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Leena Aho

Amyloid-β Deposition in the Brains of Subjects with Cerebrovascular Disease, Diabetes, Synucleinopathy and Alcohol Abuse: a Human Post-mortem Study

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ABSTRACT

With the aging population, neurodegenerative diseases are becoming major and extremely expensive health problems. The pathological cascade leading to neuronal death encountered in neurodegenerative diseases is still unclear, making the development of curative treatments challenging. Alteration in amyloid-β (Aβ) metabolism and ultimately in the deposition of Aβ is considered to be a key element in the pathogenesis of the most common age-related neurodegenerative disorder, Alzheimer's disease (AD). However, Aβ deposits are also seen in the brains of elderly cognitively intact subjects and a recent finding indicated that Aβ plaque removal does not prevent the progressive neurodegeneration challenging the presumption that Aβ represent the key factor in the ethiopathogenesis of AD. The deposition of Aβ in the brain parenchyma is however strongly linked with AD, and thus the risk factors for AD found in epidemiological studies have also been considered to be linked with the accumulation of Aβ.

The purpose of the present study was to investigate the proposed associations between Aβ deposition and cerebrovascular disease, diabetes, alcohol abuse and concomitant α-synuclein pathology in a large autopsy sample (n=1720) applying immunohistochemical methodology. The study cohort included both symptomatic and neurologically unimpaired subjects who had been autopsied which included a neuropathological examination in the Kuopio University Hospital.

The prevalence of cortical Aβ deposition varied from 19 to 60 % in the subjects in the different study groups. The prevalence of extracellular Aβ increased with age but the influence of gender was not entirely clear. Risk factors such as cerebrovascular disease, diabetes, and alcohol abuse did not have any influence on the accumulation of extracellular Aβ. Moreover, Aβ, HPτ and αS pathology were commonly seen concomitantly in elderly individuals.

The present study confirms that a substantial proportion of cognitively intact elderly individuals display extracellular Aβ in their cortical regions. Cerebrovascular disease, diabetes, or alcohol abuse had no influence on the deposition of Aβ, a finding that is at odds with proposal that these alterations are risk factor for AD via Aβ deposition. The strong association between age and the prevalence of Aβ deposition may indicate that the age-related changes in the formation and elimination of Aβ are significant for the accumulation of Aβ. The observed regular co-occurrence of all three studied age-related pathologies, i.e. Aβ, HPτ and αS does not necessarily indicate that one alteration is caused by one of others. These findings question the assumption that Aβ deposition is the initiator of the pathological cascade leading to neuronal death. Thus, the question of role of extracellular and intracellular Aβ aggregates is still open; are they harmful, inert, or protective?

National Library of Medicine Classification: WL359; QZ140; WH400

Medical Subject Headings (MeSH): Alcoholism; Alzheimer disease; Amyloid beta-Protein; Autopsy; Cerebrovascular Disorders; Diabetes Mellitus; Humans; Immunohistochemistry; Neurodegenerative diseases

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TIIVISTELMÄ

Rappeuttavat aivosairaudet lisääntyvät voimakkaasti väestön ikääntyessä, ja ovat yksi yhteiskuntamme kalliista kansantaudeista. Rappeuttavien aivosairauksien etiologia ja patogeneesi ovat edelleen epäselviä, minkä vuoksi parantavan hoidon kehittäminen on haastavaa. Yleisin rappeuttava aivosairaus on Alzheimerin tauti (AT), jonka neuropatologisia tunnusmerkkejä ovat amyloid-β-proteiini (Aβ) ja hyperfosforyloitunut-τ (HPτ). Aβ:n kertymisen solun ulkopuolelle on arveltu laukaisevan tapahtumaketjun, joka johtaa hermosolujen vaurioitumiseen ja solukatoon. Suuret väestöpohjaiset tutkimukset ovat kuitenkin osoittaneet, että osalla oireettomista ikääntyneistä nähdään merkittäviä määriä Aβ:n kertymiä aivoissa, ja toisaalta Aβ:n määrä aivoissa ei korreloi oireiden vaikeuden kanssa. Nämä tulokset kyseenalaistavat yksinkertaisen syy-seuraussuhteen Aβ:n kertymisen ja kliinisten oireiden välillä.

Epidemiologisissa ja eläintutkimuksissa on todettu aivohalvauksen, diabeteksen ja runsaan alkoholin käytön kasvattavan rappeuttavan aivosairauden riskiä lisäämällä Aβ:n kertymistä aivoihin. Tämän tutkimuksen tavoitteena oli arvioida esitettyjen riskitekijöiden vaikutusta Aβ:n kertymiseen aivoihin ihmisillä. Ikääntyneillä esiintyy aivoissa usein samanaikaisesti Aβ:n ja HPτ:n lisäksi myös α-synukleiinia (αS), mutta näiden patologioiden keskinäinen vaikutus toisiinsa on epäselvä. Työn toisena tavoitteena oli arvioida Aβ:n, HPτ:n ja αS:n keskinäisiä vaikutuksia toisiinsa. Tutkimusmateriaali koostuu yhteensä 1720 oireettomasta ja dementoituneesta henkilöstä, joille on tehty ruumiinavaus ja neuropatologinen tutkimus Kuopion yliopistossa. Metodina käytettiin immunohistokemiaa.

Tutkimuksessa havaittiin Aβ:n kertymiä aivokuorella valintakriteereistä riippuen 19–60 %:lla tutkituista. Aβ:n esiintyminen korreloi voimakkaasti iän kanssa, mutta sukupuolen vaikutus ei ollut yhtä selvä. Aivohalvaus, diabetes tai runsas alkoholin käyttö eivät vaikuttaneet Aβ:n esiintymiseen. Ikäihmisillä nähtiin aivoissa usein kaikkia kolmea patologiaa, Aβ:ta, HPτ:ta ja αS:a.

Nämä tulokset vahvistavat, että suurella joukolla oireettomista ikääntyneistä on Aβ-kertymiä aivoissa. Aivohalvaus, diabetes tai runsas alkoholin käyttö eivät näytä lisäävän Aβ:n määrää aivoissa, mikä kyseenalaistaa hypoteesin, että nämä kliiniset tilat lisäisivät dementian riskiä vaikuttamalla Aβ:n aineenvaihduntaan. Voimakas korrelaatio Aβ:n esiintymisen ja iän välillä viittaa, että ikääntymiseen liittyvät muutokset Aβ:n tuotossa ja eliminaatiossa ovat tärkeitä Aβ:n kertymiselle. Aβ:n, HPτ:n ja αS:n yhdenaikainen esiintyminen aivoissa ei välttämättä viittaa siihen, että ne ovat suorassa syy-seuraussuhteessa toisiinsa. Nämä tulokset kyseenalaistavat oletuksen, että Aβ on laukaiseva tekijä, joka aloittaa hermosolujen kuolemaan johtavan tapahtumaketjun. Aβ:n rooli ja merkitys rappeuttavissa aivosairauksissa on edelleen epäselvä; ovatko Aβ-kertymät haitallisia, haitattomia vai suojelevia.

Luokitus: WL359; QZ140; WH400

Yleinen suomalainen asiasanasto (YSA): aivosairaudet; aivoverenkiertohäiriöt; alkoholinkäyttö; amyloidi; diabetes; ihminen; ruumiinavaus

To my grandmother Elli
who suffered from Alzheimer's disease

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Kuopio, July 2010

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by the Roman numerals I-V**.**

I Aho L, Jolkkonen J, Alafuzoff I. β-amyloid aggregates in post-mortem human brains with cerebrovascular lesions. Stroke 2006; 37:2940-2945.

II Aho L, Parkkinen L, Pirttilä T, Alafuzoff I. Systematic appraisal using immunohistochemistry of brain pathology in aged and demented subjects. Dementia and Geriatric Cognitive Disorders 2008; 25:423-432.

III Alafuzoff I, Aho L, Helisalmi S, Mannermaa A and Soininen H. β**-**Amyloid deposition in brains of subjects with diabetes. Neuropathology and Applied Neurobiology 2009; 35:60-68.

IV Aho L, Karkola K, Juusela J, Alafuzoff I. Heavy alcohol consumption and neuropathological lesions: a post-mortem human study. Journal of Neuroscience Research 2009; 87:2786-2792.

V Aho L, Pikkarainen M, Hiltunen M, Leinonen V, Alafuzoff I. Immunohistochemical visualization of amyloid-β protein precursor and amyloid-β in extra- and intracellular compartments in the human brain. Journal of Alzheimer's Disease 2010;20:1015-1028.

The publishers of the original publications have kindly granted permission to reprint the articles in this dissertation**.**

Contents

ABBREVIATIONS

αS	α-synuclein
Aβ	amyloid-β
Aβ40	amyloid-β 40 residues in length
Aβ42	amyloid-β 42 residues in length
Ab	antibody
AD	Alzheimer's disease
APOE	apolipoprotein E
APOJ	apolipoprotein J
APP	amyloid precursor protein
CAA	Cerebral amyloid angiopathy
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
CVD	cerebrovascular disease
CVL	cerebrovascular lesion
DLB	dementia with Lewy bodies
DM	diabetes
FTDP	Frontotemporal dementia and parkinsonism
HAC	heavy alcohol consumers
HE	hematoxylin-eosin
HPτ	hyperphosphorylated tau protein
IDE	insulin degrading enzyme
IHC	immunohistochemistry
IR	immunoreactivity
LB	Lewy body
LN	Lewy neurite
mAb	monoclonal antibody
MMSE	Mini-Mental state examination
NFT	neurofibrillary tangle
NIA- RI	National Institute on Aging and Reagan Institute
NP	neuritic plaque
NT	neuropil threads
PD	Parkinson disease
PDD	Parkinson disease with dementia
PIB PET	Pittsburgh Compound B positron emission tomography
PM	post-mortem delay

PS1	presenilin 1
PS2	presenilin 2
SE	standard error
TDP-43	TAR DNA-binding protein
TMA	tissue microarray technique
VCI	vascular cognitive impairment

1 Introduction

The proportion of elderly people is increasing as the increase in human lifespan increases the susceptibility to live long enough to suffer neurodegenerative disorders (Jagger et al., 2007). This increase in the incidence of neurodegenerative disorders is responsible for social and economical cost for public health and as well individual suffering (Gorshow, 2007). Alzheimer's disease (AD) is the best known old age associated disease affecting millions of individuals in the Western countries and it is one of the leading causes of death in the elderly population (Heron and Smith, 2007; Lobo et al., 2000). The pathogenesis of AD is still unclear and there is no curative treatment available, and therefore it is still essential to examine the underlying mechanisms of AD.

The neuropathological diagnosis of AD is based on the detection of two hallmark proteins, amyloid-β (Aβ) and hyperphosphorylated-τ (HPτ), in the neuropathological examination (Braak and Braak, 1997). For decades, the extracellular accumulation of Aβ has been considered as the trigger for eliciting a cascade leading to neuronal dysfunction and death in AD. However, the role and function of Aβ are still under debate, as some researchers believe that Aβ has a physiological function, others believe that Aβ is an acute phase protein, and finally many researchers are convinced of the validity of the amyloid cascade hypothesis of AD (Haass et al., 1992; Hardy and Allsop, 1991; Jendroska et al., 1995). It is noteworthy that a substantial proportion of elderly population displays extracellular Aβ without expressing any neurological or psychiatric symptoms, indicating that the accumulation of Aβ is an age-related phenomenon (Bennett et al., 2006; Davis et al., 1999; MRC CFAS, 2001; Snowdon, 1997).

In addition to aging, epidemiological and experimental animal studies have observed some connection between the accumulation of Aβ and general diseases such as stroke, diabetes and alcohol abuse. It has been suggested that ischemic stress caused by stroke or toxic stress caused by alcohol abuse, or the metabolic alteration which accompany diabetes may increase the load of extracellular Aβ and consequently play a role in neurodegeneration. However, in human post-mortem studies, the association between Aβ and these proposed conditions has been far from clear (Arvanitakis et al., 2004; Janson et al., 2004; Paula-

Barbosa and Tavares, 1984; van Groen et al., 2005). One explanation for discrepant results is that there are physiological differences between humans and animals that impact on all studies that use animals as a model for human biology, and thus it is essential to re-evaluate the findings obtained from animal studies with human material (Odom et al., 2007).

Access to a large autopsy material including diseased and healthy individuals provided a unique opportunity to investigate the putative connection between the accumulation of Aβ and the proposed risk factors, and to assess the clinical relevance of Aβ pathology in human brains by applying immunohistochemical methods.

2 Review of the literature

2.1 AMYLOID β-PROTEIN

2.1.1 Historical aspects of Aβ

Alois Alzheimer, a German psychiatrist, was the first to describe neuropathological findings of Alzheimer's disease (AD). In 1906, he examined a 51-year old woman with a five year history of progressive memory impairment. When he autopsied her brain, Alois Alzheimer detected neurofibrillary tangles in nerve cells and senile plaques all over the cerebral cortex while applying the silver impregnation method (Alzheimer, 1907; for review Jellinger, 2006a). Later, these two lesions have been recognized as the hallmark lesions of AD. In 1984, Glenner and Wong isolated a 4200 dalton polypeptide, Aβ from amyloidotic vessels of Alzheimer disease and Down Syndrome patients (Glenner and Wong, 1984a) and one year later Masters and co-workers found the same polypeptide Aβ in senile plaques (Masters et al., 1985) and finally in the late 1980's Aβ was also reported to be seen in intracellular compartments in human brain (Grundke-Iqbal et al., 1989).

2.1.2 Structure and formation of Aβ

Aβ is a hydrophobic, self-aggregating peptide consisting of 40-43 amino acids and it is a cleavage product of the larger transmembrane amyloid precursor peptide (APP), encoded as a single-copy gene on chromosome 21 (Glenner and Wong, 1984a; Haass et al., 1992; Masters et al., 1985; Tanzi et al., 1988). APP with unknown function consists of 695-770 amino acids, and it is widely expressed in the brain (Kinoshita et al., 2003). The cleavage of APP has been reported to occur, when APP is located to the plasma membrane, endoplasmic reticulum, endosomal and lysosomal membranes (Kinoshita et al., 2003), trans-Golgi network (Xu et al., 1995) and mitochondrial membrane (Mizuguchi et al., 1992). The cleavage of APP can be divided into a non-amyloidogenic and an amyloidogenic pathway as demonstrated in figure 1. In the non-amyloidogenic pathway, APP is cleaved by the α-secretase and subsequently by the γ-secretase within the Aβ domain which leads to the formation of a 16 amino acid shorter peptide termed P3 (Bayer et al., 2001; Kojro and Fahrenholz, 2005). This is the major processing pathway in most cell types. In the amyloidogenic pathway, Aβ peptide

is cleaved from APP by two sequential enzymatic activities. The initial cleavage is mediated by the β-secretase identified as the novel aspartyl protease (Evin and Weidemann, 2002). This cleavage results in the release of sAPPβ into the extracellular space leaving a fragment called C99 within the membrane. The final cleavage is mediated by γ-secretase, a multimeric complex containing presenilins, which releases Aβ peptide (Evin and Weidemann, 2002). Soon after generation of Aβ, the peptide is secreted from the cell into the extracellular pool (Walsh et al., 2002). Most of the produced Aβ is 40 residues in length (Aβ40) and it is generated solely within the transgolgi network (Xu et al., 1995). Approximately 10 % of Aβ are 42 residues in length (Aβ42) and this form is generated in the endoplasmic reticulum and transgolgi network (Greenfield et al., 1999). The Aβ42 residue is more hydrophobic and in vitro it polymerizes into fibrils more readily than the Aβ40 residue (Bitan et al., 2003; Jarrett et al., 1993). This longer form is the predominant component in parenchymal plaques (Younkin, 1998) and generally Aβ42 is considered to be more toxic than Aβ40, whereas Aβ40 is a major form under physiological conditions (Bitan et al., 2003; Jarrett et al., 1993). However, this is controversial, as both in vitro and in vivo studies have reported that Aβ40 aggregates are also toxic (Walsh et al., 2002).

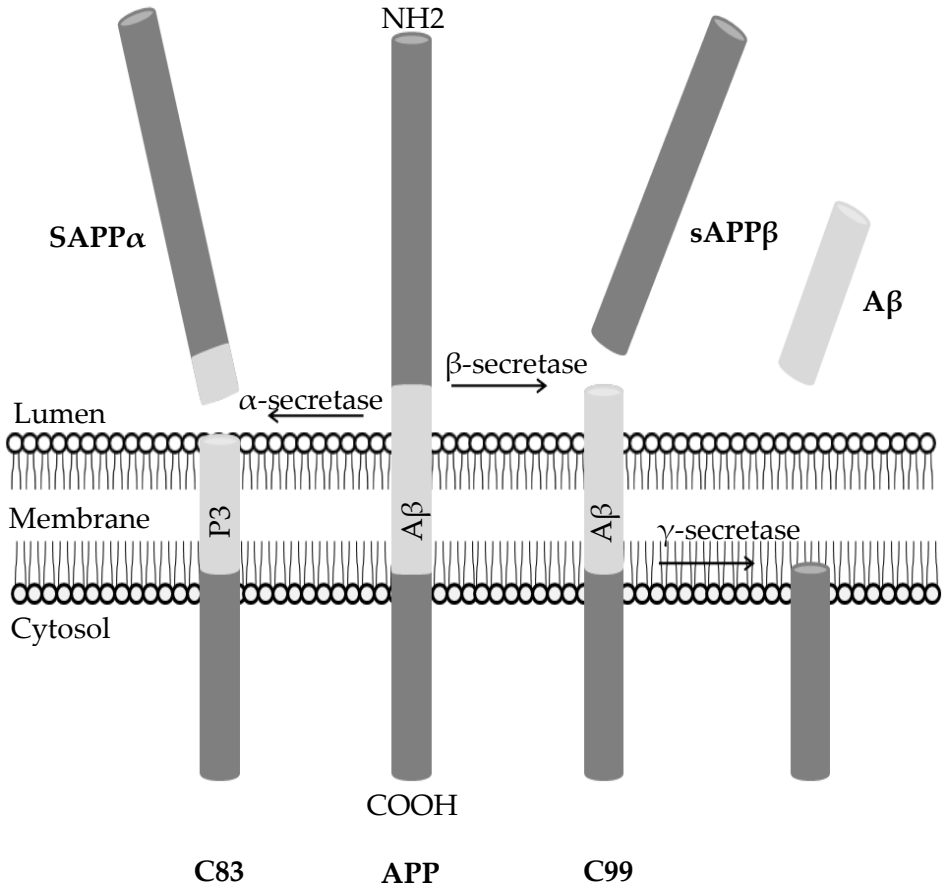


Figure 1. APP proteolysis

The Aβ peptide has a spontaneous tendency to oligomerize and it can exist in multiple assembly states: monomers, oligomers, protofibrils and fibrils (Figure 2). It has been demonstrated in cells derived from human brains that Aβ oligomerization begins within the cell rather than in the extracellular matrix, but the mechanism of the oligomerization remains a mystery (Walsh et al., 2000). The presence of multiple Aβ assembly forms highlights the difficulty in attributing toxicity to one single Aβ state (Shankar et al., 2009). Recently, it has been argued that it is soluble oligomers that evoke neurotoxicity (Haass and Selkoe, 2007). However, it is possible that the toxicity of Aβ is mediated by its multiple different assembly states (Hoshi et al., 2003; Walsh et al., 2002).

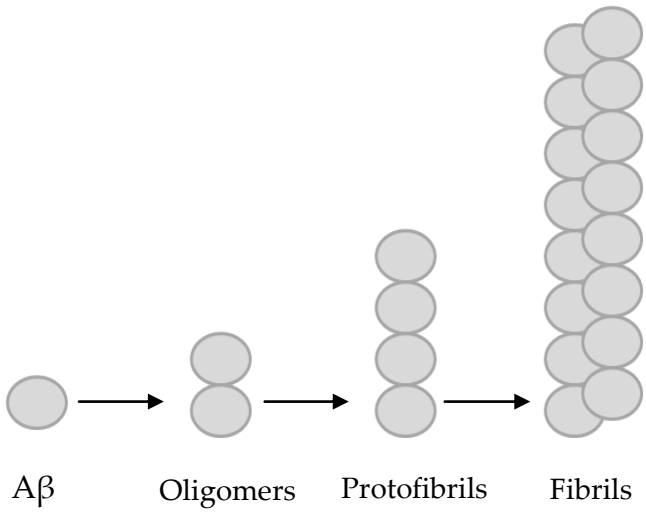


Figure 2. Aβ assembly states

2.1.3 Elimination of Aβ

In normal healthy individuals, Aβ is rapidly eliminated from brain (Pluta et al., 1999). There are several different mechanisms and pathways to eliminate Aβ from the brain, as Aβ has been reported to be degraded by multiple enzymes such as neprilysin and insulin-degrading enzyme (IDE), and by microglia and astroglia (Evin and Weidemann, 2002; Iwata et al., 2002; Wyss-Coray et al., 2003). In brain microglia are resident cells of the phagocyte system and they can slowly degrade limited amounts of Aβ. Similar to microglia, astroglia seem to have some phagocytic capabilities under certain conditions (Akiyama et al., 1996; Funato et al., 1998; Thal et al., 1997; Yamaguchi et al., 1998). It has been suggested that astrocytes take up Aβ and degrade it within their lysosomes (Funato et al., 1998). It seems that the phagocytic cells can internalize exogenous Aβ and clear it from brain into blood or cerebrospinal fluid. The most significant route of elimination of Aβ in young animals and probably in young humans is its clearance across the blood-brain barrier by vascular transport which

is mediated by low-density lipoprotein receptor related protein and α_2-macroglobulin (Shibata et al., 2000). All of these above-mentioned mechanisms appear to fail with age, at least in animals, and possibly also in humans (Weller et al., 2004). In older animals and probably also in humans, Aβ is mainly eliminated from the brain via the perivascular interstitial fluid drainage pathways (Weller and Nicoll, 2003). Aβ appears to enter the perivascular drainage pathway mainly at the level of capillaries and drain along perivascular spaces around arteries and passes out of the walls of arteries (Preston et al., 2003). The levels of Aβ in the walls of leptomeningeal arteries, middle cerebral arteries and the basilar artery are greatly increased in the elderly population and individuals with AD, but no Aβ is detected in the walls of the extracranial arteries (Weller et al., 1998). At least in AD brains Aβ accumulates around arteries five times more commonly than around veins (Weller et al., 1998). To summarize, insufficient elimination of Aβ from the brain results in its accumulation over time.

2.1.4 Accumulation of Aβ

The accumulation of Aβ is related to an imbalance between its production and elimination. The extracellular aggregates are of neuronal origin and are secreted as soluble peptides. The transgolgi network is a major reservoir of peptides from which the secreted Aβ is packaged into secretory vesicles and transported to the extracellular compartment (Greenfield et al., 1999). The extracellular Aβ deposits can be classified as fleecy, diffuse or, compact aggregates (Alafuzoff et al., 2008). Diffuse aggregates are usually large, amorphously shaped and their immunoreactivity is weak. They are believed to be the precursor for compact aggregates. The compact aggregates are typically surrounded by dystrophic neuritis (Duyckaerts et al., 2009; Ingelsson et al., 2004). The extracellular Aβ deposits are associated with several proteins, lipids and cells such as apolipoprotein E (APOE) and J, zinc, copper, iron and various components of the extracellular matrix (Duyckaerts et al., 2009).

In 2002, Thal and colleagues demonstrated the distinct phases of parenchymal Aβ deposition in the human brain (Thal et al., 2002). The Aβ deposition seems to progress in a sequential pattern and the evolution can be classified into 5 different phases (Thal et al., 2002). Aβ deposition spreads out anterogradely into regions that receive neuronal projections from regions already displaying Aβ. In the first phase, Aβ

deposits are found in the neocortex. In the second phase, Aβ spreads out of the allocortical brain regions. In phase 3, Aβ is also found in diencephalic nuclei, the striatum and the cholinergic nuclei of the basal forebrain. In phase 4, Aβ deposits expand into several brainstem nuclei and in phase 5, the cerebellum is involved. It is noteworthy that the distribution of Aβ deposition does not always follow the above phases. It has been demonstrated that at least in presenilin-1 mutation carriers, Aβ deposition seems to begin in the striatum (Klunk et al., 2007).

In addition to extracellular Aβ deposits, in 1989, Grunke-Iqbal and co-workers reported the presence of intracellular Aβ for the first time (Grundke-Iqbal et al., 1989). Since this initial report there have been several publications reporting the presence of intracellular Aβ not only in cell culture, but also in the brains of wild and transgenic animals and the brains from subjects with Down's syndrome, AD, human immunodeficiency virus, young drug abusers as well as in children and aged individuals without any known neurological disorder (Achim et al., 2009; Akiyama et al., 1999; Cataldo et al., 2004; Cruz et al., 2006; D'Andrea et al., 2001; 2002a; 2002b; 2003; Gomez-Ramos and Asuncion Moran, 2007; Gouras et al., 2000; Green et al., 2005; Grundke-Iqbal et al., 1989; Gyure et al., 2001; LaFerla et al., 1997; Mochizuki et al., 2000; Mori et al., 2002; Nagele et al., 2002; Oakley et al., 2006; Ohyagi et al., 2007; Ramage et al., 2005; Sheng et al., 2003; Wang et al., 2002; Wegiel et al., 2007). Mainly because of technical reasons, it has been difficult to provide evidence for the presence of intracellular Aβ within neurons. The main problem has been the extent of antibody cross-reactivity, as Aβ antibodies may also recognize full-length APP or its fragments. However, antibodies against neoepitopes have made it possible to distinguish Aβ from APP. In 2000, Mochizuki and colleagues demonstrated the presence of Aβ42 immunoreactivity in non-pyramidal neurons and in 2001 it was noted that Aβ42 could also accumulate in the perikaryon of pyramidal cells (D'Andrea et al., 2001; Mochizuki et al., 2000). There is also evidence that Aβ42 can be found in multivesicular bodies of neurons in the human brain by applying immunogold electron microscopy (Takahashi et al., 2002). Today it has been accepted that Aβ may accumulate intracellularly but it still remains to be confirmed whether the Aβ accumulates intracellularly because the produced Aβ is not secreted, or alternatively, whether the previously secreted Aβ is internalized from the extracellular pool of Aβ (for review Wirths et al., 2004).

In addition to intra- and extracellular accumulation of Aβ, Aβ accumulates also in the walls of capillaries and arteries within the brain and in the walls of the leptomeningeal arteries in the subarachnoid space. The accumulation of Aβ in the walls of small and medium sized arteries including arterioles and less often veins is a characteristic for cerebral amyloid angiopathy (CAA) (Vinters, 1987). The most common type of CAA is the sporadic form that frequently co-occurs with AD and appears to increase with age. This form of CAA is characterized by the accumulation of Aβ in the media and adventitia of parenchymal and leptomeningeal vessels (Glenner and Wong, 1984b). Other forms of CAA include the heritable CAA types and CAA due to transthyretin variants or prion disease (Revesz et al., 2003).

2.1.5 Genetics and Aβ

Mutations in three genes have so far been clearly associated with increased production of Aβ (for review Brouwers et al., 2008). APP located on chromosome 21 was the first gene identified in autosomal dominant early-onset AD families leading to massive overproduction of Aβ (Goate et al., 1991). In Down syndrome, there are three copies of this chromosome and this leads the accumulation of Aβ early in the life of Down syndrome patients (Gyure et al., 2001; Mori et al., 2002). Since the original report in 1991, at least 21 different missense mutations have been identified in APP (Brouwers et al., 2008). The mutations in APP have an influence on production and the alterations in Aβ sequence and Aβ properties lead to an increased aggregation propensity of Aβ and increased production of Aβ42 compared to Aβ40 (Brouwers et al., 2008). In addition to mutations in APP mutations in presenilins PS1 and PS2 on chromosomes 1 and 14 increase levels of Aβ42 (Jankowsky et al., 2004). Mutations in these three genes are known to cause autosomal dominant AD (St George-Hyslop and Petit, 2005). There are also several other mutations in additional genes that are suspected to have an influence on the accumulation of Aβ such as angiotensin converting enzyme, prion protein and sortilin-related receptor (Bertram and Tanzi, 2008).

2.1.6 The role and function of Aβ

The role and function of Aβ is still an unresolved issue, although it has been the focus of intensive investigation for decades. There are at least three different hypothesis of the role of Aβ. In 1992, Haass and co-

workers used cultured cells and demonstrated that Aβ is generated continuously as a soluble peptide during normal cellular metabolism. In 2003, Kamenetz and colleagues examined hippocampal slice neurons and observed that neuronal activity could modulate the formation and release of Aβ and they concluded that Aβ may play a role in normal synaptic physiology. The hypothesis that Aβ has a role in normal cellular mechanism was further supported by Cirrito and colleagues in 2005. They demonstrated that neuronal release of Aβ was physiologically regulated by synaptic activity throughout life by using microdialysis probes to measure Aβ levels in brain interstitial fluid and simultaneously recording local brain activity in freely moving mice (Cirrito et al., 2005). In 2005 Buckner and co-workers examined 764 participants using amyloid imaging and they found that the spatial pattern of amyloid deposition in elderly individuals with AD correlated remarkably well with brain areas of high default brain activity (posterior cortical regions including posterior cinculate, retrospenial, lateral parietal cortex, and frontal regions along the midline) in young adults, supporting the hypothesis that Aβ has also a physiological function (Buckner et al., 2005). Interestingly, in these same regions myelination seems to happen during late stage of brain development (Marsh et al., 2008). Furthermore, it has been demonstrated by examining post-mortem human brain tissue with immunohistochemistry that intraneuronal Aβ immunoreactivity appears in the first year of life, increases in childhood, stabilizes in the second decade of life, and remains high throughout adulthood suggesting that the presence of intraneuronal Aβ reflects normal cell metabolism rather than a pathological change (Wegiel et al., 2007). Taken together the findings from the cell culture, animal and post-mortem human studies, suggest that neuronal activity modulates local Aβ production or secretion or both and that it is possible that Aβ plays a role in normal physiology (Cirrito et al., 2005; Haass et al., 1992; Kamenetz et al., 2003; Wegiel et al., 2007).

Another hypothesis is that Aβ is an acute phase protein produced in stress situations. This hypothesis is supported by studies focusing on traumatic brain injuries. Traumatic brain injury has been shown to result in the rapid and long-term accumulation of several key proteins including APP and Aβ (Smith et al., 2003). An up-regulation in APP gene expression leads to the accumulation of APP in axons and this further leads to the increased generation of Aβ (Smith et al., 2003).

However, it has also been demonstrated that stress-induced Aβ deposits are later degraded and no permanent accumulation of Aβ is seen (Nihashi et al., 2001).

The third hypothesis is that Aβ is a pathological protein. In this hypothesis the aggregation of Aβ is believed to trigger a series of steps that leads to dementia. The strongest evidence for Aβ being a cause of dementia comes from genetics. Missense mutations in the APP or PS1 or 2 genes have been shown to lead to a massive overproduction of Aβ resulting in the accumulation of Aβ in the parenchyma as plaques (Goate et al., 1991; Jankowsky et al., 2004). Extracellular Aβ deposition has been claimed to lead to synapse and neuron dysfunction and loss of neurons, subsequently to atrophy of distinct brain areas and finally to dementia (Hardy and Allsop, 1991). However, this hypothesis is controversial, as a substantial proportion of elderly population display extracellular Aβ deposition without expenencing any neurological symptoms and further the extracellular Aβ load does not correlate with the severity of cognitive impairment (Bancher et al., 1996, Bennet et al., 2006; Bierer et al., 1995, Giannakopoulus et al., 2003; Jellinger, 2006b; MRC CFAS, 2001). Thus, today the emphasis has switched to the soluble Aβ. The term soluble Aβ refers to all forms of Aβ that remain in aqueous solution following high-speed centrifugation of physiological buffer extracts of brain (Lue et al., 1999; McLean et al., 1999; Wang et al., 1999). A more robust correlation has been reported between the levels of soluble, and not the insoluble Aβ form, and the extent of synaptic loss and severity of cognitive impairment (Lue et al., 1999; McLean et al., 1999; Wang et al., 1999). In cultured cells, it has been demonstrated that soluble, pre-fibrillar aggregates of Aβ may evoke toxicity (Lambert et al., 1998). Moreover, in 2002 Walsh and colleagues demonstrated that soluble Aβ oligomers and monomers inhibited hippocampal long-term potentiation in rats in vivo corroborating the toxicity of soluble Aβ (Walsh et al., 2002). In human post-mortem brain tissue, it has been shown that the mean level of soluble Aβ is increased threefold in AD cases compared to controls, suggesting that soluble Aβ best explains the neurotoxicity of Aβ (Lue et al., 1999; McLean et al., 1999). Despite extensive research, the final cascade leading to neuronal death is not fully understood and it still remains unclear what role Aβ plays in cellular metabolism.

2.1.7 Detection of Aβ

Initially, Aβ was detected by staining with congo red that is relatively unspecific chemical staining method. Later thioflavin-S was used to visualize Aβ but thioflavin-S was unable to detect diffuse protein aggregates. In the late 80's, a more specific method, immunohistochemistry (IHC), was introduced, which detected even diffuse protein aggregates. Since then, IHC has been the most commonly used method to monitor the accumulation of Aβ in the brain tissue. IHC is based on antibodies (Abs) which recognize a specific sequence of amino acids (epitopes). An Ab recognizes usually only a small part of a longer peptide (Figure 3). Some of the amino acid sequences are shared by Aβ and APP and therefore an Ab directed to Aβ may also recognize full-length APP or its other derivatives (Figure 3.). This Ab cross reactivity mainly causes problems when the presence of Aβ is assessed in the intracellular compartments where APP is physiologically located. In the 1990's Abs directed to neoepitopes, i.e. Abs which recognize the site of a terminal sequence, made it possible to differentiate Aβ from APP (LaFerla et al., 2007).

The antigen retrieval method, i.e. re-exposing and re-shaping of critical epitopes, is significant in determining the staining result (Alafuzoff et al., 2008; Beach et al., 2008; Christensen et al., 2009; D'Andrea et al., 2003; Ohyagi et al., 2007; Sheng et al., 2003). Formic acid is a widely used antigen retrieval method to enhance the immunoreactivity of extracellular Aβ, but the effect of formic acid pretreatment on the intracellular Aβ is less clear. There are publications stating that heat pretreatment is essential for the staining of intracellular Aβ, whereas formic acid pretreatment alone is not sufficient to visualize the intracellular Aβ (D'Andrea et al., 2003; Ohyagi et al., 2007). Formic acid pretreatment is a widely accepted method to enhance the immunoreactivity of extracellular Aβ deposits, whereas there is no agreement of the optimal antigen retrieval method for visualizing the intracellular accumulation of Aβ.

Until recently the only reliable method to visualize Aβ aggregates in the brain was the histological analysis of tissue samples obtained from brain biopsy or at autopsy. At the beginning of 2000s, it became possible to assess Aβ aggregates in a living patient with a noninvasive method positron emission tomography (PET) using Pittsburgh Compound B (PIB) (Klunk et al., 2004). Later it was confirmed by

frontal cortical biopsy that PIB PET did reflect brain Aβ deposition (Leinonen et al., 2008).

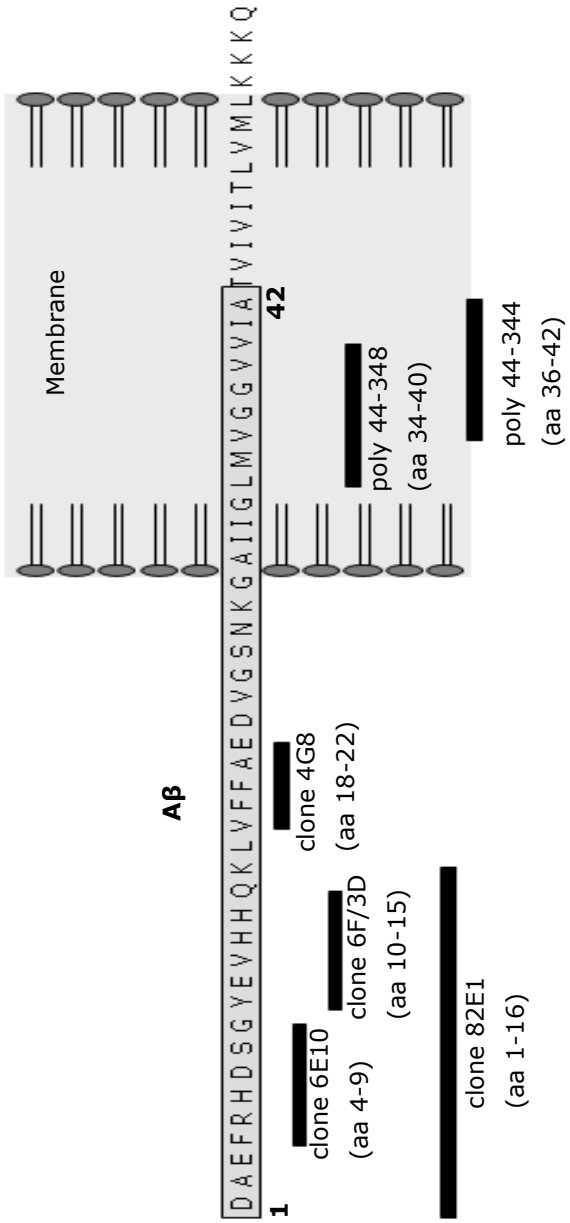


Figure 3. A schematic presentation of the Aβ. The epitope regions recognized by the antibodies are marked with black

2.2 CONCOMITANT BRAIN PATHOLOGIES

2.2.1 Hyperphosphorylated tau-protein

Another important protein, in addition to Aβ, seen in neurodegenerative diseases is hyperphosphorylated tau (HPτ). Tau proteins are encoded by a single gene on chromosome 17 and alternative splicing of the exons generates six different tau isoforms ranging from 352 to 441 amino acids in length which can be found in human brain (Goedert et al., 1989; Neve et al., 1986). Tau protein is one of microtubule associated proteins and its main function is to stabilize the microtubules (Ebneth et al., 1998). Under normal conditions, tau is constantly being phosphorylated and dephosphorylated and there is a physiological equilibrium between these two processes. Hyperphosphorylation of tau leads it to dissociate from the microtubules and to aggregate which makes it neurotoxic (Fath et al., 2002). HPτ is a hallmark lesion of neurodegenerative primary tauopathies exemplified by sporadic corticobasal degeneration, progressive supranuclear palsy, Pick's disease and hereditary frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Lee et al., 2001). Mutations in tau gene cause FTDP-17 where the disruption of tau function directly leads to neurodegeneration in the absence of extracellular Aβ deposition, corroborating the toxicity of tau (Poorkaj et al., 1998; Spillantini et al., 1998).

AD is the best-known secondary tauopathy where HPτ coexists with extracellular Aβ deposition. It has been proposed that an alteration of APP metabolism leading to an increased formation and deposition of Aβ is the trigger that induces early pathological phosphorylation of τ-proteins. Later, this assumption was supported by a finding that mutations in APP and presenilins cause familial AD with abundant HPτ pathology (Hardy and Selkoe, 2002). It was later demonstrated in primary cultures of hippocampus and in neuroblastoma cells that Aβ oligomers could promote the phosphorylation of τ-protein (De Felice et al., 2008). These findings indicate that HPτ pathology is secondary to Aβ pathology. However, there are reports indicating that HPτ pathology precedes the Aβ deposits by several decades and therefore HPτ pathology does not appear to be secondary but a prologue to Aβ deposition (Braak and Braak, 1991; Zhou et al., 2006). It is still unclear

how these two proteins affect each other and which one is the initiator of pathological cascade leading to neuronal death.

2.2.2 α-Synuclein

α-Synuclein (αS) is an abundant presynaptic protein that is mainly expressed in the brain as an isoform of 140 residues (Goedert, 2001). The physiological function of αS is not well established, but it has been postulated that αS may have a role in physiological regulation of certain enzymes, transporters, and neurotransmitter vesicles, as well as in neuronal survival (Dev et al., 2003; Lotharius and Brundin, 2002). αS is natively unfolded in a manner similar to tau (Weinreb et al., 1996). The most common type of αS-containing inclusion is the Lewy body (LB), a rounded eosinophilic inclusion in the cell soma (Spillantini et al., 1997). LBs and Lewy neurites (LNs) are a common pathological hallmark of Parkinson disease but they are also detected in brains with other neurodegenerative diseases such as AD and dementia with Lewy bodies (DLB) and they are quite commonly detected in the brains of the elderly population who do not display any neurological or psychiatric symptoms (Jellinger, 2004; Parkkinen et al., 2005). αS pathology seems to progress in a sequential pattern and this regional distribution of αS immunoreactivity is the basis of neuropathological staging of PD/DLB related pathology (Braak et al., 2003; McKeith et al., 1996). It has been speculated that during the early stages, αS pathology would be confined to the brainstem regions, resulting in a clinical presentation of PD and later, the pathology spreads to the allo- and neocortex and the clinical presentation at this stage is DLB/Parkinson disease with dementia (PDD) (Braak et al., 2003; Perry et al., 1990).

αS-positive lesions are common in familial and sporadic AD patients, estimates ranging from 50-60% and in Down syndrome patients (Arai et al., 2001; Hamilton, 2000; Lippa et al., 1999). The overlap in the pathological and clinical features suggests that these two types of pathologies may be linked (Giasson et al., 2003). In vitro models it has been demonstrated that αS can induce the fibrillation of τ-protein and further αS and τ-protein can promote eact other's polymerization (Giasson et al., 2003; Jensen et al., 1999). Furthermore, increased levels of Aβ, at least in the transgenic mouse model, can promote the accumulation of fibrillar αS into inclusions and vice versa αS has been shown to enhance the aggregation of Aβ in vitro models (Jensen et al., 1997; Masliah et al., 2001). Thus, αS, HP τ and Aβ

pathology regularly co-exist and it seems that these different proteins can affect each other either directly or indirectly. However, their functions under normal physiological conditions as well as their role in disease still remain to be clarified.

2.3 NEURODEGENERATIVE DISEASE AND NORMAL AGING

2.3.1 Alzheimer's disease

AD is the most common progressive neurodegenerative disease worldwide resulting in severe cognitive impairment and leading to death within about 10-15 years. AD is the leading cause of late-life dementia accounting for approximately 54 % of all dementias (Lobo et al., 2000). The lifetime risk for AD is 33% for men and 45% for women between the age 65 and 100 years. The prevalence of AD increases strongly with age. It is approximately 1,5 % in the seventh decade and increases to as high as 50 % in the tenth decade (van der Flier and Scheltens, 2005). Typical clinical symptoms are memory problems, executive dysfunction, visuospatials difficulties, aphasia, apraxia and agnosia.

Neuropathology is proposed to be the golden standard for diagnosing AD. Aβ deposition in the form of diffuse and neuritic plaques (NPs) and accumulation of HPτ in the form of neurofibrillary tangles (NFTs) in neuronal cell bodies and neuropil threads (NTs) in neuronal processes are the classic neuropathological features of AD (Bancher et al., 1989; Braak et al., 1986; 1994; Braak and Braak, 1997; Grundke-Iqbal et al., 1986). The first consensus guidelines for the assessment of AD-related hallmark lesions, NPs, NFTs and NTs, were published in 1985 (Khachaturian, 1985). The neuropathological diagnosis of AD was based on the quantitative assessment of both NPs and NFTs in relation to the patient's age and the clinical history. However, this quantification proved to be overtly complicated, and thus in 1991 revised guidelines were launched known as CERAD strategy which provided details on the likelihood of AD (Mirra et al., 1991). The CERAD strategy did not pay any attention to the regional distribution of HPτ and Aβ. In the same year, the Braak staging was introduced which was based on hierarchical and topographical distribution of HPτ pathology (Braak and Braak, 1991). At that time, the Braak staging system was based on an assessment of two 100 μm thick sections processed according to the silver technique proposed by

Gallyas (Braak and Braak, 1991; Gallyas, 1971; Gallyas, 1979; Iqbal et al., 1991). In 2006, this staging strategy was modified to incorporate the use of HPτ IHC and 5-15μm thick paraffin embedded sections (Braak et al., 2006). In the Braak staging system, the trans-entorhinal and entorhinal cortex (stages I-II), then the hippocampus (stages III-IV) and finally the isocortex (stages V-VI) are sequentially involved (Braak and Braak, 1991). When the lesions reach the isocortex, overt clinical symptoms of dementia can be observed. The Braak staging procedure is still commonly used in routine neuropathology. The currently used consensus recommendations for post-mortem diagnosis of AD were launched in 1997 by the National Institute on Aging and Reagan Institute (NIA-RI) working group. These recommendations are based on Braak staging for HPτ pathology and CERAD classification for NPs (infrequent, moderate and frequent) resulting in a statement of likelihood that dementia is caused by AD.

The pathogenesis of AD is still unclear. The amyloid cascade hypothesis has been the dominant theory for more than a decade. It has been postulated that the generation of Aβ initiates a pathological cascade leading to dementia (Hardy and Allsop, 1991). The cascade begins when some unknown factor increases the formation of Aβ or decreases its clearance from the brain. Aβ oligomerization and accumulation occur first and the accumulation of HPτ pathology and neuronal dysfunction are secondary events. The strongest evidence for this hypothesis comes from genetics. The mutations in APP and enzymes that mediate the cleavage of Aβ (PS1 and 2) lead to massive overproduction of Aβ and early onset of AD. However, PS1 mutations can also lead to tauopathy without the presence of Aβ deposition (Baki et al., 2004; Dermaut et al., 2004). Patients carrying the tau mutation display severe NFT pathology and cognitive impairment without exhibiting Aβ indicating that HPτ is neurotoxic. The strongest evidence against the amyloid cascade theory comes from clinicopathological studies, as no correlation between the extracellular Aβ load and the severity of cognitive decline has been found (Bierer et al., 1995; Giannakopoulos et al., 2003). In contrast, a strong correlation between the HPτ pathology and the severity of cognitive impairment has been observed in many studies (Bancher et al., 1996; Berg et al., 1998; Bierer et al., 1995; Giannakopoulos et al., 2003; Gold et al., 2001). Furthermore, the removal of extracellular Aβ plaques did not prevent the progression

of AD challenging the role of Aβ in the pathogenesis of AD (Holmes et al., 2008).

Several studies have described alterations in the immune system in AD. These studies have implied that the immune system is capable of recognizing as abnormal the proteins that accumulate in brain in AD (for review Boche and Nicoll, 2008). Inflammation in AD has been considered to contribute to disease progression specifically in the form of microglia activation (Boche and Nicoll, 2008; Duyckaerts et al., 2009). An association between activated microglia and the counts of NFTs has been reported but not between activated microglia and the load of NPs (DiPatre and Gelman, 1997; Overmyer et al., 1999b). However, this association is modified by APOE ε4 allele, as the correlation has been reported to be significant only in those subjects without the ε4 allele (Overmyer et al., 1999b). Currently, the exact role of microglia is unknown and whether they are harmful or helpful in pathogenesis of AD is still unclear. In contrast to microglia, an increase in the load of reactive astrocytes has been reported to co-occur in parallel with an increase in load of the extracellular Aβ, suggesting that Aβ deposition may stimulate astroglia to become reactive (Overmyer et al., 1999a; Pike et al., 1994). It has also been proposed that Aβ deposition can induce cytokine expression in astrocytes but the role of cytokines in the pathogenesis of AD remains unclear (Gitter et al., 1995; Hu et al., 1998). As well as the microglia and astroglia, the complement system is activated in AD (Rogers et al., 1992) and it has been suggested that the complement system may have a protective role in AD (Boche and Nicoll, 2008). Furthermore, a decrease of anti-Aβ antibodies in AD patients compared with healthy controls suggests that some individuals are able to immunize themselves against Aβ. In summary, in AD alterations in the immune system are seen, but it is still unclear whether this is beneficial or harmful. The molecular cascade leading to neurodegeneration and neuronal death in AD is not fully understood which prevents the development of a curative treatment.

2.3.2 Dementia with Lewy Bodies

Already in the early 1960's Okazaki and co-workers described two patients with widespread LB pathology throughout the cortex and with progressive cognitive impairment (Okazaki et al., 1961). In the mid 70's, this finding was confirmed by Kosaka and co-workers indicating that diffuse cortical spread of LB pathology could be responsible for

cognitive impairment (Kosaka et al., 1976; Kosaka, 1978). After these original studies, numerous reports have described widespread LBs in the cerebral cortex and brain stem in demented Parkinson patients and in patients who were demented with a clinical picture atypical of AD associated with mild parkinson features (Dickson et al., 1987; Gibb et al., 1987; Perry et al., 1989; Perry et al., 1990). At a consensus conference held in 1996, the clinical and neuropathological features of DLB were identified (McKeith et al., 1996). The key cognitive features of DLB are fluctuating attentional deficit, severe visuoperceptual impairments, and relative preservation of episodic memory as compared with AD. Before one can make a neuropathological diagnosis of DLB it is essential to observe αS immunopositive LNs and LBs in the cerebral cortex (McKeith et al., 1996). However, LBs are often found in the cerebral cortex in elderly individuals who do not exhibit any neurological or psychiatric symptoms (Colosimo et al., 2003; Lindboe and Hansen, 1998; Parkkinen et al., 2005; Yoshinobu et al., 2003) and therefore the assumption that the absolute number of cortical LBs alone would be responsible for the cognitive impairment is oversimplistic. Recent studies have indicated that the presence of Aβ deposition in the cerebral cortex was associated with extensive αS load leading to the conclusion that Aβ enhances the development of LBs (Jellinger, 2006b; Pletnikova et al., 2005). These results may point to some synergistic reactions between Aβ and αS but the molecular background to the pathogenesis of DLB remains to be elucidated.

2.3.3 Vascular cognitive impairment

VCI is considered the second most common cause of age-related dementia after AD accounting for 15-20% of all dementias worldwide (Lobo et al., 2000). The prevalence figures of VCI vary considerably depending on which diagnostic criteria are applied (Bowler, 2007; Jellinger, 2007). VCI is a heterogenous disorder that refers to all forms of mild to severe cognitive impairment presumed to be caused by stroke, multiple cortical and/or subcortical infarcts, silent infarcts, ischemic lesions in functionally important brain areas, small vessel disease with white matter lesions (O'Brien et al., 2003). Multiple small infarcts and small vessel disease are more often a substrate of VCI than a single major infarct (Bowler, 2007). Lacunar infarcts and multiple microinfarcts in the basal ganglia, thalamus, brainstem and white matter are often seen in VCI cases consistent with subcortical VCI

which is the most common variant of VCI accounting for 40% of VCI cases (Bowler, 2007; Erkinjuntti et al., 2000; Erkinjuntti, 2003).

The relationship between stroke and dementia is complex and controversial. There are three pathological types of stroke: ischemic stroke accounting for 80% of cases, primary intracerebral hemorrhage (about 15%), and subarachnoid hemorrhage (about 5%) (Warlow et al., 2003). Approximately half of ischemic strokes are due to atherothrombotic disease, 20 % are caused by cardioembolus, about 25 % are due to intracranial small-vessel disease, with the remaining 5% of strokes being due to other rare causes (Warlow et al., 2003). Cognitive impairment is frequently encountered after ischemic stroke. The estimates of the prevalence of cognitive decline after a stroke vary from 5-30% (Barba et al., 2000; Kokmen et al., 1996; Leys et al., 2005; Rasquin et al., 2004; Tatemichi et al., 1990). It has been estimated that 25-30% of patients with stroke meet the criteria for dementia at 3 months after a stroke (Barba et al., 2000; Pohjasvaara et al., 1997) and the majority of subjects who become demented after stroke will develop the symptoms in the first year (Desmond et al., 2000; Pohjasvaara et al., 1998). In a longitudinal study, the risk of incident dementia was increased 4-fold among stroke patients who were initially cognitively intact when compared with clinically stroke-free elderly control subjects (Desmond et al., 2002). In a population based study the risk of dementia was increased 9-fold in the first year after stroke and the risk for dementia each year thereafter in a cohort was about twice the risk of the general population (Kokmen et al., 1996).

Furthermore, white matter lesions are commonly seen in VCI, but their contribution to cognitive impairment is not explicit (Kalaria et al., 2004), because they are frequently observed in magnetic resonance imaging scans in both cognitively intact and demented elderly persons (Longstreth et al., 1996; Scheltens et al., 1992).

2.3.4 Combined brain pathologies

The majority of the elderly population exhibits brain pathology and those with dementia most often have multiple brain pathologies (Schneider et al., 2007). Schneider and co-workers evaluated 141 well-characterized community-dwelling older individuals and they found that those subjects with more than one neuropathological diagnosis (AD, DLB, infarcts) have an almost threefold increase in their odds of developing dementia compared to those with a single pathology

(Schneider et al., 2007). Already, in 1970 Tomlinson and co-workers reported that most demented males exhibited the pathological features of AD and arteriosclerotic dementia when they examined the brains from 50 demented subjects (Tomlinson et al., 1970). One year later Roth and colleagues reported that the two types of pathological change increase the risk of dementia (Roth et al., 1971). In the elderly population most commonly infarcts and AD co-exist (Kalaria, 2000). In patients with CVD who also have AD type pathology have a worse cognitive outcome and it is also known that the interaction between these two components more than doubles the risk of cognitive impairment (Snowdon et al., 1997; Tyas et al., 2007). However, the association between CVD and AD is complex. Epidemiological studies suggest that there are overlaps and some synergistic effects between AD and CVD pathology, but the results of these studies have been controversial. Jellinger and Attems examined 730 AD patients and 535 age-matched controls and they found that the prevalence of CVD pathology in AD cases was significantly higher than in controls (Jellinger and Attems, 2003). CVD seems to lower the threshold of AD pathology required for the clinical expression of dementia (Esiri et al., 1999; Mungas et al., 2001; Snowdon et al., 1997). However, the underlying interaction between CVD and AD is still unknown (Jellinger and Attems, 2005). It has been proposed by Jendroska and colleagues that ischemic episodes may lead to stress-induced production of Aβ and favour the accumulation of Aβ in the gray matter of cerebral artery boundary zones and in this way cerebral ischemia may contribute to the pathogenesis of AD (Jendroska et al., 1995). Similar results were recorded in the study of Nihashi and co-workers. They found that Aβ was up-regulated in reactive astrocytes after ischemic stress, but this did not result in permanent Aβ deposition (Nihashi et al., 2001). It has also been proposed that CVD may inhibit the elimination of Aβ along arteria walls and change the distribution of Aβ in the cerebral cortex (Weller et al., 2002). However, in human post-mortem studies, no association between the presence of CVL and the load of Aβ deposits or NFTs has been found (Honig et al., 2005; Mastaglia et al., 2003). In theory, small vessel disease may impair the elimination of Aβ along perivascular route and consequently lead to the accumulation of Aβ. However, there are no human post-mortem studies where an association between small vessel disease and the load of Aβ deposits

and NFTs would have been detected. Today, the interaction of CVD and AD remains to be clarified.

In the study of Jellinger, 1050 elderly demented individuals were studied. Among those individuals only 45 % displayed a pure form of AD, 23 % displayed additional CVL and 11% exhibited also LB pathology indicating that most demented elderly subjects display multiple pathologies (Jellinger, 2006b). In conclusion, all additional pathologies that frequently occur in the aged brain seem to lower the threshold for the appearance of the pathology required for the clinical expression of dementia (Snowdon, 1997; Snowdon and Nun, 2003).

2.3.5 Aging and brain pathology

A wide variety of morphological and functional alterations are common in the brain of aged individuals. It has long been known that a substantial proportion of the elderly population displays neuropathological changes of AD such as NFTs, NTs and NPs (Tomlinson et al., 1968). In community based studies, the prevalence of neocortical HPτ pathology, i.e. Braak stage V-VI, has varied between 4-34% of cognitively intact subjects (Bennett et al., 2006; McKee et al., 2006; MRC CFAS, 2001). Some NFTs are present in the enterorhinal cortex and hippocampus of most elderly individuals irrespective of their cognitive status (Haroutunian et al., 1999). These results provide evidence that some type of neural reserve, or some other defense or adaptive mechanism can allow a sizeable percent of aged individuals to tolerate a significant amount of AD pathology without the appearance of the clinical symptoms of dementia (Bennett et al., 2006; Snowdon and Nun, 2003). In the Nun Study, a longitudinal study of aging, as many as 8% of participants who, on autopsy displayed severe AD pathology (Braak stage V-VI) while alive, did not show any signs of dementia and stroke-free participants seemed to tolerate even more AD pathology in their brain before manifesting dementia than subjects with CVL (Snowdon et al., 1997). Therefore the absence of other co-morbid conditions may help an individual to resist the clinical appearance of symptoms that could be expected in view of the existing neuropathology (Snowdon, 1997; Snowdon and Nun, 2003). Likewise AD related pathology αS can be detected in the neocortex in elderly subjects who do not display any neurological or psychiatric symptoms (Jellinger, 2004; Parkkinen et al., 2005). The concept of successful

ageing has provoked considerable interest, but little is known about the neuropathology of healthy aging (Tyas et al., 2007).

2.4 FACTORS INFLUENCING ON THE ACCUMULATION OF Aβ

2.4.1 Apolipoprotein E

The only well-established gene associated with an increased risk of late onset AD is APOE ε4 allele. APOE is a plasma protein mainly produced by astrocytes in the brain. It regulates lipid transport and metabolism and neuronal repair in the central nervous system but it also has other functions including immunoregulation and modulation of cell growth and differentiation (Dik et al., 2001; Duyckaerts et al., 2009; Mahley, 1988). APOE has a role in the regenerative response of injured nerves but it is also associated with neurodegenerative diseases (Strittmatter et al., 1993). APOE is encoded by the APOE gene on chromosome 19 (Emi et al., 1988). There are three different alleles for APOE: ε2, ε3, and ε4 and these three different alleles give rise to three different APOE isoforms which means that there are six possible genotypes (Emi et al., 1988; Mahley, 1988). A well-known risk factor for late-onset AD is the APOE ε4 allele (Corder et al., 1993; Martinoli et al., 1995; Saunders et al., 1993). The presence of the APOE ε4 allele is associated with increased total cholesterol levels leading to an increase in the risk of atherosclerosis (Davignon et al., 1988; Slooter et al., 1999). Therefore, it could be predected that APOE would be associated with AD through atherosclerosis. However, atherosclerosis has not proved to be an intermediate factor and it does not explain the association between APOE and AD (Alafuzoff et al., 1999; Slooter et al., 1999). APOE ε4 allele has been proposed to promote the formation of Aβ by regulating proteolytic degradation of Aβ or to affect the clearance of Aβ, since an increased number of NPs has been found in the cerebral cortex of APOE ε4 homozygotes (Alafuzoff et al., 1999; Mahley et al., 2006; Schmechel et al., 1993; Tiraboschi et al., 2004). In PS1 mutation carriers the load of Aβ is also higher in subjects with the APOE allele ε4 than with the other alleles (Martikainen et al., 2010).

The role of APOE ε2 allele in AD is controversial. In 1995 van Duijn and colleagues reported that ε2 allele is a risk factor for early onset AD (van Duijn et al., 1995). This finding was contradicted in 1997 when Farrer and co-workers concluded that the ε2 allele could protect from AD (Farrer et al., 1997). In other studies no relationship between ε2

allele and the risk of AD has been found (Frikke-Schmidt et al., 2001; Scott et al., 1997; Yip et al., 2002). The biological mechanism by which different APOE isoforms are related to the pathogenesis of AD is still under debate, even though there is agreement that APOE ε4 is a strong risk factor for sporadic AD.

2.4.2 Atherosclerosis

The deposits of cholesterol in the walls of arteries are the culprits in atherosclerosis. In an epidemiological study, an association between atherosclerosis and AD has been revealed (Hofman et al., 1997). Studies investigating the association between atherosclerosis and the hallmark lesions of AD such as Aβ deposits and NFTs have reported conflicting results. In 2005, Honig and colleagues analyzed 921 subjects with a neuropathologic diagnosis of AD and 133 subjects considered as neuropathologically normal (Honig et al., 2005). They found that atherosclerosis was associated with an increased frequency of Aβ deposits, though other neuropathological studies have not corroborated this finding (Alafuzoff et al., 1999; Luoto et al., 2009).

It has been postulated that an atherosclerotic occlusion of the circle of Willis and leptomeningeal arteries plays an important role in the pathogenesis of AD (Kalback et al., 2004) and further, in cross-sectional studies, the extent of high-grade carotid stenosis has been found to correlate with cognitive impairment (Johnston et al., 2004; Mathiesen et al., 2004) The stenosis may result in a decrease in perfusion pressure, and these hemodynamic disturbances have been suggested to contribute to sporadic AD (Kalback et al., 2004). Hypoperfusion has also been considered as the main etiology of white matter lesions that have been suggested predicting dementia (Pantoni and Garcia, 1997; Prins et al., 2004). To summarize, it seems do not accelerate the formation of NFTs or Aβ deposits.

2.4.3 Diabetes

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia. Chronic hyperglycemia is associated with long-term damage of several organs. DM is caused by an inherited or acquired defect in insulin production by the pancreas, or by the ineffectiveness of the released insulin to exert its physiological effects (insulin resistance). Type I DM results from autoimmune destruction of the β-cells of the pancreas leading to absolute insulin deficiency, whereas type II DM

predominantly results from insulin resistance with relative insulin deficiency (American Diabetes Association, 2005). The prevalence of DM is increasing with this increase mainly attributed to an increase in the numbers of patient with type II DM that is strongly associated with lifestyle changes, obesity, inactivity and urbanization (American Diabetes Association, 2005).

DM is a well established risk factor for stroke and it associates with small and large vessel diseases such as white matter lesions and infarctions (Arvanitakis et al., 2006). Thus, it is consistent that DM has been shown to increase the risk of VCI (Xu et al., 2004). A causative association between DM and AD has been proposed based on clinical and epidemiological studies, but the results have been controversial. According to population based studies, the risk of AD in diabetics is elevated (Leibson et al., 1997; Ott et al., 1999). In contrast large clinical studies have not been able to corroborate these findings (Akomolafe et al., 2006; MacKnight et al., 2002; Xu et al., 2004). Regardless of numerous epidemiological and clinical studies where a connection between AD and DM has been claimed, there are few studies where this relationship has been explored at the molecular level. If DM elevates the risk of AD, it could be anticipated that an association between AD related pathology such as HPτ and Aβ could be found. However, in post-mortem studies no association between DM and AD related neuropathological changes has been detected (Arvanitakis et al., 2006; Heitner and Dickson, 1997).

In 2004, Luchsinger and colleagues reported an association between a higher risk of AD and hyperinsulinemia (Luchsinger et al., 2004). Hyperinsulinemia precedes hyperglycemia by many years before the onset of type II DM and after the onset of disease, many of patients still exhibit hyperinsulinemia (Laakso, 1993; Weyer et al., 2000). The link between hyperinsulinemia and AD is the insulin-degrading enzyme (IDE) which degrades insulin, pancreatic amylin as well as extracellular Aβ (Qiu and Folstein, 2006). In the brain IDE is involved in eliminating of extracellular Aβ. IDE is inhibited by insulin and it has been suggested that this inhibition results in an increase in the levels of Aβ and leads to increased AD pathology (Luchsinger et al., 2004; Qiu and Folstein, 2006). Furthermore, it has been proposed that insulin can stimulate the phosphorylation of τ-protein and hence, hyperinsulinemia may increase the formation of NFTs and decrease the elimination of Aβ, resulting in a total increase in AD pathology

(Gasparini et al., 2002). However, there is no consenus in the findings from post-mortem studies to confirm this hypothesis, and thus the association of DM and AD still needs to be clarified.

2.4.4 Alcohol abuse

Epidemiological and case-control studies have shown that excessive consumption of alcohol, meaning an alcohol intake of more than 300 grams of ethanol per week, is a risk factor for stroke and furthermore it can lead to an impairment of cognitive function (Hillbom, 1998; Letenneur, 2004; Saunders et al., 1991). When specific alcohol related disorders, such as Wernicke's syndrome, hepatic encephalopathy and pellagra are excluded; there is still a group of heavy alcohol consumers (HAC) exhibiting cognitive impairment (Harper and Scolyer 2004). A direct toxic effect of ethanol on brain has been postulated as the primary cause of alcohol related dementia (Sun and Sun, 2001) However, the specific and discrete pathological mechanism remains a mystery and thus, the existence of primary alcohol related dementia is still a matter of debate.

Epidemiological and case-control studies have provided conflicting results as to whether alcohol intake plays a role in the risk of AD. Several studies have indicated that alcohol consumption has no effect on the development of AD (Graves et al., 1990; Huang et al., 2002; Rosen et al., 1993; Tanaka et al., 2002) though opposite results have also been reported (Fratiglioni et al., 1993). In very few studies has the relationship between alcohol consumption and the hallmark lesions of AD been evaluated. Already in 1984, Paula-Barbosa and Tavares reported that neuritic plaques were observed in rats following chronic alcohol consumption but subsequently Freund and Ballinger in 1992 reported that neuritic plaque counts were not increased in the brains of alcoholics compared with non-alcoholics, when they investigated human autopsy brains from 20 alcoholics and 20 controls (Paula-Barbosa and Tavares, 1984; Freund and Ballinger, 1992). Later, Gendron and coworkers demonstrated that ethanol could enhance HPτ accumulation in neuroblastoma cells and Sun and colleagues showed that, in rats, bilateral injection of isoproterenol into hippocampus could induce hyperphosphorylation of τ suggesting that alcohol intake could accelerate the development of neurofibrillary tangles i.e. the hallmark lesions of AD (Gendron et al., 2008; Sun et al., 2005). Thus, there is no consensus agreement about the role of alcohol consumption in the

development of AD. Despute the many epidemiological studies where a connection between dementia and alcohol intake has been claimed, there are very few studies where this relationship has been explored at the molecular level.

2.5 ANIMALS AS MODELS FOR NEURODEGENERATIVE DISEASES

Animal models have played a major role in helping to understand the mechanisms of neurodegenerative diseases and in developing potential therapeutic approaches. However, due to the differences between animals and humans, results obtained from animal studies as such cannot be directly generalized to humans (Harding et al., 1997). It has been shown in vivo that transcription factors responsible for gene expression diverge between humans and mice (Odom et al., 2007). Similar to the situation with the transcription factors, it can be assumed that epigenetic factors, DNA methylation and histone modifications, also diverge between species. In addition to these physiological species differences, in AD research most findings emerge from animal models that overproduce Aβ based on mutations seen in familial AD, and thus they do not provide information about the more common sporadic AD. Moreover, transgenic mice models based on APP mutations, that are known to influence the production and aggregation of Aβ42, exhibit Aβ peptides which are in a higher aggregation state when compared to humans (Christensen et al., 2009). Due to these species differences, the results obtained from animal models invariably need to be re-evaluated in human studies.

3 Aims of the study

The extracellular accumulation of Aβ is linked to several neurodegenerative diseases and it is thought to be a key factor in the pathogenesis of AD. However, Aβ deposits are commonly seen in the brains of the elderly population who do not seem to suffer from any neurological disorders, challenging the role of Aβ as an initiator of the pathological cascade. Epidemiological and animal studies have postulated that there is a connection between the accumulation of extracellular Aβ and different risk factors such as CVL, DM, alcohol abuse and synucleinopathy indicating that ischemic stress, metabolic disturbances, toxic stress, or concomitant pathology would elevate the load of Aβ, and accelerate the neurodegenerative process. The objective of this study was to assess these proposed associations in a large human post-mortem cohort.

The specific aims were:

1. To assess if CVL would have any influence on the accumulation of extracellular Aβ in the cortex and thalamus (study I).

2. To estimate the prevalence of Aβ and HPτ pathology in subjects who display αS pathology, and to investigate the relationship between different pathological processes as underlying subsrates for cognitive impairment, and further to assess the clinical relevance of Aβ deposits in the striatum (study II).

3. To test the proposed hypothesis that DM can influence the accumulation of Aβ (study III).

4. To evaluate the proposed association between alcohol abuse and the neuropathological hallmark lesions of AD, DLB and VCI (study IV).

5. To test the reliability of the immunohistochemistry technique while assessing intracellular Aβ in routine diagnostic human

post-mortem material, and to investigate the influence of antibody choice and antigen retrieval method on the obtained staining result (study V).

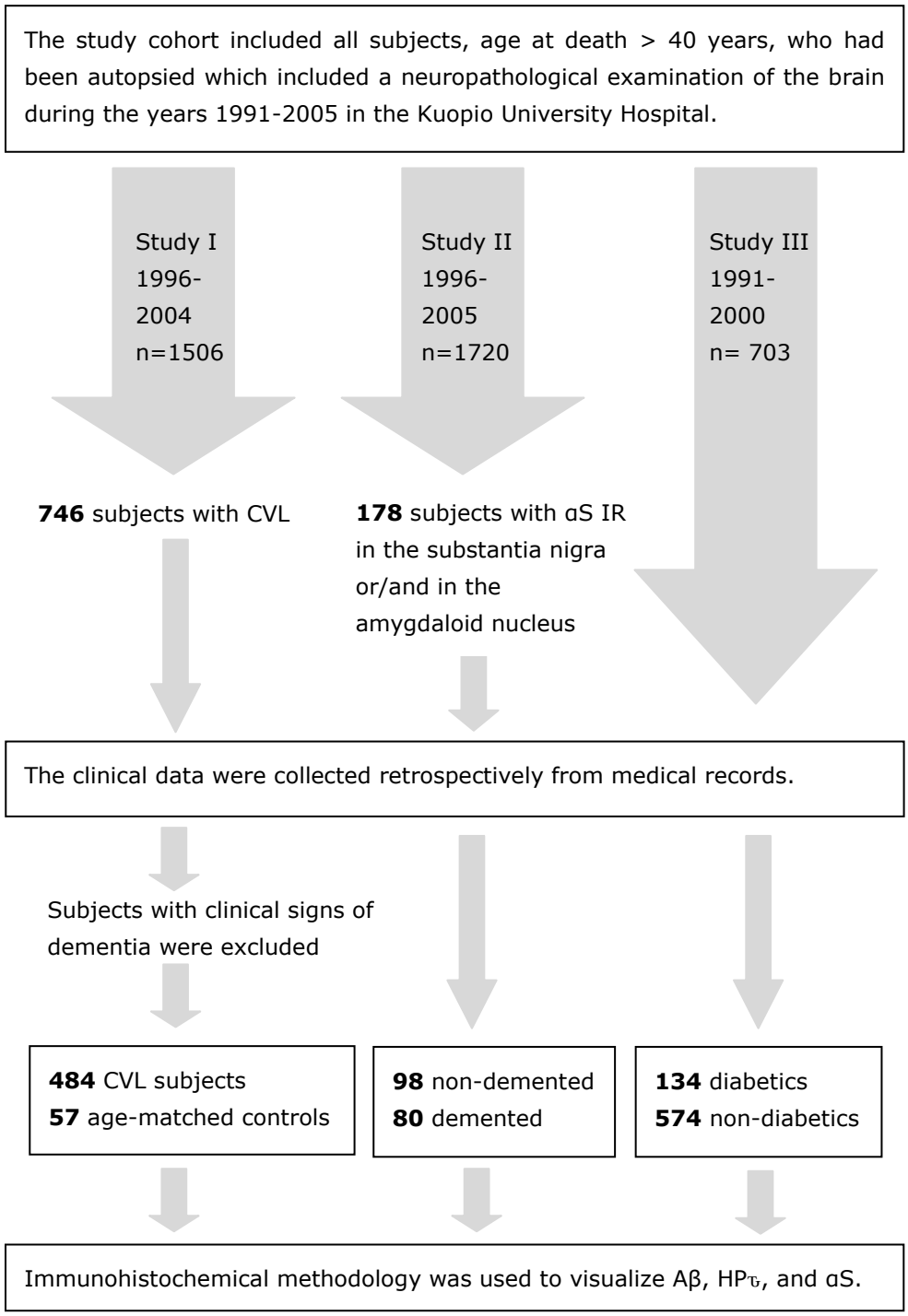
4 Subjects and methods

4.1 CASE SELECTION

The study cohort included individuals who underwent an autopsy including a neuropathological examination in the Department of Pathology of the Kuopio University Hospital that serves a catchment area of approximately 250 000 people. The mortality rate is around 10 %, given an annual rate of death at 2500 individuals. The autopsy rate is estimated to be 32 % and hence approximately 800 individuals undergo a medical or forensic autopsy in any year in the Kuopio University Hospital. The study cohort was collected during the years 1991-2005, but only from 1996 were the brain samples systematically taken from different brain regions. During a 10-year period (1996-2005), 1720 individuals underwent an autopsy including an examination of the brain and therefore the study cohort represents approximately 8 % of all deaths over 10 years in the catchment area of the Kuopio University Hospital.

Figure 4 describes the scheme involved in the selection of the material in studies I-III. In study IV, all individuals were included who were subjected to a forensic autopsy including a neuropathological examination during a six month long period in 1999. Following the legislative protocols, a forensic autopsy is carried out on all subjects dying at home when there is a lack of sufficient medical history which would permit assessment of the cause of death, on all subjects who had died in accidents, as victims of homicide or suicide or who were suspected to be victims of malpractice.

Study V was performed on surgical biopsy material and post-mortem human brain tissue. The surgical sample had been obtained from a patient who had undergone intracranial pressure monitoring with frontal cortical biopsy for suspected normal pressure hydrocephalus.



Figue 4. Study flow chart

4.2 CLINICAL ASSESSMENT

The clinical data were collected retrospectively from the medical records in studies I and V by LA and for study III by HS. In study IV, the information of alcohol consumption was collected from medical records, police reports and from close relatives by JJ. The diagnosis of dementia was based on the Diagnostic and Statistical Manual of Mental disorders, Revised Third Edition, 1987 (DSM-III-R criteria), the most widely used criteria for dementia. However, some of the cases, who were classified as non-demented, might have displayed very mild cognitive impairment that had not been recognised during their lifetime, though it is unlikely that the presence of moderate or full-blown dementia would have been overlooked. Nearly all subjects included in studies II and III had been under continuous clinical follow-up due to some chronic disease and most subjects had been examined by a neurologist and all had at least visited a general physician. In study III, the subjects were considered to have diabetes if a random or post-load serum glucose level had been greater than 11 mmol/l and the medical records revealed that they had been on medication because of diabetes. In study IV, an individual with socially disabling alcohol use, daily alcohol use, and continued drinking in spite of the indisputable health-related and / or social damage was classified as a heavy alcohol consumer (HAC).

4.3 NEUROPATHOLOGICAL ASSESSMENT

Following a standardized dissection protocol, all brains were routinely weighed, evaluated for grossly detectable lesions, fixed in 4 % buffered formaldehyde for at least 1 week, and cut into coronal slices. Prior to slicing, the severity of atherosclerosis of arterial circle of Willis was graded on a four step scale from none, mild, moderate to severe. Macroscopically detectable lesions were assessed on all fixed coronal slices (thickness = 1 cm) and noted as being present or not. For all cases, the brain specimens were systematically taken from the following 15 brain regions: frontal, temporal, parietal, precentral, occipital cortices, gyrus cinguli, striatum, basal forebrain including amygdala, thalamus, hippocampus, and midbrain including substantia nigra, pons including locus ceruleus, medulla, vermis, cerebellar cortex, and additionally from any grossly notable lesion. Neocortical sections were routinely

taken from the right hemisphere and in a case where grossly detectable lesions were seen, additional sections were taken from the non affected left hemisphere. In study IV, subjects underwent a medico-legal autopsy including an examination of brain. The brains were weighed, evaluated for grossly detectable lesions and cut into 1 to 2 cm-thick coronal slices. A central 2 cm thick coronal slice was retrieved and placed in formalin to permit a detailed neuropathological assessment. The anterior cut was through the mamillary bodies. Two weeks later, brain specimens were taken from 7 neuroanatomical regions including temporal cortex, cingulated gyrus, parietal cortex, striatum, basal forebrain including amygdala, hippocampus, and subthalamic nucleus with the adjacent substantia nigra. The brain specimens were fixed in 10 % buffered formalin for an additional week and then embedded in paraffin. The 7-μm-thick sections were cut from formalin-fixed, paraffin-embedded blocks. All sections were routinely stained by applying hematoxylin-eosin (HE).

In study V, the tissue microarray technique (TMA) was used. The HE stained slides of 7-μm thick sections were used to select regions for the core samples to be embedded into the TMA blocks. Each core sample measured 2.0 mm in diameter as recommended by Kauppinen and colleagues (Kauppinen et al., 2006). The core tissue samples from the donor blocks were taken using Beecher Instrument's Manual Tissue Arrayer 1 instrument and each core sample was inserted into previously made holes into the wax surface of the recipient block. The maximum depth of the punch used was 8 mm, and the thickness of the recipient block was 10 mm. The resulting TMA blocks were warmed in an oven for 10 minutes at 57 °C to promote the adherence of the core sample to the paraffin of the recipient block. Finally, the TMA blocks were placed upside-down in stainless steel molds for cooling.

In studies I, III and IV cerebrovascular microscopically detectable lesions in gray matter were estimated in HE stained slides and the extent of vascular lesions was graded on a three step scale 0-no vascular lesions found, 1 - only microscopic and 2- both microscopic and macroscopic vascular lesions detected. Microscopically detectable lesion included lacunar lesion and small infacrts. The features of CVLs are given in study I, figure 2. In study I, II, III and IV AD-related lesions were classified into 7 stages (0-VI) as proposed by the guidelines established by Braak and Braak and for staging the modified Bielschowsky silver impregnation technique and as well HPτ IHC were

applied (Braak and Braak, 1991; Braak et al., 2006). In studies II and IV, the distribution of αS pathology was categorized into 7 stages (0-6) according to the scheme recommended by Braak and co-workers (Braak et al., 2003).

4.4 BRAIN REGIONS EXAMINED

As Thal and co-workers have demonstrated, the Aβ deposition are usually first seen in the cortical regions, indicating that cortex is the most vulnerable region for Aβ deposition (Thal et al., 2002). In studies III and IV, the objective was to evaluate the influence of proposed risk factors on the accumulation of Aβ in a large study cohort, and thus the accumulation of Aβ was assessed in the most susceptible regions i.e.cortical regions. In study I, the purpose was to test the proposed hypothesis that focal cerebral ischemia could lead to the aggregation of extracellular Aβ in thalamic nuclei at least in rats, and thus Aβ IR was assessed in the thalamus and in the watershed area i.e. parietal cortex which is also one of the predisposes regions for the accumulation of Aβ. In study II, Aβ deposition was evaluated in cortex and striatum because these have been reported to be vulnerable regions both for Aβ deposition and αS pathology, and especially in LB disorders, there is claimed to be an extensive burden of αS pathology in the striatum.

Table 1. The Aβ immunoreactivity was evaluated in the following brain areas in each study

Brain area	Study I	Study II	Study III	Study IV
Frontal cortex			X	
Gingulate cortex				X
Temporal cortex		X	X	X
Parietal cortex	X	X	X	X
Striatum		X		
Thalamus	X			

4.5 IMMUNOHISTOCHEMISTRY

Aβ, HPτ and αS were visualized using immunohistochemical (IHC) methodology. The sections were deparaffinized and rehydrated according to the standard procedure. Following the pretreatment, the

sections were incubated with non-immune serum for 30 min at room temperature to eliminate nonspecific reactions. After the epitope unmasking procedures, the antibody was applied. The antibodies, dilutions and pretreatments are given in Table 2. The sections were incubated overnight at 4 °C. On the following day, the sections were incubated with biotinylated second antibody for 30 min followed by streptavidin enzyme conjugate (LABSA Zymed laboratories, South San Francisco, CA) for 30 min at room temperature. The presence of the streptavidin biotin complex was visualized using VectorRed (Vector Laboratories, Burlingame, CA), romulin 3-amino-9-ethyl carbazole, or diaminobenzidine. All immunostained sections were counterstained with Harris' haematoxylin, dehydrated, and mounted in DePex.

Table 2. Antibodies, pretreatments and dilutions

Name	Source, code	Clone	Pretreatment	Dilution
Hyperphosphorylated τ	Innogenetics, BR-03	AT8	none	1:500
α-Synuclein	Novocastra, NCL-ASYN	KM51	autoclave + 80% FA, 5 min	1:1000
Amyloid-β	Dako, M0872	6F/3D	80 % FA, 6 h	1:100
Amyloid-β 40	Invitrogen, 44-348	polyclonal	80 % FA, 6 h	1:1000
Amyloid-β 42	Invitrogen, 44-344	polyclonal	80 % FA, 6 h	1:1000
Amyloid-β	Immuno-Biological Laboratories, 10323	82E1	*	1:1000
Amyloid-β	Signet, 9320	6E10	*	1:2000
Amyloid-β	Signet, 9220	4G8	*	1:2000
Amyloid-β 42	Signet	12F4	*	1:1000
Amyloid precursor protein A4	Chemicon, MAB348	22C11	*	1:100
Amyloid precursor protein	Novocastra , NCL-APP	40.10	*	1:40
Amyloid precursor protein C-terminal	Sigma , A8717	polyclonal	*	1:1000

*The following antigen retrieval methods were applied: 80% formic acid for 2, 10, 60 minutes and for 6 hours or microwave in citrate buffer pH 6.0 for 3 x 5 minutes or a combination of microwave in citrate buffer pH 6.0 for 3 x5 min and 80 % formic acid for 10 minutes

4.6 SEMIQUANTITATIVE ASSESSMENT

The presence of Aβ, HPτ and αS was assessed blinded to the clinical data. The Aβ score, used in study I, was based on the number of stained extracellular aggregates in the total microscopic field (x100 magnification). The numbers of aggregates were semiquantitatively assessed on a scale from 0 to 3 and the final score, ranging from 0 - 3 for each subject, was obtained by calculating the average of scores obtained in the two most affected areas. In studies III and IV, a computer-

assisted image analysis, the NIH image system for PC, was used in the quantification of Aβ expression. The Aβ labeling was estimated in all three neocortical sections. In each neocortical section, the assessment was carried out in three randomly selected fields of gray matter with light microscopy at x40 magnification. The load of Aβ was given as mean stained area fraction in frontal, temporal and parietal cortices (Kraszpulski et al. 1998).

4.7 STATISTICAL ANALYSIS

SPSS for Windows program was used for the statistical analyse. In studies I, II and IV, the relationships between various clinical and pathological parameters were assessed by adjusted logistic regression. The strength of association was estimated by the odds ratio, and this is presented with the 95% confidence interval. Furthermore, the non-parametric Mann-Whitney U-test was used to assess differences between study groups. In study III, also Kruskal-Wallis and χ^2 tests were applied and further the correlations between different variables were tested by the non-parametric Spearman's correlation test. The level of significance was $p<0.05$ in all analyses.

4.8 ETHICAL ASPECTS

The study has been autohorized by the ethics committee of Kuopio University Hospital and in study IV a permission for sampling of the analyzed brain material was granted by the National Board of Medicolegal affairs in Finland 3485/(32/200/98).

5 Results

5.1 INFLUENCE OF ANTIBODY AND ANTIGEN RETRIEVAL METHOD ON THE STAINING OF EXTRACELLULAR Aβ (V)

The influence of Ab and antigen retrieval method on the staining results is demonstrated in Table 3. The extracellular staining of Aβ deposits was excellent with all commercial Abs directed to the amino acid residues present in Aβ (82E1, 6E10, 6F3D, 4G8, Aβ40, Aβ42, 12F4) irrespective of the antigen retrieval method. The fleecy/diffuse aggregates were seen to the same extent, independent of which commercial Aβ Ab was used. Formic acid treatment enhanced the intensity of extracellular IR and the contrast increased with up to 60 minutes of formic acid pretreatment. The combination of boiling in a microwave oven for 3x 5 min in citrate buffer and incubation in 80% formic acid for 10 min intensified the extracellular labeling but the concomitant increase in the background IR decreased the contrast. If the antigen retrieval method was restricted to only boiling in a microwave oven, this led to an overall weaker IR staining of the extracellular deposits.

Antibodies directed to APP or its derivatives (APP C-terminal, APP40.10) did not label the extracellular aggregates in any specimens.

Table 3. Influence of antibody and antigen retrieval method on the staining results in cognitively intact subjects

Immunoreactivity	Antigen retrieval method																	
	80 % formic acid for 2,10, 60 min or 6 h						Microwaving in citrate buffer pH 6.0 3 x 5 min						Microwaving in citrate buffer pH 6.0 3 x 5 min+ 80% formic acid for 10 min					
	82E1	6E10	6F3D	4G8	Aß 40	Aß42	82E1	6E10	6F3D	4G8	Aß 40	Aß42	82E1	6E10	6F3D	4G8	Aß 40	Aß42
e																		
i																		

light gray = no immunoreactivity was detected, dark gray = immunoreactivity was detected, e-extracellular immunoreactivity, i-intracellular immunoreactivity

5.2 GENERAL CLINICAL CHARACTERISTIC OF THE STUDY POPULATION (I-IV)

The demographic of study population is given in table 4. The inclusion criteria for each study were different and since study cohorts may include same subjects, the study groups are not directly comparable with each other. The gender distribution was rather equal in studies I-III, whereas in study IV, most cases were male. The oldest mean age at death was 77 years in study II where the inclusion criterion was the presence of αS pathology, whereas the mean age at death was lowest in study IV. All cases with clinical signs of dementia were excluded from studies I and IV, and further all cases displaying Braak stage V-VI were also omitted from study I to ensure that none of the cases had full blown dementia. The highest prevalence of dementia was seen in subjects who also displayed αS pathology (study II).

Table 4. General characteristics of study cohort

		n	Gender Fe/Ma	Age at death, Mean ±SE	Clinical diagnosis of dementia, %	Braak stage				Cortical Aβ IR, %
						0	I-II	III-IV	V-VI	
Study I	CVL+	484	235/249	68±1	0	245	213	26	0	35
	Controls	57	22/35	71±2	0	34	22	1	0	25
Study II	Subjects with αS	178	87/91	77±1	45	30	98	32	18	60
Study III	DM+	134	68/66	74±1	21	38	63	23	10	60
	DM-	567	312/255	73±1	31	181	222	96	68	60
Study IV	HAC	54	7/47	54±2	0	34	19	1	0	19
	Controls	54	7/47	53±2	0	38	15	1	0	19

5.3 GENERAL PATHOLOGICAL CHARACTERISTIC OF THE STUDY POPULATION (I-IV)

The Braak stage and the prevalence of Aβ in the neocortex in different study cohorts are given in table 4. Most cases were in Braak stage 0-II, even in study II which included subjects with αS pathology. Overall, the prevalence of cortical Aβ varied from 19% to 60% in the different study groups. In studies III and IV, the prevalence of Aβ was assessed only in the neocortex. In study I, 14 % and in study II, 19 % of subjects displayed extracellular Aβ deposits only in the cortical regions corresponding to Aβ phases 1-2. In study I, Aβ deposits were detected in 19 % of the subjects in the thalamus and parietal cortex representing Aβ phase 3 or over. Interestingly, 5 % of subjects exhibited Aβ in the thalamus but not in the parietal cortex. In study II, 41 % of subjects displayed both neocortical and striatal Aβ deposits representing Aβ phase 3 or over, whereas 2 % of subjects had only striatal Aβ deposits.

5.4 INFLUENCE OF AGE AND GENDER ON THE PREVALENCE OF Aβ (I-IV)

In all study cohorts, the prevalence of Aβ deposition in the cortex was associated with age at death (logistic regression, $p<0.05$). The association between gender and the prevalence of Aβ IR varied in the different study cohorts. In study I, no association between gender and Aβ IR could be found, whereas in studies II and III, a statistical significant association was found and a higher odds ratio for female subjects was estimated (logistic regression, $p <0.05$). In study III, when adjusted for age, the association between gender and the prevalence of Aβ disappeared, whereas in study II, the association remained statistically significant ($p=0.03$). In study IV, a tendency toward a higher odds ratio was noted for female subjects but the value was not statistically significant ($p>0.05$).

5.5 INFLUENCE OF CVL ON THE PREVALENCE OF Aβ DEPOSITION (I)

In study I, where the influence of CVL on the accumulation of extracellular Aβ was assessed, 35 % of subjects with CVL and 25 % of controls displayed extracellular Aβ deposits in the parietal cortex. Aβ deposition in the thalamus was detected in 25 % of subjects with CVL

and 21 % of control subjects. The load of Aβ in the parietal cortex and thalamus did not significantly differ between controls and subjects with CVL. Even after adjusting for age, there was no association between CVL and the load of Aβ. However, a tendency toward higher odds ratios for Aβ deposits, though not statistically significant, was noted in subjects with acute CVL (logistic regression).

5.6 INFLUENCE OF DIADETES ON THE PREVALENCE OF Aβ DEPOSITION (III)

In study III, 60 % of diabetics and non-diabetics displayed Aβ in the neocortex. The load of Aβ in the brain was not influenced by hyperglycemia when comparing diabetics to non-diabetics. The load of Aβ was significantly higher in subjects carrying the APOE allele ε4 when compared subjects with the other APOE alleles but this finding was not influenced by diabetes (χ^2, $p<0.001$).

5.7 INFLUENCE OF ALCOHOL ABUSE ON THE PREVALENCE OF Aβ DEPOSITION (IV)

In study IV, cortical extracellular Aβ deposits were seen in 19 % of subjects. Aβ deposits were equally seen in heavy alcohol consumers, referring an alcohol intake of more than 300 grams of ethanol per week, and control subjects. To evaluate further the influence of alcohol abuse on the accumulation of extracellular Aβ, regression analysis was used to control for confounding factors such as gender and age. Even when adjusted for age and gender, the load of extracellular Aβ did not differ between the groups ($p>0.05$).

5.8 RELATIONSHIP BETWEEN Aβ PATHOLOGY AND CONCOMITANT HPτ AND αS PATHOLOGY (I-IV)

The prevalence of co-existence of Aβ and HPτ pathology is given in table 5. The prevalence of AD-related HPτ pathology varied extensively between the different study cohorts, ranging from 33 % to 83 %. The prevalence was highest (83%) in study II which included subjects with *α*S pathology and the oldest mean age at death. The prevalence was lowest (33%) in study IV that had the youngest mean age at death. There was a strong association between the age at death and the extent

of HPτ pathology in all study cohorts (Kruskal Wallis test, p=0.001). As seen in table 5, HPτ and Aβ pathology often co-existed. The prevalence of cortical Aβ deposits increased in parallel with increasing Braak stage in all four study cohorts. When adjusted for age, there was a significant association between Braak stage and the prevalence of Aβ deposits in the cortex (logistic regression analysis, $p<0.05$).

Table 5. The co-existence of Aβ and HPτ pathology in different study cohorts

Braak stage	**The prevalence of cortical Aβ deposits, %**			
	Study I	Study II	Study III	Study IV
0	20	20	31	8
I-II	45	53	61	38
III-IV	78	97	89	
V-VI		100	100	50
All	34	60	60	19

In study II, all subjects displayed αS IR in the amygdaloid complex or/and in the substantia nigra and in 52 % of subjects αS IR was also seen in the cortical regions. Moreover, 35 % of subjects displayed both Aβ and αS pathologies in the neocortex. All three pathologies, αS, Aβ and HPτ, were detected in 53 % of subjects. In study III, 16 % of subjects displayed αS IR in the midbrain and all 3 pathologies were seen in 10 % of subjects. In study IV, αS was detected in 3 % of subjects and 2 % of subjects displayed all three pathologies.

5.9 INTRACELLULAR STAINING (V)

The influence of antibody and antigen retrieval method on the staining results is given in table 3. In study V, intracellular labeling could be observed with three of seven Abs directed to the amino acid residues present in Aβ (82E1, 6E10, 4G8) already at the age of two years and the IR remained high in all samples over the entire age range of 2 to 100 years. With the mAb 4G8, intracellular labeling was seen irrespective of the antigen retrieval method, but with mAbs 6E10 and 82E1 intracellular labeling was detected only when the combination of heat and formic acid was applied. With Abs Aβ40, Aβ42 and 12F4, which are directed to Aβ neoepitopes, intracellular labeling was only detected in AD cases, regardless of which antigen retrieval method was utilized.

Interestingly, with the mAb 6F3D, intracellular labeling was not detected in any of the specimens under any conditions.

Antibodies directed to APP or its derivatives (APP C-terminal, APP40.10) detected intracellular labeling regardless of the antigen retrieval method.

6 Discussion

6.1 THE STRENGHTS AND LIMITATIONS OF THE STUDY

The present study has several strengths but also some limitations which are due to methodological aspects. When assessing material from human subjects, a major limitation is the variable of the material due to many factors such as genetic variation, systemic diseases, medications and various environmental factors. The study cohort was selected from the eastern Finnish population. The Finnish population is unique in that it originates from a small number of founders, and therefore the genetic variation is smaller than in most other populations. On the other hand, due to the special nature of the population, any results may be limited merely to this population. The study cohort was selected from the subjects who underwent an autopsy including an examination of the brain, and thus bias in the sample, due to selection for autopsy, is possible. Therefore, the results may not be generalized to the entire population. However, the study population included approximately 8 % of all deceased and it included both symptomatic and unimpaired individuals who had been living in the area of eastern Finland over the ten years that the survey encompassed, and therefore it is fairly representative (Tilastokeskus). The strength of the present study lies in a large study cohort and its sampling strategy, as the selection of material was based on the presence of a pathological finding regardless of the clinical phenotype.

The use of human post-mortem tissue also gives rise some methodological problems. Agonal and post-mortem events may influence the staining results obtained. In study V, the mode of death i.e. agonal events, did not seem to alter the amount of Aβ IR. Post-mortem delay (PM) and fixation time may have an impact on staining results, however, it has been shown that variations in PM have no qualitative effect on the staining patterns (Kuusisto et al., 2003) and recently it was shown that good Aβ IR was obtained even after 14 years of fixation (Pikkarainen et al., 2010). In order to minimize the variability attributable to the methodology, all Aβ stainings were carried out manually, the staining procedures were standardized and positive control sections were always used. The specificity of immunodetection

was ensured by using well-characterized and widely used commercial Abs such as AT8 and 6F3D.

The weakness of this study is its retrospective nature. Only cases with adequate clinical information were included. Most cases had been examined by a neurologist and nearly everyone had at least visited a general physician at a maximum of one year before death. Some cases classified as cognitively intact might have displayed mild cognitive impairment that had not been recognized during their lifetime. However, it is unlikely that the presence of moderate or full-blown dementia would have been overlooked. It has been evaluated that approximately 4 % of Finnish population have undiagnosed type 2 DM, and therefore some cases classified as non-diabetics might have had DM (Diabetesliitto). Also, reliable information on the duration of diabetes in study III was not available. In study IV, the information of alcohol consumption was collected retrospectively, which includes the possibility of misclassification.

In study I the load of Aβ was semiquantitatively assessed, whereas in study III and IV the computer based NIH Image system was used to quantification of Aβ. However, it has been shown in an inter-laboratory study that the quantification of Aβ deposits is not reliable, and thus a dichotomous assessment is recommended if one wishes to achieve reproducible results (Alafuzoff et al., 2008).

6.2 INFLUENCE OF ANTIBODY AND ANTIGEN RETRIEVAL METHOD ON THE STAINING RESULTS (V)

Immunohistochemistry is a widely used method to visualize Aβ in the brain and it has been shown that antigen retrieval method is significant with respect to the staining results (Alafuzoff et al., 2008; Beach et al., 2008; Christensen et al., 2009; D'Andrea et al., 2003; Ohyagi et al., 2007; Sheng et al., 2003). In study V, it was shown that all commercial Abs stained extracellular Aβ deposition excellently irrespective of which antigen retrieval method was used. The extracellular accumulation of Aβ was visualized by applying mAb 6F3D in studies I-IV. The mAb 6F3D labels the extracellular deposits excellently and the positivity was easily identified because of the excellent contrast. Six hours' incubation in formic acid was used as an antigen retrieval method, because it has been shown to be the optimal pretreatment strategy for visualization of Aβ deposits (Kraszpulski et al., 1998). However, in study V, we

showed that after 60 minutes of formic acid pretreatment the contrast no longer increased suggesting that 60 minutes of formic acid treatment is enough to visualize extracellular Aβ aggregates.

In contrast to the extracellular labeling, the intracellular labeling was strongly dependent on the choice of Ab and antigen retrieval method. With Aβ Abs 4G8, 6E10 and 82E1 intracellular staining was detected in all cases irrespective of disease or age and the staining was already seen at the age of two years. The amino acid sequence that is recognized by clones such as 4G8, 6E10 and 82E1 is also found in the full-length APP, and thus these Abs fail to distinguish Aβ from APP (Horikoshi et al., 2004; Knobloch et al., 2007; LeBlanc et al., 1996; Lord et al., 2006). Intracellular staining was seen with Abs directed to the C-terminus of Aβ such as Aβ40, Aβ42 and 12F4, which have been reported to be specific for Aβ, only in the AD cases with widespread neuronal degeneration. The lack of intracellular immunoreactivity in unimpaired subjects may be due to its absence or that there are such low levels of the protein to be undetectable at light microscopic level by immunohistochemistry method. If only the Abs against the N- terminus or the mid-portion of Aβ had been applied, the conclusion would have been that the intracellular accumulation of Aβ is seen in all cases irrespective of age or condition. On the contrary, if only those Abs directed to the C-terminus of Aβ had been used, the conclusion would have been that the intracellular Aβ would only have been present in the brains of subjects with AD. These results emphasize that the staining results and their interpretation are strongly dependent on the choice of Ab.

Furthermore, the intracellular staining with Abs 6E10 and 82E1 was only detected when the sections were both heated and incubated in formic acid indicating that, in addition to the choice of Ab, the antigen retrieval method is significant. These results indicate that multiple Abs including Abs directed to the N- and C-terminal neoepitopes should be used when assessing the presence of intracellular Aβ and the influence of antigen retrieval method should be borne in mind.

6.3 IMPACT OF AGE AND GENDER ON THE EXTRACELLULAR ACCUMULATION OF Aβ (I-IV)

Age is the best-known risk factor for neurodegenerative diseases (Fratiglioni et al., 2000; Lobo et al., 2000). In the present study, a strong association was found between age and the prevalence of Aβ pathology in all study cohorts. The presence of cortical Aβ deposits in cognitively intact subjects varied from 19 % in study IV to 45 % in study II. This variation is mainly due to the differing inclusion criteria, as in study IV, the mean age at death was lowest, whereas in study II, the mean age at death was highest and all subjects also displayed αS pathology which has been reported to enhance the aggregation of Aβ (Jensen et al., 1997; Masliah et al., 2001). Therefore, it is not surprising that the prevalence of Aβ was highest in study II. In population based studies, the prevalence of cortical Aβ in non-demented subjects has varied from 33 % to 59 % (Bennett et al., 2006; Knopman et al., 2003; MRC CFAS, 2001). The mean age at death in these studies was over 80 years. These prevalence figures are rather similar to those found here. As shown in these studies, a substantial proportion of the elderly non-demented subjects display Aβ in the neocortex (Bennett et al., 2006; Knopman et al., 2003; MRC CFAS, 2001). The increase in the prevalence of cortical Aβ with age supports the hypothesis that the elimination of Aβ may be insufficient in elderly population leading to the accumulation of Aβ with age.

Current studies suggest that female gender is a risk factor for AD, however, in some studies, the difference between men and women has been age dependent (Fratiglioni et al., 2000; Lobo et al., 2000). In this study, the role of gender was not entirely clear, as in study I no association was found between gender and the accumulation of Aβ but in studies II-IV, a higher odds ratio for female gender was found. When adjusted for age only in study II, the association between gender and the prevalence of Aβ was found indicating that the accumulation of Aβ is age rather than gender dependent.

6.4 THE EVOLUTION OF EXTRACELLULAR Aβ DEPOSITIONS (I-II)

Thal and colleagues proposed the evolution of Aβ deposition in the brain follows a distinct sequence in which the regions are hierarchically involved (Thal et al., 2002). The majority of our cases (studies I and II) exhibited Aβ deposition only in the neocortex representing Aβ phase I-II. However, some of the cases displayed a different pattern in the regional distribution of Aβ. In study I, 5 % of cases displayed Aβ deposits in the thalamus but not in the parietal cortex and in study II, in 2 % of cases Aβ deposits were seen in the striatum but not in the neocortex. Thus, the evolution of Aβ does not invariably seem to follow the described phases. On the other hand, we did not assess the Aβ immunoreactivity systematically in all brain areas, and thus we were able to classify the subjects only into two groups according to the evolution of Aβ deposition. Moreover, in study II, Aβ deposits were more often detected from the striatum in demented than cognitively intact subjects indicating a significance of striatal involvement in dementia. In line with this already in 1999 in the Nun study, a significance of striatal involvement in the early phase of AD was emphasized (Wolf et al., 1999). More recently, it was reported that in the PS1 mutation carriers, the accumulation of Aβ appeared to begin in the striatum while using PIB PET to visualize the amyloid (Bacskai et al., 2007; Klunk et al., 2004; Klunk et al., 2007). However, since the majority of the cases followed the described Aβ phases, it seems that concomitant pathology i.e. CVD, αS or some kind of genetic predisposition, may be responsible for the differing evolution of Aβ deposition.

6.5 IMPACT OF CVL ON THE Aβ PATHOLOGY (I)

It has been proposed that ischemic brain damage may induce the accumulation of Aβ in the human brain, and in animal studies the deposition of Aβ has been detected after occlusion of the middle cerebral artery in rats (Jendroska et al., 1995; van Groen et al., 2005). In study I, no association between the presence of CVL and Aβ deposition could be found. This finding is in agreement with other human post-mortem studies (Honig et al., 2005; Mastaglia et al., 2003). However, a tendency towards a higher odds ratio for Aβ deposition was noted for subjects with acute rather than chronic CVL, though this was not

statistically significant. This would support the hypothesis that Aβ represents an acute phase protein produced in a stress situation and which will be normally eliminated with time. This finding is in line with an animal study where Aβ deposits were seen in the surrounding of acute infarcts but the deposits vanished within a few weeks (van Groen et al., 2005; Hiltunen et al., 2009). It has further been shown in rats that focal cerebral ischemia in the cortex causes permanent aggregation of Aβ in the thalamus and this has been suggested to result in corticothalamic degeneration, alteration in APP trafficking, processing, and Aβ degradation in the thalamus (van Groen et al., 2005; HIltunen et al., 2009). In study I, the hypothesis that CVL induces the accumulation of Aβ in the thalamus was not corroborated as there were no differences in the amounts of Aβ in the thalamus when CVL subjects were compared to controls. The discrepant results may be due to physiological and anatomical differences between animals and humans (Harding et al., 1997; Rouiller and Welker, 2000). It has been shown that morphology of corticothalamic projections diverge between rats and monkeys, and thus it can be expected that these projections also differ between rodents and humans (Rouiller and Welker, 2000). Furthermore, the transcriptor factors for gene expression has been shown to diverge between humans and rodents (Odom et al., 2007)

A second hypothesis is that CVL may cause functional changes in the cerebral blood vessels and in this way may influence the perivascular elimination route and consequently lead to the accumulation of Aβ (Pluta et al., 1999). If this assumption were to be correct, it would be predicted that subjects with chronic CVL would display more Aβ than subjects with acute CVL. The load of Aβ increased with age but this phenomenon was not influenced by CVL. This result indicates that it is diffuse age related changes in the walls of blood vessels or perivascular elimination route rather than CVL which results in the insufficient elimination of Aβ. In study I the purpose was to evaluate the proposed hypothesis that a focal ischemia leads to the accumulation of Aβ in the thalamus, and therefore the accumulation of Aβ was only evaluated in the thalamus and parietal cortex. With our study design it was not possible to evaluate the influence of small vessel disease on the accumulation of Aβ. The white matter lesions frequently seen in patients with VCI were neither evaluated. The association between small vessel disease, and Aβ load remains to be elucidated in the future studies.

6.6 THE ASSOCIATION BETWEEN Aβ PATHOLOGY AND DIABETES (III)

Epidemiological studies have proposed that there is an association between AD and DM, however neuropathological studies have so far failed to detect any associations between AD-related pathology, such as Aβ deposits and NFTs, and DM (Arvanitakis et al., 2004; Arvanitakis et al., 2006; Heitner and Dickson, 1997; Janson et al., 2004; Leibson et al., 1997; Ott et al., 1999; Peila et al., 2002). In the prospective Framingham clinical study, DM did not increase the risk for incidental AD (n=2210) (Akomolafe et al., 2006). In study III, in agreement with previous studies no association was found between AD-related pathology and DM, and thus the hypothesis is not supported that DM would elevate the load of Aβ by influencing the clearance of Aβ from the brain.

Diabetes is a well-known risk factor for peripheral vascular pathology, and it may be assumed that the vessels are the primary target also in the brain, and thus it seems more likely that it is the microvascular changes that are responsible for the cognitive impairment rather than AD-related pathology in demented diabetics.

6.7 THE RELATIONSHIP BETWEEN Aβ PATHOLOGY AND ALCOHOL ABUSE (IV)

Numerous epidemiological studies have described the U-shaped relationship between alcohol intake and dementia, but the actual association between alcohol intake and neurodegenerative lesions is unclear (Huang et al., 2002; Marmot and Brunner, 1991; Ruitenberg et al., 2002). In study IV, when evaluating 108 post-mortem brains alcohol intake did not have any influence on the extent of Aβ deposition. In agreement with our results, Freund and Ballinger reported already in 1992 that NP counts were not increased in the brains of alcoholics. In 2002, Lahiri and colleagues demonstrated that alcohol may influence the processing of APP and an abnormal level of APP may lead to increased cleavage of Aβ. It could be anticipated that if alcohol consumption were to increase the level of APP, the load of Aβ would be increased in the brains of alcoholics as compared to non-alcoholics. However, we did not find any statistically significant association between the occurrence and load of Aβ and alcohol intake and thus

heavy and chronic alcohol consumption does not seem to stimulate the production of Aβ nor does it diminish the elimination of Aβ or influence the accumulation of Aβ.

6.8 SYNERGISTIC EFFECT OF Aβ, HPτ AND αS PATHOLOGY (I-IV)

A large majority of older individuals have brain pathology and those with dementia most commonly have multiple brain pathologies (Schneider et al., 2007). Therefore, numerous studies have tried to unravel the complex relationship between all three pathologies i.e. Aβ, HPτ and αS and there has been an intense debate about which Aβ or HPτ pathology is the initiator of AD pathology. In study II, Aβ deposits became more common in both the neocortex and striatum in parallel with the increasing severity of HPτ pathology and similarly in studies I, III and IV, the prevalence of Aβ deposits correlated with Braak stage. These findings support the assumption that the Aβ phase correlates with Braak stage and that Aβ and HPτ may be linked (Hardy and Selkoe, 2002; Thal et al., 2002). Since only half of the cases in Braak stage I-II displayed cortical Aβ corresponding to Aβ phase I-II this indicates that the formation of HPτ is not necessarily secondary to Aβ deposition. Even though these pathologies often co-exist, it does not necessarily indicate that one is caused by other and this proposal is also supported by the fact that the spatial distribution of Aβ and HPτ is different and that HPτ pathology may precede cortical Aβ pathology by decades (Braak and Braak, 1991; Delacourte et al., 2002). These findings support the hypothesis that Aβ and HPτ are independent but they may have synergistic effects once they co-occur, as was demonstrated in experiments with mice carrying mutations in tau and APP which developed more abundant HPτ pathology in those brain areas that were expressing both APP and tau in comparison to those areas which expressed only tau (Delacourte et al., 2002; Lewis et al., 2001; Zhou et al., 2006).

Numerous studies have reported the very high incidence of concomitant HPτ and αS pathologies (Hamilton, 2000; Uchikado et al., 2006; Wakisaka et al., 2003). Accordingly, in study II where all subjects displayed αS pathology at least in the substantia nigra or/and in the amygdaloid complex, 83 % of cases displayed HPτ pathology and 60 % displayed also cortical Aβ deposition. There is compelling evidence to suggest that all three pathologies can affect each other directly or

indirectly and the interaction of these pathologies may explain how dementia develops (Mandavilli, 2006; Masliah et al., 2001).

Recently, abnormal intracellular accumulation of TAR-DNA-binding protein (TDP-43) has been reported in AD and DLB (Amador-Ortiz et al., 2007). TDP-43 is a major component of the tau negative and ubiquitin positive inclusions and is the hallmark lesion of frontotemporal lobar degeneration and amyotrophic lateral sclerosis (Arai et al., 2006). Furthermore, it has been suggested that the co-occurrence of TDP-43 in AD and DLB might contribute to neurodegeneration or modify the clinical course (Arai et al., 2009; Pikkarainen et al., 2009). Taken together, the existence of one pathology may lower brain reserve capacity against other pathological events and the co-occurrence of pathologies may lower the threshold of pathology required to evoke clinical dementia (Schneider et al., 2007). In the elderly population, all three pathologies often co-exist, and thus it is important to evaluate all pathological changes before the significance of any lesions in relation to clinical symptoms can be assessed.

6.9 THE PATHOGENIC ROLE OF Aβ

The pathogenic role of extracellular Aβ has been controversial as the load of extracellular Aβ does not correlate with the severity of cognitive impairment and the majority of elderly population display extracellular Aβ as was also confirmed in the present study (Bennet et al., 2006; Jellinger, 2006b; MRC CFAS, 2001; Tyas et al., 2007). Moreover, the finding that the removal of Aβ plaques is not sufficient to stop the progressive neurodegeneration challenges the importance of Aβ in the pathogenesis of AD (Holmes et al., 2008). Again, it is possible that the presence of Aβ plaques might be necessary to initiate the process but not to maintain it once it has started. There is an increasing body of evidence against the pathogenic role of extracellular Aβ and thus, the Aβ hypothesis was modified in 2004 (Wirths et al., 2004). In the modified Aβ cascade hypothesis the accumulation of intraneuronal Aβ is a trigger for the cascade leading to neuronal death (Wirths et al., 2004). Intraneuronal Aβ has been reported to be detectable in the first year of life, its level increases in childhood and remains high throughout adulthood even in healthy brains, and thus it is likely that it has a physiological function (Wegiel et al., 2007). However, most studies reporting the presence of intracellular Aβ are based on IHC and

Abs such as 4G8, 6E10 and 82E1 which do not distinguish Aβ from APP or its derivates, and thus it is possible that the intracellular labeling is APP rather than Aβ. APP is widely expressed in the brain and it has physiological functions. In study V, intracellular labeling was only detected in the brains of those AD subjects who also displayed severe HPτ pathology when this was visualized with Abs directed to neoepitopes. This finding could be interpreted to mean that intracellular Aβ can be seen only when the neuronal function is already damaged which challenges the role of Aβ as an initiator of the pathological cascade.

It has been reported that the amount of soluble Aβ rather than insoluble Aβ correlates better with the severity of cognitive impairment suggesting that soluble Aβ may be the trigger (Figure 5.) (Lue et al., 1999; McLean et al., 1999; Wang et al., 1999). Furthermore, it has been argued that intraneuronal Aβ must exist in a distinct assembly state for it to induce pathological alterations, as in vivo studies it has been demonstrated that soluble oligomers but not monomers can inhibit hippocampal LTP and alter memory function (Selkoe, 2008; Walsh et al., 2002; Walsh et al., 2002).

Not only in AD but also in many neurodegenerative disorders has the formation of insoluble inclusions been considered to be responsible for synaptic dysfunction and neuronal cell loss. However, recent evidence indicates that these deposits may function as a reservoir and may be either inert or even protective (Baglioni et al., 2006; Haass and Selkoe, 2007). It is possible that Aβ is produced as a compensatory response to the disease. Immunization may even increase the concentration of oligomeric Aβ during the active phase of disintegration, and therefore the process of removing Aβ might be harmful (Holmes et al., 2008). At present, there has been no systematic evaluation of the prevalence of Aβ in the human brain with Abs directed to Aβ oligomers.

Our findings question the assumption that Aβ deposition is the initiator of the pathological cascade leading to neuronal death, however the present series of studies are descriptive in character, and thus no firm conclusions about the function of Aβ can be made. The role and the toxicity of Aβ remain to be determined.

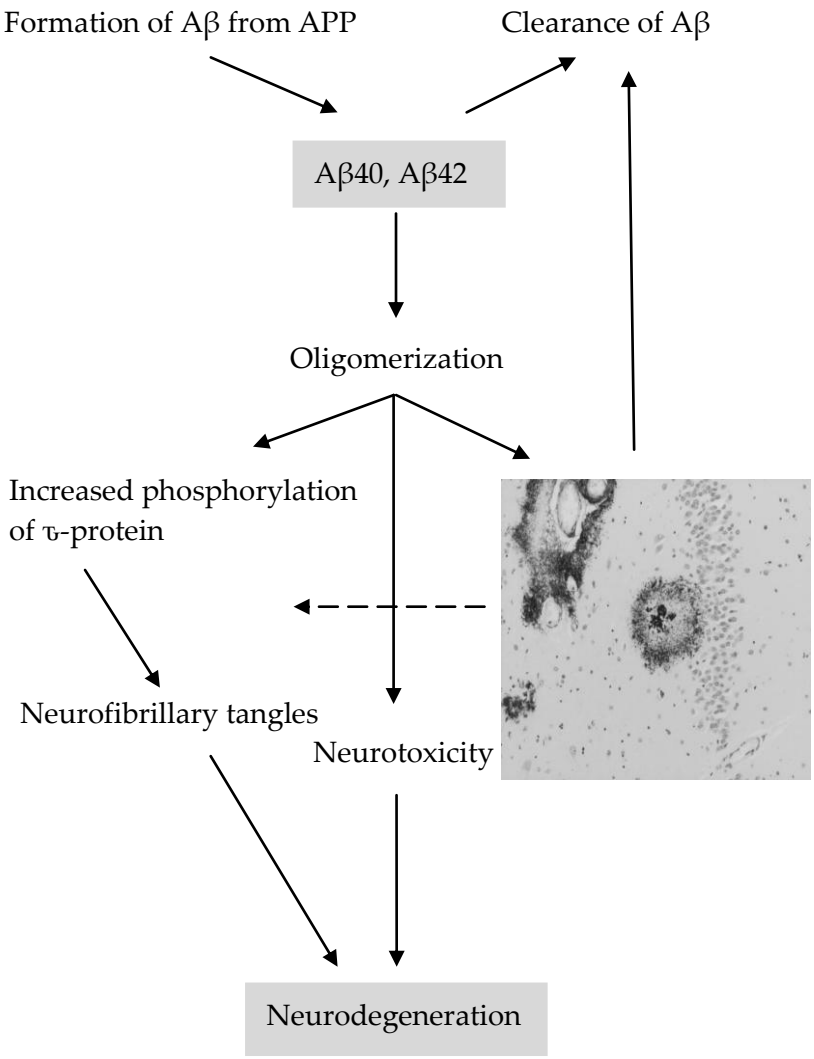


Figure 5. Molecular pathogenesis of AD (Modified from Jellinger 2006)

6.10 FUTURE ASPECTS

Despite considerable progress in AD research, there are many issues that still need to be clarified. The role of extracellular Aβ deposition is still open. It is still unclear whether Aβ deposits are harmful, inert, or protective. Also, the role of intracellular Aβ need be clarified and especially the presence of intracellular Aβ in different diseases applying Abs specific for Aβ should be assessed. In addition to the role of Aβ deposits the co-existence of intra- and extracellular Aβ needs to be evaluated and whether or not there is a connection between intracellular Aβ and HPτ pathology. The influence of small vessel disease on the accumulation of Aβ needs to be clarified. Furthermore, hyperinsulinemia has been reported to be linked with Aβ via IDE. IDE degrades pancreatic amylin as well as extracellular Aβ, and thus the level of pancreatic amylin should be compared to the level of extracellular Aβ.

7 Conclusions

Epidemiological and animal studies have pointed to a connection between the accumulation of Aβ and CVD, DM, and alcohol abuse. However, in the present series of studies, no statistically significant association was found between the occurrence and load of extracellular Aβ and these putative risk factors. The following conclusions can be made:

1. CVL does not influence the load of extracellular Aβ in the thalamus and parietal cortex.

2. All three pathologies: Aβ, HPτ, and αS, often co-exist in the elderly population. They can occur independently but once they co-exist, they may have synergistic effects. Furthermore, the presence of one pathology may lower brain reserve capacity to withstand a second or third pathology.

3. DM does not elevate the load of extracellular Aβ.

4. Heavy and chronic alcohol intake does not influence the accumulation of extracellular Aβ, HPτ, or αS pathology.

5. When IHC is applied to visualize Aβ, especially intracellular Aβ, the staining results and the conclusions will depend strongly on the chosen antibody and the pretreatment strategy, and thus multiple antibodies should be used when assessing the intracellular accumulation of Aβ.

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Leena Aho

Amyloid-β Deposition in the Brains of Subjects with Cerebrovascular Disease, Diabetes, Synucleinopathy and Alcohol Abuse: a Human Post-mortem Study

Deposition of amyloid-β (Aβ) is considered to be a key element in the pathogenesis of Alzheimer's disease (AD). In this study no association between Aβ deposition and cerebrovascular disease, diabetes, alcohol abuse and synucleinopathy was found, a finding that is at odds with proposal that these alterations are risk factor for AD via Aβ deposition. The strong association between age and Aβ deposition indicates that the age-related changes are significant for the accumulation of Aβ. The role of Aβ deposits remains open - are they harmful, inert, or protective?

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