MARGIT OVERMYER

Gliosis in relation to Alzheimer's hallmark lesions in aging and Alzheimer's disease

A postmortem immunohistochemical study

Doctoral dissertation

To be presented with the permission of the Medical Faculty of the University of Kuopio for public examination in Auditorium L1, Canthia building of the University of Kuopio on Thursday 29th of August, 2002 at 1 pm.

Department of Neurology, University of Kuopio Department of Neurology, Kuopio University Hospital

Distributor	Kuopio University Library PO Box 1627 FIN-70211 KUOPIO FINLAND Tel. +358 17 163 430 Fax +358 17 163 410
Author's address:	Department of Neuroscience and Neurology University of Kuopio PO Box 1627 70211 Kuopio FINLAND Tel. +358 162877 Fax +358 162048
Supervisors:	Docent Irina Alafuzoff, M.D., Ph.D. Department of Pathology Kuopio University Hospital Department of Neuroscience and Neurology University of Kuopio
	Professor Hilkka Soininen, M.D., Ph.D. Department of Neurology Kuopio University Hospital
Reviewers:	Docent Matias Röyttä, M.D., Ph.D. Department of Pathology University of Turku
	Professor Irina Elovaara, M.D., Ph.D. Department of Neuroimmunology Tampere University Hospital
Opponent:	Docent Hannu Kalimo, M.D., Ph.D Department of Pathology University of Turku

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia among the elderly and accounts for 50-70% of all the demented cases. The major AD hallmark lesions are senile/neuritic plaques (SP/NP) and neurofibrillary tangles (NFT). The ApoE ϵ 4 genotype is one of the riskfactors for AD.

The present study showed that the prevalence of the ApoE ϵ 4 genotype was higher among AD cases and the number of major AD hallmark lesions was higher in the AD subjects carrying this deleterious ApoE ϵ 4 allele. The number of reactive astrocytes (RA) was significantly higher in AD subjects compared to nondemented ones. However, the number of activated microglia (AM) did not differ significantly between the demented and the non-demented group in this study. The ApoE genotype modified the positive correlation between the number of glial cells (RA and AM) and AD hallmark lesions. The correlation was stronger in AD subjects without the ApoE ϵ 4 allele.

When AD cases without NSAID treatment were studied, there were no significant differences in the amount of NFTs, RA or AM between different ApoE genotypes. The number of SP/NPs was only slightly higher in cases with the ApoE ϵ 4. The number of RA correlated significantly with the β A load and the number of AM correlated significantly with the extent of NFT's in AD cases without NSAID treatment. Regular NSAID treatment in AD subjects was associated with a significantly lower number of RA compared to the subjects without NSAID use.

DNA-fragmentation was more pronounced in definite AD cases compared to probable AD. There was a significant correlation between DNA-fragmentation and NFT count in cases with the ApoE ϵ 4 allele. The SP/NPs correlated with DNA-fragmentation in cases without the ApoE ϵ 4 allele.

The present study shows a complex association between the glia and AD lesions. This association seems to be modified by several risk factors e.g. the ApoE genotype. Epidemiological studies have proposed that NSAID treatment might be of benefit to an AD patient. Results obtained by this retrospective postmortem study support this notion. From the methodological point of view, however, it should be emphasized that a patient sample of 95 individuals is still rather small when analyzing pathological lesions influenced by several riskfactors and this influence being modified by the ApoE genotype.

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Medical subject headings: Alzheimer's disease; aging; gliosis; apolipoprotein E/ genetics; apoptosis; immunohistochemistry; human

This book is dedicated to Kirk

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Helsinki, June 2002

Margit Overmyer

ABBREVIATIONS

AD	Alzheimer's disease		
ADRDA	Alzheimer's Disease and Related Disorders Association		
AM	activated microglia		
ApoE	apolipoprotein E		
APP	amyloid precursor protein		
BBB	blood brain barrier		
βΑ	beta amyloid		
BS	Bielschowsky silver stain		
CAA	Cerebral amyloid angiopathy		
CERAD	Consortium to Establish a Registry for Alzheimer's disease		
CNS	central nervous system		
СТ	computer tomography		
DefAD	definitive Alzheimer's disease		
DSM	Diagnostic and Statistical Manual for Mental Disorders		
EEG	electroencephalography		
GFAP	glial fibrillary acidic protein		
HE	hematoxyline-eosin		
HLA	human leukocyte antigen		
HP-τ	hyperphosphorylated tau		
IHC	immunohistochemistry		
MHC	major histocompatibility complex		
MMSE	mini mental status examination		
MRI	magnetic resonance imaging		
NFT	neurofibrillary tangle		
NIA	The National Institute of Aging		
NINCDS	The National Institute of Neurological and Communicative		
	Disorders and Stroke		
NP	neuritic plaque		
NSAID	non-steroid anti-inflammatory drugs		
PET	positron emission tomography		
PHF	paired helical filament		
ProAD	probable Alzheimer's disease		
PosAD	possible Alzheimer's disease		
RA	reactive astrocytes		
SP	senile plaque		
SPECT	single photon emission computed tomography		
Thio-S	thioflavin-S staining		
TUNEL	terminal-unit nick-end-labeling		



LIST OF ORIGINAL PUBLICATIONS

This work is based on the following publications, which are referred in the text by Roman numerals I-V

- I Overmyer M, Helisalmi S, Soininen H, Laakso M, Riekkinen Sr.P,
 Alafuzoff I. Astrogliosis and the ApoE genotype. An immunohistochemical study of postmortem human brain tissue.
 Dement Geriatr Cogn Disord 1999:10:252-257
- II Overmyer M, Helisalmi S, Soininen H, Laakso M, Riekkinen Sr.P, Alafuzoff I. Reactive microglia in aging and dementia: an immunohistochemical study of postmortem human brain tissue. Acta Neuropath 1999:97:383-392
- III Overmyer M, Krazpulski M, Helisalmi S, Soininen H, Alafuzoff I. DNA fragmentation, gliosis and histological hallmarks of Alzheimer's disease. Acta Neuropathol 2000:100:681-687
- IV Alafuzoff I, Overmyer M, Helisalmi S, Riekkinen Sr.P, Soininen H. β-amyloid load, astroglia and microglia in Alzheimer's disease: association with ApoE genotype.
 Alzheimer's Reports 2000:2:283-289.
- V Alafuzoff I, Overmyer M, Helisalmi S, Soininen H. Lower counts of astroglia and activated microglia in patients with Alzheimer's disease with regular use of non-steroidal anti-inflammatory drugs. JAD 2000: 2:37-46

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I. INTRODUCTION

Alzheimer's disease (AD) in general. AD is the most common form of dementia among the elderly. General estimated prevalence of AD is 3-11 % and it accounts for 50-70% of all the demented cases in an autopsy material (Katzman 1976, Alafuzoff et al. 1987). This age-associated neurodegenerative disorder is characterized by a broad impairment of cognitive performance (Alzheimer 1906, Sulkava and Amberla 1982, Khachaturian 1985, Soininen et al. 1996). The people affected are normally over 65 years of age although the rare familial forms are seen in as early as the 5th to 6th decades (Khachaturian 1985).

Clinical examination and criteria for clinical diagnosis. The patients go through clinical examination, laboratory testing, neuropsychological testing and electroencephalography (EEG) to exclude other possible causes of dementia. The diagnosis is supported by atrophy seen on computer tomography (CT) (Knopman et al. 2001) and magnetic resonance imaging (MRI) (Laakso et al. 1996, Pearlson et al. 1992, Sencakova et al. 2001). The possible history of other AD cases in the patient's family supports the diagnosis (McKhann et al. 1984).

The most commonly used criteria for clinical diagnosis are those published by the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) Work Group (McKhann et al. 1984) and the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R, DSM-IV) (American Psychiatric Association, 1987,1995).

Hallmark lesions in AD. The major diagnostic histopathological lesions are betaamyloid (β A) aggregates in senile/neuritic plaques (SP/NP) (Alzheimer 1906, Khachaturian 1985, Dickson et al. 1988, Mann et al. 1990, Lue et al. 1996), hyperphosphorylated tau (HP- τ) in paired helical filaments (PHF) in neurofibrillary tangles (NFT) (Iqbal et al. 1975, Dickson et al. 1988, Lue et al. 1996) and neuronal loss (Mizutani et al. 1990, Gomez-Isla et al. 1996a). These changes are also seen in cognitively unimpaired elderly individuals (Dickson et al 988, Braak & Braak 1991, Langui et al. 1995). *The criteria used for neuropathological diagnosis.* The neuropathological diagnosis of AD is primarily based on evaluation of the extent of neocortical SP/NP's and NFT's. Most commonly used histopathological diagnostic criteria such as those published by Khacharturian (1985) and Mirra et al. (CERAD 1991) take into account the extent of NP's in the neocortex as well as the patient's age and information concerning the clinical symptoms. Merely an estimation of the amount and localization of NFT's is used in the staging of AD published by Braak and Braak (1991). The latest diagnostic criteria published in 1997 by the National Institute of Aging (NIA) includes information from both CERAD and the Braak and Braak classifications.

Occasional changes seen in AD. In some of AD patients, histologic examination reveals Lewy bodies (Parkkinen et al. 2001) or cerebrovascular pathology (Mirra et al. 1991, Ince et al. 1998) concomitant with AD pathology.

Reactive astrocytes. The human cortex normally contains very few astrocytes expressing GFAP immunoreactivity referred to below as reactive astrocytes (RA). The number of RA increases with aging and the number of RA is also higher in AD and other neurodegenerative diseases when compared to age matched controls (Paetau 1988, Miyazono et al. 1993, Åström et al. 1994, Biernat et al. 1995, Janss et al. 1996, Wallin et al. 1996). GFAP immunoreactivity is limited to the subpial area and into white matter in non-demented cases (Dickson et al. 1988) whereas in AD brains GFAP labeled RA have been seen in the surrounds of SP/NP's (Duffy et al. 1980, Dickson et al. 1988) or diffusely in gray matter (Renkawek et al. 1994).

Activated microglia. The number of microglia is increased in AD cases compared to normally aged controls (Carpenter et al. 1993). Activated microglia (AM) are seen in association with neurodegenerative changes like NP's (McGeer et al. 1987, Styren et al. 1990) and NFT's (Perlmutter et al. 1993). The presence of both RA and AM supports the theory that an inflammatory mechanism might be involved in the progression of AD.

Apoptosis. Neuronal loss has been described as a phenomenon seen in aging (Anglade et al. 1997, Naoi et al. 1999) and the neuronal loss is accentuated in AD

(Engidawork et al. 2001). The two major pathways leading to cell death are necrosis and apoptosis (Bosman 1996). Apoptosis has been proposed as the major pathway leading to cell death in AD (Loo et al. 1993, Su et al. 1994, Dragunow et al. 1995, Smale et al. 1995).

Risk factors. Only three risk factors for sporadic AD can be regarded as confirmed old age (Alzheimer 1906, Tyas et al. 2001), female gender (Tyas et al. 2001), and the ApoE ϵ 4 allele (Saunders et al. 1993, Kuusisto et al. 1994, Gearing et al. 1995, Soininen 1996). Other disputed risk factors include, head trauma (Mortimer et al. 1991), aluminum in drinking water (Rondeau et al. 2000), cardiovascular disease (Hofman et al. 1997, Kivipelto et al. 2001), diabetes (Ott et al. 1999, Luchsinger et al. 2001), the α -macroglobulin genotype (Blacker et al. 1998), the interleukin-1 genotype (Nicoll et al. 2000, Grimaldi et al. 2000, Overmyer et al. 2001) and smoking (Graves et al. 1991). Possible protective factors have also been suggested, such as the ApoE ϵ 2 allele (Yamada 1996), a high educational level (Tyas et al. 2001), estrogen replacement therapy (Henderson et al. 1994, Brenner et al. 1994, Ohkura et al. 1995, Kawas et al. 1997), and the use of NSAID's (Stewart et al. 1997), int'Veld et al 1998).

Aims. The aim of this study was to focus on the association between the major hallmark lesions of AD (NP and NFT) and glia (RA and AM). These changes were also studied in relation to cell death. Furthermore, the influence of the confirmed risk factors such as of age, gender and the ApoE genotype were evaluated on the activation of glia. Finally, it was also investigated whether or not the use of NSAID's was related to neuropathological changes in AD.

2. REVIEW OF THE LITTERATURE

2.1. CLINICAL DIAGNOSIS OF AD

Dementia is usually easily distinguished from normal aging since in healthy aging, intellectual performance remains relatively unimpaired over time (Morris et al. 1991). The clinical diagnosis is based on DSM-III-R, DSM-IV (American Psychiatric Association 1987, 1995) and NINCDS-ADRDA (McKhann et al. 1984) criteria. The DSM criteria for primary degenerative dementia include impairment of short-term and long-term memory as well as at least one of the following: problems with abstract thinking, poor or decreased judgment, disturbances in the higher brain functions (aphasia, apraxia, agnosia), constructional difficulty and personality changes (Reisberg et al. 1987). A person with the disorder also shows difficulties with work or usual social activities. Evaluation of all these symptoms must be done when the subject is in normal state of consciousness, and other underlying causes of the dementia-like symptoms must be excluded (alcohol intoxication, metabolic and psychiatric disorders, intracranial mass, trauma, infections etc.). The NINCDS-ADRDA classification further divides cases into three degrees: possible, probable and definite AD. The diagnosis of definite AD requires neuropathological investigation.

2.2. NEUROPATHOLOGY OF AD

2.2.1. β-Amyloid (βA)

Amyloid is formed from the amyloid precursor protein (APP) which is a transmembrane glycoprotein (Weidemann et al. 1989). The APP protein is produced in the CNS by neurons, astrocytes, microglia (Banati et al. 1993, LeBlanc et al. 1996), endothelial cells and smooth muscle cells. The synthesis of APP is upregulated after various CNS lesions. Proteolytic processing of APP gives rise to β A-peptide (LeBlanc et al. 1996). APP constitutes a family of different isoforms that are produced by alternative splicing (König et al. 1991). Differential splicing yields eight isoforms of APP. The constitutive secretory pathway results in cleavage within the β A domain of the APP by " α -secretase". This pathway precludes the formation of β A and is considered nonamyloidogenic (Busciglio et al. 1993, Haass et al. 1993a). The endosomal-lysosomal pathway consists of reinternalization of cell surface APP. It results in the formation of five

C-terminal peptides, the largest of which contains the entire βA domain and can be cleaved to produce βA (Estus et al. 1992, Haass et a 1992a,1993b). The third pathway involves endocytosis of APP and yields to secreted 4-kDa βA from β and γ -secretase cleavage of APP (Haass et al. 1992b, Busciglio et al. 1993).

One of the central events in AD is considered to be the conformational change of the soluble β A peptide into insoluble amyloid fibrils found in NP's as well as in vessel walls as congophilic amyloid angiopathy (CAA) (Wisniewski et al. 1992). The shorter protein, β A 1-40 mainly deposits on blood vessel walls (Gravina et al. 1995) whereas the longer β A 1-42 is mostly associated with NPs (Fukumoto et al. 1996). The mechanism by which the amyloid deposition is initiated in the diseased brain is not clearly understood. The β A protein forms different morphologic subtypes of plaques (Dickson et al. 1997). Diffuse plaques are composed primarily of β A protein and NP are composed of both β A protein and dystrophic neurites.

2.2.2. Tau (τ)

Microtubules are essential for the structure and function of the neuronal cells. The major microtubule-associated protein tau (τ) promotes the assembly of microtubules by binding to microtubules and stabilizing their structure.

 τ is phosphoprotein and the degree of phosphorylation regulates its biological activity. It belongs to a family of closely related polypeptides with a molecular mass ranging between 50-85 kDa (Iqbal et al. 1975, Cleveland et al. 1977). τ is found in three different forms: cytosolic normally phosphorylated-τ (C-τ), soluble hyperphosphorylated-τ (HP-τ) and HP-τ polymerized into paired helical filament-τ (PHF-τ).

Unlike normal τ , which contains two or three phosphate groups, the HP- τ contains 5-9 mol of phosphate per mol of the protein (Köpke et al. 1993). The hyper- phosphorylation of τ is a likely cause of the breakdown of the microtubule system in a nerve cell (Alonso et al. 1994). The HP- τ does not bind to tubulin and inhibits the in vitro assembly of normal τ and tubulin into microtubules (Alonso et al. 1994).

The PHF- τ is a common finding in degenerative diseases such as AD, progressive Supranuclear palsy, Cortico-basal ganglionic degeneration, Pick's disease, frontotemporal dementia and multisystem tauopathy (Grundke-Iqbal et al. 1986, Spillantini & Goedert 1998). The protein is found in both nerve and in glial cells (Mirra et al. 1991). In AD the PHF- τ is detected as NFT in neurons, as threads in the neurophil and as dystrophic neuritis in SP/NP's.

2.2.3. Histopathological lesions in AD

The histopathological hallmark lesions of AD are SP/NPs (Khachaturian 1985, Dickson et al. 1988, Mann 1990, Lue et al. 1996) and NFT's (Iqbal et al. 1975, Dickson et al. 1988, Lue et al. 1996). SP/NP consists of β A aggregates intermingled with HP- τ neurites, and reactive glial cells (Arai et al. 1990, Fukumoto et al. 1996). The plaques are considered to start as diffuse plaques (β A aggregates only) and develop later into neuritic plaques (β A aggregates and dystrophic neurites containing HP- τ). Finally they appear as burn out plaques (β A core). NFT's seen in neurons consist of PHF- τ . These lesions are also seen in cognitively unimpaired elderly individuals (Dickson et al. 1988, Braak & Braak 1991, Langui et al. 1995). In AD the number of SP/NP's is significantly higher when compared to age matched controls and isocortical NFT's are a common finding (Crystal et al. 1988).

The histopathological diagnostic criteria used are almost all based on the counts of the SP/NP's. In the diagnostic criteria proposed by Khachaturian (Khachaturian 1985) and those advanced by CERAD (Mirra et al. 1991) a semiquantitative estimate of SP/NP's in silver stained sections is related to the patients age and clinical symptoms. Another way to estimate the histopathological lesions of AD is to evaluate the amount and regional distribution of NFT's (prediction of the progress of the pathology). In 1991, Braak and Braak described the staging of AD lesions where the extent of NFT was evaluated specifically in the hippocampal region. They divided the progression into 6 defined stages. Stages 1-2 were designated as the entorhinal stage, 3-4 as the limbic stage and 5-6 as the isocortical stage. The Braak staging is used in addition to the CERAD histopathological classification in a recent set of criteria proposed by NIA (1997) (Table 1).

Neuropathological	Lesions of interest	Age	MMSE	Classification	Criteria for definite AD
classifications					
currently used					
1976	SP/NP in silverstain,	< 50	< 26	-	SP/NP & NFT counts $> 2-5/field$
Khacharturian	Thio-S or Congo red	50-65			some NFTs & SP/NP > 8/field
		66-75			some NFTs & SP/NP > 10/field
		>75			some or no NFTs & SP/NP > 15/field
1991	SP/NP in Bs	<50	-	Definite AD	age-related SP/NP score and dementia and
CERAD		50-75		Probable AD	presence or absence of other neuropathologic
		>75		Possible a, b	lesions likely to cause dementia
				Normal a, b, c	(see Tables 6 and 7)
1991	NFT in Bs	-	_	Isocortical V-VI	Isocortical V-VI
Braak and Braak				Limbic III-IV	
				Entorhinal I-II	
1997	CERAD	-	-	1. High likelihood:	CERAD: defAD and Braak & Braak V/VI
NIA	Braak & Braak			CERAD: posADa, defAD and	
	In addition to these			Braak & Braak V/VI	
	IHC recommended			2. Intermediate:	
				CERAD posADb, proAD and	
				Braak & Braak III/IV	
				3. Low likelihood:	
				CERAD control b/c, posADc,	
				Braak & Braak I/II	

Table 1. Neuropathological diagnostic criteria used for AD. - = not used.

2.2.4. Glia

2.2.4.1. Astrocytes

Astrocytes comprise one-third of the cerebral cortex volume forming the most numerous cellular element of the brain tissue. They are divided into different subclasses according to their morphology and location. The fibrous astrocytes are mostly seen in the white matter whereas protoplasmic astrocytes fill the gray matter (Frederickson et al. 1992). However there is still an enormous heterogeneity within fibrous and protoplasmic astrocytes based on the profile of enzymes, antigenic markers, transporters, receptors and ionic channels (Norenberg 1994).

Astrocytes clearly serve a supportive purpose in normal brain tissue, but there is a wide diversity of different functional properties (Picture 1). Astrocytes are involved in many major brain functions like neuronal migration, neurite outgrowth, synaptogenesis and synaptic plasticity, maintenance of the blood-brain barrier (BBB), regulation of water, ion and aminoacid metabolism, energy and nutrient support, modulation of immune/inflammatory responses, and phagocytic functions (Norenberg 1994).

The astrocyte response to the central nervous system (CNS) injury is described by increase in the number and size of astrocytes expressing the glial fibrillary acidic protein (GFAP) referred to as reactive astrocytes (RA) (Neary et al. 1994, Bodjarian et al. 1997). GFAP is an intermediate cytoskeletal protein expressed by astroglia. The glia maturation factor (GMF) as well as S100β increases astrocyte proliferation and induce morphologic changes (Norenberg 1994). Definite stimuli leading to an increase in GFAP expression is not yet fully understood, but increased production of TGF- β in RA raises the expression of GFAP (Krohn et al. 1999). The biochemical stimulus leading to astrocyte activation can also come from microglia, neurons and oligodendrocytes among others (Norenberg 1994). In vitro studies have shown that release of the cytokine IL-1- β from activated microglia (AM) is an especially an important activator (Fredrickson 1992).

The human cortex normally contains very few RA (Fredrickson 1992). Their number increases with aging in some brain regions (Hansen et al. 1987, Beach et al. 1989). Compared to age matched controls the number of RA is higher in different neurodegenerative diseases, demyelinating disease, CNS infections, tumors, traumas (Paetau 1988, Miyazono et al. 1993, Åström & Stoner 1994, Biernat et al. 1995, Janss et al. 1996, Wallin et al. 1996) and especially in AD (Dickson et al. 1988, Paetau 1988, Beach et al. 1989, Miyazono et al. 1993, Brun et al. 1995, Arnold et al. 1996, Janss et al. 1996, Wallin et al. 1996).

The GFAP immunoreactivity is normally limited to the subpial area and to the white matter in non-demented cases (Dickson et al. 1988, Beach et al. 1989) whereas in AD brains RA have been seen in the surrounds of plaques (Dickson et al. 1988) or diffusely all around the tissue (Renkawek et al. 1994). Kato et al. 1998 showed that the processes of astrocytes also penetrated into SP/NP's. The amount of GFAP protein in brain homogenates has been shown to correlate with counts of both NFTs (Harpin et al. 1990) and NPs (Harpin et al. 1990, Le Prince et al. 1993) and the classic β A plaques shown by immunohistochemistry (IHC) (Harpin et al. 1990).

The role of astrocytes is not fully understood in AD. It has been proposed that A β can induce cytokine expression in astrocytes (Gitter et al. 1995, Forloni et al. 1997, Hu et al. 1998). The cytokine (IL-1 β , TNF- α , IL-6, α -1-antichymotrypsin, C1q, C5b-9, MIP-1 α and - β , MCP-1) secretion in reactive astrocytes might be one of the factors leading to neurodegeneration (Frederickson 1992). On the other hand it has been suggested that reactive astrocytes may also take part in amyloid production. The basic fibroblast growth factor (bFGF) stimulates APP production which is increased in activated astrocytes. Furthermore amyloid stimulates bFGF production in astrocytes forming a continuous cycle. Astrocytes may also be associated with APP processing through production of proteases and their inhibitors (i.e. cathepsin D and α -1-antichymotrypsin) (Norenberg 1994).

However it has further been proposed that astrocytes also provide support for neuronal function. They can for example take up and remove synaptically released glutamate (the major excitatory neurotransmitter), thus ending its stimulatory action and preventing neuronal damage (Vesce et al. 1999). In 1989 Rosenberg and Aizenman showed that neurons grown in astrocyte-poor cultures were much more vulnerable to glutamate toxicity compared to cell cultures with astrocytes.



b) Some functional properties of microglia



Picture 1. Some functional properties of an astrocyte (a) and microglia (b).

(ACT=al-antichymotrypsin, bFGF=basic fibroblast growth factor, CNF=ciliary neurotrophic factor, CSF=colony stimulating factor, GM=glial maturation factor, IL=interleukin, MHC=major histocompatibility complex, NGF=nerve growth factor, PDGF= platelet derieved growth factor, TGF=transforming growth factor, TNF=tumor necrosis factor)

a) Some functional properties of astrocytes

2.2.4.2. Microglia

In 1933 del Rio-Hortega identified microglial cells with silver impregnation. Microglia comprise up to 20 % of the total glial cell population and are the first cell line to respond to a CNS insult leading to formation of activated microglia (AM) (Gehrman & Banati 1995, Plata-Salaman 1991). Microglia are the resident macrophage resembling cells of the CNS (Davis et al. 1994) and represents the immune system of the brain tissue (McGeer et al. 1993). It has been suggested that AM are sources of trophic factors known to support also the development and normal function of CNS cells (Elkabes et al. 1996, Rappolee et al. 1988).

Microglia originates from the bone marrow-derived precursor cells (monocytes) that populate the CNS after it has been vascularized (Hickey et al. 1988, Perry et al. 1988, Gehrman & Banati 1995). Microglia assume many forms in the adult human CNS such as ramified, amoeboid and rod form (Davis et al. 1994). Microglia do not show any preferential localization to neuronal or vascular surfaces, as is often true for astrocytes (Duffy et al. 1980, Dickson et al. 1988). They are present in nearly equal numbers in the gray and white matter (Akiyama & McGeer 1990), although quantitative studies suggest that slightly more microglia is seen in the gray matter than in the white matter.

Resident resting microglia are activated in response to trauma, infection and inflammation of the CNS (Dickson et al. 1991, Gehrman & Banati 1995) and after activation express class I and II MHC (major histocompatibility complex) antigens (Picture 1). HLA-DR is a class II MHC antigen commonly used to label the AM in IHC (McGeer et al. 1988, Cras et al. 1990). After activation the cells proliferate in the diseased brain, exhibit phagocytic activity, release free radicals and nitric oxide, produce lysosomal cysteine proteinases important for the metabolic processing of APP and secrete cytotoxic agents inducing death of neurons and oligodendrocytes. IL-1 α , IL-1 β , IL-6, tumor necrosis factor (TNF)- α and TNF- β are inflammatory cytokines secreted by AM in the human brain (Gehrman et al. 1995).

2.2.4.3. Inflammatory reaction in AD

The inflammatory reactions seen in AD i.e. the increase in the number of RA and AM and the complement activation indicate that inflammation might be of importance in neurodegeneration (McGeer et al. 1996, Combs et al. 2000).

AM serves in brain tissue as immunocompetent cell and phagocytates alien material like amyloid (Davis et al. 1994, Elkabes et al. 1996). The AM can slowly degrade limited amounts of β A but the degradation mechanism can be overwhelmed by an excess of β A protein (Paresce et al. 1998, DeWitt et al. 1998). In vitro studies have implicated that microglial activation leads to APP production and that the overload of APP in the tissue might lead to abnormal proteolysis, aberrant cleavage of APP and β A formation.

Another aspect implying the importance of AM in the pathogenesis of AD is the suggestion that AM provoked by β A (Araujo et al. 1992, Meda et al. 1995, Yamada et al. 1995) releases IL's and INF's causing neurodegeneration (Brown et al. 1996) i.e. formation of HP- τ and consequently PHF- τ .

2.2.5. Apoptosis

There are two major pathways that lead to cell death i.e. apoptosis and necrosis (Hale et al. 1996) which can be recognized according to the morphological differences (Table 2). The major biochemical features of apoptosis are DNA-fragmentation and a requirement for de novo protein and nucleic acid synthesis, which are not seen in necrosis (Bosman et al. 1996).

Apoptosis	Necrosis
Cell shrinkage	Cell swelling
Organelles intact	Organelles damaged
Chromation marginated	Chromation fragmented
Cytoplasmic extensions	Cytoplasm fragmented
Membrane intact	Membrane damaged

Table 2. Some morphologic features of apoptosis and necrosis.

In necrosis cells die due to some extrinsic factors whereas in apoptosis they die due to an intrinsic mechanism (Lesort et al. 1997). It has been suggested that oxidative stress, excitatory toxicity, calcium toxicity and survival factor deficiency may induce apoptosis in neuronal cells. The pathway leading to apoptosis is strictly regulated by activation or inactivation of specific genes (e.g. c-myc, bcl-2 and p53). Currently in histology apoptosis is detected by means of TUNEL technique whereby fragmentation of DNA is labeled and visualized. (Gavrieli et al. 1992, van Lookeren Campagne et al. 1995).

Apoptosis plays an important role in cell turnover (Bredesen 1995), embryogenesis (Bosman et al. 1996, Hensey & Gautier 1999), immunogenesis (Bosman et al. 1996, Bredesen 1995), hormonal regulation (Bosman et al. 1996, Bredesen 1995), cancer (Bosman et al. 1996, Hensey & Gautier 1999), etc. For example, during embryogenesis neurons are produced in excess (Bosman et al. 1996). The only neurones surviving are the ones receiving sufficient amounts of nerve growth factor (NGF) that is produced by target-cells that they will innervate. During embryogenesis, neuronal cell death appears to adjust the number of cells to the size of the target they innervate.

Neuronal loss has been reported to occur in the neocortex with aging (Anglade et al. 1997, Naoi & Maruyama 1999) but it has also been reported that no such age-related neuronal loss occurs (Braak & Braak 1986). It is now suggested that the instead of neuronal loss the age-related changes may be more likely i.e. due to shrinkage in the size of the neurons rather than real loss. Neuronal loss is common in neurodegenerative disease and is accentuated in AD (Rose & Henneberry 1993, Smale et al. 1995, Bobinski et al. 1997) specifically in the limbic structures. The pathway leading to cell death in AD has been suggested to be apoptosis (Loo et al. 1993, Su et al. 1994, Dragunow et al. 1995, Smale et al. 1995). However, Stadelmann et al. 1998 suggested that the observed DNA fragmentation in AD indicates increased neuronal vulnerability rather than apoptosis.

In Alzheimer disease the stimulus leading to apoptosis may be βA . It has been demonstrated that cultured neurons undergo apoptosis induced by βA (Loo et al. 1993, Paradis et al. 1996, Gschwind et al. 1996). The mechanism by which β A leads to apoptosis or cell death is not yet clear. However, the downregulation of bcl-2 in the presence of β A might be of importance (Paradis et al. 1996). On the other hand LeBlanc et al. (1999) suggested that increased production of β A is a consequence of neuronal apoptosis and that apoptosis actually activates proteases involved in APP processing (Jordan et al. 1997).

Various results have been reported when studying the association between apoptosis and the hallmark lesions of AD (Kiatipattanasakul et al. 1996, Lassmann et al. 1995, Sheng et al. 1998, Rohn et al. 2001). A positive association as well as a lack of association between βA , SP/NP's, NFT's and apoptotic cells has been reported. Lassmann et al. 1995 found that several AD patients exhibit a large number of βA associated with no morphological signs of apoptotic cells (Lassmann et al. 1995). It has been suggested that failure to demonstrate apoptotic cells is due to the low number of cells undergoing apoptosis at the given moment (Anderson et al. 1996) or that there might be additional other forms of cell death operating in AD (Migheli et al. 1994).

2.3. GENETIC AND ENVIROMENTAL FACTORS

2.3.1 Causative genes

A small percentage of the all AD cases are due to mutations in the amyloid precursor protein (APP) gene on chromosome 21. The presenilin genes i.e. presenilin 1 (PS1) in chromosome 14 and presenilin 2 (PS2) in chromosome 1 are also considered to influence the processing of β A-protein as well (Levy-Lahad & Bird 1996, Haltia et al. 1994, Barinaga 1995). Mutations in these genes cause the majority (40%) of early-onset familial AD (FAD).

2.3.2 Risk factors

2.3.2.1. Aging

The incidence of AD increases as the average life expectancy rises. Age is the major demographic risk factor for the development of AD (Tyas et al. 2001) and the aging of our society has contributed to the growing importance of the disease (Forstl 1998).

2.3.2.2. Gender

All reports indicate that most of AD patients are females. Clinical studies have also revealed that females have a significantly lower age at onset, and a more rapid course of the disease (Raghavan et al. 1994, Payami et al. 1996, Tang et al. 1996, Tyas et al. 2001). It has been suggested that this is related to the decline of estrogen levels in the menopause (Bachman et al. 1992). Administration of a higher dose of estrogen has been shown to enhance attention and memory in postmenopausal women with AD (Asthana et al. 2001). Estrogen has not been directly linked to a lower risk of AD, but the significantly lower rate of oral estrogen use among AD patients compared with nondemented control subjects lends credence to the hypothesis that estrogen replacement therapy in postmenopausal women may reduce the relative risk of developing AD (Henderson et al. 1994, Kawas et al. 1997). Recently a link between estrogen and apoptosis has been suggested when it was shown that estrogen significantly increases the expression of the anti-apoptotic protein Bcl-XL in cultured hippocampal cells (Pike 1999) and in 2001 Hosoda showed that estrogen protected neurons from βA-induced apoptotic cell death.

2.3.2.3 Apolipoprotein

ApoE is a lipid metabolism regulating polymorphic glycoprotein defined by three alleles, $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$, of a gene located in chromosome 19q13.2 (Steinmetz 1987, Pericak-Vance et al. 1991). It is found in neuronal cytoplasm and the expression of mRNA for ApoE is detected in glial cells, especially astrocytes (Oropeza et al. 1987, Diedrich et al. 1991). Biological effects of ApoE favor myelination and modulates βA and tangle formation (Holtzman et al. 2000). It also modifies neuronal plasticity in both healthy and diseased brains (White et al. 2001).

The ApoE ε 4 allele has been linked to memory impairments (Bartres-Faz et al. 2001). The ApoE ε 4 allele is more frequent among subjects with age-associated memory impairment (AAMI) (Bartres-Faz et al. 1999) and in AD compared to controls (Saunders et al. 1993, Strittmatter et al. 1993, Heinonen et al. 1995, Martinoli et al. 1995, Lehtovirta et al. 1996). Several studies indicate that ApoE ε 4

is a risk factor in AD (Saunders et al. 1993, Gearing et al. 1995, Soininen 1996, Kuusisto J et al. 1994). The severity of clinical symptoms related to memory and judgment increase proportionally with the ApoE ε 4 allele dosage.

The ApoE ε 4 has been suggested to influence the AD pathology by promoting amyloid formation (Heinonen et al. 1995, Martinoli et al. 1995, Nagy et al. 1995, Polvikoski et al. 1995). ApoE ε 4 promotes aggregation of amyloid fibrils (Ma et al. 1994, Castano et al. 1995) as well as enhancing NFT formation (Nagy et al. 1995). The presence of THE ApoE ε 4 allele is associated with more severe degeneration in AD (Martinoli et al. 1995, Nagy et al. 1995, Gomez-Isla et al. 1996b). The results from Ghebremedhin (2001) confirm the association between the ε 4 allele and both types of AD-related lesions and show that this association is differentially modified by age and gender.

The ApoE genotype has also been linked to glia. Egensperger et al. (1998) showed that the amount of AM increased significantly with ApoE ε 4 gene dose and Saitoh et al. (1997) reported that this increase is dependent on the localization of microglia i.e. the ApoE ε 4 allele was associated with an increase in the scattered microglia.

2.3.2.4 Cardiovascular disease

Several studies have implied that the cardiovascular status is another factor influencing depression, cognitive decline and amyloid accumulation in brain tissue (Prince 1995, O'Brien et al. 1996, Wisniewski & Maslinska 1996). Epidemiological studies have indicated that hypertension is significantly linked to AD (Hofman et al. 1997, Kivipelto et al. 2000), but a lack of association between the hallmark lesions of AD and the end stage cardiovascular status has also been reported (Alafuzoff et al. 1999).

2.3.2.5 Diabetes

Stewart et al. (1999) showed association between diabetes mellitus (DM) type II and cognitive impairment. Recent studies have implicated that diabetes is a risk factor for dementia in the elderly (Ott et al. 1999, Luchsinger et al. 2001). A moderate association between diabetes and AD was reported by Bruce et al. (2001), but contrary results have also been reported (Curb et al. 1999, Tariot et al.

1999). Retrospective clinico-pathological studies have not shown any direct association between the hallmark lesions of AD and diabetes (Alafuzoff et al. manuscript).

2.4. PHARMACOLOGICAL INTERVENTION

So far there is no cure for AD. The disease progresses invariably and leads to severe impairment and finally death. Treatment of AD patients with acetylcholinesterase inhibitors, the most commonly used medication, can improve cognition and reduce neuropsychiatric problems to some extent (Francis et al. 1999, Perry et al. 1999).

At the beginning of the 1990's it was proposed that the use of NSAID's might influence the progression of AD. Epidemiological evidence suggests that patients on long-term NSAID therapy for rheumatoid arthritis had an unexpectedly low incidence of AD (McGeer et al. 1992) and a separate twin study supported this hypothesis (Breitner et al. 1994). Later studies showed that the relative risk for AD decreased with increased duration of NSAID use (Stewart et al. 1997, int'Veld et al. 1998) and some clinical trials indicated an arrest or slowing of cognitive decline with NSAID use (Beard et al. 1998). Halliday et al. (2000) suggested that NSAID use demonstrated enhanced cognitive function but did not show any reduction in pathological markers of the disease in postmortem brain tissue. The mode of action of NSAID's, i.e the influence of the treatment on the AD related pathology is still unclear.

3. AIMS OF THE STUDY

The inflammatory reaction through glial activation is of great importance in the development of AD changes. The number of pro-inflammatory proteins (cytokines, complement factors etc.) is high in glial cells surrounding plaques. It is believed that activated glia is most likely the major source of the pro-inflammatory proteins. β A can activate microglia (Araujo and Cotman 1992, Meda et al. 1995) and astrocytes (Gitter et al. 1995, Forloni et al.. 1997, Hu et al. 1998). This leads in microglia to production of neurotoxins such as nitric oxide (Ii et al. 1996), reactive oxygen species (Klegeris and McGeer 1997) and glutamate (Klegeris and McGeer 1997). Astrocytes have also been shown to respond to β A by producing nitric oxide (Rossi and Bianchini 1996). All these biochemical compounds secreted by activated glia are suspected to lead to the neurodegeneration in AD. Further support for the inflammatory cascade hypothesis comes from epidemiological studies that show reduced risk for individuals using non-steroidal anti-inflammatory drugs (McGeer et al. 1992, Breitner et al. 1994).

According, the present retrospective neuropathological immunohistochemical study aimed to elucidate the important association between glia and the hallmark lesions of AD by doing the following:

1) investigating the association between NP, NFT and RA and AM (I, II)

2) investigating the association between NP, NFT, RA, MA and TUNEL-labeled cells (III)

3) investigating the influence of some risk factors (age, gender, ApoE genotype) of AD on glia (I, II, III, IV)

4) evaluating whether or not the glial reaction is altered by the use of non-steroid anti-inflammatory drugs (V)

4. MATERIAL AND METHODS

4.1. SUBJECTS

The material used in this study was from the Kuopio Brain Bank. The patients were members of clinical studies on dementia and the autopsies were carried out at the University Hospital of Kuopio during 1991-1996. Prior to their death the patients were either living at home, in geriatric departments or in old age homes. All patients were examined by a neurologist. This study was approved by the ethics committee of the University Hospital and University of Kuopio and by the National Board of Medical Legal Affairs.

4.2. CLINICAL METHODS AND DIAGNOSTIC CRITERIA USED

The patients underwent a general clinical and neurological examination including a wide range of various neuropsychological tests and imaging (Table 3). The information regarding the use of NSAID's was obtained retrospectively from medical records.

Neuropsychological testing	Minimental State Examination		
	Blessed score		
	Hamilton score		
	Webster scale, Testing of memory, verbal skills,		
	visuospatial functions, executive functions		
Cardiovascular risk factors	Hachinski score		
Laboratory tests	CBC, ESR, K, Na, Ca, Mg, G, C, ALT, AST,		
	TSH, T ₄ , B ₁₂ , folate, UA		
Electrophysiology	EKG, EEG		
Imaging	CT, MRI		
Diagnosis	NINCDS-ADRDA*, DSM-III-R criteria**		

Table 3. Methods used for examination of the patients.

(MMSE=mini-mental state examination, CBC=complete blood cell count, ESR=erythrocyte sedimentation rate, K=potassium, Na=sodium, Ca=calcium, Mg=magnesium, G=glucose, C=creatine, ALT=alanine aminotransferase, AST=aspartate aminotransferase, TSH=thyroid stimulating hormone, T₄=thyroxine, B₁₂=cyanocobalamin, folate=folic acid, UA=urine analysis, MRI=magnetic resonance image, was performed using a 1.5T Magnetom (Siemens, Erlagen) by using the standard head coil and a tilted coronal 3D gradient echo sequence. This resulted in 128 T1-weighted partitions)

* McKhann et al. 1984, **American Psychiatric Association 1987

4.3. APO E GENOTYPING

10 ml samples of venous blood were collected in EDTA-tubes. DNA was extracted by the standard phenol-chloroform extraction (Isola et al. 1994). The ApoE genotypes were analyzed using polymerase chain reaction as described earlier (Hixon et al. 1990, Tsukamoto et al. 1993, Heinonen et al. 1995) with primers 5'-GCACGGCTGTCCAAGGAGCTGCAGGC-3'(forward) and 5'-GGCGCTCGCGGA TGGCGCTGAG-3'(reverse). The PCR products were digested and then analyzed through non-denaturing polyacrylamide gel. Electrophoresis was performed and separated DNA fragments were visualized through ethidium bromide staining.

4.4. AUTOPSY

The necropsy was performed at Kuopio University Hospital within 48 hours postmortem. The brains were removed, weighed, evaluated for grossly detectable lesions and vessel abnormalities. 4 % buffered formalin was used for fixation for at least one week. Later the brains were cut in coronal slices and each slice was examined for macroscopically detectable lesions. The specimens for histological evaluation were taken according to a standardized procedure from 15 different brain areas (Table 4). The brain specimens were embedded in paraffin and five µm thick sections were stained by applying hematoxylin-eosin (HE) and modified Bielschowsky silver (BS) impregnation.

Ν	Brain area		
1.	Frontal cortex=Brodmann 9		
2.	Temporal cortex=Brodmann 41		
3.	Gyrus cinguli=Brodmann 33		
4.	Parietal cortex=Brodmann 40		
5.	Precentral cortex=Brodmann 6		
6.	Occipital cortex=Brodmann 17		
7.	Hippocampus on the level of lateral geniculate body		
8.	Striatum including capsula interna anterior limb		
9.	Basal fore brain including basal nucleus of Meynert and amygdala		
10.	Thalamus including subthalamic nucleus		
11.	Midbrain including substantia nigra and oculomotor nucleus		
12.	Pons including locus ceruleus		
13.	Medulla including hypoglossal nucleus		
14.	Vermis including dentatus		
15.	Cerebellar cortex		

Table 4. Sampling of brain tissue.

4.5. HISTOPATHOLOGICAL EVALUATION

SP/NPs and NFTs were evaluated on BS stained sections taken from the frontal, temporal and parietal cortices and the hippocampus. The lesions were scored using the quantification methodology described by Mölsä et al. (1987) and also following the instructions published by CERAD for the histopathological classification of AD (Mirra et al. 1991).

As described by Mölsä et al. in (1987) the BS stained sections were evaluated under light microscopy at 100x magnification of the area (0.92mm²) in five randomly selected fields. The counts of SP/NPs and NFTs were graded as described in Table 5.

Score	Number of lesions in Bilschowsky silver stain
0	0
1	1-3
2	4-6
3	7-9
4	10-12
5	13-15
6	16-18
7	19-21
8	22-24
9	25-27
10	28-30

Table 5. Neocortical score of SP/NPs and NFTs (Mölsä et al. 1987). The results, the extent of lesions in individual subjects are given as the sum of scores in the frontal, temporal and parietal cortices.

All subjects were also classified into histopathological diagnostic groups according to CERAD (Mirra et al. 1991). In this classification, first the quantity of NPs (some, moderate and numerous) is related to the age of the subjects (see Table 6). This gives the age-related NP classes: A=uncertain, B=suggestive, C=indicative for the diagnosis of AD. In the second stage the age-related NP class is related to the patients clinical symptomatology (demented/not-demented) (Table 7) resulting in 3 categories of demented subjects namely those with possible, probable or definite AD.

*Numerous NP	С	С	С
*Moderate NP	С	С	В
*Some NP	С	В	А
*None NP	0	0	0
	<50 years	50-75 years	>75 years

Table 6. Age-related NP classes according to the CERAD criteria (Bielshowsky silver stain used): 0=no evidence of AD, A=uncertain evidence of AD, B=suggestive evidence of AD, C=certain evidence of AD

С	PosADb	DefAD
В	PosADb	ProAD
Α	normal-b	PosADa
0	normal-a	normal-c
Age-related NP classes	Not demented	Demented
(see table 6)		

•

Table 7. CERAD histopathological classes of AD. 0=no evidence of AD, A=uncertain evidence of AD, B=suggestive evidence of AD, C=certain evidence of AD.

4.6. IMMUNIHISTOCHEMSITRY AND TUNEL

Immunohistochemical staining and 3'-end labeling using the TUNEL detection method were carried out on the specimens from the frontal, parietal and temporal areas. Detailed labeling protocol is given on Table 8.
a) Immunohistochemistry

Primary antibodies:

1) monoclonal anti human major histocompatibility complex II HLA-DR (DAKO M775) at a dilution 1:100 in TBS

2) polyclonal anti cow glial fibrillary acidic protein, GFAP (DAKO Z0334) at a dilution 1:1000 in TBS

3) monoclonal anti human βA (DAKO M872) at a dilution 1:100 in TBS

4) monoclonal antihuman HP- τ , AT8 (Innogenetics BR 03) at a dilution 1:100 in TBS

Pretreatments:

1) HLA-DR - autoclaving in 0.01M citrate buffer (pH 6.0) at 121°C for 10 minutes

2) βA - 80% formic acid pretreatment at room temperature for 6 hours

Staining procedure:

1. mounting of 5 µm thick paraffin sections on 3-aminopropyl-trilthoxy-silane (Sigma chemicals) coated slides

- 2. deparaffinization
- 3. pretreatments

4. incubation with normal goat-serum (Histomark, no. 71-00-27) (room temperature for 10 minutes)

- 5. incubation with the primary antibody in the refrigerator overnight
- 6. TBS washes

7. incubation with biotinylated secondary antibody (Histomark, no. 71-00-37) for 60 minutes

- 8. TBS washes
- 9. streptavidin-alkalinephosphatase (Histomark, no. 71-00-45) incubation for 45 minutes
- 10. vector-Blue (Vector Labs, SK-5300) or Vector-Red (Vector Labs, SK-5100) for color development
- 11. mounting with crystal/mount (Biomeda corp. no. MO3) and coverslipping

b) 3' end labeling of the DNA using a modified TUNEL method

1. deparaffinization

2. incubation with proteinase K (Boehinger Mannheim GmbH, Mannheim, Germany)-at room temperature for 15 minutes

- 3. dH₂O washes
- 4. 3% hydrogen peroxide treatment
- 5. TBS wash
- 6. inqubation in equalibritium buffer for 30 minutes
- 7. TdT-enzyme incubation in the oven at 37°C for one hour
- 8. stop/wash buffer for 10 minutes
- 9. TBS wash
- 10. anti-digoksigenin-peroxidase incubation
- 11. TBS wash
- 12. diaminobenzidine (DAB)-hydrogen peroxide incubation

13. crystal/mount mount (Biomeda corp. no. MO3) and Depex (BDH, no. 361254D) and coverslipping

Table 8 a,b. The procedure used for IHC and 3' end labeling of the DNA using modified TUNEL method

4.7. MOPRPHOMETRICAL ANALYSIS

The quantification of HLA-DR and GFAP expression was performed using the Quantimet 570 Image Analysis System (Leica Cambridge Ltd, Cambridge, England). The HLA-DR and GFAP expression were estimated in five randomly selected fields. The subpial region and the borderzone between white and gray matter were excluded from the estimation. The quantification of GFAP and HLA-DR was carried out under light microscopy at 100x magnification per standard unit field (0.5 mm²). All labeled features were included for the measurement of the stained area of GFAP and HLA-DR. A size limitation (unit larger than 30 microns²) was used for cell counts, i.e. density of features. In additional to stained area, stained area fraction (stained area: 0.5 mm²) was used to describe the GFAP and HLA-DR expression.

The quantification of A β was carried out under light microscopy at 40x magnification using the NIH Image System for PCs. The A β staining was detected within the whole cortical thickness and the results were given as stained area fraction. The severity of CAA was evaluated in both the leptomeninges and brain parenchyma and graded on a four-stage scale from 0 to 3 (0-none, 1-some, 2-moderate, and 3-severe). Similarly, the PHF- τ expression, was scored on a four step scale from 0 to 3 (0-none, 1-some, 2-moderate or 3-extensive). In a case scored 1, occasional positively stained fibrils were noted, in a score of 2, several stained fibrils were noted with additional threads, and in a score of 3, numerous fibrils and threads were noted.

For DNA fragmentation the number of labeled cells was estimated under light microscopy at 40x magnification per standard unit field (0.5mm^2) in five fields. The fields were chosen within the areas where labeled cells were seen.

4.8. NSAID USE

The information on NSAID use was collected retrospectively from medical records. For inclusion in the NSAID non-user or regular-user group, strict criteria were applied: 1) the patient was hospitalized and a nurse gave the daily prescriptions; 2) the patient fulfilled the CERAD criteria for definite AD. Only the patients with a minimum 12 month's registered daily NSAID-use were considered as regular-users (the average exposure to NSAID's was much longer). The

indication for the daily dose of NSAID was mostly various musculo-skeletal pain conditions. The used drugs were ketoprophen, ibuprophen and naproxen (variable doses: 50 mg x 2, 200 mg x 3, 250 mg x 2). Precise evaluation of the effect of a used drug or dose of drug was not possible due to the retrospective nature of the reported study. The non-user group did not have any records of NSAID use. Occasional NSAID use was ruled out since the patients were hospitalized for at least 5 years.

4.9. STATISTICAL ANALYSIS

Applied statistical methods are given in table 9.

Study: -questions addressed in studies	PTS	Non/Dem (number of cases)	Methods	Statistics
I Astrogliosis and the apoE genotype studied astrogliosis in non-demented and demented the amount of astroglia in different apoE genotypes	All 95	22/73	IHC, morphometry	Student's t-test, multiple regression analysis, Pearson's correlation test
II Reactive microglia in aging and dementia the extent of microglia in aging and dementia the relationship between microgliosis and AD lesions	All 95	22/73	IHC, Morphometry	Student's t-test, multiple regression analysis, Pearson's correlation test
III DNA fragmentation, gliosis and histological hallmark lesions of AD the extent of DNA fragmentation correlation: DNA fragmentation and gliosis or AD lesions	24 of 95	0/24	IHC, TUNEL, Morphometry	Student's t-test, Pearson's correlation test
IV β-amyloid load, astroglia and and microglia in AD: association with apoE genotype apoE genotype and the β A load, the number of astroglia, microglia in cases with AD lesions	51 of 95	8/43	IHC, Morphometry	Student's t-test, Pearson's correlation test, multiple linear regression analysis
V Lower counts of astroglia and activated microglia in patients with AD patients with regular use of NSAID relationship between glia and NSAID use	42 of 95	0/42	Review of clinical records, IHC, Morphometry	Student's t-test, one-way ANOVA and multivariate analysis

Table 9. Material and methods used in studies I-V. PTS=the number of cases, Non/Dem=Non-demented/Demented, IHC=immunohistochemistry, TUNEL=method to detect DNA-fragmentation, NSAID=non-steroidal anti-inflammatory drug

5. RESULTS

5.1. DESCRIPTION OF THE STUDY-GROUP (I-V)

A total of 95 patients were investigated (22 non-demented and 73 demented subjects) The number of females/males (10/12 respectively) was almost equal in the non-demented group, whereas most of the demented subjects were females (61 females out of 73 cases). The age at death did not differ significantly between demented (85 ± 9) and non-demented subjects (78 ± 9). The clinical physical and clinical neurological examination in all cases was relatively normal. The cases with significant clinical findings had already been excluded from this study. The clinical data concerning the patients included in each separate study is given in Table 10.

5.2. DISTRIBUTION OF APO E GENOTYPE (I,II)

The ApoE ε 4 allele was found in 58 % of demented cases (42 out of 73) and in 32 % of the non-demented subjects (7 out of 22) (p<0.05) (Table 10). The prevalence of the ApoE ε 4 allele did not differ significantly between sporadic (51 cases) and familial (22 cases) AD subjects (55% vs. 56% respectively) or between subjects with presenile (12 cases) or senile (61 cases) (67% vs. 56% respectively) AD.

The subjects with ApoE $\varepsilon 4/\varepsilon 4$ had significantly lower age at on set and age at death when compared to the group without ApoE $\varepsilon 4$ (p<0.01 and p<0.05 respectively). The duration of the disease did not differ when subjects with different Apo E genotypes were compared.

Study	Patient groups	F/M (number of cases)	Age at death (years)	Duration (years)	ApoE -ε4/+ε4 (number of cases)
I Astrogliosis and the apoE genotype	Non	10/12	78 <u>+</u> 9	-	15/ 7*
	Dem	61/12	84 <u>+</u> 9	10 <u>+</u> 5	31/42*
II Reactive microglia in aging and dementia	Non	10/12	78 <u>+</u> 9	-	15/ 7*
	Dem	61/12	84 <u>+</u> 9	10 <u>+</u> 5	31/42*
III DNA fragmentation, gliosis and histological	PrAD	5/1	75 <u>+</u> 5	10 <u>+ 6</u>	2/ 4*
hallmarks of AD	DefAD	18/0	83 <u>+</u> 5	10 <u>+</u> 7	4/14*
IV β-amyloid load, astroglia and and microglia in	Non	3/5	83 <u>+</u> 3	-	4/4*
AD: association with the apoE genotype	Dem	34/9	84 <u>+</u> 9	11 <u>+</u> 4	18/25*
V Astroglia and activate microglia in AD patients	- NSAID	16/6	83 <u>+</u> 10	11 + 3	6/16
with and without regular use of NSAIDs	+ NSAID	19/ 1	80 <u>+</u> 8	8 <u>+ 5</u>	6/14

Table 10. Clinical description of the patients. Non/Dem=non-demented/demented, F=female, M=male, Pr=probable AD, Def=definitive AD, -NSAID=no NSAID use, +NSAID=regular daily NSAID use, mean +/- SD, *significant difference in the prevalence of the ApoE ε 4 allele between non-demented and demented cases: p<0.05.

5.3. THE AD HALLMARK LESIONS

AD related lesions were common in the whole material (Table 11). SP/NPs were seen in 55 % of non-demented and in 100 % of demented subjects. In 32 % of the non-demented subjects the extent of lesions was sufficient to fulfill the histopathological diagnosis of posADb. In the demented group 7 % fulfilled the histopathological diagnosis of posADa, 18 % for proAD and 75% for defAD.

The number of SP/NPs and NFTs was significantly higher in the demented group when compared to the non-demented cases (p<0.001 for both).

The amount of SP/NPs as well as the amount of NFTs was highest in the demented with ApoE ϵ 4 allele (significance for both SP/NPs and NFTs p<0.01).

5.4. REACTIVE ASTROCYTES (RA)

Aging did not correlate with GFAP expression in non-demented subjects (n=22). Furthermore, the ApoE ϵ 4 allele (7 out of 22) was not associated with GFAP expression in non-demented cases (Table 11). The non-demented cases with SP/NPs (12 out of 22) showed significant correlation between the GFAP expression and SP/NP scores (r=0.8, p<0.05). However, this correlation seemed to be influenced by the presence of ApoE ϵ 4. The correlation was significant only in cases without ApoE ϵ 4 (r=0.8; p<0.01).

GFAP expression was significantly higher in the demented (73 cases) subjects compared to controls (22 cases) in all analyzed cortical regions (parietal p<0.002, temporal p<0.02 and frontal p<0.005 cortices) (Table 11). In the demented patients the GFAP expression was more intensive in ApoE ϵ 4 allele carriers (42 out of 73 cases) compared to non carriers (p<0.05). The cases with defAD diagnosis (55 cases out of 73) showed significant correlation between GFAP expression and the duration of the disease (r=0.45, p<0.05). This correlation was seen in the subgroups with and without the ApoE ϵ 4 allele (r=0.4, p<0.05; r=0.6, p<0.01 respectively). The GFAP expression also correlated with the NFT score in the demented group (r=0.5, p<0.01) (Table 12). The correlation was stronger for cases lacking the ApoE ϵ 4 allele compared to those carrying the ApoE ϵ 4 allele (r=0.5;p<0.01 and r=0.3;p<0.05

GFAP expression was not influenced by the age at onset, familial clustering, gender or vascular lesions.

	Patient groups:	Neurofibrillary tangle count	Senile/neuritic plaque count	Aβ protein load	The number of TUNEL-	The number of reactive astrocytes	The number of activated microglia
					labeled cells		
Ι	Non (ApoE-ε4/+ε4)	0.8 <u>+</u> 2.1*/2.3 <u>+</u> 3.9*	9.8 <u>+</u> 7.0 /15.3 <u>+</u> 3.4	-	-	55.4 <u>+</u> 49.7/ 65.3 <u>+</u> 10.2* ^b	-
	Dem (ApoE-ε4/+ε4)	9.1 <u>+</u> 8.8*/16.2 <u>+</u> 9.3*	17.5 <u>+</u> 8.9*/22.7 <u>+</u> 6.6*	-	-	80.4 <u>+</u> 85.5/113.8 <u>+</u> 105.1* ^b	-
Π	Non	0.7 <u>+</u> 2.1*	6.3 <u>+</u> 7.5*	-	-	-	58.4+46.5 ^b /151.7 <u>+</u> 130.5 ^b
	Dem	13.1 <u>+</u> 9.1*	20.5 <u>+</u> 8.0*	-	-	-	69.3+53.3 ^b /168.8 <u>+</u> 121.9 ^b
III	ProAD	1.3 <u>+</u> 0.4**	3.7 <u>+</u> 0.6**	6.5 ± 1.90^{b}	2.3 <u>+</u> 1.1**	0.6 ± 0.2^{a}	1.10 ± 0.50^{a}
	DefAD	6.6 <u>+</u> 0.8**	7.2 <u>+</u> 0.6**	7.9 <u>+</u> 1.10 ^b	20.0 <u>+</u> 3.5**	1.1 ± 0.2^{a}	1.00 ± 0.20^{a}
IV	 ΑpoE ε4 	7.7 <u>+</u> 9.9	17.9 <u>+</u> 9.4*	0.04 ± 0.03^{a}	-	0.81 ± 0.84^{a}	1.01 ± 1.42^{a}
	+ ApoE ε4	14.5 <u>+</u> 9.5	22.8 <u>+</u> 6.2*	0.05 ± 0.03^{a}	-	1.28 <u>+</u> 1.21 ^a	1.14 <u>+</u> 0.95 ^a
V	- NSAID	16.5 <u>+</u> 8.6	-	6.0 ± 2.50^{b}	-	82.5 <u>+</u> 69.7*	69.3 <u>+</u> 47.0 ^b
	+ NSAID	16.0 <u>+</u> 9.2	-	7.5 <u>+</u> 3.60 ^b	-	47.7 <u>+</u> 36.3*	56.1 <u>+</u> 45.5 ^b

Table 11. Summary of histopathological findings in studies I-V. S=studies

Non/Dem=non-demented/demented, proAD=probable AD, defAD=definite AD, -NSAID=no NSAID use, +NSAID=regular daily NSAID use, TUNEL=method to detect DNA-fragmentation, -=not studied, ^a=stained area fraction used to describe the expression, ^b= cell count in the neocortex used

TUNEL=method to detect DNA-fragmentation, -=not studied, ^a=stained area fraction used to describe the expression, ^b= cell count in the neocortex used to describe the expression (in study II activated microglia are given in the gray matter/white matter)

Significant differences *p<0.05, ** p<0.01, ***p<0.001

S:	The number of reactive astrocytes (RA)	The number of activated microglia (AM)	The number of TUNEL-labeled cells	Amyloid β protein load (Aβ)	Plaque count (SP/NP)	Tangle count (NFT)
Ι	Dem>non** Dem: ApoE ε4> ApoE εx*	ND	ND	ND	Significant correlation with RA*	Significant correlation with RA**
II	ND	No difference: Dem/non No correlation with aging Correlation with duration*	ND	ND	No correlation with AM	Significant correlation with NFT**
III	No correlation with the number of TUNEL- labelled cells	No correlation with the number of TUNEL-labelled cells	defAD>proAD **	No correlation with the number of TUNEL labelled cells	Significant correlation with the number TUNEL labelled cells *	Significant correlation with the number TUNEL labelled cells**
IV	No difference ApoE εx vs ApoE ε4	No difference ApoE εx vs ApoE ε4	ND	No difference ApoE ɛx vs ApoE ɛ4 Significant correlation with SP/NP,NFT,RA(**all)	ApoE ε4> ApoE εx* Significant correlation with Aβ**&NFT**	Significant correlation with Aβ**, SP/NP**, RA** and AM**
V	+NSAID<-NSAID**	No difference: +NSAID vs -NSAID	ND	Cases with ApoE ɛx/4: +NSAID>-NSAID*	ND	No significant difference +NSAID vs NSAID

Table 12. Summary of main results (S=Studies I-V): correlation between major AD lesions in different patient groups. Non/Dem=non-demented/demented, TUNEL=method to detect DNA-fragmentation, -NSAID=no NSAID use, +NSAID=regular daily NSAID use, defAD=definite AD, proAD=probable AD, -=not studied. Significancies: p<0.05*, p<0.01***, p<0.001***

5.5. ACTIVATED MICROGLIA (AM) (II)

The HLA-DR expression was slightly higher in demented subjects compared to nondemented (Table 11), but the difference was not statistically significant. Some nondemented subjects displayed high expression of HLA-DR without any significant AD changes and some subjects with numerous AD lesions showed hardly any HLA-DR expression.

The age at death did not correlate directly with the HLA-DR expression. However, subjects over 75 years of age had higher mean values of HLA-DR expression in the gray matter compared to younger ones. This was seen in both nondemented (p<0.01) and demented subjects (p<0.05). The level of HLA-DR expression also varied according to the gender. The non-demented males showed higher HLA-DR expression than the females (p<0.05) whereas in the demented subjects higher expression was found in females compared to males (p<0.05). However, the difference was significant only in the white matter (p<0.05).

There was a significant correlation between HLA-DR expression and the duration of the disease in the demented females (61 out of 73 cases) (r=0.3; p<0.05). Furthermore, it was noted that the ApoE genotype, familial clustering and the age at onset had an influence on this correlation in subjects with defAD. The correlation between the duration of the disease and the HLA-DR expression was significant in cases lacking the ApoEɛ4 allele (r=0.8; p<0.01), in cases with sporadic disease (r=0.4; p<0.01) and in subjects with senile onset of the disease (r=0.4; p<0.01).

No correlation was found between the SP/NP and the AM in demented or non-demented subjects, whereas the NFT counts correlated significantly with HLA-DR expression in demented cases (r=0.4; p<0.01) (Table 12). This correlation was, however, influenced by the ApoE ε 4 genotype, the age at onset and familial clustering. The correlation was significant only in patients not carrying the ApoE ε 4 allele (r=0.5; p<0.01), in senile cases (r=0.3; p<0.05) and in patients with sporadic disease (r=0.3; 0.05).

5.6. TUNEL-LABELED CELLS (III)

For methodological reasons only cases with a post-mortem delay of 9 hours or less were included (24 cases). The mean age at onset of the disease was 72 ± 10 years and the age at death 83 ± 8 . Most of the cases died of cardiovascular dysfunction.

The definite AD cases (n=18) showed significantly more SP/NPs, NFT and TUNEL labeled cells than probable AD cases (n=6) (p<0.01 for all changes) (Table 11) and the highest number of SP/NPs as well as NFTs were found in cases with two ApoE ϵ 4 alleles (p<0.05 in both changes).

The SP/NP score correlated significantly with the NFT score (r=0.8, p<0.01) and there was also a significant correlation between SP/NP and β A load (r=0.6, p<0.01). There was a significant correlation between TUNEL-labeled cells and both SP/NPs and NFTs (r=0.5, p<0.05 and r=0.6, p<0.001 respectively) (table 12).

The correlation between the counts of TUNEL labeled cells and AD lesions was influenced by the ApoE genotype. Significant correlation was found between the TUNEL labeled cells and SP/NP's in subjects not carrying the ApoE ε 4 allele (r=0.9; p<0.01) whereas the correlation between TUNEL labeled cells and NFT's was significant only in patients with the ApoE ε 4 allele (r=0.6; p<0.01).

There was no correlation between the TUNEL-labeled cells, βA load, AM or RA.

5.7. βA LOAD AND GLIA (IV)

The ApoE ε 4 allele caused a slight but not significant increase in the β a load and the GFAP and HLA DR expression in 43 demented subjects with neuropathologically verified AD without any NSAID use. β A load correlated significantly with the GFAP expression (r=0.5, p<0.01) and the counts of NFT's correlated significantly with the HLA DR expression (r=0.4, p<0.01). The correlation between NFT and AM was influenced by the ApoE genotype since it was significant only in patients without the ApoE ε 4 allele (r=0.5; p<0.01).

5.8. NSAID (V)

Twenty-two patients without regular NSAID use and 20 patients on regular NSAID treatment were investigated. The used compounds (ketoprofen, ibuprofen, naproxen) and the daily doses varied (from 50mg to 250 mg twice daily).

The main cause of death was bronchopneumonia (60%) in patients not receiving NSAIDs and myocardial infarction (55%) in patients on NSAID medication. The duration of the disease was significantly shorter in patients receiving regular NSAID treatment (p<0.05).

The NFT, AM and RA counts were lower in subjects on regular NSAID treatment when compared to non-users. The difference was significant only for RA (p<0.05). Surprisingly the regular NSAID users with the ApoE $\epsilon x/4$ genotype had a higher βA load than the cases without any NSAID use (p<0.05).

6. DISCUSSION

6.1. METHODOLOGICAL CONSIDERATIONS

6.1.1 Subjects

The brain material used in this study came from the Kuopio Brain Bank, which was established for dementia research. Since the number of younger subjects was limited reliable conclusions concerning the gliosis can be made only for the older age group (subjects older than 75 years). The results of this study are also influenced by the gender distribution. Most of the cases were women. The dominance of females in the group of demented subjects is however in line with the fact that most of demented patients are females (Jorm et al. 1987).

The significantly higher frequency of the ApoE ε 4 allele in the demented group (I,II) is comparable with previous results showing that independent of racial group ApoE ε 4 allele frequency is higher in AD patients compared to age-matched controls (Garry 2001, Farrer et al. 1997).

6.1.2 Methodology

The methods used in this study included routine histological staining, immunohistochemistry (IHC) and the TUNEL method. The fixation time and the paraffin embedding are known to influence the staining results both in IHC and TUNEL methodology. Specifically the GFAP and HLA DR labeling are known to be sensitive. In order to minimize the variability in the staining results the fixation time was standardized as well as the procedure of paraffin embedding. In order to optimize the staining results various pretreatment strategies were used including autoclaving and incubation in formic acid. All staining was carried out manually, the staining procedures were standardized and control sections were always used. In general the IHC staining results were even in intensity and the background staining was close to zero making the morphometric analysis possible. The morphometric methodology was used in order to minimize the variability in the results evaluating the extent of staining in up to 285 GFAP, 285 HLA DR and 285 β A stained sections.

The TUNEL technique labels DNA breaks not only in apoptotic cells but also in necrotic cells (Wijsman et al. 1993, Thomas et al. 1995). It has been suggested that some labeling is a results of the postmortem delay and tissue preparation procedures such as fixation and paraffin embedding. In order to avoid over interpretation of the staining only those cells with strong nuclear staining were evaluated as labeled.

6.2. THE HALLMARK LESIONS OF AD

These results (I,II) support previous studies demonstrating that AD lesions are also frequently seen in cognitively unimpaired elderly individuals (Dickson 1988, Braak & Braak 1991, Langui et al. 1995). Half of the non-demented cases (55 %) showed SP/NPs and 32% of the cases showed lesions sufficient to fulfill the histopathological CERAD criteria for possible ADb (I,II). Aa early as 1988 Crystal et al. showed that the SP/NP count did not distinguish well between demented and non-demented subjects. They suggested that non-demented subjects with SP/NP might be considered as AD patients in the "preclinical/presymptomatic" stage of the disease. On the other hand they also suggested that numerous cortical SP/NPs might be seen in some elderly subjects who would never develop clinical signs of dementia. Some of the clinically non-demented subjects with SP/NPs also displayed some neocortical NFT's (see Table 11). The finding of NFT's in the neocortex is highly alarming. These subjects with NFTs, fulfilling the Braak & Braak criteria for the isocortical stage of AD and the NIA criteria for high likelihood of AD, should be considered as AD patients in their pre-symptomatic stage of the disease. Gold et al. (2001) showed that the criteria based on evaluation of the SP/NP count i.e. the CERAD criteria, are insufficient to distinguish mild cognitive changes from dementia when compared to criteria based on the HP- τ load i.e. the NFT count based on the Braak & Braak criteria.

The ApoE ε 4 allele favors amyloid deposition and tangle formation. In line with results from the present study it has previously been reported that ApoE ε 4 allele is associated with more pronounced neurodegeneration (Nagy et al. 1995, Polvikoski et al. 1995). The isoform ApoE ε 4 is more efficient in enhancing amyloid formation in cell cultures (Castano et al. 1995) and may act as a chaperone. However this allele can not be used as a predictor of a possible disease later in the subjects life.

6.3. ASTROCYTES

Aging is associated with astrogliosis (Beach et al. 1989, Hansen et al. 1987), i.e. an increase in the size of GFAP expressing cells. Some experimental studies suggest

that the also the number of GFAP positive cells increases with the age. Hansen et al. (1987) suggested that astrogliosis may correlate with the development of fibrous astrocytes from protoplasmic forms. Age related gliosis is still speculative and the many different reasons have been suggested (e.g. neuronal shrinkage, neuronal depopulation). Astrocytic responses to age-related changes in cortical neurons are under continuous study.

The immunohistochemical study by Arnold et al. 1996 showed that the correlation between age and GFAP positive expression can vary in different parts of cortical areas. They detected a positive correlation only in the visual cortex.

The GFAP expression did not vary significantly between men and women either in AD cases or in non-demented cases. The non-demented subjects in this study did not show a correlation between aging and GFAP expression in the neocortex. However this correlation was different in the pre-elderly cases and elderly cases. Pre-elderly cases (up to 75 years at death), showed a slight correlation between age and GFAP positive cells (r=0.4; p<0.05). This finding indicates a possible age related astrogliosis. No correlation between GFAP positive cells and aging was seen in the elderly subjects (75 years and older). This might be due to changes in the astrocytic function in very old age. The astrocytes in the oldest subjects might have lost some functional properties. Cell culture studies have already shown that there are differences in the function of old and young astrocytes. In addition, Beach et al. (1989) showed in their immunohistochemical study that there were differences in the GFAP immunoreactivity between the pre-elderly and elderly. The GFAP immunoreactivity was diffuse and strong in pre-elderly subjects whereas in the elderly subjects (over 73 years) the immunoreactivity appeared to be slightly decreased but also localized differently.

Several groups studying GFAP mRNA expression have reported that GFAP mRNA correlates with aging (Delacourte 1990, Harpin et al. 1990, Nichols et al. 1993, David et al. 1994). Nichols et al. (1993) suggested that astrogliosis with age may result from altered transcriptional regulation and that the basis for increased GFAP mRNA expression during aging could be due to many humoral and ionic changes. Their study also reported differences in mRNA levels in brain homogenates between pre-elderly patients (42 ± 2) and elderly patients (70 ± 1 years) (Nichols et al.

1993). They concluded that the GFAP mRNA was seen especially after 60 years of age.

The results reported here support the studies showing that astrogliosis is one characteristic feature of AD (Beach et al. 1986, Dickson et al. 1988, Brun et al. 1995, Arnold et al. 1996). Astrogliosis was associated with the duration of dementia in this study and similar results are shown by Renkawek et al (1994). It is not completely clear whether or not astrocytes are directly involved in the neurodegeneration in dementia or only responding to the primary insult in dementia i.e. βA , HP- τ or anoxia/ischemia (Suenaga et al. 1994, Tomimoto et al. 1996). It has been suggested that βA might stimulate astroglia to become reactive (Pike et al. 1994). GFAP labeling correlated with the βA load in the present study. This indicates that the increase in the β A load is concomitant with an increase in RA count. Employing IHC methodology it is however impossible to estimate whether the deposition of βA is followed by astrocytosis or vice versa. No definite clarity of the order of event has been reached. Histologically, the astrocytes are seen in the surrounds of the βA deposits making the interpretation of astrocytosis as a response to the βA deposits highly persuative. The astrocytosis might be an appropriate response to the accumulation of the pathologic protein βA . However, the RA surrounding the plaque might still have a deleterious effect as once they are in an active state, they may deposits large amounts of molecules that are inhibitory to neurite outgrowth (Nieto-Sampedro 1999.). The other mechanism leading to neurodegeneration is that amyloid can impair the astrocytic glutamate uptake system in the early stages of the disease. The glutamate can accumulate at the synapses and reach an exitotoxic level with a consequent degeneration (Fredricsson 1991).

Previous studies have shown a positive correlation between GFAP levels, and densities of NFTs, SP/NPs and β A aggregates (Harpin et al. 1990) but this correlation has been shown to display variability both in respect to the brain region (Le Prince et al. 1993) and duration of the disease (Pike et al. 1995). When the GFAP labeling was compared with the β A, SP/NP or NFT in demented subjects with numerous lesions (proAD & defAD), a significant positive correlation was detected and this correlation was found to be modified by the ApoE genotype. The results of this study indicate that astrogliosis is proportional to the AD hallmark lesions in cases without the ApoE ϵ 4 allele, whereas neurodegeneration and astrogliosis are

independent of each other i.e. out of control, in the cases carrying the ApoE ε 4 allele. The ApoE genotype dependent astroglial reaction was also proposed in a experimental set of studies by Vincent et al. (2001) who concluded that astrocytes strongly regulate neuronal APP expression and that astrocytes promote the amyloidogenic pathway in an ApoE ε 4 dependent manner. Thus this human immunohistochemical study suggests that the mode of modulation of the pathogenesis of AD by the ApoE genotype might be via the function of astrocytes.

6.4. MICROGLIA

Activated microglia (AM) might play an important role in the progression of AD because of their capability to synthesize cytokines and other inflammatory mediators. The close association of AM with β A aggregates (Fukumoto et al. 1996), the clear increase of AM in the AD cortex (Carpenter et al. 1993) and parallel increase of AM and NFT's (DiPatre & Gelman 1997) indicates that AM are likely to play a role in the pathogenesis of AD.

This study shows that AM increases with age in non-demented subjects as has been shown earlier (Rogers et al. 1988, Mattiace et al. 1990, Sasaki et al. 1992, Perlmutter et al. 1993, DiPatre & Gelman 1997). The aged non-demented subjects (age at death > 75 years) showed a higher number of AM in the gray matter when compared to younger subjects (unpublished data). Comparable results have been reported by DiPatre & Gelman (1997). However, this phenomenon decreased towards the very old age (no concomitant AD pathology was seen). The increase in the HLA-DR expression with aging and the reduction of this up-regulation in the very old might indicate that there is a decrease in the general inflammatory response in very old individuals.

Microglia are contiguous with dense amyloid deposits in the brain. In 1995 it was shown in cell culture that microglia can be activated by βA (Meda et al. 1995) and even by APP (Barger & Harmon 1997). Moreover, Muehlhauser et al. (2001) showed that the fibrillary βA (insoluble), but not the non-fibrillary βA (soluble) peptide induced microglial activation in vivo. The present IHC analysis of human brain tissue, however did not indicate a significant correlation between the AM and the insoluble aggregated βA load as was also described by Rozemuller et al. (1989). It has been suggested that the HLA DR expression is associated with SP/NPs (Rogers

et al. 1988, Grundke-Iqbal et al. 1991) rather than with the diffuse plaques i.e. β A deposits. DiPatre and Gelman (1997) reported an association between AM and NFT but not between AM and SP/NP load which is in line with this study. Interestingly this correlation was significant only in cases without ApoE ϵ 4 once again indicating that the ApoE genotype might modulate the function of glia in AD. The positive correlation between neuronal degeneration i.e. NFT's and AM is in line with a study by Sheffield et al. (2000).

The non-demented males in this study (no AD lesions) showed significantly higher HLA DR expression in the white matter compared to the females. Contrary to this, in AD cases in both in white and gray matter, AM were significantly more numerous in females than in males. The influence of gender on AM might be due to the regulation of the immune response by sex hormones (Cutolo et al 1995a). These findings cannot be explained by this histological study but imply that more research is needed on hormonal influence and AM function. This study also emphasize that, one should note the distribution of females and males when interpreting results from histopathological studies concerning AM.

This study shows that the HLA-DR expression increased with the duration of the disease. This is in line with the increase in GFAP expressions and the hallmark lesions of AD with the duration of the disease. The HLA-DR expression correlated with the NFT counts in sporadic and senile cases but not in the familial or presenile cases. In familial cases this correlation might be distorted by unknown genetic regulation in the progression of the pathology of the disease whereas, the difference found between senile and presenile cases might be related to various immunological response in middle age and senility.

In 1998 Egensperger et al. reported a significant increase in AM in AD with increasing the copy numbers of the ApoE ε 4 allele. The subjects with two ApoE ε 4 alleles had the highest HLA-DR expression but the differences were not significant. The discrepant results might be due to the different sizes of the studied populations, to different distribution of genders or different distribution of the allele. The ApoE ε 4 allele influence on microglial activation can also be dependent on the localization. Saitoh et al. (1997) showed that only the scattered microglia were influenced by the ApoE ε 4. Scattered microglia are known to represent a supply for clustered microglial cells that localize closely around the plaques.

Moreover, a common phenomenon noted here was that AM varied extensively between one AD subjects and another. Large numbers of AM were seen in both subjects with few and those with numerous SP/NP's. One explanation might be that there is cell death in cases with extensive AD changes and microglial cells die as well.

In line with other studies, this study indicates that the activation and the function of microglia, the inflammatory mononuclear cells of the CNS, are influenced by many factors. These findings indicate an association between AM and AD hallmark lesions in patients with senile onset of the disease, female gender and not carrying the ApoE ϵ 4 allele. In a subject of male gender or a disease of presenile onset or carrying the ApoE ϵ 4 allele the prediction of microglial activation based on the AD related pathology is not possible.

6.5. APOPTOSIS

Apoptotic cell death can be triggered in cell culture by external signals like βA peptide (Loo et al. 1993, Paradis et al. 1996, Gschwind et al. 1996). In 1998 Sheng et al. reported an association between the number of βA deposits and TUNEL positive cells. Contrary to this Lassmann et al. (1995) studying apoptosis in AD, found no correlation between βA deposits and apoptotic cells comparable to the present findings. These findings imply that the cells labeled with TUNEL might also be partly necrotic cells. Behl et al. (1993) suggested that βA induces necrosis rather than apoptosis. This study showed no significant correlation between TUNEL labeled cells and βA load even though the extent of DNA fragmentation seemed to increase with βA load.

Price et al. (2001) suggested in their study that there is little or no neuronal loss in aging or preclinical AD but already substantial neuronal loss in very mild AD. They argued that clinical deficits in AD are a result of a significant neuronal loss.

It has been mentioned above that the extent of the hallmark lesions of AD is influenced by the ApoE genotype. Comparable to this a higher number of TUNEL labeled cells were found in ApoE ε 4 carriers (Nagy et al. 1995). This study showed a similar but not significant phenomenon. There was, however, considerable variation between individual cases. Some cases with extensive β A deposition exhibited few TUNEL-labeled cells. Lassmann et al. (1995) produced similar findings in their study. This might be explained by the fact that only a low number of cells undergo cell death at a given time or that many cells have already died for example for apoptosis. Furthermore, other forms of cell death than apoptosis might occur in AD.

A significantly higher number of TUNEL labeled cells were found in patients with numerous AD lesions (definite AD) compared to those with a moderate number of lesions. Parallel results indicating a correlation between NFT counts and apoptotic cells (Lassmann et al. 1995, Bobinski et al. 1997) have previously been reported. A possible explanation for this correlation was proposed by Sheng et al. (1998) who speculated that NFT formation was partly responsible for DNA fragmentation and neuronal injury in AD. Apart from βA , apoptosis can also be triggered by different cytokines released by AM or RA. However, no correlation was found between glia and TUNEL-labeled cells in the present study.

6.6 NSAID

The very first indications that NSAID treatment could be of use in AD came from epidemiological studies when a lower risk of dementia was found in patients with rheumatoid arthritis compared to controls (McGeer et al. 1992). In 1998 experimental studies indicated that microglial activation by βA was modified by NSAID (Netland et al. 1998) indicating an effect between βA protein, microglia and NSAID's.

Regular NSAID use was associated with a significantly lower number of RA, a trend towards a lower number of AM and a significantly higher load of β A in the brain tissue when 42 clinically and histopathologically defined AD cases were analyzed. The NFT counts were only slightly lower in NSAID users than in nonusers. The influence of NSAID was noted in all ApoE genotypes and in patients with the ϵ 4/4 allele the results were closest to being statistically significant. These results suggest that if NSAID proves to be effective in treating AD in the future, the mechanism may be through the suppression of glial activity.

This study indicates that some patients with AD hallmark lesions might benefit from NSAID use. Especially in a patient carrying the ApoE ϵ 4 allele who receives the treatment at an early stage the suppression of the microglial and astroglial activation by NSAIDs might slowe the progression of the disease.

7. CONCLUSIONS

This study carried out on postmortem human brain tissue employing immunohistochemical methodology indicates that glia may modify the Alzheimer's disease (AD) pathology. The central findings are:

1) The number of reactive astrocytes (RA) was significantly higher in AD patients when compared to age matched controls (p<0.05). Furthermore, the number of RA as well the extent of AD hallmark lesions were significantly higher in AD cases with the ApoE ε 4 allele compared to the ones without this deleterious allele (p<0.05). The correlation between RA and neurofibrillary tangles (NFTs) was modified by the ApoE genotype being stronger for the cases without the ApoE ε 4 allele (r=0.6, p<0.01).

2) The number of activated microglia (AM) was altered by the gender (female > male; p<0.05) and age of the subject (highest in subjects with age at death > 75 years; p<0.05) in AD cases. A correlation was found between AM and NFTs that was modified by gender, the ApoE genotype, familiality and the age at onset of the disease. The correlation between AM and NFTs existed only for females (R=0.4; p<0.01), cases without the ApoE ϵ 4 allele (r=0.5; p<0.01), sporadic cases (r=0.3; p<0.05).

3) The intensity of TUNEL-labeled cells in AD was related to the extent of the hallmark lesions of AD (SP/NP: r=0.5, p<0.05, NFT: r=0.6, p<0.001). Contrary to this the intensity of apoptosis did not correlate with the load of the insoluble β A protein and was not modified by the ApoE genotype.

4) In subjects in the terminal stage of AD the extent of RA correlated with the extent of β A load (r=0.5, p<0.01) and the extent of AM correlated with the NFT counts (r=0.4, p<0.01). Furthermore, the correlations between AM and NFTs were modified by the ApoE genotype and gender. The correlation was strongest for cases without ApoE ϵ 4 (r=0.5; p<0.01) and females (r=0.5; p<0.05).

5) In subjects in the terminal stage of AD the regular NSAID use reduced the expected the number of RA (p<0.05). The amount of microglia and NFTs was also slightly lower in cases on regular NSAID treatment. The effect was noted in subjects with various ApoE genotypes, but the effect was most notable in subjects with the ApoE ϵ 4/4 genotype.

These findings suggesting that glia modifies the brain pathology in AD may open new avenues for possible pharmacological treatment of the disease.

The present study shows there is no doubt that inflammatory mechanisms are an important factor in AD pathogenesis. A relationship is seen between astrocytes and βA protein but also between microglia and NFT's. An interesting observation was that the relationships above were modified not only by the ApoE genotype but also by the gender and the age of the patient.

These findings indicate that subjects with AD form a heterogeneous population. One AD patient might benefit from an anti-inflammatory or an anti-apoptotic drug, whereas in another patient no effect can be detected at the same stage of the disease. On the basis of this histological study, the subjects chosen for the clinical drug trials should be carefully selected and categorized.

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APPENDIX Original publications I-V

Ι

Astrogliosis and the ApoE genotype.

An immunohistochemical study of postmortem

human brain tissue.

Overmyer M, Helisalmi S, Soininen H, Laakso M, Riekkinen Sr.P,

Alafuzoff I. Dement Geriatr Cogn Disord 1999;10: 252-257

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Π

Reactive microglia in aging and dementia:

an immunohistochemical study of postmortem human brain tissue.

Overmyer M, Helisalmi S, Soininen H, Laakso M, Riekkinen Sr.P,

Alafuzoff I. Acta Neuropath 1999; 97: 383-392

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III

DNA fragmentation, gliosis and histological hallmarks

of Alzheimer's disease.

Overmyer M, Krazpulski M, Helisalmi S, Soininen H, Alafuzoff I.

Acta Neuropathol 2000;100:681-687

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IV

β-amyloid load, astroglia and microglia in Alzheimer's disease: association with ApoE genotype.

Alafuzoff I, Overmyer M, Helisalmi S, Riekkinen Sr. P, Soininen H. Alzheimer's Reports 1999;2: 283-289.

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V

Lower counts of astroglia and activated microglia in patients with Alzheimer's disease with regular use of nonsteroidal anti-inflammatory drugs

Alafuzoff I, Overmyer M, Helisalmi S, Soininen H. JAD 2000;2:37-46

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PUBLICATIONS

SERIES OF REPORTS, DEPARTMENT OF NEUROLOGY

- **1. Juhani Partanen (1978):** Time-locked phenomena of human motor unit potentials. An electromyographic study of satellites and doubles.
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