ELOISE KOK

Alzheimer’s Disease Neuropathology and Inflammation

A genetic and immunohistochemical study

ACADEMIC DISSERTATION
To be presented, with the permission of the board of the School of Medicine of the University of Tampere, for public discussion in the Small Auditorium of Building B, School of Medicine of the University of Tampere, Medisiinarinkatu 3, Tampere, on June 3rd, 2011, at 12 o’clock.

UNIVERSITY OF TAMPERE
“Only an academic could state the obvious and pass it off as wisdom”
From Terry Pratchett’s *Going Postal*

“Back off man! I’m a scientist!”
Dr Peter Venkman from *Ghostbusters*

“Nothing shocks me, I’m a scientist!”
Indiana Jones from *Indiana Jones & the Temple of Doom*

To my wonderful family
Contents

List of Original Communications ................................................................. 7
Abbreviations .................................................................................................. 8
Abstract ........................................................................................................... 10
Tiivistelmä ..................................................................................................... 12
Introduction ................................................................................................... 14

Literature Review ......................................................................................... 17

1. The history and definition of Alzheimer’s disease .................................... 17
2. Alzheimer’s disease types ........................................................................ 18
   2.1 Familial AD & genetic mutations ......................................................... 18
   2.2 Sporadic AD ...................................................................................... 18
3. Alzheimer’s disease diagnosis ................................................................. 19
4. Alzheimer’s disease symptoms .............................................................. 22
5. Risk factors ............................................................................................. 23
   5.1 Environmental risks .......................................................................... 23
   5.2 Concomitant diseases ....................................................................... 25
   5.3 APOE & Lipidomics .......................................................................... 26
   5.4 Polymorphisms & genes ................................................................... 28
   5.5 Epigenetics ....................................................................................... 30
6. Causal theories ......................................................................................... 31
   6.1 Brief history of causes ...................................................................... 31
   6.2 Cholinergic hypothesis ..................................................................... 31
   6.3 Amyloid theory ................................................................................. 32
   6.4 Tau theory ........................................................................................ 35
7. Other potential causes ............................................................................. 37
7.1 Inflammation ............................................................................................................ 37
7.2 Oxidation and Mitochondrial dysfunction ............................................................... 39
7.3 Metal imbalance ....................................................................................................... 41
7.4 Viruses & bacteria ................................................................................................... 42

8. Summary ....................................................................................................................... 43

Aims of the study ......................................................................................................... 44

Study Subjects .............................................................................................................. 45

Methods ......................................................................................................................... 46

1. Neuropathological tissue samples ................................................................. 46
2. Immunohistochemistry .......................................................................................... 46
3. Genotyping .............................................................................................................. 47
4. Statistics ................................................................................................................... 49

Results ........................................................................................................................... 51

1. Study cohort & genotyping (I-III) ............................................................... 51
2. Senile plaques (I-III) ............................................................................................ 52
3. Neurofibrillary tangles (I-III) .............................................................................. 54
4. CRP genotypes & neuropathological lesions (II) ............................................... 55
5. CRP genotypes & immunohistochemistry (II) .................................................... 56
6. CLU, CR1 & PICALM, and SP (III) ................................................................. 57
7. USF1 genotypes & neuropathological lesions (unpublished data) ..................... 59

Discussion ...................................................................................................................... 65

1. Study subjects ...................................................................................................... 65
2. Methodological considerations ............................................................................. 65
3. APOE & neuropathological lesions (I) .............................................................. 66
4. CRP & neuropathological lesions (II) ................................................................. 68
5. CLU, CR1 & PICALM, and neuropathological lesions (III) ............................... 69
6. USF1 & neuropathological lesions (unpublished data) ...................................... 70
Summary and Conclusions ............................................................................................................. 73
Acknowledgements ..................................................................................................................... 75
References ........................................................................................................................................ 77
Original Communications ........................................................................................................... 115
This dissertation is based on the following original communications, which are referred to in the text by their Roman numerals (I-III).


In addition, this thesis contains unpublished data.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ</td>
<td>amyloid beta</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>APOE</td>
<td>apolipoprotein E</td>
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<tr>
<td>APOEε4</td>
<td>apolipoprotein E epsilon 4</td>
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<tr>
<td>AβPP</td>
<td>amyloid beta precursor protein</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>BIN1</td>
<td>bridging integrator 1</td>
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<tr>
<td>CAA</td>
<td>cerebral amyloid angiopathy</td>
</tr>
<tr>
<td>CERAD</td>
<td>Consortium to Establish a Registry for Alzheimer’s Disease</td>
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<tr>
<td>CLU/APOJ</td>
<td>clusterin/apolipoprotein J</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CR1</td>
<td>complement component (3b/4b) receptor 1</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebro-spinal fluid</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>DAPI</td>
<td>4’6-diamidino-2-phenylindole</td>
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<tr>
<td>DM2</td>
<td>diabetes mellitus type 2</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EXOC3L2</td>
<td>exocyst complex component 3-like 2</td>
</tr>
<tr>
<td>F-IHC</td>
<td>fluorescent immunohistochemistry</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>GWAS</td>
<td>genome wide association study</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
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<tr>
<td>HP-tau</td>
<td>hyperphosphorylated tau</td>
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<td>HSV1</td>
<td>herpes simplex virus 1</td>
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<tr>
<td>IDE</td>
<td>insulin-degrading enzyme</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<td>IL8</td>
<td>interleukin 8</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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<tr>
<td>LpL</td>
<td>lipoprotein lipase</td>
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<tr>
<td>LTP</td>
<td>long term potentiation</td>
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<tr>
<td>MAPT</td>
<td>microtubule-associated protein tau</td>
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<tr>
<td>MBDs</td>
<td>methyl-CpG binding domain proteins</td>
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<tr>
<td>MCI</td>
<td>mild cognitive impairment</td>
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<tr>
<td>MMSE</td>
<td>mini-mental state examination</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NAD+</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>NFT</td>
<td>neurofibrillary tangles</td>
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<tr>
<td>NKκB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>NSAIDs</td>
<td>non-steroidal anti inflammatory drugs</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PGC1α</td>
<td>proliferator-activated receptor gamma coactivator-1 alpha</td>
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<td>PHF</td>
<td>paired helical filaments</td>
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<td>PICALM</td>
<td>phosphatidylinositol binding clathrin assembly protein</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PSEN1 or PS1</td>
<td>presenilin 1</td>
</tr>
<tr>
<td>PSEN2 or PS2</td>
<td>presenilin 2</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
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<tr>
<td>SNPs</td>
<td>single nucleotide polymorphisms</td>
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<tr>
<td>SORL1</td>
<td>sortilin-related receptor</td>
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<tr>
<td>SP</td>
<td>senile plaques</td>
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<tr>
<td>TASTY</td>
<td>Tampere autopsy study</td>
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<tr>
<td>TMA</td>
<td>tissue microarray</td>
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<tr>
<td>TNFα</td>
<td>tissue necrosis factor alpha</td>
</tr>
<tr>
<td>TNK1</td>
<td>tyrosine kinase, non-receptor, 1</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>USFs</td>
<td>upstream stimulatory factors</td>
</tr>
<tr>
<td>USF1</td>
<td>upstream transcription factor 1</td>
</tr>
<tr>
<td>VLDLs</td>
<td>very low density lipoproteins</td>
</tr>
</tbody>
</table>
Abstract

Background. Alzheimer’s disease (AD) affects a large proportion of the elderly population and will be one of the most challenging problems of public health in the future, as the population ages. It is imperative to determine effective treatments towards not only the symptoms, but also the causes of the disease.

To date, the only conclusive risk gene for sporadic AD is APOE. The e4 allele of APOE is associated with the accumulation of amyloid beta (Aβ) peptide in the brain. The role of many other genes and polymorphisms has been associated with the disease, but the effects have been small. The pathogenic hallmarks that are found in postmortem AD brains are senile plaques (SP) and neurofibrillary tangles (NFT), related to the accumulation of Aβ and hyperphosphorylated tau (HP-tau) in neurons. The exact function or mechanism by which these lesions appear is not completely understood and studies also suggest contradictory roles for them, such as being protective or not related to the course of the disease at all. It has also been proposed that slow central nervous system inflammation might be involved in the pathogenesis of SP and NFT.

Objectives. The objective of this thesis was to study the occurrence of these neuropathological lesions and their genetic risk factors in the brains of a non-demented population, and try to find new information about the causes and pathogenesis of these lesions. In this study we have evaluated the association of SP and NFT phenotypes with polymorphisms of the genes of Apolipoprotein E (APOE), C-reactive protein (CRP) and upstream transcription factor 1 (USF1). We have also studied the association of neuropathology with variations in the newly identified genes (clusterin, CLU; complement component 3b/4b receptor 1, CR1; phosphatidylinositol binding clathrin assembly protein, PICALM) found in large genome wide association studies in patients suffering from probable clinical AD.

Subjects and methods. The Tampere Autopsy Study (TASTY) series comprised of 603 men and women, aged 0 to 97 years, who were subjected to medicolegal autopsy at the Department of Forensic Medicine, University of Tampere, in Finland, during the years 2002 to 2004, covering approximately 25% of the medicolegal autopsies performed in the Tampere region.

Data pertaining to the autopsies were obtained from hospital records and interviews of family members in police reports. Females within the cohort accounted for 35.8% (215 cases) and the average age of the entire cohort was 63 years (59 years for males and 68 years for females).

None of the cases died of AD, but 6 (1.0%) had been diagnosed with the disease whilst alive. Additionally 16 cases (2.7%) were reportedly suffering from undefined dementia, 10 (1.7%) had memory disorders, and 1 (0.2%) case was diagnosed with Parkinson’s disease prior to death, according to available hospital records and next of kin reports.

At autopsy, samples from four (middle frontal gyrus, gyrus cinguli with corpus callosum, hippocampus, and cerebellum) different areas of the brain were placed in Tissue-Tek boxes and fixed in a phosphate-buffered 4% formaldehyde solution for at least 2 weeks. The tissue blocks were then embedded in paraffin from which 10µm sections were cut and stained using hematoxylin & eosin, Bielschowsky’s argyrophilic silver impregnation methods, and fluorescent immunohistochemical staining for Aβ
peptide and CRP protein. The immunohistochemical studies utilised cylindrical samples from paraffin-embedded tissue blocks collected together to create brain tissue microarrays.

Common genetic polymorphisms and haplotypes for the genes \textit{CRP}, \textit{USF1}, and the putative novel AD risk genes \textit{CLU}, \textit{CRI} and \textit{PICALM} were determined from blood samples of the cases.

\textbf{Results.} In this thesis, the \textit{APOE} ε4 allele was strongly associated with the presence of SP in the TASTY series, as compared to the most common ε3/ε3 genotype. The ε2 allele appeared to show some form of protection, however this was not significant. There were no associations between the \textit{APOE} genotypes and NFT. Assuming that NFT and SP indicate disease progression, our results on the common occurrence of these brain changes suggest that interventions for AD may need to be initiated in middle age in individuals carrying the \textit{APOE} ε4 allele, especially if they have a family history of dementia.

A number of \textit{CRP} SNPs and haplotypes that associated with elevated CRP protein levels were associated with early stage ‘non-neuritic’ SP, as determined by Bielschowsky staining, with a trend in most cases for late stage ‘neuritic’ SP. There were no associations between the \textit{CRP} SNPs or haplotypes and NFT. Both CRP IHC stains and Aβ peptide IHC staining correlated with each other, as did CRP IHC staining with \textit{CRP} SNPs and haplotype pairs. Interestingly, Aβ peptide IHC staining did not correlate with any \textit{CRP} SNPs or haplotypes. Our data suggest that \textit{CRP} genotypes may modify initial SP formation in the brain and may participate in the slowing down or enhancement of early stage SP, after which other factors come into play to effect conversion to late stage SP and therefore clinical AD.

Whilst \textit{CLU}, \textit{CRI} and \textit{PICALM} did associate with some variables of SP, they did so sparingly and raise questions about the involvement of SP in the aetiology of AD. The studied SNPs did not correlate with NFT either, however previous reports cement their involvement in the pathogenesis of the disease. Our results suggest that whilst these SNPs associated with probable AD cases in recent GWAS, they do not strongly relate to SP prevalence in an autopsy series representative of the general population, possibly indicating their complex involvement in the disease.

A number of \textit{USF1} SNPs and haplotypes associated with variables of SP and also with NFT in the TASTY series. This suggests a strong role of USF1-mediated effects in the development of both neuropathological lesions and warrants further investigations. \textit{USF1} polymorphisms may contribute to development of brain lesions possibly through disturbances in lipid metabolism or other mechanisms by which USF1 is known to operate, thus participating in AD pathogenesis.

\textbf{Conclusions.} Based on these results, it can be concluded that a number of inflammatory genes may influence the development of the neuropathological lesions associated with AD and may therefore participate in the initiation or progression of the disease. This is of course, assuming that these characteristic hallmarks are in fact a detrimental part of disease pathogenesis and not simply bystanders of the disease. Because these results were accumulated from an autopsy series consisting primarily of non-demented cases, there remains the question of the involvement of these AD-related lesions in disease aetiology. Further detailed studies investigating this much-discussed topic will be required and help to elucidate their contribution to Alzheimer’s disease.
Tiivistelmä

Taustaa. Alzhemerin taudin yleisyys ja yleistyminen ikääntyneessä väestössä kuormittaa jo nyt terveydenhuoltoa ja tulee aiheuttamaan tulevaisuudessa erään hyomattavimmista kansanterveydeslisistä ongelmista. Tämän vuoksi tutkimukset tehokkaiden ennaltaehkäisevien ja parantavien lääkehoitojen kehittämiseksi ovat äärimmäisen tärkeitä.


Tavoitteet. Tämän väitöskirjatyön tarkoitus on kartoittaa amyloidikertymämuutosten esiintymistä oireettomalla väestöllä, niiden perinnöllisiä riskitekijöitä sekä löytää uutta tietoa varhaisten kertymämuutosten syntymisestä. Tutkimuksessa tarkasteltiin C-reaktiivinen proteiini (CRP) geenin sekä upstream stimulatory faktorin (USF1) geenin polymorfioiden yhteyttä varhaisten neuropatologisten muutosten esiintyvyyteen. Lisäksi selvitettiin äskettäin genominlaajuuisissa tutkimuksissa esiin tulleiden uusien geenien polymorfioiden yhteyttä näihin muutoksiin verrattuna APOE-geenin vaikutukseen.


Aineiston keski-ikä oli 63 vuotta ja tapauksista 215 (35.8%) oli naisia (miesten keski-ikä oli 59 ja naisten 68 vuotta). Kaikkien tapausten kuolinsyy oli muu kuin Alzheimerin tauti. Tapauksista 6 (1.0%) diagnozisoitiin heidän elinaikanaan. Lisäksi 16 tapauksella (2.7%) oli määrättelemätön dementia, 10 (1.7%) tapauksella oli muistihäirioita ja yhdellä (0.2%) oli Parkinsonin tauti.

Neljältä eri aivoalueelta (keskiavopoiu, aivokurkiaisessa, hippocampus ja pikkuaivot) kerätty kudosnäytteet fiksattiin Tissue-Tek-laatikoissa fosfaattipuskuroidussa 4% formaldehidiijuoksessa vähintään kahden viikon ajan. Tämän jälkeen paraaffinoiduista kudoksista leikattiin 10µm kudosleikkeet, jotka värjättiin hematoksyliniumiäsoiniviärjäksellä ja Bielschowskyn hopeavärjäksellä. Histologisista blokeista irrottetut kudosylinteriöstä rakennettiin monikudodosblokit (TMA) immunohistokemiollisia tutkimuksia varten.

Verinäytteistä eristetyistä DNA:stä määritettiin yhden emäisen muutoksia (SNP) tai näiden SNP:den yhdistelmiä eli haplotyypppejä seuraavissa geeneissä: apolipoproteiini E (APOE), C-reaktiivinen proteiini (CRP), upstream transcription faktori (USF)-1 sekä äskettäin sairastumisriskiihin yhdistetyt amyloidin aineenvaihduntaan osallistuvat clusterin
(CLU), komplementti reseptori 1 (CRI) sekä hermostollisen impulssin väliittämiseen liittyvä fosfatidylinositolia sitova clathrin proteiini (PICALM).

Tulokset. SP:t olivat selkeästi yleisempiä APOE4 alleelin kantajilla ja hieman harvinaisempi APOE2-alleelin kantajilla kuin niillä henkilöillä joilla oli kaksi kopioita yleisimmästä ε3-geenimuodon. APOE geneettinen vaihtelu ei ollut yhteydessä NFT:n esiintymiseen. Mikäli NFT ja SP ovat liittyvät Alzheimerin taudit etenemiseen, näiden tulosten perusteella Alzheimerin taudit etenemisen ehkäisemisen aloittaminen APOEε4-geenimuodon kantajilla voi olla tarpeellista jo keski-äissä, etenkin jos heidän suvussaan on aiemmin esiintynyt dementia.


Johtopäätökset. Tämän väitöskirjatöyön perusteella tulehdusvarastetta säätellevät geenit saattavat vaikuttaa Alzheimerin tautiin liittyvien aivojen kertymämuutosten kehittymiseen ja voivat sitä olla yhteydessä myös sairauden syntyyn tai etenemiseen.

On kuitenkin huomiotava että ruumiinavusten yhteydessä koottu aineisto koostui enimmäkseen ei-dementoituneista henkilöistä. Kertymämuutosten yhteyks kliniksen Alzheimerin taudin puhekeamiseen on siten kyseenalaista ja lisätutkimukset syysseuraussuhteen selvittämiseksi ovat tarpeellisia.
Introduction

Alzheimer’s disease (AD) is the most common and well-known form of dementia affecting the increasingly elderly population, including families of those afflicted. Characterised by behavioural, psychological and cognitive degeneration, including memory loss, the disease affects the lives of patients and their families. With no effective treatments and no curative therapies, AD requires research to elucidate the mechanisms behind the disease.

AD affects approximately 70 – 80,000 individuals in Finland with approximately 10,000 new cases each year (according to KELA medication data and Statistics Finland), accounting for 60-80% of elderly dementia cases. AD increases in prevalence with age, with 5-10% over 65 affected increasing to 45% in those over 85 years old (Lobo et al. 2000), with more than 30% of those over 85 years old having neuropathological lesions (Polvikoski et al. 2001). The disease leads to death within 10-15 years from diagnosis, with approximately 3000 cases dying each year in Finland alone (according to Statistics Finland).

Clinical diagnosis of AD involves cognitive testing of patients, however diagnosis can only be confirmed at postmortem after neuropathological investigation. The golden standard involves measuring the characteristic brain lesions of amyloid beta (Aβ) peptide senile plaques (SP) and neurofibrillary tangles (NFT) consisting of hyperphosphorylated tau (HP-tau) (Braak, Braak 1991, Braak, Braak 1997, Khachaturian 1985, Alafuzoff et al. 2008).

The accumulation of SP and the build up of neurotoxic forms of the Aβ peptide such as protofibrils and oligomers, having been implicated in AD pathogenesis through familial genetic mutations of AD, is thought to lead to the cascade of neurodegeneration in AD (Hardy, Higgins 1992, Rosenblum 2002, O’Nuallain et al. 2010). Whilst this train of thought has sufficed for many years, the actual function of Aβ is still unknown, with researchers also believing it may be an acute phase protein (Soscia et al. 2010, Kontush 2005), or have another physiological role within the brain, such as an apolipoprotein involved in transporting metals, or regulating synaptic formation and transmission (Kontush 2005, Cirrito et al. 2005).

SP and Aβ peptide deposits are found also in cognitively normal elderly individuals, sometimes at the levels of those warranting an AD diagnosis, however without any cognitive decline (Blair et al. 2005, Knopman et al. 2003). SP are highly associated with age and emerge in early middle age, continuing to accumulate as an individual gets older (Braak, Braak 1997). It is unknown what allows some individuals to remain free of cognitive deficits in the presence of high numbers of these brain lesions, although there are suggestions of ‘cognitive reserve’ obtained through the benefit of education (Dumurgier et al. 2010).

The risks and causes associated with AD are not completely known or understood (see table 1). Age is the strongest known risk factor (van der Flier, Scheltens 2005), along with less strong risks of female gender and a family history of the disease (discussed in (Kukull, Ganguli 2000)), cardiovascular disease (Kivipelto 2002, Stampfer 2006), diabetes (Kroner 2009, Figaro 2006), inflammation (Finch, Morgan 2007, Giunta 2008, Grant et al. 2002), head injury, and low educational levels, as examined in this thesis.
Table 1. Theories pertaining to the cause(s) of Alzheimer’s disease.

<table>
<thead>
<tr>
<th>Theory</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholinergic hypothesis (Martorana, Esposito &amp; Koch 2010, Contestabile 2010, Eskander et al. 2005)</td>
<td>Decreased numbers of cholinergic-producing neurons</td>
</tr>
<tr>
<td>Amyloid theory (Hardy, Higgins 1992, Hardy, Selkoe 2002)</td>
<td>Amyloid plaques found in AD patients’ brains and mutations in AβPP/PS1/PS2 causative for familial AD</td>
</tr>
<tr>
<td>Tau theory (Brunden et al. 2010)</td>
<td>Neurofibrillary tangles found in AD patients’ brains</td>
</tr>
<tr>
<td>Metal imbalance (Roberts et al. 1998, Crouch, White &amp; Bush 2007)</td>
<td>Imbalance in metals of postmortem AD brains, evidence of effective treatment with metal chelators</td>
</tr>
<tr>
<td>Pathogens including viruses and bacteria (Finch, Morgan 2007, Kamer et al. 2008, Miklossy 2008)</td>
<td>Presence in AD brains, evidence for causing SP in cell culture studies</td>
</tr>
</tbody>
</table>

The most consistent and strongest genetic predisposition to the disease comes from the gene for apolipoprotein E (APOE) (Farrer et al. 1997, Ghebremedhin et al. 1998, Gomez-Isla et al. 1996, Roses, Saunders 1994, Corder et al. 1993, Polvikoski et al. 1995), with an increase in the risk of developing AD reported to be up to 30 times higher with two ε4 alleles (Farrer et al. 1997, Corder et al. 1993, Corder 1995). Other genes have also been implicated as disease risk factors and a website (www.alzgene.org) has been set up to meta-analyse their effects (Bertram et al. 2007). Many of the top findings of genetic risk results are attributed to inflammation, cholesterol metabolism, transport/trafficking proteins and neurotransmission signalling (Bertram et al. 2007).

AD research has mostly focussed on the actions of Aβ peptide in cell culture studies, animal studies, and genetic risk in cohorts consisting of heterogeneous AD-affected cases with underlying (possibly unknown) diseases, thus making causes difficult to elucidate. It was therefore the focus of this thesis to investigate the associations of genetic polymorphisms related to the prevalence of SP and NFT, to try to uncover genetic associations relating to the pathophysiology and emergence of these purported AD-related lesions.

The results of this thesis are based on an autopsy series collected during the years 2002 – 2004, in the Tampere Autopsy Study (TASTY) consisting of 603 cases who died out-of-hospital and were thought to be representative of the general population. The first
article investigated the associations of SP, NFT and their variables with the common risk allele for AD – APOE. The second set of results discussed the observations of the AD-related lesions and the C-reactive protein (CRP) gene, an inflammatory marker molecule, including both immunohistochemical and genetic aspects. The third manuscript examined the prevalence of SP and NFT with three recently identified potential AD-risk polymorphisms (CLU, CR1 and PICALM). Finally, the association of polymorphisms in the upstream transcription factor 1 (USF1) gene – a transcription factor affecting the function of AD-related genes such as APOE and AβPP – were explored with relation to the AD brain lesions and their variables.
Literature Review

1. The history and definition of Alzheimer’s disease

Alzheimer’s disease (AD) was described by Oskar Fischer, Francesco Bonfiglio and Graetano Perusini (Lage 2006), however the disease is known today as AD because the 8th edition of the book Psychiatrie (by Emil Kraepelin, published in 1910) included a description of work done by Alois Alzheimer. Kraepelin was the supervisor of Alzheimer (a psychiatrist and neuropathologist), and in the description of the symptoms and pathology of the disease, Kraepelin coined the name Alzheimer’s disease, which has remained ever since. Alzheimer gave a lecture on Mrs. Auguste Deter in 1907 during the 37th Conference of South-West German Psychiatrists in Tubingen (Alzheimer 1907), describing the observation of the neuropathological lesions, neurofibrillary tangles in her brain at autopsy, in the 55 year old patient. Her case presented with memory impairment, aphasia, psychosocial incompetence and disorientation, which progressed gradually over the remaining years of her life, including experiencing hallucinations and worsening cognitive function.

This was not the first case of cognitive degeneration that Alzheimer encountered, however the case of Auguste Deter was interesting due to her younger age, as previous patients encountering such cognitive decline were in their seventies. So at her death, Alzheimer requested her brain be sent to him, from which he examined tissue sections stained with a silver staining technique. From these microscopic analyses, he observed and described the presence of ‘fibrillary bundles’ and ‘small miliary foci,’ nowadays recognised as neurofibrillary tangles (NFT) and senile plaques (SP) (Lage 2006, Alzheimer 1907).

AD itself wasn’t considered a disease separate from dementia until the late 1960’s, after studies (Blessed, Tomlinson & Roth 1968) showed that there was a connection between the characteristic hallmarks, SP and NFT, and cognitive decline, as discussed in the review by Lage (Lage 2006). Additionally researchers indicated that AD was different from normal aging (Kay, Beamish & Roth 1964) and identified mutations involved in hereditary forms of the disease (Tanzi et al. 1996). These studies lead to the revelation that AD was its own disease and that diagnosis could be achieved by eliminating other causes of dementia and monitoring progression of the symptoms (Khachaturian 1985).

Unfortunately, due to the elusive nature of the disease, clinical diagnosis consists of terms such as ‘possible’ and ‘probable’ AD, with definite diagnosis only available at autopsy after verification of the presence of specific neuropathological lesions – SP and NFT. According to most sources, the definition of AD consists of irreversible deterioration of language, judgement and memory skills that progress over 10 to 15 years and are associated with the accumulation of neuropathological SP and NFT at postmortem evaluation (Braak, Braak 1991, Braak, Braak 1997, Khachaturian 1985, Mirra et al. 1991).

AD remains a difficult disease to study due to its long term progression and the inability to reliably detect the initial stages of the disease. Today, brain scans and improving imaging techniques have given researchers further insight to the aetiology of the disease, but reaching agreement on the pathological aspects and causes of AD, in addition to the lack of ways to confirm these, have caused impediments to treatments and cures for the disorder.
2. Alzheimer’s disease types

A minority (less than 1%) of those affected with AD are dominant familial forms (van der Flier et al. 2011), caused by mutations in one of three genes and having an early age of onset before 65 years (van der Flier et al. 2011, Miyoshi 2009). The more common sporadic version has no commonly acknowledged causes and the risks pertaining to the disease are not well understood.

2.1 Familial AD & genetic mutations

Familial AD, whilst rare, has provided researchers with much information about the causes of the disease, including the sporadic form. Discoveries of families with early onset, dominant forms of the disease lead researchers to connect the β-amyloid precursor protein (AβPP, the gene of which was found on chromosome 21) (Wisniewski, Wisniewski & Wen 1985) and two enzymes that cleave it (Presenilin 1 – gene of PSEN1, found on chromosome 14 (Levy-Lahad et al. 1995) and Presenilin 2 – gene of PSEN2, found on chromosome 1 (St George-Hyslop et al. 1992)), with the Aβ peptide found in SP within the brains of AD sufferers. Most mutations within the three genes (AβPP, PSEN1 and PSEN2) increase the levels of Aβ, thought to lead to excess amounts of toxic forms of Aβ peptide, which may aggregate into SP and supposedly disrupt neuronal messaging, ultimately causing the death of neurons (Zhang et al. 2001). Further studies have also suggested that oligomers or protofibrils of Aβ peptide are to blame and disrupt synapses (Gouras et al. 2010, Takahashi et al. 2004).

2.2 Sporadic AD

The more common sporadic form of AD has no directly known causes and is considered a multifactorial disease where many risk factors add up to instigate the dysfunction that results in the symptoms recognised as AD, as reviewed in (Kukull, Ganguli 2000, Iqbal, Grundke-Iqbal 2010).

There have been recent suggestions that there needs to be differentiation between subtypes of AD (Iqbal et al. 2005), which may have implications for treatments and progression of the disease. The levels and abundance of the characteristic hallmarks of AD – SP and NFT – have been observed occurring disproportionately in different cases, indicating there may be SP- or NFT-dominant forms (Jellinger, Attems 2007, Katzman et al. 1988, Duyckaerts, Delatour & Potier 2009). Others have proposed that there may be up to five subgroups of the disease (Iqbal et al. 2005), differing with regards to cerebrospinal fluid (CSF) levels of Aβ peptide, ubiquitin and tau, including early and late onset, high and low Aβ peptide and tau levels, prevalence of APOEɛ4 allele, and incidence of concomitant neuropathological lesions such as Lewy bodies, as seen in figure 1.

Many things have been attributed to triggering AD, but as with many complex diseases, it may require a certain threshold to be surpassed before actual disease manifestation occurs. Multiple factors including genetic, environmental, dietary, or a combination of these could determine disease initiation, as well as disease progression. These factors will be dealt with later in the following chapters on the topic.
3. Alzheimer’s disease diagnosis

The diagnosis of AD can be separated into two parts. The first deals with the symptoms seen during a patient’s life and involves measuring the deterioration of cognition, including memory, behaviour, speech and understanding. Numerous tests are utilised by doctors to identify the extent of the damage, and when repeated frequently enough can provide indications of the progression of the disease.

Tests of cognition are performed, often beginning with the simple screening test Mini-Mental State Examination (MMSE)(Folstein, Folstein & McHugh 1975) and complemented by more sophisticated neuropsychological tests e.g. WAIS or CERAD, to observe impairments in memory, language, perceptual skills, attention, constructive abilities, orientation, problem solving and functional abilities. These tests are performed in combination with brain imaging techniques, such as MRI and PET scans, to exclude other types of dementia and thus give a diagnosis of possible or probable AD(Khachaturian 1985).

Whilst a clinical diagnosis of probable AD is considered up to 90% accurate by professional experienced doctors, confirmation of diagnosis must be carried out postmortem (Polvikoski et al. 2001, Braak, Braak 1991, Khachaturian 1985, Mirra et al. 1991, The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease, 1997) and includes substantial measurements of the two main hallmarks of the disease: extracellular amyloid beta deposits known as SP and intracellular NFT, as seen in figure 2.

The golden standard for assessing these measurements utilise the staging protocols suggested by Braak and Braak (Braak, Braak 1991) in conjunction with the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD)(Mirra et al. 1991) neuropathology scoring system, correlating well with AD prediction and diagnosis(Nagy et al. 1998).
Studies have investigated postmortem brains from AD patients to measure a number of molecules thought to be involved in the pathogenesis of the disease, utilising immunohistochemistry. Whilst silver impregnation techniques used in the historical detection of SP and NFT are effective at identifying these characteristic hallmarks, researchers have utilised numerous antibodies (Aho et al. 2010, Wirths et al. 2001) to specifically identify the different molecules that define them and thereby have tried to narrow down on the neurotoxic components that potentially cause AD.

In addition to attempting to identify the harmful constituent of the major brain lesions, postmortem immunohistochemical studies have also shed light on other possible participants in disease aetiology.

**Figure 2.** Senile plaque (SP; arrows) and neurofibrillary tangles (NFT; arrowheads) stained with Bielschowsky silver staining – the two primary hallmarks of Alzheimer’s disease. Image kindly supplied by Professor Hannu Kalimo.

Immunohistochemical data indicate the presence of other neurodegenerative structures – although not exclusively related to AD neuropathology – including spongiform changes similar to those in Creutzfeldt-Jakob disease, intracellular Hirano bodies, synaptic loss and disturbances in many neurochemical systems (Duyckaerts, Delatour & Potier 2009). Additionally, atrophy of the hippocampus and amygdala, as well as cortical atrophy of the gyri and sulci is observed (Kidd 2008). What causes these
characteristics to occur in some cases and not others is not clearly understood, however it could be related to subtypes of the disease (Duyckaerts, Delatour & Potier 2009).

Large amounts of dysfunctional proteins have also been identified within the affected regions of the AD brain, suggesting that inefficient protein processing and maintenance could be one of the causes for the disease (Haapasalo et al. 2010, Cuervo, Wong & Martinez-Vicente 2010). From DNA to the production of protein, many mechanisms are utilised to form a complete, correct product. Messenger RNA (mRNA) editing is thought to be one of the mechanisms behind phenotypic variability, although if not closely controlled, problems can arise (van Leeuwen et al. 1998).

Deletions within GAGAG motifs are the most common modifications that proteins undergo to develop variability. The $\alpha$PP gene has seven such sequences and whilst there may not be mutations in the DNA itself, errors in processing cause mutated proteins, which can then disrupt subsequent pathways (van Leeuwen et al. 1998). These mutant proteins have been detected in the brains of AD, as well as Down’s syndrome patients, who also develop SP and NFT (Wisniewski, Wisniewski & Wen 1985). These transcriptional errors affect postmitotic neurons, such as those in the brain, more often due to their sensitivity and their inability to compensate for these faults (van Leeuwen et al. 1998).

Researchers have suggested that some individuals are more susceptible to these errors, either through lifestyle habits or genetic backgrounds, leading to less effective mechanisms preserving protein formation integrity (van Leeuwen et al. 1998). Whilst evidence for this theory is scant and treatment for these options possible, but not yet available, this could be another avenue of treatment.

In addition to immunohistochemical staining of brain tissue and in an attempt to determine which individuals will eventually develop the disease and provide confirmation of diagnoses, studies have investigated the levels of particular substances within patients’ CSF and blood (Finch, Morgan 2007, Iqbal et al. 2005, Fiala 2009). Whilst a number of studies have published contradictory results and have questioned the ability of individual biomarkers (Fiala 2009) to decipher between memory problems and actual AD, results suggest that combined measurements of tau protein, prostanes, $\alpha$β peptide species and inflammatory molecules from CSF could provide detailed information on disease progression and subtype (Fagan, Holtzman 2010).

Whilst immunohistochemistry has increased the number of molecules possibly involved in AD aetiology, it has also generated many more questions in terms of deciphering which are mere bystanders and which are actual participants in dictating disease manifestation. Future research will no doubt help to narrow down this field of potential suspects.

Currently, therapies to treat Alzheimer’s disease do nothing more than treat symptoms and barely slow the disease, let alone undo the damage that has been accumulated over the years of brain injury. In this way, research continues to investigate the mechanisms participating in disease aetiology and as will be seen in this thesis, there is a wide range of theories and studies into different pathways and treatments. The most likely candidate for treating or even possibly curing AD will be one that attacks all the symptoms and addresses the ultimate cause or causes of the disease. The number of research avenues promises that we are getting close to that day soon.
4. Alzheimer’s disease symptoms

Although AD is characterised by the progressive cognitive deterioration of a patient, each individual advances through the course of the disease uniquely. Initial symptoms are often mistaken for age-related problems or stress-induced indicators, including the inability to form new memories and recall recently learnt facts. These subtle problems in the ‘pre-dementia’ stage usually go unnoticed or may be identified as ‘mild cognitive impairment’ (MCI).

Early stages of the disease see behavioural, psychological changes, as well as a gradual inability to handle normal daily activities, including newly learned skills. Usually a patient is able to manage their own affairs, although their vocabulary and language fluency tend to be noticeably affected at this point of the disease.

Later phases of AD require full-dependency on a caregiver, leading in some cases to a complete loss of speech and accompanied by a loss in muscle mass due to lack of mobility, ultimately causing the patient to become bedridden. Delusional symptoms and irritability, confusion, aggression and wandering tend to become less common than in the intermediate stages of the disease.

Postmortem analyses of AD patients’ brains (see figure 3) show losses of neurons and synapses in the cerebral cortex, atrophy of the hippocampi, temporal and parietal lobes, parts of the frontal cortex and cingulate gyrus, as well as a presentation of large numbers of SP (in the cortex) and NFT (beginning in the hippocampus region and extending into the cerebral cortex of different lobes), thus giving a diagnosis of AD(Khachaturian 1985, Alafuzoff et al. 2008, Polvikoski et al. 1995, Mirra et al. 1991, Duyckaerts, Delatour & Potier 2009, The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease, 1997, Braak et al. 2006).

Figure 3. Diagram showing the shrinkage of an AD patient’s brain (right) compared to a normal undemented individual’s brain (left). The primary affected areas are the cortex and hippocampus. Image kindly supplied by Professor Hannu Kalimo.
5. Risk factors

As part of identifying the causes of AD, studies have investigated which factors can increase or decrease the risk of an individual’s potential to develop the disease. Many years of research have narrowed down on a number of mechanisms by which we can reduce the risk of getting AD, including our diet (Grant et al. 2002, Smith, Petot & Perry 1999), happiness (Berger et al. 1999, Chen et al. 1999) and exercise (Larson et al. 2006). Additionally, there are other risk factors which we cannot change, but could provide therapeutic avenues including gender (Behl 2002) and genetics (Bertram et al. 2007). A few of the primary impacting factors are discussed below.

5.1 Environmental risks

A number of risks can be considered to environmental risks in the form of things that an individual exposes him or herself to over the period of their life. Longitudinal or retrospective studies (discussed in (Kukull, Ganguli 2000, Grant et al. 2002)) have produced many ideas for ways to maintain ones’ cognitive reserve and allow an individual to live healthily into old age with intact brain function. Whilst clinical trials have failed to confirm some beneficial lifestyle habits, most likely due to the difficulty in designing such a long-lived study, and some argue against the presence of these risk factors (Daviglus et al. 2010), the general consensus is that if you live healthy through middle age, you will preserve cognitive function into old age and therefore prevent AD (as discussed in (Kukull, Ganguli 2000, Grant et al. 2002, Qiu, Kivipelto & Fratiglioni 2011)).

Exercise is foremost thought to be the most beneficial way to retain cognitive function (Larson et al. 2006, Scarmeas et al. 2009). Beneficial in so many ways, exercise keeps the body operating effectively and keeps hormones and the immune system in check, as well as reducing body fat and keeping the cardiovascular system healthy.

Along with exercise, keeping your brain functioning with mind games and puzzles is thought to retain cognitive reserve by allowing your brain to use and maintain all regions (Kidd 2008). Epidemiological studies have also suggested that knowing more than one language (Chertkow et al. 2010) is also beneficial to brain maintenance. Cholesterol lowering medications (Wolozin et al. 2000) have additionally hinted at being beneficial in the fight against AD, as studies indicate AD patients have significantly lower mean plasma concentration of HDL-cholesterol (Kuo et al. 1998), larger mean waist circumference, and higher mean plasma concentrations of triglycerides and glucose, compared with controls (Razay, Vreugdenhil & Wilcock 2007, Altman, Rutledge 2010). This enforces the idea that exercise and a healthy heart both contribute to healthy cognition and also prevent other diseases.

Treatments with the female sex hormone oestrogen (Oestrogen replacement therapy) may also decrease the risk of developing AD (Behl 2002), as the drop in oestrogen levels during and after menopause has been observed to increase the incidence of AD in post-menopausal women (Sunday et al. 2007). Oestrogen is considered a neuroprotective hormone (Hua et al. 2007) in that it can act as an antioxidant, transcription regulator, enhance synaptic plasticity and connectivity (Candore et al. 2010), and has been shown to protect neuronal cells against brain insults including viral proteins and the Aβ peptide (Gottfried-Blackmore, Croft & Bulloch 2008). Studies have also shown a lower incidence
in pre-menopausal women of ischemic stroke, suggesting that oestrogen can also protect against neuro-trauma (Garcia-Segura, Azcoitia & DonCarlos 2001).

Additionally, there are many foods that are suggested to be beneficial towards maintaining cognitive functions in old age, including those that contain antioxidants and vitamins (Smith, Petot & Perry 1999, Grant 1999), such as vitamin E (Morris et al. 2005), that the body requires to keep oxidation at bay. Foods high in these substances and proposed to help retain cognition throughout old age include berries, fruits such as apples, vegetables, nuts (Kidd 2008, de Rekenere 2006, Kannappan et al. 2011) and moderate consumption of alcohol (Mukamal et al. 2003, Criqui, Ringel 1994) especially red wine (Di Matteo et al. 2007), which contains the antioxidant resveratrol (Kim et al. 2006). These foods also help the body prevent cardiovascular diseases (CVD), as indicated by studies that show, for example, in France, which has high consumption of these foods, has some of the lowest incidences of these diseases (Criqui, Ringel 1994). Additionally, Indian cohorts show lower incidences of AD, most likely due to their consumption of curries, with the active ingredient identified as being curcumin (Ganguli et al. 2000, Chandra et al. 2001, Awasthi et al. 2010).

Other foods or diets, and beverages which are purportedly beneficial for preventing AD are coffee (Arendash, Cao 2010), liquorice (Kannappan et al. 2011), and a Mediterranean diet (Scarmeas et al. 2009), which lower cholesterol levels and prevent heart disease by providing dietary vitamins, antioxidants, anti-inflammatory molecules, omega-3 fatty acids and minerals (Grant et al. 2002). The fat composition and fat quantity within a diet also warrant monitoring (Grant et al. 2002, Altman, Rutledge 2010), as studies indicate fatty acids can initiate Presenilin-1 generation and saturated fatty acids can induce HP-tau formation (Altman, Rutledge 2010).

New research also suggests that deficiencies in certain vitamins – required by the body as we cannot manufacture them ourselves – for example B_12, can increase the risk of developing AD (Thomas, Fenech 2007, Aisen et al. 2003). B_12, also known as cobalamin, is an essential organic micronutrient that is required to maintain healthy nervous and circulatory systems, due to its function as a cofactor for two enzymes involved in the tricarboxylic acid cycle, and as a methyl carrier, involved in DNA metabolism (Thomas, Fenech 2007). The latter includes catalysing the conversion from methylenetetrahydrofolate to homocysteine creating methionine, which forms the universal methyl donor S-adenosylmethionine (SAM) and is involved in gene regulation (through DNA methylation) and the repair and regulation of proteins (Thomas, Fenech 2007, Wagner et al. 1995, Scarpa et al. 2006). Sufficient levels of this vitamin are necessary to maintain these essential pathways, and supplements have indicated cognitive impairment improvements and lowered brain atrophy rates in MCI patients (Aisen et al. 2003).

Family history (Breitner, Folstein & Murphy 1986, Breitner, Murphy & Folstein 1986, Breitner et al. 1990), education (Addae, Youssef & Stone 2003), gender (Gao et al. 1998), a high fat diet (Solfrizzi et al. 2008), hypertension (Kalariya 2003), diabetes (Kroner 2009, Carlsson 2010), a history of head trauma (Guo et al. 2000, Mayeux et al. 1995), and susceptibility from particular genes (Bertram et al. 2007) are risk factors for AD. Taking these into account and including the above recommendations for preventative measures, such as lifestyle changes including avoiding toxins, overcoming depression (Berger et al. 1999, Chen et al. 1999) and being married (Helmer et al. 1999), are all suggested to stave off the onset of dementia in old age and AD. Whilst they may not be the major cause of
the disease, and identifying substantial risks might prove difficult due to the challenges related to inconsistencies in defining AD (Daviglus et al. 2010), research suggests they may have significant effects on AD progression.

5.2 Concomitant diseases

Epidemiological studies have raised questions as to why some individuals can survive to old age with intact cognition, yet at autopsy show numerous SP and NFT (Iacono 2009), suggesting that they should have presented with AD during their life. These issues have suggested that some persons have higher ‘cognitive reserve (Stern 2009)’ and handle brain insults better than those with lower cognitive reserve. This is a favoured theory, however it is also suggested that those who are unable to deal with large amounts of the neuropathological lesions may have other diseases present (Kivipelto et al. 2005, Martinez et al. 2002), which are either asymptomatic, or chronic, and contribute to the pathogenesis of AD.

Whilst the body is thought to be quite capable of fighting off diseases, aging brings about a gradual decrease in efficiency at the mechanisms required, allowing normal functions to be compromised. Newer theories suggest that the general chronic inflammation (Finch, Morgan 2007, Giunta 2008) brought on by continual infections can cause dysfunction within homeostatic pathways, however more specific diseases are also suggested to participate. Interestingly, two of the main diseases thought to affect AD risk are lifestyle diseases, cardiovascular diseases (CVD) (Stampfer 2006, Kalaria 2003) and diabetes (Kroner 2009). In most cases these are preventable, or at the least treatable.

CVD share many risk factors with AD and are also suggested to enhance AD progression when present (Stampfer 2006, Luoto et al. 2009). The uncanny number of similarities between the diseases in terms of genetic and environmental risk factors (Stampfer 2006, Martins et al. 2009) indicates the close relationship that these diseases have. The mechanisms by which CVD are proposed to cause AD, or at least participate in pathogenesis are not clearly understood, however lipid dysfunction (Kalaria 2003) is thought to play a large role.

Vascular dysfunction involving endothelial injury contribute to atherosclerotic CVD and this dysfunction within the brain is a key mediator of stroke and vascular dementia (Kalaria 2003), which are thought to contribute to disease development, progression or even cause AD (Kivipelto et al. 2005, Luoto et al. 2009). In addition, cerebral amyloid angiopathy (CAA) is found in up to 80% of AD patients (Altman, Rutledge 2010, Kalaria 2003), even without atherosclerotic CVD. Studies have reported increased cognitive impairment in patients with concomitant CAA and AD, including vascular effects such as capillary occlusion and blood flow alterations (Stampfer 2006, Altman, Rutledge 2010). Suggested mechanisms for disease progression have included indirect effects from CVD, which predisposes the brain to neurodegeneration, as well as the direct effect from vascular factors on neuronal death (Stampfer 2006, Altman, Rutledge 2010).

Recent evidence has proposed that lipid lowering drugs may be beneficial to AD prevention, again connecting these diseases (Wolozin et al. 2000, Jick et al. 2000). Additionally, the main risk allele for both diseases is ε4 of the APOE gene (Stampfer 2006), suggesting that the underlying genetic factors are also closely linked (Bertram et al. 2007). This could indicate that by treating or preventing common risk factors such as
hypertension and dyslipidaemia, patients could also be reducing their likelihood of developing AD.

Type 2 diabetes (diabetes mellitus type 2; DM2) also shares many risk factors with AD (Stampfer 2006, Figaro 2006, Lovestone 1999) and it has even been suggested that AD is a ‘type 3’ of the diabetes family of diseases (Kroner 2009). Treatments for diabetes have even indicated a reduction in AD neuropathology (Beeri et al. 2008). The early metabolic syndrome dysfunction seen in NMR spectroscopy studies (Tukiainen et al. 2008) indicates that glucose metabolism is affected early on in the disease and may initiate the aetiology through breakdown in the normal functioning of these pathways. APOEε4 carriers have also been observed to have reduced brain glucose metabolism in middle age in PET studies (Reiman et al. 2005), suggesting that these elements are connected in some way.

5.3 APOE & Lipidomics

As previously mentioned, the only well confirmed and commonly accepted AD risk factor is the ε4 allele of the APOE gene, with approximately 40-65% of AD patients having at least one copy of the detrimental allele (Finch, Morgan 2007, Farrer et al. 1997, Altman, Rutledge 2010). Gene dose also has an impact on AD risk, with a relative risk of 3.2 for ε3/ε4 carriers and 14.9 for ε4/ε4 carriers to develop the disease (Farrer et al. 1997), as well as affecting the age of onset (Khachaturian et al. 2004). The APOE gene encodes a 34kDa glycoprotein, apolipoprotein E, which has many roles in brain development, growth, function, maintenance, and anti-inflammatory properties, including repair (Horsburgh et al. 2000). Additionally, APOE is a component of very-low-density lipoproteins (VLDLs) and serves as a receptor that participates in distribution of cholesterol and helps to control lipid levels within the brain and around the body (Finch, Morgan 2007, Altman, Rutledge 2010).

These facts, as well as the brain being the most lipid-rich organ of the body, have lead researchers to believe that lipid dysfunction is an essential starting point and initiates disease pathogenesis (Altman, Rutledge 2010, Burns et al. 2003). With this in mind, studies investigating lipid levels in longitudinal studies found those with high cholesterol levels in blood are more susceptible to developing AD later in life (Altman, Rutledge 2010, Kivipelto et al. 2005).

This would open up an avenue of preventative medicine already in use: cholesterol lowering 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, or statins. Whilst this is a relatively new area of study, the research indicates that there is evidence that this treatment may be feasible and lower incidences of the disease (Wolozin et al. 2000, Jick et al. 2000).

APOE is generated within the brain by glial cells (Altman, Rutledge 2010, Pitas et al. 1987) and has been reported to facilitate the pathophysiology of AD through promotion of NFT formation, amyloid deposition, neurotoxicity and oxidative stress, as well as increasing the permeability of the BBB (Altman, Rutledge 2010, Burns et al. 2003). The ε4 allele has also been shown to augment these factors to a greater extent that the ε3 allele, including a study that showed the ε4 allele amplified brain inflammation through increased TNFa-induced cell injury (Altman, Rutledge 2010) and APOEε4 macrophages have an altered inflammatory response (Jofre-Monseny et al. 2007). APOEε4 has also been suggested to impair vitamin E delivery (Mas et al. 2006) and evidence indicates
APOE may determine the occurrence and severity of many concomitant diseases by pathogens such as hepatitis C (Wozniak et al. 2002) and HSV-1 (Itzhaki et al. 1997).

Many studies have investigated the mechanisms through which APOE<sub>ε4</sub> may contribute to AD compared to other alleles, and there is a wealth of information suggesting methods towards which treatments could be directed. The APOE protein created from the ε4 allele increasingly forms a more linear conformation after a high-fat meal as seen in figure 4, which may increase permeability of the BBB, and the lipidation state of APOE is also reported to affect degradation and clearance of Aβ peptide (Altman, Rutledge 2010, Tetali et al. 2006). Additionally it has been shown that the APOE<sub>ε4</sub> allele is more prone to degradation itself and has reduced stability, as well as preferential binding to larger triglyceride-rich lipoproteins than the ε3 allele (Dong et al. 1994, Morrow et al. 2002, Hatters, Peters-Libeu & Weisgraber 2006).

APOE, as part of VLDLs, is hydrolysed by lipoprotein lipase (LpL), located at the brain microvascular endothelium, and could potentially directly damage the BBB and facilitate production of pro-inflammatory mediators due to the high concentrations of lipolysis products it creates (Altman, Rutledge 2010). This lipid accumulation which causes cell dysfunction and death, also known as lipotoxicity, has been associated with apoptosis, and dysfunction of mitochondria, as well as the lysosomal and autophagy pathways (Altman, Rutledge 2010).

To further support the involvement of lipid dysfunction in the aetiology of AD, researchers investigated genes involved in lipid, and specifically cholesterol metabolism. As well as regulating lipid and glucose pathways (Corre, Galibert 2005), USF1 has been shown to manipulate genes involved in immune response and cell cycle control as well as AβPP transcription, synaptic plasticity, and neuronal survival and differentiation (Corre, Galibert 2005, Kovacs et al. 1995, Yang et al. 2002, Naukkarinen et al. 2005). A single study investigating polymorphisms within USF1 however, was negative for association with AD (Shibata et al. 2006), although questions still remain as to its involvement in pathogenesis, due to its important role as a master transcriptional regulator.

Some studies have already suggested improvements in AD risk reduction through the use of statins (Wolozin et al. 2000, Jick et al. 2000, Sparks 2005, Solomon 2009), although meta-analyses have generally been negative (Zhou 2007, McGuinness 2010). It cannot be detrimental however, for an individual to reduce high cholesterol levels, considering the benefits obtained from a healthy heart. The beneficial effects of statins may be better suited to prevention of Alzheimer’s disease (Kivipelto 2005).

**Figure 4.** Schematic representation of APOE<sub>ε4</sub> confirmation before (left) and after (right) a high-fat meal. Dotted lines indicate the salt bridge between the amino acids R61 and E255.
5.4 Polymorphisms & genes

Studies of the disease have indicated that a large part of AD is hereditary and passed on through genetic polymorphisms (Myllykangas et al. 2005, Peuralinna et al. 2008) or differences between individuals in genes (Wesson Ashford, Mortimer 2002). Up to 80% of disease risk is thought to be hereditary (Wesson Ashford, Mortimer 2002) and affect disease occurrence and determine whether an individual will develop AD. The only accepted AD risk gene, APOE, is claimed to account for approximately 65% of this genetic risk (Bertram et al. 2007).

Initial studies into the genetic risk of AD focussed on pathways that were known to be involved in the disease, however due to the large number of controversial studies, resulting in a large number of potential disease factors, and the small impact of the identified risk genes, the tide is changing. Newer studies are now utilising genome wide association studies (GWAS), where up to 500,000 single nucleotide polymorphisms (SNPs) can be detected simultaneously (Lambert JC et al. 2009, Beecham et al. 2009, Harold et al. 2009).

In order to assess the new data that is accumulating from these large studies, a website was developed (www.alzgene.org), which records associations (positive/negative) and risk assessments for identified AD risk factors. In addition, the data is meta-analysed and generates an overall risk assessment for revealed polymorphisms and genes, and the likelihood of their effects and ability to cause AD (Bertram et al. 2007).

Due to the large number of AD risk genes currently in the literature, only the top ten most likely AD-risk factors will be discussed, according to the www.alzgene.org website (accessed 11.1.2011). The list includes (from most likely to least) APOE, CLU, PICALM, EXOC3L2, BIN1, CR1, SORL1, GWA 14q32.13, TNK1 and IL8. See table 2 for the complete data accessed the same date.

Table 2. The top AD risk genes from the www.alzgene.org website (accessed 11.1.2011).


As APOE has been previously discussed, focus will shift to the remaining nine risk genes. Of the nine revealed genes from the www.alzgene.org website, two are
inflammatory, two are related to lipid metabolism and transport, three are linked with endocytosis and vesicular transport, and two have been associated with cellular signalling according to the NCBI protein database (http://www.ncbi.nlm.nih.gov).

CLU or clusterin, found on chromosome 8, is also known as APOJ and as suspected, encodes a lipid transport molecule, able to bind Aβ peptide like APOE(Jenne, Tschopp 1992, Jones, Jomary 2002). CLU most likely participates in Aβ peptide transportation both out of and possibly back into the brain(Jenne, Tschopp 1992, Jones, Jomary 2002, Calero et al. 2000, DeMattos et al. 2004). According to the NCBI protein database, CLU has been reported as being involved in cell death, tumour progression and neurodegenerative disorders. It has small effects on AD risk and most likely participates in disease pathogenesis through gene expression modulation or damage induced expression(Guerreiro et al. 2010).

PICALM, found on chromosome 11, encodes the phosphatidylinositol binding clathrin assembly protein and is thought to be involved in synaptic neurotransmitter release and intracellular trafficking(Dreyling et al. 1996, Tebar, Bohlander & Sorkin 1999, Yao et al. 2005). According to NCBI, PICALM has many names and is involved in endocytosis. Researchers suggest its involvement in AD relates to PICALM’s location in endothelial cells and is most likely associated with transporting Aβ peptide through the BBB(Baig et al. 2010).

EXOC3L2 encodes exocyst complex component 3-like 2, is located on chromosome 19, and its function according to NCBI is unknown. Studies indicate it is transactivated by the Hepatitis B virus X antigen(Seshadri et al. 2010), which suggests it may participate in the inflammation process.

BIN1 encodes bridging integrator 1 and is found on chromosome 2. NCBI reports the protein is involved in synaptic vesicle endocytosis, including vesicle formation, and studies indicate BIN1 facilitates apoptosis and has a role in membrane organisation(Seshadri et al. 2010).

CR1 produces complement component (3b/4b) receptor 1, which is the main receptor for the complement C3b protein, a major part of the innate immune system and binds to Aβ peptide (Rogers et al. 2006, Wyss-Coray et al. 2002). Through this mechanism it is thought to promote clearance of Aβ peptide and therefore affect AD risk. The gene for it is found on chromosome 1, and the membrane glycoprotein CR1 mediates cellular binding to immune complexes or particles that have activated the complement system(Rogers et al. 2006, Wyss-Coray et al. 2002, Kuo et al. 2000, Zhou et al. 2008).

SORL1 (also known as LR11, SORLA or SORLA1), or sortilin-related receptor, is located on chromosome 11q23 and produces a receptor for neuronal APOE, low density lipoprotein receptor class A. SORL1 binds AβPP and regulates its sorting into endocytic- or recycling- pathways(Rogaeva et al. 2007). High levels of this receptor have been associated with lower Aβ peptide production due to SORL1 promoting recycling of AβPP, instead of transporting it to endosomes or lysosomes where Aβ peptide is produced(Rogaeva et al. 2007). Many SORL1 SNPs have been associated with AD risk(Bertram et al. 2007, Rogaeva et al. 2007), however further factors, both genetic and non-, are also thought to affect expression of this receptor, although the implications of these are not fully understood.
GWA 14q32.13 (rs11622883) was identified in GWAS and does not locate to any known gene loci and therefore its function is unknown (Grupe et al. 2007). Further research will be required to determine the link SNP rs11622883 has with AD.

TNK1 encodes ‘tyrosine kinase, non-receptor, 1’ and is located on chromosome 17. It mediates intracellular signalling subsequent to receptor activation, according to (Azoitei et al. 2007, Felschow, Civin & Hoehn 2000). Studies have determined that TNK1 is a molecular switch that determines the properties of TNFα signalling. By inhibiting NFκB, TNK1 facilitates the TNFα apoptotic pathway leading to cell death (Azoitei et al. 2007).

IL8 or interleukin 8, located on chromosome 4, produces an inflammatory molecule known for its pro- and anti-inflammatory actions as a chemokine (Li et al. 2009). IL8 is a major mediator of inflammatory responses through its functions as a chemoattractant and properties as an angiogenic factor (Li et al. 2009).

Many more genes have been associated with AD risk, however the risk effects are small. Many studies suggest it is most likely a combination of multiple genes, as well as environmental effects that impact on an individual’s AD risk, however studies revealing associations do reveal pathways that may be involved in the aetiology of the disease.

5.5 Epigenetics

Whilst whole genome association studies try elucidate the underlying genetic causes of AD, researchers have started investigating a much more subtle – and less understood – mechanism for the disease to manifest (Vanyushin 2007, Calvanese et al. 2009). Epigenetics is a relatively new field of study (Vanyushin 2007) and involves the cell’s way of turning on and off genes, which can be manipulated by food intake and other external pressures, as well as affecting the developmental stages of an individual’s life cycle. Epigenetics also manipulate upregulation and down-regulation of genes, as well as regional changes in different organs due to local stimuli, including pathogens (Vanyushin 2007).

When gene promoters become methylated, they are physically blocked from binding to transcription factors and essentially inactivated, the gene silenced and protein production inhibited (Lee, Ryu 2010). The methylated DNA is associated with methyl-CpG-binding domain proteins (MBDs), which recruit other components such as histones that inactivate the gene region or locus, forming what is known as chromatin (Lee, Ryu 2010, Suzuki, Bird 2008).

Whilst Aβ peptide production is usually normal, it has been suggested that through epigenetic modifications of the AβPP gene promoter – hypomethylation (Tohgi et al. 1999, West, Lee & Maroun 1995) due to aging mechanisms or other insults – the protein is upregulated and consequently generates the neuropathology seen in AD brains.

Further studies have supported evidence for this mechanism, including observations of inflammatory genes being hypomethylated in AD cortex (Akiyama et al. 2000), and investigations into epigenetic modifications in monozygotic twins discordant for AD, indicating that DNA methylation was reduced in the AD-twin (Mastroeni et al. 2009). The suggestions for causes behind these epigenetic changes vary, with some proposing age-related effects that gradually lower the methylation content of genes, to others that suggest a decrease in SAM levels – the primary methyl donor of cells – could be to blame (Scarpa et al. 2006).
Other genes have also been reported to be affected by epigenetics in AD-brains, although the cause for these modifications is unknown and will require further studies before it can be concluded as to whether these changes are causative or mere bystanders in the initiation and progression of AD, although they propose an interesting therapeutic target (Lee, Ryu 2010).

6. Causal theories

6.1 Brief history of causes

Since the description of the disease in 1907 by Alois Alzheimer (Alzheimer 1907), there have been many theories as to the cause of AD. In the 1960’s a correlation was found between the numbers of SP and NFT and cognitive decline, opening up investigations into the causes behind these neuropathological lesions (Lage 2006). It was not until the 1970’s however, that AD was officially listed as a disease and no longer considered a part of normal aging, as discussed in the review (Lage 2006). Classified as a disease, AD attracted intensive research into its causes. Evidence of large amounts of the metal aluminium found in NFT in AD patients’ brains (Perl, Brody 1980, Terry, Pena 1965) lead to theories that excess of this metal caused AD. This theory is generally dismissed nowadays, due to mostly circumstantial evidence.

Most current theories surrounding AD pathogenesis involve the identification of abnormal or large amounts of the molecules found in SP and NFT within AD patients’ brains, such as Aβ peptide. Some researchers have suggested imbalances in the brain’s homeostatic environment could cause AD (Crouch, White & Bush 2007), along with viruses and bacteria (Kamer et al. 2008, Itzhaki, Wozniak 2004) that are able to cross the blood-brain-barrier, as well as the possibility that the immune system loses the ability to perform properly and effectively (Giunta 2008, Miklossy 2008).

The following sections discuss some of the foremost theories thought to cause the brain to degenerate and develop AD.

6.2 Cholinergic hypothesis

The cholinergic hypothesis (Martorana, Esposito & Koch 2010, Contestabile 2010) was theorised because investigations showed that AD brains had lower levels of acetylcholine – a major neurotransmitter in the brain – than non-demented elderly cases. As one of the oldest causal theories of AD, the disturbances in the cholinergic system have also been the focus of most treatments available on the market, although they fail to provide much improvement in delaying clinical symptoms of the disease.

Treatments of AD based on the cholinergic hypothesis (donepezil, rivastigmine and tacrine etc.) are cholinesterase inhibitors, which act simply by deterring the actions of cholinesterase – an enzyme which breaks down the neurotransmitter acetylcholine. By reducing cholinesterase, the brain would be able to retain acetylcholine and thus function properly.

Although newer compounds treating this avenue of dysfunction have been developed with fewer side effects (in order to provide higher drug doses) (Martorana, Esposito & Koch 2010), this is not the only thing that goes wrong in an AD brain and thus
researchers have looked to other causal theories and newer treatments that will have more improvement on patients’ symptoms.

6.3 Amyloid theory

The most commonly supported hypothesis for the cause of AD relates to a protein expressed in many cells, of unknown function and implicated in familial AD due to mutations in the gene that code for it(Wisniewski, Wisniewski & Wen 1985). Although its function is not completely understood, β-amyloid precursor protein (AβPP) is suggested to be critical for neuron growth(Turner et al. 2003, Vasto et al. 2008, Priller et al. 2006), signalling, and may also function as an antioxidant(Crouch 2007) and a metalloprotein, modulating copper transport and metabolism(Turner et al. 2003, Priller et al. 2006, Kong et al. 2007).

The parent protein, the 695-770 amino acid AβPP, in most cell types undergoes the non-amyloidogenic pathway. Cleavage of the AβPP protein can occur at many sites within the cell, including the trans-Golgi network, mitochondrial membrane and plasma membrane(Rogaeva et al. 2007), with location and reagents present said to dictate which pathway is followed. The non-amyloidogenic pathway results in the production of the P3 peptide fragment, which consists of 16 amino acids and involves α-secretase cleavage followed by a γ-secretase cut within the Aβ domain of the AβPP protein(Selkoe 2001, Findeis 2007) (see figure 6).

The amyloidogenic version of the pathway involves cleavage by β-secretase followed by the γ-secretase (see figure 6), releasing the 40-43 amino acid amyloid beta (Aβ) peptide, thought to cause the neurodegenerative disease(Selkoe 2001, Findeis 2007). The enzymatic action of β-secretase leaves a C-terminal fragment known as APP-CTFβ or C99, within the membrane and releases APPsβ into the extracellular space. After Aβ peptide generation by γ-secretase from the C99 fragment, the Aβ peptide is extracellularly secreted(Rogaeva et al. 2007).

Most of the Aβ peptides produced are 40 amino acids long, however it is thought in the diseased state the usual 10% of Aβ42 peptide production increases, causing havoc within the cell and the surrounding environment(Vasto et al. 2008). This longer form (Aβ42) is more hydrophobic and fibrillates more easily, and is also controversially thought to be more neurotoxic than the Aβ40 peptide(Selkoe 2001, Findeis 2007).

These peptides can be found as monomers, oligomers, protofibrils and fibrils and in the latter stages form the SP seen in the brains of AD patients. Much debate has surrounded the identification of the actual toxic form of Aβ, ranging from the aggregation of the SP themselves, to the new idea that soluble forms (ref McLean 1999, Crouch 2008), specifically oligomers of the peptide, are the detrimental form(Hardy, Selkoe 2002, Gandy et al. 2010). This is supported by studies indicating the neurotoxicity of Aβ oligomers against neurons and their elusiveness in current detection methods (reviewed in (Gandy et al. 2010)).

Recent studies have also suggested that intraneuronal Aβ, or even truncated forms of the peptide are responsible(Wirths et al. 2010, Miravalle et al. 2005), although this revelation is currently not fully understood. Research has confirmed the difficulty in identifying the toxic form of Aβ, due to its different assembly states(Lesné et al. 2006, Tew et al. 2008), with this ability in itself being a potential mechanism of toxicity(Smith, Cappai & Barnham 2007).
Figure 6. The amyloid beta component is formed by subsequent cleavage by the enzymes \( \beta \)-secretase and \( \gamma \)-secretase of the amyloid precursor protein (A\( \beta \)PP), whilst initial cleavage with the \( \alpha \)-secretase results in the formation of the P3 peptide fragment and inhibits the formation of the neurotoxic amyloid beta protein.

Researchers have suggested that SP are a storage mechanism by the brain to contain the neurotoxic oligomers (Feng et al. 2009), or even suggested that A\( \beta \) peptide itself may be related to immune function, with researchers noticing its similarity to proteins of the innate immune system (Soscia et al. 2010). Mechanisms by which A\( \beta \) peptide is suggested to accomplish this destruction is through the physical interruption of neuronal networks, blocking signalling pathways and initiating oxidative stress and inflammatory responses through free radical production, which cause further damage to the affected cells, as reviewed in (Hardy, Higgins 1992, Hardy, Selkoe 2002, Duyckaerts, Delatour & Potier 2009).

Apart from a suggested increase in A\( \beta \) peptide production, the accumulation of the peptide in older age can be attributed to the malfunction or ineffectiveness of A\( \beta \) peptide removal from the brain. Elimination of A\( \beta \) peptide can be instigated by phagocytosis of the peptide by the resident immune cells – microglia and astrocytes – said to be capable of degrading limited amounts and also transporting A\( \beta \) peptide from the brain into blood.
or CSF (Perry, Nicoll & Holmes 2010). Other mechanisms of reducing Aβ peptide include enzymatic degradation such as that by neprilysin and insulin-degrading enzyme (IDE) (Duyckaerts, Delatour & Potier 2009). Additionally, removal of the peptide across the blood brain barrier (BBB) can be mediated by proteins such as low-density lipoprotein receptor related protein and P-glycoprotein (discussed in (Duyckaerts, Delatour & Potier 2009)). Researchers have also suggested drainage of Aβ peptide through perivascular pathways may contribute to its removal from the brain (Hawkes et al. 2011, Weller et al. 2009).

Traditional consensus indicates that Aβ peptide accumulations occur extracellularly and that this is the cause of AD degeneration, comprising the basis of the amyloid theory (Hardy, Higgins 1992, Hardy, Selkoe 2002) of pathogenesis. There is newer theories suggesting that intraneuronal Aβ peptides may be the cause of the neurotoxicity (Gouras et al. 2010, Takahashi et al. 2004). Aβ peptide phases of progression within AD brains are suggested to follow a pre-determined pattern (Thal et al. 2002), although exceptions to the rule have been observed. In 2002, Thal and colleagues (Thal et al. 2002) described the regional distribution of SP, stating that initial deposits are found in the neocortex, followed by the allocortical regions and expanding to the diencephalic nuclei, striatum and cholinergic nuclei of the basal forebrain, involving the brainstem and finally spreading to the cerebellum. Following SP accumulations, secondary NFT formation and neuronal dysfunction occur, causing the symptoms recognised as AD (Thal et al. 2002).

SP appear to have subtypes, enabling researchers to measure different stages of Aβ peptide accumulation and progression. Various techniques can detect diverse forms of SP (discussed in (Duyckaerts, Delatour & Potier 2009)), and those most commonly referred to are non-neuritic and neuritic SP, of which the latter are more commonly associated with cognitive dysfunction and AD (Price, Morris 1999). Other descriptions include four stages of SP, suggesting a life cycle starting from ‘diffuse’ SP and progressing through ‘primitive’ and ‘classic’ forms, finally visible in stains as a shell termed ‘burnt out’ (Armstrong, Myers & Smith 1993). Throughout the progression of Aβ peptide accumulation, SP are described as having particular immunoreactive elements and some subtypes involve immune cells, finally ending their life cycle in the ‘burnt out, neuritic’ form consisting of just an amyloid core (see review by (Duyckaerts, Delatour & Potier 2009)), although there have been indications that not all SP behave identically (Verkkoniemi et al. 2001).

Studies have identified substituents in SP, such as APOE, CRP, tau protein, ubiquitin, α1-antichymotrypsin, protein kinase C, complement proteins, fibroblast growth factor, advanced glycation end products, proteoglycans, copper ions, cholinesterases and cholesterol, amongst others (Duyckaerts, Delatour & Potier 2009, Burns et al. 2003, Takeuchi, Yamagishi 2008, Yasojima et al. 2000). The implications of these are unknown within the context of disease pathology.

A notable characteristic of SP, which has been suggested to involve a bigger part of immune cell interaction, is some mechanism of stability in SP growth. Studies have observed SP size unchanged over long periods of time (Christie et al. 2001) and SP completely covering the cortex have never been witnessed. These factors – including evidence that although diffuse SP are thought to be early stages of SP formation (Dickson et al. 1988), they always exist in some numbers regardless of duration and stage of
AD(Gearing et al. 1993, Iwatsubo et al. 1994, Wolf et al. 1999) – indicate the elusive aetiology of SP and leaves conclusions as to their disease-causing status unresolved.

Recent work has also suggested that Aβ peptide can also be detected within cells(Aho et al. 2010, Wirths et al. 2001), however the implications of this are currently unknown. Furthermore, the validity of the amyloid hypothesis has recently been questioned due to failed vaccines developed to remove SP, which although successful, did not remove NFT or improve patients’ cognition(Holmes et al. 2008). Another study also suggested that APOE 4 was associated with SP, CAA and NFT, although not with dementia, after adjusting for these pathologies(Nicoll et al. 2010). The common occurrence of these lesions also in non-demented elderly cases also sheds doubt upon their involvement in causing AD(MRC-CFAS Neuropathology Group 2001, Polvikoski et al. 2006, Savva et al. 2009, Matthews et al. 2009). Many studies have been investigating these avenues of research, however conclusions remain elusive.

Alzheimer’s disease treatments have long been sought, although only treating symptoms that occur in the latter stages of the pathology. Unfortunately these treatments have not been successful in remitting or halting the disease and research continues to try to elucidate the fundamental mechanisms that are involved in AD.

Current therapies have included more drastic measures such as developing antibodies against Aβ peptide and immunising patients to remove SP, which although it was thought that the heart of the problem was being tackled, has so far not been the ‘magic bullet' treatment it was designed to be(Holmes et al. 2008). This does not necessarily discredit the ‘Amyloid theory’, as further research to identify the actual component or form of the Aβ peptide that is neurotoxic may lead to successful treatments.

6.4 Tau theory

As SP and neuron loss do not correlate well(Gómez-Isla et al. 1997), the attention has turned towards the other well known characteristic of AD brains, supporting the idea known as the ‘tau hypothesis’(Iqbal 2009). The other major hallmark of AD involves a protein that supports tubulin assembly into microtubules, which support the structure and flexibility of the cell(Lace, Wharton & Ince 2007). Abundant in the central nervous system and specifically in neurons, tau utilises alternative splicing isoforms and phosphorylation to perform its functions(Lace, Wharton & Ince 2007).

Tau has six isoforms in the brain, which are all created from the one gene: MAPT, which is found on chromosome 17(Lace, Wharton & Ince 2007, Andreadis, Brown & Kosik 1992). Differences between the isoforms relate to different numbers of binding domains and differential splicing of the gene’s 16 exons, creating six proteins ranging from 352 – 441 amino acids in length. Although some studies have suggested there are different ratios of particular isoforms in diseased states, all forms are present in AD(Iqbal 2009, Lace, Wharton & Ince 2007, Andreadis, Brown & Kosik 1992).

Phosphorylation and de-phosphorylation of tau occurs under normal homeostatic conditions, with so-called hyperphosphorylation occurring in diseased brains(Lace, Wharton & Ince 2007). This phosphorylation disequilibrium causes the phosphorylated tau (HP-tau) to dissociate from microtubules and aggregate into dense compact paired helical filaments (PHF) within the cell – ultimately killing the neuron that it is supposed to be supporting and resulting in the NFT observed in postmortem AD brains(Iqbal 2009). It has also been observed that the tau present in PHF has higher D-forms of
aspartate and serine than L-forms, suggesting it is long lived and thus may enhance aggregation (Shapira, Austin & Mirra 1988, Kenessey et al. 1995). A subsequent stage in the life cycle of NFT occurs when they survive after the death of the neuron, leaving behind ‘ghost’ tangles that are easily seen with silver staining or immunohistochemical techniques (Duyckaerts, Delatour & Potier 2009).

NFT consist mostly of HP-tau, but are also known to include casein kinase II, protease nexin I, heparin sulphate proteoglycan, fibroblast growth factor, microtubule association protein-5, and ubiquitin, amongst other molecules (Duyckaerts, Delatour & Potier 2009, Hasegawa, Arai & Ihara 1990, Perry et al. 1987, Rosenblatt, Geula & Mesulam 1989, Baum et al. 1992). The participation of these molecules in NFT development is currently undetermined, however they could also be a secondary mechanism to the emergence of these brain lesions.

A staging system developed by Braak and Braak (Braak, Braak 1991) monitors the progression of AD into six so-called Braaks’ stages, based on the location and density of NFT. Stages I and II describe neuropathology containing NFT found in the entorhinal, transentorhinal and CA1/subicular portions of the hippocampus. Increasing numbers of tau pathology in the limbic system represent stages III and IV, where higher numbers in the hippocampus normally correspond with the severity of dementia. NFT found in the isocortical areas resolves stages V and VI and complete the progression of the disease. Newer staging systems have utilised immunohistochemistry (Braak et al. 2006) although some are yet to be validated (Alafuzoff et al. 2008).

HP-tau and NFT are not only seen in the brains of AD patients; they are also characteristic lesions of other neurodegenerative diseases, collectively termed ‘tauopathies’ (Lace, Wharton & Ince 2007). Such diseases include several entities in the major tau molecular class of frontotemporal lobar degenerations (FTLD-tau), including for example sporadic corticobasal degeneration, progressive supranuclear palsy, Pick’s disease and argyrophilic grain disease (Mackenzie et al. 2010). Some noted mutations in the gene that code for tau, also directly cause neurodegeneration in the form of hereditary frontotemporal dementia and Parkinsonism linked to chromosome 17 (Lace, Wharton & Ince 2007), without the presence of SP. The tau mutations directly disrupt the normal functioning of the tau protein and also substantiate the neurotoxicity of HP-tau. Other mechanisms causing the development of NFT are proposed to be through an imbalance between the activities of kinases and phosphatases that control the phosphorylation of the tau protein (Iqbal 2009).

Whilst neurotoxic itself, HP-tau is thought to be secondary to Aβ peptide accumulation in AD (Iqbal 2009, Lace, Wharton & Ince 2007). This is the generally accepted theory, although there are some researchers who believe the tau protein is responsible for the initiation of the neurodegeneration, due to its obvious neurotoxicity and its ability to be predictive of dementia severity (Thomas, Fenech 2007, Iqbal 2009, Lace, Wharton & Ince 2007). HP-tau pathology is often observed several decades prior to Aβ peptide deposition and thus advocates that tau could be the cause behind SP and thus AD (Lace, Wharton & Ince 2007, Braak, Del Tredici 2011), however studies have also demonstrated that Aβ oligomers are capable of instigating phosphorylation of the tau protein (Lace, Wharton & Ince 2007), corroborating the Aβ theory and leaving this topic undecided.
The exact mechanisms behind these lesions’ roles are still to be determined, including whether they are causative in AD, or just secondary events to some other underlying and as yet unknown pathology.

7. Other potential causes

7.1 Inflammation

Whilst plaques and tangles are more specific to AD, there are other characteristics of the diseased brains that are broader in their disruption of brain function and causing neurodegeneration. Inflammation, seen in many diseases of the elderly, is observed in AD brains and has long been thought to initiate the recognised pathology. New theories suggesting Aβ peptide is an acute phase protein(Soscia et al. 2010, Kontush 2005) and involved in immunity also support the participation of inflammation in AD aetiology. In addition, a common risk factor for the disease is brain injury(Guo et al. 2000, Mayeux et al. 1995), suggesting that chronic inflammation could initiate or at least partake in the course of AD.

Epidemiological studies in the early 1990’s(McGeer et al. 1990, Beard, Kokman & Kurland 1991) suggested that exposure to anti-inflammatory drugs (more specifically non-steroidal anti-inflammatory drugs – NSAIDs) reduced the risk of developing AD in later life. Whilst contradictory results have stemmed from this, including failed clinical trials of NSAID use in AD patients(Aisen et al. 2002), it is evident that inflammation plays some part in the disease. Much discussion surrounds the type of effect that inflammation plays however, as there is evidence for both a beneficial (Sunday et al. 2007) and detrimental(Giunta 2008) role in the disease aetiology.

Multiple inflammatory markers are observed in postmortem AD brains, including pro-, anti- and post-acting molecules(Finch, Morgan 2007, McGeer, McGeer 2007), as well as the activation of the resident immune (glial) cells in the central nervous system – microglia and astrocytes(Finch, Morgan 2007, McGeer, McGeer 2007), although this is not always the case(Verkkoniemi et al. 2001). These have been found in the AD-affected regions of the brain and also localised with SP and NFT(Tuppo, Arias 2005). Other researchers have even suggested that SP and NFT are byproducts of the host response to basic pathogenic processes(Castellani et al. 2008). Although still other researchers have suggested that microglia lose functionality and this may participate in disease aetiology(Graeber, Streit 2010).

Some examples of upregulated inflammatory components found in AD brains are prostaglandins, complement component proteins, anaphylotoxins, adhesion molecules, cytokines, chemokines, proteases, protease inhibitors, free radicals, pentraxins such as CRP and nuclear factor-kappa B (NFκB), as well as acute phase proteins, amongst other inflammatory responses(Moreira 2008, Yasojima et al. 2000, McGeer, McGeer 2007, Casadesus et al. 2007, McGeer, Klegeris & McGeer 2005, Terai, Matsuo & McGeer 1996). These in turn can initiate further inflammatory pathways and indicate that inflammation definitely participates in disease progression, although to what extent is still undetermined.

Microglia make up 12% of brain cells and are normally in a ‘resting’ state monitoring the brain, however recent studies contradict the terminology and suggest that these cells are in fact in a low state of activity(Block, Zecca & Hong 2007). Fully activated
microglia on the other hand, are essential for brain development and maintenance, although once activated, they can remain in this state(Zilka, Ferencik & Hulin 2006) and enhance neuronal damage in affected regions(Tuppo, Arias 2005). The presence of activated microglia has been observed to increase throughout AD progression(Block, Zecca & Hong 2007) and studies suggest that Aβ peptide deposits are capable of stimulating this activation, due to the observation that microglia cluster around Aβ peptide aggregation sites(Tuppo, Arias 2005).

Microglia reportedly produce, or signal other cells to produce pro-inflammatory molecules such as interleukin-1β, interleukin-6 and tumour necrosis factor-alpha (TNFα), which recruits lymphocytes to inflamed areas by altering vascular cell adhesion(Ghoshal et al. 2007). As well as being capable of directly killing cells(Ghoshal et al. 2007), the excretion of neurotoxins and excitatory neurotransmitters can escalate and spiral out of control in the AD brain.

Astroglia cells are major players in the maintenance of the BBB, including regulating its permeability(Altman, Rutledge 2010). Studies have reported BBB integrity is key to AD progression, although the cause of this permeability has not been determined as solely the effect of astroglial actions, or a combined effort of other more elusive factors(Altman, Rutledge 2010).

Whilst microglia and astrocytes are the resident immune cells within the brain, the neurons themselves have also been observed responding to inflammatory signals, which could participate in disease pathology. Evidence also suggests that the risk allele APOEε4 incites an altered inflammatory reaction in macrophages, compared to APOEε3, with lower anti-inflammatory mediators(Jofre-Monseny et al. 2007), thus tying together these two mechanisms. The APOEε4 allele has also been proposed to increase chronic inflammation in carriers(Wozniak et al. 2002, Itzhaki, Wozniak 2006, Itzhaki, Wozniak & Dobson 2002), as compared to APOEε3 carriers.

There is discussion as to whether inflammation found in AD brains is causative of the disease, or a response to the breakdown of normal functioning mechanisms. Evidence for the former theory includes exposed genetic polymorphisms of inflammatory molecules that associate with the risk of AD, such as IL8(Li et al. 2009) and CR1(Lambert JC et al. 2009) (as designated on the www.alzgene.org website – 22.11.2010).

Further indications of inflammation’s involvement in AD come from studies showing increased levels of inflammatory markers in diseased brains, as well as in blood(Finch, Morgan 2007, Schmidt et al. 2002). Additionally, high levels of these markers can also indicate progression of the disease(Schram et al. 2007), although what this effect has on initiation of the disease is still debated. Such markers as CRP are capable of activating clearance of cellular debris through the complement pathway and macrophages(Bottazzi 2006), as well as functions pertaining to the host defence system. Although thought to be produced only in the liver during inflammatory responses(Pepys, Hirschfield 2003), immunohistochemistry studies have shown CRP is also produced locally within the brain and studies have also suggested an upregulation of CRP is present near SP and NFT(Yasojima et al. 2000).

Additional regulatory molecules involved in inflammatory responses such as the COX enzymes(Ho et al. 2006), which produce prostaglandins by converting arachidonic acid, have been suggested to tie inflammation in with increased production of Aβ peptide through increased prostaglandin E2 production(Ho et al. 2006). Prostaglandin E2 has been
shown to increase γ-secretase cleavage of AβPP, producing Aβ40-42 and can be reduced with COX inhibitor drugs (Ho et al. 2006).

The mechanism by which AD could develop with inflammatory responses participating is no doubt a complex one and probably includes many other factors. Genes or polymorphisms that enhance an individual’s inflammatory response or susceptibility to a particular disease(s), in addition to the decrease in effectiveness of maintenance systems in old age, could create a system of chronic inflammatory activation and allow insults from additional diseases. This aging immune system with enhanced innate immune responses has been suggested by those backing the ‘inflammaging’ theory (Giunta 2008).

Whilst observations of inflammation’s part in AD are evident, it is still unknown as to whether abnormal functioning of our immune system is to blame in disease aetiology, or secondary to other detrimental events prior to the appearance of inflammatory responses.

### 7.2 Oxidation and Mitochondrial dysfunction

Prior to inflammatory pathways, oxidation is thought to initiate the immune reaction that causes cascades of mechanisms to go wrong in AD (Casadesus et al. 2007). As the body ages, increasing levels of oxidative stress markers are produced and the efficient functioning of molecules to clean up and repair these errors also deteriorate with time (Casadesus et al. 2007). This in turn leads to further creation of oxidation and destruction continues in a cyclic manner. These processes themselves are reported to cause inflammation and the hallmarks of AD – SP and NFT (Moreira 2008). How and why are still unanswered questions in this debate, although some researchers suggest that the SP and NFT are the end result of a ‘mopping up’ system that reduces oxidative stress within the brain (Castellani et al. 2006).

The creation of reactive oxygen species (ROS) with age results from many homeostatic mechanisms – from producing energy to replication of DNA for cellular growth. During aging, the processes that maintain protein integrity and cleanup systems reduce in efficiency and cause the build up of numerous errors (van Leeuwen et al. 1998). These in turn cause further mistakes to occur and the system continues to suffer with the build-up of dysfunctional proteins and ROS. ROS themselves can directly oxidise and damage DNA, lipids and proteins and induce stress-responses, as well as facilitate apoptosis through mitochondrial pathways (Altman, Rutledge 2010, Casadesus et al. 2007).

Levels of oxidative stress have been linked with AD (Casadesus et al. 2007), leading researchers to investigate clinical trials in anti-oxidant therapies. Whilst trials with vitamin E (Morris et al. 2005) have been inconclusive, it may be that the window of treatment has been surpassed in patients who have converted to late stages of AD. This has been the suggested mechanism for most failed treatments and it is proposed that patients have exceeded the threshold for resolving the disease and its causes.

It is difficult to determine whether oxidation is causative in AD, due to the fact that it is a normal part of aging (Casadesus et al. 2007). It is difficult to determine which individuals will be affected, and also challenging to undo years of oxidative damage. Measures for reducing excess levels of ROS and oxidation would have to be initiated early and could explain the lack of improvement in clinical trials on AD patients towards these mechanisms.
A proposed theory related to oxidation and other supposedly causative agents of AD suggests that some individuals have a higher threshold for the damage (Stern 2009) caused during aging. This higher tolerance to oxidative (and other) damage prevents the individual from succumbing to the build-up of ROS and other affected molecules (proteins, lipids, Aβ peptide etc.) and therefore protects from the development of AD. This higher tolerance could be related to diet, environmental factors or genetics (Dumurgier et al. 2010), but as yet remains an uncertain area that is difficult to prove without highly detailed longitudinal studies.

Mitochondria are the energy production factories of the cell (Cheng, Hou & Mattson 2010) and any defects within these usually cause serious disorders (Gibson, Sheu & Blass 1998). Having to deal with large amounts of energy generation for the cell, including ATP and NAD\(^+\) production, these industrial units have also developed ways of managing the vast amounts of waste that are created. During aging however, these systems become less efficient and can break down and this is one of the proposed mechanisms for which AD is thought to initiate – through mitochondrial dysfunction (Gibson, Sheu & Blass 1998, Onyango et al. 2010, Swerdlow, Khan 2009, Twig, Hyde & Shirihai 2008).

The large amount of ROS and other oxidised molecules, such as lipids and glucose, are taxing to the mitochondrial machinery and become more difficult to clean up or remove with age, causing the mitochondria to function less effectively, which can have drastic implications for both the individual cell and body as a whole (Swerdlow, Khan 2009). In addition to the mechanism of aging in general, some studies have suggested haplotypes and alleles of mitochondrial genes are more susceptible to mitochondrial dysfunction (Swerdlow, Khan 2009, Maruszak et al. 2009, van der Walt et al. 2004). Studies have found associations between mitochondrial haplotypes H, U, K, J, T and decreased mitochondrial function, as well as associations with AD itself (Maruszak et al. 2009, van der Walt et al. 2004).

Mitochondria contain 37 genes encoding 22 tRNAs, two rRNAs and 13 polypeptides – the latter of which encode subunits of the electron transport protein complex (Cheng, Hou & Mattson 2010). These are complimented by about 1000 further mitochondrial proteins, which are found within nuclear genes (Cheng, Hou & Mattson 2010). In addition to polymorphisms in mitochondrial genes that have reportedly been associated with increased AD risk, the mitochondrial biogenesis regulator peroxisome-proliferator-activated receptor γ co-activator 1α (PGC1α) provides an interesting mechanism for disease aetiology (discussed in Cheng, Hou & Mattson 2010). As a transcription factor, it co-activates and interacts with other transcription factors such as peroxisome-proliferator-activated receptor γ (PPARγ), thyroid hormone and oestrogen, and is involved in lipid metabolism and responses by antioxidants. It has been suggested that through mitochondrial biogenesis (Onyango et al. 2010), which can be stimulated by exercise (Cheng, Hou & Mattson 2010), synaptic plasticity may be enhanced and thus improve cognitive function or provide the ability for the brain to retain cognitive reserve (Stern 2009).

In addition to generating energy for the cell, mitochondria regulate subcellular calcium signalling and homeostasis as well as having purported roles in neuroplasticity through their role as a signalling station (Cheng, Hou & Mattson 2010). Distributed throughout the cell, including presynaptic terminals and the axons of neurons, mitochondria also have the ability to undergo fusion and fission, move rapidly between
subcellular compartments, function as signalling platforms and respond to electrical, neurotransmitter and growth factor receptor stimuli (Cheng, Hou & Mattson 2010). Mitochondrial fission and fusion pertain to the regulation of location, number, function and morphology of these organelles (Cheng, Hou & Mattson 2010).

Changes in mitochondrial dynamics, as described above, have been associated with the processes of learning and memory such as long term potentiation (LTP) and recovery of synapses in phases of high synaptic activity, which when not functioning correctly have been marked with oxidised DNA and RNA and structural mitochondrial abnormalities (Cheng, Hou & Mattson 2010). Additionally, mitochondrial DNA mutations accumulated over the cell’s lifecycle for whatever reason may also contribute to aging and disease aetiology as suggested by animal studies (Trifunovic 2004), and evidence of the presence of deformed mitochondria, including abnormal shapes and sizes (Trifunovic 2005). Additionally, decreased numbers of mitochondria were found in aged individuals (Trifunovic 2005). Studies have suggested therapies to enhance mitochondrial function could be beneficial to AD, and include such treatments as creatine, manganese superoxide dismutase mimetics, coenzyme Q10 and mitochondrial uncoupling agents, although results are as yet inconclusive (discussed in (Cheng, Hou & Mattson 2010)).

Whilst the evidence of mitochondrial involvement in AD is substantial, it is unlikely that it is the (only) process that initiates the disease and it is suggested that perhaps it is a secondary event to the actual dysfunction that causes AD. Whilst research still continues on this topic, it is hard to conclusively agree on the mitochondrial role in AD pathogenesis.

### 7.3 Metal imbalance

Whilst early studies suggested excess aluminium accumulated from for example food and present in the brain was the cause of AD (Perl, Brody 1980), nowadays it is proposed that an imbalance in the naturally occurring metals (Crouch, White & Bush 2007, Adlard et al. 2008) found in the body could be to blame. Some other theories also still suggest metals absorbed from the environment could be to blame, such as animal studies indicating copper found in drinking water combined with a high cholesterol diet initiated Aβ peptide accumulation (Marx 2003, Sparks, Schreurs 2003), and lead exposure during development which increases Aβ peptide production in the brain later in life (Wu et al. 2008).

Metals are required in the body, especially in the brain, in order to perform redox reactions and also as components of some enzymes (Crouch 2007). An imbalance in metals can cause oxidative stress and neurodegeneration (Crouch 2007), although evidence suggests that this is probably not the initial cause of AD.

Research has showed that metal chelators (Adlard et al. 2008) are capable of disrupting SP and other amyloid deposits, although their toxicity and inability to cross the BBB has limited their use as therapeutic treatments for the disease. Research on the metal chelator clioquinol (for the treatment of malaria) has proven to be at least partially successful in both animal and human studies (Adlard et al. 2008). Metals that have been identified in AD brains to be in dyshomeostasis are trace metals such as iron, zinc, and copper (Crouch 2007, Adlard et al. 2008). These metals instigate aggregation of Aβ peptide, a mechanism of which may be due to the fact that Aβ peptide itself has been
shown to bind copper and zinc (Curtain et al. 2001), although can have antioxidant capabilities in the correct amounts (Thomas, Fenech 2007).

The evidence substantiating metal imbalance as the cause of AD is limited, however further research should be pursued in this area due to some improvement in patients in results from treatments with metal chelators in clinical trials (Adlard et al. 2008).

7.4 Viruses & bacteria

Due to a lack in definitive answers as to the causative factors behind both the characteristic brain lesions, SP and NFT, and AD itself, researchers have begun to look at pathogenic agents as potential causes and thus therapeutic targets in the fight against the disease (Urosevic, Martins 2008). Whilst epidemiological studies have found it difficult to pinpoint the exact causes, further immunohistochemical studies have proven useful in the detection of pathogens within brain tissue samples.

Correlations between brain levels of certain bacteria have suggested that perhaps invading pathogens are capable of facilitating systemic inflammatory responses and thus setting off the suspected irreversible mechanisms behind AD (Kamer et al. 2008). Examples of suggested microbes that correlate with either SP, dementia or AD prevalence include viruses such as cytomegalovirus, herpes simplex virus (HSV)-1 (Itzhaki, Wozniak 2006, Jamieson 1992), bacteria such as the Chlamydia (Gérard et al. 2005), Helicobacter, Treponema and Borrelia species, and periodontal infections (Kamer et al. 2008) with bacteria including Aggregatibacter, Tannerella and Porphyromanos species, amongst others, as reviewed by Kamer (Kamer et al. 2008). By infecting the brain and inciting inflammation in an aged system, these normally usually asymptomatic or non-serious infections are thought to cause the dysfunction and chaos seen in an AD brain.

The involvement of a common viral infection with AD, usually causes minimal symptoms, but is known to cause a rare encephalopathy and affect similar brain regions, was found co-localised with the SP in diseased-brains (Wozniak 2009). HSV-1 affects approximately 80% of the population, although mostly asymptomatically, and is suggested to access the brain through its normal infection route into neural networks (Itzhaki, Wozniak 2008b, Wozniak et al. 2007). Once there, it hides within cells, integrating its DNA into the host cell’s genome and can later reactivate and taking over the cell’s machinery, initiating inflammatory cascades and oxidative damage (Itzhaki, Wozniak 2006, Itzhaki, Wozniak 2008b).

Cycles of reactivation and dormancy can be detrimental to an aging brain and the virus itself is capable of using autophagy systems for its own purposes, which would normally allow the cell to survive starvation and degrade microbes (Itzhaki 2008). It is suggested through disrupting this mechanism and others such as cell cycle manipulation, HSV-1 causes damage and can even kill neurons by leaving them susceptible to further insult, leading to similar neuropathology as that seen in AD (see figure 7) (Wozniak et al. 2007, Itzhaki, Wozniak 2008a, Wozniak, Frost & Itzhaki 2009). Additionally, an envelope glycoprotein of HSV-1 has a high sequence homology to Aβ peptide and is capable of facilitating its aggregation (Watts 2008). Another virus protein has functional similarities to tau, which promotes hyperphosphorylation of tau (Itzhaki, Wozniak 2006).
Lack of definitive evidence for these mechanisms, and clinical trials based on these theories have not yet taken place; therefore conclusive data on the participation of these pathogens in the aetiology of AD has yet to be ascertained.

**Figure 7.** Proposed mechanisms for HSV-1 to cause Alzheimer’s disease, as suggested by (Itzhaki, Wozniak 2008b).

\[\beta \gamma \uparrow \beta\]

### 8. Summary

Alzheimer’s disease affects a large proportion of the elderly population and will continue to do so, increasing in numbers and stress on healthcare systems as the population ages. It is imperative to determine effective treatments towards not only the symptoms, but also the causes of the disease and only through laborious studies and research can this become a reality.

The vast amounts of research already performed have opened up new pathways and causal mechanisms, but the ‘magic bullet’ that investigators are after has so far remained elusive. To date, the strongest conclusive risk-increasing allele that has been determined is the \(APOE^4\) allele and whilst many other genes and polymorphisms have been associated with the disease, effects have been small (Bertram et al. 2007).

In addition to the \(APOE\) gene, focus for AD research has revolved around the pathogenic hallmarks that are found in postmortem AD brains – SP and NFT. The exact function or mechanism by which these lesions appear is not completely understood and studies also suggest contradictory roles for them, such as being protective or not related to the course of the disease at all (discussed in (Nelson 2009)).

Due to the confusion over the exact role or roles that these brain lesions may have, the prevalence of these lesions were investigated in the context of associative studies related to genes (single nucleotide polymorphisms or SNPs, and haplotypes) and pathways involved in inflammation that may lead to the development of these brain lesions and thus AD pathogenesis.
Aims of the study

The specific aims of the present study were:

1. To investigate the prevalence of the apolipoprotein E (APOE) gene alleles with both senile plaques and neurofibrillary tangles within the context of an autopsy series consisting of a mostly non-demented, population that lived outside of institutions – the Tampere Autopsy Study (TASTY) collection.

2. To analyse the association/s between the C-reactive protein (CRP) gene (individual SNPs and haplotypes) and senile plaques and neurofibrillary tangles within the TASTY series, thereby revealing potential mechanisms for which these brain lesions develop, and providing answers on their links with AD.

3. To examine whether there are correlations between recently identified AD-risk genes (CLU, CR1 and PICALM) and the senile plaques and neurofibrillary tangles present in our non-demented population within the TASTY series.

4. To explore whether senile plaque or neurofibrillary tangle prevalence is dependent on the genotypes of upstream transcription factor (USF)-1 (SNPs and haplotypes) within the TASTY cohort.
Study Subjects

The Tampere Autopsy Study (TASTY) series comprised of 603 women and men, aged 0 to 97 years (see figure 8), who were subjected to medicolegal autopsy at the Department of Forensic Medicine, University of Tampere, in Finland, during the years 2002 to 2004. The population in the Tampere region during this time grew from 452,362 people in 2002 to 462,923 in 2004, and medicolegal autopsies covered 22.1% of all deaths registered during this time. The TASTY series consisted of approximately 25% of the medicolegal autopsies performed in the Tampere region.

Data pertaining to the autopsies were obtained from hospital records and interviews of family members in police reports. Females within the cohort accounted for 35.8% (215 cases) and the average age was 63 years (59 years for males and 68 years for females).

None of the cases died of AD, but 6 (1.0%) had been diagnosed with the disease whilst alive. Additionally 16 cases (2.7%) were reportedly suffering from undefined dementia, 10 (1.7%) had memory disorders, and 1 (0.2%) case was diagnosed with Parkinson’s disease prior to death, according to available hospital records and next of kin reports. The study was approved by the Board of Medicolegal Affairs of Finland (Dnro: 1239/32/200/01).

![Figure 8. Age distribution of the TASTY cohort.](image-url)
Methods

1. Neuropathological tissue samples

At autopsy, samples from four different areas of the brain (middle frontal gyrus, gyrus cinguli, corpus callosum, hippocampus, and cerebellum) were placed in Tissue-Tek (Sakura, Torrance, CA, USA) boxes and fixed in a phosphate-buffered 4% formaldehyde solution for at least 2 weeks. The tissue blocks were then embedded in paraffin from which 10µm sections were cut and stained using hematoxylin & eosin and Bielschowsky’s argyrophilic silver impregnation methods.

The entire neocortical area of each sample was screened at 100x magnification to find the area with the greatest frequency of silver-positive SP. This was performed by two researchers using a bifocal microscope (with two eyepieces), to ensure consistency. The scoring was performed using a square microscopic graticule (10 horizontal and 10 vertical lines with 100 intersections, covering an area 1mm$^2$ in size), extending in contiguous cortical microscopic fields from the molecular layer of the gray matter to the white matter, along a line perpendicular to the pial surface. All intersections that overlaid a silver-positive SP were counted. To determine the area of cortex covered by SP, the number of intersections overlying a silver-positive SP was divided by the total number of intersections, as performed by Polvikoski et al (Polvikoski et al. 2001). Multiplying this number by 100 then gave the average percentage of cortex in which SP were seen.

SP were analysed for neuropathological stage (diffuse, primitive, classic, and burnt-out), or phenotype, under the supervision of a neuropathologist (Hannu Haapasalo). These were also grouped into non-neuritic (the categories of diffuse and primitive SP) and neuritic (categories classic and burnt-out) SP. The same area with the greatest frequency of SP was also scored according to the CERAD semi-quantitative assessment of SP. CERAD scoring using the definitions No Plaques, Sparse Plaques, Moderate Plaques, and Frequent was then given to each case.

The quantity of NFT was estimated from the hippocampus. The entire hippocampus of each sample stained with Bielschowsky’s technique was screened to find the CA1 area with the highest number of NFT. In five (4–6) random columns of a grid (size 0.5 x 0.5mm at a magnification of 200x), all NFT were counted. The average number of NFT in 1mm$^2$ was recorded and used in the statistical analyses.

2. Immunohistochemistry

For immunohistochemical confirmation of the Bielschowsky staining, tissue microarrays (TMAs) were constructed from the same paraffin tissue blocks of each case, using the Bielschowsky-stained slides as guides to locate regions. Between nine and 24 cases were placed in each TMA, with six regions of the brain sampled from each case (hippocampus CA1 and CA2; CA3; cerebellum; gray matter from frontal cortex; gray matter from gyrus cinguli; and cerebral white matter). The TMA block was constructed (see Figure 9) with a custom-built instrument (Beecher Instruments, Silver Spring, MD). When all samples were inserted, the TMA was heated at 37°C for 30 minutes to promote the attachment of tissue to the paraffin. The diameter of the tissue cylinder punch was 1mm, and the thickness of slices from the completed TMA was 1–2µm. TMA slides were then stained separately with Bielschowsky’s argyrophilic silver impregnation method and rabbit (epitope against amino-terminus of human Aβ peptides) anti-Aβ peptide antibody
1:150 (Cell Signaling Technology, Danvers, MA, USA). Due to technical difficulties and sample damage, only 92.5% of cases from TASTY that were stained with Bielschowsky’s method could be included in the TASTY TMA construction. For TMA analyses (both Bielschowsky and Aβ peptide staining), the sphere on the TMA slide corresponding to gray matter of the cortex was screened as above.

Fluorescent immunohistochemical (F-IHC) staining was performed on the TASTY-TMAs in the hippocampal CA1/2 area and utilised DAPI (Sigma-Aldrich, Schnelldorf, Germany), rabbit anti-CRP (BioLegend, San Diego, CA, USA), mouse anti-Aβ peptide (Acris Antibodies, Hiddenhausen, Germany), anti-mouse IgG FITC conjugated (Novus Biologicals, CO, USA), anti-rabbit IgG rhodamine conjugated (Antibodies-online, Aachen, Germany), all according to manufacturer’s instructions.

![Diagram illustrating the process used to create the tissue microarrays (TMAs) for the TASTY series.](image)

**Figure 9.** Diagram illustrating the process used to create the tissue microarrays (TMAs) for the TASTY series. 1. Samples are taken from the donor paraffin block, matching up the required sections with a slide from the same sample. 2. The cylinder of tissue is then inserted into a recipient paraffin block. Samples are added in a similar way until the recipient paraffin block is complete. 3. After preparing the TMA, slices of the recipient paraffin block are cut. 4. The slices are then placed on a slide and ready for staining.

### 3. Genotyping

DNA was extracted from 5ml of postmortem blood samples using the standard salt precipitation method (see results in table 3). From this, 1µL was used for polymerase chain reaction amplification. APOE genotyping was performed in the department of Forensic Medicine at the University of Tampere, as described by Hixson et al., 1990(Hixson, Vernier 1990).

CRP genotyping was performed at Biomedicum, Helsinki on the Sequenom MassArray system with the homogeneous Mass Extension (hME) reaction (Sequenom, San Diego, USA) for 6 reported haplotype tagging single nucleotide polymorphisms (SNPs), including rs2794521 (T>C), rs3091244 (C>T>A), rs1800947 (G>C), rs1130864 (C>T), rs1205 (C>T) and rs3093075 (C>A) (see figure 10 for a schematic diagram of the SNP locations within the CRP gene).
Table 3. The frequencies of genotyped SNPs and haplotypes within the TASTY cohort.

<table>
<thead>
<tr>
<th>APOE haplotypes</th>
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<tbody>
<tr>
<td>ε3/ε3</td>
<td>59.2%</td>
</tr>
<tr>
<td>ε2 carriers*</td>
<td>9.7%</td>
</tr>
<tr>
<td>ε4 carriers*</td>
<td>31.1%</td>
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<table>
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<th>rs1130864</th>
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<th>rs3093075</th>
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<td>H1</td>
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<td>T</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>-</td>
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</tr>
<tr>
<td>H4</td>
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<td>C</td>
<td>C</td>
<td>T</td>
<td>-</td>
</tr>
<tr>
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<td>T</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>-</td>
</tr>
<tr>
<td>H6</td>
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<td>C</td>
<td>C</td>
<td>C</td>
<td>-</td>
</tr>
<tr>
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<td>A</td>
<td>C</td>
<td>C</td>
<td>C</td>
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| T>C C>T>A G>C C>T C>A |

<table>
<thead>
<tr>
<th>CLU, CR1 and PICALM genotyping</th>
<th>CLU (rs11136000)</th>
<th>CR1 (rs1408077)</th>
<th>PICALM (rs3851179)</th>
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<tr>
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<td>4.2%</td>
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<td>CA</td>
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<td>TT</td>
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<table>
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<th>rs2774276</th>
<th>rs2516839</th>
<th>rs1556259</th>
<th>rs2774279</th>
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<tbody>
<tr>
<td>H1</td>
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<td>C</td>
<td>C</td>
<td>T</td>
<td>A</td>
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<td>C</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td>H7</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>A</td>
<td>C</td>
</tr>
</tbody>
</table>

| (C>G) (C>T) (C>G) (T>C) A>G) (C>T) |

* carriers here refers to those with at least one of the afore mentioned allele. Those with APOEε2/ε4 were designated as ε4 carriers.

Haplotyping was calculated with 5 SNPs (SNP order: rs2794521, rs3091244, rs1800947, rs1130864 and rs1205; rs3093075 was excluded as it produced too many low prevalence haplotypes) using the PHASE program (version 2.1.1) and indicated five haplotypes with prevalence above 5%.

The ABI Prism 7900HT Sequence Detection System at the University of Tampere used PCR primers (Applied Biosystems) for rs11136000 (CLU), rs1408077 (CR1) and rs3851179 (PICALM). All SNPs were in Hardy-Weinberg equilibrium. Genotyping for the polymorphisms of CLU, CR1 and PICALM were successful for 94%, 97% and 97% of the TASTY cases, respectively.

Six reported haplotype-tagging single nucleotide polymorphisms (SNPs) of the USF1 gene were genotyped (rs10908821, rs2073658, rs2774276, rs2516839, rs1556259 and rs2774279) to capture all reported common allelic variants, using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in the University of Tampere at the department of Clinical Chemistry.
Figure 10. Approximate locations of the genotyped SNPs of the CRP gene.

Estimation of USF1 haplotype frequencies was performed using PHASE v2.1.1 (Stephens, Smith & Donnelly 2001, Stephens, Donnelly 2003). SNP order was rs10908821, rs2073658, rs2774276, rs2516839, rs1556259 and rs2774279 and created the haplotypes CCCTAC (1), CCCTAT (2), CCCCGC (3), CCGCAC (4), CTCTAC (5), CTCTAT (6) and GCGCAC (7). Haplotypes 1 and 6 were excluded from analyses based on their low prevalence (0.001 % and 0.1 %, respectively).

4. Statistics

For statistical analysis in the first paper (I), we used three genotype groups: 1) APOE ε2/ε2, ε2/ε3 (APOEε2 group), 2) APOEε3/ε3 group (reference group), and 3) APOE ε4/ε2, ε4/ε3, and ε4/ε4 (APOEε4 carriers), and divided these into age groups ranging 0–49, 50–59, 60–69, 70–79, 80–89 and >90 years. Analyses for SP (and NFT) assembled the cohort into two sets: those with one or more SP and those without. Further analyses grouped SP into non-neuritic (diffuse and primitive) and neuritic (classic and burnt-out). Spearman rank correlation (rS) was used to determine the association between SP and age, and NFT and age. Binary logistic regression analysis was used to reveal the occurrence of SP (and NFT) among age groups, and their association with the APOE genotype. The Bielschowsky and Aβ peptide staining association was analysed using the Pearson correlation. The statistical analyses were performed using the SPSS programme, version 14.0 (SPSS, Inc., Chicago, IL).

Statistical analyses for the second manuscript (II) were also performed with SPSS program version 14.0 on the CRP SNPs and haplotypes, and used the most common genotype or previously reported ‘risk’ allele as the reference group, including APOEε4 carriership and age as covariates where possible. Their associations were analysed using logistic regression. Chi square analysis was used to determine the association between IHC staining and SNPs/haplotypes.

Logistic regression analyses, with continuous age and APOEε4 carriership as covariates (where possible), were used with SPSS (version 14.0 for Windows) in the third manuscript (III) to determine associations between the three SNPs (CLU, CRI1 and PICALM) and the AD-related neuropathological lesions. For all SNPs, the most common homozygous genotype was used as the reference group. The cohort was also split by age
groups, with the following categories: 0-49 years, 50-59 years, 60-69 years, 70-79 years, 80+ years. The cohort was also split by gender, where mentioned.

Statistics in the unpublished analyses used SPSS v16.0 for Windows. The variables used were: USF1 SNPs (genotypes versus the common homozygous genotype, and also carrierhip of the rare allele versus the common homozygous genotype), haplotypes (yes/no carrierhip), SP (yes/no) and NFT (yes/no). SP were further grouped into non-neuritic (diffuse and primitive) and neuritic (classic and burnt out). We also used a variable with percentage of SP cortex coverage, which was assessed semi-quantitatively in the categories ‘no SP’, and equal sized groups ‘<1.05% SP coverage’ and ‘≥1.05% SP coverage’, in order to create the strongest statistical power assessments. Logistic regression analyses used age and APOEε4 carrierhip as covariates. The data was also split by gender, where mentioned. Because the prevalence of SP and NFT are dependent on age, the analyses were also performed splitting the entire data series into 2 age groups: 0-64 (49.4 %) and ≥ 65 (50.6 %) years.
Results

1. Study cohort & genotyping (I-III)

Of 603 individuals, 388 were male and 215 were female (see Table 4). The average brain weight was 1408 grams for the entire cohort. Cause of death was grouped into disease (59.1%), accident (26.8%), suicide (11.8%), homicide (0.5%) or unknown (1.5%). Of age grouping, there were 136 in the 0-49 year old group, 109 (50-59 years), 95 (60-69 yrs), 140 (70-79 yrs), 96 (80-89 yrs) and 20 in the 90 years old and over.

SP frequency was available for 548 (90.9%), NFT counts for 484 (80.3%), and APOE genotypes for all (n = 603).

APOE genotyping indicated that there were no significant differences in the distribution of allele frequencies compared to normal Finnish populations. CRP genotypes were acquired for 537 cases (89%) and there were no significant differences in the distribution of allele frequencies, following Hardy-Weinberg proportions. Genotyping for the polymorphisms of CLU, CRI and PICALM were successful for 94%, 97% and 97% of the TASTY cases, respectively. USF1 genotyping was available for SNPs rs10908821 (87.6 %, n=528), rs2073658 (87.7 %, n=529), rs2774276 (88.4 %, n=533), rs2516839 (87.7 %, n=529), rs1556259 (87.7%, n=529) and rs2774279 (87.4 %, n=527).

Missing cases for neuropathological analyses were due to extensive brain or sample storage damage. Of the 603 cases, 558 were included in the TASTY TMA construction (92.5%), of which 206 were female and 352 male. SP frequency was available in 536 and 558 cases for Bielschowsky and Aβ peptide staining, respectively.

Table 4. Characteristics of the TASTY cohort (n=603).

<table>
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<tr>
<th>Age (years)</th>
<th>0 - 97</th>
<th>(62.7)</th>
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<tbody>
<tr>
<td>Females</td>
<td>215</td>
<td>35.7%</td>
</tr>
<tr>
<td>Males</td>
<td>388</td>
<td>64.3%</td>
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<table>
<thead>
<tr>
<th>Cause of Death</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Disease</td>
<td>59.1%</td>
</tr>
<tr>
<td>Accident</td>
<td>26.8%</td>
</tr>
<tr>
<td>Suicide</td>
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</tr>
<tr>
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<table>
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<tr>
<th>Dementia Status</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>6</td>
</tr>
<tr>
<td>Dementia</td>
<td>16</td>
</tr>
<tr>
<td>Memory Problems</td>
<td>10</td>
</tr>
<tr>
<td>Parkinson’s Disease</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SP type</th>
<th>Diffuse</th>
<th>21</th>
<th>3.7%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primitive</td>
<td>35</td>
<td>6.2%</td>
</tr>
<tr>
<td></td>
<td>Classic</td>
<td>83</td>
<td>14.7%</td>
</tr>
<tr>
<td></td>
<td>Burnt Out</td>
<td>25</td>
<td>4.4%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SP type 2</th>
<th>Non-neuritic SP</th>
<th>56</th>
<th>9.9%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neuritic SP</td>
<td>108</td>
<td>19.1%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Semi quantitative SP coverage</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sparse SP</td>
<td>90</td>
</tr>
<tr>
<td>Moderate SP</td>
<td>62</td>
</tr>
<tr>
<td>Frequent SP</td>
<td>32</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>APOEε4 carriership</th>
<th>187</th>
<th>31.1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFT</td>
<td>204</td>
<td>42.1%</td>
</tr>
<tr>
<td>SP</td>
<td>172</td>
<td>31.1%</td>
</tr>
</tbody>
</table>

SP = senile plaques; NFT = neurofibrillary tangles; AD = Alzheimer’s disease; number in parentheses refers to the average.
2. Senile plaques (I-III)

At least one SP was found in 30.8% of all cases with a clear female preference (41.4% versus 24.5%, p < 0.0001). SP were found in some individuals around 30 years of age, and their occurrence increased with age, reaching almost 100% in the oldest age groups. The SP density varied between 0 and 5.41% for all cases, with a median of 0% and a mean of 0.44%, and also had a strong correlation with age (rS = 0.46, p < 0.001). When the cohort was divided into 5 approximately equal-sized groups according to age, each group was consistently more likely to have SP compared to the youngest group, with each association also highly statistically significant.

As expected, APOE ε4 carriership was significantly associated with increased risk of having SP (OR 2.52, CI 1.72 – 3.68, p < 0.0001); having both non-neuritic (OR 2.42, CI 1.54 – 4.13, p < 0.0001) and neuritic SP (OR 2.58, CI 1.66 – 4.02, p < 0.0001) compared to no SP; and having primitive (OR 2.52, CI 1.52 – 5.10, p = 0.010), classic (OR 2.52, CI 1.54 – 4.13, p < 0.0001) and burnt out SP (OR 2.77, CI 1.22 – 6.27, p = 0.014) compared to no SP, when evaluated against non ε4 carriers. Results showed similar trends when the cohort was split by gender.

Of 320 cases with the most common APOE ε3/ε3 genotype, 97 (24.7%) had at least one SP, which was more common among females (OR 1.4, 95% CI 0.95–2.26, p = 0.087 with age as a covariate) (see table 5). Twelve (23.5%) of the 51 cases with the genotypes ε2/ε2 or ε2/ε3 had SP, again with a slight tendency for female preference (31.8% versus 17.2%, p = 0.22). The highest percentage of SP-positive cases was seen among carriers of APOE ε4, where 76 (44.5%) of 171 cases had SP. In this group, female preference was not as clear (50.7% vs 40.2%, p = 0.17) as among the APOE ε3/ε3 carriers.

### Table 5. Senile plaque prevalence by APOE genotype.

<table>
<thead>
<tr>
<th>Age groups</th>
<th>All</th>
<th>APOE ε3/ε3</th>
<th>APOE ε2+</th>
<th>OR (95% CI)</th>
<th>p-value</th>
<th>APOE ε4+</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>83/339 (24.5%)</td>
<td>37/208 (17.8%)</td>
<td>5/29 (17.2%)</td>
<td>0.66 (0.22 – 1.95)</td>
<td>0.451</td>
<td>41/102 (40.2%)</td>
<td>3.34 (1.87 – 5.96)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Females</td>
<td>84/203 (41.4%)</td>
<td>42/112 (37.5%)</td>
<td>7/22 (31.8%)</td>
<td>0.64 (0.22 – 1.91)</td>
<td>0.421</td>
<td>35/69 (50.7%)</td>
<td>2.50 (1.21 – 5.18)</td>
<td>0.0131</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age groups</th>
<th>All</th>
<th>APOE ε3/ε3</th>
<th>APOE ε2+</th>
<th>OR (95% CI)</th>
<th>p-value</th>
<th>APOE ε4+</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>7/118 (7.9%)</td>
<td>4/73 (5.5%)</td>
<td>0/7 (0%)</td>
<td>-</td>
<td>-</td>
<td>3/38 (7.9%)</td>
<td>1.48 (0.31 – 6.98)</td>
<td>0.62</td>
</tr>
<tr>
<td>Females</td>
<td>17/100 (17.0%)</td>
<td>5/66 (7.6%)</td>
<td>1/7 (14.3%)</td>
<td>2.03 (0.20 – 20.38)</td>
<td>0.55</td>
<td>11/27 (40.7%)</td>
<td>8.39 (2.55 – 27.62)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Males</td>
<td>21/87 (24.1%)</td>
<td>7/47 (14.9%)</td>
<td>1/8 (12.5%)</td>
<td>0.82 (0.09 – 7.70)</td>
<td>0.86</td>
<td>13/32 (40.6%)</td>
<td>3.91 (1.34 – 11.39)</td>
<td>0.012</td>
</tr>
<tr>
<td>Females</td>
<td>55/129 (42.6%)</td>
<td>23/68 (33.8%)</td>
<td>5/16 (31.3%)</td>
<td>0.89 (0.28 – 2.87)</td>
<td>0.84</td>
<td>27/45 (60.0%)</td>
<td>2.94 (1.35 – 6.40)</td>
<td>0.007</td>
</tr>
<tr>
<td>Males</td>
<td>56/89 (62.9%)</td>
<td>34/58 (58.6%)</td>
<td>3/10 (30.0%)</td>
<td>0.30 (0.07 – 1.29)</td>
<td>0.11</td>
<td>19/21 (90.5%)</td>
<td>6.71 (1.43 – 31.53)</td>
<td>0.016</td>
</tr>
<tr>
<td>Females</td>
<td>11/19 (57.9%)</td>
<td>6/8 (75.0%)</td>
<td>2/3 (66.7%)</td>
<td>0.67 (0.04 – 11.94)</td>
<td>0.78</td>
<td>3/8 (37.5%)</td>
<td>0.20 (0.02 – 1.71)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Logistic regression analyses with age as a covariate. Bold p-values indicate statistically significant results.
The difference between SP prevalence of the APOE\(\varepsilon4\) carriers compared with the APOE\(\varepsilon3/\varepsilon3\) reference group was significant in every age group except the youngest and oldest groups, with an OR of 1.48 (95% CI, 0.31–6.98) and 0.20 (0.02–1.71), respectively (see figure 11). The difference was most evident in 50–59-year-olds, among whom 40.7% of \(\varepsilon4\)-carriers had SP, compared to 8.2% of non-carriers (OR, 8.39; 95% CI, 2.55–27.62). Although a protective tendency was observed, no statistically significant difference between SP prevalence was discovered for any of the age groups carrying the APOE \(\varepsilon2/\varepsilon2\) and \(\varepsilon2/\varepsilon3\) genotype when compared to the reference group with the \(\varepsilon3/\varepsilon3\) genotype, perhaps due to the small number of cases with the \(\varepsilon2\) genotype. In the age group 0–49 years, no SP were found in the \(\varepsilon2/\varepsilon2\) and \(\varepsilon2/\varepsilon3\) genotype groups.

![Figure 11. Senile plaque prevalence according to age and APOE genotype. Axes represent age and percentage of those with SP. APOE\(\varepsilon3/\varepsilon3\) genotype carriers were considered the reference group. Small numbers above columns represent the number of cases with SP.](image)

The severity of CERAD plaque score in the cortex increased with age. The association between age and CERAD plaque score gave a correlation of \(r_S = 0.45\) (\(p < 0.001\)) and showed significant differences between CERAD scoring and APOE genotypes in all age groups, except the youngest and oldest. Two-sided Fisher exact analyses produced \(p < 0.001\) (50–59 years), \(p = 0.046\) (60–69 years), \(p = 0.070\) (70–79 years), and
p = 0.005 (80–89 years). TMA analysis comparing Bielschowsky and Aβ peptide staining showed that both were correlated to each other (Pearson correlation 0.231, p = 0.004), thus validating our analyses on Bielschowsky-stained slides. Similar significant associations between plaques and APOE genotype were found for each age group in both staining methods.

3. Neurofibrillary tangles (I-III)

In 42.1% of all cases, at least one NFT was seen; this had a strong (p < 0.0001) relationship with age. Those with NFT were on average older than those without (71.4 versus 66.1 years, p = 0.041). The density of NFT varied between 0 and 29.60 tangles/mm², with a median of 0 tangles/mm² (mean 3.17 tangles/mm²), and also showed a clear increase with age (rS = 0.49, p < 0.0001). Figure 12 shows the prevalence of NFT across age groups and includes those also with SP.

![Figure 12. Neurofibrillary tangle prevalence across age groups. Of those with NFT, cases also with SP are shown in grey.](image)

The difference in prevalence of NFT between the different APOE genotype groups compared with the reference group ε3/ε3 was not statistically significant. Of the cases with NFT, 47% also showed SP, and of the cases that had SP, 61% also possessed NFT. Cases with some form of dementia (including AD and memory problems) reported prior to death had significantly more SP (see table 6) when compared to the rest of the cohort (those considered non-demented). The coverage (percentage) of SP however, overlapped
with those cases without reported dementia. There was a similar trend for tangles (data not shown).

### Table 6. SP prevalence according to dementia status.

<table>
<thead>
<tr>
<th></th>
<th>Total n (affected*)</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non demented</td>
<td>570 (148)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>6 (5)</td>
<td>1.8 (1.04-3.15)</td>
<td><strong>0.037</strong></td>
</tr>
<tr>
<td>Dementia</td>
<td>16 (13)</td>
<td>2.2 (1.56-3.06)</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
<tr>
<td>Memory problems</td>
<td>10 (6)</td>
<td>1.5 (0.90-2.60)</td>
<td><strong>0.116</strong></td>
</tr>
</tbody>
</table>

* Those affected are those with SP. p-values in bold are statistically significant.

### 4. CRP genotypes & neuropathological lesions (II)

Fisher’s exact test analyses revealed statistically significant associations between CRP SNPs and the prevalence of SP (see table 7). Univariate logistic regression analysis showed SNP rs2794521 (p = 0.067) was associated with SP prevalence (yes/no SP presence), however including age and APOEε4 carriership as covariates weakened the association (p = 0.096). When we took into account the phenotype of SP, two high CRP level-linked SNPs – rs3091244 (TA carriers; OR 6.7, p = 0.007) and rs3093075 (CA carriers; OR 3.5, p = 0.003) – appeared to convey an increased risk for early non-neuritic SP, compared to no SP. There was also a tendency towards increased risk for late neuritic SP (OR 4.5, p = 0.072; OR 2.1, p = 0.080, respectively). Comparatively, carriers of the low CRP level-linked C allele of SNP rs2794521 (OR 0.46, CI 0.22 – 0.96, p = 0.039) were less likely to have non-neuritic SP, derived from an association with the common CT genotype (OR 0.43, p = 0.037). A trend towards the same associations was seen with neuritic SP. Conversely, the high CRP level SNPs rs1130864 (TT carriers; OR 0.26, p = 0.076) and rs1205 (CC carriers; OR 0.39, p = 0.056) showed a non-significant trend towards protection for non-neuritic compared to no SP.

In multivariate logistic regression, CRP haplotypes composed of alleles related to high CRP levels, such as TAGCC, associated with the presence of non-neuritic SP (OR 2.99, p = 0.007), significantly increasing the risk of occurrence. On the other hand, haplotype carriership of alleles linked with lower CRP levels, such as CCGCC, reduced (OR 0.45, p = 0.034) the risk of possessing non-neuritic SP. Similar, but non-significant tendencies towards these associations were also seen for both haplotypes and neuritic SP.

Haplotype pair analyses compared all haplotype pairs with prevalence above 6% against the most common pair (TTGTC/TCGCT). None of the haplotype pairs were associated with SP prevalence. Analyses with SP phenotype suggested a trend towards protection for the haplotype pair TTGTC/TTGTC (p = 0.065) and TCGCT/CCGCC (p = 0.070) with non-neuritic SP, although the association weakened with the inclusion of age and APOEε4 carriership as covariates.

NFT prevalence (yes/no presence) showed an association only with SNP rs2794521 using univariate logistic regression (p = 0.059). Inclusion of APOE genotype and age as covariates weakened the association (p = 0.107). Semi-quantitative SP analyses did not reveal any significant associations with any of the CRP genotypes and splitting the data by gender did not provide any additional results.
Table 7. Senile plaques and CRP SNP associations.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Plaque Type</th>
<th>Plaque Type (Neuritic)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p=0.042 (F.E. p=0.024)</td>
<td>p=0.049 (F.E. p=0.045)</td>
</tr>
<tr>
<td>rs2794521</td>
<td>genotype no SP diffuse primitive classic burnt out</td>
<td>genotype no SP non neuritic neuritic</td>
</tr>
<tr>
<td></td>
<td>CC 60.0     8.0     0.0     24.0    8.0</td>
<td>CC 60.0     8.0     32.0</td>
</tr>
<tr>
<td></td>
<td>TT 67.6     3.1     8.1     16.8    4.4</td>
<td>TT 67.6     11.2    21.2</td>
</tr>
<tr>
<td></td>
<td>CT 78.6     3.8     3.3     9.9     4.4</td>
<td>CT 78.6     7.1     14.3</td>
</tr>
<tr>
<td>rs3091244</td>
<td>genotype no SP diffuse primitive classic burnt out</td>
<td>genotype no SP non neuritic neuritic</td>
</tr>
<tr>
<td></td>
<td>TT 71.2     2.7     26.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC 72.6     9.1     18.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TA 50.0     31.3    18.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC 72.1     10.1    17.9</td>
<td></td>
</tr>
<tr>
<td>rs1205</td>
<td>genotype no SP diffuse primitive classic burnt out</td>
<td>genotype no SP non neuritic neuritic</td>
</tr>
<tr>
<td></td>
<td>CC 70.5     3.6     3.1     14.3    8.5</td>
<td>CC 70.5     3.6     3.1     14.3    8.5</td>
</tr>
<tr>
<td></td>
<td>TT 67.7     1.5     12.3    13.8    4.6</td>
<td>TT 67.7     1.5     12.3    13.8    4.6</td>
</tr>
<tr>
<td></td>
<td>CT 71.4     4.6     7.1     15.5    1.3</td>
<td>CT 71.4     4.6     7.1     15.5    1.3</td>
</tr>
<tr>
<td>rs3093075</td>
<td>genotype no SP diffuse primitive classic burnt out</td>
<td>genotype no SP non neuritic neuritic</td>
</tr>
<tr>
<td></td>
<td>CC 72.3     8.3     19.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA 100.0    0.0     0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC 57.9     21.1    21.1</td>
<td></td>
</tr>
</tbody>
</table>

Analyses performed used Chi square and Fisher exact tests. SP = senile plaques. Numbers refer to percentages, unless otherwise stated.

5. CRP genotypes & immunohistochemistry (II)

CRP immunohistochemistry (IHC) staining (positive/negative) was found to be significantly correlated with Aβ peptide (amyloid-beta) staining (positive/negative) in all studied brain regions (see figure 13) in the cohort, (Chi square p < 0.0001). Aβ peptide IHC staining however was not found to be associated with any of CRP SNPs or haplotypes. In univariate analyses CRP IHC staining was significantly associated with the high protein CRP level TT genotypes of SNPs rs3091244 (OR 5.9, CI 1.20 – 28.87, p = 0.029) and rs1130864 (OR 5.9, CI 1.21 – 28.95, p = 0.028). Individual haplotype (yes/no carriernesship) analyses were not, but haplotype pair TTGTC/TTGTC analyses were significantly associated (OR 5.5, CI 1.03 – 29.48, p = 0.047) with CRP IHC staining. This relationship strengthened on inclusion of APOEε4 carriernesship and age as covariates (OR 14.9, CI 1.14 – 196.37, p = 0.040), however the CI were extremely large.
Figure 13. Fluorescent immunohistochemistry indicating the co-localisation of a) Aβ peptide, b) CRP and c) merged together. Magnification 100x.

6. CLU, CR1 & PICALM, and SP (III)

In APOEε4 adjusted analyses, 80+ year old carriers of the rare TT genotype of PICALM had a significantly lower incidence of SP compared to the common CC carriers (OR 0.18, CI 0.04 – 0.81, p = 0.025). This association was not seen among younger age groups. There were no significant associations between genotypes of CLU and CR1 and SP prevalence (see figure 14).

Figure 14. Graph representing the senile plaque prevalence within PICALM genotypes and divided by age.
Grouping the rare homozygote and heterozygotes versus the common homozygotes for the SNPs uncovered statistically significant associations between the T allele of \textit{PICALM} and SP (OR 0.62, CI 0.41 – 0.95, p = 0.028, versus CC genotype) as seen in figure 15.

When we divided the SP into diffuse, primitive, classic and burnt out phenotypes, we found that the rare C allele of \textit{CLU} was significantly associated with the presence of late stage SP (OR 4.4, CI 1.61 – 12.2, p = 0.004) compared to the common TT genotype. In that setting, the statistically significant association of the \textit{PICALM} T allele was lost.

When analyses were performed with SP frequency as the dependent variable, \textit{PICALM} TC genotypes (versus CC genotype) were significantly less likely to have moderate SP compared to no SP (OR 0.42, CI 0.21 – 0.83, p = 0.012), whilst \textit{CR1} CC genotype carriers (compared to AA genotype) were more likely to have sparse SP than no SP (OR 2.1, CI 1.01 – 4.43, p = 0.048). When we grouped the rare homozygote and heterozygotes together versus the common homozygotes, significance was lost for \textit{CR1}, however \textit{PICALM} T allele carriers remained less likely to have coverage of SP versus no SP compared to CC genotype, however again statistical significance was only reached for moderate SP (OR 0.43, CI 0.23 – 0.82, p = 0.010). Reanalysing the significant associations with the cohort split by gender gave similar results, with females generally more strongly associated, most likely due to their older age. No further significant associations were uncovered.

\textbf{Figure 15.} Graph of the prevalence of senile plaques according to carriersonship of genotypes in the SNPs studied and \textit{APOE}.
7. USF1 genotypes & neuropathological lesions (unpublished data)

A lower tendency for having SP could be seen in USF1 SNP rs10908821 G allele carriers compared to the common homozygous CC genotype carriers (OR 0.619, 95% CI 0.377 – 1.016, p = 0.058), although not significant. The corresponding haplotype GCGCAC showed the opposite trend compared to non-carriers (OR 1.526, CI 0.949 – 2.454, p = 0.081). Haplotype GCGCAC carriers also had an increased risk of SP coverage in the cortex compared to non-carriers, although only significant for SP coverage above 1.05% (OR 1.820, CI 0.999 – 3.316, p = 0.050). We also investigated SP type, dividing those with non-neuritic and neuritic SP, however no significant associations were found without comparing gender or age.

![Figure 16. USF1 SNP rs10908821 with SP and NFT prevalence according to gender (males in grey and females in black).](image)

The previously unreported SNP rs10908821 female minor G-allele carriers were found to associate with SP in the simplest analysis (OR 0.25; p=0.003), as shown in Figure 16, compared to the common CC genotype. Additionally, the female G-allele carriers were statistically less likely to have classic (p=0.024) and burnt out (p=0.001) SP, and neuritic SP (p=0.003) when SP phenotype was grouped together. In a CERAD-like analysis, female carriers of the G-allele were less likely to have increasing levels of SP coverage. In analyses investigating NFT prevalence within gender, there were no statistically significant values. With age division analyses, rs10908821 minor G-allele carriers in the older age group (≥65 years) were less likely to have SP (p=0.013) and higher SP coverage, compared to the common CC genotype (see Figure 17).

Male carriers of the SNP rs2073658 TT genotype were more likely to have neuritic SP versus CC carriers (OR 2.82, CI 0.99-7.98, p=0.050), however no statistically significant values were discovered when comparing the minor allele T carriers against the common homozygous CC carriers in analyses with gender and age divisions (data not shown).
Female carriers of the minor G-allele of rs2774276 had increased risk of late stage burnt out SP, compared to the common CC homozygotes ($p=0.019$), whilst male carriers of the minor G-allele were more likely to have early stage non-neuritic SP ($p=0.030$), as seen in Figure 18. Analyses with age division revealed that younger (0-64 years) individuals were more likely to have diffuse SP if they had the minor G-allele, compared to CC homozygotes (see Figure 19).

**Figure 17.** USF1 SNP rs10908821 with SP and NFT prevalence according to age grouping (0-64 years in grey and 65+ in black).

**Figure 18.** USF1 SNP rs2774276 with SP and NFT prevalence according to gender (males in grey and females in black).
Female carriers of the rs2516839 minor C allele were more likely to have late stage burnt out SP (p=0.046), however age division analyses did not reveal statistically significant associations, as seen in Figures 20 and 21. Statistically significant results were also not seen for the SNPs rs1556259 or rs2774279, and SP or NFT variables.
Haplotypes 2, 3, 5 and 6 did not reveal any statistically significant associations with any of the SP or NFT variables in simple analyses, or with gender or age divisions in our cohort. Haplotype 4 (CCGCAC) carriers were less likely to have SP ($p=0.053$) and NFT ($p=0.024$) in older ($\geq 65$ years) persons. Conversely, younger individuals carrying the CCGCAC haplotype were more likely to have non-neuritic ($p=0.022$) and diffuse ($p=0.002$) SP (see Figures 22 and 23).
Figure 23. USF1 Haplotype 4 (CCGCAC) with SP and NFT prevalence according to age grouping (0-64 years in grey and 65+ in black).

Additionally, female carriers of the GCGCAC haplotype (7) were more likely to have SP (p=0.013), neuritic (p=0.011) and late stage burnt out SP (p=0.001), as well as increased risk for increasing SP cortical coverage (≤1.05% p=0.029, >1.05% p=0.047) as seen in Figure 24. When concerning age division, older (≥65 years) individuals with the GCGCAC haplotype were more likely to have SP (p=0.017) and higher SP coverage (>1.05% p=0.020), seen in Figure 25. No further associations were found.

Figure 24. USF1 Haplotype 7 (GCGCAC) with SP and NFT prevalence according to gender (males in grey and females in black).
Figure 25. USF1 Haplotype 7 (GCGCAC) with SP and NFT prevalence according to age grouping (0-64 years in grey and 65+ in black).
Discussion

1. Study subjects

The TASTY series was an autopsy collection originally designed to investigate epidemiological aspects of coronary artery disease and cardiovascular aspects (Norja et al. 2007). Because of this original design plan, there are some disadvantages in studying this cohort within the context of neuropathological disease. Samples are incomplete in terms of neuropathological evaluation and cerebro-spinal fluid (CSF) samples are non-existent. Tissue sample analyses were also done on a routine basis and therefore not as detailed as other studies.

The population of the TASTY series consisted of Finnish subjects and thus genotyping investigations will not only reflect this, but results may only be applicable to the Finnish population because of this. This is a caveat of any genetic population study however, and this collection of cases is probably the most representative of a living undemented population of the Tampere/Pirkanmaa region. Additionally, Finland contains a well-studied and known isolated population phenomenon due to starvation bottlenecks, making it particularly suitable for genetic studies (Jakkula et al. 2008, Nelis et al. 2009).

Whilst this series has limitations, including the lack of confirmed dementia status of cases, the large number and unselected nature suggests that the TASTY cohort has some useful advantages. Collected over a number of years and consisting of a large age range, the cases are useful if their context is understood. Additionally, medication use, illness information and blood sample analyses would have been useful to fully complement the data, the results achieved with the TASTY cohort are interesting and provide grounds in the future for more effective collections.

2. Methodological considerations

With a large autopsy series and the common prevalence of both SP and NFT within this so-called ‘normal’ population of non-demented cases, it seemed logical to investigate the genetic associations with these neuropathological lesions to try to elucidate the mechanisms by which they develop. Genetic studies have become quite a powerful tool in science and are commonly used to determine the involvement of pathways and mechanisms within disease aetiology and other disorders (Lambert JC et al. 2009, Harold et al. 2009).

Genetic studies do however, have the disadvantage that they are often performed with low statistical power, which when many variables are investigated simultaneously, can in most cases not show predicted associations or provide significant correlations in the form of false positives. For example in the TASTY series (and in many others), it was extremely difficult to visualise significant associations with the APOEε2 allele, due to its rarity – in the entire cohort there were only 51 individuals who had at least one ε2 allele, and only one that had two ε2 alleles. It is for this reason that many studies are rigorously subjected to multiple testing corrections. It is important to note however, that some forms of multiple testing, such as the method referred to as Bonferroni, are in fact too conservative and override significant results that lie on the border of such limitations (Perneger 1998).

Another important aspect of the studies contained in this thesis involves the evaluation of neuropathological tissue stains. Bielschowsky’s staining was the original
staining method used to count neuropathological lesions related to AD, providing a conclusive diagnosis at death of the disease. More specific immunohistochemical stains however, are more commonly used nowadays, especially in research environments, although the TASTY series was only subjected to Bielschowsky staining for simplicity, considering the large number of cases.

Alafuzoff et al. (Alafuzoff et al. 2006) showed the need for standardised procedures to ensure as little discrepancies between research groups as possible. These staining methods, as well as the evaluation by a neuropathologist have indicated the necessity for these systems to be accurately set up and remain consistent. A difference in personal opinions, for example what semi-quantitative SP coverage classification was to be assigned, is just one of the many problems facing this field.

Additionally, there were limited samples and sample types to take advantage of, which provided difficulties when reviewers or editors requested further experiments to back up our results. This was due to the time constraints and storage limitations placed on sample collection, which was to be performed during routine autopsies, and thus did not include whole brain fixation. It is with these considerations in mind that a new autopsy series has been created and will utilise these relevant findings as it is collected over the next few years.

3. APOE & neuropathological lesions (I)

The occurrence of an early prodromal phase in AD that includes the development of brain changes in the general population is not well understood, as studies have usually been comprised of older individuals and it is difficult to determine even if this phase exists at all, let alone when it begins. We found that the accumulation of AD-related changes starts already around 30-40 years of age in some individuals. In general, there was a strong relationship with age for the prevalence of both SP and NFT, which reached almost 100% in the oldest age group. In APOE ε4 carriers, SP were more frequent compared with noncarriers in every age group, except the youngest (0-49 years) and oldest (>90 years). The difference was most evident among 50-59 year olds, suggesting that a significant percentage – in this study 40.7% of APOE ε4 carriers – had brain changes related to AD, whereas the SP frequency was <10% among the reference group (ε3/ε3 genotype).

This may indicate that the process leading to the development of AD begins in middle age, and that the effect of APOE genotype can be seen during these age groups more clearly than in later life. Our results are supported by other reports (Gomez-Isla et al. 1996, Baum et al. 2000, Pirttilä et al. 1997), confirming that the ε4 allele is a risk factor for SP formation within the brain. Additionally, our studies show the effect of the APOE ε4 allele begins earlier than previously thought. The difference between APOE genotypes of NFT prevalence was not found to be statistically significant. Of our cases, only a few had a clinical history of dementia, but the numbers of SP and NFT found among those overlapped with the counts of those without reported dementia.

We chose to assess NFT from the hippocampal area, where they are usually discovered preceding amyloid formation (Braak, Braak 1997). On the other hand, neocortical areas, which are located at some distance from the allocortex are the first (phase 1) brain regions to show precipitations of Aβ peptide as reported by Thal et al., 2002 (Thal et al. 2002) Thus, our selection of middle frontal gyrus was well motivated.
SP and NFT were detected in 6% and 12%, respectively, of brains of individuals <50 years of age, suggesting that the preclinical stage could be even longer than previously thought(Yamaguchi 1998). Some studies(Ghebremedhin et al. 1998) analysing younger patients have suggested that these cases are a rare form, or the beginning of the neuropathological development of the disease(Braak, Del Tredici 2011), although this is not supported by our results. In fact, the common occurrence of these features discovered in our series suggests that these brain changes might actually belong to normal aging within the human brain. New theories of AD also suggest that the SP and NFT seen might be the result of oxidative stress, a known aging mechanism(Thomas, Fenech 2007).

The risk for SP development was largest for the 50-59 year old group carrying the APOE\(\varepsilon4\) genotype. This means that middle age is possibly the period when the \(\varepsilon4\) allele exerts its effect on modifying SP formation. Other factors such as inflammatory processes may potentially be activated at this age period. The involvement of APOE in neuronal repair(Mahley, Rall 2000) also gives support to this theory.

In our study, the association between SP and APOE\(\varepsilon4\) allele carriernship weakened in older age groups, possibly due to other effects on morbidity that are associated with the APOE gene, which would ultimately exclude APOE\(\varepsilon4\) carriers from our series.

Concurring with the SP results, the CERAD SP scoring also clearly shows the expected decrease in the percentage of cases with no plaques, and corresponding increase of those with higher densities of plaques. Crosstabulation of significant values revealed that the APOE\(\varepsilon4\) allele was linked with increasing the occurrence of higher SP densities. The loss of a well-defined increase in moderate and frequent SP densities with age is most likely due to these cases being omitted from our study for reasons discussed earlier.

The percentage area of cortex covered by SP showed a general trend of increasing with age, dropping off after >90 years, consistent with a loss in samples for analysis. APOE\(\varepsilon4\) carriers had a higher percentage of SP coverage than those with both \(\varepsilon2\) and \(\varepsilon3\) alleles, providing further evidence of the \(\varepsilon4\) effect on SP formation. High SP coverage among “normal” undemented cases agrees with previous data on similar “discordant” results. Presence of abundant SP may indicate “pathological aging” or “premise of full-blown AD”(Duyckaerts et al. 2009). In any event, in the NIA-Reagan criteria all lesions of AD type are considered pathological(The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer’s Disease, 1997). On the basis of pathology alone however, no definite conclusion should be drawn as to whether a person has had disturbances in cognition.

The relationship between NFT and AD, as well as between NFT and APOE\(\varepsilon4\), has remained unclear, with contradictory reports in many studies(Sparks et al. 1996, Ghebremedhin et al. 2001). Although NFT occurrence increased with age, there was no statistically significant difference between the APOE allele genotypes and NFT prevalence, and although NFT and SP often occurred together, half of the cases with NFT did not show any SP, and more than one third of SP were not accompanied by NFT.

There was a tendency toward the typical protective effect of the \(\varepsilon2\) allele, as has been seen by others(Farrer et al. 1997, Corder et al. 1994, Tiraboschi et al. 2004). This study sustains the assumption that the APOE\(\varepsilon4\) allele is an age-dependent risk factor for SP formation, but not NFT, concurring with other studies that have found no such relationship between APOE and NFT(Gomez-Isla et al. 1996, Berg et al. 1998).
4. CRP & neuropathological lesions (II)

The inflammation theory was proposed after epidemiological studies revealed a 6 times smaller incidence of AD in a cohort of patients receiving NSAIDs for rheumatoid arthritis, compared to a control group(Aisen et al. 2002, McGeer et al. 1990). Whilst the effectiveness of NSAIDs is controversial in the treatment of AD(McGeer et al. 1990), there is still strong support for the hypothesis that inflammation is an important part of the AD process, although it remains controversial(Streit et al. 2009).

CRP is an acute phase inflammatory marker of plasma and CRP levels have been shown to be upregulated in affected areas of AD brains(Yasojima et al. 2000). Polymorphisms of the CRP gene associated with elevated CRP levels have also been shown to increase the mortality of patients(Hurme et al. 2007). Research has implicated genetic factors as determining 27 – 40% of CRP levels(Teng et al. 2009, Wang et al. 2009). We did not find a relationship between CRP genotypes and NFT. NFT formation is presumed to be secondary to SP production(Duyckaerts, Delatour & Potier 2009), thus the lack of an association with CRP genotypes and NFT in our study supports the idea that CRP polymorphisms would be related only to SP.

SNP rs2794521 has been previously reported to affect expression levels of CRP with the C allele reducing transcription levels of the protein(Teng et al. 2009, Wang et al. 2009), compared to the T allele. In our cohort, this was the only SNP that associated with the occurrence of SP, with the most common CT genotype borderline significantly associated with a reduced risk of having at least one SP (p=0.067).

When we further analysed the associations taking into account the early or late SP phenotype, it was found that CRP SNP rs2794521 (C carriers) was significantly associated with a reduced risk of harbouring non-neuritic SP. It may be possible that the CT genotype associates with lower levels of CRP, thus interfering with formation of SP. In contrast, high CRP level SNPs (rs3091244, TA carriers and rs3093075 CA carriers) were strongly associated with an increased risk of non-neuritic SP. However as a sign of the complex relationship between SNPs and CRP levels, we found that other high CRP level SNPs rs1130864 (TT genotype) and rs1205 (CC carriers) also showed trends toward protection for non-neuritic compared to no SP.

The CCGCC haplotype contains the protective, low CRP protein-linked C allele for both rs2794521 and rs3091244, whilst TAGCC has the high CRP level T and A alleles for the same SNPs. The effects of these SNPs were corroborated in haplotype analyses showing CCGCC carriership reduces the risk and TAGCC carriership increases the risk of non-neuritic SP, with tendencies in the same directions for neuritic compared to no SP.

Our immunohistochemical results showing that CRP and Aβ peptide IHC staining were correlated, supports the involvement of inflammation in AD and corresponds with other studies(Yasojima et al. 2000). In line with previous reports and our results above, the high CRP SNP rs3091244 (TT genotype), was significantly associated with CRP IHC staining in the CA1/2 region. In contrast, the previously reported high CRP level TT genotype of rs1130864 was significantly associated with positive staining, although our SP results would suggest it has some protective effect in non-neuritic SP formation. This could suggest that this SNP may be more effective at clean-up and its higher levels in this case, may be beneficial.

The absence of an association between Aβ peptide staining and CRP genotypes could be explained by CRP affecting only SP formation and not the presence of the Aβ peptide
itself, which is the product of normal AβPP processing (Haass et al. 1992). This makes sense, given the revealed associations between CRP genotypes and SP types in our study. As the majority of the TASTY series are non-AD cases, correlative findings between CRP genotypes and SP prevalence reveal an interesting view into the early development of AD neuropathology.

It is possible that these SP positive cases could be in a prodromal phase of the disease and may later have developed AD had they lived. Our data suggest that CRP genotypes may modify initial SP formation in the brain. This is an interesting finding that will need to be investigated further in cohorts comprising only AD cases and replicated in larger epidemiological studies. It may be that CRP polymorphisms associate with or participate in the slowing down or enhancement of early stage SP, but after this, other factors come into play to effect conversion to late stage SP.

As end-stage SP are more likely to be associated with dementia than other types (Duyckaerts, Delatour & Potier 2009), this could explain why NSAID treatments in clinical AD patients have proven ineffective at slowing or reversing the disease, as inflammation may have already played its part. It could be assumed that other factors aside from CRP genotypes participate in the conversion from these ‘benign’ SP, to pathological SP types related to AD, assuming that SP are more than just tombstones in the pathogenesis of the disease.

The common occurrence of these brain lesions and subclinical elevations in elderly patients of inflammatory markers (Gallicchio et al. 2008), as well as our current results, suggest that these may be simply a consequence of brain aging without any relationship to clinical AD. The conversion of these pathways into those causing AD however, are yet to be ascertained and remain controversial.

5. CLU, CR1 & PICALM, and neuropathological lesions (III)

Genome wide association studies (GWAS) investigating AD have in the past not been powerful enough to reveal anything except APOE. Two recent large GWAS however (Lambert JC et al. 2009, Harold et al. 2009), collectively investigated over 30,000 individuals (with almost 12,000 probable AD cases) and examined around 500,000 SNPs that may influence AD risk.

NFT were found to associate with age and gender, however they were not associated with any of the SNPs investigated. We hypothesised that the three SNPs investigated would associate with SP, as they are involved in AD pathways (Jenne, Tschopp 1992, Jones, Jomary 2002, Calero et al. 2000, DeMattos et al. 2004, Dreyling et al. 1996, Tebar, Bohlender & Sorkin 1999, Yao et al. 2005, Rogers et al. 2006, Wyss-Coray et al. 2002, Kuo et al. 2000, Zhou et al. 2008), however we did not find as many correlations as for APOE.

In our study, genetic variants of CLU, PICALM and CR1 genes were associated with SP and remained so with the inclusion of APOE4 carriers and age as covariates. The appearance of an increased risk for CLU C carriers versus TT is unusual in that it only applies for burnt out SP – a group in the cohort that is relatively small and are largely female. This suggests that the effect of CLU could be on the later stages of SP development and related to the removal of Aβ peptide from cells and the brain (Jenne, Tschopp 1992, Jones, Jomary 2002, Calero et al. 2000, DeMattos et al. 2004) being reduced in efficiency.
The *PICALM* T allele appears to have a protective effect on SP prevalence, true also for TT genotypes versus CC genotypes in the oldest age group. This may be due to more efficient intracellular trafficking and clear-up of Aβ peptide, or the components or pathways that induce Aβ peptide build-up or production(Dreyling et al. 1996, Tebar, Bohlander & Sorkin 1999, Yao et al. 2005, Kuo et al. 2000). The protective effect of the T carriers was seen also for SP coverage, however only significant for moderate SP.

We found that *CR1* CC genotype carriers were associated with an increased risk of having sparse compared to no SP, however the trend was not consistent for the increasing coverage of SP. This was also true for the analysis grouping the rare homozygote with heterozygotes. This suggests the effect of *CR1* is complex and not as straightforward as increasing SP risk and requires further investigation.

SP treatments have so far failed to improve patients’ cognitive abilities(Holmes et al. 2008) and current theories are moving away from SP and suggest soluble oligomeric Aβ may be the culprit in AD(Gandy et al. 2010, Moir et al. 2005, Tomiyama et al. 2010). The scarcity of associations may be due to the small number of cases with SP within the TASTY series (31.1%), resulting in low power, however we have a 600 case-strong cohort, which revealed strong associations between *APOE* with SP.

Further studies should replicate these findings in a larger autopsy series of the general population, with and without AD cases. Our results suggest that whilst these SNPs associated with probable AD cases, they do not strongly relate to SP prevalence in an autopsy series representative of the general population, possibly indicating their complex involvement in the disease.

6. **USF1 & neuropathological lesions (unpublished data)**

USF1 has been previously identified as a crucial and general transcription factor with multiple roles in the transcriptional regulation of several genes involved in lipid and glucose metabolism, stress and immune responses, cell cycle and proliferation. The number of USF1-dependent genes is extremely high (Corre, Galibert 2005). We discovered associations between the *USF1* SNPs and haplotypes with both SP and NFT, indicating a possible role in the pathogenesis of AD-related lesions.

The rs10908821 C allele associated with a higher tendency of SP prevalence, especially neuritic SP, and a higher SP coverage within the cortex. Although rs10908821 has not previously been linked to disturbances in lipid metabolism, the rs2774276 GG genotype has been linked to higher total serum cholesterol and LDL-levels, and the G allele with higher waist-to-hip ratios(Komulainen et al. 2006). In our study the G allele was associated with a higher tendency for early stage SP. The rs2516839 T allele has been previously linked to lower HDL-levels, higher triglyceride levels(Laurila et al. 2010) and a risk factor for calcification of coronary arteries and development of coronary atherosclerosis(Kristiansson et al. 2008).

The rs2073658 C allele has been previously linked to a decreased risk of CVD and female carriers of rs2073658 T allele have higher risk for CVD and mortality(Komulainen et al. 2006). In our study the rs2516839 T allele and rs2073658 TT genotype were associated with a higher tendency for having later stage SP. These findings fit to the hypothesis that disturbances in lipid metabolism might connect the development of AD-related lesions.
Younger carriers (<65 years) of the haplotype CCGCAC were more likely to have non-neuritic SP indicating an increase in the risk of early stage SP. On the other hand, a trend towards a lower tendency for SP was seen for older carriers of the same haplotype, possibly indicating that different effects come into play at different ages. Carriership of haplotype GCGCAC was associated with SP, especially neuritic SP, prevalence and higher SP cortical coverage. Although this risk haplotype contains the low risk alleles of rs10908821, rs2073658 and rs2516839, it contains the high risk rs2774276 G allele, possibly indicating its strong effect. The haplotype CCCTAT (which only contains risk allele rs10908821) was associated with a lower tendency of early stage SP in men. A lower prevalence of NFT was seen for the rs2774276 GG genotype and the same trend was observed for the corresponding haplotype CCGCAC.

USF1 might affect the development of AD-related lesions through differential expression of USF1 target genes such as ABCA1 and $\alpha$PP. USF1 represses the gene encoding the ABCA1 transporter protein, which has an essential role in the cellular efflux of cholesterol and phospholipids. Disturbances in its production might cause diminished cellular efflux of lipids resulting in disturbances in cell function, possibly leading to cell death. Another possible mechanism might be the overproduction of $\alpha$P peptide, since USF1 upregulates transcription of the $\alpha$PP gene.

USF1 might also cause disturbances in neuronal functioning. Upstream stimulatory factors (USFs) have been found to mediate calcium responsive transcription and to have a role in the regulation of activity-dependent transcription in neurons(Chen et al. 2003). USFs activate the brain derived neurotrophic factor (BDNF) gene promoter, which has an essential role in promoting neuronal survival, differentiation and synaptic plasticity(Tabuchi et al. 2002). USFs also interact with GABA$_B$-receptors, which play an essential role in synaptic plasticity(Steiger et al. 2004).

Changes in cholesterol metabolism have been suggested to be involved in the pathological process behind AD(Martins et al. 2009) and supported by studies on $\alpha$PP transgenic mice on a cholesterol-rich diet which had increased levels of brain $\beta$-amyloid deposition(Refolo et al. 2000). A significant suppression of de novo synthesis of cholesterol and decreased generation of $\beta$ peptide has also been suggested(Simons et al. 1998). Cholesterol is actively turned over among neurons and glial cells, with the help of apolipoproteins and their receptors, and cholesterol has an essential role in synaptic plasticity in the central nervous system(Yanagisawa 2002). Studies investigating the connection between cholesterol levels in serum and AD have found an association between high serum levels of cholesterol and the incidence of AD(Jarvik et al. 1995, Notkola et al. 1998). Also a significant increase in the levels of LDL cholesterol, as well as a significant decrease in the levels of HDL cholesterol was found postmortem in AD patients(Kuo et al. 1998).

It has also been suggested that tau phosphorylation might also depend upon an alteration in intracellular signalling, which is closely associated with cell cholesterol metabolism(Yanagisawa 2002). The use of statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) has been suggested to potentially suppress the development of AD(Wolozin et al. 2000), but this observed effect might be due to other beneficial effects(Tan et al. 2003).

USF1 has also been found to regulate the transcription of APOE(Naukkarinen et al. 2005) and contains a USF1 low-risk dyslipidemia allele, which has been associated with
higher plasma C-reactive protein (CRP) and interleukin-6 levels (Reiner et al. 2007). Since USF1 regulates genes involved in immune responses, this offers yet another possible mechanism by which USF1 contributes to AD.

Our data suggests that USF1 genotypes play a part in the development of AD-related lesions. This interesting finding will need to be investigated further in AD patient cohorts and replicated in larger epidemiological studies. USF1 polymorphisms may contribute to development of AD related lesions possibly through disturbances in lipid metabolism, and therefore might play a part in the formation of AD related lesions.
Summary and Conclusions

Alois Alzheimer first described the NFT, one of the major pathological hallmarks of the disease that now bears his name, way back in 1907. A world of research has delved into the pathways that may potentially be involved in the disease since then, and continue to report new results including theories and possible treatments to reduce the symptoms or cure AD.

We now step into a new era where the original (old) theories are starting to be seriously questioned and new ideas are being developed, utilising GWAS and functional studies. We may now start to be somewhat hopeful about the elusive cause or causes of the disease and perhaps even in our lifespan be able to see another disease beaten by the human race.

In this thesis, a candidate gene approach was utilised to investigate the possible associations with the neuropathological lesions SP and NFT, in a 600-case population representative of a normal, non-institutionalised cohort – the TASTY series. With a focus on inflammatory mechanisms and previously reported AD-risk genes, we studied the correlations of \textit{APOE}, \textit{CLU}, \textit{CR1}, \textit{PICALM} and \textit{USF1}. The main results and conclusions can be found below:

1. The \textit{APOE}^\epsilon4 allele was strongly associated with the presence of SP in the TASTY series, as compared to the most common \textit{\epsilon}3/\textit{\epsilon}3 genotype, especially in early middle aged individuals. The \textit{\epsilon}2 allele appeared to show some form of protection, however this was not significant. There were no associations between the \textit{APOE} genotypes and NFT. Assuming that NFT and SP indicate disease progression, our results on the common occurrence of these brain changes suggest that interventions for AD may need to be initiated in middle age in individuals carrying the \textit{APOE}^\epsilon4 allele, especially if they have a family history of dementia.

2. A number of \textit{CRP} SNPs and haplotypes were associated with early stage ‘non-neuritic’ SP, with a trend in most cases for late stage ‘neuritic’ SP. There were no associations between the \textit{CRP} SNPs or haplotypes and NFT. Both CRP IHC stains and A\beta peptide IHC staining correlated with each other, as did CRP IHC staining with \textit{CRP} SNPs and haplotype pairs. Interestingly, A\beta peptide IHC staining did not correlate with any \textit{CRP} SNPs or haplotypes. Our data suggest that \textit{CRP} genotypes may modify initial SP formation in the brain and may participate in the slowing down or enhancement of early stage SP, after which other factors come into play to effect conversion to late stage SP and possibly AD.

3. Whilst \textit{CLU}, \textit{CR1} and \textit{PICALM} did associate with some variables of SP, they did so sparingly and raise questions about the involvement of SP in the aetiology of AD. The studied SNPs did not correlate with NFT either, however previous reports have supported their involvement in the pathogenesis of the disease. Our results suggest that whilst these SNPs associated with probable AD cases in recent GWAS, they do not strongly relate to SP prevalence in an autopsy series representative of the general population, possibly indicating their complex involvement in the disease.
4. A number of *USF1* SNPs and haplotypes associated with variables of SP and also with NFT in the TASTY series. This suggests a strong role of USF1-mediated effects in the development of both neuropathological lesions and warrants further investigations. *USF1* polymorphisms may contribute to development of brain lesions possibly through disturbances in lipid metabolism or other mechanisms by which USF1 is known to operate, thus participating in AD pathogenesis.

Based on these results, it can be concluded that a number of inflammatory genes may influence the development of the neuropathological lesions associated with AD and may therefore participate in the initiation or progression of the disease. This is of course, assuming that these characteristic hallmarks are in fact a detrimental part of disease pathogenesis and not simply bystanders of the disease. Because these results were accumulated from an autopsy series consisting primarily of non-demented cases, there remains the question of the involvement of these AD-related lesions in disease aetiology. Further detailed studies investigating this much-discussed topic will be required and help to elucidate their contribution to Alzheimer’s disease.
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Original Communications

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Apolipoprotein E–Dependent Accumulation of Alzheimer Disease–Related Lesions Begins in Middle Age

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Objective: To study the prevalence and age dependency of senile plaques (SP) and neurofibrillary tangles (NFT), the brain changes characteristic of Alzheimer disease (AD), and their association with apolipoprotein E (APOE) genotypes in a community-dwelling normal population.

Methods: This neuropathological study used both silver staining and Aβ immunohistochemistry in brain tissue microarrays, including SP coverage and NFT counts from frontal cortex and hippocampus, and APOE genotyping, and was performed on a consecutive prospective series of 603 subjects (aged between 0 and 97 years) of an unselected population living outside of institutions. Cases were subjected to autopsy following sudden or unexpected out-of-hospital death, covering 22.1% of the mortality of Tampere, Finland and its surroundings. None died of AD, although 22 (3.7%) were demented and 10 (1.7%) had memory problems.

Results: Of the series, 30.8% had SP, and 42.1% had NFT; these occurred more commonly among females and showed a strong relationship with age. Both changes had already appeared at around 30 years of age, reaching an occurrence of almost 100% in the oldest. SP were more frequent in APOE ε4-carriers compared with noncarriers in every age group except the oldest (>90 years). The difference was most evident during the ages 50 to 59 years, where 40.7% of ε4-carriers had SP, compared with 8.2% in noncarriers (odds ratio, 8.39; 95% confidence interval, 2.55–27.62). The difference in NFT prevalence between APOE genotypes was not statistically significant in any age group.

Interpretation: The brain changes associated with AD may already begin developing early in middle age, especially among APOE ε4 carriers.


Several studies have demonstrated that brain changes typical of AD are relatively common even in cognitively normal individuals.4–6 These studies include retrospectively collected patient series from the files of pathological institutes, or epidemiological studies7 comprising elderly participants aged ≥65 years, which have been subjected to neuropathological study. To our knowledge, there are no studies on the occurrence of these brain changes in a normal community-dwelling population including all ages.

The only commonly accepted gene affecting the risk of the sporadic form of AD is apolipoprotein E (APOE), which is involved in lipid transport and most probably participates in the neuronal repair process.8 Variation at two different polymorphic sites code for three alleles (ε2, ε3, ε4), which determine six genotypes. It has been demonstrated that carriers of APOE

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ε4 have a decreased cerebral metabolic rate for glucose, an increased risk of AD, and an earlier age of onset compared to noncarriers, whereas the ε2 allele seems to be slightly protective. 

Most but not all longitudinal studies of nondemented persons have reported that APOE ε4 carriers have greater cognitive decline with aging. However, the disease itself does not appear to progress more rapidly in APOE ε4 carriers than in noncarriers. Until now, only a few studies focusing retrospectively on the relationship between APOE genotype and Alzheimer disease–related brain changes have included younger or middle-aged individuals. There are no population-based epidemiological data on the relationship between APOE and brain changes.

We studied the prevalence of brain changes related to AD and their association to APOE genotypes in different age groups in a normal community-dwelling population, without age as a restriction. The cases were a consecutive series of medicolegal autopsies, collected over 3 years (2002–2004) from the city of Tampere and its surroundings, representing a geographical area of southern Finland. The cases were autopsied due to sudden or unexpected death of a previously healthy person, and thus represented the best available cross section of the general population. None was living within a dementia unit or institution, and there were therefore no persons with severe dementia among the cases.

## Materials and Methods

### Autopsy Series

The Tampere Autopsy Study (TASTY) series comprised of 603 men and women, aged ≤97 years, who were subjected to medicolegal autopsy at the Department of Forensic Medicine, University of Tampere, Finland, from 2002 to 2004. The population at this time grew from 452,362 in 2002 to 462,923 in 2004, and medicolegal autopsies covered 22.1% of deaths in the region. None of the cases had died of AD, but 6 had been diagnosed with the disease (1.0%) while alive. Additionally 16 cases (2.7%) were suffering from undetermined dementia, 10 (1.7%) had memory disorders, and 1 (0.2%) was diagnosed with Parkinson disease prior to death, according to available hospital records and next of kin reports. The study was approved by the Board of Medicolegal Affairs of Finland.

At autopsy, samples from four (middle frontal gyrus, gyrus cinguli with corpus callosum, hippocampus, and cerebellum) different areas of the brain were placed in Tissue-Tek (Sakura, Torrance, CA) boxes and fixed in phosphate-buffered 4% formaldehyde solution for at least 2 weeks. Tissue blocks were then embedded in paraffin from which 10µm sections were cut and stained using Bielschowsky’ argyrophilic silver impregnation and hematoxylin & eosin methods.

The entire neocortical area of each sample was screened at 100× magnification to find the area with the greatest frequency of silver-positive senile plaques. This was performed by two researchers (T.L. and S.H.) using a bifocal microscope (with two eyepieces). The scoring was performed using a square microscopic graticule (10 horizontal and 10 vertical lines with 100 intersections covering an area 1mm² in size), extending in contiguous cortical microscopic fields from the molecular layer of the gray matter to the white matter, along a line perpendicular to the pial surface. All intersections that overlaid a silver-positive plaque were counted. To determine the area of cortex covered by plaques, the number of intersections overlying a silver-positive plaque was divided by the total number of intersections, as performed by Polvikoski et al. 17 Multiplying this number by 100 then gave the average percentage of cortex in which SP were seen. Plaques were analyzed for neuropathological stage (diffuse, primitive, classic, and burnt-out). The same area with the greatest frequency of plaques was then scored according to the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) semiquantitative assessment of plaques. 18 CERAD scoring using the definitions No Plaques, Sparse Plaques, Moderate Plaques, and Frequent was then given to each case.

The quantity of NFT was estimated from the hippocampus. The entire hippocampus of each sample stained with Bielschowsky’s method was screened to find the CA1 area with the highest number of NFT. In five (4–6) random columns of a grid (size 0.5 × 0.5mm at a magnification of 200×), all NFT were counted. The average number of NFT in 1mm² was recorded and used in the statistical analyses.

For immunohistochemical confirmation of the Bielschowsky staining, tissue microarrays (TMA)s were constructed from the same paraffin tissue blocks of each case, using the Bielschowsky-stained slides as guides to locate regions. Between nine and 24 cases were placed in each TMA, with six regions of the brain sampled from each case (hippocampus CA1, CA2; CA3; cerebellum; gray matter from frontal cortex; gray matter from gyrus cinguli; cerebral white matter). The TMA block was constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD). When all samples were inserted (Fig 1), the TMA was heated at 37°C for 30 minutes to promote the attachment of tissue to paraffin. The diameter of the tissue cylinder punch was 1mm, and the thickness of slices from the completed TMA was 1–2µm. TMA slides were then stained separately with Bielschowsky’s argyrophilic silver impregnation method and amyloid β antibody 1:150 (Cell Signaling Technology, Danvers, MA).
that were stained with Bielschowsky’s method could be included in the TASTY TMA construction. For TMA analyses (both Bielschowsky and Aβ staining), the sphere on the TMA slide corresponding to gray matter of the cortex was screened as above.

**Genotyping**

DNA was extracted from 5ml of postmortem blood samples using the standard salt precipitation method. From this, 1μL was used for polymerase chain reaction amplification. APOE genotyping was performed as described elsewhere in detail.21

**Statistical Analyses**

For statistical analysis we used three genotype groups: 1) APOE ε2/ε2, ε2/ε3 (APOE ε2 group), 2) APOE ε3/ε3 group (reference group), and 3) APOE ε4/ε2, ε4/ε3, and ε4/ε4 (APOE ε4 carriers), and divided these into age groups ranging 0–49, 50–59, 60–69, 70–79, 80–89 and >90 years. Analyses for SP (and NFT) assembled the cohort into two sets: those with one or more SP and those without. Further analyses grouped plaques into non-neuritic (diffuse and primitive) and neuritic (classic and burnt-out).

Spearman rank correlation (rS) was used to determine the association between SP and age, and NFT and age. Binary logistic regression analysis was used to reveal the occurrence of SP (and NFT) among age groups, and their association with the APOE genotype. The Bielschowsky and Aβ staining association was analysed using Pearson correlation. The statistical analyses were performed using the SPSS programme, version 14.0 (SPSS, Inc., Chicago, IL).

**Results**

Of 603 individuals, 388 were male and 215 were female (see Table 1 for cohort data), ranging in age from a few days old to 97 years (average, 62.7 years). SP frequency was available for 548 (80.3%), and APOE genotypes for all (n = 603). APOE genotyping indicated that there were no significant differences in the distribution of allele frequencies. Missing cases for neuropathological analyses were due to extensive brain damage. Combined SP and APOE genotype was available for 542 cases, and NFT and APOE genotype was available for 478 cases. Of the 603 cases, 558 were included in the TASTY TMA construction, of which 206 were female and 352 male. APOE genotyping was available for 556 cases, and SP frequency was available in 536 and 558 cases for Bielschowsky and Aβ staining, respectively.

**Senile Plaques**

At least one SP was found in 30.8% of all cases with a clear female preference (41.4% vs 24.5%, p < 0.0001). SP were already found in some individuals around 30 years of age, and their occurrence increased with age, reaching almost 100% in the oldest age groups (Fig 2). The SP density (Fig 3) varied between 0 and 5.41% for all cases, with a median of 0% and a mean of 0.44%, and also had a strong correlation with age (rS = 0.46, p < 0.001 for both).

The occurrence of SP was modified strongly by APOE genotype. Of 320 cases with the most common APOE ε3/ε3 genotype, 97 (24.7%) had at least one SP, which was more common among females (odds ratio [OR], 1.4; 95% confidence interval [CI], 0.95–2.26; p = 0.087 with age as a covariate). Twelve (23.5%) of the 51 cases with the genotypes ε2/ε2 or ε2/ε3 had SP, again with a slight tendency for female preference (31.8% vs 17.2%, p = 0.22). The highest percentage of SP-positive cases was seen among carriers of APOE ε4, where 76 (44.5%) of 171 cases had SP. In this group, female preference was not as clear (50.7% vs 40.2%, p = 0.17) as among the APOE ε3/ε3 carriers.

<table>
<thead>
<tr>
<th>Table 1. Characteristics of the TASTY Cohort</th>
</tr>
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<tbody>
<tr>
<td><strong>Age Group</strong></td>
</tr>
<tr>
<td><strong>Male</strong></td>
</tr>
<tr>
<td>Brain, g</td>
</tr>
<tr>
<td><strong>CoD</strong></td>
</tr>
<tr>
<td>Disease</td>
</tr>
<tr>
<td>Accident</td>
</tr>
<tr>
<td>Suicide</td>
</tr>
<tr>
<td>Homicide</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td><strong>SP count/mm²</strong></td>
</tr>
<tr>
<td><strong>NFT count/mm²</strong></td>
</tr>
</tbody>
</table>

Values in parentheses are standard deviations.

TASTY = Tampere Autopsy Study; CoD = cause of death; SP = senile plaques; NFT = neurofibrillary tangles.
The difference between SP prevalence of the APOE ε4 carriers compared with the APOE ε3/ε3 reference group was significant in every age group (see Table 2) except the youngest and the oldest groups, with an OR of 1.48 (95% CI, 0.31–6.98) and 0.20 (0.02–1.71), respectively. The difference was most evident in 50–59-year-olds, among whom 40.7% of ε4-carriers had SP, compared to 8.2% of noncarriers (OR, 8.39; 95% CI, 2.55–27.62).

Although a protective tendency was observed (see Fig 2), no statistically significant difference between SP prevalence was discovered for any of the age groups carrying the APOE ε2/ε2 and ε2/ε3 genotype when compared to the reference group with the ε3/ε3 genotype, perhaps due to the small number of cases with the ε2 genotype. In the age group 0–49 years, no SP were found in the ε2/ε2 and ε2/ε3 genotype groups.

The severity of CERAD plaque score in the cortex increased with age (Fig 4). The association between age and CERAD plaque score gave a correlation of $r_S = 0.45$ ($p < 0.0001$) and showed significant differences between CERAD scoring and APOE genotypes in all age groups except the youngest and oldest. Two-sided Fisher exact analyses produced $p > 0.001$ (50–59 years), $p = 0.046$ (60–69 years), $p = 0.070$ (70–79 years), and $p = 0.005$ (80–89 years).

TMA analysis comparing Bielschowsky and Aβ staining showed that both were correlated (Pearson correlation 0.231, $p = 0.004$), thus validating our analyses on Bielschowsky-stained slides. Similar significant associations between plaques and APOE genotype were found for each age group in both staining methods (see Table 2).

**Neurofibrillary Tangles**

In 42.1% of all cases, at least one NFT was found; this had a strong ($p < 0.0001$) relationship with age (see Fig 5). Those with NFT were on average older than those without (71.4 vs 66.1 years, $p = 0.041$). The density of NFT varied between 0 and 29.60 tangles/mm², with a median of 0 tangles/mm² (mean 3.17 tangles/mm²), and also showed a clear increase with age ($r_S = 0.49$, $p < 0.0001$). The difference in the prevalence of NFT between the different APOE genotype groups compared with the reference group ε3/ε3 was not statistically significant.

Of the cases with NFT, 47% also showed SP, and of the cases that had SP, 61% also possessed NFT.

Cases with some form of dementia reported prior to death had significantly more plaques (Fig 6) when compared to the rest of the cohort. However, the percentage of plaques overlapped greatly among those cases without reported dementia. There was a similar trend for tangles (data not shown).

**Discussion**

The occurrence of an early prodromal phase in AD that includes the development of brain changes in the general population is not well understood, as studies have usually been comprised of older individuals. We found that the accumulation of AD-related changes starts already around 30–40 years of age in some individuals. In general, there was a strong relationship with age for the prevalence of both SP and NFT ($p < 0.0001$ for both), which reached almost 100% in the oldest age group. In APOE ε4-carriers, SP were more frequent compared with noncarriers in every age group, except the youngest (0–49 years) and oldest (≥90 years). The difference was most evident among 50–59-year-olds, suggesting that a significant percentage—in
this study 40.7% of APOE ε4-carriers—had brain changes related to AD, whereas the SP frequency was <10% among the reference group (ε3/ε3 genotype). This may indicate that the process leading to the development of AD begins in middle age, and that the effect of APOE genotype can be seen during these age groups more clearly than in later life. The difference between APOE genotypes of NFT prevalence was not found to be statistically significant. Our results are supported by other reports,22,23 confirming that the ε4 allele is a risk factor for SP formation within the brain.

The random selective nature of this study, including the broad age range and sampling of a normal community-dwelling population (not from institutions or hospitals), gives an advantage over previous studies investigating the nature of brain changes related to AD. Of our cases, only a few had reports of dementia, but the numbers of SP and NFT found among those overlapped with the counts of those without reported dementia. Recent estimations indicate that AD is much more common than previously thought,24 and our results agree with the suggestion that there may possibly be many cases with undiagnosed AD living among

Table 2. Occurrence of Plaques and Their Association With APOE Genotypes, Split by Age, for Both Bielschowsky and Aβ Staining in the TASTY TMA Series

<table>
<thead>
<tr>
<th>Age Group, y</th>
<th>Staining Method</th>
<th>APOE ε3/ε3</th>
<th>APOE ε2+ vs APOE ε3/ε3</th>
<th>APOE ε4+ vs APOE ε3/ε3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Affected</td>
<td>N Affected</td>
<td>OR 95% CI</td>
<td>p</td>
</tr>
<tr>
<td>0–49 Bielschowsky</td>
<td>73</td>
<td>4 (5.5%)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Beta-amyloid</td>
<td>75</td>
<td>2 (2.7%)</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>50–59 Bielschowsky</td>
<td>66</td>
<td>5 (7.6%)</td>
<td>6</td>
<td>1 (16.7%)</td>
</tr>
<tr>
<td>Beta-amyloid</td>
<td>68</td>
<td>4 (5.9%)</td>
<td>7</td>
<td>1 (14.3%)</td>
</tr>
<tr>
<td>60–69 Bielschowsky</td>
<td>47</td>
<td>7 (14.9%)</td>
<td>9</td>
<td>1 (11.1%)</td>
</tr>
<tr>
<td>Beta-amyloid</td>
<td>51</td>
<td>6 (11.8%)</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>70–79 Bielschowsky</td>
<td>67</td>
<td>19 (28.4%)</td>
<td>16</td>
<td>5 (31.3%)</td>
</tr>
<tr>
<td>Beta-amyloid</td>
<td>70</td>
<td>18 (25.7%)</td>
<td>16</td>
<td>5 (31.3%)</td>
</tr>
<tr>
<td>80–89 Bielschowsky</td>
<td>59</td>
<td>33 (55.9%)</td>
<td>10</td>
<td>3 (30.0%)</td>
</tr>
<tr>
<td>Beta-amyloid</td>
<td>59</td>
<td>27 (45.8%)</td>
<td>10</td>
<td>3 (30.0%)</td>
</tr>
<tr>
<td>90+ Bielschowsky</td>
<td>7</td>
<td>5 (71.4%)</td>
<td>2</td>
<td>1 (50.0%)</td>
</tr>
<tr>
<td>Beta-amyloid</td>
<td>7</td>
<td>5 (71.4%)</td>
<td>3</td>
<td>1 (33.3%)</td>
</tr>
</tbody>
</table>

Logistic regression analysis with age as a covariate.
*Statistically significant figures.

TASTY = Tampere Autopsy Study; TMA = tissue microarray; APOE = apolipoprotein E; N = number valid; OR = odds ratio; CI = confidence interval.
community-dwelling populations but not institutionalized, who die of causes unrelated to AD.

We chose to assess NFT from the hippocampal area, where they are usually discovered preceding amyloid formation. On the other hand, neocortical areas, which are located at some distance from the allocortex, may show precipitations of β-amyloid before the first appearance of NFT, which is why we chose to count SP from the middle frontal gyrus. We separated cases based on whether they had at least one SP compared with those with none, due to the idea that although the illness remains in a preclinical or prodromal stage for an extended period of time, it has been proposed that once the destructive process has begun, it inevitably progresses, and no remissions occur during the course of the disease.

SP and NFT were detected in 6% and 12%, respectively, of brains of individuals <50 years of age, suggesting that the preclinical stage could be even longer than previously thought. Some studies analyzing younger patients have suggested that these cases are a rare form of the disease, which is not supported by our results. In fact, the common occurrence of these features discovered in our series suggests that these brain changes might actually belong to normal aging within the human brain, and might only be associated with the APOE genotype, as supported by results from Reiman et al, who found APOE ε4 carriers had a reduced cerebral metabolic rate for glucose. New theories of AD also suggest that the brain lesions seen might be the result of oxidative stress, a known aging mechanism.

The risk for SP development was largest for the 50–59-year-old group carrying the APOE ε4 genotype. This means that middle age is possibly the period when the ε4 allele exerts its effect on modifying SP formation. Other factors such as inflammatory processes or unknown mechanisms may potentially be activated at this age period, providing a means for AD to take hold and progress into the clinical disease. The involvement of APOE in neuronal repair also gives support to this theory.

The association of female sex with the occurrence of SP in our cohort agrees with other studies dealing with AD, as discussed in the review by Behl. However, the lack of an association with female sex among APOE ε4 carriers agrees with studies that have found similar results, especially in Finnish populations. This could indicate that female susceptibility is independent of APOE genotype, as suggested by Farrer et al, that the effect of APOE ε4 is not as prevalent in younger females due to the protective effect of estrogen, or that the ε4 allele may have a stronger effect in males with regards to initial SP development.

In our study, the association between SP and APOE ε4 allele carriership weakened in older age groups, possibly due to other effects on morbidity that are associated with the APOE gene, excluding APOE ε4 carriers from our series. The most obvious explanation is the link between the APOE ε4 allele and AD that requires institutionalization of patients; such patients were excluded from our cohort. Additionally, the development of coronary heart disease (CHD) could contribute to these cases being excluded, as those diagnosed with clinical CHD prior to death do not usually require autopsies.

Concurring with the SP results, the CERAD plaque scoring also clearly shows the expected decrease in the percentage of cases with no plaques, and corresponding increase of those with higher densities of plaques. Cross tabulation of significant values revealed that the APOE ε4 allele was responsible for increasing the occurrence of higher plaque densities. Although the CERAD plaque scoring procedure did not include dementia scores for complete assessment, the crude semiquantitative measure of “sparse,” “moderate,” and “frequent” adequately describes the abovementioned SP phenomenon with age. The loss of a well-defined increase in moderate and frequent SP densities with age is most likely due to these cases being omitted from our study for reasons discussed earlier.

The percentage area of cortex covered by SP showed a general trend of increasing with age, dropping off after ≥90 years, consistent with a loss in samples for analysis. APOE ε4 carriers had a higher percentage of SP coverage than those with both ε2 and ε3 carriership, providing further evidence of the ε4 effect on SP formation within the cortex. High SP coverage among “normal” undemented cases indicated that many could be neuropathologically classified as having dementia. Moreover, AD patients had a smaller range of SP cov-
erage to those suffering undefined dementia or memory problems, conflicting with the conventional concept of an association between SP and dementia, including AD. This reignites the issue of the relevance that measurements of SP coverage have in AD diagnosis.

The relationship between NFT and AD, as well as between NFT and APOE ε4, has remained unclear, with contradictory reports in many studies. Although NFT occurrence increased with age, there was no statistically significant difference between the APOE allele genotypes and NFT prevalence, and although NFT and SP often occurred together, half of the cases with NFT did not show any SP, and more than one third of SP were not accompanied by NFT. This study sustains the assumption that the APOE ε4 allele is an age-dependent risk factor for SP formation, but not NFT, concurring with other studies that have found no such relationship between APOE and NFT. As the results of this study were derived from a large random cohort of noninstitutionalized cases, it tends to be more representative of the general population than other studies involving only institutionalized cases. This subsequently raises questions of the relevance of NFT and also SP in the neuropathological diagnosis of AD, especially when investigating other neurodegenerative diseases that coexist with AD. Considering the relatively common occurrence of both brain changes, they might alternatively originate from other processes, which might be pathological or simply associated with normal aging.

There was a tendency toward the typical protective effect of the ε2 allele, as has been seen by others. The ApoE ε2 isof orm is attributed to more effective cleanup of amyloid levels, and where previous studies related the APOE ε2 protective effect to cases of AD, our study looked only at the occurrence of brain changes at a population level. In our series, the ε2 group was perhaps too small to reveal any statistically significant effect.

One of the drawbacks of our observations was that examinations were performed in only three sections of brain tissue, which may not be as comprehensive as other studies focusing on neuropathological changes. However, these locations are informative according to the CERAD protocol, which suggests that not all regions of the brain need to be investigated to obtain a reliable count of the lesions involved in AD, allowing us to compare SP and NFT loads between individuals. In any case, our study most likely underestimates the number of lesions within the brain, causing the actual lesion load to be more apparent.

The relatively simple Bielschowsky staining protocol is used regularly for routine diagnosis of brain changes related to AD. Although some authors have suggested that it is unreliable due to differences between labora-

tories, in our series restaining of the samples using TMA with both Bielschowsky and Aβ staining methods showed concurrent results for SP counts.

At present, the underlying pathogenic processes in AD and the significance of these lesions occurring in middle age and even younger individuals are unclear. It is also unknown what their relevance is in predicting the development of advanced AD and the dementia that accompanies the disease. According to a recent study, the effect of APOE ε4 on the risk of AD can be attenuated by keeping in check blood pressure and cholesterol levels. Because these are also risk factors for the development of cardiovascular diseases, it proposes an interesting relationship, as vascular changes frequently appear together with both NFT and SP, suggesting a common mechanism or pathway underlying both pathologies. Assuming that NFT and SP indicate disease progression, our results on the common occurrence of these brain changes suggest that interventions for AD may need to be initiated in middle age in individuals carrying the APOE ε4 allele, especially if they have a family history of dementia.

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The guarantor for this research was P. Karhunen.

Approved by the National Authority for Medicolegal Affairs of Finland.

References

CLU, CR1 and PICALM genes associate with Alzheimer’s-related senile plaques

Eloise H Kok1*, Teemu Luoto1, Satu Haikonen1, Sirkka Goebeler2, Hannu Haapasalo3 and Pekka J Karhunen1

Abstract

Introduction: APOE is the strongest risk gene for sporadic Alzheimer’s disease (AD) so far. Recent genome wide association studies found links for sporadic AD with CLU and CR1 involved in Aβ clearance, and PICALM affecting intracellular trafficking.

Methods: We investigated the associations of senile plaques (SP) and neurofibrillary tangles (NFT) with the proposed risk genes and APOE, in the Tampere Autopsy Study (TASTY) series (603 cases), a sample of the general population (0 to 97 yrs), who died out-of-hospital.

Results: Age and the APOEε4 allele associated strongly with all phenotypes of SP, as expected. In age and APOEε4 adjusted analyses, compared to the most common homozygous genotype, burnt out SP were more common among carriers of the C-allele of CLU, whereas the T-allele of PICALM and C-allele of CR1 were linked with lower SP coverage. We found no significant associations between any of the genetic variants and NFT.

Conclusions: Marginal effects from CLU, CR1 and PICALM suggest that these genes have minimal effects on the development of AD lesions.

Introduction

Alzheimer’s disease (AD) is the most common form of dementia in Western society and is, and will continue to be, a burden on health systems in the future as the population ages. Age is the largest risk factor for the disease, with higher incidences in older populations [1,2].

Identification of genes related to sporadic AD risk has been slow with study groups isolating only one strongly associated gene: APOE [3,4]. The epsilon 4 allele of apolipoprotein E (APOEε4) provides odds ratios (ORs) of between 3 and 25 [5,6] for disease association. APOEε4 is suspected to have a lower effectiveness at transporting cholesterol and is not as efficient at repairing neuronal damage as APOEε3 [7]. One or even two copies of the allele, however, are not sufficient to cause the disease, as many carriers of two ε4 alleles do not develop AD [5].

Studies aiming to detect genes associated with disease risk have used heterogeneous AD cohorts and ascertained few polymorphisms with only a minor impact on disease incidence. One of the problems is to distinguish between pure AD, vascular dementia and other dementia types in clinical cohorts [8-10]. The only consistent and currently accepted method for confirming AD is with post-mortem assessment of the neuropathological lesions neurofibrillary tangles (NFT) and senile plaques (SP) [11-14].

Demented individuals do not always exhibit large enough numbers of SP to warrant an AD diagnosis [15] and NFT and SP are both relatively common in the general population [16-19]. Furthermore, these lesions do not provide a clear-cut explanation as to the cause of AD, with different theories advocating amyloid beta (Aβ) accumulation [12,20] or hyperphosphorylated NFT-causing tau protein [21] as the underlying initiating mechanisms that trigger the disease.

Two recent extensive genome-wide association studies (GWAS), comprising 12,000 probable AD cases and 18,000 age-matched non-demented controls [22,23], revealed three new candidates for genetic risk of developing late onset or sporadic AD: CLU, CR1 and PICALM. Phosphatidylinositol-binding clathrin assembly protein (PICALM) is involved in synaptic neurotransmitter release and intracellular trafficking [24-26], whilst
complement component (3b/4b) receptor 1 (CR1), the main receptor of complement C3b protein, binds Aβ and thus may promote clearance [27-30]. Clusterin (CLU, and also known as ApoJ), was replicated independently in the two studies and is thought to bind and remove Aβ from the brain, as well as assist in re-entry of Aβ [31-34].

We have previously shown that as many as one-third of non-demented individuals in an autopsy series-based sample carry SP and more than 40% NFT, with strong age dependence [16]. This suggests that in clinical study series, non-demented control patients may not be free of AD-related neuropathological lesions. Utilising this same cohort, we aimed to investigate whether SP and NFT are associated with any of the recently identified GWAS single nucleotide polymorphisms (SNPs); CLU, CR1 and PICALM to examine their involvement in the development of these brain lesions.

Materials and methods

Autopsy series

The Tampere Autopsy Study (TASTY) cohort consisted of 603 autopsy cases, of which the majority died out-of-hospital within Tampere, Finland and surroundings, collected during the years 2002 to 2004 (described in detail elsewhere [16]). The study was approved by the Board of Medicolegal Affairs of Finland. Females within the cohort accounted for 35.8% and the ages for the entire series ranged from 0 to 97, with an average of 63 years (59 years for males and 68 years for females). Of the cases, 6 (1%) had a clinical AD diagnosis, 16 (2.7%) undefined dementia, 10 (1.7%) had memory disorders and 1 (0.2%) had Parkinson’s disease prior to death (according to available hospital records and next of kin reports). In some cases it was impossible to obtain all variables due to technical difficulties and sample damage.

Alzheimer-related lesion measurements

SP and NFT staining and measures have be portrayed previously [16]. Briefly, the Bielschowsky argyrophilic silver impregnation method was performed on samples and measured by two researchers to acquire SP (neocortex) and NFT (hippocampus) counts. Each area was screened to find the highest density of SP (neocortical area at 100 x magnification) and NFT (hippocampus - CA1 area at 200 x magnification) and then scored using a square microscopic grid (SP - 100 intersections covering 1 mm², NFT - four to six random columns), before creating an average percentage of coverage (SP) or average number in 1 mm² (NFT). Bielschowsky staining was highly correlated with Amyloid beta (Aβ) staining (antibody from Cell Signaling Technology, Danvers, MA, USA; concentration 1:150), which was used to verify our previous results [16], and used the same cortical coverage of SP method. SP and NFT variables included the following categorisations as measured by a neuropathologist: SP (No, Yes), SP type (No Plaques, Diffuse, Primo-tive, Classic, Burnt Out), SP type 2 (No Plaques, Non-neuritic SP, Neuritic SP), NFT (No, Yes), where reference groups were those with ‘No SP’ or ‘No NFT’ and those with either brain lesion were considered ‘affected’. Semi-quantitative data for SP utilised the categories ‘no’, ‘sparse’, ‘moderate’ and ‘frequent’ SP.

Genotyping

The ABI Prism 7900HT Sequence Detection System used 1 µl DNA with PCR primers (Applied Biosystems, Espoo, Finland) for rs1136000 (CLU), rs1408077 (CR1) and rs3851179 (PICALM). All SNPs were in Hardy-Weinberg equilibrium and genotyping confirmed using SDS version 2.2 (Applied Biosystems). Genotyping for APOE has been previously described [16]. Genotyping for the polymorphisms of CLU, CR1 and PICALM were successful for 94%, 97% and 97% of the TASTY cases, respectively.

Statistics

Logistic regression analyses, with continuous age and APOEε4 carriership as covariates (where possible), were used with SPSS (version 14.0 for Windows; SPSS Finland Oy, Espoo, Finland) to determine associations between the SNPs and AD-related neuropathological lesions. For all SNPs, the most common homozygous genotype was used as the reference group. As previously mentioned, those unaffected by SP or NFT were considered the reference group for the brain lesion categories. When analysing with the cohort split by age groups, the following categories were used: 0 to 49 years, 50 to 59 years, 60 to 69 years, 70 to 79 years, 80+ years, with the youngest group (0 to 49 years) considered the reference group with respect to age, in analyses. The cohort was also split by gender, where mentioned.

Results

Autopsy series characteristics

The Tampere Autopsy Series (TASTY) (n = 603) comprises consecutive autopsies on males and females aged 0 to 97 years that lived outside institutions or hospitals (see Table 1). Females were on average 10 years older than males, but males were more likely to have SP compared to females (odds ratio (OR) 2.15, 95% confidence intervals (CI) 1.49 to 3.11). When age was divided into five equal-sized groups, each age group was consistently more likely to have SP compared to the youngest group, with each association also highly statistically significant (see Table 2). This was also true for NFT prevalence (see Table 2), with females more likely...
than males to have NFT (OR 2.18, \(P < 0.0001, \text{CI} 1.49\) to 3.18).

APOE, CLU, CR1 and PICALM associations with SP

As expected, APOE\(\varepsilon\)4 carriership was significantly associated with increased risk of having SP (OR 2.52, \(P < \text{0.0001, CI 1.72 to 3.68}\); having both non-neuritic (OR 2.42, \(P = 0.003, \text{CI} 1.36\) to 4.29) and neuritic SP (OR 2.58, \(P < \text{0.0001, CI 1.66}\) to 4.02) compared to no SP; and having primitive (OR 2.53, \(P = 0.010, \text{CI} 1.25\) to 5.10), classic (OR 2.52, \(P < \text{0.0001, CI 1.54}\) to 4.13) and burnt out SP (OR 2.77, \(P = 0.014, \text{CI} 1.22\) to 6.27) compared to no SP, when evaluated against non \(\varepsilon\)4 carriers (see Table 3). Results showed similar trends when the cohort was split by gender (data not shown).

APOE\(\varepsilon\)4 carriers, compared to \(\varepsilon\)3-\(\varepsilon\)3 carriers, were significantly associated with an increased risk of having SP in all age groups except the youngest and oldest (Figure 1). There was a trend of age-related increases in SP, especially of the neuritic type, across all studied genotypes. The APOE\(\varepsilon\)2 carrier group was too small to investigate supposed protective effects, although previously published results suggest tendencies towards protection [16]. In APOE\(\varepsilon\)4 adjusted analyses, 80+ year old carriers of the rare TT genotype of PICALM had a significantly lower incidence of SP compared to the common CC carriers (OR 0.18, \(P = 0.025, \text{CI} 0.04\) to 0.81) (see Figure 1). This association was not seen among younger age groups.

There were no significant associations between genotypes of CLU and CR1 and SP prevalence.

Grouping the rare homozygote and heterozygotes versus the common homozygotes for the SNPs uncovered statistically significant associations between the T allele of PICALM and SP (OR 0.62, \(P = 0.028, \text{CI 0.41}\) to 0.95, versus CC genotype). When we divided the SP into diffuse, primitive, classic and burnt out phenotypes (to investigate the particular phases of the SP life cycle), we found that the rare C allele of CLU was significantly associated with the presence of late stage SP (OR 4.4, \(P = 0.004, \text{CI 1.61}\) to 12.2) compared to the common TT genotype (Table 3). In that setting, the statistically significant association of the PICALM T allele was lost.

### Table 1 TASTY cohort characteristics

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>0 to 97</th>
<th>62.67</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>388</td>
<td>64.5%</td>
</tr>
<tr>
<td>Cause of Death</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease</td>
<td>59.1%</td>
<td></td>
</tr>
<tr>
<td>Accident</td>
<td>26.8%</td>
<td></td>
</tr>
<tr>
<td>Suicide</td>
<td>11.8%</td>
<td></td>
</tr>
<tr>
<td>Homicide</td>
<td>0.5%</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1.5%</td>
<td></td>
</tr>
<tr>
<td>Dementia Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>6</td>
<td>1%</td>
</tr>
<tr>
<td>Dementia</td>
<td>16</td>
<td>2.7%</td>
</tr>
<tr>
<td>Memory Problems</td>
<td>10</td>
<td>1.7%</td>
</tr>
<tr>
<td>Parkinson’s Disease</td>
<td>1</td>
<td>0.2%</td>
</tr>
<tr>
<td>SP</td>
<td>172</td>
<td>31.1%</td>
</tr>
<tr>
<td>NFT</td>
<td>204</td>
<td>42.1%</td>
</tr>
<tr>
<td>SP type</td>
<td></td>
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<tr>
<td>Diffuse</td>
<td>21</td>
<td>3.7%</td>
</tr>
<tr>
<td>Primitive</td>
<td>35</td>
<td>6.2%</td>
</tr>
<tr>
<td>Classic</td>
<td>83</td>
<td>14.7%</td>
</tr>
<tr>
<td>Burnt Out</td>
<td>25</td>
<td>4.4%</td>
</tr>
<tr>
<td>SP type 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-neuritic SP</td>
<td>56</td>
<td>9.9%</td>
</tr>
<tr>
<td>Neuritic SP</td>
<td>108</td>
<td>19.1%</td>
</tr>
<tr>
<td>Semi quantitative SP coverage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sparse SP</td>
<td>90</td>
<td>16.2%</td>
</tr>
<tr>
<td>Moderate SP</td>
<td>62</td>
<td>11.2%</td>
</tr>
<tr>
<td>Frequent SP</td>
<td>32</td>
<td>5.8%</td>
</tr>
<tr>
<td>APOE(\varepsilon)4 carriership</td>
<td>187</td>
<td>31.1%</td>
</tr>
</tbody>
</table>

### Table 2 Senile plaque and neurofibrillary tangle prevalence in the TASTY cohort by age group

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Total</th>
<th>Affected (%)</th>
<th>P-value</th>
<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Senile plaques</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 49</td>
<td>119</td>
<td>7</td>
<td>5.9</td>
<td>Ref</td>
<td>-</td>
</tr>
<tr>
<td>50 to 59</td>
<td>101</td>
<td>17</td>
<td>16.8</td>
<td>0.013</td>
<td>3.24</td>
</tr>
<tr>
<td>60 to 69</td>
<td>89</td>
<td>21</td>
<td>23.6</td>
<td>0.001</td>
<td>4.94</td>
</tr>
<tr>
<td>70 to 79</td>
<td>130</td>
<td>56</td>
<td>43.1</td>
<td>&lt;0.0001</td>
<td>12.11</td>
</tr>
<tr>
<td>80+</td>
<td>114</td>
<td>71</td>
<td>62.3</td>
<td>&lt;0.0001</td>
<td>26.42</td>
</tr>
<tr>
<td><strong>Neurofibrillary tangles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 49</td>
<td>103</td>
<td>13</td>
<td>12.6</td>
<td>Ref</td>
<td>-</td>
</tr>
<tr>
<td>50 to 59</td>
<td>90</td>
<td>28</td>
<td>31.1</td>
<td>0.002</td>
<td>3.13</td>
</tr>
<tr>
<td>60 to 69</td>
<td>82</td>
<td>23</td>
<td>28.0</td>
<td>0.010</td>
<td>2.70</td>
</tr>
<tr>
<td>70 to 79</td>
<td>109</td>
<td>62</td>
<td>56.9</td>
<td>&lt;0.0001</td>
<td>9.13</td>
</tr>
<tr>
<td>80+</td>
<td>100</td>
<td>78</td>
<td>78.0</td>
<td>&lt;0.0001</td>
<td>24.55</td>
</tr>
</tbody>
</table>

*Those ‘Affected’ refers to those cases in the TASTY cohort that are affected by senile plaques or neurofibrillary tangles, accordingly.*
Table 3 Association of senile plaque type with APOE, CLU, CR1 and PICALM genotypes

<table>
<thead>
<tr>
<th></th>
<th>Valid N</th>
<th>SP</th>
<th>OR (95% CI)</th>
<th>P-value</th>
<th>Diffuse</th>
<th>OR (95% CI)</th>
<th>P-value</th>
<th>Primitive</th>
<th>OR (95% CI)</th>
<th>P-value</th>
<th>Classic</th>
<th>OR (95% CI)</th>
<th>P-value</th>
<th>Burnt out</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>APOE6</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>388</td>
<td>88</td>
<td>(22.7)</td>
<td>-</td>
<td>-</td>
<td>12 (3.1)</td>
<td>-</td>
<td>19 (4.9)</td>
<td>-</td>
<td>-</td>
<td>44</td>
<td>-</td>
<td>-</td>
<td>13 (3.4)</td>
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<tr>
<td>APOE4+</td>
<td>174</td>
<td>74</td>
<td>(42.5)</td>
<td>3.2 (2.05 to 4.90)</td>
<td>&lt;0.0001*</td>
<td>9 (5.2)</td>
<td>2.4 (0.97 to 5.86)</td>
<td>0.059</td>
<td>16 (9.2)</td>
<td>2.8 (1.37 to 5.74)</td>
<td>0.005*</td>
<td>37</td>
<td>3.2 (1.83 to 5.48)</td>
<td>&lt;0.0001*</td>
<td>12 (6.9)</td>
<td>3.8 (1.56 to 9.01)</td>
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<tr>
<td><strong>CLU TT</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>336</td>
<td>89</td>
<td>(26.5)</td>
<td>-</td>
<td>-</td>
<td>13 (3.9)</td>
<td>-</td>
<td>22 (6.5)</td>
<td>-</td>
<td>-</td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>6 (1.8)</td>
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<tr>
<td><strong>CLU C+</strong></td>
<td>194</td>
<td>60</td>
<td>(30.9)</td>
<td>1.1 (0.73 to 1.76)</td>
<td>0.570</td>
<td>6 (3.1)</td>
<td>0.79 (0.29 to 2.14)</td>
<td>0.641</td>
<td>11 (5.7)</td>
<td>0.9 (0.39 to 1.84)</td>
<td>0.680</td>
<td>27</td>
<td>0.99 (0.56 to 1.77)</td>
<td>0.988</td>
<td>16 (8.2)</td>
<td>4.4 (1.61 to 12.2)</td>
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<tr>
<td><strong>CR1 AA</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>186</td>
<td>56</td>
<td>(30.1)</td>
<td>-</td>
<td>-</td>
<td>8 (4.3)</td>
<td>-</td>
<td>9 (4.8)</td>
<td>-</td>
<td>-</td>
<td>28</td>
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<tr>
<td><strong>CR1 C+</strong></td>
<td>361</td>
<td>102</td>
<td>(28.3)</td>
<td>0.9 (0.62 to 1.54)</td>
<td>0.924</td>
<td>12 (3.3)</td>
<td>0.71 (0.28 to 1.80)</td>
<td>0.475</td>
<td>24 (6.6)</td>
<td>1.2 (0.55 to 2.77)</td>
<td>0.611</td>
<td>53</td>
<td>0.85 (0.48 to 1.49)</td>
<td>0.572</td>
<td>13 (3.6)</td>
<td>0.57 (0.24 to 1.40)</td>
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<tr>
<td><strong>PICALM CC</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>219</td>
<td>71</td>
<td>(32.4)</td>
<td>-</td>
<td>-</td>
<td>7 (3.2)</td>
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<td>19 (8.7)</td>
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<tr>
<td><strong>PICALM T+</strong></td>
<td>327</td>
<td>87</td>
<td>(26.6)</td>
<td>0.6 (0.41 to 0.95)</td>
<td>0.028*</td>
<td>12 (3.7)</td>
<td>1.09 (0.42 to 2.84)</td>
<td>0.864</td>
<td>16 (4.9)</td>
<td>0.5 (0.26 to 1.09)</td>
<td>0.086</td>
<td>42</td>
<td>0.73 (0.42 to 1.25)</td>
<td>0.253</td>
<td>17 (5.2)</td>
<td>1.36 (0.55 to 3.39)</td>
</tr>
</tbody>
</table>

Abbreviations: APOE, Apolipoprotein E; CI, confidence intervals; CLU, Clusterin; CR1, complement component (3b/4b) receptor 1; N, number of cases; OR, odds ratio; PICALM, phosphatidylinositol-binding clathrin assembly protein; SP, senile plaques; No SP was the reference group. Numbers in brackets are percentages, unless otherwise stated. In some cases it was impossible to obtain all variables due to technical difficulties and sample damage.

* Denotes statistically significant values.
1 Denotes reference group used for statistical analyses. Age was used as a covariate.
2 Denotes most common homozygous genotype and used as the reference group for statistical analyses. APOE=4 carriership and age were used as covariates.
**APOE, CLU, CR1 and PICALM associations with SP frequency**

When analyses were performed with SP frequency as the dependent variable, APOE ε4 carriership was again found to be highly significantly associated with increasing SP coverage, compared to ε3-ε3 carriers (see Table 4). PICALM TC genotypes (versus CC genotype) were significantly less likely to have moderate SP compared to no SP (OR 0.42, \( P = 0.012, \) CI 0.21 to 0.83), whilst CR1 CC genotype carriers (compared to AA genotype) were more likely to have sparse SP than no SP (OR 2.1, \( P = 0.048, \) CI 1.01 to 4.43).

When we grouped the rare homozygote and heterozygotes together versus the common homozygotes (Table 4), significance was lost for CR1, however PICALM T allele carriers remained less likely to have coverage of SP versus no SP compared to CC genotype, however again statistical significance was only reached for moderate SP (OR 0.43, \( P = 0.010, \) CI 0.23 to 0.82).

**Associations with gender**

Reanalysing the significant associations with the cohort split by gender gave similar results (data not shown), with females generally more strongly associated, most likely due to their older age. No further significant associations were uncovered.

**Associations with Aβ staining**

A subpopulation of the cohort were assessed for associations with immunohistochemical staining (\( n = 152 \)). None of the newly identified SNPs were statistically significantly associated with Aβ staining, as seen in Figure 2. APOE ε4 carriership, however, was significantly associated with higher cortical coverage of Aβ staining (\( P < 0.0001 \)).

**Discussion**

AD is the most common form of dementia, but to date its aetiology has remained elusive, despite intensive research. The proposed causes of AD relate to neuropathological findings post-mortem, which is the only way to definitively confirm a patient’s diagnosis [11-14]. Diagnosis of the first AD patient, back in 1906, revealed large numbers of SP and NFT; however, although new treatments aimed at reversing the disease by reducing SP have proven successful, they have been without improvements in cognitive abilities of patients [35].
Table 4 Association of senile plaque coverage with APOE, CLU, CR1 and PICALM genotypes

<table>
<thead>
<tr>
<th></th>
<th>Valid N</th>
<th>No SP</th>
<th>Sparse SP</th>
<th>Moderate SP</th>
<th>Frequent SP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>OR (95% CI)</td>
<td>P-value</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>APOEε4-1</td>
<td>378</td>
<td>281 (74.3)</td>
<td>-</td>
<td>-</td>
<td>29 (7.7)</td>
</tr>
<tr>
<td>APOEε4+</td>
<td>174</td>
<td>89 (51.1)</td>
<td>39 (22.4)</td>
<td>2.86 (1.73 to 4.74)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>CLU TT2</td>
<td>329</td>
<td>227 (69.0)</td>
<td>48 (14.6)</td>
<td>-</td>
<td>33 (10.0)</td>
</tr>
<tr>
<td>CLU C+</td>
<td>192</td>
<td>123 (64.1)</td>
<td>37 (19.3)</td>
<td>1.26 (0.76 to 2.09)</td>
<td>0.378</td>
</tr>
<tr>
<td>CR1 AA2</td>
<td>182</td>
<td>125 (68.7)</td>
<td>22 (12.1)</td>
<td>-</td>
<td>21 (11.5)</td>
</tr>
<tr>
<td>CR1 C+</td>
<td>355</td>
<td>234 (65.9)</td>
<td>65 (18.3)</td>
<td>1.48 (0.85 to 2.58)</td>
<td>0.167</td>
</tr>
<tr>
<td>PICALM CC2</td>
<td>217</td>
<td>136 (62.7)</td>
<td>33 (15.2)</td>
<td>-</td>
<td>32 (14.7)</td>
</tr>
<tr>
<td>PICALM T+</td>
<td>319</td>
<td>222 (69.6)</td>
<td>54 (16.9)</td>
<td>0.97 (0.58 to 1.60)</td>
<td>0.898</td>
</tr>
</tbody>
</table>

Abbreviations: APOE, Apolipoprotein E; CI, confidence intervals; CLU, Clusterin; CR1, complement component (3b/4b) receptor 1; N, number of cases; OR, odds ratio; PICALM, phosphatidylinositol-binding clathrin assembly protein; SP, senile plaques. No SP was the reference group. Numbers in brackets are percentages, unless otherwise stated. In some cases it was impossible to obtain all variables due to technical difficulties and sample damage.

* Denotes statistically significant values.
1 Denotes reference group used for statistical analyses. Age was used as a covariate.
2 Denotes most common homozygous genotype and used as the reference group for statistical analyses. APOEε4 carriership and age were used as covariates.
Furthermore, studies have shown cognitively normal elderly can also have large numbers of these brain lesions [16-19] and not all AD cases have the required amounts to corroborate cognitive dysfunction [15].

Genome wide association studies (GWAS) investigating AD have in the past not been powerful enough to reveal anything except APOE. Two recent large GWAS [22,23], however, collectively investigated over 30,000 individuals (with almost 12,000 probable AD cases) and examined around 500,000 SNPs that may influence AD risk.

We recently showed that SP and NFT were surprisingly common in a non-demented autopsy series, which represents the closest model to a population sample and that the occurrence of SP, but not NFT, was strongly affected by the APOEε4 allele, regardless of age [16]. Because of the GWAS’ discoveries of three potential new candidates for AD risk, we decided to look at their associations with the neuropathological lesions SP and NFT in our cohort to investigate their involvement in the development of these brain lesions.

SP associated with both age and gender, and the APOEε4 allele was highly associated with SP in many of our analyses. Additional analyses showed that the APOEε4 associations were extremely robust in the TASTY series, thus validating our cohort’s ability to detect associations with the measured brain lesions. However, whilst NFT were found to associate with age and gender, they were not associated with any of the SNPs investigated. The strong association seen between males and SP prevalence, and females and NFT occurrence may be a confounding factor, due to the older average age of females in the cohort. Additionally, our cohort may over-represent early and violent deaths; however, all cases were included to best represent a population-based investigation.
We hypothesised that the three other SNPs (CLU, CR1 and PICALM) would also associate with SP, as they are involved in AD pathways and most likely would be associated with the development of brain lesions [24-34]. Our results indicate that we did not find as many robust correlations as for APOE.

Genetic variants of CLU, PICALM and CR1 genes were associated with SP and remained so with the inclusion of APOEε4 carriers and age as covariates. The appearance of an increased risk for CLU C carriers versus TT is unusual in that it only applies for Burnt Out SP - a group in the cohort that is relatively small and are majority females. This suggests that the effect of CLU could be on the later stages of SP development and related to removal of Aβ [31-34] being reduced in efficiency.

The PICALM T allele appears to have a protective effect on SP prevalence, true also for TT genotypes, versus CC genotypes in the oldest age group. This may be due to more efficient intracellular trafficking and clear-up of Aβ, or the components or pathways that induce Aβ build-up or production [24-27]. The protective effect of the T carriers was seen also for SP coverage; however, it was only significant for moderate SP.

CR1 CC genotype carriers were associated with an increased risk of having sparse compared to no SP; however, the trend was not consistent for increasing coverage of SP (data not shown), which was also true for the analysis grouping the rare homozygote with the heterozygotes. This suggests the effect of CR1 is complex and not as straightforward as increasing SP risk and requires further investigation.

The lack of robust and numerous associations with the GWAS SNPs and brain lesions, alongside the strong APOEε4 results, questions the involvement of SP in AD pathology. It may be a coincidence that SP are found in AD brains with evidence suggesting that they may be a part of normal aging [16,17]. In light of this, SP treatments have so far failed to improve patients’ cognitive abilities [35] and current theories are moving away from SP and suggest soluble oligomeric Aβ may be the culprit in AD [36-39]. The scarcity of associations may be due to the small number of cases with SP within the TASTY series (31.1%), resulting in lower power; however, we have a 600 case-strong cohort, which revealed strong associations between APOE with SP. It may also be due to the low strength of these prior associations in the original studies, which should be investigated in future cohorts of a similar nature.

Some may question the use of an autopsy series to investigate an age-dependent illness such as AD; however, the TASTY cohort provides a unique view into the early midlife prevalence of well-defined neuropathological lesions, showing their common prevalence.

Conclusions
We have an interesting window into the development of neuropathological lesions and their associations with AD-risk genes in the general population, and as far as we know, this is the first study of its kind. SP were found to associate with age, gender, and APOEε4, but not consistently with CLU, CR1 or PICALM, suggesting that these SNPs most likely do not affect the development of the studied neuropathological lesions. Further studies should replicate these findings in a larger autopsy series of the same kind, both with and without AD cases, to define the occurrence of these neuropathological lesions within the context of normal aging.

Our results suggest that whilst these SNPs are associated with probable AD cases (in the GWAS), they do not strongly relate to SP prevalence, or at all to NFT, in an autopsy series most representative of the general population, possibly indicating their complex involvement in the disease.

Abbreviations
AD: Alzheimer’s disease; APOE: apolipoprotein E; CI: confidence interval; CLU: clusterin; CR1: complement component (3b/4b) receptor 1; GWAS: genome wide association studies; NFT: neurofibrillary tangles; OR: odds ratio; PICALM: phosphatidylinositol binding clathrin assembly protein; SNPs: single nucleotide polymorphisms; SP: senile plaques; TASTY: Tampere autopsy study.

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Authors’ contributions
All authors contributed to this manuscript. EHK performed experiments and analyses and wrote the manuscript. HH, TL and SH measured the neuropathological lesions. SG and PJK collected the autopsy series. SG, HH and PJK provided comments and discussions on the progress of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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