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The effect of the *APOE* genotype on Alzheimer's pathology in pathological ageing and Alzheimer's disease post-mortem brain samples.

Degree Project in Medicine

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Abstract

Background: The pathological hallmarks of Alzheimer's disease (AD) are amyloid-β (Aβ) plaques and neurofibrillary tau tangles. The *APOE* ε4-allele is the most important genetic risk factor behind AD and has been linked to the metabolism of AD neuropathology. For unknown reasons, individuals with pathological ageing (PA) remain cognitively intact despite substantial amounts of AD pathology.

Aims: To investigate the effect of the *APOE* genotype on AD-related neuropathology in brains with pathological ageing and brains with Alzheimer's disease.

Methods: A cross-sectional neuropathological study investigating frontal cortex brain tissue from 73 cognitively healthy individuals (57 of whom classified as PA cases) and 120 AD cases. Frontal cortex slides were stained for Aβ and tau pathology, using immunohistochemistry, and analysed digitally with a macro in the software ImageJ. The study was conducted at Queen Square Brain Bank, London, and had ethical approval.

Results: The *APOE* ε4-allele was significantly more frequent among AD cases than controls (p-value <0.001), while the *APOE* ε2-allele was more frequent among controls (p-value 0.013). AD cases had significantly higher loads of Aβ and tau pathology than controls (p-values <0.001). The *APOE* ε4-allele was associated with higher Aβ and tau pathology loads in general, irrespective of cognitive function (p-values 0.001 & <0.001). The *APOE* genotype did not appear to affect AD pathology loads when PA cases were analysed separately from AD cases.

Conclusion: Our results indicate that *APOE* genotype affects both AD risk and AD pathology in the population at large. We were not able to confidently determine whether the effect of the *APOE* genotype on AD-related pathology loads differs in pathological ageing compared to its effect in AD cases. This remains an important question to answer in light of its implications for our understanding of AD pathogenesis.

Key words: Alzheimer's disease, pathological ageing, *APOE*, amyloid-β, tau pathology

1. Introduction

Dementia and Alzheimer's disease

Dementia is currently a big threat to public health worldwide and apart from being devastating to those affected it is also highly strenuous on national economies in that these patients lose many professionally active years to the disease whilst requiring extensive and costly care. According to a systematic review on dementia, carried out by the Swedish Council on Health Technology Assessment (SBU), almost fifty percent of dementia patients end up in assisted living facilities a mere 2-3 years after being diagnosed (1). The total societal cost of dementia care in Sweden in 2012 was estimated to approximately 60 billion SEK according to a report from The National Board of Health and Welfare in Sweden (2). The same report states that about 17% of the total costs comes from the informal care carried out by relatives or close acquaintances of the afflicted. Naturally, not everything can be measured in money which is why it is also important to consider the psychological burden of dementia for both the patients themselves and their surroundings. Several studies have previously shown an increased prevalence of depression among people with dementia (3, 4), as well as among older people living in nursing homes (5). Over 50% of caregivers for dementia patients reported a negative impact on their health as a result of their caregiving role (6). According to the most recent World Alzheimer Report, published in September 2019, an estimated 50 million people suffer from dementia worldwide (6). The general assumption is that the observed increase in life expectancy in most western countries during the 20th century is a reflection of a still ongoing trend that will contribute to a large shift in the World population age demographics (7, 8). Since age is generally acknowledged as the most

important risk factor for developing dementia, it is not surprising that the prevalence of dementia is expected to increase further during the coming decades. The aforementioned World Alzheimer Report from 2019 estimates that the number of people with dementia will rise to about 150 million by the year 2050 (6). All these facts combined led the World Health Organization (WHO) to proclaim dementia as a global public health priority in both 2012 and 2015 (9), and to develop a global action plan for tackling this health threat (10).

The most common cause of dementia is Alzheimer's disease (AD), which accounts for approximately 50-75% of all cases (11, 12), although dementia with mixed pathology is known to occur – typically combining AD pathology with vascular brain injury (13-15). AD is divided into two main categories; late-onset AD, the main topic of this paper, and early-onset AD, including Familial AD (FAD). Dementia in general is a group of diseases that all contribute to a progressive loss of cognitive function, typically beginning with light memory loss and ending with severe impairment of most cerebral functions, leaving the afflicted unable to function in daily life. Likewise, the initial symptom of AD is most often a progressive loss of episodic memory (12). Later stages of the disease are generally characterised by a cognitive decline in most cerebral functions, leading to an inability to independently manage the tasks of daily life. The affected cerebral functions include impaired linguistic abilities, mobility and behavioural changes (12). However, there are a few other known clinical presentations of AD, where the initial symptoms are either visuospatial difficulties (Posterior Cortical Atrophy, PCA), linguistic impairments (Logopenic Aphasia, LPA), or early personality changes (Frontal AD) (12). These different variants of AD eventually become more similar in their clinical presentations as they progress to their final stages. The average life expectancy after symptom onset is approximately 8,5 years (16).

Alzheimer's pathology

The most recent consensus guidelines on the neuropathologic evaluation of AD were agreed upon in 2011 by researchers from both the United States and Europe (17). These guidelines were an updated and revised version of the former consensus criteria from 1997 (18), and were published in an article describing their factual basis. Since these articles have successfully contributed to a standardization of the neuropathological evaluation of AD, they constitute the main factual basis of the following description of AD pathology.

The main pathological hallmarks of AD are senile plaques and neurofibrillary tangles (NFTs). These two separate entities were first discovered by Alois Alzheimer and were both described in his original article from 1907 (19). Senile plaques are made of 40 to 42 amino acid-long β-amyloid (Aβ) peptides and are found extracellularly in the cerebral cortex (20). According to the predominant "amyloid cascade hypothesis" (21, 22), which has been widely acknowledged for lack of a better pathogenic model, the deposition of Aβ is the central upstream event in the pathogenesis of AD and causes the accumulation of tau as well as cognitive deterioration. However, due to conflicting evidence it is also clear that this hypothesis, if indeed true, is a gross simplification of the truth as there are cognitively intact individuals with Aβ deposits (23), as well as people with substantial tau pathology in the relative absence of Aβ (24). This last phenomenon has been named "primary age-related tauopathy" (PART) (24). A potential clue as to how this might be possible comes from the fact that there are different subtypes of Aβ deposits, some of which seem more neurotoxic than others (17). Neuritic plaques are a subtype of senile plaques, which has been generally acknowledged as more neurotoxic since it is associated with more synapse loss and glial

activation (17). Neuritic plaques are Aβ deposits surrounded by dystrophic neurites which often are immunoreactive for phospho-tau (17). Other types of senile plaques are diffuse plaques, cotton wool plaques, subpial bands and amyloid lakes (17). NFTs, on the other hand, are intraneuronal fibril structures made of abnormal tau (17). Unlike Aβ plaques, both NFTs and neuritic plaques better correlate with the progression of cognitive deterioration (17).

The neuropathological disease progression in AD generally follows predetermined patterns throughout the brain, which to some extent correlate with the clinical degeneration (17). These patterns of progression have been separately characterized for Aβ, NFTs and neuritic plaques and are now part of the diagnostics and staging of AD. The Braak and Braak model (25) is used to stage the accumulation of NFTs, which usually first appear in the entorhinal cortex or its proximity (stages I/II), to then primarily spread to the hippocampus and amygdala (stages III/IV) until the NFTs reach their final stages where they are widespread throughout the neocortex (stages V/VI) (17). The Thal model is used to stage the accumulation of Aβ deposits, which first arise in the neocortex to then spread “inwards and downwards” until they reach their final stage in the cerebellum (26). The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuritic plaque scoring system measures the density of neuritic plaques in various neocortical brain regions (27). These three different scores are then merged into “ABC scores” which are finally translated into four different categories of AD neuropathologic change; [1] Not, [2] Low, [3] Intermediate and [4] High (20). An “Intermediate” or “High” level of AD neuropathologic change, along with antemortem cognitive impairment, is required for the clinical AD diagnosis (20).

Apart from the abovementioned essential pathological features of AD, there are other commonly occurring pathological changes. One such is cerebral amyloid angiopathy

(CAA) which is common alongside AD pathology (20). In addition, neuron loss, synapse loss, gliosis and atrophy commonly occur in brains with AD (17). Since the full stream of events in the pathogenesis of AD is not yet fully understood, it cannot be ruled out that any of the aforementioned pathological changes also have critical parts to play in the development of AD.

In addition to the documentation of AD-associated neuropathology, the new consensus guidelines also encourage the pathological investigation and documentation of other concurrent neurological diseases (17). The three most frequent comorbidities of AD are vascular brain injury (VBI), Lewy body disease (LBD), and hippocampal sclerosis (HS) (17). As all of them can exist independently of AD, it is important to chart their presence in order to grasp the full picture behind each case of cognitive decline (17).

Pathological ageing

While the main neuropathological features of Alzheimer's disease (AD), senile plaques and neurofibrillary tangles, have been well-characterised and known for over 100 years, there are still uncertainties as to how the pathological changes relate to and contribute to the development of symptoms. One such uncertainty arises from the fact that certain individuals who present with the neuropathological hallmarks of AD, still manage to stay free of symptoms during their entire lifetime (25, 28-35). A relative lack of consensus on how to interpret these findings has led to a similar dissent on what to call this phenomenon (23). Thus, there are several different terms used to describe the same thing, examples of which are: pathological ageing, preclinical/insipient/presymtomatic AD, nondemented high pathology

controls and intermediate probability mismatches, among others (23). In this text, the term pathological ageing will be used henceforth.

The main characteristics of pathological ageing seem to be a significant degree of amyloid-β (Aβ) deposits accompanied by limited to no NFT pathology in cognitively normal individuals (23). Findings from several independent studies suggest that the correlation between cognitive function and Aβ load is weaker than the correlation between cognitive function and tau pathology (35-37). Indeed, in a meta-analysis (which encompassed more than 60 studies and 7000 subjects) of the correlation between Aβ load and cognition in pathological ageing-cases, only a small association between the two was found (38). This explains why the Aβ deposits often are widespread in pathological ageing (23), while neurofibrillary tangles most often are limited to the entorhinal cortex (35). Also of note is that, out of the two most common types of senile plaques, diffuse plaques are more frequent in pathological ageing and are known to be less associated with cognitive impairment than dense-core neuritic plaques (36, 39). Another important feature of pathological ageing is the relative lack of neuronal loss and synaptic or dendritic anomalies, compared to AD (29, 40). This converges with the findings that nondemented patients with positive Aβ PET imaging might lack structural changes on magnetic resonance imaging (MRI) despite substantial Aβ pathology loads (35).

As previously mentioned, there are several different views on pathological ageing. One hypothesis is that the Aβ strain present in pathological ageing is less neurotoxic than the one in AD cases (41). An example of such an Aβ strain with different pathogenic qualities is the Aβ variant that is produced from amyloid precursor protein (APP) containing a protective mutation (A673T) (42) which leads to a decreased tendency to aggregate (43).

Other studies have shown differences in the concentrations of various types of Aβ peptides between pathological ageing and AD (44, 45). Another view on pathological ageing is that they lack a mandatory pathological feature that combined with Aβ would lead to their developing AD, for example neurofibrillary tau pathology or cerebrovascular disease (23).

Others have proposed that there might be some kind of inherent resilience in pathological ageing cases, which protects them from contracting the fully developed disease – a very intriguing prospect for those in search of a remedy to AD (31). The main suggestion of such a protective factor at this moment is a genotype that provides these subjects with a larger cognitive reserve (41). Last but not least is the hypothesis that the pathological ageing cases merely represent the pre-symptomatic AD cases who, had they not died of other causes, would eventually have developed symptomatic AD (30). Since neuropathological studies necessarily are cross-sectional, it is impossible to know what would have happened to the pathological ageing cases if they had survived a few more years. In recent years, however, it has become possible to investigate neuropathology non-invasively with the development of positron emission tomography (PET) aimed at detecting Aβ and tau. A recent longitudinal study investigated the Aβ and tau pathology loads in clinically normal people over 7 years (46), and found a significant association between decrease in PACC (Preclinical Alzheimer Cognitive Composite) cognition score and increase in tau pathology, but not in Aβ pathology load. Out of the 60 participants, who ranged from low-high Aβ signalling at baseline, only 6 individuals had developed mild cognitive impairment (MCI) at the end of the study (46).

Nevertheless, regardless of whether pathological ageing represents an early stage of asymptomatic AD or exists as a separate entity entirely, no one can dispute the fact that certain people can tolerate heavier Aβ loads than others without developing symptoms. It

is of importance to elucidate the reason behind this apparent resilience as this might lead to clues about the pathogenesis of AD as well as possible future treatment targets.

APOE genotype

The *APOE* ε4 allele is the most important genetic risk factor for developing late-onset AD (47, 48) – in fact, the conferred risk is so apparent that this correlation was established as early as in the 1990s (49), when genotyping was still an elaborate and excessively time-consuming process. The *APOE* gene encodes a glycoprotein called apolipoprotein E (apoE). Since its discovery, apoE and its role in the pathogenesis of AD have been extensively studied. In spite of this, the exact mechanisms behind its contribution to the development of AD remain unclear (50). However, numerous possible mechanisms have been proposed and it seems likely that the *APOE* ε4 allele entails both gain of pathological function as well as loss of neuroprotective qualities (51, 52). In contrast, the *APOE* ε2 allele has been shown to lower the risk of contracting AD (53), as well as decrease the severity of the disease in *APOE* ε2-positive patients with AD (54). There are three different isoforms of the *APOE* gene; ε2, ε3 and ε4 (50). Since each person inherits one allele from each parent, there are six possible allele combinations; ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4. In the general population worldwide, the ε3 allele is the most common, with a frequency of 77.9%, while the frequencies for ε2 and ε4 are 8.4% and 13.7%, respectively (53). Among patients with AD, however, the ε4 allele is more than twice as common as in the general population and the ε2 allele is about twice as rare (53).

The apoE glycoprotein mainly functions as a transporter of cholesterol and other lipids to cells in different parts of the body (55), but has also been implicated as an important

part of several other biochemical processes in the human body (56). It is produced by hepatocytes and macrophages and is then released into the blood circulation from whence it reaches most other parts of the body (52). The apoE protein is also present in the central nervous system (CNS), but because of the blood-brain barrier, which impedes the passage of apoE to the brain from the blood stream (57), most brain apoE has to be produced locally by astrocytes (52). In the brain, it seems the most important function of apoE is to transport cholesterol from astrocytes to neurons (51). Cholesterol constitutes a main building block of membranes and myelin sheaths (58), and is thus essential for synapse maintenance and neuronal function (51). Several studies have provided evidence that suggests that the apoE E4 isoform is less efficient at supplying neurons with cholesterol than its counterparts (59-61).

The apoE protein is made up of 299 amino acids and consists of two main sections; the N-terminal domain and the C-terminal domain, as well as a smaller section that conjoins the other two (50). The N-terminal domain contains the main receptor-binding portion of the protein, whereas the C-terminal domain contains the lipid-binding portion (62, 63). A simplified schematic overview of the structure of apoE is shown in Figure 1.1 below.

The three isoforms of apoE differ from each other on position 112 and 158, where you find two cysteine amino acids in the E2 isoform, two arginine amino acids in the ε4-isoform and one of each in the E3 isoform (cysteine at 112 and arginine at 158) (64). These amino acid variations occur on either side of the receptor-binding portion of the N-terminal domain (63). Thus, it is not surprising to find that the different apoE isoforms show different affinity to the various apoE receptors, most of which are part of the low-density lipoprotein receptor (LDLR) family (50). For instance, the apoE E2 isoform has a lower affinity to the LRP1-receptor than the other two isoforms and is often described as a loss of

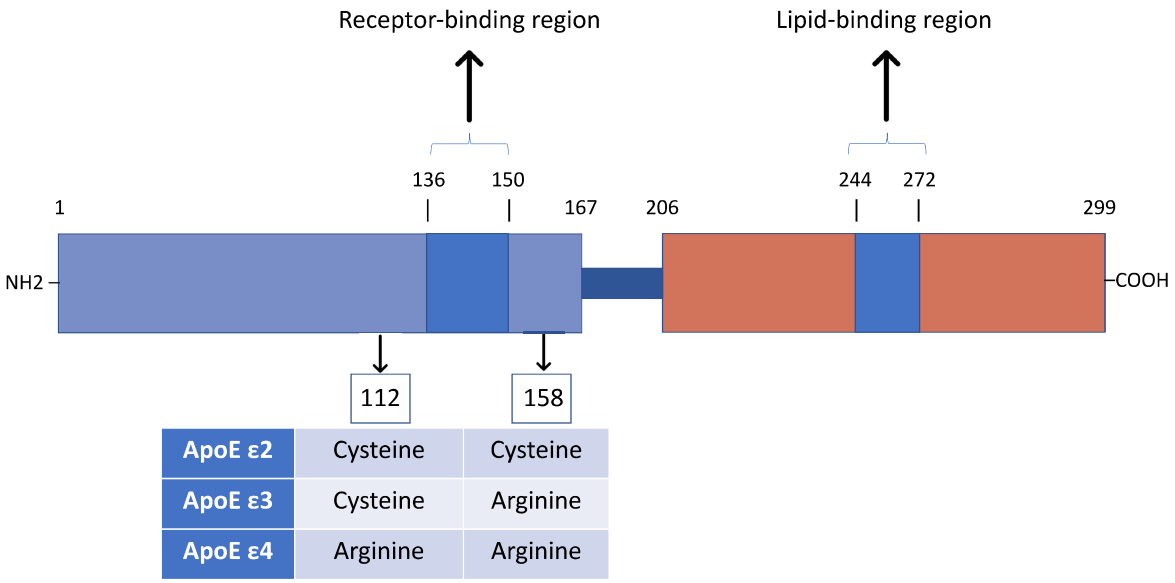

Figure 1.1. A simplified overview of the structure of apolipoprotein E (apoE). The N-terminal contains the principal receptor-binding region and the C-terminal contains the main lipid-binding region. The three isoforms of apoE differ at residues 112 and 158. Figure adapted from *Liu et al, 2013* (52).

function variant (65). The LRP1 receptor has been implicated in AD pathogenesis since it has been shown to accelerate APP endocytic trafficking and, thus, increase Aβ production (66, 67). In addition, it has been shown that the E4 isoform of apoE combined with LRP1 stimulates APP internalization, and consequently Aβ production, more than E3 (68). The fact that apoE E4 binds most effectively to LRP1 also promotes Aβ pathology in an Aβ clearance pathway that depends on the binding between LRP1 and Aβ in order to function: the E4 isoform gets in the way of that binding (69). Moreover, various other apoE receptors are also implicated in Aβ production and the different apoE isoforms have been shown to differentially influence protease-mediated Aβ clearance (50). There is also evidence to support that apoE and its three isoforms are involved in the Aβ metabolism and thus affect what kinds of Aβ oligomers are formed (70, 71).

However, the aforementioned effects of apoE on both Aβ accumulation and cholesterol-dependent neuronal function are not the only ways in which apoE has been linked to AD pathogenesis. In an article from 2001, apoE end products from the protein's catabolism were shown to induce neurofibrillary tangle-like inclusions (72), thus linking apoE to the second most important pathological hallmark of AD. In addition, several apoE receptors have been shown to interact with other important synaptic receptors, such as the NMDA (N-methyl-D-aspartate) and glutamate receptors (50). Another perplexing aspect of the *APOE* genotype is that it has been indicated to affect therapeutic responses in several independent drug trials aimed at finding treatments against AD (73-75).

All things considered, although the exact mechanisms behind its contribution to AD remain unclear, there is no denying the importance of the seemingly central role of the *APOE* genotype in the development of late-onset AD.

Other known risk factors for Alzheimer's disease

Late-onset Alzheimer's disease (AD) is, by all accounts, a multifactorial disease where environmental and genetic factors combine in predisposing an individual to contracting the disease. Because of the large prevalence of the disease as well as the hitherto lack of any effective disease-modifying treatments, the importance of identifying risk factors for AD has been highlighted as a means of eventually being able to define effective prevention strategies. Thus, a plethora of large multicentre studies have been conducted over the years and have managed to identify a multitude of possible risk factors for AD.

It is often said that what is good for the heart is likewise beneficial for the brain, and this seems to hold true for AD, as many of its risk factors are cardiovascular (11).

Hypertension, hypercholesterolemia and obesity have all been identified as risk factors for AD, particularly when present in midlife (76). Diabetes (77), smoking and lack of physical exercise have also been recognized as risk factors for AD (78, 79).

On a global scale, lack of secondary education emerges as one of the most important risk factors, as it is thought to be a key factor behind a significant amount of dementia cases (80). On a similar note, cognitive inactivity has also been linked to an increased risk for AD (78), while bilingualism has been shown to be protective against dementia (81, 82). The general interpretation of these combined facts is that they provide proof of the concept of cognitive reserve as a defence against dementia (83).

Other potentially modifiable risk factors for AD or dementia include depression (78) and nutritional deficiencies (11). On the contrary, social engagements (79) and a Mediterranean diet (84, 85) have been associated with a reduced risk for AD but with small and inconsistent effect sizes.

However, modifiable risk factors for AD only account for about a third of all AD cases worldwide (86). The remaining two thirds are believed to be mostly due to unmodifiable risk factors such as age and genetic predispositions. Age is the single most important risk factor for developing AD (11) and the heritability for AD has been found to be high (87). As previously mentioned, the *APOE* ε4 allele confers the greatest genetic risk of contracting sporadic late-onset AD, but thanks to the recent surge in Genome Wide Association Studies (GWAS), a multitude of other susceptibility genes have been identified in the last decade (11). Several of these susceptibility genes confer a very small increase in AD risk but are so frequent in the general population that they can still be expected to contribute to a substantial amount of cases: for example *PICALM* (48), *CLU* (48, 88) and *CR1* (88).

Other susceptibility genes for AD have a relatively high increase of risk ratio but are less common in the population (11), examples of which are *TREM2* (89) and *PLD3* (90). The aforementioned genetic susceptibilities for late-onset AD are all examples of recessive disease genes which generally demand additional pathogenic factors in order to lead to the actual disease – unlike the gene mutations in *APP* (91), *PSEN1* (92) and *PSEN2* (93) which are known to cause the dominantly inherited familial AD. Familial AD (FAD), however, is a separate entity from the late-onset AD discussed above, and accounts for less than 5% of the total amount of AD cases (11).

Aim

The main aim of this project is to elucidate the role of the *APOE* genotype in the accumulation of AD-related pathology in pathological ageing cases presenting with AD neuropathology, while lacking clinical symptoms of AD.

Primary specific research question: Does *APOE* genotype have an effect on the amount and type of AD-like pathology in pathological ageing cases?

Secondary specific research questions: Is there a difference in pathology type and load between cognitively normal individuals compared with cases with fully developed AD? What is the effect of the *APOE* genotype on AD-like pathology, irrespective of clinical diagnosis?

2. Material and Methods

Case demographics

All cases included in this study come from the collection of donated brain tissue kept by Queen Square Brain Bank for neurological disorders (QSBB) in London. The cases were recruited from a list of all cases where immunohistochemistry had been performed on brain tissue slides to achieve a diagnosis, possibly including slides with frontal cortex brain tissue stained for Aβ and tau pathology. The list contained 251 cases; 131 AD-cases, 16 FAD cases and 104 control cases. Control cases in this context refers to all individuals which did not meet the clinical and pathological criteria to be classified as AD cases. Thus, the control group contains both pathological ageing cases as well as cognitively normal individuals without AD neuropathology. Pathological ageing in this study was defined as the control cases with a Thal score > 0. The earliest cases on the list had arrived at QSBB in 1993 and the latest were donated in 2018.

A power analysis was performed ahead of the case recruitment process, using an excel file where we simply had to fill in our desired values into an already prepared equation. To discover a difference in pathology between the *APOE* allelic subgroups that is at least as big as the standard deviation for each pathological variable, we would have needed a sample size of 16 cases in each group to reach a power of 80%. However, since we cannot control the genetic makeup of those who decide to donate their brains for research, there was no way to compensate if the required group sizes were not met. Thus, the goal was to get at least 16 cases per subgroup, but the realistic expectation was that we simply had to investigate what was available. The inclusion criteria for this study are described in Table 2.1 below.

Table 2.1. Inclusion criteria for this study.

Inclusion criteria	Alzheimer's disease/AD	Pathological ageing
Dementia symptoms in life & pathologically verified AD (not Familial AD):	Yes	No
No dementia symptoms in life:	No	Yes
Known *APOE* genotype:	Yes	Yes
Have given their consent for this kind of study:	Yes	Yes
Available slides of frontal cortex stained for Aβ and/or AT8:	Yes	Yes

After reviewing the case list with these inclusion criteria in mind, 120 AD-cases and 73 control cases remained (in total 193). The sixteen FAD cases were excluded due to the fact that the disease-modifying effect of the *APOE* genotype pales in comparison to the influence of the FAD-related gene mutations. A total of twelve cases were excluded because they lacked available DNA needed for *APOE* genotyping. Out of the 73 control cases, 57 qualified as pathological aging cases since they had Thal scores above 0. However, not all of the 193 cases had frontal cortex slides stained for both Aβ and tau pathology, and there was not enough time to make new slides for all of them. The flowchart in Figure 2.1 gives an overview over the case recruitment process.

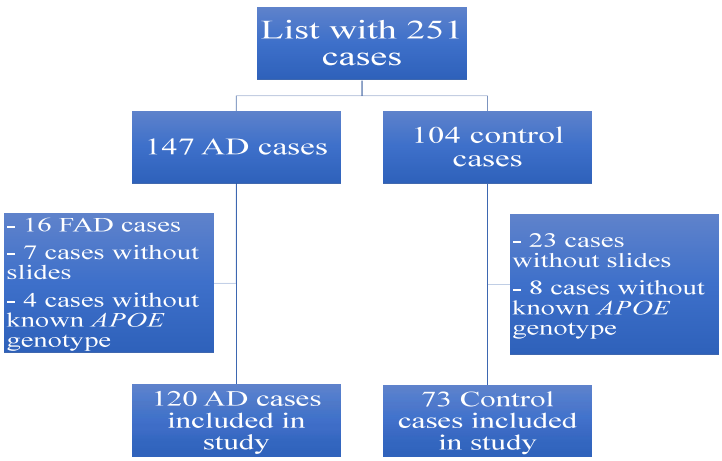

Figure 2.1. Flowchart of the case recruitment. (AD = Alzheimer's disease, FAD = Familial AD.)

Owing to the fact that this study relies on voluntary brain donation, it was not possible to recruit cases in a way that would allow our subgroups to be matched in regard to age, gender and *APOE* allele distributions. Table 2.2 below gives an overview over the case demographics in each subgroup.

Table 2.2. An overview of number of cases, percentage of women, mean age at death and mean post-mortem delay in the various subgroups. Here, cases are divided according to diagnosis as well as AD risk, as determined by their *APOE* genotype.

	CONTROLS				ALZHEIMER'S DISEASE			
	22&23	**24&33**	**34&44**	**Total**	**22&23**	**24&33**	**34&44**	**Total**
Total no of cases:	12	46	15	**73**	5	41	74	**120**
Mean age at death (years):	81.6	84.4	82.3	**83.5**	76.8	71	71.3	**71.5**
Mean post-mortem delay (h):	54 h	60 h	61 h	**59 h**	65 h	64 h	62 h	**63 h**
Percentage of women:	67%	60%	53%	**60%**	20%	34%	47%	**42%**

22&23, 24&33 and 34&44 refer to the *APOE* genotypes ε2/ε2 & ε2/ε3, ε2/ε4 & ε3/ε3, and ε3/ε4 & ε4/ε4.

Tissue processing

The donated brain tissue typically arrives fresh at Queen Square Brain Bank where it is hemi-dissected. The aim is to retrieve the brains as soon as possible post-mortem, but naturally, there is some variability in the delay. After arrival, the right hemisphere is flash frozen at -80ºC and stored for future use. Meanwhile, the left hemisphere is put into a 10% buffered formalin solution in order to fix the tissue and thus preserve its morphology. Tissue fixation is attained through formalin's ability to stabilize the tissue by cross-linkages between proteins, to inhibit proteolytic enzymes and to act as a disinfectant which prevents microbial growth (94). After three weeks in the formalin solution, the brain tissue is examined by a

neuropathologist who dissects it into five mm coronal slices before separating predetermined areas of interest into labelled blocks (95).

These smaller blocks are then processed according to a 6-day protocol, shown in Table 2.6 below, with the aim of embedding the tissue in paraffin wax. At first, the tissue blocks are immersed in ethanol solutions of increasing concentrations in order to dehydrate the tissue. Chloroform is then used to clear the tissue of alcohol to leave room for paraffin to take its place. When the tissue blocks have been successfully embedded in paraffin, a microtome is used to cut eight µm sections which can be put on slides and stained for various proteins of interest using immunohistochemistry (95).

Table 2.6. The 6-day protocol for tissue processing. Table modified from (95).

Step	Agent	Time spent
1	70% ethanol	12 hours
2	90% ethanol	12 hours
3	90% ethanol	12 hours
4	≥ 99% ethanol	12 hours
5	≥ 99% ethanol	12 hours
6	≥ 99% ethanol	12 hours
7	≥ 99% ethanol	12 hours
8	Chloroform	12 hours
9	Chloroform	12 hours
10	Paraffin wax	12 hours
11	Paraffin wax	12 hours
12	Paraffin wax	12 hours

Immunohistochemistry

The slides used in this study were cut from formalin-fixed paraffin-embedded (FFPE) brain tissue from the frontal cortex of all cases. Most slides included in this study had already been prepared before the conception of this project since there would not have been enough time to

cut and stain them all in addition to getting them scanned and analysed in ImageJ. Thus, some of the slides used had been cut and stained in the early 1990s whereas the most recently made slides were prepared in 2019. However, the slides were all prepared according to the same immunohistochemistry protocol, regardless of when they were made. This general immunohistochemistry procedure is described below.

An eight μm thick section from frontal cortex tissue is put on a slide and allowed to dry in a 37 ºC warm oven overnight. It is then put back into the oven at 60 ºC for at least 24 hours in order to properly adhere the tissue section to the slide. All slides are labelled to indicate which case and what part of the brain they are from, as well as containing information about their staining. Next, the paraffin is removed from the slide using xylene. To rehydrate the tissue, the slide is sequentially immersed in ethanol solutions of decreasing concentrations, starting with absolute alcohol and ending with a 70% ethanol solution at which point it is considered sufficiently rehydrated. The slide is then put into a methanol and hydrogen peroxidase solution to avoid any effects of endogenous peroxidase on the staining. Slides meant for Aβ staining are pre-treated with 98% formic acid at room temperature for ten minutes before being washed in running tap water for another 10 minutes. All slides, regardless of staining type, undergo heat mediated epitope retrieval in citrate buffer in a pressure cooker for 10 minutes. Both the formic acid incubation and the use of a pressure cooker are necessary steps in the antigen retrieval process, in which the antigens targeted by the staining are once more made available for antibody binding. To block unspecific antibody binding, the slides are immersed in 10% milk/TBS solution for half an hour at room temperature. After the antigen retrieval process is complete and unspecific antibody binding has been blocked, the primary antibodies are applied on the slides for one hour. Primary

antibodies used were Aβ (stains beta-amyloid, catalogue number: Dako, M0872) and AT8 (stains hyper-phosphorylated tau, catalogue number: Thermo, MN1020). Both primary antibodies are monoclonal mouse antibodies. Slides are then gently washed with TBS-Tween solution to remove excess primary antibodies and are incubated with biotinylated secondary antibodies for thirty minutes. The secondary antibodies used for Aβ and AT8 staining are rabbit-derived antibodies which bind to the C-region of the primary mouse antibodies, thus indirectly binding to the antigen of interest in the staining process. Slides are washed once more in TBS-Tween solution and then dried before an Avidin-Biotin Complex solution is applied onto each section for another thirty minutes. This complex binds to the biotinylated secondary antibodies with great affinity. A solution of 3,3'-di-aminobenzidine-TBS-H_2O_2 is then used as a chromogen since it creates a brown insoluble precipitate when activated. Slides are then washed in warm tap water for 10 minutes before being counterstained with Mayer's haematoxylin to visualise cell nuclei. Slides are dehydrated by being immersed in alcohol baths of increasing concentration before being put in Xylene. Finally, the slides are mounted using DPX mounting medium and then left to dry. (96)

Image analysis

Quantitative analysis of the amount of Aβ and Tau pathology in the frontal cortex of all cases was conducted digitally with the help of the software ImageJ (https://imagej.nih.gov/ij/). In order to do this, the slides containing frontal cortex brain tissue all had to be scanned using an Olympus VS120 slide scanner at 4x magnification. Olympus VS120 software was used to extract the regions of interest from the original files, thus enabling the images to be stored onto the computer as TIF files.

The extracted TIF-images were uploaded into ImageJ where the grey matter was outlined manually using the program's Polygon tool, as shown in Figure 2.2 below. Once the grey matter ribbon had been selected as the region of interest, a macro was installed into ImageJ that could calculate the percentage of the grey matter area that stained positive for pathology. As shown in Figure 2.2, the neuropathology identified by the macro turned red upon analysis, and a window appeared with the results presented as a %area stained. As a general rule, all grey matter visualised on the scanned images was included in the analysis except grey matter areas that had been blurred in the scanning or that contained large artefacts which would have affected the results. Artefacts, most often dirt on the slides, and blurred areas were easily identified since they both differed greatly from the general histological colour and structure of brain tissue.

The ImageJ software is a widely used and well-established tool in this field of research, and its validity and reproducibility are therefore generally accepted. The macro used in this project was previously published (97).

The pathology types of interest in this study were Aβ plaques and neurofibrillary tangles. However, the Aβ and AT8 immunohistochemistry targets the proteins in general, regardless of the formation they are in. To ensure that the macro primarily included Aβ plaques and neurofibrillary tangles respectively, different threshold settings were tried and evaluated in the macro. A good balance between the macro's sensitivity and specificity for the respective neuropathology types was reached with macro thresholds set at 0-150 saturation for Aβ plaque analysis, and at 0-120 saturation for the analysis of neurofibrillary tangles. However, a small portion of the slides stained for tau were found to have a significantly less intense staining which led to their pathology being misrepresented in ImageJ. Thus, these tau

slides with a less intense staining were analysed using the macro with thresholds set at 0-150 saturation. Out of the 179 slides stained for tau pathology, a total of 8 slides (7 AD cases and 1 control case) were found to have a less intense staining upon scrutiny.

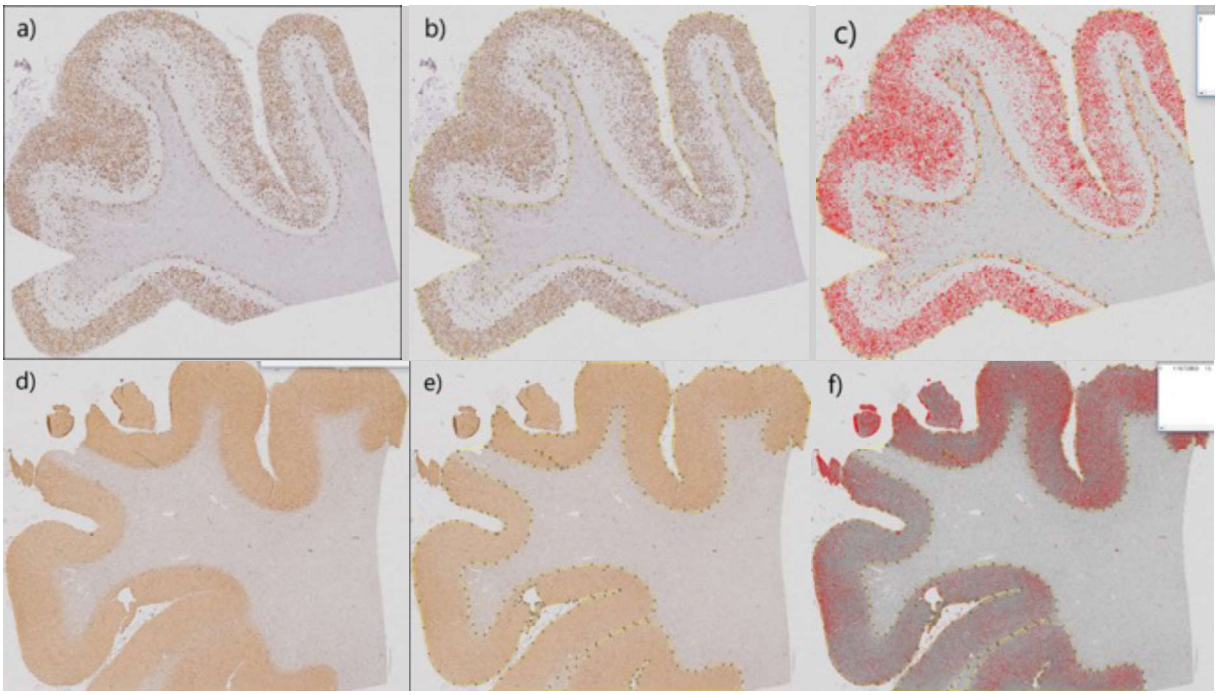

Figure 2.2. Showing the ImageJ analyses of slides stained for Aβ (a-c) and tau (d-f) pathology respectively. At first, the grey matter was outlined using the Polygon tool in the software ImageJ (images b and e), and then a macro was used to analyse the pathological loads as a % area of the grey matter (images c and f).

Statistical methods

All data retrieved from the ImageJ analyses was put into IBM SPSS Statistics software for analysis. Fisher's exact test was used to compare sex distribution, *APOE* ε2-positivity and *APOE* ε4-positivity between AD cases and controls. Mann Whitney U test was used to compare age distribution and differences in the average post-mortem delay between our subgroups. The non-parametric Kruskal-Wallis H test was utilized to compare pathology loads between the various subgroups. Finally, both post-mortem delay and age were investigated as potential confounders using linear regression.

Ethics

The test material used in this project consisted of donated post-mortem brain tissue collected by the Queen Square Brain Bank (QSBB) in London. The QSBB conducts research on various types of neurodegenerative disorders. Informed consent is obtained from the subjects themselves or a lawful representative prior to donation. The work conducted at the QSBB has been approved by ethical committees, and research projects conducted in the UK on brain tissue from their bank are automatically covered by their 'QSBB ethics approval'(98). Ethical approval for this study was obtained from the Local Research Ethics Committee of the National Hospital for Neurology and Neurosurgery. There were no conflicts of interest in this study.

Since this study investigated the brain tissue of already deceased individuals, there was no risk that this project would violate the human right to the highest attainable physical and mental health, as declared by the United Nations, UN (99). The right to autonomy was protected through a strict adherence to the subjects' wishes regarding study participation, recorded before donation. The greatest ethical risk in this project consisted in safeguarding the participants' right to privacy and confidentiality, but this potential problem was easily taken care of by making all cases anonymous from the start. Thus, since all data was stored, handled and published using de-identification, the ethical principle of confidentiality, as stated in the WMA Declaration of Helsinki (100), was also adhered to. In addition, we naturally ascertained that test material was handled with respect and in accordance with the relevant legislature.

3. Results

Table 3.1. Group demographics table comparing distribution of gender, *APOE* ε2 and ε4 positivity between Alzheimer's disease (AD) cases and control cases, as well as comparing mean age at death and post-mortem delay between the groups.

Variables	AD (p = 120)	Controls (p = 73)	p-value
Gender, females (%)	42%	60%	0.017*
Age at death (years)	71.5 ± 8.1	83.5 ± 10.1	<0.001°
Post-mortem delay (h)	63 h	59 h	0.366°
APOE ε4 positivity (%)	63%	22%	<0.001*
APOE ε2 positivity (%)	6%	18%	0.013*

*Fisher's Exact test, 2-sided
°Mann Whitney U test, Exact Sig. 2-tailed

As demonstrated in Table 3.1, there were significant differences in age at death and gender distribution between the AD cases and the control cases. The AD group was significantly younger at death and had a significantly smaller proportion of women than the control group. There was no significant difference in post-mortem delay. The AD group also had a significantly higher proportion of *APOE* ε4-positive individuals than the control group, while the opposite was true for *APOE* ε2-positivity which was significantly more common among the controls. Post-mortem delay and age at death were investigated as potential confounders, using linear regression, but were both found not to influence our results.

Table 3.2. Comparison of pathology load between Alzheimer's disease (AD) cases and control cases.

Variable	AD cases	Control cases	p-value
Aβ load*	7.4 ± 4.1 (p=117)	2.1 ± 3.9 (p=61)	<0.001°
Tau load*	5.6 ± 5.7 (p=111)	0.086 ± 0.222 (p=68)	<0.001°

*pathology load expressed as a mean % area of total grey matter area ± standard deviation
°calculated using Kruskal Wallis H test for independent samples

The AD cases were found to have significantly higher Aβ and tau pathology loads than control cases (p-values <0.001, Table 3.2).

Table 3.3. Comparison of pathology load between *APOE* ε4-positive and *APOE* ε4-negative cases, regardless of whether they have Alzheimer's disease (AD) or not.

Variable	*APOE* ε4 positive	*APOE* ε4 negative	p-value
Aβ load*	6.5 ± 4.3 (p=86)	4.8 ± 5.1 (p=92)	0.001°
Tau load*	4.8 ± 5.0 (p=83)	2.5 ± 5.3 (p=96)	<0.001°

*pathology load expressed as a mean % area of total grey matter area ± standard deviation
°calculated using Kruskal Wallis H test for independent samples

To investigate the impact of *APOE* ε4 positivity in general, regardless of diagnosis, the AD cases and control cases were merged before being divided into an *APOE* ε4-positive and an *APOE* ε4-negative group for comparison (Table 3.3). The *APOE* ε4-positive cases had significantly higher pathology loads of both Aβ and tau than the *APOE* ε4-negative cases.

Table 3.4. Comparison of pathology load between three risk groups determined by their *APOE* genotype, where the first group (ε2/ε2 & ε2/ε3) equals lowered Alzheimer's disease (AD) risk, the second group (ε2/ε4 & ε3/ε3) is neutral and the third group (ε3/ε4 & ε4/ε4) has a higher AD risk.

Variable	ε2/ε2 & ε2/ε3	ε2/ε4 & ε3/ε3	ε3/ε4 & ε4/ε4	p-value
Aβ load*	3.6 ± 5.7 (p=15)	5.0 ± 4.9 (p=80)	6.6 ± 4.3 (p=83)	0.001°
Tau load*	0.49 ± 0.79 (p=16)	2.9 ± 5.6 (p=83)	4.8 ± 5.0 (p=80)	<0.001°

*pathology load expressed as a mean % area of total grey matter area ± standard deviation
°calculated using Kruskal Wallis H test for independent samples

To further investigate the impact of *APOE* genotype variation on pathology loads in general, all cases were divided into three different risk groups determined by *APOE* allele set (Table 3.4). The first group, containing allele sets ε2/ε2 & ε2/ε3, represents a lower risk for AD, the second group (containing ε2/ε4 & ε3/ε3) represents risk neutrality, while the last group (containing allele sets ε3/ε4 & ε4/ε4) represents an increased risk for AD. Using Kruskal-Wallis H tests, significant differences were found between the groups for both Aβ and tau.

Post hoc analysis of the Kruskal-Wallis H tests further revealed that risk group three (allele sets ε3/ε4 & ε4/ε4) had a significantly higher Aβ load than risk group one (allele sets ε2/ε2 & ε2/ε3)(p-value 0.003, not adjusted for multiple testing), and two (ε2/ε4 & ε3/ε3)(p-value 0.005, not adjusted for multiple testing) respectively. There was no significant difference between group one and two (p-value 0.165). The post-hoc analysis for tau pathology revealed similar results, *i.e.*, that risk group three had significantly more tau pathology than group one (p-value <0.001 not adjusted for multiple testing), and two (p-value <0.001, not adjusted for multiple testing), respectively. The difference between tau pathology between group one and two was not significant here either (p-value 0.082, not adjusted for multiple testing).

Table 3.5. Comparison of pathology loads between *APOE* ε4-positive and *APOE* ε4-negative cases; pathological ageing cases analysed separately from AD cases.

ALZHEIMER'S DISEASE CASES				PATHOLOGICAL AGING CASES			
	***APOE* ε4 positive**	***APOE* ε4 negative**	**p-value**		***APOE* ε4 positive**	***APOE* ε4 negative**	**p-value**
Aβ load*	7.4 ± 4.0 (p=73)	7.5 ± 4.4 (p=44)	0.960°	Aβ load*	2.1 ± 2.2 (p=10)	2.9 ± 4.7 (p=37)	0.443°
Tau load*	5.7 ± 4.9 (p=69)	5.5 ± 6.9 (p=42)	0.241°	Tau load*	0.097 ± 0.1 (p=12)	0.095 ± 0.3 (p=42)	0.169°

*pathology load expressed as a mean % area of total grey matter area ± standard deviation
°calculated using Kruskal Wallis H test for independent samples

When the AD cases and pathological ageing cases were divided and analysed separately, no significant differences in pathology loads were found between *APOE* ε4-positive cases and *APOE* ε4-negative cases among either the pathological aging cases or AD cases, as shown in Table 3.5 above.

4. Discussion

Results

General results

In this study, we evaluated the Aβ and tau pathology loads in the frontal cortex region of 120 cases with a clinical diagnosis of AD and 73 cases without cognitive impairment. We then compared pathology loads between different subgroups determined both by their diagnoses (AD or controls) and by their *APOE* genotypes. There was a significant difference in *APOE* ε4 allele frequencies among the groups – it was a lot more common among AD cases than among control cases (p-value <0.001). The opposite was true for the *APOE* ε2-allele, which was more common among controls (p-value 0.013). We found significant differences in both Aβ and tau pathology load between AD cases and controls (p-values; <0.001 and <0.001). At one point, all cases were mixed and then divided into an *APOE* ε4-positive and an *APOE* ε4-negative group. Comparison of pathology load between *APOE* ε4-positive and *APOE* ε4-negative cases in general, yielded significant results for both Aβ and tau (p-values 0.001 and <0.001 respectively). Significant differences in Aβ and tau pathology loads were also found when all cases, regardless of diagnosis, were divided into three AD risk groups determined by their *APOE* genotype (p-value 0.001 for Aβ, p-value <0.001 for tau). In this instance, *APOE* genotypes ε2/ε2 & ε2/ε3 were interpreted as a lowered AD-risk and were put in group one, whereas genotypes ε2/ε4 & ε3/ε3 were considered risk neutral and the third group, containing genotypes ε3/ε4 & ε4/ε4, entailed a higher risk. Post hoc analyses revealed that the high-risk group had significantly higher pathology loads than the low-risk group and the risk-neutral group, respectively. However, when AD cases and pathological ageing cases were analysed

separately, no statistically significant differences were shown in comparisons of pathology loads between *APOE* ε4-positive and *APOE* ε4-negative cases.

APOE allele frequency

The fact that the *APOE* ε4-allele was significantly (p-value <0.001) more common among AD cases (63%) than among control cases (22%) was not surprising as it has long been a known risk factor for AD (49). The higher frequency of the *APOE* ε4 allele among AD cases has been shown in several previous studies (53, 101, 102). Likewise, it was not surprising that the *APOE* ε2 allele, which is known to be protective against AD (53), was more common among the control cases in this study (p-value 0.013).

Pathology loads in AD compared to controls

We found that AD cases had significantly higher Aβ and tau pathology loads than controls. Several studies have indicated that the correlation between Aβ pathology load and cognitive decline is dubious (29, 46) or, at best, quite small (38). Particularly the amount of diffuse amyloid deposits tends to show little impact on a patient's cognitive state (103). Thus, it happens that a cognitively normal individual is found to have a rather substantial Aβ pathology load (23, 31), sometimes exceeding the load of certain AD cases – a phenomenon referred to as *pathological ageing*. On the other hand, although cognitively healthy individuals of a certain age commonly have some AD-pathology (30), evidence has shown that the extent of this pathology load most often does not exceed that of the average AD patient (26). As for tau pathology loads, NFT distribution has been found to better correlate with cognition (46). Also, tau pathology tends to not be very widespread in cognitively normal subjects (29), although cognitively normal individuals with substantial amounts of NFTs, while lacking Aβ deposits, have been described in the context of the syndrome PART

(24). Furthermore, since an “intermediate” or “high” degree of AD-related neuropathologic change, according to the ABC scoring system, is required for an AD diagnosis (17), the individuals in the AD group can be expected to have a high average pathology load. Thus, the fact that there was a highly significant difference in pathology loads between AD cases and controls is logical and also in line with previous findings (26, 102, 104).

***APOE* ε4-positivity and AD-related pathology**

When looking at the entire collection of cases as a whole, regardless of diagnoses, we found a significant association between the *APOE* ε4-allele and higher pathology loads of Aβ (p-value 0.001) and tau (p-value <0.001). The *APOE* ε4-allele has long been known to be associated with an increased risk for AD and it is also known for being more frequent among AD cases compared with the population at large (49). However, whether the *APOE* ε4 allele affects the AD-associated neuropathological load, which was investigated in this instance, is a separate question from how it affects the risk of AD. The *APOE* ε4 allele has been implicated in both Aβ production (68), Aβ clearance (69) and the accumulation of NFTs (72) – thus it is not farfetched to assume that its genetic presence results in a higher pathological load of Aβ and tau. Previous studies on this subject, using PET neuroimaging, have shown that occurrence of the *APOE* ε4 allele results in a higher pathological load of Aβ in cognitively normal individuals (105) and a higher density of neuritic plaques in AD cases (106). A similar study showed that the *APOE* ε4 allele in cognitively normal individuals led to an earlier debut of amyloid imaging positivity (107). A rather extensive neuropathological study, with over 1000 cases, found an association between the *APOE* ε4 allele and increased tau pathology in brains with Aβ (108). Another neuropathological study, with over 400 cases, found an association between the *APOE* ε4 allele and a heavier Aβ load in clinically uncharacterised subjects

(109). These results seem to concur quite well with our findings that the *APOE* ε4 allele increases the AD neuropathology in general, in addition to increasing the risk of AD. Another aspect to take into account when interpreting these results, is the potentially interfering effect of the *APOE* ε2 allele which not only decreases the risk of contracting AD (53), but also has been shown to result in milder cases of AD as well as milder AD-related neuropathology (54, 108). Since the *APOE* ε4-negative group in our analysis contained individuals with *APOE* ε2 alleles, it can be said that our analysis measured both the negative impact of *APOE* ε2 on pathology as well as the positive impact of *APOE* ε4. However, there is conflicting evidence on the *APOE* ε2 allele's effect on AD pathology. While certain studies show that the *APOE* ε2 allele lowers the AD pathology load (54, 108), another study showed a significant increase in AD neuropathology among *APOE* ε2 carriers in the oldest old population (110). However, since we were not able to find other studies that validated the increase in AD neuropathology among old *APOE* ε2 carriers, we must conclude that this is still open to debate. Another relevant point to consider is that we included the *APOE* ε2/ε4 genotype in the *APOE* ε4-positive group. The AD risk profile of the *APOE* ε2/ε4 genotype is somewhat uncertain. It is often perceived as AD risk neutral but has also previously been associated, at least among Caucasians, with an increased AD risk (53). This ought, however, not have been a problem since we had so few individuals with the *APOE* ε2/ε4 genotype in our analysis (only three cases). The most important potential issue with this analysis, however, is the fact that it could be perceived simply as an extension of the comparison of pathology loads between AD cases and controls. This is because of the fact that the *APOE* ε4 allele is so much more common among AD cases than among controls, which results in a larger proportion of AD cases in the

APOE ε4-positive group (>80% AD cases) and a larger proportion of control cases in the *APOE* ε4-negative group (>50% controls). Thereby, we also have an influence on pathology loads from the diagnosis and not only from the *APOE* genotype, which makes it difficult to determine their separate effects on the results. With this in mind, it might be suggested that the difference in proportion of AD cases between the ε4-positive and ε4-negative groups accounted for the entire difference in pathology loads. Subsequently, it could be speculated that the *APOE* genotype did not affect the pathology loads at all in this instance. Because of the knowledge gained from previously mentioned studies (105-109), however, it is relatively safe to assume that both *APOE* genotype and diagnosis affected the results of this analysis. The slight difference in significance between the differences in tau pathology compared with Aβ pathology might be a result of there being a larger variance in the means of tau pathology between AD cases and controls, compared to Aβ.

***APOE* genotype-conferred AD risk and AD-related pathology**

We also made a similar analysis of the association between *APOE* genotype and AD-related pathology load in general, regardless of diagnosis, but with three *APOE*-determined risk groups instead of two. This analysis yielded significant results for the association between *APOE* genotype and both Aβ pathology (p-value 0.001) and tau pathology (p-value <0.001). Thus, this analysis was undeniably rather similar to the aforementioned analysis, but with the important difference that this one allowed us to investigate the effects of the *APOE* ε2 allele and the *APOE* ε4 allele independently, with very little interference between the two. Here we were able to demonstrate that occurrence of the *APOE* ε4 allele was associated with a higher pathology load compared to risk neutral individuals (with *APOE* allele sets of ε2/ε4 or ε3/ε3), for both Aβ (p-value 0.005) and tau (p-value <0.001) pathology. As previously mentioned,

one study found that the *APOE* ε2/ε4 genotype increased AD risk among Caucasians (53). These results contradict our decision to put that genotype into the risk neutral group. However, seeing as there were so few of those cases in this study (<4% in the risk neutral groups, <2% in total), it ought not to have greatly influenced our results – even if the genotype indeed does increase the AD risk. Furthermore, the fact that a particular genotype confers AD risk does not necessarily entail that it also affects the AD pathology load. Unsurprisingly, there was an even more significant difference in pathology loads between those with a protective *APOE* genotype compared to those with an *APOE* genotype with conferred AD risk, both when comparing Aβ (p-value 0.003) and tau (p-value <0.001) pathology. However, we were not able to show a protective effect of the *APOE* ε2 allele against pathology compared with the risk neutral genotypes. There are two possible explanations for this. As previously mentioned, studies have shown that while the *APOE* ε2 allele protects against AD, it has been shown to increase AD-related pathology in the oldest old (110) and can therefore not be considered as protective against AD pathology. However, by that logic we might instead have expected to see a significantly higher pathology load among the *APOE* ε2-positive individuals compared to the risk neutral group. Furthermore, other studies have shown a protective effect of the *APOE* ε2 allele against AD pathology (111, 112). Thus, these insignificant results are probably due to the fact that we did not have a sufficient amount of cases who classified as having protective *APOE* genotypes.

The effect of *APOE* genotype on pathology loads in AD vs pathological ageing (PA)

When we analysed the impact of the *APOE* genotype on pathology load in AD cases and pathological ageing (PA) cases separately, we did not get any significant results.

There are two possible explanations for this. Firstly, it cannot be ruled out that no significant differences were found because *APOE* genotype does not in fact affect the pathology load in AD cases and/or PA cases. This reasoning would go hand in hand with the aforementioned possibility that the significant differences in pathology loads between *APOE* genotypes, irrespective of diagnosis, were just a reflection of the fact that there was a significant difference in pathology load between controls and AD cases. An extensive search for similar results as ours was rather fruitless, possibly due to the tendency not to publish negative results. However, one study reported that the *APOE* genotype did not affect Aβ nor tau pathology in AD cases, and that the *APOE* ε4 allele was associated with an increased senile plaque density in non-demented individuals but not an increase in NFT density (102).

The other possible explanation is that the *APOE* genotype actually does affect AD-related pathology loads in both PA cases and AD cases, but that our analysis lacked the power to prove it. The separation of AD cases from PA might have left us with too few cases in each subgroup to be able to detect differences in pathology. Another contributing factor to the difficulty of getting significant results in this way was probably that the individual samples became a lot more homogenous after the separation and, thus, that the differences in means between each group became narrower – which in turn increases the number of cases needed to gain enough power to be able to detect differences. The skewed distribution of different *APOE* alleles among both PA and AD cases also made the various group sizes uneven and resulted in small *APOE* ε4-positive groups among PA cases and small *APOE* ε2-positive groups among AD cases. This latter alternative is probably true given the fact that several previous independent studies have indicated the effect of the *APOE* genotype on Aβ

and tau pathology loads, both among AD cases (54, 106, 108) and cognitively intact individuals (105, 107, 108, 110, 113).

Strengths and weaknesses

As well as analysing the results in the manner employed above, it is always of importance to consider the methodological aspects of a study as well, which we will strive to do in this section. The primary concern for the vast majority of studies is the amount of cases included in the analysis, and this holds true here as well. Naturally, the goal is always to collate as many cases as possible but there are always limitations both in time and logistics. To be able to detect small effects of the *APOE* genotype on the amount of AD-related pathology in non-demented individuals, we may have needed more cases. Thus, future studies in this area ought to try to enrol more cases to be able to investigate this satisfactorily.

Another important aspect to take into consideration is how alike the various subgroups are in relation to other qualities that are not investigated but, nonetheless, might influence the results. This is commonly referred to as potential confounders. On this basis, we investigated if there was a significant difference in the number of women among the control cases compared to the AD cases. Since the cases were all recruited in a de-identified manner in this study, it was assumed that there would not be a difference in sex between the two groups. It became apparent, however, that other factors were at play since there turned out to be a significant difference in the proportion of women among the controls compared to the AD cases upon analysis (p-value 0.017). There were significantly more women among the controls (60%) than among the AD cases (42%). This is rather surprising given the fact that prevalence of AD generally is found to be higher in women than men (114, 115), although

incidence rate of AD is comparable between the sexes (116-118) – possibly a result of the fact that women tend to live longer than men on average (119). There are several plausible reasons behind this difference in sex distribution. Since women generally live longer than men it can also be presumed that there are more widows than widowers with dementia, which might lead to the fact that fewer demented women than men have a representative who can consent to their being enrolled in studies – an issue that has been found to be true in stroke research (120). Another possible explanation could be that women in general tend to be less investigated (121) and receive less expensive care (122) in the medical sciences than men. Whatever the cause, the most interesting aspect of this difference, in this context, is whether or not it is likely to affect the outcomes of the pathology comparisons in this study. There is conflicting evidence on the impact of sex on AD-related pathology load, both among AD cases and non-demented elders. Both neuropathological (123) and PET neuroimaging (124-126) studies have shown a difference in AD-pathological loads between the sexes – however, some of these studies indicate a higher prevalence of AD pathology in men (124, 126), and others in women (123, 125). Several other studies have shown no difference in AD pathology load at all between the sexes (37, 127-129). Likewise, there is no consensus on the interplay between sex and *APOE* genotype, with some studies suggesting a greater sensibility among women towards the disease-provoking mechanisms of the *APOE* ε4-allele (130, 131), while other studies suggest that there is no difference between the sexes in regard to their pathological response to *APOE* genotype (127, 128). If female sex indeed does amplify the neuropathological effect of the *APOE* ε4 allele, then this might definitely have affected our results, but there does not seem to be a consensus on this subject yet. Thus, it is unclear

whether sex could have been a confounder in this study, and further studies are needed to elucidate the impact of sex on AD-related neuropathology.

Further analysis on the case demographics also revealed a significantly older age at death in control cases (p-value <0.001) compared to AD cases. Given that AD is an acknowledged cause of death and is known to cause a large number of years lost to disability (YLD) and years of life lost (YLL) globally each year (132), it is not surprising to find that this holds true for our AD group as well. Since increased age, by all accounts, leads to a higher AD-related pathology load, the older age among controls might plausibly have contributed to a smaller difference in AD-related pathology between controls and AD cases, than we would have seen if they had been age-matched. It also cannot be ruled out that there might be a difference between age groups in the response to the disease-modifying mechanisms of the *APOE* genotype, which also might have affected our results. Indeed, the *APOE* ε2 allele has been shown to result in an increase in neuropathology – but only among the oldest old (110) (although these results need to be replicated). There does not, however, seem to be strong grounds to suspect that age was a significant confounder in this study. In fact, age at death and post-mortem delay were investigated as potential confounders, using linear regression, but were both found not to influence our results. Whether other differences in demographics existed between our subgroups remains unknown, since we did not have access to other information than what is listed above.

As in most other studies, the human factor must be considered, because where humans are involved, mistakes will inevitably follow. The human factor in this study was perhaps most prominent in the outlining of the grey matter of the frontal cortex during the image analysis. Since this outlining was done manually, there was naturally a variability

although efforts to be as uniform as possible were made. However, upon investigation of images that, for different reasons, had to be reanalysed, very similar results were consistently found between the independent image analyses of the same slides. This is shown in Figure 4.1 below. Thus, it seems that the image analysis was performed in a reliable and replicable manner, which ought not to have greatly affected the validity of the final results.

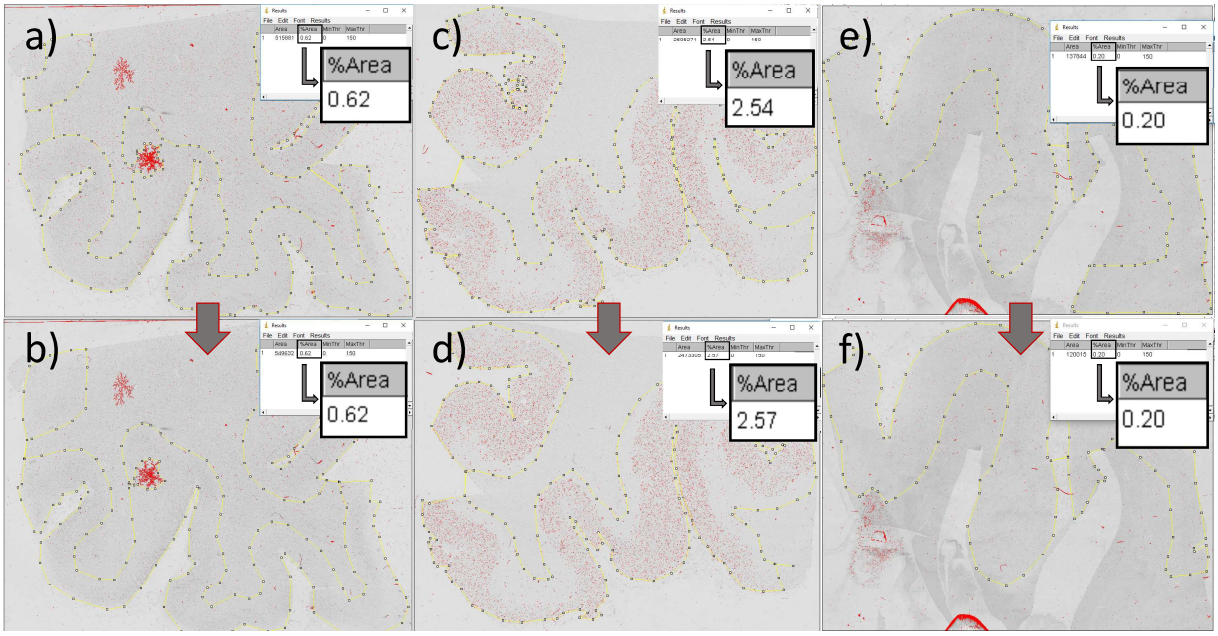

Figure 4.1. Showing separate analyses of the same slides. Although the analyses were made at different times, they yielded identical (a & b, and e & f) or very similar results (pictures c & d).

Another way in which the human factor made itself known in this project was in the assessment of the variability of the staining intensity. We found that certain slides, stained for tau, had a less intense staining than others and thereby decided to adapt the macro to these slides so that it would accurately detect the pathology. In this way, although we of course strived to be as objective as possible, an element of subjectivity was added in the image analysis, which could have affected the results. On the other hand, not adapting the macro to these paler slides would definitely have given us unrepresentative pathology estimates for those slides, since we found that the regular macro did not accurately detect all of the tau

pathology. In any case, the vast majority of our tau slides did not require this measure as only 8 slides, out of 179, were analysed with the adapted macro.

One final thing to take into account, regarding the methodological aspects of this study, is that we only looked at frontal cortex tissue from our cases. This was not a big issue regarding the analysis of Aβ pathology, since the accumulation of Aβ deposits throughout the brain is generally considered to follow a pattern of distribution where the deposits first appear in the neocortex (26), of which the frontal cortex is a part. The tau pathology, however, generally does not spread to the frontal cortex until the final stages of its accumulation pattern (25). Thus, by looking at the frontal cortex we probably obtained lower tau pathology loads in general than if we had chosen for example the entorhinal cortex, and in this way, we received a smaller spread in tau pathology data. This might have made it more difficult for us to discover significant differences in pathology load between our subgroups.

Conclusions and implications

This study successfully managed to further validate several previously shown differences between AD cases and controls. We demonstrated that the *APOE* ε2 allele is significantly more common among cognitively normal elderly people than among individuals with AD, and that the opposite is true for the *APOE* ε4 allele which is more common among AD cases. We also showed a highly significant difference in tau and Aβ pathology loads between AD cases and controls. Our analyses of the general impact of *APOE* genotype on AD-related pathology load indicated that the occurrence of the *APOE* ε4 allele results in increased amounts of Aβ and tau pathology.

Unfortunately, we were not able to confidently determine whether the effect of the *APOE* genotype on AD-related pathology in pathological ageing differs from its effect on AD cases. This remains an important question to answer in light of its possible implications for the understanding of the pathological mechanisms of both the *APOE* genotype and AD in general. If studies were able to show that the effect of the *APOE* genotype on AD-related pathology is identical among pathological ageing cases and AD cases, it would have to be assumed that there is an additional unknown pathological mechanism among AD cases that the pathological ageing cases lack. This might prove an essential clue in elucidating key pathological mechanisms in AD. On the other hand, if studies managed to confidently show that the *APOE* genotype does not have an effect on AD-related pathology in pathological ageing, then this would suggest that there is some kind of protective factor against *APOE*-related pathogenesis in pathological ageing. Consequently, this protective factor could be a clue to finding effective treatment against AD. In short, this is a fascinating research area where more studies are needed.

5. Populärvetenskaplig sammanfattning

APOE-genens påverkan på Alzheimers-relaterade hjärnvävsförändringar i patologiskt åldrande och Alzheimers sjukdom

Förutom att innebära en stor sorg för patienter och dess anhöriga, så utgör demens även en stor belastning på samhället och beräknades kosta Sverige över 60 miljarder kronor år 2012. Alzheimers sjukdom är den vanligaste typen av demens. Det som karaktäriserar Alzheimers sjukdom vid undersökningar av hjärnvävnaden postmortem, är förekomsten av så kallade *senila plack* och *neurofibrillära nystan*. Senila plack består av β-amyloid medan neurofibrillära nystan består av tau-proteiner. Den så kallade *APOE*-genen har visat sig vara

tätt sammankopplad med Alzheimers sjukdom och olika varianter, så kallade *alleler*, av *APOE*-genen medför olika stor risk att insjukna. Det finns tre olika alleler: *APOE* ε2-allelen minskar risken, *APOE* ε3-allelen anses riskneutral och *APOE* ε4-allelen ökar risken att insjukna i Alzheimers sjukdom. Av okänd anledning så lyckas vissa individer förbli symtomfria trots att deras hjärnor drabbats av betydande mängder senila plack och neurofibrillära nystan (Alzheimers-relaterade hjärnvävsförändringar). Detta fenomen kallas för *patologiskt åldrande* (patologiskt = sjukligt).

I denna studie ville vi undersöka huruvida de Alzheimers-relaterade hjärnvävsförändringarna (senila plack och neurofibrillära nystan) påverkas av *APOE*-alleluppsättningen hos individer med patologiskt åldrande på samma sätt som vid Alzheimers sjukdom. Därför undersökte vi mängden β-amyloid och tau-proteiner i hjärnvävnaden från 73 mentalt friska personer (varav 57 stycken uppvisade patologiskt åldrande) och 120 individer med Alzheimers sjukdom. Föga förvånande visade våra analyser att Alzheimerssjuka hjärnor uppvisar betydligt mycket mer hjärnvävsförändringar än mentalt friska hjärnor. Dessutom såg vi att *APOE* ε2-allelen var mycket vanligare bland de mentalt friska, samtidigt som *APOE* ε4-allelen var nästan 3 gånger vanligare hos Alzheimerssjuka. För övrigt såg vi att individer med *APOE* ε4-allelen hade mer Alzheimers-relaterade hjärnvävsförändringar än individer med antingen *APOE* ε2- eller *APOE* ε3-alleler, oavsett diagnos. Enligt våra analyser så fanns det däremot inget samband mellan *APOE* ε4-allelen och ökad mängd Alzheimers-relaterade hjärnvävsförändringar, när fall med patologiskt åldrande analyserades separat. Detta kan tolkas på två olika sätt: Antingen är individer med patologiskt åldrande skyddade mot den *APOE*-relaterade sjukdomsprocess som bidrar till Alzheimers sjukdom; eller så finns det

egentligen ett samband som vi helt enkelt inte kunde upptäcka i vår analys på grund av att vi inte hade tillräckligt många fall i analysen.

Om våra resultat bekräftas av större studier i framtiden så skulle nästa steg vara att försöka identifiera eventuella skyddande faktorer i fall med patologiskt åldrande för att se ifall det kan vara till hjälp i utvecklingen av läkemedel mot Alzheimers sjukdom. Ifall det däremot skulle visa sig att *APOE*-genotypen faktiskt påverkar Alzheimers-patologin även hos individer med patologiskt åldrande så skulle detta indikera att det krävs ytterligare en sjukdomsbidragande faktor för att utlösa själva sjukdomen. Oavsett om det finns ett samband eller inte så skulle ett definitivt svar på frågan leda till ytterligare ledtrådar om sjukdomsprocessen bakom Alzheimers sjukdom, vilket i förlängningen kanske även skulle leda till effektiva behandlingar som faktiskt påverkar sjukdomens förlopp. Idag är mekanismerna bakom Alzheimers sjukdom inte helt klarlagda och det saknas fortfarande effektiva läkemedel. Därför krävs ytterligare studier inom detta fascinerande område.

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