ALZHEIMER'S DISEASE RELATED SENILE PLAQUE ACCUMULATION AND APOLIPOPROTEIN-E GENOTYPE IN A NON-DEMENTED POPULATION

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MIETTINEN TIIA: ALZHEIMER'S DISEASE RELATED SENILE PLAQUE ACCUMULATION AND APOLIPOPROTEIN-E GENOTYPE IN A NON-DEMENTED POPULATION

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Alzheimerin tauti (AT) on yleisin dementian muoto. Sen yksi klassisimmista histopatologisista muutoksista on beeta-amyloidista koostuvat seniilit plakit (SP), joiden kertyminen aivoihin on hermostolle haitallista, vahingoittaen ympäröivää kudosta. Erityisen haitallisena pidetään SP:ien kehittyneimmän muodon, "burnt out" -plakkien (BO), kertymistä aivoihin. AT:a on kahta eri muotoa; familiaarista ja sporadista, joista jälkimmäisen puhkeamiseen vaikuttavat ympäristötekijät ja geenit. Tärkein geeni sporadisessa taudinkuvassa on apolipoproteiini E (*APOE),* jonka alleelin ε4 on arvioitu lisäävän riskiä sairastua AT:iin nelinkertaiseksi. Genotyypin ε4/ε4 on todettu liittyvän aikaisempaan taudin puhkeamiseen ja vaikeampiin neuropatologisiin muutoksiin. *APOE* ε4 on todettu lisäävän ja aikaistavan SP:n kertymistä aivoihin.

Tässä tutkimuksessa tarkasteltiin *APOE*:n vaikutusta SP:ien kertymiseen kotona asuneilla, sairaalan ulkopuolella kuolleilla, yli 17-vuotiailla henkilöillä, joille tehtiin oikeuslääketieteellinen ruumiinavaus Tampereen yliopistollisessa sairaalassa. Vainajilta kerättiin etuaivokuorelta aivokudosnäyte, joka siirrettiin mikroskopointilasille ja värjättiin beeta-amyloidi -vasta-aineella. Tiedot APOE-genotyypistä kerättiin DNA:sta suolasaostusmenetelmällä. Tiedot henkilöiden muistiongelmista kerättiin läheisiltä tutkimuskaavakkeen avulla.

SP:ien ja BO:ien määrän havaittiin lisääntyvän iän myötä. SP:ien kertyminen alkoi iästä 29, kun taas BO:ien iästä 54. *APOE* ε4 -alleelin havaittiin lisäävän sekä SP:ien että BO:ien muodostusta, sekä *APOE* ε4/ε4 -genotyypin lisäävän riskiä entisestään. Lisäksi havaittiin, että muistiongelmilla ja SP:ien määrällä ei ollut juurikaan yhteyttä, kun taas *APOE* ε4 -alleelin ja varsinkin *APOE* ε4/ε4 -genotyypin havaittiin lisäävän muistiongelmien riskiä.

Tutkimuksen mukaan SP:ja esiintyy myös terveillä, muistisairauksia sairastamattomilla henkilöillä. *APOE* ε4 -alleelin SP:ja lisäävä vaikutus oli nähtävissä 60. ikävuoden jälkeen. Vaikuttaa siltä, että *APOE* ε4 -alleeli lisää SP:ien muodostusta sekä muistiongelmien riskiä, kun taas SP:ien havaittiin olevan huono muistiongelmien ennustaja. Tarkempia tutkimuksia koskien AT:n neuropatologiaa tarvitaan.

CONTENTS

1. INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia (1). The biggest risk factor for AD is aging (1) and the world population is now older than ever (2). The number of patients with some form of memory disorder is estimated to grow, as well as the burden on social health care and countries' economy (3). There is still no treatment for AD that would halt the disease and medication barely slows the progression (1).

AD is diagnosed in cases where no other reason for memory problems and cognitive impairment can be found, although it can only ever be a clinical diagnosis, confirmed at autopsy through neuropathological staining of the brain. AD usually follows a typical clinical course which reflects the underlying neuropathology of the disorder. The two main histopathological features observed are neurofibrillary tangles and senile plaques (SP), although brain atrophy and other characteristics are also seen. (1) Histopathological changes in the brain first occur in the neocortex (4) and then spread mainly throughout the mediotemporal limbic area, the posterior inferior temporal areas, the adjoining parieto-occipital lobes and the posterior cingulate gyrus (1).

SP consist of an amyloid core with a corona of argyrophilic axonal and dendritic processes, amyloid fibrils, glial cell processes and microglial cells. Amyloid beta is the main constituent of SP and their neurotoxicity is proposed to be based on the amyloid beta protein. The formation of SP is a process of amyloid beta oligomerization, fibril formation, aggregation and precipitation occurring in several stages of which each has a different impact on the surrounding neurons. Initial precipitation leads to diffuse amyloid plaques, followed by increasing complexity leading to a "burnt out" plaque, which are those considered the end stage pathological changes of AD. (1)

The accumulation of SP in the brain is thought to be neurotoxic; especially end stage plaques ("burnt out") (5), although the mechanism how plaque formation leads to AD is not fully understood (6). One mechanism suggested may be the oxidative stress caused by caspase production triggered by the presence of plaques (6-8). It has also been suggested that amyloid beta causes a decrease in mitochondrial enzyme activity, respiration and membrane potential, and it has been shown that mitochondrial abnormalities are a pathological sign of AD. Additionally, the relationship between amyloid beta, AD and Calcium-ion signalling may be imbalanced. (9) Amyloid beta may interact and disturb plasma membrane Ca2+ transport (10), or it may cause the sensitization of neurons to calcium signals mediated by glutamate that might increase neuronal vulnerability to excitotoxicity and increase neuronal cell death. Some theories also suggest that alterations in calcium signalling in microglia generate an inflammatory response and initiate pathological

changes in neighbouring neurons (9), but there are many other theories related to immune system deterioration, glucose metabolism, chronic inflammation, and infections from common viruses and parasites.

There are two main types of AD; familial and sporadic AD, which are generally known also as early and late onset AD. The rare usually early onset familial AD is highly heritable, with single gene mutations leading to many cases. The more common generally late onset, sporadic AD is affected by genetics, as well as environmental factors. (1) The most important gene in late onset AD is *APOE,* coding the apolipoprotein E - protein (3). The gene lies on chromosome 19 (11) and has three common alleles; ε2, ε3 and ε4 (1), which form six different genotypes (11). *APOE* ε3 is present in 50-90% of the population; *APOE* ε4 is present in 5-35% and *APOE* ε2 in 1-5% (12). The ε4 allele has been suggested to increase the risk of AD by about 4 times (3) and is present in about 50% of patients who have the sporadic disease (12). The *APOE* ε4/ε4 genotype is associated with an earlier onset of AD, earlier death and more severe neuropathological changes, and is more detrimental than heterozygosity for ε4 (13). The *APOE* ε4 allele increases the severity of atrophy, particularly in the inner temporal lobe (3). There is evidence that AD patients who are *APOE* ε4 carriers have higher levels of amyloid beta plaque deposition than patients without the *APOE* ε4 allele (14). The *APOE* ε2/3 and possibly the ε2/2 genotypes have also been shown to provide a protective effect against AD (15).

The APOE glycoprotein consists of 299 amino acids and is involved in lipid transportation and neuronal repair. In humans it is synthesized and secreted primarily by liver, brain and skin cells. Three alleles encode three different proteins: APOE2, APOE3 and APOE4, which produce six phenotypes E2/E2, E2/E3, E3/E3, E4/E2, E4/E3 and E4/E4. APOE3 and APOE2 are effective at the repair process and protecting neurons from excessive damage, whereas APOE4 is less effective. APOE3 also protects cells against oxidative damage better that APOE4. The APOE3 and E2 have been associated with good neuronal repair, while APOE4 with an inhibition of neuronal outgrowth. (11) The mechanism of how APOE isoforms affect the risk of AD is not totally understood, but there is evidence that APOE isoforms modulate amyloid beta metabolism and accumulation differently (12). The APOE protein is associated with the extracellular amyloid plaques in the brains of AD patients and APOE4 has been shown to result in the formation of insoluble, high-molecular-weight complexes with the amyloid peptide, leading to the formation of dense amyloid monofibrils *in vitro*. APOE3 is less reactive and does not result in such extensive complexes. (11)

The AD related accumulation of SP already starts before clinical symptoms appear and some individuals develop SP already at a surprisingly young age (16). The accumulation starts in early middle age, especially among *APOE* allele ε4 carriers (17). It has been proposed that only some individuals live long enough to develop AD (16).

The prevalence and age and sex dependency of senile plaques and their association with apolipoprotein E genotypes will be investigated in this study, within a post-mortem cohort of community-dwelling 'normal' individuals – that most closely resembling the general population. Kok et al. (2009) performed a similar study with a population of 603 men and women. A larger cohort is currently being acquired (n_{final}=1000) in order to replicate these findings, of which this project is a partial analysis of the first 200 cases. On completion of the entire collection a complete analysis of both SP and neurofibrillary tangles of the entire cohort will be performed.

2. MATERIAL AND METHODS

The cohort was a post-mortem study of 200 individuals who underwent autopsy at the University of Tampere and University Hospital of Tampere from 2010 to 2011, a subset of the Tampere Sudden Death Study (TSDS) cohort (n_{final}=1000, collected from 2009 - ongoing). The inclusion criteria were age 17 or older of an individual that had died suddenly or unexpectedly out of hospital within Tampere and the surrounding regions. In this way, the cohort represents a normal sampling of the population (as close as can be expected considering they are deceased individuals), and no cases had been living in institutes. Data on age, gender and APOE genotyping were collected. A survey was voluntarily filled by relatives including information about the deceased's health conditions, memory problems and other lifestyle factors. Six brain regions (frontal cortex, insula-putamen, hippocampus, substantia nigra, pons and cerebellar cortex) were excised by trained pathologists from medical forensic autopsies, of which samples from frontal cortex were utilised in this study. Samples were placed in TissueTek boxes, and stored in formaldehyde for at least 24 hours, after which they were transferred to PBS for four days, followed by ethanol until they were paraffinated in batches and stored at room temperature until sectioning. DNA isolation was performed on frozen blood samples with the salt precipitation method. The samples were genotyped using Illumina HumanCoreExome chip (Illumina Inc., San Diego, CA, USA) at the Helmholtz Zentrum in Munich, Germany.

Brain sections from frontal cortex were stored overnight in 4˚C, after which 5 µm thick sections were cut with a microtome and placed on glass slides and subjected to staining using a Labvision LV-1 autostainer and Monoclonal Mouse Antihuman Beta-Amyloid Clone 6F/3D antibody (DakoCytomation) at a dilution of 1:100. The endogenous enzyme blocker used was GPR Rectapur H_2O_2 3%, protein blocker was colostrum, post-staining blocker was Immunologic's Post antibody blocking for Brightvision plus, the labelled polymer was Immunologic's Poly-HRP-GAM/R/R IgG, the substrate used was ImmPact™ DAB Peroxidase Substrate Kit SK-4105, and the diluent for the antibody was Normal Antibody Diluent – Immunologic. Positive and negative controls were included and slides digitalised with an Operio Scan Scope XT, followed by analysis with computer programs ImageJ 1.45s and JVSview 1.2, where SP were counted manually.

Ethical permission for this study was granted by the Tampere University Hospital and the national ethics committee Valvira.

3. STATISTICS

Statistical analyses were performed using the IBM statistical program SPSS version 22. Five covariates were utilised: SP counts, *APOE* genotype, age, gender and memory problems. The amount of the plaques compared to the area of the slide was calculated for both diffuse and burnt out plaques. Because of the small number of cases in most of the *APOE* genotype groups (due to this being a subset of a larger cohort), *APOE* genotype was divided into ε4 carriers versus not, and also *APOE* ε4/ε4 carriers versus not. Some analyses were also performed while dividing the cohort into three age groups with similar numbers of individuals: under 60, 60-72 and 73+. The Mann-Whitney U-test was used to investigate associations between the amount of plaques and *APOE* ε4 carriership, and also for *APOE* ε4/ε4 carriership. Spearman rank correlation was used to determine the correlation between SP and age. The Mann-Whitney U-test was used to study association with memory problems and the amount of SP, and Chi-Square test to analyse the connection between *APOE* genotyping and memory problems. Chi-Square tests were used when comparing the data in age groups. $P \leq 0.05$ was considered statistically significant. The results for Mann-Whitney tests are shown as medians and lower and upper quartiles (25% and 75%).

4. RESULTS

The population consisted of 200 individuals where 145 were males and 55 females. SP counts were available for 183 cases and *APOE* genotype for 183. Cases overlapping with this data were 169. Ages ranged from 19 to 94, and averaged 63.41 years. Of those with SP data, 76 had SP and 39 had burnt out plaques. The amounts of SP varied from 0.00 plaques/mm^2 to 10.89 plaques/mm^2 (median 0.28 plaques/mm^2) and the amounts of burnt out plaques from 0.00 plaques/mm^2 to 0.94 plaques/mm^2 (median 0.10 plaques/mm^2) (Figure 1). Individuals with an *APOE* ε4 allele numbered 63 and there were 9 with the *APOE* ε4/ε4 genotype. According to relatives' reports on individuals with memory problems there were 27, with 64 reporting none, and 26 reporting unknown. (Table 1) The most common *APOE* genotype was ε3/ε3 (n=111), followed by ε3/ε4 (n=51), ε4/ε4 (n=9) and ε2/ε3 (n=9), ε2/ε4 (n=3), with no ε2/ε2 carriers in this population subset. The population was in Hardy-Weinberg equilibrium. All cases were used in analyses.

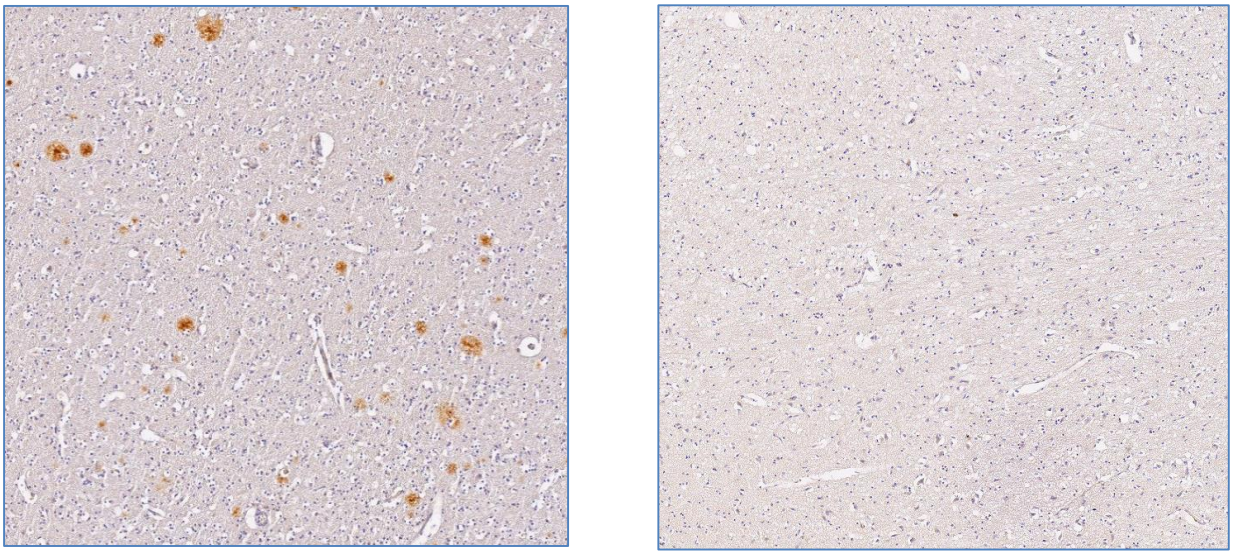

Figure 1: *Two different slides with one (left) with a high amount of plaques (SP: 6.75 plaques/mm^2, Burnt Out: 0.12 plaques/mm^2) and another with none (0.00 plaques/mm^2, 0.00 BO plaques/mm^2).*

Table 1: *Population characteristics of different age groups.*

Age group	Under 60 (N=65)		60-72 (N=69)		73+ (N=66)		Total (N=200)	
male	53	81,5 %	53	76,8 %	39	59,1 %	145	72,5 %
SP	14	25,0 %	28	43,8 %	34	54,0 %	76	41,5 %
burnt out plaques	2	3,6 %	10	15,6 %	27	42,9 %	39	21,3 %
memory problems	7	20,6 %	10	26,3 %	10	22,2 %	27	23,1 %
APOE ε4	23	39,0 %	22	34,9 %	18	29,5 %	63	34,4 %
APOE ε4/ε4	2	3,4 %	5	7,9 %	2	3,3 %	9	4,9 %

The youngest individual with SP was a 29 year old with the numbers of plaques and affected individuals rising with age ($p<0.001$, $r_s=0.385$). A similar pattern was seen with burnt out plaques (youngest was 54 years) and age ($p<0.001$, $r_s=0.453$). When dividing the population into equal-sized age groups, the connection between age and SP was only noticeable in the oldest (73 years +) age group with median M=0.061 plaques/mm^2 and quartiles [0.000, 1.996], (p=0.007). Women and men didn't differ statistically significantly with regards to SP coverage. There was no significant difference in APOE genotype among age or gender. Females in this cohort tended to be a bit older, but the difference wasn't statistically significant.

SP was associated with *APOE* genotype (see Figure 2), with *APOE* ε4 carriership associated with a higher amount of plaques (*APOE* ε4 carriers M=0.055 [0.000, 1.3194] versus non-carriers M=0.000 [0.000, 0.056]; p=0.001), with the association even stronger among ε4/ε4 carriers (M=1.360 [1.054, 3.998] vs. M=0.000

[0.000, 0.111]; p<0.001). Dividing the population into equal-sized age groups revealed the youngest had no associations between SP and *APOE* genotype. The association with SP was seen in the 60-72 year old age group with both ε4 (p=0.002) and ε4/ε4 (p<0.001). In the oldest age group the association between SP and *APOE* ε4 (p=0.001) was seen, but not with *APOE* ε4/ε4 (p=0.254).

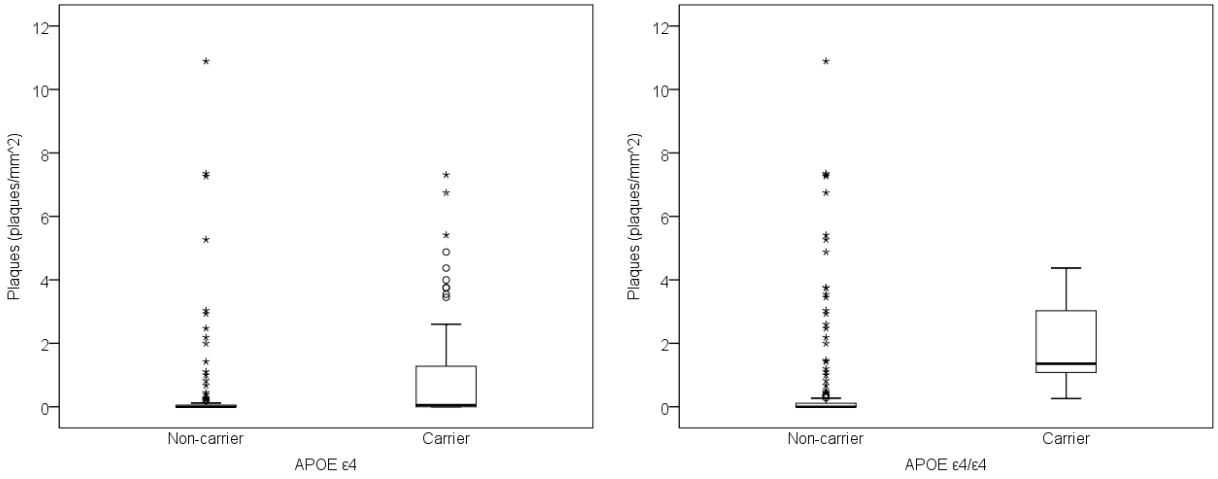

Figure 2: *APOE ε4 carriers had more plaques per measured area than non-carriers (p=0.001), and APOE ε4/ε4 carriers were even more strongly affected (p<0.001).*

A similar pattern was seen with burnt out plaques: there was an association between *APOE* ε4 allele carriership and the amount of burnt out plaques (M=0.000 [0.000, 0.065] vs. M=0.000 [0.000, 0.000]; p=0.007), and also between *APOE* ε4/ε4 genotype and burnt out plaques (M=0.792 [0.287, 0.832] vs. M=0.000 [0.000, 0.000]; p<0.001). When dividing the cohort by age, the amount of *APOE* ε4 related burnt out plaques was not statistically significant in the youngest group (under 60 years), but was in the middle aged group (60-72 years) with both *APOE* ε4 (p=0.023) and *APOE* ε4/ε4 (p=0.010). In the oldest age group (73 years +) there was a connection between burnt out plaques and *APOE* ε4 (p=0.007), but not with *APOE* ε4/ε4 (p=0.392).

There was no association between SP and memory problems, or with burnt out plaques and memory problems when comparing plaque amounts in the entire population. When comparing SP (yes/no) and memory problems in the different age groups, SP presence still failed to show any association. In the oldest age group however, there was a trend towards having both memory problems and burnt out plaques (yes/no), where 80.0% of those who had memory problems also had burnt out plaques (p=0.017).

45.8% (N=11) of those who had had memory problems were *APOE* ε4 carriers, slightly less than the percentage (54.2%, N=13) having memory problems and being a non-carrier (p=0.121), but the difference wasn't statistically significant. Among ε4/ε4 carriers the results were reversed and all ε4/ε4 carriers had

memory problems reported with none without (100.0% vs. 0.0%; p=0.007, Table 2). Those who had had memory problems in the oldest 73+ age group 7 (77.8%) were *APOE* ε4 carriers versus 2 (22.2%) who were not (p=0.004) and with the *APOE* ε4/ε4 genotype all carriers in the oldest age group had memory problems (100.0% vs. 0.0%, N=2; p=0.027). In other age groups the percentages between *APOE* genotype and memory problems didn't differ statistically significantly. No association was seen between memory problems and age.

***Table 2**: APOE ε4/ε4 and memory problems, p=0.007.*

	Memory problems		Total
	No	**Yes**	
Non ε4/ε4	57	21	78
	73,1 %	26,9 %	100,0 %
ε4/ε4	0	3	3
	0,0 %	100,0 %	100,0 %
Total	57	24	81
	70,4 %	29,6 %	100,0 %

5. DISCUSSION

SP presence was seen already at age 29. Our results indicate that also "healthy, non-demented" populations have SP, confirming the results of the previous study by Kok et al. (2009) (17). The length of a prodromal or pre-dementia AD phase cannot be reliably established with today's clinical research tools, but evidence suggests that this stage may extend over several decades (1). The results of this study suggest that the accumulation of SP starts already in the thirties while the accumulation of burnt out plaques follows after about 20 years. General understanding of SP indicates they are one of the main hallmarks and involved in the pathology of AD (18), however the exact nature of their involvement remains to be proven with new therapies directed at their removal unsuccessful at alleviating symptoms of AD. This study and its predecessor are advantageous in that they explore a wide range of ages and can thus investigate the prevalence of brain lesions over the whole age spectrum.

Age was correlated with the amount of SP and burnt out plaques. However, the effect of age was only seen in the oldest age group (73+). *APOE* genotype affected the accumulation of SP in our cohort, with *APOE* ε4/ε4 conferring more risk than ε4 alone, confirming results seen in previous studies (13). The results were similar with *APOE* genotype and burnt out plaques. The association between *APOE* ε4 and SP was seen in individuals aged 60 and over, as was the association between *APOE* ε4 and burnt out plaques. The effect of *APOE* ε4/ε4 was also seen in the age group 60-72 regarding both SP and burnt out plaques, but interestingly an association between the *APOE* ε4/ε4 genotype and SP, and *APOE* ε4/ε4 and burnt out plaques was not seen in the oldest age group (73+). One possible reason for this could be that the individuals over 72 years old

carrying the *APOE* ε4/ε4 were demented or for another reason institutionalized, or died in hospital and thus excluded from this cohort. It may also be that individuals with the *APOE* ε4/ε4 genotype die at a younger age, reducing the numbers of this genotype in older age groups. It is known that the *APOE* ε4 genotype is also a risk factor for cardiovascular disease (11). The causes of death were not investigated in this study, but perhaps further analysis could reveal interesting results.

There was no statistical difference between women and men having SP or burnt out plaques in this study. It is known that females are affected more often by AD than men (1), but the mechanisms are not well understood. A higher life-expectancy of women and also the higher dementia risk at very old age could explain this (19). In this cohort females tended to be older, however the difference wasn't statistically significant. *APOE* genotype didn't differ significantly among genders and possible differences between men and women were not seen in this study.

In this material SP weren't a good predictor of memory problems, and this is in line with previous studies (20). The only connection between SP and memory problems was in the age group over 73 when comparing individuals with and without burnt out plaques, suggesting plaques must be end-stage in order to cause cognitive impairment. Interestingly, *APOE* genotype appeared to have more to do with memory problems, as the connection between *APOE* ε4/ε4 and memory problems was seen in the whole material and there was also a statistically significant association in the oldest age group (73+) with *APOE* ε4 allele and memory problems. In our cohort *APOE* genotype appeared to be a better predictor of memory problems than the amount of SP. It has been suggested that *APOE* ε4 is also a risk factor for vascular dementia, so the connection between *APOE* ε4 and memory problems may also be related to that (21). It has been suggested *APOE* allele ε2 carriership has a protective effect against AD (11), but unfortunately our cohort was too small to investigate this.

One disadvantage considering the memory problems is that the information came from the relatives and not from an accurate diagnosis. It can't be known how intimate the relationship between the relative and the deceased was and how precise the fact is about memory problems. On the other hand, information regarding memory problems might not have been mentioned otherwise. In this study cohort only people living outside institutions were included and so it is assumed that none had a major dementing disorder. Some of the reported memory problems may be related to alcohol abuse or vascular changes, which are not known to be related to the accumulation of SP. However the exclusion criteria didn't include having dementia so there may also be some individuals with mild AD in this cohort. One limitation to this study is the size of the cohort and the small number of APOE ε4/ε4 carriers (N=9), although this will hopefully be rectified in the analysis of the full cohort.

As the development of SP is very individual and related to age it may also be that the development of SP is just a part of the normal aging process while *APOE* ε4 speeding up the appearance of SP. It has also been suggested that everybody would get SP and possibly even AD if they lived long enough (16). Not all *APOE* ε4 carriers develop SP, at least in their lifetime, and at the same time some individuals have SP without the *APOE* ε4 allele. It is an interesting factor that SP are one of the main hallmarks of AD and the *APOE* ε4 allele seems to speed up the accumulation of SP, as was seen in this study, but when combining these with memory problems it seems SP isn't a good predictor of cognitive impairment, whilst the *APOE* ε4 allele seems to predict memory problems better. A deeper investigation into the neuropathology associated with AD and the pathways leading to their development are needed to fully understand their nature and develop effective therapies towards their reduction or removal.

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