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Implications of cholesterol and cholesterol-lowering therapy in Alzheimer's disease

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To my children with love

The beginning of all science is wondering why things are the way they are.

All vetenskaps början är förvåningen över att tingen är som de är.

— Aristotle

Abstract

BACKGROUND: Alzheimer's disease (AD) is a severe neurodegenerative disease that mainly afflicts elderly persons, with a characteristic progressive decline of cognitive functions and dementia. It is believed that the majority of all AD patients are affected by the sporadic form, thus caused by the combined effects of several risk factors, such as elevated cholesterol levels in midlife and deficiencies in the lipoprotein transporters apolipoprotein E (ApoE). Cholesterol play an essential role in the central nervous system (CNS) were it maintain normal physiological conditions, and is a requirement for the ability of neurons to communicate. It has previously been demonstrated that the cholesterol homeostasis in the CNS is maintained by a slow conversion of brain cholesterol into 24(S)-hydroxycholesterol, with a net flux of 27-hydroxycholesterol from the circulation into the brain. The importance of a preserved cholesterol homeostasis has been supported by the observation that dietary cholesterol may induce an inflammation in the CNS, and increase the levels of pro-inflammatory mediators. It has, in direct association with these observations, been demonstrated that plasma levels of pro-inflammatory proteins, such as interleukin-6, are increased before the clinical onset of AD, after which a chronic state of inflammation often is found. Accordingly, it has been suggested that memory and cognitive functions could benefit from a cholesterol-lowering therapy. **STUDIES:** The research presented in this thesis has examined the effect of cholesterol and cholesterol-lowering therapy with rosuvastatin, on key factors associated with AD. The experimental setup consisted of in vitro-models with human neuroblastoma SH-SY5Y cells (paper I–II), and in vivo-models with wild type (WT) and ApoE knockout (ApoE-/-) mice, provided with high levels of dietary cholesterol for 18 weeks (paper III–IV). The hydrophilic compound rosuvastatin is an effective inhibitor of cholesterol synthesis and has been demonstrated to be an effective treatment for hypercholesterolemia, with an indirect effect on microglial activation and inflammation through isoprenoid depletion. **RESULTS:** Paper I describes the selective non-amyloidogenic processing (α-secretase activity) of the amyloid precursor protein (APP), induced by 24(S)-hydroxycholesterol, with a subsequent increased ratio of α-/β-secretase activity, and increased levels of soluble APPα and total soluble sAPP; effects significantly decreased by the presence of 27-hydroxycholesterol. Paper II describes the effect of pre-treating in vitro-cultures with rosuvastatin prior exposure to Aβ oligomers. The treatment was found to decrease caspase-3 activity and promote cell survival, with a selective non-amyloidogenic processing of APP. However, no influence was observed on cell viability, with a potential explanation in a downregulated metabolism. Paper III–IV describes the effect of high levels of dietary cholesterol in vivo, with increased microglial activation in WT mice, increased gliosis in ApoE-/- mice and subsequent increased plasma IL-6 levels in WT and ApoE-/- mice. The elevated levels of total cholesterol (TC) were found to be caused by increased levels of HDL and LDL in WT mice, whereas only LDL where increased in ApoE-/- mice. Plasma TC levels were correlated with body weight gain in WT and ApoE-/- mice. Furthermore, the selective and ApoE-associated influence induced by the rosuvastatin therapy, on the effects of dietary cholesterol, is described. We observed a decreased microglial activation and gliosis in WT and ApoE-/- mice, with plasma IL-6 levels decreased by 45% in WT mice, without reaching significance. The elevated levels of TC and LDL were decreased in WT mice, whereas no effect was observed on the levels of TC, HDL or LDL in ApoE-/- mice. Body weight gain was decreased in WT mice, with a previously unpublished age- and ApoE-associated declining response to the therapy observed in ApoE-/- mice. **SUMMARY:** The presented thesis has examined the connection between Alzheimer's disease and cholesterol, and demonstrated a selection of detrimental effects which may be induced by high levels of dietary cholesterol. Furthermore, the thesis has demonstrated the potential for a cholesterol-lowering therapy with rosuvastatin to exercise a preventive influence, with an age- and ApoE-associated response. The latter is an important observation, which indicates that rosuvastatin may be an effective therapy up to a certain point in the progression of the disease, after which the effects decline. In conclusion, the results discussed in this thesis offer a hypothetical explanation, at least in part, for the discrepancies observed in clinical trials on statins. In addition, this thesis have been able to support the theoretical connection between AD and plasma cholesterol levels with the observation that 27-hydroxycholesterol may exhibit an inhibitory influence on 24(S)-hydroxycholesterol and *non-AD* processes. It is suggested that persons with high risk of developing AD may benefit from a rosuvastatin therapy, in combination with lifestyle precautions taken in early midlife. The approach could be an effective *lifestyle-preventive strategy,* with preserved cognitive functions and quality of life at high age.

KEYWORDS: Alzheimer's disease, dietary cholesterol, rosuvastatin, apolipoprotein E, α-secretase, β-secretase, α-/β-secretase ratio, soluble APPα, gliosis, microglial load, interleukin-6, caspase-3, body weight gain, lifestyle-preventive strategy, 24(S)-hydroxycholesterol, 27-hydroxycholesterol

Sammanfattning

BAKGRUND: Alzheimers sjukdom (AD) är en allvarlig neurodegenerativ sjukdom som framförallt drabbar personer i hög ålder, med en karakteristisk progressiv försämring av kognitiva funktioner och demens. Med undantag för vissa riskfaktorer har man inte funnit någon direkt ärftlig koppling till sporadisk AD som är den vanligaste formen av AD. Istället anser man att den främsta bakomliggande orsaken är den kombinerade effekten av flera olika riskfaktorer; en kombinerad risk där yttre och inre faktorer tillsammans är av avgörande betydelse. Två av dessa riskfaktorer är ökade kolesterolnivåer i medelåldern, och en medfödd brist i lipoproteintransportören apolipoprotein E (ApoE), där isoform ApoE ε4 har visat sig vara en av de viktigaste indikationerna för sporadisk AD. Kolesterol har en essentiell roll i det centrala nervsystemet (CNS), där det under normala förhållanden hjälper till att underhålla de fysiologiska funktionerna och är nödvändigt för att nervcellerna ska kunna kommunicera. Tidigare studier har visat att kolesterolnivåerna i CNS kontrolleras av en långsam omvandling av kolesterol till 24(S)-hydroxykolesterol, med ett netto flux av 27-hydroxykolesterol i motsatt riktning, från cirkulationen in i hjärnan. Betydelsen av en bevarad kolesterolhomeostas har förstärkts av observationerna att en kolesterolrik diet kan framkalla en inflammation i CNS, och orsaka en förhöjning av inflammatoriska agenter. I direkt association till denna iakttagelse har man även observerat att plasmanivåerna av inflammatoriska cytokiner, däribland interleukin-6 (IL-6), ökar före den kliniska definitionen av AD, vid vilken man ofta finner en kronisk inflammation i hjärnan. Följaktligen har det föreslagits att minne och kognitiva funktioner skulle gynnas av kolesterolsänkande terapier. **STUDIER:** Den forskning som den här avhandlingen bygger på har undersökt hur kolesterol och kolesterolsänkande terapier med rosuvastatin påverkar olika nyckelfaktorer associerade med AD. Det experimentella upplägget bestod av in vitro-modeller med humana neuroblastoma SH-SY5Y celler (artikel I–II), och in vivo-modeller med kontrollmöss av 'vildtyp' (WT), samt möss med en 'knockout'-mutation av ApoE (ApoE-/-) (artikel III–IV). Det hydrofila substansen rosuvastatin är en effektiv hämmare av kolesterolsyntesen och har visat sig vara en effektiv behandling mot förhöjda kolesterolnivåer, med indirekta effekter på inflammationer genom en uttömning av isoprenoiderna. **RESULTAT:** Artikel I beskriver hur 24(S)-hydroxykolesterol selektivt inducerar den icke-amyloidogena bearbetningen (α-secretase aktivitet) av amyloid prekursor protein (APP), med ett ökat ratio mellan α-/β-secretase aktivitet och ökande nivåer av lösligt APPα och totalt lösligt sAPP; effekter som tydligt förminskades i närvaro av 27-hydroxykolesterol. Artikel II beskriver effekterna av att förbehandla in vitro-kulturer med rosuvastatin, före exponering av Aβ oligomerer. Behandlingen ledde till en minskning av caspase-3 aktivitet och främjade cellöverlevnaden, med en selektiv inducering av den icke-amyloidogena bearbetningen av APP. Cellernas viabilitet påverkades emellertid inte, vilket eventuellt kan förklaras av en nedreglerad metabolism. Artikel III–IV beskriver effekterna av en kolesterolrik diet *in vivo*, med en ökad aktivering av mikrogliaceller i WT möss, ökad gliosis i ApoE-/- möss och ökade plasma IL-6 nivåer i både WT och ApoE-/- möss. De ökade nivåerna av totat kolesterol (TC) i WT möss var relaterat med ökade nivåer av HDL och LDL, medan TC nivåerna i ApoE-/- möss endast var relaterat med ökade nivåer av LDL. De ökade TC nivåerna var emellertid korrelerade med den relativa ökningen av kroppsvikt i både WT och ApoE-/- möss. Här beskrivs även den selektiva och ApoE-associerade inverkan som rosuvastatin utövade på effekterna av den kolesterolrika dieten. Vi observerade en minskad aktivering av mikrogliaceller och gliosis i WT och ApoE-/- möss, med en 45% sänkning av plasma IL-6 nivåerna i WT möss, utan att nå signifikans. De förhöjda nivåerna av TC och LDL i WT möss sänktes av behandlingen med rosuvastatin, parallellt med ett svagt svar på behandlingen i ApoE-/- möss och oförändrade nivåer av TC, HDL och LDL. Den relativa ökningen av kroppsvikt i WT möss påverkades av behandlingen, med en tidigare opublicerad ålders- och ApoE-associerad minskad respons på behandlingen i ApoE-/- möss. **SUMMERING:** Den presenterade avhandlingen har undersökt kopplingen mellan Alzheimers sjukdom och kolesterol, med en demonstration av ett urval skaldiga effekter som kan framkallas av en kolesterolrik diet. Vi har även presenterat de förebyggande effekter som kan induceras av en kolesterolsänkande behandling med rosuvastatin, och de rön som tyder på att behandlingen är associerad med ålder och ApoE. Detta är en viktig observation som indikerar att rosuvastatin är en effektiv behandling fram till ett visst steg i sjukdomsprocessen, efter vilket effekterna avtar. Denna avhandling erbjuder därmed en hypotetisk förklaring till en del avvikelser som har observerats i kliniska studier på statiner. Nya rön har också presenterats som stödjer koppling mellan AD och plasmakolesterolnivåer, där 27-hydroxykolsterol har befunnits utöva ett hämmande inflytande på 24(S)-hydroxykolesterol och *icke-AD* processer. Vi föreslår att personer med hög risk att utveckla AD skulle kunna gagnas av en rosuvastatin terapi, i kombination med förebyggande åtgärder i livsstil från och med tidig medelålder. Metoden skulle kunna vara en effektiv *livsstilsförebyggande strategi* för att bevara kognitiva funktioner och livskvalitet i hög ålder.

NYCKELORD: Alzheimers sjukdom, kolesterolrik diet, rosuvastatin, apolipoprotein E, α-secretase, β-secretase, α-/β-secretase aktivitets ratio, lösligt APPα, gliosis, microglia celler, interleukin-6, caspase-3, relativ ökning av kroppsvikt, livsstilsförebyggande strategi, 24(S)-hydroxykolesterol, 27-hydroxykolesterol

Se även ***Populärvetenskaplig översikt.***

List of Abbreviations

24S-OHC	24(S)-hydroxycholesterol
27-OHC	27-hydroxycholesterol
Aβ	amyloid-β
ABComplex/HRP	avidin-biotin horseradish peroxidase complex
AD	Alzheimer's disease
ADAM	a disintegrin and metalloprotease
AICD	APP intracellular domain
APP	amyloid precursor protein
ApoE	apolipoprotein E
ApoE-/-	apolipoprotein E knock-out
BACE	β-site amyloid precursor protein-cleaving enzyme
BBB	blood-brain barrier
BSA	bovine serum albumin fraction V
CTFα; CTFβ	membrane-bound C-terminal fragment of 83 or 99 residues
CNS	central nervous system
CR	caloric restriction
CSF	cerebrospinal fluid
CYP46A1; CYP46a1	cholesterol 24-hydroxylase of human or murine origin
DAB	diaminobenzidine
DMEM:F12	Dulbecco's Modified Eagle's Medium:Ham's F12
F4/80	microglia cell marker
FAD	hereditary (familial) AD
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
HC	western type-high cholesterol (diet)
HCRS	western type-high cholesterol (diet) and rosuvastatin (therapy)
HDL	high density lipoproteins
HMG-CoA	3-hydroxy-3-methylglutaryl-Coenzyme A
IL-6	interleukin 6
LDL	low density lipoproteins
NMDA	N-methylD-aspartate
MCI	mild cognitive impairment
NFE	nitrogen-free extract
NPC	Niemann-Pick Type C disease
NSAIDs	non-steroidal anti-inflammatory drugs
NTFs	neurofibrillary tangles
P3	short peptide fragment
PBS	phosphate buffered saline
PHFs	paired helical filaments
PI	propidium iodide
RS	rosuvastatin (therapy)
PS1; PS2	presenilin-1 or presenilin-2; presenilins
RT	room temperature
SAD	sporadic (non-familial) AD
sAPPα; sAPPβ	soluble fragments of APP, cleaved at the α- or β-secretase cleavage site
TACE	tumour necrosis factor-α converting enzyme
TC	total cholesterol
WT	wild-type

List of Publications

This thesis is based on the following papers, which will be refereed to in the text by their roman numerals.

I. **Daniel Famer***, Steve Meaney*, Malahat Mousavi, Agneta Nordberg, Ingemar Björkhem and Milita Crisby. Regulation of α- and β-secretase activity by oxysterols: Cerebrosterol stimulates processing of APP via the α-secretase pathway. *Biochemical and biophysical research communications*, 2007 Jul 20; 359(1):46–50.

II. **Daniel Famer** and Milita Crisby. Rosuvastatin reduces caspase-3 activity and up regulates alpha-secretase in human neuroblastoma SH-SY5Y cells exposed to A beta. *Neuroscience Letters*, 2004 Nov 23; 371(2–3):209–214.

III. **Daniel Famer** and Milita Crisby. Rosuvastatin reduces gliosis and the accelerated weight gain observed in WT and ApoE-/- mice exposed to a high cholesterol diet. *Neuroscience Letters*, 2007 May 23; 419(1):68–73.

IV. **Daniel Famer**, Lars-Olof Wahlund and Milita Crisby. The effect of rosuvastatin on microglia and IL-6 expression in the brain of WT and ApoE knockout mice. *Journal of Neuroimmunology* (submitted).

* Shared first authorship.

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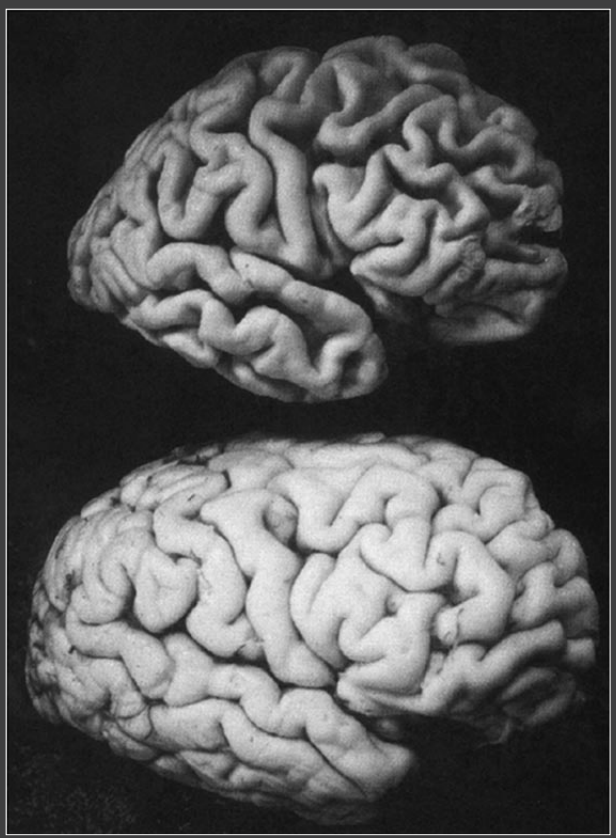


Introduction

ALZHEIMER'S DISEASE

General background

In 1906, the German psychiatrist Alois Alzheimer (1864–1915) presented the case history of a middle-aged demented woman named Auguste D. The diagnosis described 'a peculiar, severe disease process of the cerebral cortex', which eventually would be known as Alzheimer's disease (AD) [Alzheimer, 1907]. Since this initial discovery, AD has become recognized as one of the most important diseases amongst the elderly. It is a complex disorder, characterized by a major neurodegeneration causing a progressive cognitive decline and dementia. Although research on dementia and neurodegeneration has developed a number of symptomatic anti-dementia drugs to ease the burden, we are still far from finding a cure.

The worldwide population of demented persons was estimated to approximately 30 million people in 2003 [Wimo et al., 2006], with an estimated prevalence exceeding 10% in people over the age of 65 years [Evans et al., 1989]. Interestingly, demographic studies have revealed that prevalence is associated with age; below 5% between the age of 65 and 74 years, compared with almost 50% among those over the age of 85 years [Evans et al., 1989]. We still have to discover what effects the general awareness about health and life style will have on prevalence of AD, but the median age is increasing world-wide, and providing care for the increasing number of demented patients is going to present an enormous challenge; it has been estimated that the number of demented persons in Europe will increase by at least 45% between the years 2000 and 2050, assuming that mortality remains constant [Wancata et al., 2003].

Dementia (from *Latin* [de-] *apart, away* and [mens] *mind*) is, in its clinical definition, the progressive decline of cognitive function, due to damage or disease of the brain, beyond what might be expected from normal aging. Dementia is a non-specific term encompassing many disease processes, in the same

way that many neuropathological hallmarks in AD per se, not are AD-specific events. Closely related with AD is the condition mild cognitive impairment (MCI), a diagnosis believed to affect up to a third of the people over the age of 65 years. Whilst first considered a separate syndrome, it is now recognized as a frequent prelude to AD – a transitional phase from which 20–50% will develop full AD within 3 years [Kluger et al., 1999; Schmidtke et al., 2007].

As a consequence of the disease process it is commonly assumed that patients with dementia and severe cognitive disabilities are no longer "really there", with their selfhood open to question despite the persistence of an animate body. Patients with severe AD require such amounts of informal care from their care givers, and inflict such a deep psychological effect on their families, that what we have is a situation that could be described in terms of burden, stress and depression [Jonsson, 2003; Wimo, 2004]. Thus, we have a situation where care givers and families may suffer more from the disease than the patients. In order to accurately estimate the growing cost of dementia care for the society, we need to consider the quality of life for patients, care givers and families alike.

Classification of Alzheimer's disease

AD can be classified into hereditary (familial) AD (FAD) or sporadic (non-familial) AD (SAD), depending on the age of onset and underlying cause of the disease. However, they have nearly identical clinical symptoms, with similar neuropathological hallmarks, and are regarded as the same disease in clinical treatment and research.

FAD is a hereditary disease, comprising 10–15% of the diagnosed AD patients, with a disease onset in early midlife; sometimes with symptoms becoming evident as early as age 28 [Vetrivel and Thinakaran, 2006]. A genetic causal link can be found in a small percentage of the patients, with mutations in three particular genes associated with FAD: the amyloid precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2). Among these three the majority have been found in the presenilins, with more than 160 established mutations in PS1, causing the most aggressive forms of FAD [Vetrivel and Thinakaran, 2006; Goedert and Spillantini, 2006].

SAD has a symptomatic onset after age 65 and comprises the remaining 85–90% suffering from AD. The only known genetic linkage with SAD is an isoform of apolipoprotein E [Goedert and Spillantini, 2006], although a number of risk factors have been identified that may increase the likelihood of developing the disease.

Common risk factors

AD is in the majority of cases caused by the effects of several and often separate

factors which interacts and affects the brain. It is believed that when the combined influence of these, so called, environmental and biological risk factors, cross a yet undefined threshold level, they overwhelm the natural self-repair mechanisms in the brain, thus causing the disease. Most prominent of these risk factors are: high age; family history; apolipoprotein E ε4 (ApoE ε4); mild cognitive impairment (MCI); midlife elevated cholesterol level; chronic inflammatory conditions; obesity; head injuries; low levels of formal education; diabetes; Down's syndrome; clinical depression; stroke; high blood pressure; stress and inadequate exercising of the brain [Kivipelto et al., 2001; Turner, 2006; Ni et al., 2006]. Many of these are further included in the metabolic syndrome. Risk factors that are less firmly associated with AD include smoking, excessive alcohol consumption and narcotic drugs.

Epidemiological studies have reported a higher prevalence and incidence of SAD in women, but the biochemical basis for this gender-disparate susceptibility is still largely unknown. Some risk factors that deserve further explanation and not will be discussed in greater detail elsewhere are:

Family history

AD occurs in most cases regardless of family history. A significantly higher risk is only conceivable if the family history contains two generations or more of first degree relatives (i.e. grandparents and parents, or parents and siblings on the same side of the family), with diagnosed AD (and not any other form of dementia) and if the age of onset was before 65 years of age in both cases.

Down's syndrome

Down's syndrome is caused by a trisomy of chromosome 21, the same chromosome that contains the APP gene. The number of people with Down's syndrome that survive to middle age and develop AD is high, as expected, but the disease is not inevitable [Turner, 2006; Margallo-Lana et al, 2007], which emphasizes the importance of further risk factors.

Metabolic syndrome

The metabolic syndrome is a syndrome characterized by abnormalities in insulin, glucose, and lipoprotein metabolism, as well as hypertension and obesity. It is an acknowledged risk factor for cardiovascular diseases and has been significantly associated with AD [Vanhanen et al., 2006; Razay et al., 2007].

In a comparison study of patients with and without the metabolic syndrome, it was revealed that AD was more frequently found in patients with the syndrome. An interesting and unexpected finding was the occurrence of a gender-specific difference. The prevalence of AD was found to be higher in women with the metabolic syndrome, whilst no difference was found in men [Vanhanen et al., 2006]. The association between the metabolic syndrome and

AD, as well as the gender specific risk association, is of high significance, given that it might be possible to target by relatively simple lifestyle interventions [Scott, 2003].

Mild cognitive impairment

Individuals with MCI are regularly able to function quite normally, with few complaints about their cognitive capacity, regardless of the fact that a major neurodegeneration has often occurred [Tronsco et al., 1998; Morris et al., 2001]. Individuals with MCI is neither normal nor demented; there is evidence of cognitive deterioration; and activities of daily life are preserved, with complex instrumental functions either intact or minimally impaired [Winblad et al., 2004]. Researchers are investigating the use of neuropsychological assessment, peripheral and CSF biomarkers, as well as structural and functional MRI (magnet resonance imagining) to distinguish MCI from normal age-related memory changes, and identify subjects with pre-clinical AD [Zetterberg et al., 2003; Korf et al., 2004]. For example, recent studies have found that the $A\beta_{1\text{-}42/1\text{-}40}$ ratio may become a useful biomarker for identifying cognitively normal aging subjects who are at increased risk for developing MCI or AD [Graff-Radford et al., 2007]. But MCI is not necessarily AD. Instead, a MCI diagnosis may serve as a valuable early warning signal for many treatable symptoms such as stress, depression, heart disease, hearing loss, nutritional deficiencies and inactivity.

Clinical symptoms and diagnosis

Language and memory are recognized as two of the major building blocks in the construction of selfhood, and any disorder that impairs these abilities is considered to be both frightful and devastating. The clinical case notes on Auguste D. reveal some classic symptoms; the agitated suspicion she felt towards her husband; her increasing loss of memory and interests; her inability to interact socially; and her increasingly abnormal and disruptive behaviours [Alzheimer, 1907].

In order to set a definitive diagnosis we need to fulfil certain neuropathological criteria such as the presence of neuritic plaques and/or NFTs [Tierney et al., 1988; Khachaturian, 1985; Mirra et al., 1991]. These major anatomical changes in the brain were accurately described by Dr. Alzheimer [Graeber et al., 1998; Graeber, 1999], but occur in a number of different neurological disorders and are not AD-specific events as such. Accordingly, the criteria must be fulfilled but are by themselves not sufficient to set an accurate diagnosis; they need to be supported by clinical symptoms and a clinical diagnosis to rule out other types of dementias and neurodegenerative disorders.

Symptoms and general progression

The general progression of the disease can be outlined in three progressive stages, stretching over a period of 3–10 years depending on the age at onset [Brookmeyer et al., 2002; Bonsignore and Heun, 2003]. However, this is only a general progression and AD is affecting all patients differently.

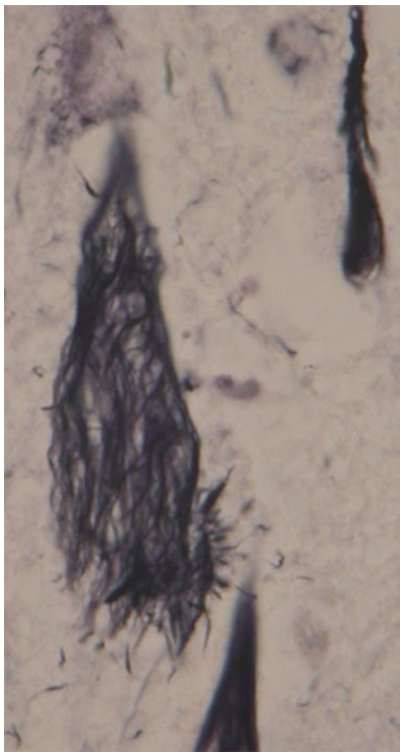
(i) Early stage in the disease progression (mild AD); symptoms may not yet be noticeable. The patients tend to have less energy and spontaneity. They exhibit minor memory loss, personality changes and mood swings, and are slow to learn and react. After a while they start to shy away from anything new and prefer the familiar. Memory loss begins to affect job performance. The patients are confused, get lost easily, and demonstrate poor judgment.

(ii) Middle stage; the patient can still perform tasks independently, but may need assistance with more complicated activities. Speech and understanding become slower, and patients often lose their train of thought in mid-sentence. They may also get lost while travelling or forget to pay bills. As the patients become aware of this loss of control, they may become depressed, irritable and restless. The individual is clearly becoming disabled. The distant past may be recalled, while more recent events are difficult to remember. The progressing disease affects the patients' ability to understand where they are, the date and the time. Caregivers and family must give clear instructions and repeat them often. As the mind continues to slip away, the patients may invent words and not recognize familiar faces.

(iii) Late stage (severe AD); the patients lose their ability to chew and swallow. Their very self-hood seems to be vanishing. Memory is often very poor and the patients can no longer recognize anyone. They lose bowel and bladder control, and will eventually need constant care. They become vulnerable to pneumonia, infection and other diseases. Respiratory problems get worse, especially when the patients become confined to bed, and eventually, death is inevitable.

Neuropathological hallmarks and diagnosis

The neuropathological changes found in the AD brain include shrinkage of gyri, widening of sulci and enlargements of the ventricles. Brain regions most affected are the enthorinal cortex, hippocampus, amygdala, neocortex and some basal forebrain nuclei [Braak and Braak, 1994; Gosche et al., 2002], but neurodegeneration will soon occur in other regions, including the temporal and parietal lobes, and in some cases also the frontal and occipital lobes [Braak



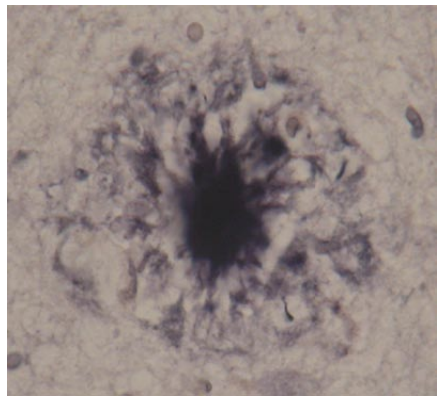


Figure 1B. *Hallmark of AD; extracellular mature ("classic") plaque composed of a dense central core of Aβ (Bielschowsky silver staining), by courtesy of Associate professor Nenad Bogdanovic.*

Figure 1A. *Hallmark of AD; intracellular neurofibrillary tangle composed of hyperphosphorylated tau, aggregated into paired helical filaments (Bielschowsky silver staining), by courtesy of Associate professor Nenad Bogdanovic.*

and Braak, 1994]. Of particular concern for the classic symptoms occurring in AD, are the neuropathological changes in the hippocampus, important as they affect the area of the brain involved in learning and memory tasks.

Histopathological examinations reveal a widespread neuronal degeneration, loss of synapses, intracellular neurofibrillary tangles (NFTs; Figure 1A) and neuritic plaques with clusters of activated microglial cells and macrophages (Figure 1B) [Alzheimer, 1907; Tarkowski et al., 2003]. Considering the strong correlation between synaptic loss and dementia, it has been suggested that AD could be regarded as a disease of synaptic failure [Sorrentino and Bonavita, 2007].

Neurodegeneration in Alzheimer's disease

The neuronal death found in the AD brain is believed to be executed primarily by apoptosis, which is a highly regulated physiological event [Tarkowski, 2003] and, to a lesser degree, necrosis, which is a passive pathological event arising from insult or trauma. However, it appears that these processes may have an overlap in neuronal death, as Aβ peptides have been suggested to induce a

rapid response in neurons, representing necrosis, followed by a long term apoptosis [Ankarcrona et al., 1995]. Several hypotheses have been suggested to explain the selectivity of the neurodegeneration, including the possibility that Aβ peptides could function as an extracellular signal molecule for caspase-3 activation [Takuma et al., 2004].

The activation of caspase-3 represents an early physiological marker for apoptosis and determines the apoptotic pathway [Janicke et al., 1998; Slee et al., 1999; Nicotera et al., 1999; Riedl and Shi, 2004], with characteristic cleavages of vital cellular substrates, apoptotic morphology and internucleosomal fragmentation of DNA. Typical morphological features are shrinkage of the cell body, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighbouring cells [Saraste and Pulkki, 2000].

Neuritic plaques

Any single neuritic (amyloid) plaque in AD may contain dystrophic neuritis of varying neurotransmitter specificities, reflecting the normal distribution of cell bodies and axons [Struble et al., 1987]. Even though the pyramidal glutamatergic hippocampal neurons seem to be especially vulnerable, and are affected early in the pathological process [Beach et al., 1982], it is believed that the progressive accumulation of neuritic plaques and synaptic failure involve local fibres of intrinsic and projection neurons in a non-specific fashion [Selkoe, 1991].

The classic neuritic plaque in AD is a complex lesion of the cortical neuropil containing several abnormal features; a central deposit of extracellular amyloid-β (Aβ) fibrils, surrounded by dystrophic neuritis, activated microglial cells and astrocytes in a manner that reminisces of glial scarring [Selkoe, 1991]. However, all plaques are not identical. They change their morphology during development, which allows us to distinguish between plaques at different stages:

- Diffuse (*early*) plaques have a loose accumulation of Aβ and no surrounding dystrophic neuritis.
- Immature (*primitive*) plaques have a loose accumulation of Aβ and are surrounded by dystrophic neuritis.
- Mature (*classic*) plaques consists of a dense central core of Aβ, surrounded by a halo measuring about 0.2 millimetres in diameter, activated glial cells and a ring of dystrophic neuritis associated with wisps of Aβ.
- Hypermature (*burned out*) plaques with a dense core of Aβ surrounded by reactive astrocytes and no dystrophic neuritis.

Interestingly, inflammatory mediators produced by activated glial cells, are more likely to occur in the *early* diffuse plaques without neuritic pathology, rather than in the late stage *burned-out* plaques, which is consistent with their alleged lesion-promoting role [Bauer et al., 1991; Gruol and Nelson, 1997; Fassbender et al., 2000].

Amyloid-β peptide

The occurrence of Aβ peptides in neuritic plaques is, as previously described, one of several criteria needed for an accurate diagnosis of AD Figure 1B. It is also undisputable that many of the known mutations in AD are affecting APP processing and the production of Aβ. However, the total Aβ load does not necessarily predict the degree of dementia, as large deposits of Aβ peptides can be found also in cognitively intact brains [Webster et al., 1997].

Interestingly, the gender-specific aspect of AD has recently been linked with the processing of APP, suggesting that BACE-activity may be one of the factors that contributes to the observed increase in prevalence and incidence of AD in women [Schäfer et al., 2007].

Processing of the amyloid precursor protein

APP is a transmembrane protein found mostly at nerve terminals. It is located on chromosome 21, contains 770 amino acids, and is ubiquitously expressed in all mammalian cells with a high degree of evolutionary conservation. The general processing of APP is carried out by two competing pathways, of which the amyloidogenic pathway is associated with AD. APP is here initially cleaved at the β-secretase site by the β-site cleaving enzyme (BACE), producing a large soluble extracellular fragment of APP (sAPPβ), and an Aβ-bearing membrane-associated C-terminal fragment (CTFβ) of 99 residues. The CTFβ is subsequently cleaved within the APP transmembrane domain by the γ-secretase complex, composed of at least four integral membrane proteins: presenilins (PS1/PS2), nicastrin, Aph1, and Pen2 [Kaether et al., 2006]. The cleavage of CTFβ subsequently generates Aβ peptides of varying length (39–43 amino acids), and a fragment called APP intracellular domain (AICD) (Figure 2).

Interestingly, the localization of CTFβ during cleavage might be the determining factor for the length of the generated peptide. The most frequently occurring peptide, $A\beta_{1\text{-}40}$, is generally believed to be generated in the cell membrane, while the more toxic $A\beta_{1\text{-}42}$ peptide is believed to be generated in either the endoplasmic reticulum or the golgi complex, early in the biosynthetic pathway [Klein et al., 1999; Selivanova et al., 2007]. It has recently been proposed that newly synthesized APP is subject to amyloidogenic processing during the initial phases of the secretory pathway [Selivanova et al., 2007], which might be an explanation for the different cleavage sites. In addition, APP processing

is highly associated with cholesterol and the cholesterol-rich *lipid rafts* located within the cell membrane [Ehehalt et al., 2003]. Thus, alterations in cholesterol transport may have an effect on the localization of the presenilins in the γ-secretase complex [Runz et al., 2002].

The non-amyloidogenic pathway constitutes an alternative pathway, initiated by the cleavage at the α-secretase site by a member of the ADAM (a disintegrin and metalloprotease) family, of which ADAM10, ADAM17 (TACE), ADAM9 and ADAM19 have been identified as having α-secretase activity [Fahrenholz et al., 2000; Asai et al., 2003]. The cleavage occurs within the Aβ region (between residues 16 and 17), thereby preventing the formation of Aβ, generating a soluble extracellular fragment of APP (sAPPα), and a membrane-associated C-terminal fragment (CTFα) of 83 residues.

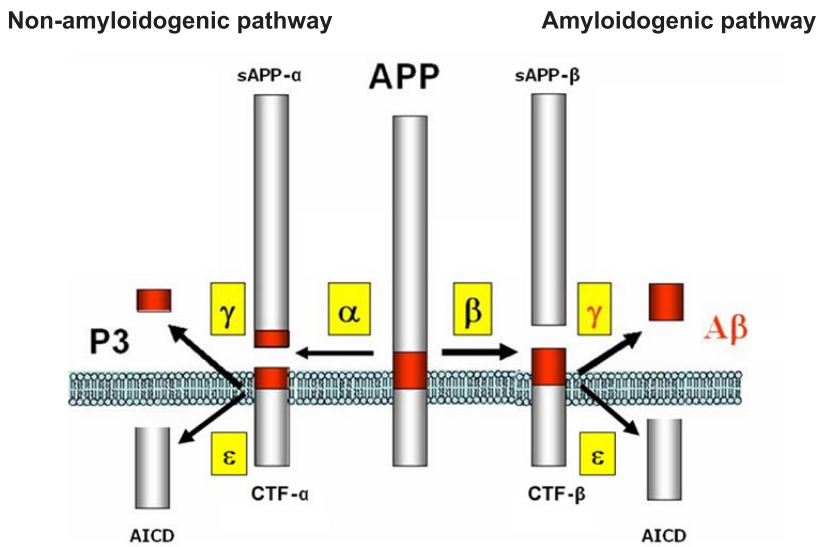


Figure 2. *Schematic overview of the general processing of the amyloid precursor protein; the non-amyloidogenic pathway constitutes a 'non-Aβ' pathway, with a cleavage with the Aβ region, whereas the amyloidogenic pathway generates Aβ peptide of lengths between 39–42 amino acids.*

The α-secretase cleavage does not only constitute a *non-Aβ* pathway by the cleavage within the Aβ region, but it has also been suggested that α-secretase activity occurring in the golgi complex may compete directly with the β-secretase pathway [Skovronsky et al., 2000], and the resulting sAPPα fragment has been found to have neurotrophic effects that counteract apoptotic signalling and promote synaptogenesis [Selkoe, 2000; Farenholz and Postina, 2006]. Cleavage of the sAPPα fragment by the γ-secretase complex generates a short peptide fragment (P3) unable to form aggregates, and in parallel with the amyloidogenic pathway, an AICD fragment (Figure 2).

The AICD fragments are believed to be liberated by a final cleavage named the ε-cleavage, executed by the γ-secretase complex, [Weidemann et al, 2002]. It has been demonstrated that AICD fragments have a transcriptional potential, with a suggested important function in the regulation of cellular actin dynamics [Cao and Südhof, 2001; Müller et al., 2007], but the physiological role and target of AICD-dependent gene regulation remain largely unknown.

Among the well-known mutations in the APP gene, we find the *Swedish* mutation, located close to the BACE cleavage. FAD patients with this inherited form of APP have been found to have increased levels of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in plasma [Mullan et al., 1992]. Another mutation of APP that has been associated with FAD is the *Arctic* mutation, located in the Aβ sequence. This mutation appears to decrease the production of Aβ whilst enhancing the formation of fibrils [Nilsberth et al., 2001].

Amyloid cascade hypothesis

The clinical symptoms and the neuropathological events in both FAD and SAD are, according to the amyloid cascade hypothesis, caused by the toxic effects of overproduction, aggregation and deposition of Aβ oligomers in neuritic

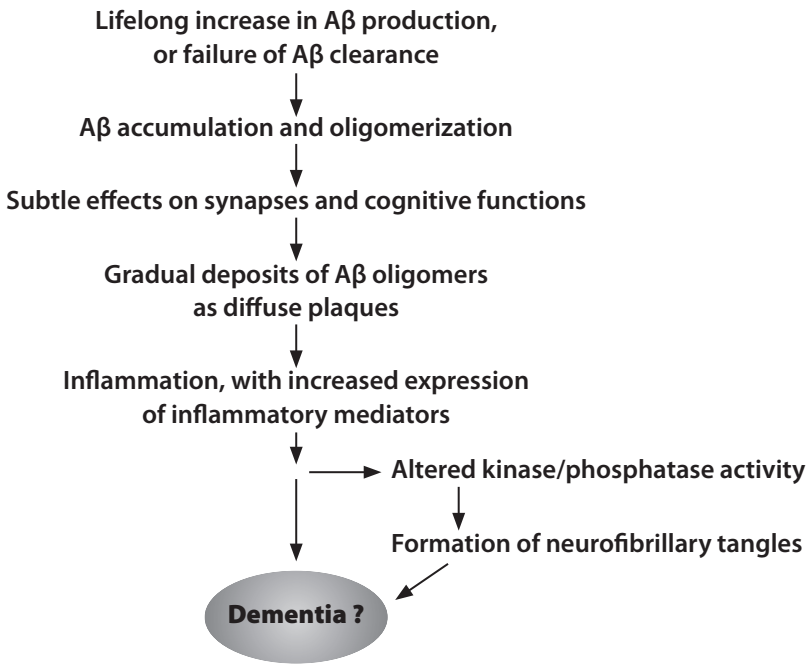


Figure 3. *Schematic overview of the amyloid cascade hypothesis. The hypothesis suggests that the clinical symptoms and the neuropathological events in AD are caused by the toxic effects of overproduction, aggregation and deposition of Aβ oligomers in neuritic plaques [Hardy and Higgins, 1992].*

plaques [Hardy and Higgins, 1992]. It has been demonstrated, under controlled conditions, that $A\beta_{1\text{-}42}$ peptides aggregate and create a mixture of Aβ fibrils, protofibrils and soluble oligomers [Hartley et al., 1999; Ferrari et al., 2003]; a mixture with an established toxic effect on neurons in *vitro* [Pike et al., 1991]. The amyloid cascade hypothesis elaborates that the plaques, with these toxic Aβ oligomers, are causing a disruption of synaptic connections; reduction of neurotransmitters; activation of glial cells with accompanying inflammatory responses; tau phosphorylation; formation of NFTs; and finally dystrophic neuritis and neurodegeneration [Hardy and Higgins, 1992] (Figure 3).

The main objection to the theory of a disturbed Aβ homeostasis as a central event in AD is the finding that cognitively intact persons have revealed large Aβ deposits at autopsy, without any occurrence of clinical symptoms [Webster et al., 1997]. This implicates that solid Aβ depositions (fibril Aβ aggregates) alone, are insufficient to cause cognitive decline, and should not be correlated with the progression of the clinical disease. Furthermore, the genes required for Aβ generation (APP, PS1, and BACE) are expressed at high levels in areas that develop senile plaques in AD, but they have also been found at high levels in the cerebellum, which only develop insignificant amounts of Aβ depositions [Fukumoto et al., 2002]. These results indicate that a hitherto unknown region-specific factor, or the combined effect of several separate factors, is necessary for Aβ deposition and plaque formation to occur.

Neurofibrillary tangles and tau phosphorylation

The formation of neurofibrillary tangles (NFTs) is caused by an abnormal phosphorylation of the microtubule-binding tau protein, which aggregates into paired helical filaments (PHFs) in the cytoplasm of degenerating neurons. These PHFs are the main constituent of NFTs found within the neuronal perikaryon, the neuropil threads and within dystrophic neuritis surrounding the plaques. The observation that NFTs have been found in degenerating neurons, adjacent to neurons free of any morphological indication of the disease, has led to the suggestion that it is the NFTs that are responsible for the synaptic failure in AD [Callahan and Coleman, 1995].

Tau phosphorylation is regulated by the balance and interaction between several kinases and phosphatases [Iqbal et al., 2005], but the molecular mechanisms affecting this balance are not fully understood. However, NFTs may occur in a number of different neurodegenerative diseases such as AD, Parkinson's disease and Niemann-Pick Type C disease (NPC) [Lee et al., 2001]. These findings imply that the abnormal hyperphosporylation of tau, and subsequent occurrence of NFTs, may constitute a set of factors, which increase the risk of neurodegeneration, without specifying the exact course of action. Accordingly, the hyperphosphorylated tau in NFTs of patients with NPC has been found to be indistinguishable from the ones found in AD [Auer et al., 1995; Distl et al., 2003].

Inflammation and cytokines

The definition of inflammation is a 'non-specific host response to ubiquitous noxious stimuli'. Thus, when we consider the occurrence of an inflammation in various neuropathological conditions, we have to consider whether inflammation and its inflammatory mediators have beneficial or detrimental functions or if it may even be an irrelevant observation. Proof in support of the detrimental effects is substantial, even though a number of reports have suggested otherwise.

Microglia and astrocytes

The microglia are the resident immune cells of the CNS, and in the healthy non-AD brain they are ramified resting cells that have lost their macrophage-like properties [Nakajima and Kohsaka, 1993; Kreutzberg, 1996]. When an injury or a microbial infection occurs, the microglial cells become activated with a macrophagic phenotype, proliferation, phagocytosis, and expression of MHC-II and antigen presentation [Rogers et al., 1988].

In contrast, the AD brain suffers from a severe chronic inflammation which is causing a chronic activation of the injury-responsive microglial cells in the vicinity of neuritic plaques [Gordon, 1993; Kalaria et al., 1996; Tarkowski et al., 2003; Liu and Hong, 2003]. The neuroprotective effects of the inflammation in AD have been supported by the finding that Aβ peptides may be phagocyted by activated microglia [Mentlein et al., 1998]. Though, it has been suggested that the inflammation and presence of microglial activation in AD, is not only a reaction to Aβ deposits and neurodegeneration, but that it may also contribute to disease progression. Some studies have implied an active role of microglia in the mediation of Aβ toxicity and subsequent secondary tissue damage, via release of pro-inflammatory cytokines and cytotoxic molecules [McGeer et al., 1988; McGeer and McGeer, 2004]. Accordingly, it has been hypothesized that the inflammation observed in AD is a chronic, self-perpetuating neuroinflammation that stimulates an autotoxic process that aggravates the pathogenic mechanism and influences the disease progression [McGeer and McGeer, 2004]. In another hypothesis, the microglia becomes functionally deficient with a subsequent decline in neurotropic support of neurons and impaired phagocytic functions, resulting in an excessive release of cytokines and defective clearance of Aβ deposits [Blasko et al., 2004; Flanary et al., 2007].

Astrocytes are the most abundant glial cells in the CNS and apart from producing cholesterol and ApoE, as previously described, they play an essential role in the maintenance of the BBB integrity and normal brain physiology [Rubin and Staddon, 1999]. This role becomes even more evident in light of the fact that astrocytes participate in the CNS response to injury [Eng and Ghirnikar, 1994; Gustafson et al., 2003]. Accordingly, astrocytic activity and

gliosis are associated with cognitive decline and have been described in many neurodegenerative diseases, such as AD and Parkinson's disease [Minagar et al., 2002; Kashon et al., 2004]. We can define gliosis further and describe it as an increased number of hypertrophied astrocytes, positive for the glial fibrillary acidic protein (GFAP), a major member of the class III intermediate structural filament protein and expressed in mature astrocytes [Eng and Ghirnikar, 1994].

Cytokines in the aging brain

A general decline in immunological regulation is considered to be a hallmark in the normal aging process, with an age-associated increase in microglial activation in cognitive intact patients [Sheng et al., 1998]. Considering that pro-inflammatory cytokines is secreted by a wide variety of cells, including microglia cells and astrocytes [Benveniste, 1992], it is not surprising that we also find an age-associated increase of cytokines correlated with a normal decline in cognitive function [Ye and Johnson, 1999; Weaver et al., 2002; Teunissen et al., 2003]. The cytokine homeostasis is essential for cognitive functions and the cytokines are responsible for a variety of neurotransmitter and neuroendocrine responses subserving cognition. It is thus possible that they are liable for a part of the normal cognitive decline seen in the aging brain.

The cytokines are suggested to modulate their effects by a complex signalling pathway in which the mitogen-activated protein kinases may phosphorylate and activate each another. The primary activation of the enzyme p38 is mediated by the inflammatory cytokines and environmental stresses; ERK has a function in the control of cell division; whereas JNK are a critical regulator of transcription [Johnson and Lapadat, 2002].

Interleukin-6 and Alzheimer's disease

Interleukin-6 (IL-6) is a 26,000 kDa pro-inflammatory cytokine involved in the regulation of many aspects of the inflammatory and immunological responses [Taga and Kishimoto, 1997; Dziedzic, 2006]. Findings of IL-6 and IL-6 receptor mRNA in the pyramidal cells of the rat hippocampus [Minami et al., 1991], suggest that the cytokine in addition to the previously described expression by the microglia cells and astrocytes, under certain circumstances can be expressed by neurons. Transgenic over-expression of IL-6 in astrocytes has been found to cause gliosis and neurodegeneration of hippocampal neurons, reduction of dendritic arborisation, angiogenesis and induction of acute phase proteins [Campbell et al., 1993], whereas mice with IL-6-deficieny will develop mature-onset obesity [Wallenius et al., 2002].

In the healthy brain IL-6 are regularly found at low levels [Wilson et al., 2002], with an age-associated expression as mentioned above. Increased

plasma IL-6 levels have been reported in mice and non-demented populations, with a correlation to a decline in cognitive function and increased mortality [Cohen et al., 1997; Harris et al., 1999; Ye and Johnson, 1999; Weaver et al., 2002; Teunissen et al., 2003; Yaffe et al., 2003]. These observations are interesting, particularly as the Rotterdam study has demonstrated that plasma levels of inflammatory proteins, such as IL-6, are increased before clinical onset of AD and vascular dementia, which is consistent with their alleged lesion-promoting role [Engelhart et al., 2004]. Consequently, an increased production of pro-inflammatory cytokines has been found in the vicinity of neuritic plaques in AD patients [Griffin et al., 1989; Bauer et al., 1991; Dickson et al., 1993], whereas patients with late-stage dementia demonstrate high levels of cytokine mRNA expression in the entorhinal cortex and superior temporal gyrus at autopsy [Luterman et al., 2000]. Furthermore, inflammatory mediators are more likely to occur in "early" diffuse plaques without neuritic pathology, than in late stage "burned-out" plaques [Bauer et al., 1991; Gruol and Nelson, 1997; Fassbender et al., 2000].

Interestingly, it has been demonstrated that treatment with statins, which inhibit the cholesterol synthesis, has the potential to decrease the secretion of IL-6 and reduce the cell viability of human microglial cells [Lindberg et al., 2005], as well as indirectly inhibit the inflammatory response through isoprenoid depletion [Khwaja et al., 2000]. The isoprenoids are intermediates generated in the cholesterol biosynthesis pathway and play a significant role in the activation of IL-6 mediated inflammation [Heinrich et al., 1998].

CHOLESTEROL

General background

Cholesterol can be found in all human cells where they contribute to the structural stability of the lipid bi-layers. Although the brain makes up only 2% of the human body mass, it is, with 25% of the cholesterol, the most cholesterol-rich organ in the body. Here it plays an essential role in maintaining the normal physiological conditions of the central nervous system (CNS), a role stressed by the fact that it is required for the ability of neurons to communicate.

The presence of incorporated microdomains, composed of cholesterols, sphingolipids and saturated fatty acids, is one of the characterizing features of the plasma membranes [Simons and Ikonen, 1997]. These lipid rafts are thought to exist as ordered membrane patches, surrounded by more fluid membrane mainly composed of glycerolipids. They are believed to have an important function in the lateral segregation of membrane proteins, and the spatial organization and regulation of membrane proteins involved in many cellular processes such as APP processing, cell proliferation, apoptosis, and cellular signalling [Ehehalt et al., 2003; Ma, 2007]. Thus, it has been hypothesized that the lipid rafts could be a vital component in the pathogenesis of different neurodegenerative disorders.

In addition to the lipid rafts, cholesterol serves as an important precursor and cofactor of several signalling molecules [Tabas, 2002], a barrier against sodium leakage [Haines, 2001], and as a required component for the formation of synapses [Mauch et al., 2001]. In addition, it has been demonstrated that cholesterol is a major component of the myelin sheaths, thus essential for an efficient electrical transmission [Dietschy and Turley, 2004].

Cholesterol appears in many varieties, for example, LDL (low density lipoprotein) cholesterol is considered to be increasing the risk for atherosclerosis and heart disease, whereas HDL (high density lipoprotein) cholesterol, on the other hand, may help to protect the heart and blood vessels, including those that supply food and nourishment to the brain. Plasma cholesterol are under normal circumstances not able to cross the blood-brain barrier (BBB) [Rubin and Staddon, 1999; Pfrieger, 2003], and the cholesterol found in the CNS is characterized by a very low turnover. In light of this, it is not surprising to discover that essentially all cholesterol in the CNS is synthesized in situ, with their source of synthesis shifting from the neurons during infancy, to the astrocytes in the adult brain [Poirier et al., 1993; Pfrieger, 2003; Brown et al., 2004]. (For a overview of the cholesterol synthesis, see Figure 4, page 16.)

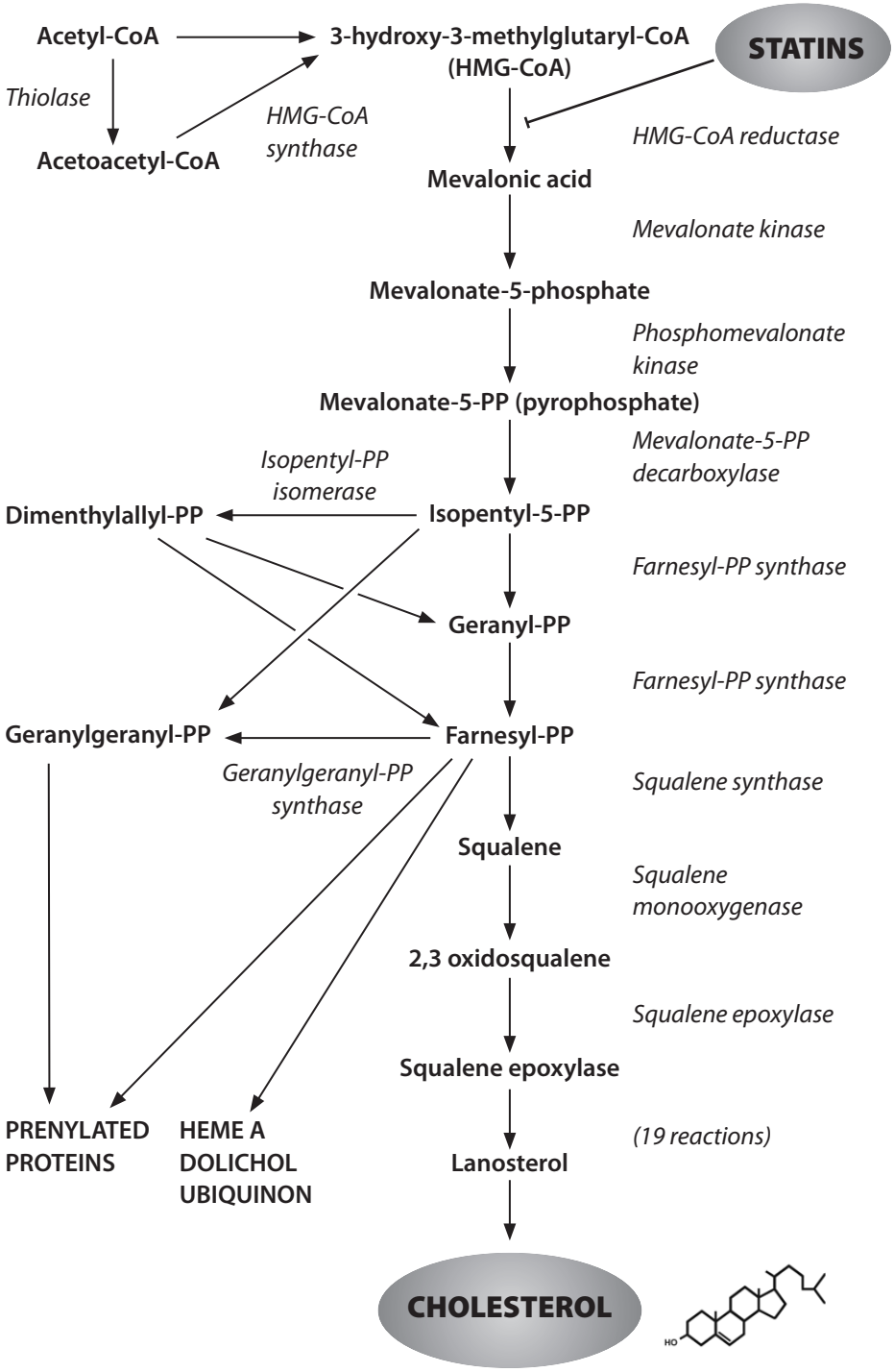


Figure 4. *Schematic overview of the cholesterol synthesis. Statins exert their inhibition on the HMG-CoA reductase, the rate-limiting step, early in the biosynthesis.*

Cholesterol homeostasis in the CNS

In the adult brain, although there is a continuous turnover of membrane, the cholesterol is efficiently re-cycled and has a remarkably high half-life up to 5 years. The homeostasis is maintained by a slow conversion of brain cholesterol into the oxysterols 24(S)-hydroxycholesterol (24S-OHC) and 27-hydroxycholesterol (27-OHC) (Figure 5). The conversion is mediated by the two enzymes cholesterol 24-hydroxylase (CYP46A1) and cholesterol 27-hydroxylase (CYP27A1), with the interesting detail that CYP46A1 only generates the (S)-stereoisomer of 24-hydroxycholesterol. The oxidation in the steroid side-chain dramatically increases the capacity of the generated oxysterols to pass the BBB and other lyophilised membranes, resulting in a rapid turnover of the small pool of oxysterols out in the circulation [Björkhem et al., 1997; Brown et al., 2004; Meaney et al., 2007]. The flux of 24S-OHC is considered to be the major pathway for excretion of excess cholesterol out of the CNS, with a half-life of 12 hours [Björkhem et al., 1997; Xie et al., 2003].

Immunocytochemical studies have shown that both CYP46A1 and CYP27A1 are expressed in neurons as well as by some astrocytes in the healthy brain [Lund et al., 1999; Brown et al., 2004]. Not surprisingly, the concentration of 24S-OHC are 30–1500-fold higher in the brain than in any other organ, except the adrenals [Ercoli and Rugieri, 1953; Lütjohann et al., 1996]. In contrast, 27-OHC has demonstrated a net flux from the circulation into the CNS, by a yet undefined cause, but is found at low levels in the brain due to an active metabolism and elimination [Heverin et al., 2005; Meaney et al., 2007]. Interestingly, the regulation of oxysterols seems to have an age-associated factor, as

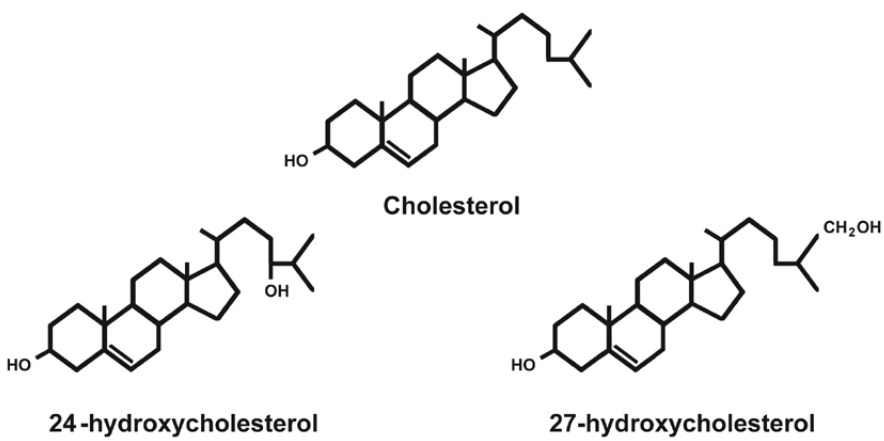


Figure 5. *Schematic overview of cholesterol (cholest-5-en-3β-ol), 24-hydroxycholesterol and 27-hydroxycholesterol. The enzyme CYP46A1 converts cholesterol into the S-isomer of 24-hydroxycholesterol, whereas the enzyme CYP27A1 converts cholesterol into 27-hydroxycholesterol.*

the ratio between 24S-OHC and plasma cholesterol was approximately 5 times higher during the first decade of life, than during the sixth decade [Lütjohann et al., 1996]. Furthermore, 24S-OHC has in addition to its contribution to cholesterol homeostasis been reported as one of the most potent endogenous ligands for the liver X receptor, the activation of which has demonstrated to have both anti-amyloidogenic and anti-inflammatory effects [Janowski et al., 1999; Abildayeva et al., 2006; Cao et al., 2007].

Cholesterol and Alzheimer's disease

It has been established that cholesterol and hypercholesterolemia are important risk factors for neurodegeneration [Sparks et al., 1994; Refolo 2000; Fassbender et al., 2001; Kivipelto et al., 2001; Puglielli et al., 2003], but the circumstances surrounding the connection are complicated, and exactly how it is manifested is still under debate. It is likely that some of the neuropathological changes found in the AD brain are linked with deficiencies in the lipoprotein transporter ApoE, implying the importance of a regulated cholesterol homeostasis in the brain.

Cholesterol has been reported to be required for the production of Aβ peptides [Simons et al., 1998], which has lead to the hypothesis that the amount of Aβ in the brain could be decreased, by decreasing cholesterol from the blood and the cerebrospinal fluid (CSF) [Buxbaum et al., 2002]. However, the requirement of cholesterol in the processing of APP is most likely connected with the lipid rafts, which supports the hypothesis that the lipid rafts play a crucial role in the cleavage [Ehehalt et al., 2003]. To further complicate the situation does the association between plasma total cholesterol (TC) and dementias, appear to be both age-associated, and bi-directional; high plasma TC levels in midlife is a risk factor for subsequent dementia and AD [Kivipelto et al., 2001; Solomon et al., 2007], wheras the decreasing plasma TC levels after midlife can be regarded as a sign of the underlying disease processes, and as such represent a potential marker for late-life cognitive impairment [Solomon et al., 2007].

The task to evaluate cholesterol levels in the brain represents another challenge. It has been demonstrated that the cholesterol homeostasis is disturbed in AD patients, with decreased levels of 24S-OHC and increased levels of 27-OHC in the brain [Heverin et al., 2004; Björkhem et al., 2006]. The reason for these changes could be a directly relationship with the decreased metabolic activity in the CNS due to the neurodegeneration; the decreased number of metabolic active neuronal cells affects the production of 24S-OHC and the elimination of 27-OHC. Among previously reported and yet not satisfyingly explained observations, we find a study in which patients with AD were found to exhibit a selective expression of CYP46A1 around neuritic plaques and in astrocytes, whereas CYP27A1 expression was found to be decreased in neu-

rons and around the plaques [Brown et al., 2004]. A possible explanation for these findings could be that they indirectly reflect the disease process, and what we see is a feed-back regulation in response to the altered cholesterol homeostasis.

Cholesterol and APP processing

The processing of APP is, as previously described, a requirement for the onset and progression of AD. An increased BACE activity can be found even in the healthy brain as a natural consequence of aging [Fukumoto et al., 2004], but AD patients demonstrate a significantly higher activity [Li et al., 2004].

The BACE complex undergoes a sequence of events for dimerization and stabilization within the membrane to occur, such as farnelysation and palmitoylation. They provides BACE both directly and indirectly with the two lipid anchors commonly found in membrane-bound proteins, and needed for the recruitment into the lipid rafts [Zhang and Casey, 1996; Westmeyer et al., 2004; Schmechel et al., 2004; Parsons et al., 2006]. The order of assembly of the BACE complex suggests that a reduction in membrane cholesterol could result in an inhibition of the dimerization of BACE. This would prevent BACE from entering the lipid rafts; reduce its association with the APP, and as a consequence, result in a decreased Aβ production [Parsons et al., 2007]. Noteworthy is the finding that cholesterol distribution – rather than total levels – has been correlated with altered APP processing after treatment with statins [Burns et al., 2006]. The connection could be the previously described association between the processing of APP and the lipid rafts [Ehehalt et al., 2003].

It has also been demonstrated that the intracellular cholesterol transport has important consequences for both APP processing and the localization of the γ-secretase associated presenilins, and could be an important factor for how cholesterol alters Aβ production [Runz et al., 2002].

Cholesterol and Apolipoprotein E

The lipoprotein transport is maintained by the apolipoproteins of which primarily apolipoprotein E (ApoE), produced by the glial cells, has an important function in the CNS not only for lipid transport, but also for maintenance and repair of neurons. Human ApoE is a polymorphic protein, 299 amino acids long and located on chromosome 19. Interestingly, the genetic polymorphisms of ApoE have been found to be among the strongest determinants of the risk and mean age of onset of SAD [Saunders et al., 2003]. It encodes three alleles that differ only at two positions; 112 and 158. ApoE ε2 has cysteines at both positions and occurs at a frequency of 5%–10%. ApoE ε3 occurs in 60%–70% of the population and has cysteine at 112 and arginine at 158. The detrimental ApoE ε4 has arginine at both sites and occurs in 15%–20% of all humans, with

an increased risk of approximately 300% for developing AD [Buxbaum, 2002]. Of great concern is the finding that the risk is age-associated, with a significant risk only below the age of 80 years [Dupuy et al, 2001]. This finding suggests that the connection between AD and ApoE ε4 is most important during the initial and middle stages of the disease. It has been suggested that the reason why ApoE ε4 persists in our genome, despite its detrimental effects, is the possibility that it may confer advantages in pathogen resistance [Charlesworth, 1996; Finch and Morgan, 2007].

The presence of ApoE ε4 has been associated with an increased concentration of cholesterol in the plasma [Notkola, 1998; Buxbaum, 2002], and an increased concentration of 24S-OHC in the CSF [Papassotiropoulos et al., 2002], while ApoE deficiency has been found to dramatically reduce the Aβ deposition [Bales et al., 1997].

The connection between ApoE-deficiencies, cholesterol and AD has been studied using ApoE knockout (-/-) mice, originally developed for studies on the pathogenesis of atherosclerosis, with a 5–10 fold increase in plasma cholesterol levels [Zhang et al., 1992]. It has also been hypothesized that an age-related BBB leakage is associated with ApoE deficiency, and that it could be one of the mechanisms linking ApoE ε4 with plasma cholesterol and the progression of AD [Hafezi-Moghadam et al., 2007], without influencing the transport of Aβ across the BBB [Matsumoto et al., 2007]. Accordingly, it has been reported that ApoE-/- mice provided with a high cholesterol (HC) diet have increased tau hyperphosphorylation and gliosis with increased levels of inflammatory mediators such as IL-6 and caspase-1 [Crisby et al., 2004; Rahman et al., 2005.a; Rahman et al., 2005.b].

The connection between cholesterol and apoptosis is supported by a significant increase of apoptotic cells and caspase-3 activity in AD patients bearing one or two alleles of the ApoE ε4, compared to non-ε4 carriers [Frey et al., 2006]. The connection might be explained by an association with the lipid rafts and the hypothesis that ApoE ε4-carriers are awarded with excessive amounts of cholesterol in the plasma membranes. Furthermore, it has been suggested that the some of the detrimental effects of ApoE ε4 are related to the inability of the isoform to interact with the microtubule associated protein tau, and thereby prevent its hyperphosphorylation. In support of this theory, it has been demonstrated that ApoE-/- mice have increased levels of phosphorylated tau in association with dietary cholesterol [Genis et al., 1995; Rahman et al., 2005.a].

TREATMENT STRATEGIES

General introduction

It is currently possible to decrease the symptoms of AD, and improve the patients' quality of life, within a few weeks. However, the majority of treatments is given first when the disease has progressed to the point of diagnostic certainty, and is purely symptomatic anti-dementia drugs without profound disease-modifying effects. Treatments with cholinesterase inhibitors may delay the breakdown of the neurotransmitter acetylcholine in the cortex and, thus, aid the preservation of memory function, decrease the degeneration of cognitive symptoms and reduce behavioural problems, whereas an alternative treatment for moderate to severe AD use a NMDA (N-methylD-aspartate) antagonist acting on glutamate, to reduce neuronal death.

Recent research on AD has focused on the possibilities to affect the progression of the disease using prevention strategies and modulations of specific aspects of the AD pathology, such as; APP processing and secretase inhibition; Aβ-aggregation inhibition; vaccination studies; and immunotherapy [Ohno, 2006; Na et al., 2007; Chauhan and Siegel, 2007; Nikolic et al., 2007]. The association between AD and inflammation has been examined using non-steroidal anti-inflammatory drugs (NSAIDs). The Rotterdam study suggested that NSAIDs can delay, or slow, the progression of AD with a protective effect up to 80% [in t' Veld et al., 2001], but reports from other trials have yielded inconclusive results [Akiyama et al., 2000; Aisen et al., 2003; McGeer and McGeer, 2007].

The connection between AD, diet, cholesterol and lifestyle factors has been examined in a number of investigations [Kivipelto and Solomon, 2006; Weih et al., 2007; Solomon et al., 2007]. The results have been unanimous in support of a strong association, which implies that relatively simple modifications could be an effective treatment. Among these modifications cholesterol-lowering therapies, with a focus on statins have, in particular, attracted rigorous attention [Sparks et al., 1994; Simons et al., 1998; Fassbender et al.,2001; Puglielli et al., 2003; Sparks et al., 2006]. The outcome has been debated, but contradictory results may in part be due to the different properties of the statins working in conjunction with other factors, such as the severity of the disease and the ApoE phenotype.

The majority of all AD patients are diagnosed very late in life, and it could be an acceptable solution for an increasingly aging population, if possible, to simply delay the symptoms. It has, in agreement with this suggestion, been hypothesized that if the onset of the disease could be delayed by just 5 years, the incidence would be cut by 50% [Pasinetti et al., 2007].

Cholesterol-lowering therapy

The association between cholesterol-lowering therapy and dementia has been supported by numerous studies [Simons et al., 1998; Fassbender et al., 2001; Puglielli et al., 2003; Sparks et al., 2006], including the observation that animals undergoing accelerated aging have improved memory when they are fed a diet that lowers their cholesterol [Chan et al., 2002]. These findings suggest that memory and cognitive functions during aging could benefit from a cholesterol-lowering therapy.

Statins

Statins were first introduced into clinical practice in the late 1980s after the accidental discovery of their lipid-lowering effects in 1976 [Endo et al., 1976]. The effect is exerted by an up-regulated expression of the LDL-receptor in the liver, which clear LDL-cholesterol and its precursors from the circulation [Brown and Goldstein, 1981]. This effect is in turn caused by the competitive inhibition of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting step early in the synthesis of cholesterol and many other non-steroidal isoprenoid compounds (Figure 4, page 16).

The first statins were produced from fungal metabolites, but the majority is now produced synthetically. Accordingly, the traditional method differentiates the two types and classifies them as either natural (fungal metabolites) or synthetic. Another classification defines the statins by their solubility: i.e. hydrophilic (rosuvastatin, pravastatin, fluvastatin to some extent) or lipophilic (atorvastatin, simvastatin, lovastatin, cervistatin). The lipid bi-layers of the cell membranes are easily penetrated by lipophilic compounds, whereas the uptake of hydrophilic compounds needs to be facilitated by organic anion transporters [Tamai, 2000; Simonson, 2004]. Accordingly, the lipophilic statins may cross the BBB and have been demonstrated to cause apoptosis in a variety of cells [Negre-Aminou et al., 1997; Kubota et al., 2004], whereas the hydrophilic statins do not [Harrison and Ashton, 1994; Knopp 1999]. With this is mind, the question have been raised if not hydrophilic statins should to be recommended for treatment purposes [Sparks et al., 2002], particularly as it has been demonstrated that hydrophilic statins exert effects on the CNS despite their inability to cross the BBB [Kilic et al., 2005].

Clinical studies on statins have produced some promising but contradictory results. On one hand the use of statins failed to prove a significant association with maintenance of cognitive function in patients with AD [Winblad et al., 2007; Summers et al., 2007], whereas, on the other hand, treatment with statins has been significantly associated with a decreased prevalence of AD [Wolozin et al., 2000]. Statins have also been found to significantly increase the α-secretase activity [Paper II, Xiu et al., 2006], and decrease the levels of both $A\beta_{1\text{-}42}$

and $A\beta_{1\text{-}40}$ [Fassbender et al., 2001; Simons et al., 2002]. Of utmost interest is the notion that decreased levels of $A\beta_{1\text{-}40}$ in the CSF were restricted to patients with mild AD, as no effect was observed in patients with more severe forms of AD [Simons et al., 2002].

Rosuvastatin

Rosuvastatin is one of the most recently approved statins for clinical use and has a structure similar to that of other synthetic statins. It has a long half-life of 20–24 hours (comparable to atorvastatin); it is hydrophilic (equivalent with pravastatin); and has no significant cytochrome P450 drug interactions. In addition, the safety of rosuvastatin at low doses has been reported to be comparable to that of other statins [Stein, 2003], and the lack of cytochrome P450 metabolism suggests that the therapy may be used without precautions in patients who are on CYP3A4 inhibiting drugs. The STELLAR study group has demonstrated that rosuvastatin is more efficient than equivalent doses of atorvastatin and simvastatin to lower the levels of LDL-cholesterol, with a beneficial effect not only on AD but also on the metabolic syndrome [Jones et al., 2003; Deedwania et al., 2005].

Pleiotropic effects

All statins have structural similarities, but they also have structural differences that may influence their pleiotropic effects. The primary cause behind these mechanisms is believed to be a reduction in protein isoprenylation, which is regulated independently of the cholesterol levels (Figure 4, page 16). The pleiotropic effects include a stimulated nitric oxide expression through an up-regulated expression and activity of endothelial nitric oxide syhthase [Kalinowski et al., 2002]; anti-inflammatory effects through an attenuation of mediators of inflammation and immunological responses [Kleeman et al., 2003; Wagner et al., 2002]; anti-oxidant effects through a reduction of lipoprotein oxidation [Crisby et al., 2001]; stabilization of atherosclerotic plaques [Crisby et al., 2001]; and some anti-angiogenic effects [Vincent et al., 2001].

The pleiotropic effects on inflammation have been supported by the finding that statins exert an effective inhibitory effect on MHC-II expression [Kwak, 2000], particularly interesting as MHC-II molecules are directly involved in the control of the immune system. Furthermore, rosuvastatin has been reported to reduce the caspase-3 levels in ischemic brain areas [Kilic et al., 2005], and the formation of dolichols which have an essential role in lipoprotein synthesis [Faggiotto and Paoletti, 2000].

Lifestyle factors

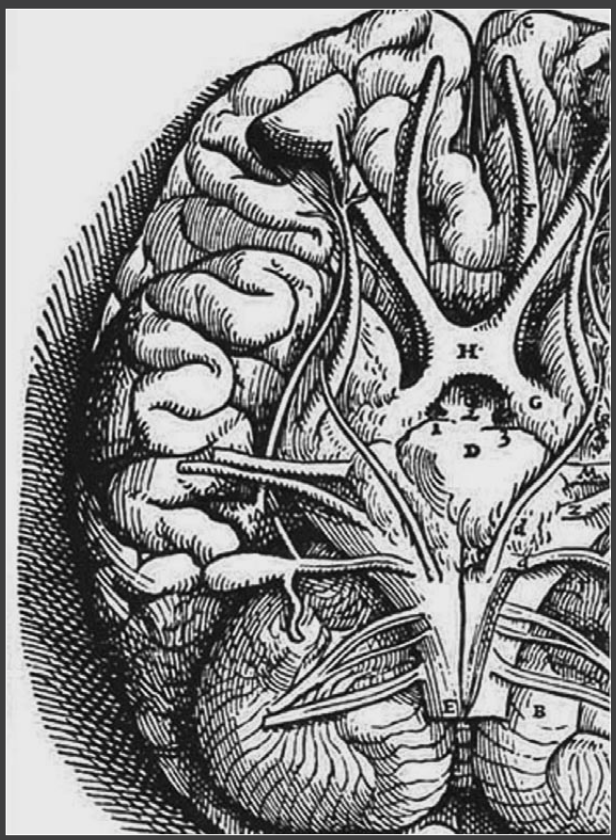
Obesity and diabetes is associated with a >4-fold increased risk of developing AD [Pasinetti et al., 2007], and both clinical and epidemiological evidence suggests that modifications of lifestyle factors such as diet and nutrition may prove crucial for the onset of AD. The importance of diet is emphasized by the findings that diets based on saturated fats may decrease learning and memory, increase oxidative stress and support Aβ load [Winocur and Greenwood, 1999; Pinilla, 2006; Pasinetti et al., 2007], whereas diets supplemented either with omega-3, vitamin E or the curry spice curcumin may be beneficial for cognitive functions [Pinilla, 2006]. Dietary supplementation of curcumin has also been found to counteract the outcome of traumatic brain injury on oxidative stress, synaptic plasticity, and cognition [Wu et al., 2006]. In a recent clinical trial on the use of omega-3, a significant effect on cognitive decline was only observed in a small group of patients with very mild AD [Freund-Levi et al., 2006], whereas an ApoE ε4-dependent effect on neuropsychiatric symptoms was observed in mild to moderate AD [Freund-Levi et al., 2007].

However, given the fact that our metabolism changes with age, and that several medications interfere with the absorption of nutrients, diet alone may not be a clinically useful treatment in elderly people. Short-term effects of a healthy lifestyle have been evaluated in a 14-day clinical study, regarding cognition and cerebral metabolism in people with mild age-related memory complaints. The program combined diet with other lifestyle factors, such as relaxation exercises, cardiovascular conditioning and mental exercises (i.e. brain teasers and verbal memory training techniques). The result of this so called *short-term healthy lifestyle program* was an association between the program and significant positive effects on cognitive function and brain metabolism [Small et al., 2006]. These findings suggest that it may be possible to prevent early memory deficiencies by a combination of relatively simple methods.

Caloric restriction

Caloric restriction (CR) has re-emerged as a remarkable strategy, if properly executed and precautions are taken to avoid malnutrition. It has been reported that if it is initiated during midlife, it can result in significantly decreased metabolic risk factors and a substantial longevity benefit independent of the effects on reproductive capabilities [Kaerberlein et al., 2006; Holloszy and Fontana, 2007].

The effects induced by CR include a potential protection of neurons against excitotoxic and metabolic insults [Mattson et al., 1997]. CR may also have a preventive effect on Aβ generation and subsequent Aβ depositions, through mechanisms associated with α-secretase activity and the non-amyloidogenic pathway [Wang et al., 2005; Pasinetti, 2007].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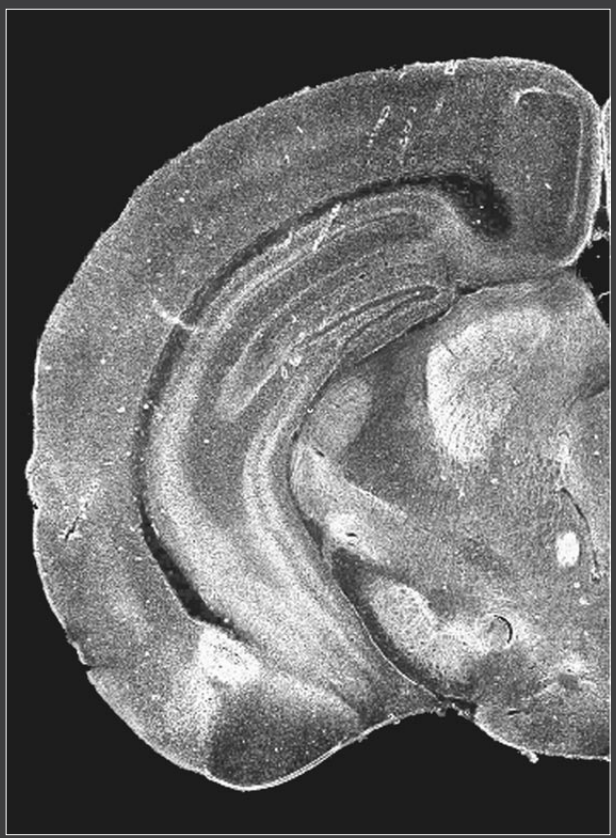
Aims

The general aim of this thesis was to study the effects of cholesterol and cholesterol-lowering therapy with rosuvastatin, on key factors associated with Alzheimer's disease.

The specific aims were as follows:

- To investigate the effects of 24(S)-hydroxycholesterol, 27-hydroxycholesterol and cholesterol 24-hydroxylase, on the processing of the amyloid precursor protein in human neuroblastoma SH-SY5Y cells, with focus on α- and β-secretase activity, and levels of soluble APPα and total soluble APP.
- To investigate the effects of pre-treating human neuroblastoma SH-SY5Y cells with rosuvastatin prior exposure to toxic aggregates of $A\beta_{1-42}$ oligomers, on neurodegeneration and the processing of the amyloid precursor protein, with focus on caspase-3 and α-secretase activity.
- To investigate the effects of rosuvastatin on inflammation in WT and ApoE-/- mice provided with high levels of dietary cholesterol for 18 weeks, with focus on gliosis, presence of activated microglial cells and levels of the pro-inflammatory cytokine interleukin-6 in plasma and brain tissue.
- To investigate the effects of rosuvastatin on lifestyle risk factors in WT and ApoE-/- mice provided with high levels of dietary cholesterol for 18 weeks, with focus on body weight gain, plasma cholesterol and plasma lipoprotein levels.



Materials and Methods

PAPER I–II

Cell culture conditions, reagents and treatments

Human neuroblastoma SH-SY5Y cells were cultured in 60-mm cell culture dishes using Dulbecco's Modified Eagle's Medium:Ham's F12 (DMEM:F12; 1:1; v/v) with Glutamax (paper I) or 365 µg/ml L-Glutamine and 50 µg/ml Gentamicin (paper II), and 10% (v/v) fetal bovine serum (FBS). All media, supplements and reagents were obtained from Gibco Invitrogen unless otherwise stated. Cells were plated at 5 x 10^4 cells/well into 96 well microtiterplates (Nunc), at 0.5 x 10^6 cells/dish into 60 mm culture dishes (Corning Costar) or at 10 x 10^4 cells/well on 8 well culture slides (Becton Dickinson, Falcon), and allowed to adhere. The cells were cultured for 6–7 days under non-differentiated conditions, until a confluence of 80% was achieved. The Trypan Blue Stain assay was used to count the cells and evaluate their viability as a percentage between viable and non-viable cells. The viability of cells was >97% prior experimental procedure. All cultures and incubations were kept in a humidified incubator at 37°C, 5% CO^2. Serum-free DMEM:F12 without antibiotics was used during all treatments and cells incubated without any treatment were used as control. Protein quantifications were made using the BCA protein assay (Pierce Biochemicals).

To the cell culture study in paper I we obtained purified 24S-hydroxycholesterol (24S-OHC) from Bio-Nuclear Scandinavia AB and 27-hydroxycholesterol (27-OHC) from Steraloids. A stock of each oxysterol was prepared in ethanol and stored in the dark at -70°C. The cells were incubated with hydroxycholesterol by incubation for 24 hours at 37°C in serum-free medium with 24S-OHC, 27-OHC or a mixture of 24S-OHC and 27-OHC (1:1) to a final concentration of 5 µM/mL.

The synthetic peptide $A\beta_{1\text{-}42}$ used in paper II was obtained from US Peptide Inc. A stock solution (25 µM) was dissolved in serum-free medium and

incubated for 24 hours at 37°C in a sealed tube to allow self-aggregation and oligomerization. Rosuvastatin Calcium was provided by AstraZeneca (UK). DL-Mevalonate Acid Lactone (mevalonate) was purchased from Sigma-Aldrich. Stock solutions of rosuvastatin (200 μM) and mevalonate (4 mM) were dissolved in serum-free medium and mixed thoroughly immediately before treatment of the cells. SH-SY5Y cells were pre-treated with 20 μM rosuvastatin for a period of 24 hours, before exposure to 2.5 μM $A\beta_{1-42}$ alone or concurrent with 400 μM mevalonate for a period of 24 hours.

Cell culture transfection with CYP46

The SH-SY5Y cells in paper I were transfected with the complete open reading frame of cholesterol 24-hydroxylase of either human (CYP46A1) or murine (CYP46a1) origin, under the control of a viral promoter, using Lipofectamine 2000. A 1:1 ratio of DNA:Lipofectamine was used in all transfections. The cells were transfected and allowed to recover for 72 hours at 37°C in DMEM:F12 with Glutamax and 10% FBS. To ensure that the effect was specific for the introduced plasmid, and not merely the addition of DNA, or cationic lipids (i.e. the transfection reagent), we performed control experiments using an unrelated plasmid (the commonly used reporter plasmid pGL3-basic=mock) or crude lipofectamine.

Determination of α- and β-secretase activity

We determined the α- and β-secretase activities using a Fluorometric α- and β-Secretase Activity Kits obtained from R&D Systems. The cell culture treatments described above in paper I, were lysated using 2 mL of cell extraction buffer, centrifuged at 10.000 x g for 1 min at 4°C and 500 μl of the supernatants was removed. In paper II the cells were lysated with a repeated freeze-thaw cycle for 15 minutes, and the supernatants were collected by centrifugation at 10.000 x g for 15 min at 4°C and stored at -70°C. The supernatants were transferred (50 μL/well; 50 μg total protein) to a 96-well microplate (Nunc F16 Black MaxiSorpTM, Nunc), with 50 μL 2× reaction buffer and 5 μL substrate added to a volume of 105 μL. The substrate consisted of the APP peptides YEVHHQKLV (α-secretase) and REEVNLDAEFKR (β-secretase) respectively, using the reporter system EDANS/DABCYL. The plate was sealed with a plastic film, gently mixed by tapping and incubated in the dark for 2 hours at 37°C. The fluorescent emission from EDANS was analyzed and measured at $\lambda = 345$ nm (excitation) and $\lambda = 500$ nm (emission). High fluorescence was correlated with high enzymatic activity. Measurements were performed using a Safire II microplate reader with Magellan PC software (v6.2; Tecan Austria Gmbh) at 37°C (paper I), or a FLUOstar Galaxy (BMG Labtechnologies Inc) at 37°C (paper II). Recombinant human TACE (0.5 μg/ml), generously provided by R&D Systems, was used as the positive control in paper II.

Sample preparation for ELISA analysis of sAPP

Cells at 80% confluence (equivalent to approximately 1x107 cells per plate) were treated with the oxysterols as described above in paper I, with the medium removed and saved for ELISA analysis. The cells were rinsed with 5 mL PBS pH 7.4 and solubilized in 500 µL lysis buffer (1x Protease inhibitor complete (Roche) and 1% Triton (Sigma-Aldrich) diluted in PBS pH 7.4). Following incubation at 4°C for 20 minutes the cell lysates were collected, mixed and centrifuged at 13.000 x rpm for 10 min at 4°C. The supernatants, containing the released proteins, were saved at -20°C until required for ELISA analyses. The protein contents of the cell lysates were measured by DC protein assay according the instructions of the manufactures (BioRad).

Double-antibody sandwich ELISA analysis of sAPPα

150 µL of the rabbit polyclonal antibody, AB5076 (Chemicon) 1:40.000, were diluted in a coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6), used to coat the individual wells of the 96-well microtiter plates (Immuno Modulies, Maxisorp, Cert Nunc 469949, VWR). Incubation was performed over night at 4°C by shaking. The coating buffer was then discarded and the wells were blocked with 250 µL/well blocking buffer (5% bovine serum albumin (BSA) in coating buffer) and incubating for 2 hours at RT by shaking horizontally. The blocking buffer was discarded and the wells were washed 3 x with 300 µL TBS-T (0.05% Tween 20, 0.01% NaN_3 [26628-22-8] (Sigma-Aldrich), in TBS pH: 7.4) buffer. Either 150 µL cell medium or 75 µL cell lysate from cells treated as described above in paper I was added to the wells and incubated over night at 4°C by shaking. These antigens were then discarded and the wells were washed two times with 300 µL/well PBS, pH: 7.4.

Soluble APPα (sAPPα) was detected by applying 100 µL/well of the mouse monoclonal antibody against human sAPP (Chemicon) 1:5000, diluted with TBT-T-BSA (0.1% BSA, 0.05% Tween 20, 0.02% NaN_3, in TBS). The plate was then incubated over night at 4°C. The detection antibodies were discarded and the wells were washed 3 x with 300 µL/well PBS-T. 150 µL/well of the alkaline phosphate labelled bovine anti mouse IgG antibodies, 1:2500 diluted in TBT-T-BSA (sc-2377, Santa Cruz Biotechnology) was applied and incubated 2.5 hours at RT. The wells were washed 3 x with 300 µL with TBS-T. 200 µL/well Substrate (4-Nitrophenyl phosphate-Na_2-6H_2O, Fluka, Biochemica, [71770]) diluted in DEA buffer (1M Diethanolamine, 0.5M $MgCl_2$. 6H_2O, 0.01 % NaN_3 in H_2O pH to 9.8) was applied and incubate at RT for 2.5 hours in darkness Absorbance was measured at A_{405} nm using a Safire II microplate reader with Magellan PC software (v6.2; Tecan Austria Gmbh) at 37°C. All measurements were calculated as percentage of control.

Direct ELISA analysis of total sAPP

In contrast to the Double-antibody sandwich ELISA, plate wells were also coated directly, with either 150 µL cell medium, or 75 µL cell lysate from cells treated as described above in paper I, and incubated over night at 4°C. Using the mouse monoclonal antibody against human sAPP and method to measure absorbance described above, we were able to detect total sAPP in these wells.

Determination of Caspase-3 activity

The caspase-3 activity was determined in paper II using a modified colorimetric assay (CaspACE™ assay, Promega). The caspase-3 specific cleavage at the C-terminal side of the aspartate residue of the amino acid sequence DEVD (Asp-Glu-Val-Asp) was measured using the Ac-DEVD-pNA (chromophore; p-nitroaniline (pNA)) substrate. The cell culture treatments described above in paper II were lysated with a repeated freeze-thaw cycle for 15 minutes after which the supernatants were collected by centrifugation at 10.000 x g for 15 min at 4°C and stored at -70°C. The supernatants were mixed individually and transferred (20µl/well; 20 µg total protein) to a 96 well microtiterplate (Nunc), with 32 µl of Caspase assay buffer, 2 µl DMSO, 10 µl DTT (100 mM), 2 µl Ac-DEVD-pNA Substrate (10 mM) and ddH20 added to a final volume of 100 µl. The plate was sealed with a plastic film, gently mixed by tapping, and incubated over night at 37°C, 5% CO^2. Absorbance was measured at A_{405} nm for the free pNA products and corrected against the blank using a Safire II microplate reader with Magellan PC software (v6.2; Tecan Austria Gmbh) at 37°C. All measurements were calculated as percentage of control, with high absorbance correlated with high activity of caspase-3.

Visualization of non-viable cells

The fluorescent dye propidium iodide (PI), used in paper II to identify necrotic cells and cells in the late stages of apoptosis, was obtained from Sigma-Aldrich. Cells cultured on 8 well culture slides were treated as described above and washed with 300 µl cold PBS (phosphate buffered saline), exposed to 200 µl PI (1.0 mg/ml), incubated for 15 min at 37°C, washed twice with 200 µl cold PBS and mounted with fluorescent mounting medium (Dako, Denmark). Nuclei of non-viable cells with damaged membrane were stained red with propidium iodide, indicating that the dye had intercalated with cellular DNA. Visualization and photography was performed using a Nikon Eclipse E800 fluorescence microscope and a Nikon FDX-35 digital camera connected to a PowerMac computer with Open Lab (v2.1.0) image processing software (Improvision). The controls were used to analyze auto fluorescence and any spontaneous neurodegeneration.

Determination of cell viability (MTT assay)

The cell viability and metabolic activity reported in paper II, was analyzed using a MTT assay obtained from Roche Diagnostics Scandinavia, which measures the ability of mitochondrial dehydrogenase to form formazan through cleavage of the tetrazolium ring of MTT. Cells cultured in 96 well microtiterplates were treated as described above. 0.5 mg/ml MTT labelling agent dissolved in phosphate buffered saline (PBS) was added to each well and incubated for 4 hours in a humidified atmosphere at 37°C, 5% $CO^{2\cdot}$ The formed formazan crystals were dissolved by the addition of 100 µL of 10% SDS in 0.1 M HCl and incubation over night in a humidified atmosphere at 37°C, 5% CO^{2}. Absorbance was measured at A_{570} nm and A_{670} nm, using a Safire II microplate reader with Magellan PC software (v6.2; Tecan Austria Gmbh) at 37°C (paper I), or a SpectraMax 250 (Molecular Devices) at 37°C (paper II). Optical imperfections in the microplate were corrected by the formula: A_{570} nm - A_{670} nm. Absorbance was correlated with high activity of live mitochondria and was used as a measurement of cell viability. All measurements were calculated as percentage of the controls.

Statistical analysis

Data was checked for normality and analyzed for statistical significance using Student's *t*-test and analysis of variance (ANOVA). Statistical significance was determined at $p < 0.05$. Homogeneity of variance was tested by Levene's test. Statistical analysis was performed using Statistica PC software (v.6.0; StatSoft). All values are expressed as percentage of the mean value of the control group ± SEM.

PAPER III–IV

Animal models and experimental treatments

Weight-matched littermates of C57BL/6/Bkl wild-type mice (WT; n=18) and B6.129P2-ApoE^{tm1Unc} N11 apolipoprotein E knockout mice (ApoE-/-; n=18), were obtained from Scanbur BK and Taconic Europe respectively. All animals were housed in the accredited animal facility at the Karolinska University Hospital Huddinge, in standard M2-cages at a constant temperature of 24–26°C under controlled lighting conditions, with food and water available ad libidum. The animals were conditioned during four weeks prior to the onset of the study. Experimental treatment was initiated at 14 weeks of age (n=6 mice/group) and withheld for 18 weeks: high cholesterol (HC) diet also known as western diet (R638 [15.6 MJ/kg; nitrogen-free extract (NFE) 43.9%, fat 21.0%, protein 17.2%, H2O 10.0%, fibres 3.9%, ash 4.1%]; Lactamin), HC diet with rosuvastatin treatment (HCRS) and normal diet (ND). Rosuvastatin was kindly provided by AstraZeneca (UK). The mice on ND were used as a control group. Rosuvastatin was provided in the drinking water at a concentration of 0.5 mg/kg body weight/day. The volume of the drinking water consumed was monitored to ensure that the dose received was as indicated above. Ethical consent was granted by the local regional ethics committee prior to the onset of the study. Body weight measurements were performed once weekly. The percent gain in body weight was calculated using the body weight values at the time of euthanasia, compared with body weight values prior to treatment. The animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (600 mg/kg body weight; Apoteket), after which plasma samples (600 µl/mice) were collected in a separation tube (BD Microtainer PST LH; Becton Dickinson), through puncture of the left ventricle of the heart and stored at -70°C until further analysis. Immediately after euthanasia, the brains were removed and snap-frozen in -40°C isopentane for 15 sec and stored at -70°C.

Tissue sectioning

Coronal sections 14 µm thick were processed using a cryostat (Microm HM 500 M) at -20°C, from -2.80 mm posterior to bregma according to the stereotaxic coordinates given by the mouse brain atlas [Paxinos and Franklin, 2004]. The sections were dried overnight at 4°C and fixed in acetone at room temperature (RT) for 10 min, dried at RT for 15 min and stored at -20°C until immunohistochemical analysis.

Immunohistochemical analysis

The frozen sections were briefly dried at RT for 2 min, rehydrated in PBS and incubated at RT for 10 min in a dark chamber with 0.3% H2O2 in Methanol.

Non-specific binding was inhibited by incubation at RT for 10 min with 1% Bovine Serum Albumin, Fraction V (BSA; Sigma-Aldrich). The sections were subsequently washed with PBS at RT for 10 min and incubated overnight in a dark humidified chamber at 4°C with antibodies against GFAP (1:2000; Z0334; Dako; paper III), microglia F4/80 (1:100; MCA479B; AbD Serotec; paper IV) or IL-6 (1:300; PM626; Pierce Endogen; paper IV) in 1% BSA. All sections were kept in a dark humidified chamber at 4°C. The sections was rinsed with PBS and incubated at RT for 30 min with respective biotinylated secondary antibodies (1:300; Dako). After washing with PBS, the antigen-antibody complexes were visualized by incubation at RT for 60 min with an avidin-biotin horseradish peroxidase complex (ABComplex/HRP; Dako). They were rinsed in PBS, exposed to diaminobenzidine (DAB; Sigma-Aldrich), rinsed again in ice-cold PBS, until the reaction stopped, and mounted with an aqueous mounting medium (S3025; Dako). Sections incubated in the absence of the primary antibody or with immunoglobulin fractions (1:300; Dako), served as negative controls.

Cell count for cells labelled with GFAP, was calculated from three randomly selected areas in the CA1 field of the hippocampus (paper III). Cell counts for cells labelled with F4/80 or IL-6, was calculated from three randomly selected areas in the capsula externa (paper IV). All calculations were made using a standardized counting grid (0.24 x 0.24 mm) at 40x magnification. Each measurement was repeated three times. Only cells with a distinct staining of the cytoplasm and a discernable nucleus were counted. Cells edging the upper and left grid lines were included, whereas cells edging the bottom and right lines were excluded from the counts. The counting was performed blinded. The results were calculated using the mean number of counted cells from each mouse (n=5 mice/group), translated to cells/mm^2 using the known grid area.

Determination of plasma lipoproteins levels

Plasma from each mouse was sampled prior to sacrifice. Plasma total-cholesterol (TC) and high-density lipoprotein (HDL) levels were measured using enzymatic techniques (Hitachi 917). Low-density lipoprotein (LDL) was calculated according to the Friedewald's Formula.

Determination of plasma IL-6 levels by ELISA

The plasma levels of IL-6 in paper IV, were analysed from 50 µl blood samples withdrawn prior to sacrifice, using an IL-6 ELISA Kit (EM2IL6; Pierce, Endogen). The samples were incubated on an anti-mouse IL-6 pre-coated 96-well strip plate according to the instructions from the manufacturer, and visualized with streptavidin-HRP and a TMB substrate solution. Absorbance was measured at A_{450} nm and A_{550} nm using a Safire II microplate reader with

Magellan PC software (v6.2; Tecan Austria Gmbh). Optical imperfections in the microplate were corrected by the formula: A_{450} nm - A_{550} nm. The amount of mouse plasma IL-6 (pg/mL) was determined using a standard curve.

Statistical analysis

Data were checked for normality and analyzed for statistical significance using Student's *t*-test and analysis of variance (ANOVA). Statistical significance was determined at $p < 0.05$. Homogeneity of variance was tested by Levene's test. Spearman's non-linear correlation was used to calculate the relation between the numbers of cells labelled with GFAP, F4/80 or IL-6, plasma total-cholesterol and body weight gain. Statistical analysis was performed using Statistica PC software (v.6.0; StatSoft). Values are expressed as mean value ± SEM or as percentage of the mean value of the control group ± SEM (n=5 mice/group).


Thermo

Results and Discussion

IN VITRO-MODELS WITH HUMAN NEUROBLASTOMA SH-SY5Y CELLS

Paper I: Selective processing of APP by 24S-OHC

The association between cholesterol and AD is, as previously described, an established connection with disturbed cholesterol homeostasis and hypercholesterolemia as important risk factors. But the question has been raised as to whether the cholesterol in the plasma affects the brain? Plasma cholesterol is under normal physiological circumstances not able to cross the BBB, and essentially all cholesterol in the CNS is synthesized in situ. It has been demonstrated that cholesterol homeostasis in the CNS is maintained by the enzyme CYP46A1 with a slow conversion of brain cholesterol into 24S-OHC, which constitutes the major pathway for excretion of excess cholesterol across the BBB. Clinical studies have further demonstrated that the level of 24S-OHC is decreased, whereas 27-OH is increased in the brain of AD patients [Heverin et al., 2004; Björkhem et al., 2006]. Intriguing and of major significance for paper I is the finding that 27-OHC has a net flux from the circulation into the CNS, but under normal conditions are found at low levels in the brain due to an active elimination process [Heverin et al., 2005; Meaney et al., 2007].

Thus, in paper I we aimed to investigate the function of brain cholesterol in the pathogenesis of AD, using a human neuroblastoma cell line and the oxysterols 24S-OHC and 27-OHC, as well as cells transfected with variants of CYP46. Although the response was less marked than in the study by Brown et al., we were able to confirm and expand on the former investigation [Brown et al., 2004]. We demonstrated that stimulation with 24S-OHC increased the α-secretase cleavage and decreased the β-secretase cleavage of APP. We suggest that 24S-OHC exercise a selective preference towards non-amyloidogenic pathway through a mechanism different from 27-OHC.

The decreased response exerted by 24S-OHC on the APP processing, ob-

served when 24S-OHC and 27-OHC were combined in the cell culture, is an important observation as the latter oxysterol represents a possible linkage with plasma cholesterol. The possibility must be considered that 27-OHC are capable of replacing the more active 24S-OHC and thus reduce the effects of 24S-OHC on the APP cleavage.

The α-/β-secretase activity ratio was calculated in order to compare the differential effects and was found to be increased after exposure to 24S-OHC, as well as in cells transfected with either human (CYP46A1) or murine (CYP46a1) cholesterol 24-hydroxylase. We suggest that the α-/β-secretase activity ratio could be a convenient method for screening alterations in the APP processing pathway in response to different pharmacological treatments.

In conclusion, we have in paper I demonstrated that 24S-OHC favours the non-amyloidogenic APP processing, with subsequently increased extra- and intracellular levels of sAPPα and total sAPP. The selective influence on APP processing by 24S-OHC and the decreased response observed with 27-OHC, provides new insight in how cholesterol affects the AD pathology. Together with the findings that the levels of 24S-OHC and 27-OHC are altered in the brain of AD patients, and that 27-OHC have a flux into the brain, the described observations in paper I may support the hypothesis that 27-OHC could be a part of a yet undefined link between plasma cholesterol levels and AD [Björkhem et al., 2006].

Paper II: Selective processing of APP and decreased apoptosis by rosuvastatin during Aβ exposure

The previously described connection between cholesterol and AD has been interpreted as having a potential for cholesterol-lowering compounds, such as statins, which could, in turn, have a beneficial effect on AD. This has so far yielded inconsistent results in clinical studies. Though, it should be noted that different statins may exert slightly different actions. Even though statins per se have been associated with a decreased prevalence of AD, we find that the lipophilic statins have been reported to cause apoptosis in a variety of cells [Negre-Aminou et al., 1997; Kubota et al., 2004], whereas to our knowledge no such effects have been reported for the statins that belong to the hydrophilic group.

Thus, in paper II we aimed to examine the preventive potential of pre-treating human neuroblastoma SH-SY5Y cells with hydrophilic rosuvastatin prior exposure to aggregates of $A\beta_{1\text{-}42}$ oligomers. Furtermore, we aimed to analyse the effects of the cholesterol-lowering therapy on α-secretase activity demonstrated to be effectively selected by 24S-OHC in paper I, and caspase-3 activity, central regulator and early physiological marker of apoptotic cell death [Nicotera et al., 1999; Riedl and Shi, 2004]. It has been hypothesized that Aβ peptides could function as extracellular signal molecules for caspase-3 activation in AD

[Takuma et al., 2004], supported by the findings of an up-regulated caspase-3 activity in the medial temporal lobe of subjects with early AD [Gastard et al., 2003].

With our results we have established that rosuvastatin therapy decreases caspase-3 activity and increases α-secretase activity in human neuroblastoma SH-SY5Y, cells without affecting cell viability. These results have been confirmed by independent in *vitro* and in *vivo* studies [Kilic et al., 2005; Xiu et al., 2006]. We can also report a significant reduction of caspase-3 activity, and an increase of α-secretase activity, in cells treated with rosuvastatin prior to exposure to toxic amyloid aggregates. The response to rosuvastatin was significantly attenuated by mevalonate, which suggests that the cholesterol synthesis were restored. We suggest that rosuvastatin exerts its effects through an inhibition of the isoprenoid pathway and regulation of prenylation of important small GTPase signalling molecules. We further suggest that the connection between statins and AD could be a link with the lipid rafts, as they have a high cholesterol-associated regulatory function on apoptosis and APP processing.

Additionally, our results suggest that treatment with rosuvastatin alone not have an effect on cell viability as measured by the MTT assay, while it was clearly demonstrated by the staining with PI that rosuvastatin may decrease the detrimental effects induced by exposure to toxic amyloid aggregates. However, the percentage of viable cells, as measured by the MTT assay, did not differ when exposed to $A\beta_{1\text{-}42}$ alone, compared with rosuvastatin treatment prior exposure to $A\beta_{1\text{-}42}$. We hypothesized that the observed discrepancy could be explained by two closely related factors; (i) the MTT assay gives a measure of the metabolic activity in the culture and can not identify the amount of surviving cells per se, and (ii) the possibility must be considered that surviving cells exhibit a down-regulated stress response with decreased metabolic activity as a reaction to the Aβ exposure [Magrané et al., 2005].

In conclusion, we have in paper II reported two mechanisms clearly associated with the pathogenesis of AD and significantly modulated by rosuvastatin therapy in *vitro*. The connection between the increased α-secretase activity and decreased caspase-3 activity, in cells treated with rosuvastatin prior to exposure to aggregated $A\beta_{1\text{-}42}$ oligomers, is an important finding, and may serve as a promising target for research on prevention treatment in AD and related neurodegenerative diseases.

IN VIVO-MODELS WITH WT AND APOE-/- MICE

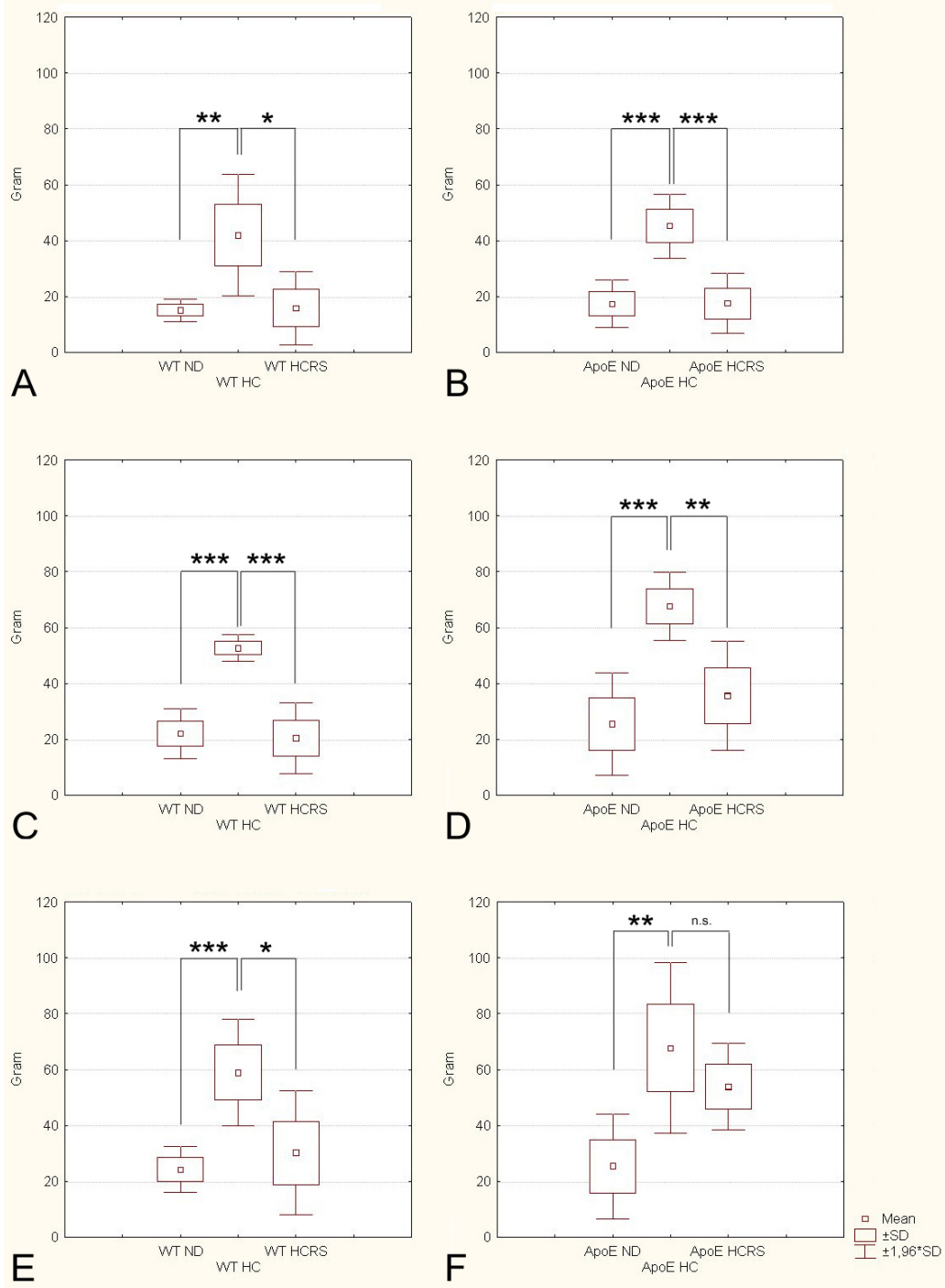
Paper III: Rosuvastatin therapy has an effect on gliosis and lifestyle risk factors

The connection between cholesterol and AD has been described previously, and in paper III we aimed to go further with the results from paper I–II and examine the association between dietary cholesterol and AD in *vivo*. A large number of clinical studies have shown consistent results in their claim that dietary restriction may have the possibility to suppress brain aging and neurodegenerative disorders such as AD. The association between AD and ApoE deficiencies have been suggested to be most critical during the early and middle stages of the disease [Dupuy et al., 2001], which further suggests that any therapy that will have effect on the disease progression must take place before this stage. Lack of ApoE has been found to dramatically reduce Aβ deposition [Bales et al., 1997], and it has been suggested that an age-related BBB leakage associated with ApoE deficiency could be one of the mechanisms that link ApoE ε4 with cholesterol [Hafezi-Moghadam et al., 2007]. In support of the detrimental effects of ApoE deficiencies it has previously been reported that ApoE-/- mice provided with a western type high cholesterol (HC) diet have increased gliosis, tau hyperphosphorylation and elevated levels of inflammatory mediators such as IL-6 [Crisby et al., 2004; Rahman et al., 2005.b; Rahman et al., 2005.a].

Thus, in paper III we analyzed whether the increased gliosis, as monitored by GFAP and previously demonstrated in WT and ApoE-/- mice provided with a HC diet, could be decreased by rosuvastatin therapy, and how this would affect lifestyle risk factors such as body weight gain and plasma lipoprotein levels.

We found that high levels of dietary cholesterol induced gliosis in WT and ApoE-/- mice in support of previous studies, with an increased body weight gain and elevated levels of plasma total cholesterol (TC). Using lipid profiles, we found the increased TC levels in the WT mice to be caused by increased

Figure 6. *Body weight gain in WT and ApoE-/- mice after 6 weeks (A-B), 12 weeks (C-D) and 18 weeks (E-F) respectively. The mice were provided with high levels of dietary cholesterol (WTHC, ApoEHC), or diet with rosuvastatin therapy (WTHCRS, ApoEHCRS). Mice on normal diet were used as control (WTND, ApoEND). Body weight gain was significantly increased by the high-cholesterol diet, and significantly decreased with the rosuvastatin therapy, in WT and ApoE-/- mice after 6 and 12 weeks. However, the significance was found to decrease between 6 to 12 weeks in ApoE-/- mice, whereas it increased in WT mice. The response on body weight gain was after 18 weeks found to be insignificant in ApoE-/- mice, whereas it remained significant in WT mice. n=5 mice/group; *=p<0.005; **=p<0.001; ***=p<0.0001; n.s=non significant.*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levels of HDL and LDL, whereas only LDL were increased in ApoE-/- mice. Treatment with rosuvastatin significantly decreased the observed gliosis in WT and ApoE-/- mice. Furthermore, we demonstrated that the therapy had a significant effect on lifestyle risk factors in WT mice, with decreased body weight gain, as well as decreased plasma TC and LDL levels.

However, despite the positive effects observed on gliosis in ApoE-/- mice, we found the therapy to have an insignificant effect on the measured life style risk factors. We suggest that the observed response are associated with the notion that ApoE-deficiency, per se, may have a negative effect on various metabolic pathways, whereas the response on gliosis could be attributed to the modulation of signalling pathways involving isoprenoids, as well a reduction of overall atherosclerosis [Crisby et al., 2002; Grosser et al., 2004]

In Figure 6 we present some hereto unpublished data from the study, which clearly illustrates the association between rosuvastatin and ApoE-deficieny, as demonstrated by body weight gain after 6, 12 and 18 weeks on high levels of dietary cholesterol, with or without, rosuvastatin therapy. Accordingly, the declining response to the therapy was primarily observed in the ApoE-/- mice, with a correlation to plasma TC levels after 18 weeks of HC diet and rosuvastatin therapy.

In conclusion, paper III is the first study to report that rosuvastatin may prevent gliosis in the brain of WT and ApoE-/- mice and affect lifestyle risk factors in WT mice provided with a HC diet. Clarification of the specific mechanisms involved and the responses observed, is a challenge for further research, but unpublished data suggests that treatment with rosuvastatin is most effective before midlife, with a clearly age- and ApoE -associated declining response to the cholesterol-lowering therapy.

Paper IV: Microglial activity and plasma IL-6 levels decreased by rosuvastatin therapy

We have in paper III demonstrated the detrimental effects of a western type high cholesterol (HC) diet on gliosis and lifestyle risk factors, and the beneficial effects exerted by rosuvastatin therapy. We extended these findings in paper IV towards the effects of dietary cholesterol and cholesterol-lowering therapy on inflammation and inflammatory mediators.

The occurrence of an inflammation, and the subsequently occurring pro-inflammatory cytokines, has been lively debated whether they have beneficial or detrimental functions in AD. Available facts clearly demonstrate that neuroinflammation is not an AD-specific event; nevertheless, AD is characterized by a chronic state of inflammation. In addition and as previously described, the Rotterdam study has demonstrated that plasma levels of inflammatory proteins such as IL-6 are increased before the clinical onset of AD. Other studies

have demonstrated elevated levels of IL-6 in the diffuse plaques formed at an early stage of the pathology. Treatment with rosuvastatin has been reported to induce isoprenoid depletion, with an indirect effect on microglial activation and inflammation, as the isoprenoids play a significant role in the activation of IL-6 mediated inflammation [Heinrich et al., 1998; Khwaja et al., 2000]. It has also been suggested that it is possible to inhibit the secretion of IL-6 and reduce the cell viability of human microglia cells, by treating them with statins [Lindberg et al., 2005]. The consequences of a HC diet on inflammation has been demonstrated, with an increased microglia load and elevated levels of IL-6 in the brain of WT and ApoE-/- mice [Rahman et al., 2005.b].

Thus, in paper IV our aim was to examine whether rosuvastatin therapy could prevent the increased microglial load and decrease the levels of IL-6 in the brain and plasma of WT and ApoE-/- mice provided with a HC diet. We found that a HC diet provided for 18 weeks increased the microglial activity in the brain of WT and ApoE-/- mice, in support of previous studies. Treatment with rosuvastatin demonstrated a clear and significant decrease in microglial activity in WT and ApoE-/- mice provided with a HC diet.

We could also report that despite the increased number of activated microglial cells, there was no increase in IL-6 positive cells observed in the brains of WT or ApoE-/- mice on a HC diet. However, even though the levels of IL-6 in the brain remained largely unaffected were the plasma IL-6 levels found to be significantly increased in WT and ApoE-/- mice provided with a HC diet. Treatment with rosuvastatin resulted in a large 45% reduction of plasma IL-6 levels in WT mice on a HC diet; although, the reduction never reached significance due to individual variations among the WT mice given dietary cholesterol without therapy.

The general response on the dietary cholesterol was found to be significantly weaker in the ApoE-/- mice, and we suggest that the observed results in paper IV are linked with the findings reported for the ApoE-deficient mice in paper III. We further hypothesize that statin therapy of its own accord is insufficient to regulate the transcription factors involved in IL-6 mediated inflammation.

In conclusion, we have in paper IV clearly demonstrated that statin treatment decrease microglial activity in WT and ApoE-/- mice on a HC diet provided for 18 weeks. The same therapy was however unable to significantly affect the diet-induced chronic inflammatory state; an inflammation possibly triggered by a defective regulation of cytokine production and related transcription factors [Libermann and Baltimore, 1990]. With these observations we support the growing body of evidence that indicates the importance of dietary cholesterol in AD, and how statins may have a beneficial influence in the prevention of AD.

Concluding Remarks and Future Perspectives

Current research suggests that the decisions we make early in life could have a profound effect on the manner in which our cognitive abilities and selfhood are preserved in old age. The research this thesis is built on has focused on the implications of cholesterol and cholesterol-lowering therapies in Alzheimer's disease, but with these basic features we have touched on more than one aspect of the AD pathology. A major concern is the fact that many of the pathological hallmarks in AD, are not AD-specific events as such, but occur in a number of different neurological disorders. In addition, with the exception that we still have to discover what effects current trends on health, life style and diet will have on dementia and prevalence of AD, it has been suggested by demographic studies that the prevalence will increase rapidly in the next 50 years [Wancata et al., 2003]. Research on AD has primarily focused on means to reduce the symptoms, but a new model has been proposed, with the aim to go beyond the clinical symptoms; the model postulates that a latent expression of specific genes, triggered at the developmental stage by environmental agents, intrinsic mechanisms, or dietary factors, could disturb gene regulation in a long-term fashion with the pathological results revealed significantly later in life [Lahiri et al., 2007].

Factors with a previously described age-associated connection with AD are midlife plasma total cholesterol (TC) levels, 24S-OHC levels and inflammation; the latter manifested by gliosis and microglial activation, as well as increased levels of pro-inflammatory cytokines. A possible connection between plasma TC levels and AD has been described in the flux of 27-OHC over the BBB, with an inhibitory influence on the selective non-amyloidogenic APP processing induced by 24S-OHC (paper I). This connection has potential for further research, aimed towards a deeper understanding of the actual mechanisms involved and how to modulate them. We have in this thesis demonstrated that dietary cholesterol have detrimental effects on inflammation and a number of *classic* life style risk factors, such as body weight gain and LDL levels (paper III–

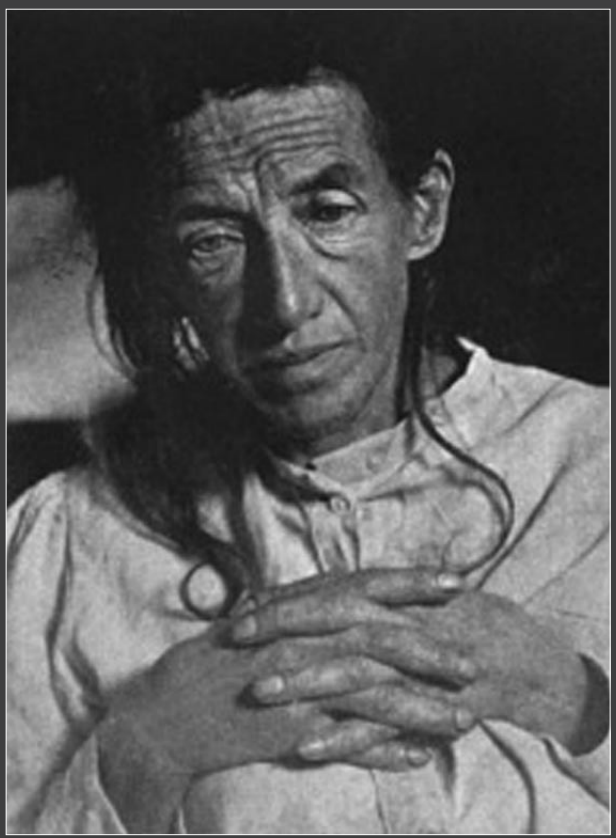
IV). Treatment with rosuvastatin has demonstrated neuroprotective properties in *vitro* (paper II), whereas cholesterol-lowering therapy with rosuvastatin in *vivo* have been able to reduce gliosis, microglial load, plasma IL-6 levels and improve the status of the lifestyle risk factors in an age- and ApoE-associated manner (paper III–IV). Unpublished data, presented in this thesis, supports the hypothesis of an *early life-preventive factor* in AD, which is suggested to be most effective before midlife (paper III). Taken together, it is evident that cholesterol-lowering therapies with rosuvastatin may have beneficial effects on AD, but when combined with ApoE-deficiencies, the therapies may lose their effects after a certain stage in the disease progression. These observations could hypothetically explain some of the discrepancies found in clinical studies, and is supported by the suggestion that the beneficial effects of statins are influenced by plasma cholesterol levels, ApoE genotype and the severity of dementia [Sparks et al., 2006].

Preventative treatment using caloric restriction (CR) can result in a substantial longevity benefit if it is initiated during midlife, and delay the onset of cognitive dysfunction [Kaerberlein et al., 2006]. Although, with the notion that our metabolism changes with age, and that ApoE-deficiencies may have a negative effect on our metabolism, it is suggested that diet alone may not be a clinically useful treatment in elderly people. As an alternative, the combined effect of dietary precautions and cholesterol-lowering therapy may have the potential to become an important preventive strategy in AD, with the aim to circumvent the inflammatory changes due to hypercholesterolemia, and decrease the impact of lifestyle risk factors.

Clinical research on health care and nursing practise have already resulted in better possibilities to improve quality of life for AD patients, but further research into the health economics of dementia, and early preventive treatments, are highly relevant. In addition, larger long-term trials are needed to study the effects of rosuvastatins on cognitive functions and how it interacts with current therapies using acetylcholinesterase inhibitors, as well as any potential interference with the absorption of nutrients.

In conclusion, this thesis has clearly demonstrated the detrimental effects induced by high levels of dietary cholesterol on a selection of Alzheimer's disease-associated factors, and that a cholesterol-lowering therapy using rosuvastatin may have a beneficial influence in the prevention of AD. Furthermore, the thesis discusses the observation of an age- and ApoE-associated response on the therapy. The latter is an important remark, which indicates that rosuvastatin may be an efficient therapy up to a certain point in the progression of the disease, after which the effects decline. These results offer a hypothetical explanation, at least in part, for the discrepancies observed in clinical trials on statins. In addition, further evidence for a potential connection between plasma cholesterol and AD has been presented, with a novel function of 24(S)-OHC in the

healthy brain found to be decreased by the presence of 27-OHC. The findings by Small et al. suggest that it may be possible to prevent early memory deficiencies through a combination of relatively simple precautions [Small et al., 2006]. An alternative strategy is proposed in which it is hypothesized that lifestyle precautions taken in early midlife, in combination with rosuvastatin therapy, could be an effective *lifestyle-preventive strategy* in persons with high risk for developing AD. An effective strategy that may have far-going consequences with preserved cognitive functions in high age, improved quality of life for patients and their families, and decreased cost of dementia care for the society. We need a re-examination of current concepts and practices among clinicians, researchers and patients, and more work on questions of dementia, selfhood, and early prevention therapy, to which the combined efforts presented in this thesis may prove to be a valuable contribution.



Populärvetenskaplig översikt

ALZHEIMERS SJUKDOM

Alzheimers sjukdom (Alzheimer's disease; AD) diagnostiserades för första gången år 1906 av den tyska läkaren Alois Alzheimer. Patienten var en medelålders kvinna vid namn Auguste D som 51 år ålder hade börjat få allvarliga symptom på demens och en gradvis försämring av kognitiva funktioner.

> Kognitiva funktioner innefattar högre hjärnfunktioner som tänkande, perception, uppmärksamhet, uppfattningsförmåga, minne och språk. Begreppet kan härledas ur det latinska ordet *cognoscere*, som betyder att lära.

Auguste D var mycket glömsk, oförmögen att ta hand om sig själv, stundtals orolig och misstänksam även mot sin man, men samtidigt medveten om sitt behov av andras hjälp. Vid obduktionen fann Alois Alzheimer en rad patologiska (sjukliga) förändringar i hjärnan. Dessa förändringar utgör idag några av de kriterier som används för att diagnostisera sjukdomen och består framförallt av små, rundade ärrbildningar (neuritiska plack; amyloid plaques) uppbyggda av giftiga ansamlingar av protein amyloid-beta (Aβ), störningar av nervcellens inre organisation med långa fibrer (neurofibrillär degeneration; neurofibrillary tangles) och förlusten av signalvägarna mellan nervcellerna [Bild 7]. Förändringarna sker i början mycket selektivt, och leder till att nervcellerna förtvinar i områden som framförallt har med minne och språk att göra. Så småningom sker emellertid en generell degeneration av hela hjärnan. Men sjukdomen är komplicerad, och dessa kriterier är i sig själv inte tillräckligt för att kunna ställa en diagnos, utan den måste även kunna stödjas av kliniska symptom och en klinisk diagnos.

Idag har omkring 160 000 personer i Sverige en demenssjukdom, varav cirka 60% utgörs av AD. Över hela världen var antalet patienter beräknat

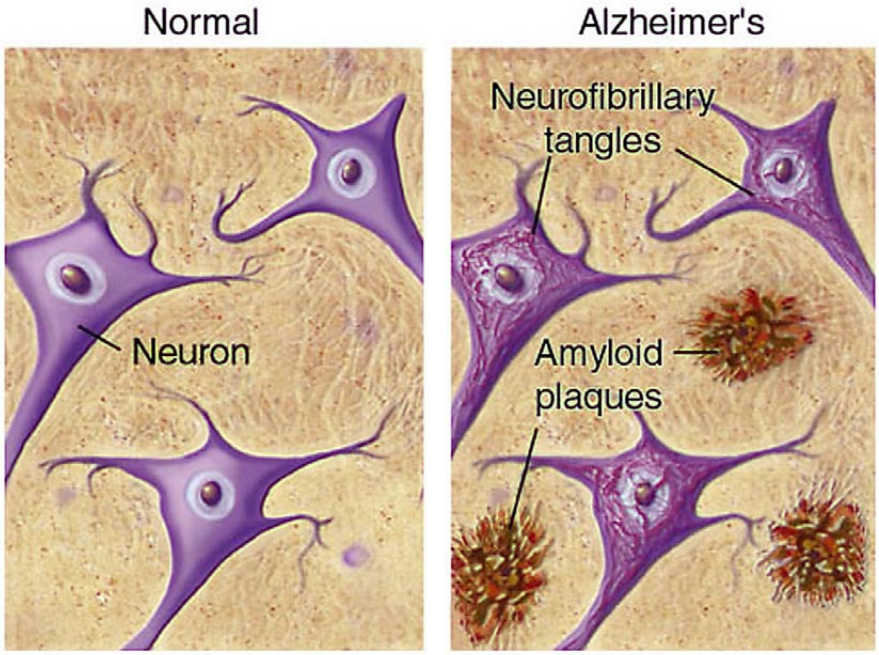


Figure 7. *Normal hjärna med frisk vävnad, jämfört med vävnaden från en hjärna som drabbats av Alzheimers sjukdom; Döende nervceller, neurofibrillary tangles och amyloid (neuritic) plaques.*

till ca 30 miljoner människor år 2003, och bara i Sverige drabbas ytterligare 20–25 000 av någon form av demens varje år. De flesta som drabbats av Alzheimer bor hemma under de tidiga stadierna av sjukdomen och vårdas av sina anhöriga. Vården av demenssjuka i Sverige beräknas kosta drygt 30 miljarder kronor per år, men de anhörigas insatser är mycket svåra att uppskatta i ekonomiska termer och av stor betydelse för samhället.

Alzheimers sjukdom förekommer i två olika varianter, som emellertid inte skiljer sig åt vad beträffar symptom eller behandling. Ärftlig (familial) Alzheimers sjukdom (FAD) drabbar ca 10–15% av alla patienter, medan resterande 85–90% drabbas av den sporadiska formen av Alzheimers sjukdom (SAD). FAD har som namnet antyder en stark ärftlig koppling och kan i vissa fall vara så aggressiv att den drabbar samtliga vuxna inom en familj. Bland dessa ärftliga kopplingar har man funnit ett antal mutationer i generna APP, PS1 och PS2, som alla har en stor betydelse för uppkomsten av de förändringar i hjärnan som definierar Alzheimers sjukdom.

De flesta fall av Alzheimers sjukdom förekommer sporadisk, utan någon tydlig genetisk orsak. Man anser därför att SAD orsakas av den kombinerade effekten av flera olika riskfaktorer, där yttre och inre faktorer tillsammans är av avgörande betydelse.

> Det finns en signifikant ökad risk för ärftlig AD, endast om det inom en och samma familj har funnits minst två generationer i direkt släktled (t.ex. mormor och mamma, eller föräldrar och syskon med samma föräldrar), som drabbats före 65 års ålder och diagnostiserats med AD bortom allt tvivel.

Den viktigaste av dessa riskfaktorer är en medfödd genetisk brist i lipoproteintransportören apolipoprotein E (ApoE), där isoform ApoE ε4 ökar risken att drabbas av sporadisk AD med 300%. Andra riskfaktorer är hög ålder; ökade kolesterolnivåer i medelåldern; diabetes; övervikt; högt blodtryck; låg formell utbildning; klinisk depression; stroke; stress; Downs syndrom och otillräcklig (kontinuerlig) träning av hjärnan. Andra riskfaktorer med en lindrigare koppling till Alzheimers sjukdom är rökning, höga mängder av alkohol och narkotiska preparat. Betydelsefullt är också att kvinnor av okänd anledning drabbas i något högre utsträckning än män.

Koppling mellan Alzheimers sjukdom och ålder är viktig. Sjukdomen kan uppträda tidigt, speciellt vid den ärftliga formen av AD, då den kan uppträda redan vid 28 års ålder, men vanligast är den efter 65 år. Skulle man undersöka alla människor över 65 år kommer ca 10% att vara drabbade av någon form av AD. Undersöker man enbart gruppen mellan 65 och 74 år kommer man att upptäcka att antalet drabbade är ca 5%, vilket ska jämföras med ca 50% bland dem som är äldre än 85 år.

Med undantag för att vi inte vet hur trenderna inom hälsa och träning kommer att påverka riskerna för AD, så har forskarna varnat för att den ökande medelåldern i världen kan medföra att antalet personer som drabbas av demenssjukdomar i framtiden kommer att öka lavinartat. Smärre minnesproblem och en sämre förståelse av komplicerade sammanhang är en naturlig del av vårt åldrande, men hälften av alla svenskar tror att även demens är en naturlig del av vårt åldrande och att det drabbar alla som blir äldre. Det här är en helt felaktig uppfattning! Demens orsakas av sjukdomar eller skador på hjärnan, och de förändringar som sker vid demens, sker utöver de förändringar som är en naturlig del av vårt åldrande.

> Demens är ett begrepp som kan härledas ur de latinska orden *de-* och *mens*, som tillsammans betyder frånvarande sinne.

De första tecknen på Alzheimers sjukdom kan ofta vara ganska svåra att upptäcka. Förutom mindre minnesproblem förekommer svårigheter att hitta ord, uttrycka sig och att förstå komplicerade sammanhang. Utmärkande är att symtomen kommer successivt. Det kan därför vara svårt att kartlägga när problemen egentligen började. Många symtom återfinns, i varierande grad, hos

alla personer med demenssjukdom, men alla personer drabbas olika och sjukdomen fortskrider olika snabbt hos olika personer. Sjukdomen påverkas också av andra sjukdomar och ökad ålder.

Några vanliga symptom på tidig Alzheimers sjukdom är problem med tidsuppfattningen, svårigheter att hitta i omgivningen, att läsa, skriva, räkna och klara av praktiska saker. En del får problem med varseblivningen och har svårt att känna igen det de ser och det blir svårare att resonera med dem. En del personer får också svåra känslomässiga störningar som påverkar humöret och beteendet.

Vid Alzheimers sjukdom dör nervcellerna i hjärnan onormalt fort. Av de områden av hjärnan som brukar drabbas är hippocampus nödvändigt för hjärnas minnesfunktioner, och tinning- och hjässloberna har funktioner för vårt språk [Bild 8]. Varför nervcellerna dör är fortfarande inte helt klarlagt, men en förklaring till många symptom är den uttalade bristen på signalsubstansen acetylkolin, som har en viktig funktion vid uppgifter som kräver minne och/eller uppmärksamhet, och även reglerar hjärnans blodflöde. Detta ledde tidigt till en rad behandlingsförsök som påverkade nivåerna av acetylkolin i hjärnan.

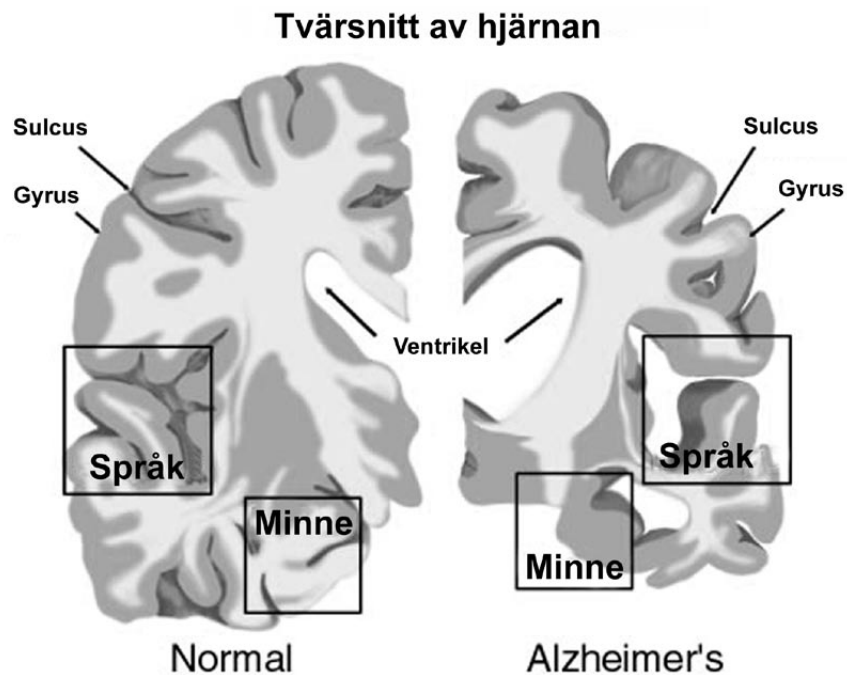


Figure 8. *Tvärtsnitt av en normal hjärna med frisk vävnad, jämfört med vävnaden från en hjärna som drabbats av Alzheimers sjukdom; Bredare sulcus* (dalgångar), *mindre gyrus* (berg) *och större ventriklar* (hålrum). *Två viktiga areor i Alzheimers sjukdom är språk och minne, med en tydlig illustration av den utbredda degenerationen.*

Behandlingar med acetylkolinesterasinhibitorer har visat sig vara en effektiv metod när det gäller att minska de symptomatiska besvären, men det finns fortfarande ingen behandling som har effekt på den huvudsakliga sjukdomsprocessen. Alzheimers sjukdom är en kronisk sjukdom med dödlig utgång.

Alzheimers sjukdom och andra demenssjukdomar är de största enskilda anledningarna till att vi idag har stora vårdbehov i hemmen och på våra institutioner. Som en direkt konsekvens av sjukdomen anser man ofta att patienter med demens och allvarliga kognitiva problem inte längre *är där*, med deras personlighet och kanske även deras människovärdighet, kraftigt ifrågasatt. Patienter med långt framskriden sjukdom kräver onekligen stor omsorg, men lika sant är det att sjukdomen medför en enorm psykologisk belastning på patienternas familjer och närstående. Vid långt framskriden sjukdom upplevs situationen ibland svårare av familjerna än av patienterna, och Alzheimers sjukdom kan därför med rätta kallas *de anhörigas sjukdom*.

KOLESTEROL OCH KOLESTEROLSÄNKANDE TERAPI

Denna avhandlig har huvudsakligen fokuserat på hur höga kolesterolvärden i maten påverkar olika faktorer som är associerade med Alzheimers sjukdom, och hur vi kan påverka dessa faktorer med hjälp av en kolesterolsänkande terapi med rosuvastatin. Rosuvastatin är en effektiv hämmare av kolesterolsyntesen, och har visat sig vara en effektiv behandling av förhöjda kolesterolvärden och inflammationer i CNS.

Vi har först och främst demonstrerat att en kolesterolrik diet har en effekt på hjärnan och det centrala nervsystemet (CNS), trots att det runt hjärnan finns en skyddande barriär som kallas blod-hjärn barriären (blood-brain barrier; BBB). Denna BBB har till uppgift att bevara hjärnas integritet och kontrollerar vilka ämnen i kroppen som påverkar nervcellerna i hjärnan. Under normala omständigheter finns ca 25% av allt kolesterol i hjärnan, trots att den bara upptar ca 2% av kroppsvolymen. Detta till synes märkliga förhållande har sin förklaring i det faktum att kolesterolet har en central roll i cellernas funktion och bl.a. är nödvändigt för att nervcellerna ska kunna kommunicera. Men i och med att kolesterolet inte kan passera BBB måste i princip allt kolesterol i hjärnan tillverkas i hjärnan, med en mycket liten andel från blodet och maten. Hur kan då en kolesterolrik diet påverka hjärnan och CNS? Det finns flera olika teorier om detta, men en hypotetisk koppling som vi har haft möjlighet att undersöka i artikel I, är konverteringen mellan kolesterol, 27-hydroxykolesterol (27-OHC) och 24(S)-hydroxykolesterol (24S-OHC). Denna konvertering gör det lättare för kolesterolet att passera BBB. Tidigare studier har också visat att 27-OHC kan röra sig över BBB, från blodet och in i hjärnan. Väl inne i hjärnan representerar 27-OHC en direkt (hypotetisk) länk mellan kolesterolnivåerna

i blodet och kolesterolet i hjärnan. Vi har i artikel I visat att 27-OHC har ett negativt inflyttande på de processer som representerar icke-AD, med signifikans för sjukdomsförloppet i Alzheimers sjukdom.

I artikel II visade vi att nervceller reagerar positivt på en behandling med rosuvastatin, vilket skulle kunna öka deras möjligheter att skydda sig mot den celldöd som framkallas av Aβ. Denna behandling har även visat sig vara effektiv som kolesterolsänkande terapi. I artikel III och IV visade vi hur kolesterolet i dieten påverkade kroppen negativt med en ökad inflammation i hjärnan, och att en kolesterolsänkande terapi med rosuvastatin kan ha en tänkbar skyddande inverkan. Men våra rön tyder på att effekterna av rosuvastatin till viss del sker via mekanismer som inte är associerade med kolesterolnivåerna i kroppen, och att de dessutom kan associeras med både ålder och defekter i lipoproteintransportören ApoE, vilket hypotetiskt skulle kunna förklara en del motsägelsefulla resultat som har rapporterats i kliniska studier på statiner.

Vi har också visat att en kolesterolrik diet har en direkt effekt på olika faktorer som berör vår livsstils, det vill säga faktorer som representerar hur vi lever och som är möjliga att påverka med relativt enkla medel. Genom att ändra livsstil ökar vi våra möjligheter att behålla våra kognitiva funktioner och vår livskvalitet även i hög ålder. En kalorisnål diet (caloric restriction; CR) har t ex visat sig kunna förlänga vår livslängd om man börjar vid medelåldern, och även vara en effektiv preventiv åtgärd mot kognitiva försämringar. Men med tanke på att vår metabolism förändras när vi blir äldre och att bristande funktioner i ApoE inte bara är associerade med AD, utan också med negativa effekter på vår metabolism, kanske CR i sig inte är en lämplig behandling mot Alzheimers sjukdom.

> De åtgärder som rekommenderas är enkla och logiska råd som generellt omfattar ett aktivt och sunt liv; fysisk aktivitet; fritidsaktiviteter; utbildning och minnesträning; strukturerad tillvaro; näringsrik mat och dryck; minskning av högt blodtryck; social samvaro och normala kolesterolvärden.

Denna avhandling har visat på några av de skadliga effekter som höga nivåer av en kolesterolrik diet orsakar i hjärnan, och de möjligheter till förebyggande behandlingar som finns med kolesterolsänkande terapier med rosuvastatin. Men rönen tyder även på att en rosuvastatin terapi sannolikt inte är en tillräckligt effektiv insats mot Alzheimers sjukdom, då effekterna av behandlingen avtar i direkt association med ålder och defekter i ApoE. Vi föreslår därför en alternativ strategi där man kombinerar rosuvastatin tillsammans med åtgärder som påverkar vår livsstil och håller vår hjärna aktiv (se ovan). Om det initieras i tidig medelålder hos personer med risk att utveckla Alzheimers sjukdom, skulle det kunna vara en effektiv livsstilsförebyggande strategi med bevarade

kognitiva funktioner, bevarad livskvalitet i hög ålder och minskade kostnader för samhället. Utvecklingen av alternativa vårdformer har visat att det går att förbättra tillvaron och livskvaliteten för patienterna och deras familjer. Men vi behöver även en omvärdering av nuvarande idéer och av vårt förhållningssätt till patienter med demenssjukdomar, och mer forskning på frågor om demens och tidiga preventions terapier, till vilket den presenterade avhandlingen förhoppningsvis ska vara ett värdefullt bidrag.

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Time glides by with constant movement, not unlike a stream.
For neither can a stream stay its course, nor can the fleeting hour.

Assiduo labuntur tempora motu, non secus ad flumen.
Neque enim consistere flumen, nec levis hora potest.

— Ovid, Metamorphoses XV