leads to increased Aβ deposition it could be either by an enhanced production or a decreased clearance of Aβ. Among other potential risk factors for developing AD besides increasing age, are reduced brain size, head injury and low educational and occupational attainment.[2]

The clinical diagnosis of AD is based on medical history together with a clinical, neurological, and psychiatric examination and depends mainly on the exclusion of other dementias. The diagnosis is often based on the criteria from the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA).[19] To attain a definitive diagnosis of AD a neuropathological examination is required.

Plaque and the amyloid cascade hypothesis

Senile plaques, one of the typical hallmarks of AD, were identified in the mid-1980s to be composed mainly of Aβ peptides.[20-22] This paved the way for cloning of the *APP* gene on chromosome 21 in 1987.[23, 24] Aβ is expressed constitutively throughout life during normal cell metabolism. In AD, Aβ forms fibrils, deposited in the brain as plaques. Much of the fibrillar Aβ found in the plaques consists of Aβ1-42, the slightly longer, more hydrophobic form that is particularly prone to aggregation.[25, 26] However, Aβ1-40 which is normally more abundantly produced by cells than Aβ1-42, is usually colocalized with Aβ1-42 in the plaque (*Figure 2*).

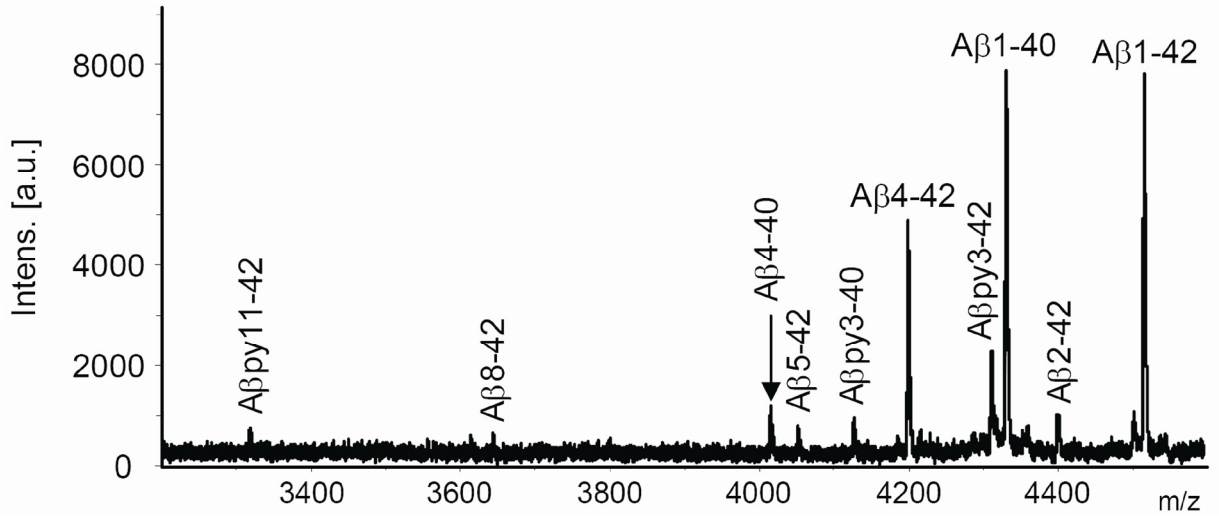

Figure 2. Mass spectrum of an AD brain sample immunoprecipitated using the antibody 4G8 from formic acid extraction of hippocampus. Several peaks corresponding to Aβ isoforms were detected including a modification on position 3 and 11 in the Aβ sequence (py = pyroglutamate).

The amyloid cascade hypothesis was first described more than 15 years ago.[27, 28] It postulates that there is an imbalance between the production and clearance of Aβ which activates a self-aggregation cascade leading to the formation of neurotoxic Aβ oligomers and larger Aβ assemblies, which drive the synaptic loss and neuronal degeneration in the disease process (*Figure 3*). The resulting accumulation of Aβ in the brain is the primary force driving AD pathogenesis.[29] However, results from cell culture data are contradictory. Synthetic Aβ peptides may show no, toxic or trophic effects on neurons, and some studies suggest that Aβ-induced toxicity only occurs in conjunction with other factors, such as metal ions or oxidative stress.[30] One explanation for this deviating data may be that the neurotoxicity of Aβ is related to its aggregation state.[5] Data supporting this view indicate oligomers of Aβ possess toxic effects that induce apoptosis[31-33] while insoluble Aβ fibrils induce negligible neuronal loss.[34] Recently it has been shown that a dodecameric form of Aβ (Aβ*56) impaired memory in a transgenic mouse model.[35] Furthermore, it even remains an open question whether the toxicity is produced by intracellular or extracellular Aβ.[36]

While researchers still do not know what causes AD, it is widely believed that the formation of amyloid plaques is a central feature. All other disease processes, including formation of neurofibrillary tangles, might therefore be the result of an imbalance between Aβ production and clearance (*Figure 3*).

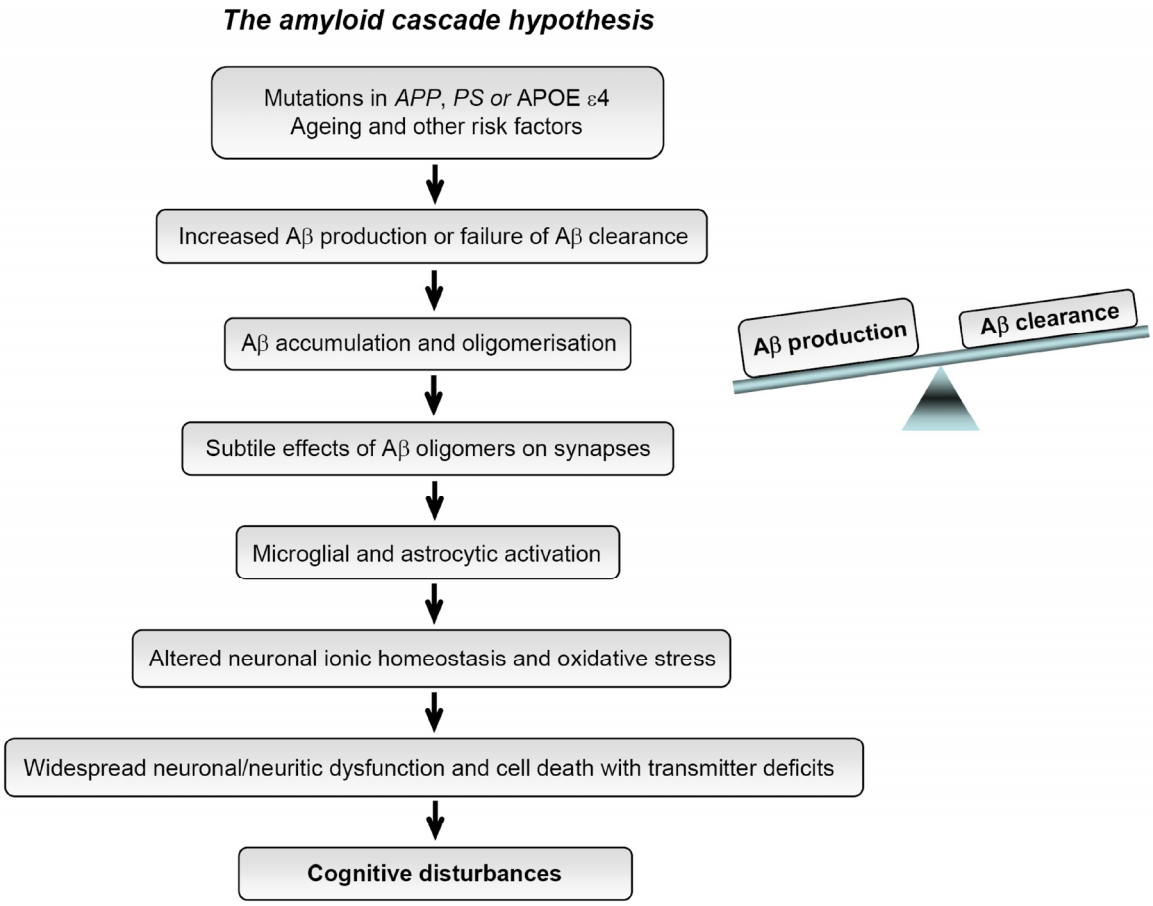

Figure 3. According to the amyloid cascade hypothesis, the central event in AD pathogenesis is an imbalance between Aβ production and clearance, with increased Aβ production in FAD and decreased Aβ clearance in SAD. Aβ oligomers might directly impair synaptic function, in addition to the inflammatory and oxidative stress caused by aggregated and deposited Aβ.

There is mounting evidence supporting this hypothesis. Trisomy 21 (Down's syndrome) for example, leads to APP over expression due to increased dosage of the *APP* gene on chromosome 21 and early onset of AD with classical AD neuropathology in the form of plaques and tangles.[21] Further, purified Aβ oligomers specifically inhibit long-term potentiation and cause memory dysfunction.[35, 37] There are also observations that do not fit with the hypothesis. One major objection is that although some studies have observed a strong correlation between dementia and senile plaques[38] others have identified only a weak correlation between degree of cognitive impairment and the number of amyloid deposits in the brain.[39] For example, some elderly people with no AD symptoms have many Aβ deposits.[40] Further, the deposition of Aβ may also be a consequence of traumatic brain injury (TBI) making TBI a risk factor for AD.[41] It has been shown that the production of APP is increased after TBI[42] which together with local accumulation of APP, as seen in traumatic axonal injury, creates a possible source for APP´s intraneuronal processing, resulting in production of Aβ.[43, 44]

Aβ production

Aβ is produced through the amyloidogenic pathway from APP by proteolytic cleavage involving β- and γ-secretase.[45] The exact biological function of APP and its known derivatives remain elusive although several cell biological studies indicate that APP has a role in cell growth and neuritic outgrowth.[46, 47] β-Secretase is a membrane-anchored aspartyl protease encoded by the β-site APP-cleaving enzyme 1 (*BACE1*) gene.[48-51] Cleavage of APP by β-secretase generates a large soluble N-terminal fragment and a 12 kDa C-terminal fragment known as C99 (*Figure 4*). This smaller fragment can be further processed by γ-secretase generating APP intracellular domain (AICD) and Aβ consisting of 40 or 42 amino acids (*Figure 4*).[52] γ-Secretase is a membrane-bound protease complex consisting of at least four essential components: the homologous PS1 and PS2, nicastrin, Aph-1 and Pen-2.[53]

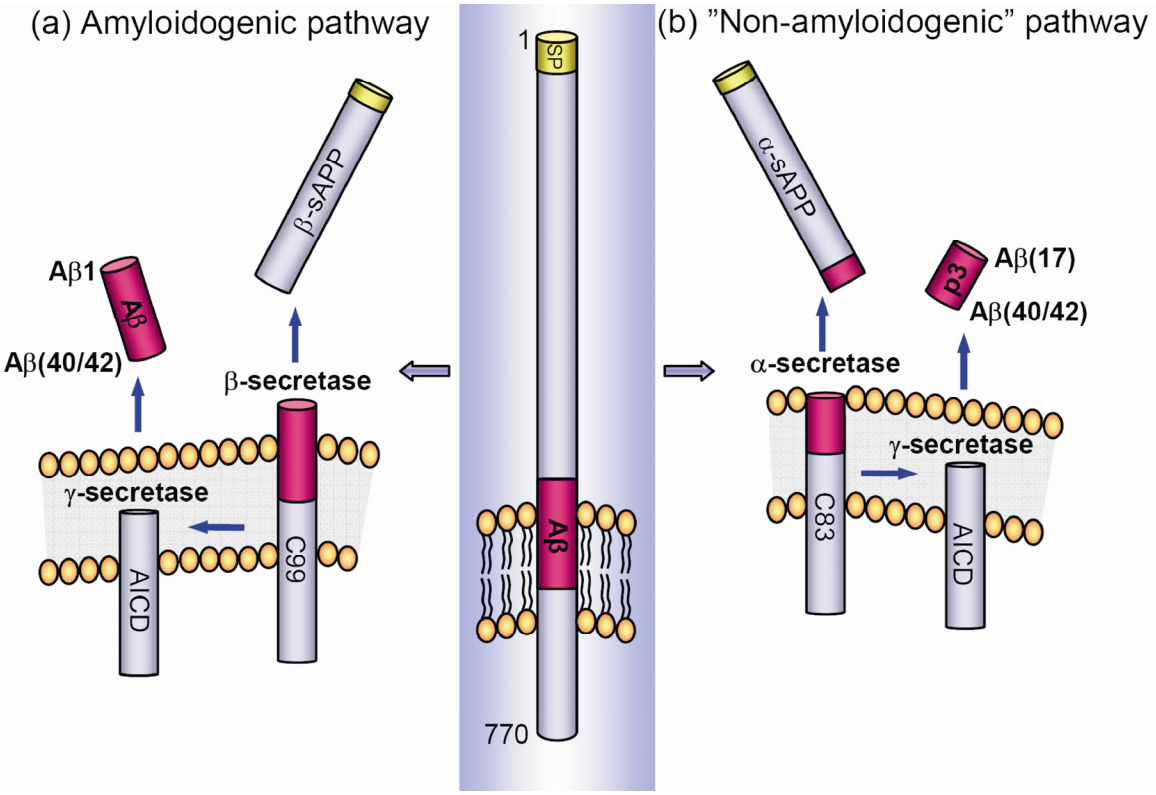

Figure 4. The metabolism of the largest isoform of APP (APP770) with Aβ and p3 generation.

In the alternative non-amyloidogenic pathway, α-secretase cleaves Aβ at amino acid 16 and, followed by γ-secretase cleavage, an Aβ17-42 fragment, also known as p3, is liberated.[54-56] The candidate α-secretases identified to date are ADAM9,[57, 58] ADAM10[59, 60] and ADAM17,[61, 62] all of which are membrane-anchored cell surface proteins belonging to the a disintegrin and metalloprotease (ADAM) family. The p3 peptide has been isolated from AD brains containing vast deposits of diffuse amyloid plaques and also from patients with Down's syndrome.[63, 64] However, describing this pathway as non-amyloidogenic might be misleading since studies have shown that p3

has neurotoxic properties, indicating that it might be a contributing factor to AD pathogenesis.[65] Further, the p3 fragment has not been detected in CSF. Instead, it may be a component of plaques in the brain or the concentration may be too low for detection by present-day analytical methods. A third possibility is that the peptide is further processed by various proteases to shorter, undetectable Aβ fragments.

A fourth potential cleavage site in APP, located 12 residues N-terminal of the β-secretase site, has been designated the δ-secretase site.[66] Yet another was found between amino acids Leu[49] and Val[50] (numbering relative to the Aβ sequence) and termed the ε-site.[67]

Aβ clearance

Numerous different Aβ-degrading enzymes have been proposed as being involved in the clearance of Aβ by cleaving Aβ at multiple sites *in vitro*.[45] Neprilysin (NEP) has more than 10 different proposed cleavage sites within the Aβ1-40 sequence (*Figure 5*).[68] Furthermore, NEP degrades Aβ1-42 and is also capable of degrading the more pathogenic oligomeric form of Aβ.[69] Strongly supporting NEP as a key player in the clearance of Aβ is the increased level of Aβ1-40 and Aβ1-42 in several different brain areas in NEP-deficient mice.[70] Insulin-degrading enzyme (IDE) has also been shown to degrade Aβ at multiple sites.[71] However, it has been shown to be a poor degrader of oligomerized Aβ, which may be of relevance in AD pathogenesis.[72] Endothelin-converting enzyme (ECE) is expressed in two different forms, ECE-1 and ECE-2, both of which seem to participate in Aβ degradation.[73] Further, recombinant ECE-1 hydrolyzes synthetic Aβ at multiple sites (*Figure 5*).[74]

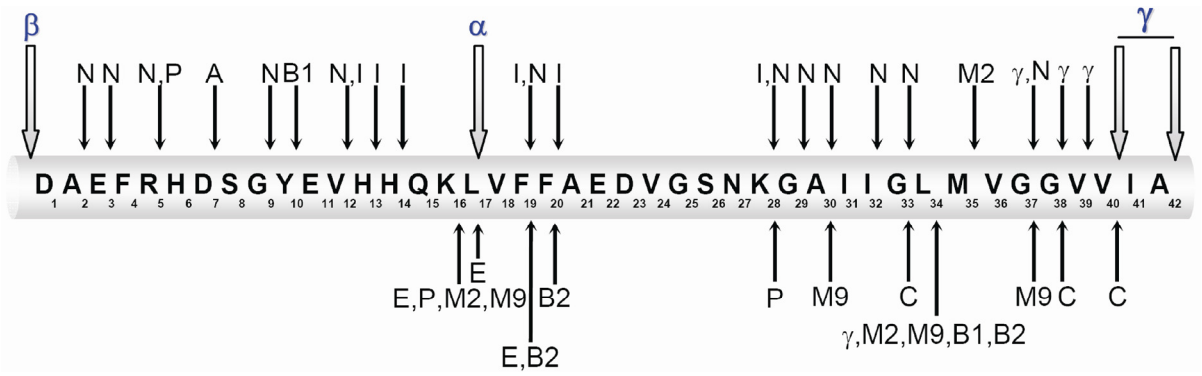

Figure 5. Aβ production involves β- and γ-secretase in the amyloidogenic pathway. α-Secretase cleaves at amino acid 16 in the sequence precluding Aβ production. Aβ can be further processed by numerous proteases; A: ACE; C: Cathepsin B; E: ECE-1 and ECE-2; I: IDE; M2: MMP-2; M9: MMP-9; N: NEP; P: Plasmin; B1: BACE1; B2: BACE2.

Plasmin has been shown to be markedly reduced in the brain of AD patients and to hydrolyze Aβ with physiological efficiency at multiple sites implying that down-regulation of this protease may cause deposition of Aβ.[75-77] Other candidate Aβ-

degrading enzymes include Angiotensin-converting enzyme (ACE), Matrix metalloproteases (2, 3 and 9) and Cathepsin B (*Figure 5*).[45]

In addition to degradation by enzymes, dysfunction in the blood-brain barrier (BBB) may contribute to the development of AD through clearance failure of toxic Aβ species from the brain.[78-80] There is mounting evidence that specialized transporters of Aβ must exist to remove the peptide from the brain into the blood stream.[81] Aβ may be removed from the brain by low-density lipoprotein receptor-related protein-1 (LRP)-mediated efflux across the BBB, where it is then degraded at peripheral sites.[80, 82] Aβ clearance is also influenced by two known ligands to LRP, α2-macroglubulin and apoE, which mediate endosomal Aβ internalization and subsequent degradation via the lysosomal pathway.[83] The level of apoE has been shown to be low in *APOE* ε4-positive AD patients which may result in a reduced clearance capacity of soluble Aβ and an increased rate of amyloid deposition in the extracellular space which has also been reported.[84]

ROUTES TO STUDY Aβ PATHOGENESIS

Cell culture

To address from which processing pathways the various Aβ peptides arise, cell cultures make valuable models since they can easily be transfected with constructs over-expressing human APP, enabling the study of Aβ in a specific manner. Cells expressing FAD mutants of APP and PS for example, are reported to secrete increased amounts of Aβ, due to altered γ-secretase cleavage sites, modified specificity of γ-secretase or because APP becomes a superior substrate for BACE, suggesting a link between this variant of Aβ and AD pathogenesis.[5] Most of our present knowledge of APP processing has come from work with cultured cells. However, one drawback with these models is that they are based mainly on the familial form of AD caused by mutations in the *APP* or *PS* genes which may not reflect the mechanism in SAD. Furthermore, they do not reflect complete AD pathology. One of the most popular cells used today is SH-SY5Y, a human neuroblastoma cell line.

Animal models

Transgenic mice (Tg) models mimicking human AD pathology have been used for more than 10 years to investigate AD pathogenesis and test potential treatments. The first Tg mouse with an AD-related phenotype was reported in 1995 and expressed mutant APP (V717F) under control of a platelet-derived growth factor promoter.[85] This line over-expresses mutant APP at levels that generate plaques. Shortly thereafter, the Tg 2576 mouse model (APPswe, K670N/M671L) was developed (hamster prion protein promoter)[86] which is one of the most well characterized strains of transgenic animals over-expressing human APP. To date, more than 20 pathogenic mutations identified in *APP*, including E693G "TgAPPArc" and "TgAPPArcSwe", have been

expressed in Tgs.[87] They develop amyloid deposits in the brain and have been the most widely used tool in the study of AD-related pathogenic mechanisms.[88-90]

The Tg 2576 can be modified to express other mutant genes, including *PS1* and *PS2*, that cause FAD and they manifest most of the signs and symptoms of AD.[91] Recently, the expression of APPSwe and P301L tau were combined on a *PSEN1* (M146V) background generating a 3xTg-AD mouse model closely recapitulating human AD pathology.[92] By genetically interfering with regulatory enzymatic pathways, the role of these enzymes in APP processing, Aβ deposition and memory impairment has been characterized.[2] For instance, by crossing APP Tg mice on a *BACE*$^{-/-}$ background, Aβ formation and deposition was reduced while Tg BACE over-expression in APP/BACE mice increased Aβ generation.[93-95] One useful feature of APP transgenic mice is the opportunity it gives to test disease-modifying strategies *in vivo* before launching clinical trials. However, at the present time, very few Tg models reproduce all the features of AD-type pathology. They display for example almost no neurodegeneration, despite plaque pathology and there is no development of tangles.[96]

Plasma

An excellent biomarker for AD would be the steady-state levels of Aβ in human plasma since no spinal tap would be needed as for CSF. There are enzyme-linked immunosorbent assays (ELISA) to measure Aβ1-40 and Aβ1-42 but the results are conflicting. Different groups report either high or low plasma Aβ1-42 levels in SAD although with a broad overlap between patients and controls, while most groups find no significant differences.[97] These variations between groups may be due to Aβ1-42 being very hydrophobic and may bind to high abundant plasma proteins including albumin and lipoproteins.[98, 99] Further, both plasma protein binding and oligomerisation may mask Aβ epitopes, resulting in reduced apparent concentration of Aβ. It should also be noted that Aβ is produced by many cell types and there is no correlation between Aβ1-42 levels in plasma and CSF.[100, 101] However, FAD patients having *PS1*, *PS2* or *APP* mutations have significantly higher plasma Aβ1-42 levels than controls, suggesting that plasma could be used for detecting changes in Aβ metabolism caused by these mutations.[102]

CSF

CSF is a valuable diagnostic window on neurodegenerative diseases for many reasons. It is in direct contact with brain interstitial fluid and its molecular composition reflects metabolic processes in the brain. Thus, CSF Aβ concentrations are more likely to reflect brain Aβ metabolism than analysis of either blood or plasma. It has been known since 1992 that Aβ is secreted to CSF as a soluble peptide, as part of normal constitutive APP metabolsim.[103] This finding made CSF Aβ a candidate biomarker for AD and more specifically Aβ1-42 which became the main target in CSF since it was

found to be the initial form deposited in plaques.[104-106] Numerous studies using ELISA methods have shown that the CSF Aβ1-42 level is significantly reduced in AD CSF compared with healthy controls, and with an even greater decrease in the Aβ1-42/Aβ1-40 ratio.[107] This lower concentration of Aβ1-42 in AD CSF has been hypothesized as reflecting the peptide deposition in the plaques, with smaller quantities diffusing to the CSF.[108] Further, it has been shown that Aβ is manifested in many isoforms in CSF having both N- and C-terminal truncations where the most abundant Aβ peptide is Aβ1-40.[52, 109, 110]

In a recent study, healthy subjects were infused intravenously with stable isotope-labelled leucine, after which CSF was continuously sampled through an intrathecal catheter for 36 h.[111] Using immunoprecipitation (IP) in combination with mass spectrometry (MS), the Aβ turnover in man was determined.[112] The production and clearance rate was 7-8% of the total Aβ per hour. The same methodology was used to determine the effect on Aβ production during treatment with a γ-secretase inhibitor, LY450139, and it was shown that the production of Aβ in the central nervous system was significantly decreased while Aβ clearance remained stable.[113] Using this method it may be possible to resolve the longstanding question whether SAD patients have an increased production of Aβ, a reduced clearance, or both.

Many researchers are trying to develop sensitive methods for specific detection of the oligomeric pool of Aβ in CSF since soluble oligomers may be the earliest effectors of synaptic compromise in AD.[114] Aggregated forms of Aβ are readily detected in senile plaques while data on biological fluids are more ambiguous. It has been reported that human CSF from both elderly controls and AD patients contains dimers of Aβ which disrupt synaptic plasticity.[115] There are also reports of quantitative detection of Aβ oligomers in CSF.[116, 117] However, these results need to be verified.

PIB-PET

An alternative to CSF analysis, Aβ deposition can be monitored *in vivo* using positron emission tomography (PET) enabling live visualization of amyloid deposits in AD brains.[118-120] A PET scan can potentially indicate biological changes in the brain attributable to AD earlier than any other diagnostic test.[121] The technique is based on an intravenous injection of a radioactive substance (tracer) that enters the brain and binds to endogenous amyloid. Using Pittsburgh Compound-B (PIB) as the tracer, the amyloid imaging technique has been used for direct visualization of amyloid plaques in the living human brain[122, 123] and accumulating evidence suggests that PIB-PET may be a useful diagnostic tool in patients with memory complaints.[118, 124] Individuals with positive PIB binding for example, also have lower CSF Aβ1-42 levels suggesting that both measurements are complementary as indicators for fibrillar Aβ burden.[125] An association between PIB retention and CSF total-Tau has also been shown.[126, 127] However, PET analysis is expensive and has limited availability.

EXPERIMENTAL SECTION

TARGETED Aβ PROTEOMICS USING IMMUNOPRECIPITATION

In proteomics, high resolution separation methods such as capillary or gel electrophoresis, isoelectric focusing and high performance liquid chromatography (LC) are used in conjunction with MS and bioinformatics to study differences in protein expression due to genetic variations, disease or drug treatment.[128]

Although it is possible to analyze proteins directly in CSF using MS, only the most abundant proteins, such as albumin, the immunoglobulins, and beta-trace protein (prostaglandin-H2 D-isomerase), representing approximately 80% of the total protein content, are detected, due to the complexity and large dynamic range of most proteomes.[129] A truly comprehensive view of all expressed proteins is not possible today, due to insufficient separation of these complex mixtures. Since many of the proteins and peptides of interest are in nanomolar concentrations, a targeted proteomic approach is often necessary, focusing on selective identification of a specific peptide or protein and its variants (isoforms) to study disease specific peptide patterns.

Many studies on Aβ in CSF have been based on immunoassays where specific antibodies directed against different epitopes have been used to discriminate between for example Aβ1-40 and Aβ1-42.[100] However, other proteolytically processed Aβ peptides (isoforms) are difficult to detect using standard methods, possibly because they comprise a heterogeneous set of both N- and C-terminally truncated peptides, some of which are present in low concentrations. IP can be used to preferentially enrich these low-abundant Aβ isoforms. IP using antibodies, also known as immunoaffinity capture, in combination with MS involves a selective capture of Aβ from solution, e.g., plasma, CSF, cell culture media, or brain tissue extracts, prior to MS analysis. It is a powerful separation method based on the specific interaction between immobilized antibodies and the target protein. Detection using MS can not only verify the expected antigen, but also enable the identification of modified and differentially processed forms of the antigen, antibody cross-reactive species and molecules that interact with the antigen. In a typical IP-MS experiment, the Aβ specific antibody is immobilized on a chromatographic medium which is subsequently incubated with for example CSF. After extensive washing, the antigen is eluted and analyzed using MS. IP-MS has previously been used for determination of the Aβ peptide profile in cultured cell media and CSF.[110, 130]

MASS SPECTROMETRY

MS is a very powerful technique that can be used to identify/characterize and quantify proteins and peptides of interest. In MS, analyte molecules are transformed into gas-phase ions and separated according to their mass-to-charge ratios (m/z). MS can also be used to determine the primary structure, i.e., the amino acid sequence of a peptide.

This can be accomplished through so-called tandem MS or MS/MS (mass selection/mass separation) experiments.[131] There are numerous methods of ion generation. However, for proteomic analysis, matrix-assisted laser desorption/ionization (MALDI)[132, 133] and electrospray ionization (ESI)[134] are at present the methods of choice and will be further discussed in the sections below.

Matrix assisted laser desorption/ionization time-of-flight mass spectrometry

MALDI is based on ions that are created by irradiating a target spot with short laser pulses from a focused laser beam (~150 μm in diameter). In standard practice, MALDI is mostly combined with a time-of-flight (TOF) mass analyzer[135] although it can be combined with other mass analyzers such as trapping analyzers.[136] The TOF analyzer separates ions according to the elapsed time between the start signal and the pulse generated when an ion hits the detector, that is, the time of flight. At present, it is possible to obtain MS/MS data by coupling two TOF analyzers, tandem time-of flight (TOF-TOF). A MALDI-TOF provides comparatively high mass accuracy and resolution and is very sensitive and fast. Furthermore, MALDI is quite tolerant of sample contamination such as relatively high concentrations of salts, buffers and other species. The main disadvantage is the practical inability to couple it online to LC even though there are increasing uses of offline-LC-MALDI-TOFMS.[137]

The sample is, in the most widespread form of MALDI-TOFMS, mixed with a matrix of highly light-absorbing, low-mass molecules and subsequently applied to a target plate and inserted into the vacuum region of the instrument.[138] The laser pulse excites the matrix molecules causing an explosive breakup of the sample and ionization of a fraction of the analyte molecules. Subsequently ejected gas-phase ions are transmitted to the analyzer where, predominately peptides and proteins, carrying one single charge $[M+H]^{1+}$, are detected.[139]

Liquid chromatography electrospray ionization mass spectrometry

In LC, separation occurs when molecules interact to different extents with a mobile and/or stationary phase where the stationary phase typically consists of small porous particles, for example silica beads coated with different ligands. Proteins and peptides that interact strongly with the stationary phase will elute later from the chromatographic system, thus have longer retention times. There are numerous different chromatographic systems which can be used, reversed-phase chromatography for example, relying on hydrophobic interactions between the analyte and the stationary phase carrying hydrophobic groups. The choice of chromatographic system depends on the molecule of interest. By combining the physical separation capabilities of LC with MS, proteins and peptides can be analyzed with very high sensitivity and specificity.

The electrospray process is electrophoretic in nature. That is, an applied electric field results in the separation of positive and negative charges existing in the solution. Further, the strong electric potential produces an aerosol spray consisting of fine charged droplets from the liquid. These aerosol droplets carry an excess positive charge if the spray needle is at positive potential. As the droplet shrinks trough evaporation the ratio of surface charge to surface area increases until the charge repulsion overcomes the surface tension and the droplet breaks apart. Finally, ions are formed in atmospheric pressure. It should be noted that there are two different theories for ion formation, the "charged residue mechanism"[140] and "ion evaporation".[141, 142] Neither of the two theories has achieved universal acceptance.[143]

The ions formed in ESI at atmospheric pressure pass through an orifice, into an intermediate vacuum region, and from there into the high vacuum of the mass analyzer. There are numerous mass analyzers available including the quadrupole mass filter, the quadrupole ion trap and the orbitrap.[136] Here a hybrid linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer (FTICR MS) was used. Details will be discussed in paper **III**.

Protein identification

Identification of smaller proteins and peptides has been very successful using tandem mass spectrometry which involves multiple rounds of mass spectrometry with some form of fragmentation of gas-phase ions occurring.[144] By using MS/MS, information of the primary structure of the peptide or protein can be gained. There are numerous different mass analyzer combinations in tandem mass spectrometry of which the most commonly used are triple-quadrupole, quadrupole-time-of-flight, quadrupole ion trap and tandem time-of-flight. Usually, from many different peptides entering the mass spectrometer, peptides of a specific m/z are selected, isolated and then fragmented. Created fragments are then analyzed in the same (tandem-in-time) or a second (tandem-in-space) mass analyzer. See *figure 6* for a diagrammatic outline of a MS/MS experiment.

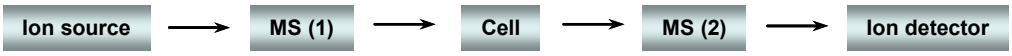

Figure 6. Diagrammatic outline of a system for MS/MS experiments. A collision cell is shown between the two mass spectrometer instruments.

There are also many different fragmentation techniques which can be used. One of the most popular methods is collision-induced dissociation (CID) which was used in paper **III** and **IV**. It is based on producing fragment ions in gas phase by colliding them with inert gas molecules (helium, nitrogen or argon). In paper **IV**, in addition to CID, infrared multiphoton dissociation (IRMPD) and electron-capture dissociation (ECD) was also used. The choice of fragmentation technique depends on the peptide or protein of interest.

AIM

The aim of this thesis was to develop a platform for targeted Aβ proteomics using mass spectrometry.

Paper I: Develop an IP-MS method for identifying novel Aβ peptides in human CSF.

Paper II: Investigate if the Aβ isoform pattern in CSF could be used to distinguish AD patients from non demented subjects.

Paper III: Optimize and automate the immunoprecipitation process followed by MALDI-TOFMS and nanoflow LC-MS.

Paper IV: Address by which processing pathways the shorter Aβ isoforms arise using a cell model.

Paper V: Further investigate the proposed processing pathway discovered in paper **IV** using a mouse model of AD.

Paper VI: Test the hypothesis that AD can be characterized by a specific CSF Aβ isoform pattern that is distinct when comparing sporadic and familial AD.

RESULTS AND DISCUSSION

PAPER I

When studying Aβ and its isoforms in human CSF, a targeted proteomics approach is necessary due to their low abundance in CSF. ELISA methods used today are unable to detect the shorter isoforms such as Aβ1-17 or differentiate between N-truncated peptides (AβX-38/40/42) and full length. Therefore, an IP method was developed which was compatible with MALDI-TOFMS for analysis of immunoprecipitated Aβ and its isoforms with high mass accuracy in a single analysis (*Figure 7*).

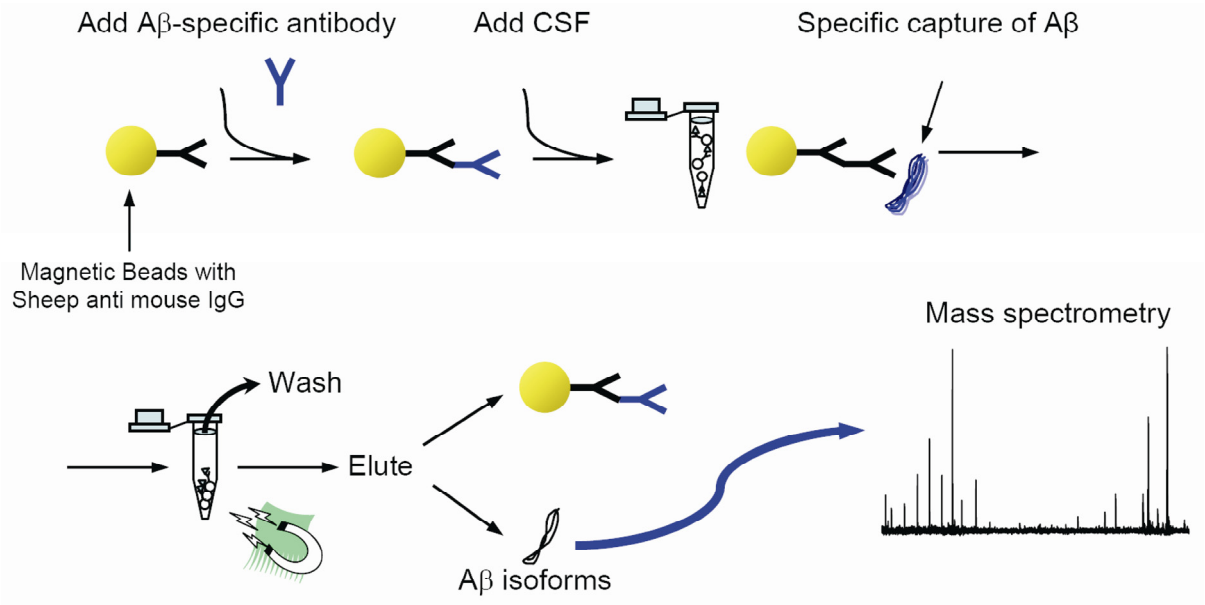

Figure 7. Schematic picture of targeted Aβ proteomics used in this thesis.

Magnetic Dynabeads, precoated by the manufacturer with sheep anti mouse IgG, were coupled to different Aβ specific monoclonal antibodies; 6E10 (epitope 4-9), 4G8 (epitope 18-22) or 11A50-B10 (reactive to the C-terminus of AβX-40) (Signet Laboratories, Inc.). These were then incubated with CSF allowing affinity capture of Aβ and its isoforms. After extensive washing, the Aβ isoforms were eluted using formic acid (FA). The collected supernatant was dried down in a vacuum centrifuge and redissolved in 5 µL 0.1% FA in 20% acetonitrile (ACN) and prepared for further analysis using MALDI-TOFMS. The FA and ACN concentrations are the key-players to optimize. The amount of acid and organic solvent greatly influences the number of Aβ isoforms detected in a single analysis since the isoforms range from hydrophilic to extremely hydrophobic. As seen in *Figure 8*, at least 19 different C- and N-terminally truncated Aβ peptides were identified. Mass detection showed high accuracy and corresponded well to calculated peptide masses (<15 ppm deviation except Aβ1-18, Aβ1-20 and Aβ1-42). Further, the 3 most intense peaks had m/z 4328.2, 2068.0 and 4130.0, corresponding to the singly protonated monoisotopic masses of Aβ1-40, Aβ1-

17 and Aβ1-38, respectively, which is consistent with Aβ1-40 being the most abundant C-terminally truncated Aβ peptide in CSF. However, it should be noted that the ratio between the Aβ1-42 and Aβ1-40 peaks in the mass spectrum cannot be interpreted as a reflection of their abundance in CSF since the ionization efficiency might be different for the two peptides and since Aβ1-42 is more hydrophobic and less soluble than Aβ1-40. By using 3 different antibodies, the risk of fragment patterns detected being the result of unspecific degradation during sample preparation, e.g. due to FA-induced degradation *in vitro*, can be excluded.

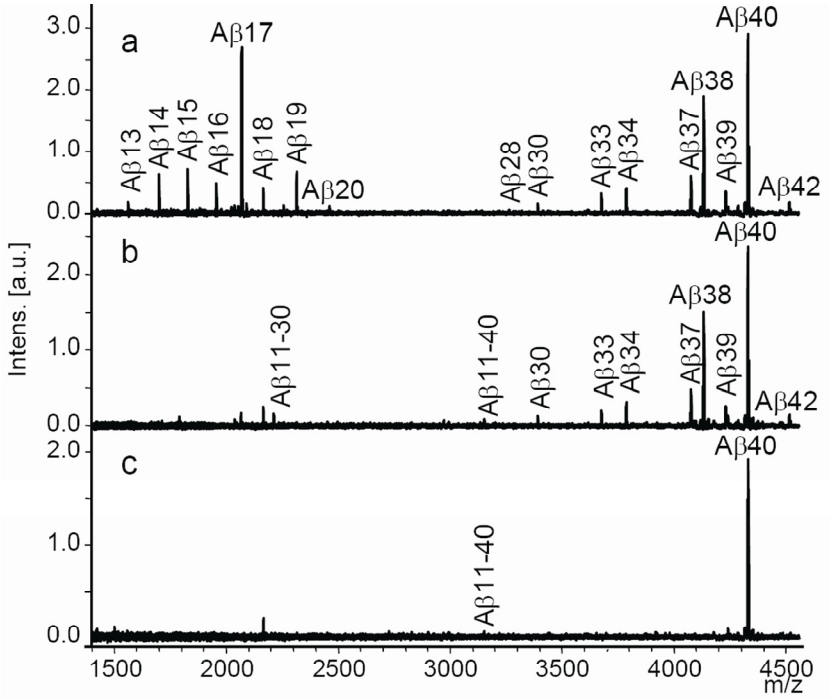

Figure 8. Mass spectra showing C- and N-terminally truncated Aβ peptides immunoprecipitated from CSF using the antibodies (a) 6E10, (b) 4G8 and (c) 11A50-B10. The m/z 2037.5, 2066.3 and 2165.7 correspond to the second charge state of Aβ1-37 [Aβ1-37 + 2H]$^{2+}$, Aβ1-38 [Aβ1-38 + 2H]$^{2+}$ and Aβ1-40 [Aβ1-40 + 2H]$^{2+}$, respectively.

Furthermore, the IP-MS method can also detect Aβ peptide modifications. Methionine, amino acid 35 in the Aβ sequence, is susceptible to oxidation, which would affect the C-terminally truncated Aβ peptides Aβ1-37, Aβ1-38, Aβ1-39, Aβ1-40 and Aβ1-42. An additional peak, which had the mass of the peptide plus 16 Da, was found for all those isoforms. The oxidized form of Aβ1-40 has been reported to be elevated in CSF from patients with Lewy body dementia compared with Parkinson's disease dementia and AD.[145] However, it should be noted that oxidized Aβ probably is extremely susceptible to artefacts caused by pre-analytical sample handling.[146]

In this paper, it has been shown that the combination IP and MS is one of the most successful analytical methods in targeted Aβ proteomics detecting several Aβ isoforms

with high mass accuracy in a single analysis. Using IP-MS, it was shown that Aβ is present in CSF in at least 20 isoforms ranging from Aβ1-13 to Aβ1-42 (Table 1).

This Aβ pattern further deepens our understanding of how the AD Aβ peptide is processed *in vivo* in humans and points to the involvement of a multitude of protease activities that may protect humans from Aβ overload in the brain. Finally, Aβ fragment signatures could potentially be used as a diagnostic test to distinguish AD patients from non-demented controls and other dementia disorders, a hypothesis that was tested in paper **II** and **VI**.

Table 1 Truncated Aβ peptides in CSF identified by IP-MS.

Aβ isoform	Mass (Da)	Aβ isoform	Mass (Da)
1-13	1561.7	1-28	3261.5
1-14	1698.7	1-30	3389.6
1-15	1826.8	1-33	3672.8
3-17	1881.9	1-34	3785.9
2-17	1952.9	1-37	4073.0
1-16	1954.9	1-37 (ox)	4089.0
1-17	2068.0	1-38	4130.0
1-18	2167.0	1-38 (ox)	4146.0
11-30	2212.1	1-39	4229.1
1-19	2314.1	1-39 (ox)	4245.1
1-20	2461.2	1-40	4328.2
11-40	3150.7	1-40 (ox)	4344.2
		1-42	4512.3

PAPER II

The same IP-MS method as described in paper **I** was used to investigate whether the relative abundance pattern of the truncated Aβ peptides detected could be used to distinguish AD patients from healthy control subjects. The shortest Aβ peptide that could be reproducibly detected using the antibody 6E10, was in all samples Aβ1-13 and the longest was Aβ1-42. The study included 18 AD patients and 18 healthy controls. The CSF samples were obtained from two different centres, analyzed on two different occasions and the results were evaluated using multivariate analysis by means of the partial least squares discriminant analysis and a permutation test for validation.

Four Aβ peptides contributed significantly to the separation of AD and controls, Aβ1-16, Aβ1-33, Aβ1-39 and Aβ1-42. The optimal cut off point in the final combined model (*Figure 9a*) allowed for correct classification of 16 of the 18 AD patients and

15 of the 18 controls, yielding a sensitivity of 89%, a specificity of 83% and an accuracy of 86%.

Aβ1-33, Aβ1-39 and Aβ1-42 have all been recognized as cleavage products involving β- and γ-secretase and all are decreased in AD. Surprisingly, Aβ1-16 was the most important C-terminally truncated peptide for separating AD from non-demented controls where the levels were elevated in AD (*Figure 9b*). One possible explanation is that this peptide may be generated by the combined actions of α-secretase and β-secretase, which would represent a novel pathway for APP processing. This pathway might be up-regulated in AD in an attempt to counteract amyloidogenic APP processing. This hypothesis will be further investigated and discussed in papers **IV** and **V**.

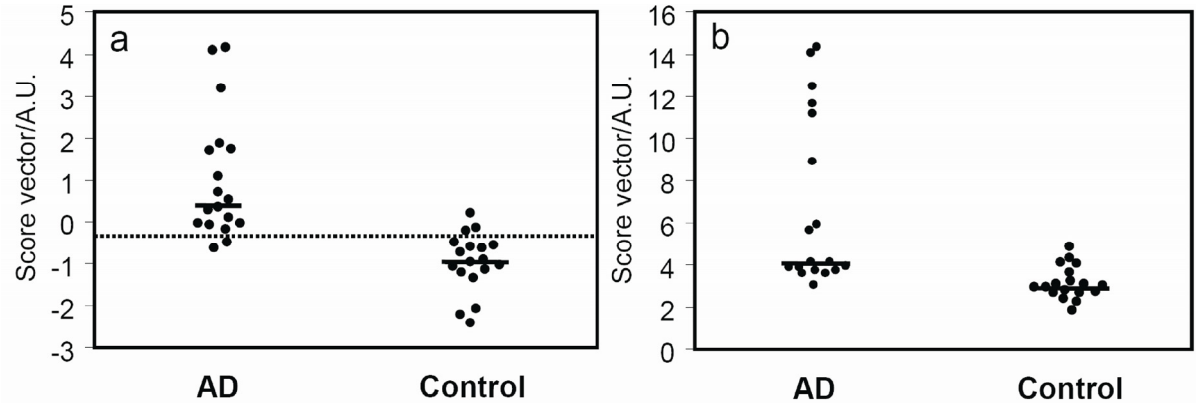

Figure 9. Aβ1-42, Aβ1-39, Aβ1-33 and Aβ1-16 were combined to a single vector where the dotted line represents the optimal cut off which results in a sensitivity of 89% and specificity of 83% (a). In (b) the median score plot showing the separation between AD and control using Aβ1-16 is displayed.

PAPER III

In paper **III**, the IP method was optimized by crosslinking the Aβ specific antibodies to magnetic beads, automating the washing and elution procedure and by the addition of detergent, thereby increasing the sensitivity for the longer hydrophobic Aβ isoforms. Furthermore, the quantitative performance of the Aβ IP-MS method was improved by using two different Aβ peptides labelled with an amino acid, enriched with stable carbon and nitrogen isotopes (Aβ1-15 $Arg^{13}C^{15}N$ and Aβ1-34 $Arg^{13}C^{15}N$). The identities of the Aβ isoforms were confirmed using MALDI-TOFMS and nanoflow LC-MS/MS with a hybrid linear trap FTICR MS.

Cross-linking of antibodies to the beads eliminates the problem of interfering antibodies that otherwise are co-eluted with the antigen. For example, when analyzing high mass components or proteolytically digested peptides or proteins, the antibodies used may interfere with the signal of interest, or may give spectral peaks that would need to be identified in order to be eliminated from consideration as being Aβ-derived. By using MALDI-TOF mass spectrometric analysis of higher m/z we were able to

verify that the risk of co-eluting the antibody with the Aβ peptides was substantially decreased or possibly even eliminated by using the crosslinking technique (*Figure 10*).

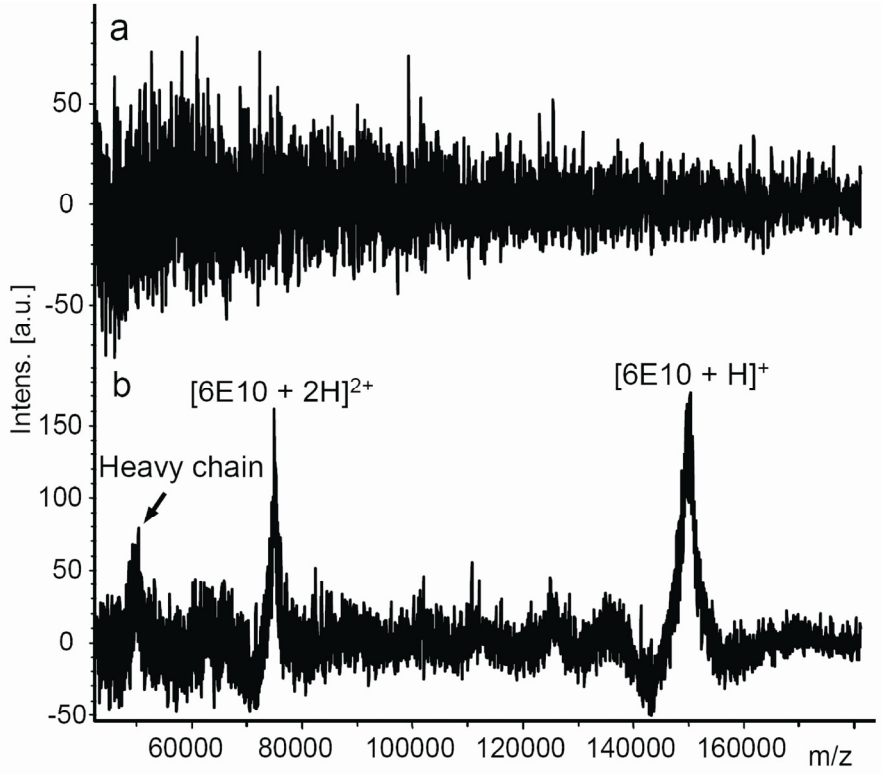

Figure 10. The antibody 6E10 was crosslinked to the magnetic beads and no peak was detected in the mass range analyzed (m/z 40-180 000) (a). In panel (b), the crosslinking technique was not used. The peak at m/z 150 000 corresponds to the molecular mass of the total immunoglobulin. The peak at m/z 75 000 is probably the doubly protonated antibody whereas the peak at m/z 50 000 could be the triply protonated molecule or the heavy chain in the antibody which also has a molecular mass of ~50 kDa.

The IP-MS method was automated by using a KingFisher magnetic particle processor which washes the beads and subsequently elutes the Aβ isoforms. The greatest advantage using this instrument is its capacity to complete the entire washing and elution procedure in less than 9 minutes compared with 20 minutes with manual handling and its ability to process 15 samples simultaneously. This enhanced through-put enables larger and clinically more relevant studies.

As seen in *Figure 11*, by adding 0.012% tween-20 to the CSF before incubating with the beads, the maximum intensity was shifted towards the more hydrophobic peptides. The intensity of the m/z corresponding to Aβ1-17 decreased whereas it increased for Aβ1-40. With increasing concentrations of tween-20 this effect became steadily more apparent. The optimum Tween-20 concentration providing the best compromise with respect to peak intensity for both the hydrophilic and hydrophobic Aβ peptides was determined as 0.025%.

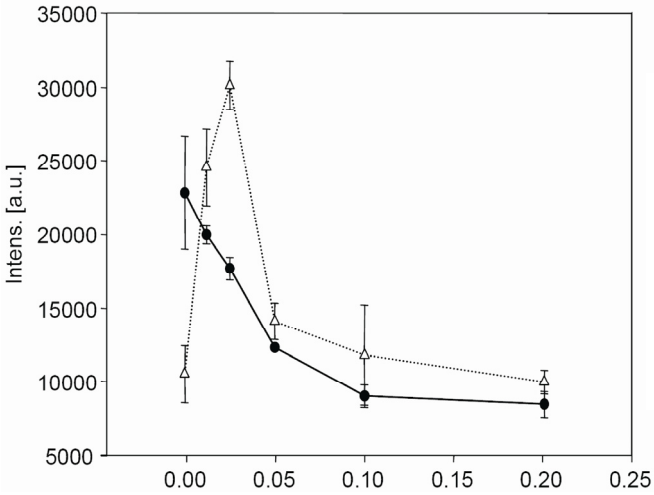

Figure 11. Plot showing the intensity average (each sample was analyzed in duplicate) with varying Tween-20 concentration. Open triangle represents the intensity curve for Aβ1-40 whereas the closed circles represent Aβ1-17. The error bars represent one standard error of the mean.

To verify the identity of the Aβ isoforms detected in the MALDI-TOFMS analysis, we used nanoflow LC-MS/MS to obtain amino acid sequence data. All Aβ isoforms (except Aβ1-42) were detected with superior mass accuracy. The most likely explanation for not detecting Aβ1-42 is that the chromatographic method used was not optimized for extremely hydrophobic peptides. Subsequent tandem MS analysis of all these Aβ peptides produced at least one fragment mass spectrum that matched an Aβ peptide amino acid sequence (*Figure 12*).

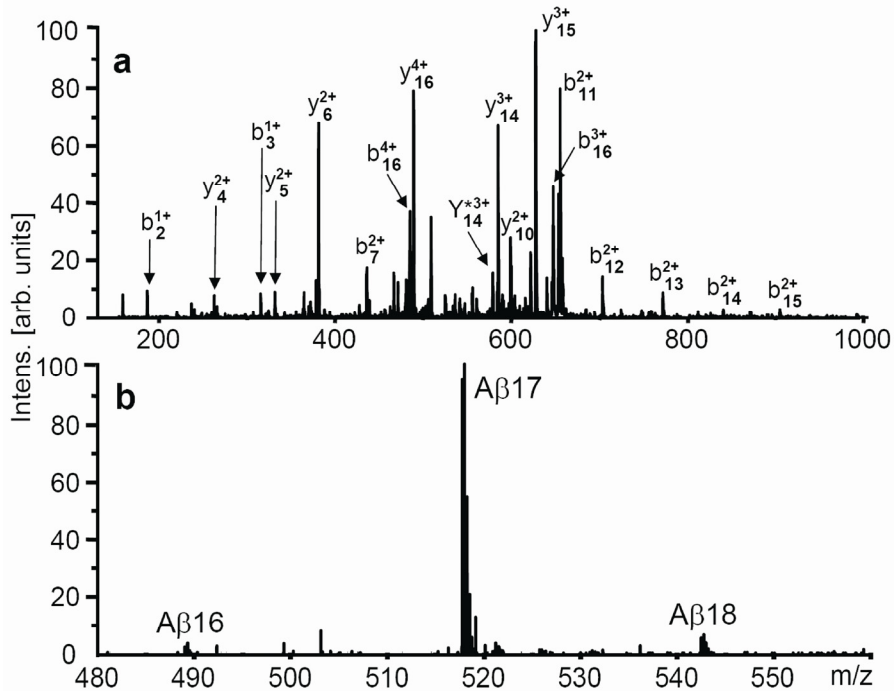

Figure 12 (a) Single scan (3 microscans) tandem mass spectrum of $[M+4H]^{4+}$ of Aβ1-17. (b) Nanoflow LC/ESI mass spectrum showing Aβ peptides from CSF, immunoprecipitated using the antibody 6E10. The spectrum is summed over the retention time interval 21-24 min and peaks corresponding to the $[M+4H]^{4+}$ ions of Aβ1-16, Aβ1-17, and Aβ1-18 are indicated.

Finally, the IP-MS method was modified by adding two isotopically labelled Aβ peptides, Aβ1-15 $Arg^{13}C^{15}N$ and Aβ1-34 $Arg^{13}C^{15}N$, as internal standards. The replacement of ^{12}C and ^{14}N with ^{13}C and ^{15}N in arginine lead to a nominal mass shift of 10 Da for both Aβ1-15 $Arg^{13}C^{15}N$ and Aβ1-34 $Arg^{13}C^{15}N$. Hence, the peaks from these internal standards do not interfere with peaks from the naturally present peptide in the mass spectrum. By adding internal standards, the CV for the method improved and at the same time the concentration of different Aβ isoforms could be estimated. One important application of using internal standards could be in identifying and monitoring biochemical effects on the APP/Aβ metabolism in treatment trials of new drug candidates, for example potential disease-modifying drugs such as secretase inhibitors and Aβ immunotherapy.

An attempt to detect the p3 (Aβ17-42) fragment in CSF was conducted. To be sure that the immunoprecipitation method was capable of extracting this peptide and that it behaved similarly in the MALDI process as the other Aβ isoforms, CSF was spiked with 10 pmol Aβ17-42 and the antibody 4G8 was used in the IP. A peak at m/z 2576, corresponding to Aβ17-42 was detected. The truncated peptide was not detected in neat CSF.

PAPER IV

The optimized and automated IP in combination with MALDI-TOFMS and LC-MS/MS was used to address by which processing pathway the shorter isoforms, e.g. Aβ1-15/16/17, arise. A cell model from Aβ-overexpressing SH-SY5Y cells carrying mutant APP, was developed and treated with different combinations of α-, β- and γ-secretase inhibitors. In addition to IP-MS of the cell media, each sample was analyzed for sAPPα and Aβ1-40 using an ELISA. The measured MALDI-TOFMS integral of the Aβ1-40 peak correlated very well with the Aβ1-40 concentration from the ELISA.

γ-Secretase inhibitor treatment induced a dramatic change in the Aβ isoform pattern abolishing the secretion of all Aβ fragments longer than and including Aβ1-17 (*Figure 13*). The reverse was seen for Aβ1-15 where a major increase was observed, which subsequently diminished after treatment with a combination of γ- and α-secretase inhibitors.

As expected, β-secretase inhibitor treated cells secreted high levels of sAPPα and substantially less of all Aβ isoforms, including Aβ1-42, Aβ1-40 and Aβ1-38 and Aβ1-14/15/16. Treatment with α-secretase inhibitor alone gave rise to the largest decrease of sAPPα whereas Aβ1-17 was unaffected in all studies by this treatment. Inhibition of α-secretase affected Aβ1-14, Aβ1-15 and Aβ1-16 by slightly decreasing the secretion of these isoforms to the cell media. Aβ1-40 as well as Aβ1-38 showed a decreased secretion to the cell media in two of the studies and an increase in a further two studies.

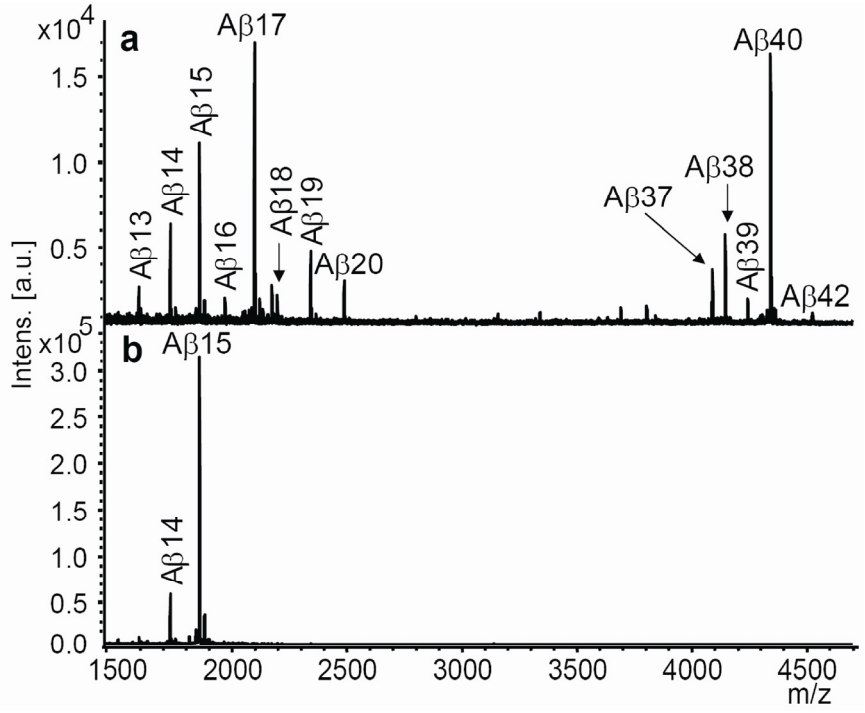

Figure 13. The mass spectrum in the upper panel (a) shows the Aβ isoform pattern in media from untreated cells and the lower panel (b) shows the Aβ isoform pattern in media from cells treated with γ-secretase inhibitor. All Aβ isoforms longer than and including Aβ1-17 are absent after treatment. All the Aβ isoforms detected using MALDI-TOFMS were also identified and verified by LC-MS/MS.

These results suggest that all isoforms longer than and including Aβ1-17 depend on γ-secretase directly or indirectly. It is well established that γ-secretase has multiple cleavage sites around amino acids 34-42 of Aβ but it is uncertain whether γ-secretase by itself can cleave APP as N-terminally as at position 17 in the Aβ sequence. Alternatively, cleavages at amino acids 17-X may depend on other, as yet unidentified enzymes that need to be activated by γ-secretase or are susceptible to the γ-secretase inhibitor.

While γ-secretase inhibitor treatment abolished all isoforms longer than and including Aβ1-17, the expression of shorter isoforms and sAPPα increased. The expression of these shorter fragments was dependent on both β- and α-secretase, as inhibitors of either enzyme resulted in the abolishment of the fragments when combined with a γ-secretase inhibitor. The combined results from α- and γ-secretase inhibitor treatment suggest that the production of Aβ1-14 and Aβ1-15 depends to a very large extent on α-secretase. While Aβ1-14 and Aβ1-15 behaved in an identical manner in all experiments, Aβ1-16 was sometimes lost upon γ-secretase inhibitor treatment, suggesting that this fragment may be generated by γ-secretase under certain circumstances.

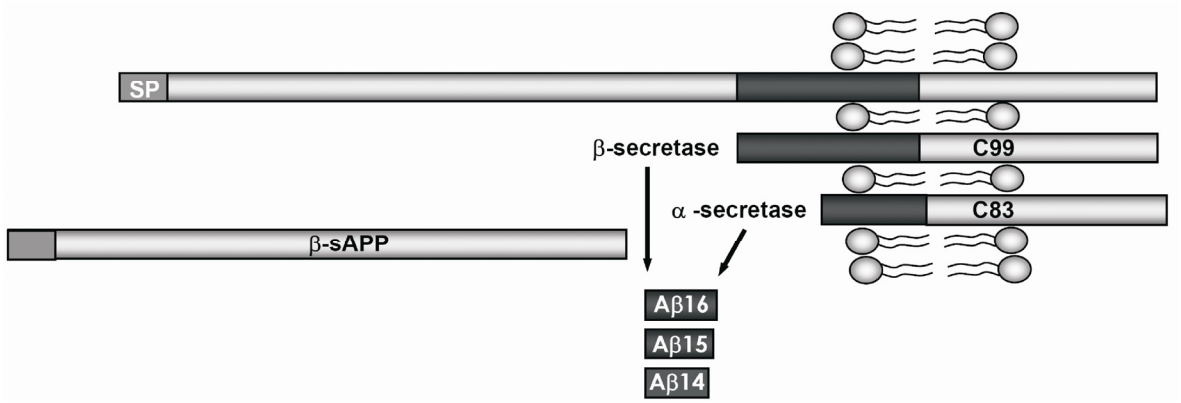

Figure 14. The novel APP degradation pathway including concerted β- and α-secretase cleavages.

Altogether, the results presented here add to current knowledge on APP processing by showing that APP can undergo processing by concerted β- and α-secretase-mediated cleavages in addition to the well established amyloidogenic and non-amyloidogenic pathways (*Figure 14*). Thus, firm evidence of a third APP processing pathway has been presented.

PAPER V

The third APP processing pathway described in paper **IV** was further investigated by exploring the effects of γ-secretase inhibition on the Aβ isoform pattern in brain and CSF from Tg 2576 mice. IP-MS was performed on 5-10 μL CSF and on neutralized Aβ extract from brain tissue (hippocampus, cortex and cerebellum) from 6-7 month old mice.

A study including 5 mice treated with γ-secretase inhibitor (DAPT) and 5 treated with corn oil (vehicle) was performed. Six different peaks at different m/z were reproducibly detected from CSF, which by mass were assigned to be six isoforms of Aβ (Aβ1-15, Aβ1-16, Aβ1-17, Aβ1-19, Aβ1-38 and Aβ1-40). As seen in *Figure 15*, treatment with DAPT greatly increased the mass spectrometric signal for Aβ1-16 ($p<0.01$, Mann-Whitney U exact test). The signal corresponding to Aβ1-15 was also increased while the signals for the longer isoforms, including Aβ1-17, were slightly reduced.

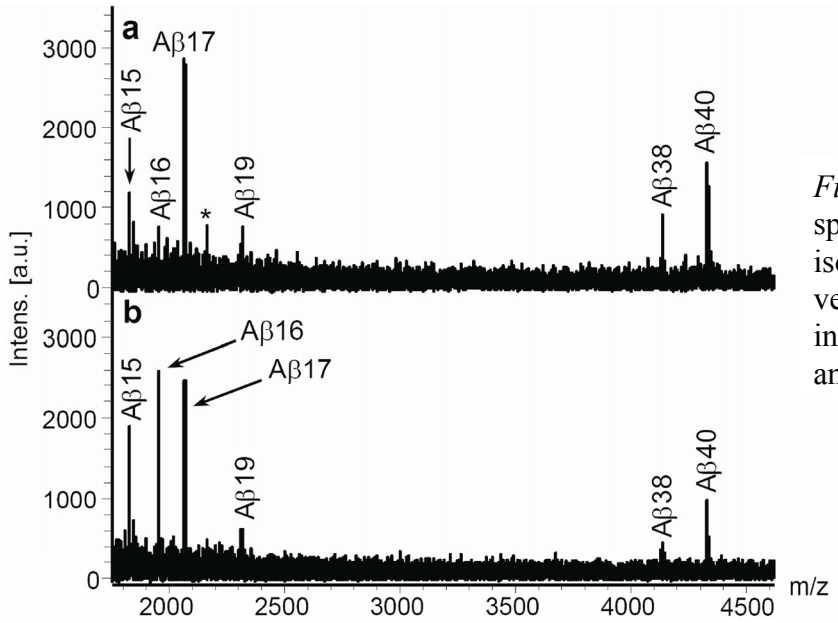

Figure 15. MALDI-TOF mass spectra showing the Aβ isoform pattern in CSF from vehicle (a) and γ-secretase inhibitor treated mice (b). * is an unidentified peak.

Further, analysis of the Aβ isoform pattern in CSF from mice of different ages revealed an age-dependent increase in the longer and more aggregation-prone Aβ peptides. If verified in human studies, this may provide a reason for the strong age-dependency of amyloid pathology in the brain.

Results from the different brain regions displayed similar trends as in CSF; the short Aβ isoforms increased, while isoforms longer than and including Aβ1-17 decreased. In the hippocampus, Aβ1-15, Aβ1-17 Aβ1-19, Aβ1-38, Aβ1-40 and Aβ1-42 were reproducibly detected (*Figure 16*) in all mice while fewer Aβ isoforms could be reproducibly detected in the cerebellum and cortex.

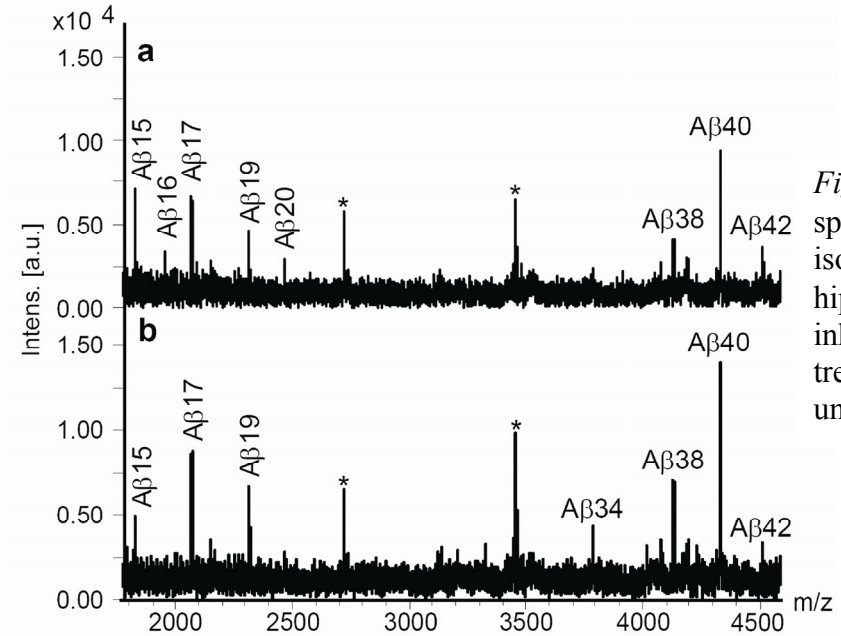

Figure 16. MALDI-TOF mass spectra showing the Aβ isoform pattern in hippocampus from γ-secretase inhibitor (a) and vehicle (b) treated mice. * are unidentified peaks

In the cortex, only 3 Aβ peptides were detected (Aβ1-38, Aβ1-40 and Aβ1-42). This may be due to either the method used being not sufficiently sensitive for detecting low abundant isoforms or that the shorter isoforms (Aβ1-15, Aβ1-17 and Aβ1-19) are not present in the cortex

As in the cell model, all fragments longer than and including Aβ1-17 decreased upon γ-secretase inhibition, whereas the shorter isoforms, e.g. Aβ1-15, increased. These data, together with the cell model data, strongly suggest that Aβ1-15 and Aβ1-16 may be generated through the third metabolic pathway by concerted β- and α-secretase cleavages of APP.

PAPER VI

In this study, the Aβ isoform pattern in CSF was investigated using IP-MS as described in paper **III**. 18 patients with SAD, 7 carriers of the FAD-associated *PSEN1* A431E mutation, 17 healthy controls and 6 patients with depression were analyzed. Representative CSF Aβ isoform mass spectra are shown in *Figure 17*.

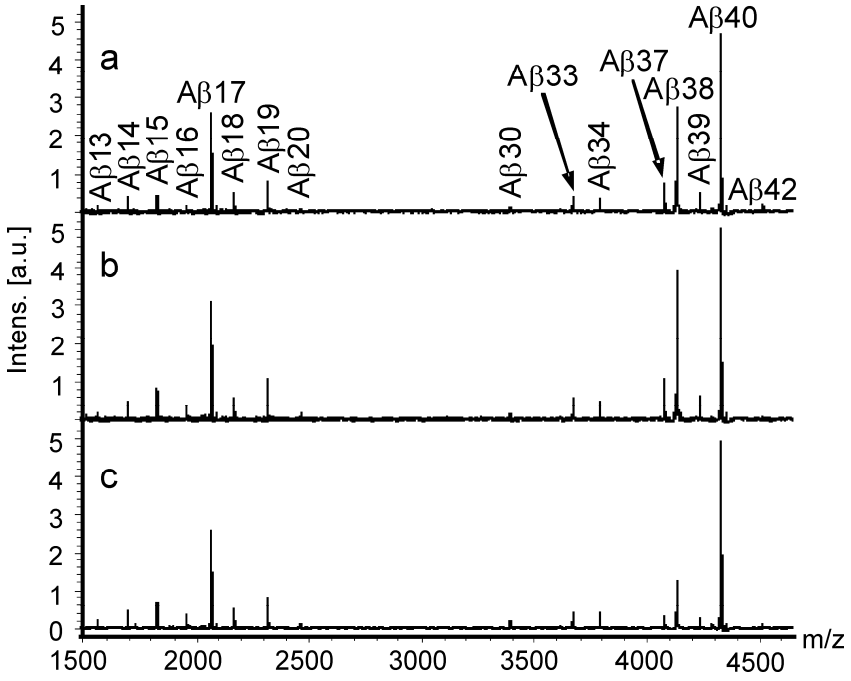

Figure 17. Representative mass spectra showing C-terminally truncated Aβ peptides immunoprecipitated from CSF using the antibody 6E10. Representative Aβ isoform patterns from (a) a control individual (b) a SAD patient and (c) a carrier of the FAD-associated *PSEN1* A431E mutation.

The data presented here confirm the results from paper **II**; low levels of Aβ1-42 and high levels of Aβ1-16 are the main contributing factors for separating AD, both SAD and FAD, from the two control groups. Both SAD and FAD were characterized by similar levels of Aβ1-42 and Aβ1-40, implying similar degrees of amyloid-related

brain pathology. However, FAD mutation carriers had considerably lower levels of Aβ1-37, Aβ1-38 and Aβ1-39 and higher levels of Aβ1-20 compared with SAD patients (see *Figure 17 b-c*). These deviations completely separated the two groups. The Aβ1-37, Aβ1-38 and Aβ1-39 isoforms are normally produced by γ-secretase, suggesting that certain *PSEN1* and *PSEN2* mutations may modulate γ-secretase function by inhibiting cleavage at Gly37, Gly38 and Val39, without significantly affecting the production of Aβ1-42 and Aβ1-40.

In conclusion, these data show that (i) SAD patients differ from cognitively normal individuals and depressed patients with regard to their CSF Aβ isoform pattern, (ii) carriers of the FAD-associated *PSEN1* A431E mutation have low CSF levels of C-terminally truncated Aβ peptides shorter than Aβ1-40, suggesting a loss of function, leading to a relative abundance of aggregation-prone Aβ1-42 and (iii) CSF Aβ1-16 is a positive biomarker for AD.

CONCLUSIONS

The overall objective of this thesis was to develop analytical techniques for studying Aβ and its isoforms in biological fluids such as CSF. The work resulted in the development of a targeted Aβ proteomics approach using IP combined with MS detection. The results presented in this thesis show that this IP-MS approach has become a successful analytical strategy for identifying markers of disrupted Aβ homeostasis in AD. Further, using the IP-MS method we have shown that Aβ exists in multiple isoforms in mice brain, cell cultures and CSF having both N- and C-terminal truncations.

The highly specific IP method was used for studying the Aβ isoform pattern in human CSF. To date, more than 20 Aβ isoforms have been identified and verified of which four have significantly contributed to the separation of AD and controls, with 89% sensitivity, 83% specificity and 86% accuracy. Further, a new potential marker for AD has been identified, Aβ1-16, which was the most important Aβ peptide for separating AD from non-demented controls. The presence and raised levels of this peptide in AD may represent a novel APP processing pathway which is up-regulated in AD in an attempt to counteract amyloidogenic APP processing. This novel pathway may involve concerted cleavage by α- and β-secretase and adds to our previous knowledge of AD pathogenesis, namely the two previously described pathways, the amyloidogenic and the non-amyloidogenic.

The use of Aβ related biomarkers will in all probability have an impact on both the design of clinical trials and the diagnoses of patients with memory disorders. We have shown that there are several shorter Aβ isoforms in CSF of which Aβ1-16 is significantly increased in AD. By designing a specific ELISA for this isoform, Aβ1-16 could become a useful biomarker in clinical diagnosis. Furthermore, Aβ and its isoforms might be used for measuring and monitoring treatment efficacy of novel amyloid-targeting drugs in the management of AD.

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