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Investigation of the expression of ATP-binding cassette sub-family A member 7 in the human brain within the context of Alzheimer's disease

Dissertation zur Erlangung des Grades eines Doktors der Medizin der Medizinischen Fakultät
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2. List of abbreviations

Aβ	Amyloid beta
ABCA7	ATP-binding cassette sub-family A member 7
AD	Alzheimer's disease
AP	Alkaline phosphatase
ApoA1	Apolipoprotein A1
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CAA	Cerebral amyloid angiopathy
CD	Cluster of differentiation
CERAD	The Consortium to Establish a Registry for Alzheimer's Disease
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computed tomography
CX3CL1	C-X3-C motif chemokine ligand 1
CX3CR1	C-X3-C chemokine receptor 1
DAB	3,3'Diaminobenzidine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FAD	Familial Alzheimer’s disease
FDA	Food and Drug Administration
FcR	Fc receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
HE	Hematoxylin and eosin
HLA-DR	Human leukocyte antigen DR isotype
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
Iba1	Ionized calcium-binding adaptor molecule 1
ICD-10	International Statistical Classification of Diseases and Related Health Problems, 10th revision
IF	Interferon
IL	Interleukin

IHC	Immunohistochemistry
IWG	International Working Group
LRP1	Low density lipoprotein receptor-related protein 1
MAP	Microtubule-associated protein
MCI	Mild cognitive impairment
MHCII	Major histocompatibility complex class 2
MLKL	Mixed lineage kinase domain-like protein
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NaOH	Sodium hydroxide
NFT	Neurofibrillary tangles
NT	Neuropil threads
NMDA	N-Methyl-d-aspartate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
pTau	Hyperphosphorylated tau protein
RIPK	Receptor-interacting serine/threonine-protein kinase
RNA	Ribonucleic acid
RT	Room temperature
SNP	Single-nucleotide polymorphism
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween
TNF-α	Tumor-necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
TRIS	Tris(hydroxymethyl)aminomethane
UK	United Kingdom
USA	United States of America
%(v/v)	Volume/volume percentage solution
%(w/v)	Weight/volume percentage solution

The dimensions of this thesis are consistent with the International System of Units (SI)

3. Abstract

Background: Alzheimer's disease is the most common cause of dementia and initially presents with a progressive deterioration of memory and orientation. As the disease's neuropathological hallmarks, amyloid beta plaques and deposits of hyperphosphorylated tau protein are found in patients' brains. In connection to the plaques, neuronal dystrophy and microglial activation occur. Multiple genome-wide association studies identified the ATP-binding cassette subfamily A member 7 (ABCA7) as a risk gene for Alzheimer's disease and found an association with amyloid deposition in the brain. The protein's function is unknown, but possible roles in lipid release from the cell and phagocytosis have been described. ABCA7 expression was found in the human brain and several other tissues. Protein expression of ABCA7 within brain cells has predominantly been found in microglia and neurons. Most evidence on ABCA7 comes from genetic studies and experiments with mouse models or cell culture. The aim of the here presented study is to examine ABCA7 expression in human brain tissue and investigate its association with pathologies related to Alzheimer's disease.

Methods: This study examined the ABCA7 expression in post-mortem brain tissue from seven cases with varying stages of Alzheimer's disease pathology. For this, I established a method for double staining of ABCA7 and a second marker via immunohistochemistry. To characterise the ABCA7-expressing cells, double labelling of ABCA7 with the phagocytic marker CD68 and the microglial marker Iba1 was performed. The association of ABCA7-positive cells to amyloid deposits in the frontal cortex and hippocampus was then analysed with a double staining using an antibody against ABCA7 and the anti-amyloid antibody 6E10.

Results: This study found a co-expression of the microglia-specific Iba1 protein and ABCA7 in all labelled cells, while only a subset of CD68-positive cells expressed ABCA7. ABCA7 expression was found in microglia of all stages of activation. The number of ABCA7-positive cells correlated positively with the amyloid beta plaque load in the grey matter of the frontal cortex. In this brain region, the majority of ABCA7-positive cells were associated with amyloid plaques. This association was particularly pronounced with dense plaques. A positive correlation between the number of ABCA7-positive cells in the frontal cortex and the hippocampus was found. In the hippocampus, the ABCA7-positive cells did not show association with amyloid beta plaques.

Conclusion: In the context of prior publications, I interpret these results as suggesting that ABCA7 influences Alzheimer's disease through microglial clearance of amyloid beta in the cortex. This hypothesis proposes that ABCA7 is upregulated in response to an increased plaque burden and holds a protective role against AD.

4. Zusammenfassung

Hintergrund: Die Alzheimer-Krankheit ist die häufigste Ursache von Demenz und beginnt oft mit einer zunehmende Verschlechterung von Gedächtnis und Orientierung. Neuropathologisch können dabei Ablagerungen von Beta-Amyloid-Plaques, welche mit neuronaler Degeneration und Mikrogliaaktivierung einhergehen, und hyperphosphoryliertem Tau-Protein nachgewiesen werden. Genomweite Assoziationsstudien konnten den ABC-Transporter ABCA7 als Alzheimer-Risikogen identifizieren. Diese Studien wiesen zudem eine Assoziation des ABCA7-Gens mit Beta-Amyloid-Ablagerungen nach. Die Funktion von ABCA7 ist bisher noch unbekannt. Möglicherweise spielt das Protein bei Lipidfreisetzung aus der Zelle und Phagozytose eine Rolle. ABCA7-Expression konnte im menschlichen Gehirn, insbesondere in Mikroglia und Neuronen, sowie in diversen weiteren Geweben nachgewiesen werden. Bisher wurde ABCA7 hauptsächlich mit Hilfe von genetischen Studien, Mausmodellen oder zellkulturellen Experimenten erforscht. In dieser Arbeit wird die ABCA7-Expression im menschlichen Gehirngewebe im Zusammenhang mit der Alzheimerpathologie untersucht.

Methoden: In dieser Studie wurde die ABCA7-Expression in postmortalem Gehirngewebe von sieben Alzheimerfällen unterschiedlicher Krankheitsstadien untersucht. Dafür entwickelte ich eine immunhistochemische Doppelfärbemethode, mit welcher ABCA7 zusammen mit einem weiteren Marker angefärbt werden kann. Um die ABCA7-positiven Zellen näher zu beschreiben, wurde das Protein zunächst zusammen mit dem Phagozytenmarker CD68 und dem Mikrogliamarker Iba1 angefärbt. Die Plaqueassoziation der ABCA7-positiven Zellen wurde mit einer Doppelfärbung von ABCA7 und Beta-Amyloid mittels 6E10-Antikörper im Frontalkortex und Hippocampus untersucht.

Ergebnisse: Alle angefärbten Zellen exprimierten ABCA7 und Iba1 gemeinsam. ABCA7 wurde jedoch nur von einem Teil der CD68-positiven Zellen exprimiert. Es konnte dabei eine ABCA7-Expression in Mikroglia aller Zellstadien nachgewiesen werden. Die Studie konnte eine positive Korrelation der Anzahl ABCA7-positiver Zellen und Beta-Amyloid-Plaques in der grauen Materie des Frontalkortex nachweisen. Zudem waren die meisten ABCA7-positiven Zellen mit Beta-Amyloid-Plaques, und dabei insbesondere mit den dichten "dense plaques", assoziiert. Die Anzahl ABCA7-positiver Zellen im Frontalkortex und Hippocampus korrelierte positiv. Im Hippocampus konnte jedoch keine Assoziation der ABCA7-positiven Zellen zu Beta-Amyloid-Plaques festgestellt werden.

Schlussfolgerung: Im Kontext früherer Veröffentlichungen gesehen, interpretiere ich die Ergebnisse so, dass ABCA7 die Beta-Amyloid-Phagozytose durch Mikroglia fördert. Diese Hypothese besagt, dass ABCA7 im Rahmen der Alzheimer-Erkrankung verstärkt exprimiert wird und eine protektive Rolle gegen Alzheimer hat.

5. Introduction

5.1 Clinical characteristics of Alzheimer's disease

5.1.1 Epidemiology and genetics

Alzheimer's disease (AD) is a neurogenerative disease characterised by a progressive cognitive decline, which especially affects short-term memory, orientation, and word-finding. AD was first described in a case report by Alois Alzheimer in 1907 (republished in translation by Stelzmann et al., 1995). According to a study from 2009, an estimated 1.2 million people in Germany have a form of dementia (Ziegler & Doblhammer, 2009). The prevalence of dementia is strongly dependent on age. In the age category of 60 to 64 years the prevalence of dementia is below 1 % (Alzheimer Europe, 2019; Ziegler & Doblhammer, 2009). That number rises to 12 % in people 80 to 84 years old and about 40 % in the age category of over 100 years (Alzheimer Europe, 2019; Ziegler & Doblhammer, 2009). It is estimated that 50 to 70 % of all dementia patients suffer from AD (Qiu et al., 2007). Women have an increased risk for AD in comparison to men (Andersen et al., 1999). Early-onset AD, defined as AD in people aged under 65 years old, has a reported prevalence of only 54 per 100,000 (Janssen et al., 2003). Calculations utilizing prevalence studies and population projections predict that, due to the continuing ageing of the population in Germany, the total number of AD patients will almost double by 2050 (Alzheimer Europe, 2019).

Besides the sporadic form of AD, there is also a familial form of AD (FAD). FAD patients develop symptoms at an unusually young age and typically have a clustering of AD cases in their families. Three genes are known to cause FAD, amyloid precursor protein (APP) gene on chromosome 21, Presenilin 1 gene on chromosome 14, and Presenilin 2 gene on chromosome 1 (Janssen et al., 2003; Raux et al., 2005; Ringman et al., 2014; L. Wu et al., 2012). Furthermore, AD pathology is closely associated to trisomy 21, which can be partially attributed to the overexpression of the APP gene on chromosome 21 (Lott & Head, 2019). The prevalence of dementia in people with trisomy 21 is highly age-dependent and has been stated as 80 % in the population aged 65 years or older (McCarron et al., 2017).

5.1.2 Diagnosis

Dementia is defined by a chronic cognitive deterioration with a duration of more than 6 months and needs to be distinguished from other forms of cognitive impairment (Bundesinstitut für Arzneimittel und Medizinprodukte im Auftrag des Bundesministeriums für Gesundheit unter Beteiligung der Arbeitsgruppe ICD des Kuratoriums für Fragen der Klassifikation im Gesundheitswesen, 2021). Delirium mainly affects attention and is often reversible. Age-

associated memory impairment describes a relative deficiency of memory that evolves in most of the elderly but does not affect everyday functioning. The more severe mild cognitive impairment (MCI) also does not disturb daily tasks, but these patients have an increased risk of developing dementia within the next years (Hacke, 2019).

According to the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10), AD is diagnosed by considering the cognitive symptoms, their progress, and chemical and pathological markers (Bundesinstitut für Arzneimittel und Medizinprodukte im Auftrag des Bundesministeriums für Gesundheit unter Beteiligung der Arbeitsgruppe ICD des Kuratoriums für Fragen der Klassifikation im Gesundheitswesen, 2021). In most cases, the disease presents with a gradual onset of short-term memory loss, other signs can be difficulty in handling complex tasks and language or visuospatial dysfunctions (Hacke, 2019). The German dementia guideline (Deutsche Gesellschaft für Neurologie, 2017) cites the International Working Group (IWG) 2 criteria by Dubois et al. (2014), as presented in Figure 1, for differentiation of AD from other forms of dementia for medical research.

In addition to dementia, the IWG-2 criteria also include MCI, which is a distinct diagnosis according to the ICD-10 classification system (Deutsche Gesellschaft für Neurologie, 2017). Apart from typical AD, which is defined in Figure 1, there are four atypical forms of AD. The posterior AD variant is dominated by early impairment of visual perception and identification of objects by eyesight. The logopenic form of AD presents with speech impairment, while the frontal form has a focus on early and progressive behavioural changes. Early-onset dementia of the AD type in people diagnosed with Down syndrome also constitutes an atypical variant of the disease (Dubois et al., 2014).

To start the diagnostic process, a thorough conversation with the patient and his or her relatives about the patient's medical history is crucial. A neurological examination is important to exclude other possible causes for the symptoms. Basic diagnostic blood tests are also essential for differential diagnostics. One or multiple cognitive tests should be conducted to further classify the patient's cognitive function. One widely used test is the mini-mental state examination which tests the patient's orientation, memory, and skills with numbers and words. This test is also fitting for usage in a general medicine setting and can be used to graduate clinical dementia into the categories mild, moderate or severe (Deutsche Gesellschaft für Neurologie, 2017).

***Panel 1*: IWG-2 criteria for typical AD (A plus B at any stage)**

A Specific clinical phenotype

- Presence of an early and significant episodic memory impairment (isolated or associated with other cognitive or behavioural changes that are suggestive of a mild cognitive impairment or of a dementia syndrome) that includes the following features:
  - Gradual and progressive change in memory function reported by patient or informant over more than 6 months
  - Objective evidence of an amnestic syndrome of the hippocampal type,* based on significantly impaired performance on an episodic memory test with established specificity for AD, such as cued recall with control of encoding test

B In-vivo evidence of Alzheimer's pathology (one of the following)

- Decreased $A\beta_{1-42}$ together with increased T-tau or P-tau in CSF
- Increased tracer retention on amyloid PET
- AD autosomal dominant mutation present (in *PSEN1*, *PSEN2*, or *APP*)

Exclusion criteria† for typical AD

History

- Sudden onset
- Early occurrence of the following symptoms: gait disturbances, seizures, major and prevalent behavioural changes

Clinical features

- Focal neurological features
- Early extrapyramidal signs
- Early hallucinations
- Cognitive fluctuations

Other medical conditions severe enough to account for memory and related symptoms

- Non-AD dementia
- Major depression
- Cerebrovascular disease
- Toxic, inflammatory, and metabolic disorders, all of which may require specific investigations
- MRI FLAIR or T2 signal changes in the medial temporal lobe that are consistent with infectious or vascular insults

AD=Alzheimer's disease. *Hippocampal amnestic syndrome might be difficult to identify in the moderately severe to severe dementia stages of the disease, in which in-vivo evidence of Alzheimer's pathology might be sufficient in the presence of a well characterised dementia syndrome. †Additional investigations, such as blood tests and brain MRI, are needed to exclude other causes of cognitive disorders or dementia, or concomitant pathologies (vascular lesions).

Figure 1: International Working Group 2 criteria for typical Alzheimer's disease

The IWG-2 criteria (Dubois et al., 2014) consider clinical symptoms typical for AD, as well as findings from cerebrospinal fluid (CSF) analyses, amyloid positron emission tomography (PET), and genetic testing. To diagnose AD, the clinical criteria A and at least one of the B criteria must be met. To exclude other causes of cognitive impairment further investigations like blood tests or brain magnetic resonance imaging (MRI) are recommended. Figure adapted from Dubois et al., 2014.

In addition to considering the clinical symptoms, the levels of amyloid beta (Aβ) in the cerebral spinal fluid (CSF) can help confirm an AD diagnosis. There are two major isoforms of the Aβ peptide, which differ in length. The CSF levels of $A\beta_{42}$, which consists of 42 amino acids, are reduced in AD, while the concentration of the shorter $A\beta_{40}$ does not differ significantly in demented individuals (Lewczuk et al., 2004). According to several studies, using the ratio of $A\beta_{42}$ to $A\beta_{40}$ provides significantly better diagnostic accuracy than evaluating the $A\beta_{42}$ levels alone (Hansson et al., 2019; Lewczuk et al., 2004, 2015). Increased levels of tau protein and phosphorylated tau (pTau) in the CSF are further laboratory indicators for AD (The British Psychological Society, 2007). Supplementary to a conventional CT or MRI for differential diagnostical purposes, an amyloid PET offers further insight to confirm the diagnosis. When using tracers like florbetaben, flutemetamol, or florbetapir, a PET scan can detect Aβ plaques in the brain with a sensitivity of more than 90 % (Clark et al., 2012). Early symptom onset or the occurrence of several cases of AD within one family are possible signs of FAD. In these cases, genetic consultation and diagnostics should be considered.

5.1.3 Treatment

Two classes of pharmaceuticals with proven effectiveness are approved for the treatment of AD in Germany and included in the guidelines published by the Deutsche Gesellschaft für Neurologie (2017), to which I refer in the following. The acetylcholinesterase inhibitors donepezil, galantamine, and rivastigmine are approved for AD cases with mild to moderate dementia. By inhibiting the enzyme that breaks down acetylcholine, the drugs raise the neurotransmitter's levels in the brain. Their effectiveness is dose-dependent, so the daily dose should be increased up to the approved maximal dose if the drug is tolerated by the patient. However, acetylcholinesterase inhibitors are not approved for patients with severe dementia and an off-label use can be difficult to realize and should be cautiously deliberated. Memantine is a non-competitive N-Methyl-d-aspartate (NMDA) receptor antagonist and inhibits the stimulating effect of the neurotransmitter glutamate. It is thought to stop the neurotransmitter's damaging effects on neurons and significantly improves cognitive function. Memantine is approved for the treatment of moderate and severe AD dementia. The effectiveness in cases with mild dementia is not proven, memantine should therefore not be used as a treatment in these patients (Deutsche Gesellschaft für Neurologie, 2017). Extracts from the leaves of the Gingko biloba tree are also commonly used by AD patients. However, a metanalysis by the Cochrane Collaboration concluded that there is no clear evidence for the effectiveness of Gingko extracts in the treatment of dementia (Birks & Grimley Evans, 2009).

The previously described treatment options are only symptomatic and do not inhibit the progress of the AD pathology. In 2021, the first therapy that affects the underlying pathology

was approved for AD in the United States of America (USA). The monoclonal antibody aducanumab binds aggregated forms of Aβ and was able to reduce Aβ plaques in the brain. Monthly infusions lead to a reduction of the Aβ plaque load as measured by florbetapir PET (Sevigny et al., 2016). However, the new treatment agent's clinical benefit for AD patients is still disputed. Initially, the two conducted phase III studies could not prove an inhibition of cognitive decline, one of them met this primary endpoint only after re-evaluation of the data (Food and Drug Administration [FDA], 2020). Nevertheless, the FDA issued an accelerated approval for the monoclonal antibody stating that the "reduction in plaques is reasonably likely to result in clinical benefit" (FDA, 2021). Aducanumab has not been approved in Europe (European Medicines Agency, 2021, 2022). With Oligomannate, another potentially disease-modifying therapy was approved in China in 2019. The oligosaccharide derived from marine algae, which is formally called GV-971, is thought to alter the gut microbiome and influence inflammatory processes in the context of AD (Syed, 2020). The drug has only been approved in China and further trials need to be awaited to evaluate the treatment's impact.

Nowadays, AD patients still have very few treatment options available, which highlights the importance of extensive research on new approaches for disease-modifying therapies. According to a review by Cummings et al. (2022), there were 143 drugs for AD treatment in development in January 2022. Thirty-one of were examined in phase III studies and 83 % of the drug mechanisms are considered disease-modifying (Cummings et al., 2022). A promising candidate is the monoclonal antibody AL002, which binds the microglial receptor TREM-2. AL002 altered the microglial response around Aβ plaques and reduced the number of neuritic plaques in a mouse model (S. Wang et al., 2020). The antibody's effect on patients is currently further examined in a phase II study (National Library of Medicine, 2022). Other approaches for new pharmaceuticals include monoclonal antibodies against Aβ and tau, a tau protein aggregation inhibitor as well as agents that are targeting inflammatory processes (Cummings et al., 2022; Hoskin et al., 2019; Reading et al., 2021; Swanson et al., 2021; Wilcock et al., 2018). Furthermore, different treatments targeting the neuropsychiatric symptoms of AD patients are currently in development and are showing promising results (Cummings et al., 2022).

5.2 Neuropathological diagnostics of Alzheimer’s disease

5.2.1 Amyloid beta

Two histopathological changes are found in post-mortem brain tissue of AD cases: extracellular Aβ deposits and intracellular tau protein. Amyloid plaques are made of aggregated Aβ, a protein consisting of 39 to 42 amino acids that is a fragment of the β-amyloid precursor protein (APP). Several versions of Aβ with differing lengths are found in the human

brain. The longer $A\beta_{42}$ is more prone to aggregate into plaques and is predominantly found in the brain parenchyma of AD patients (McGowan et al., 2005; Miller et al., 1993; Radde et al., 2006). The shorter $A\beta_{40}$ seems to inhibit Aβ aggregation, as reduced $A\beta_{40}$ levels increase amyloid deposition (Dermaut et al., 2001; Radde et al., 2006). Interestingly, $A\beta_{40}$ predominantly deposits in the blood vessels of the brain in the context of cerebral amyloid angiopathy (CAA) (Miller et al., 1993).

As an integral membrane protein, APP is found throughout the central nervous system (CNS) in different isoforms. The protein is processed by three different proteases of the secretase family, which is presented in Figure 2. There are two possible pathways for this degradation of APP. The α- and γ-secretases produce fragments that are not prone to aggregation. If APP is instead processed by the β-secretase, the Aβ fragment is released. Physiologically, Aβ is degraded by various enzymes, but in AD, it accumulates in the brain tissue and aggregates into plaques (Salminen et al., 2013).

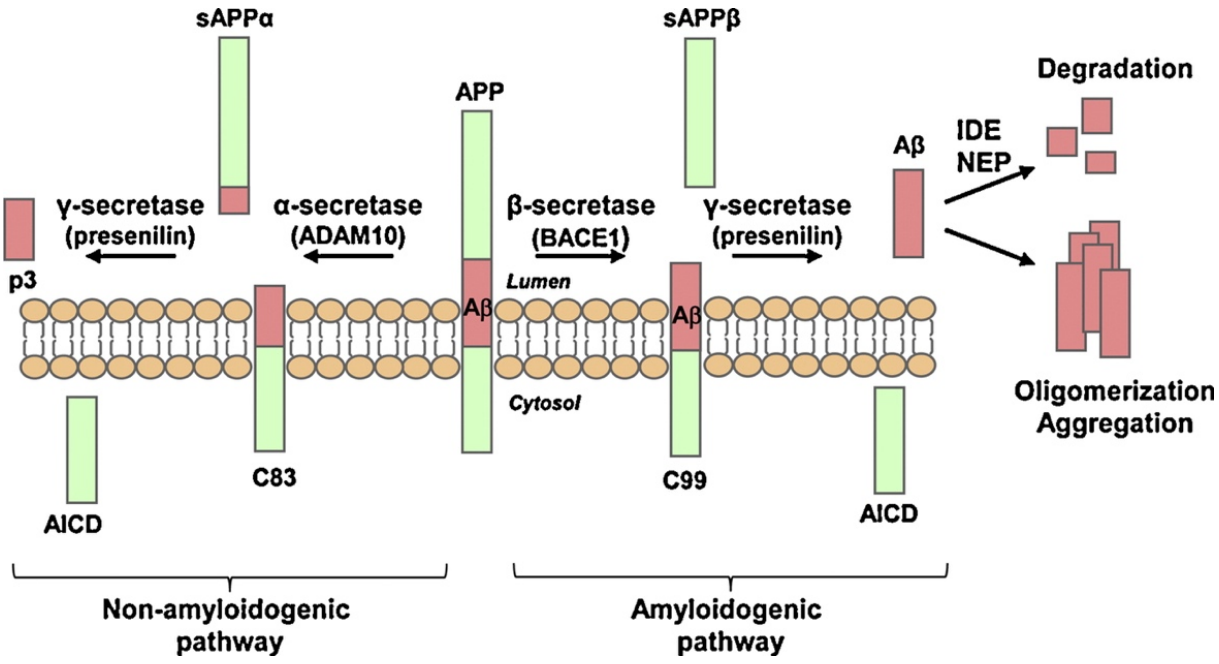


Figure 2: Processing pathways of amyloid precursor protein

In the non-amyloidogenic pathway, the α-secretase A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) releases the soluble amyloid precursor protein (APP) ectodomain α (sAPPα) and leaves the C-terminal fragment C83 in association with the membrane. C83 is then cleaved by the γ-secretase presenilin into the APP intracellular domain (AICD) and the p3 peptide. If APP is instead processed by the β-secretase beta-site APP cleaving enzyme 1 (BACE1), the soluble ectodomain β (sAPPβ) is released and the fragment C99 remains in the membrane. C99 is then cleaved into AICD and extracellular amyloid beta (Aβ). If degraded by enzymes, like insulin-degrading enzyme (IDE) or neprilysin (NEP), Aβ will not aggregate. In AD brains, Aβ pathologically accumulates, oligomerises, and eventually aggregates into Aβ plaques. Figure adapted from Salminen et al., 2013.

Aβ plaques can be made visible with traditional congo red staining or immunohistochemistry (IHC) using specific antibodies (Mirra et al., 1991). The majority of Aβ in AD is found in the grey matter, although some deposits can also be seen in the white matter (Duyckaerts et al., 2009). Several types of Aβ plaques can be categorized according to their morphology. However, it is unresolved if these different plaque types develop independently from each other or sequentially (Armstrong, 1998; Dickson & Vickers, 2001; Ikeda et al., 1990).

Cored plaques, as pictured in Figure 3A, consist of fibrillar Aβ and exhibit a dense core that is surrounded by a round Aβ deposit. They are more closely correlated with the presence of dementia (Serrano-Pozo et al., 2016). Neuritic plaques, also called senile plaques, are fibrillar plaques that contain dystrophic neurons with pTau deposits and are thought to contribute to the progression of AD (D. W. Dickson, 1997; T. C. Dickson & Vickers, 2001; Serrano-Pozo et al., 2016). Boon et al. (2020) recently described a new form of plaque, which they characterised as coarse-grained. These plaques are bigger than a classic cored plaque and exhibit several cores and Aβ-devoid pores (Boon et al., 2020).

Diffuse plaques, which are depicted in Figure 3B, are generally larger and show a homogenous Aβ distribution (Boon et al., 2020; Duyckaerts et al., 2009; Thal & Braak, 2005). These diffuse deposits are also frequently found in non-demented individuals, indicating that they do not directly contribute to AD pathology (Delaère et al., 1990; D. W. Dickson et al., 1992).

Additionally, fleecy and lake-like Aβ plaques have been described as either distinct plaque forms or subtypes of diffuse plaques (Duyckaerts et al., 2009; Thal et al., 1999; Wisniewski et al., 1998). Some publications also describe primitive plaques, in which dystrophic neurites are clearly visible (Armstrong, 1998; Marlatt et al., 2014; Probst et al., 1987). In addition to the classical extracellular deposits, Aβ can also be detected intracellularly within neurons (D'andrea et al., 2001). These deposits will be described more closely later in the introduction.

In the form of CAA, Aβ is also found around leptomeningeal and neocortical vessels (Thal et al., 2003). CAA is common in the brains of aged patients with and without dementia and is often associated with AD pathology (Arvanitakis et al., 2011; Brenowitz et al., 2015). Several studies found that CAA is present in 70 to 90 % of all AD cases with severe neuropathological changes (Attems & Jellinger, 2004; Bergeron et al., 1987; Dermaut et al., 2001; Thal et al., 2003). CAA predominantly contains the shorter peptide $A\beta_{40}$, which raises the question of whether Aβ in CAA is of a different origin than Aβ found in plaques (Greenberg et al., 2020; Kakuda et al., 2017; Miller et al., 1993). AD patients exhibit an increased extent of CAA, indicating that CAA contributes to the development of AD through vascular dysfunction

(Apátiga-Pérez et al., 2021; Thal et al., 2003). CAA is also found in elderly people without AD, but there is still a need for further research on the impact of these lesions on cognitive function (Arvanitakis et al., 2011; Thal et al., 2008). Given the overlap of vascular lesions and AD hallmarks, the differentiation between CAA, vascular dementia and AD remains a difficulty in the neuropathological diagnostic process (Thal et al., 2003).

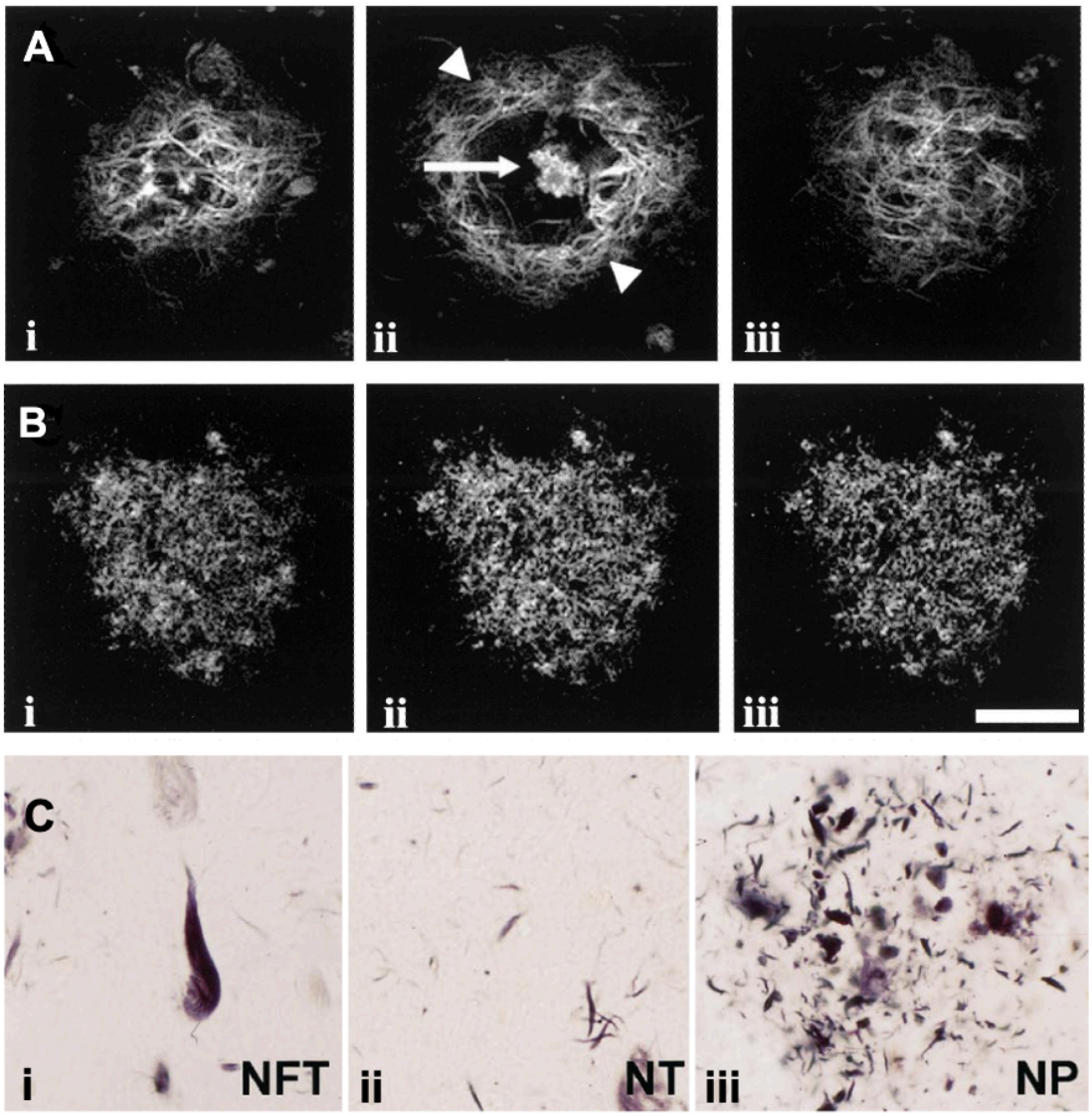


Figure 3: Neuropathological hallmarks of Alzheimer's disease

A Cored plaques have a dense core that is surrounded by loosely packed amyloid beta. **B Diffuse plaques** present with a homogenous amyloid distribution. **C** Neurofibrillary changes are made of hyperphosphorylated tau protein and are found within neurons. They can be stained with the Gallyas silver method. **Neurofibrillary tangles** (NFT) are visible within the neurons' somata and **neuropil threads** (NT) are found in dendrites. In **neuritic plaques** (NP), amyloid deposits are found around dystrophic nerve cell processes with intracellular tau protein. Figure adapted from Dickson & Vickers, 2001 (**A**, **B**) and D R Thal & Braak, 2005 (**C**).

5.2.2 Neurofibrillary changes

Changes of neurofibrils were already identified in the first reported case of AD (Alzheimer, 1907 [republished in translation by Stelzmann et al., 1995]). These neurofibrillary changes consist of pTau and can be made visible with silver staining methods, such as Bielschofsky or Gallyas, thioflavin S or specific antibodies against pTau (Thal, 2012). Tau is a microtubule-associated protein of the MAP2/Tau family and is predominantly expressed in neurons (Hu et al., 2017; Huber & Matus, 1984; Neve et al., 1986). The protein affects the polymerisation of microtubules and is essential for axonal growth and transport (Cleveland et al., 1977; Hallinan et al., 2019; Ishihara et al., 1999; Kanai et al., 1989; Lacovich et al., 2017; B. Zhang et al., 2004).

Although tau protein is encoded by one single gene, due to alternative splicing, it is expressed in six isoforms in the adult human brain (Goedert et al., 1989; Miguel et al., 2019; Neve et al., 1986). It is subject to extensive posttranslational modifications, especially phosphorylation, which affects the protein's role in microtubule stabilisation and possibly causes pTau aggregation in AD and other tauopathies (Drechsel et al., 1992; Kametani et al., 2020; Lindwall & Cole, 1984; Sato et al., 2018). In advanced AD stages, pTau was found to be three times more phosphorylated than in non-AD brains (Köpke et al., 1993).

Increased tau phosphorylation is a key mechanism in the development of neurofibrillary changes in AD (Sato et al., 2018; Y. Wang & Mandelkow, 2016). Hyperphosphorylated tau detaches from the microtubules and accumulates inside neurons in its unbound form (Bancher et al., 1989; Bramblett et al., 1993). These soluble deposits are considered pre-tangle material and are only visible with IHC methods (Bancher et al., 1989; Braak et al., 2006).

Through aggregation, the pTau material becomes insoluble and argyrophilic and can now be made visible with a silver method. Intracellular pTau is found in form of neurofibrillary tangles (NFT), bundles of fibrils within the neuronal soma, and neuropil threads, that are seen within dendrites (Braak & Braak, 1991). Figure 3C provides images of these neurofibrillary changes. Studies on AD mouse models suggest that a reduction of pTau prevents neuronal loss, which might provide opportunities for new therapeutic approaches (DeVos et al., 2017, 2018)

5.2.3 Classifications

The National Institute on Aging recommends using three scores, which are classifying NFT, Aβ plaques, and neuritic plaques, for the post-mortem neuropathological assessment of probable AD cases (Hyman et al., 2012).

The commonly-used Braak score considers the distribution of NFT to establish six stages (Braak & Braak, 1991). For this classification system, tissue sections from the entorhinal, hippocampal, and occipital area are examined. The first two Braak stages exhibit neurofibrillary changes in a specific layer of the transentorhinal region. In stages III and IV more layers and areas within the limbic system are affected. The last two stages are defined by NFT within the isocortex. Figure 4 provides more detailed information on the stages of NFT distribution according to Braak.

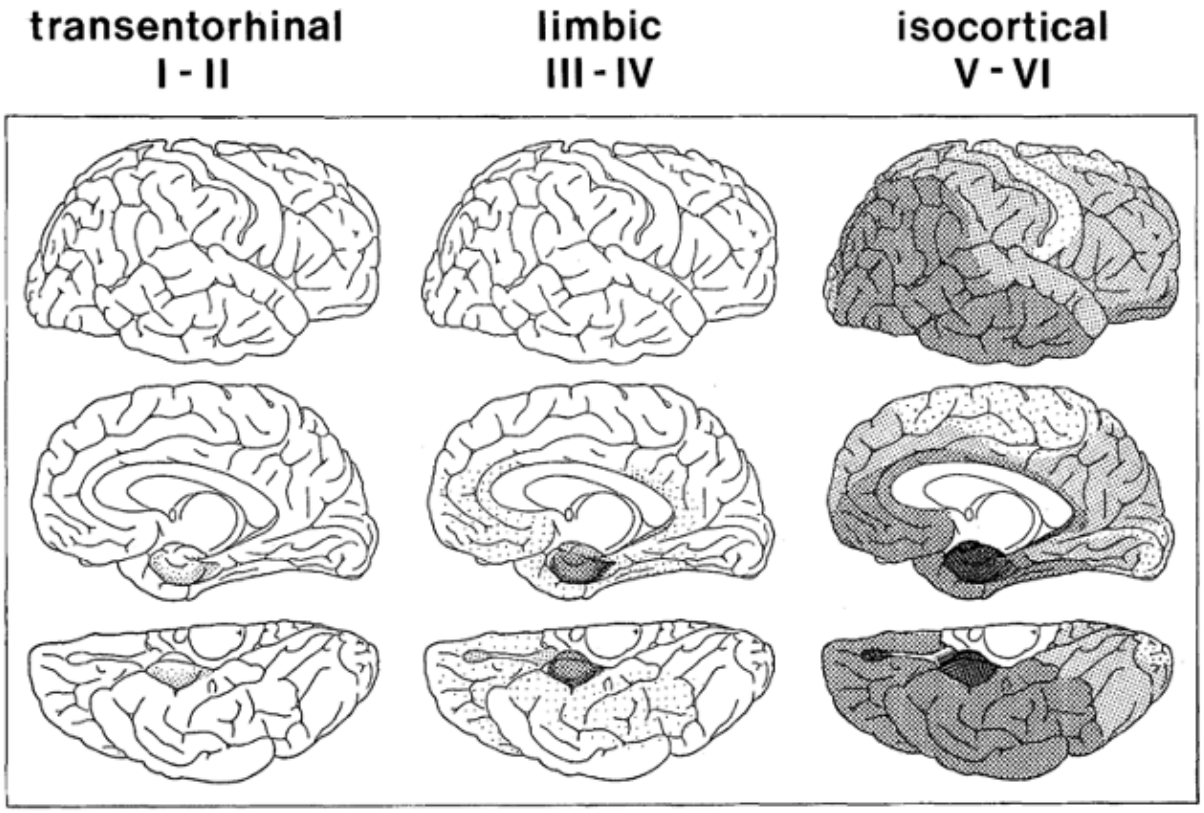
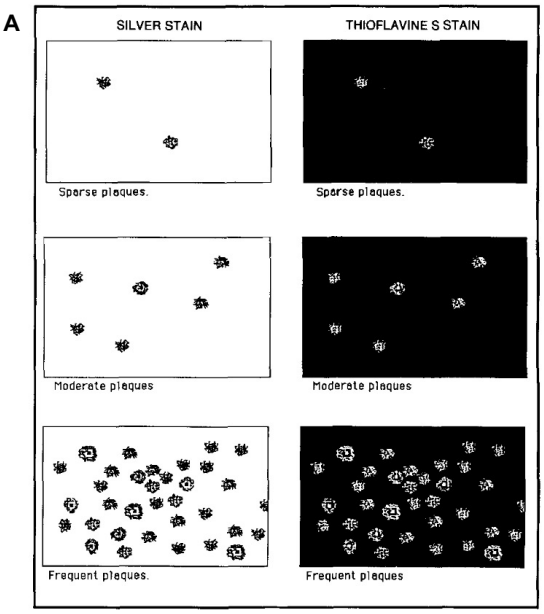


Figure 4: Neuropathological staging of Alzheimer's disease according to Braak

To identify the disease phases according to Braak & Braak (1991), the distribution of neurofibrillary tangles (NFT) within the brain is examined. This system defines six stages, which can be sorted into transentorhinal, limbic and isocortical phases. In stages I and II, mainly the transentorhinal region is affected. Stage III exhibits strong involvement of the Pre-α layer in the entorhinal and transenthorinal regions. In stage IV, NFT are predominantly found within the hippocampus and few in the isocortex. Stage V exhibits severe NFT deposits in the isocortex, which are even stronger in stage VI. Figure adapted from Braak & Braak, 1991.

Another score based on Aβ deposits was developed by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) and evaluates the frequency of neuritic plaques (Mirra et al., 1991). To establish the CERAD score, tissue from the middle frontal gyrus, superior and middle temporal gyri, and inferior parietal lobule are stained for Aβ and the region with the most visible plaques is selected. In this section, the number of neuritic plaques is assessed semi-quantitatively and defined as sparse, moderate or frequent (Mirra et al., 1991). A drawing

provided by the authors, which is included in Figure 5, is helpful to determine the plaque quantity. The original publication suggests using silver stain or thioflavin S for staining the plaques, but nowadays, IHC is the standard method for this (King et al., 2020; Mirra et al., 1991). Using a chart, which is provided in Figure 5, the frequency of plaques is then combined with the age of the patient, to create an age-related score that predicts the probability of a correct AD diagnosis.



B

Age of pt at death (yrs)	Frequency of plaques†			
	None	Sparse	Moderate	Frequent
<50	0	C	C	C
50-75	0	B	C	C
>75	0	A	B	C

* An age-related plaque score is determined using patient's age along with plaque frequency in the most heavily affected neocortical section.
† Based on section of frontal, temporal, or parietal cortex with maximum involvement.

For purpose of this protocol, the letter circled corresponds to the following assessment:

0 = NO histologic evidence of Alzheimer's disease.
A = Histologic findings are UNCERTAIN evidence of Alzheimer's disease.
B = Histologic findings SUGGEST the diagnosis of Alzheimer's disease.
C = Histologic findings INDICATE the diagnosis of Alzheimer's disease.

Figure 5: Neuropathological assessment of Alzheimer's disease according to the Consortium to Establish a Registry for Alzheimer's Disease

A The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) score, which was first published by Mirra et al. in 1991, examines the frequency of neuritic plaques in the brain area that presents with the most plaques. The number of plaques is assessed semi-quantitively as sparse, moderate, or frequent. **B** The plaque frequency is matched with the patient's age to determine the probability of a correct diagnosis of Alzheimer's disease. Figures adapted from Mirra et al., 1991.

The widely recognized five phases of Aβ deposition developed by Thal et al. (2002) are based on the sequence in which different brain regions exhibit Aβ-deposition in progressing AD. This deposition starts in the neocortex and then spreads to the allocortex, brainstem, and diencephalon. The cerebellum is eventually affected in the final phase (Thal et al., 2002). Figure 6 visualizes these stages of Aβ deposition.

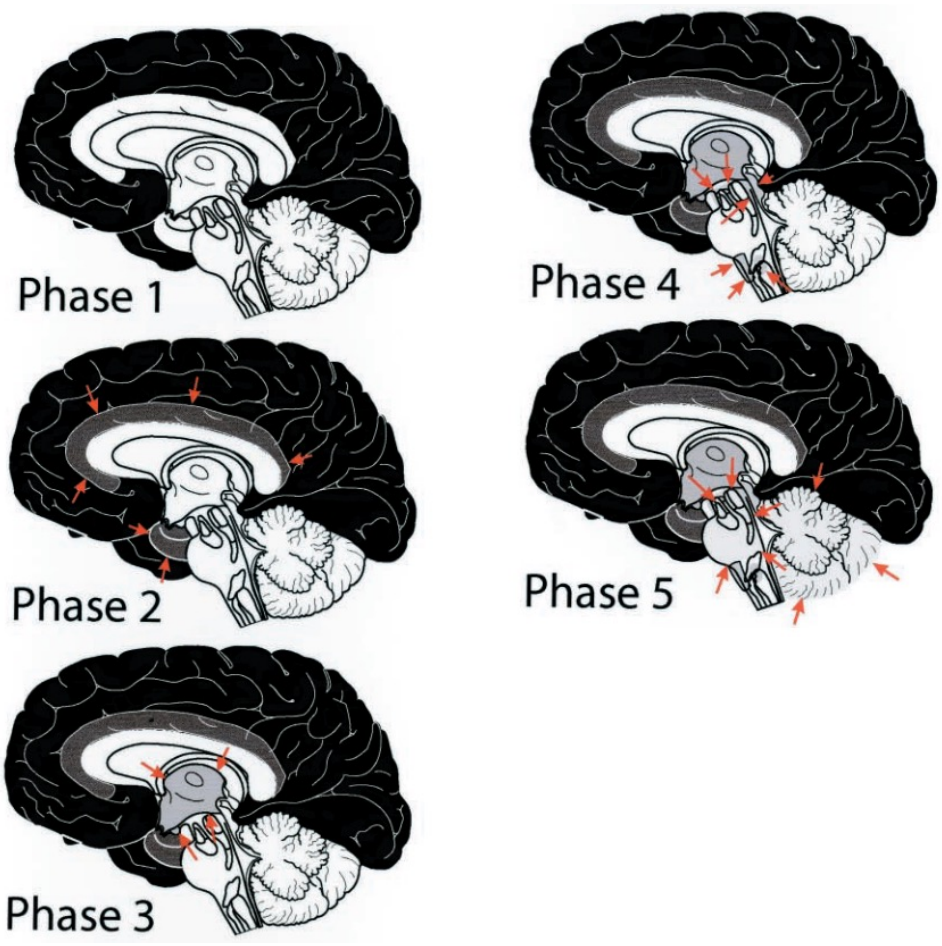


Figure 6: The five phases of amyloid deposition according to Thal et al.

Thal et al. (2002) consider the progression of amyloid deposits. In phase 1, amyloid beta is only found in the neocortex. In phase 2, the deposits are already extended into the allocortex and phases 3 and 4 are defined by the involvement of diencephalic nuclei, basal forebrain, striatum, and parts of the brainstem. Additional involvement of the cerebellum defines the last phase of amyloid deposition. Figure adapted from Thal et al., 2002.

According to the Consensus Recommendations for the Postmortem Diagnosis of Alzheimer's Disease from 1997 (The National Institute On Aging And Reagan Institute Working Group On Diagnostic Criteria For The Neuropathological Assessment Of Alzheimer's Disease, 1997), an AD diagnosis is highly likely if a post-mortem brain shows frequent neuritic plaques according to CERAD and NFT in the neocortex, which corresponds with a Braak-stage V or VI. On the contrary, infrequent neuritic plaques and a Braak-stage of I or II constitute a low likelihood of AD. An updated recommendation from 2012 proposes using the ABC score, that incorporates all three previously described scores (Hyman et al., 2012).

5.3 Cell types of the brain and their role in Alzheimer's Disease

5.3.1 Neurons

Loss of neurons occurs early in the progression of AD, even before clear dementia symptoms are detectable, and correlates with cognitive decline (Giannakopoulos et al., 2003; Gómez-Isla et al., 1996; Raji et al., 2009). This cell loss has been attributed to impaired postnatal neurogenesis and neuronal maturation, as well as increased neuronal death (Haughey et al., 2002; B. Li et al., 2008; Smale et al., 1995). It has long been thought that the adult human brain can not generate new neurons, but newer studies suggest that neurogenesis still occurs in the adult human brain (Boldrini et al., 2018; Eriksson et al., 1998; Ernst et al., 2014; Moreno-Jiménez et al., 2019). Several publications demonstrated that this process is impaired in brains with AD pathology (Haughey et al., 2002; Marx et al., 2006; Moreno-Jiménez et al., 2019; J. M. Wang et al., 2010). Furthermore, analyses of the expression of microtubule-associated protein (MAP) 2, which is important during neuronal development, suggested that recently generated neurons do not properly mature into functioning cells in AD brains (B. Li et al., 2008).

Aβ plaques are one of the most prominent pathological changes in AD, therefore early research focused on the role of extracellular Aβ. Due to technical difficulties, the existence of intracellular neuronal Aβ has long been challenged but was confirmed by using more specific Aβ antibodies (D'andrea et al., 2001; Wegiel et al., 2004). Intracellular accumulation results from neuronal production as well as internalisation of extracellular Aβ (Bahr et al., 1998; Bi et al., 2002; Greenfield et al., 1999). Using the Ntera2/D1 neuron-like cell line, a study in 1993 first demonstrated that neurons are capable of producing Aβ in cell culture (Wertkin et al., 1993). Further in vitro studies identified the secretory pathway of neurons via endoplasmic reticulum (ER) and Golgi network as an intracellular production and processing site of Aβ (Cook et al., 1997; Hartmann et al., 1997; Tan & Gleeson, 2019). Intriguingly, Clifford et al. (2007) demonstrated, by injecting mice with fluorescent Aβ, that neurons are also able to bind and take up extracellular Aβ.

Aβ treatment of neurons in cell culture resulted in Aβ internalisation and rapid cell death, which indicates that intracellular Aβ accumulation causes neuronal death in AD brains (Ditaranto et al., 2001; Yu et al., 2010; Y. Zhang et al., 2002). Remnants of neurons can be found at the core of Aβ plaques, possibly suggesting that the lysis of Aβ burdened neurons contributes to extracellular Aβ accumulation (D'andrea et al., 2001). Changes in calcium homeostasis have been connected to neurodegeneration in AD and it was shown that Apolipoprotein E (APOE) ε4 accelerates these calcium-dependent neurotoxic processes in vitro (Veinbergs et al., 2002; Wadhwani et al., 2019; X. Wang & Zheng, 2019). The exact mechanisms behind Aβ induced

neuronal death in AD are not known, but several pathways including caspase-dependent cell death and processes around the apoptosis-inducing factor have been proposed by publications (Allen et al., 2001; J.-H. Lee et al., 2012). However, the observation of intraneuronal Aβ in healthy individuals from late childhood on leaves many questions regarding the implications of intracellular Aβ for healthy and AD brains (Wegiel et al., 2007).

5.3.2 Astrocytes

The star-shaped astrocytes are found in the grey and white matter throughout the whole brain (Sofroniew & Vinters, 2010). With their foot processes, this glia cell type is in close contact with neurons and blood capillaries in the brain (Iadecola & Nedergaard, 2007). They serve a variety of functions to uphold the homeostasis of the brain. The glia cells regulate neuronal signal transmission, have complex metabolic functions and contribute to synaptic development and maintenance (Sofroniew & Vinters, 2010). Astrocyte foot processes build and control the border between the extracellular brain parenchyma and the perivascular space, which makes them an integral part of the blood-brain barrier (Bechmann et al., 2007; Y. Hayashi et al., 1997). They react to CNS damage with cell proliferation, cellular hypertrophy, and scar formation around severely damaged areas. This reaction is called astrogliosis and is accompanied by increased expression of the astrocytic specific cytoskeletal marker glial fibrillary acidic protein (GFAP) (Sofroniew, 2014).

In cell culture, astrogliosis was triggered by Aβ plaques (DeWitt et al., 1998). Furthermore, several studies have shown increased astrogliosis, quantified with the marker GFAP, in post-mortem AD cases, which might be a consequence of local neuronal damage (Beach & McGeer, 1988; Kashon et al., 2004). Simpson et al. (2010) observed that an increased Braak stage correlates with increased GFAP expression by astrocytes in brains of elderly people. In that study, astrogliosis was increasingly seen in cases with a high neuritic plaque burden, but not associated with diffuse plaques. In a triple transgenic AD mouse model, general cytoskeletal atrophy and reduction of cell body volume were observed in correlation to AD pathology progression. In later disease stages, astrocytes associated with Aβ plaques showed an increase in volume and size of the cell body, while distant cells were atrophic (Olabarria et al., 2010). Similar studies could confirm the astroglial atrophy in AD mice but could find no hypertrophic cells with correlation to Aβ plaques, which might be because different brain areas were evaluated (Kulijewicz-Nawrot et al., 2012; C.-Y. Yeh et al., 2011).

Wyss-Coray et al. (2003) discovered that astrocytes are capable of binding and degrading Aβ in a mouse model. Furthermore, an IHC study on human tissue of the entorhinal cortex found Aβ deposits within activated astrocytes around plaques (Nagele et al., 2003). The extent of

this intracellular Aβ accumulation was positively correlated to the amount of local AD pathology. Double labelling with a neuron-specific marker indicated that the internalized Aβ was taken up together with neuronal debris. The study also suggested that the lysis of these Aβ positive astrocytic cells contributes to the plaque burden in form of astrocyte-derived Aβ plaques (Nagele et al., 2003). Additionally, astrocytes can produce Aβ in cell culture and thus are possibly contributing to Aβ accumulation in AD (Busciglio et al., 1993; J. Zhao et al., 2011).

Taking the findings of the mentioned studies together, it becomes clear that astrocytes play a complex role in the pathology of AD by internalizing Aβ and developing reactive astrogliosis around plaques, while they are showing signs of atrophy further away from the plaques (Olabarria et al., 2010; Wyss-Coray et al., 2003). It has been argued that astrocyte atrophy disrupts the neuronal function and extracellular homeostasis of the brain, while astrogliosis contributes to neuronal death and chronic inflammation in the context of AD (Rodríguez et al., 2009).

5.3.3 Microglia and other phagocytic cells

Microglia are the resident mononuclear phagocytic cells of the CNS and constitute 5 to 12 % of glial cells in the CNS parenchyma (Lawson et al., 1990). It has been established that microglia are of myeloid origin, but the details of their development have long been unclear (Beers et al., 2006; McKercher et al., 1996). Ginhoux et al. (2010) proved that progenitor cells from the embryonic yolk sack migrate to the brain from early embryonic development stages until shortly after birth. Hence, a clear distinction of microglia from the peripheral macrophages, that continuously develop from bone marrow-derived monocyte precursor cells, is important. It has been established that the microglia population maintains itself by self-renewal of proliferating resident cells (Ginhoux et al., 2010; Gomez-Nicola & Perry, 2015).

However, it is still not clear to what extent bone marrow-derived monocytes or macrophages have the capacity to infiltrate the CNS and assume microglia morphology and phagocytic functions within the brain parenchyma (Ling et al., 1980). In a mouse model, bone marrow-derived phagocytic cells were attracted by Aβ plaques and were able to eliminate these deposits (Simard et al., 2006). However, a review by Heneka et al. (2015) argues that the whole-body irradiation of the mice used in this experiment impairs the blood-brain barrier and does not represent physiological processes. Other animal studies suggested that perivascular macrophages, resident brain macrophages that are closely associated with blood vessels, rather than peripheral phagocytes, can eliminate Aβ from the brain (Hawkes & McLaurin, 2009; Mildner et al., 2011). In summary, the role of peripheral phagocytic cells for the healthy and

diseased brain is not yet determined, but it seems that they do not contribute substantially to the Aβ uptake in the brain.

Physiologically, microglia are organised in a regular array and are present in the grey and white matter (Lawson et al., 1990). Depending on their state of activation, microglia can exhibit different morphologies, which are illustrated in Figure 7. In healthy brain tissue, microglia are found in a resting state with a ramified morphology. After injury or inflammation, microglia exhibit signs of activation. Initially, hypertrophy of processes can be observed, which has also been described as a bushy morphology (Paasila et al., 2019). Later stages of activation show a retraction of processes until an amoeboid shape is reached (Paasila et al., 2019; Torres-Platas et al., 2014).

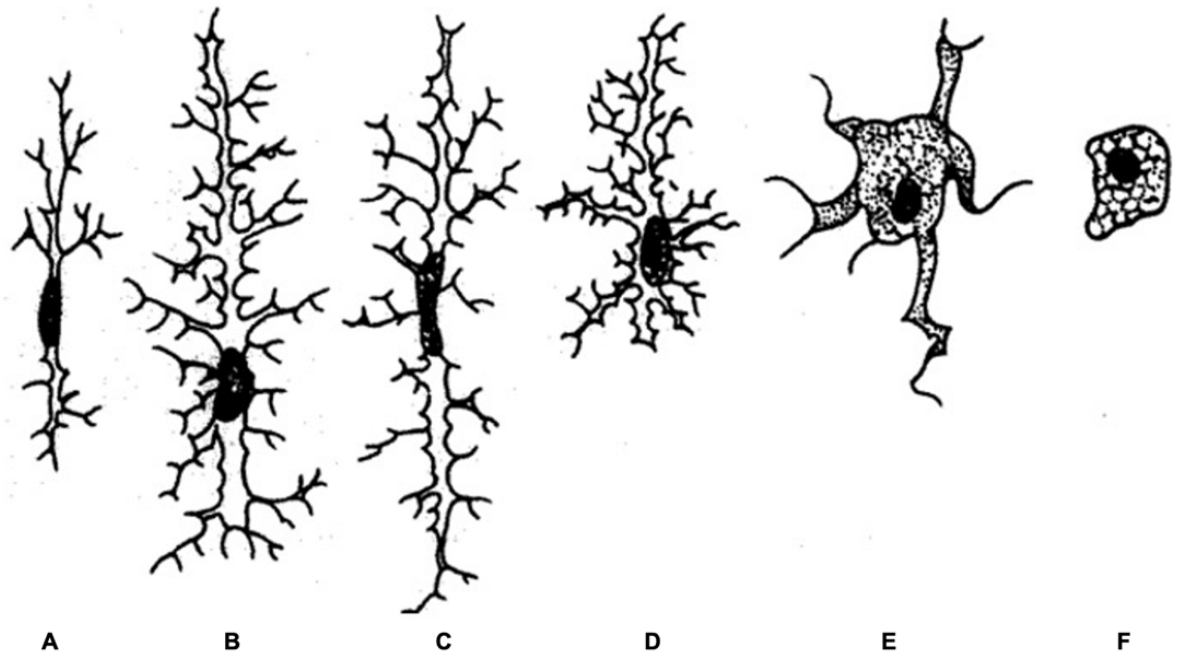


Figure 7: Morphology of microglia

A Resting microglia exhibit a ramified morphology with thin processes. **B** After activation through injury or stress the primed microglia show hypertrophy and develop larger cell bodies and thicker processes, but show fewer secondary processes. **C, D** In later activation stages, processes are increasingly retracted. **E** Reactive microglia have enlarged cell bodies with few processes. **F** Amoeboid-shaped microglia have very few or no processes and are thought of as the phagocytic form of microglia. Figure adapted from Kreutzberg, 1996.

In vivo imaging proved that the resting microglia are constantly scanning the immediate environment with their processes while keeping the soma in position (Nimmerjahn et al., 2005; Wake et al., 2009). A recent study demonstrated that microglial processes predominantly interact with neuronal cell bodies, indicating that the glial cells monitor neuronal status with these interactions (Cserép et al., 2020). These interactions between microglia and neurons are mediated through a variety of neurotransmitters and cytokines. For instance, Brain-derived

neurotrophic factor (BDNF) from microglia can regulate synaptic plasticity in mice and microglia-derived IGF-1 maintains neuronal survival (Parkhurst et al., 2013; Ueno et al., 2013). Furthermore, metabotropic glutamate receptors on microglia are able to activate neurotoxic functions in microglia (Taylor et al., 2002). Intriguingly, Hefendehl et al. (2014) found that the distribution of neocortical microglia becomes less homogeneous in aged mice. The microglial processes are shortened and their movement is slowed down, while the microglial cell bodies increase in volume and start moving, which indicates a microglial dysfunction associated with ageing (Hefendehl et al., 2014).

Synaptic pruning, a process in which functioning synapses are eliminated after birth, is essential for healthy, well-adapted brain development. Interestingly, microglia have been shown to play an active role in this process, which continues at least until adolescence (Sakai, 2020). Using mouse models, it was shown that the complement system can mediate engulfment of presynaptic inputs by microglia (Schafer et al., 2012; Stevens et al., 2007).

Mediated through direct cell contact, microglia can phagocyte apoptotic cells in the brain, without activating a proinflammatory reaction (Magnus et al., 2001). Experiments using cell cultures of neonatal rat brain demonstrated that this phagocytosis of apoptotic cells is initiated without delay by the microglia and completed within two hours of the first cell contact (Parnaik et al., 2000). Chan et al. (2001) confirmed that microglia have a high capacity for the clearance of apoptotic cells. Furthermore, microglia are able to phagocyte axonal debris, which enhances neuronal regeneration (Hosmane et al., 2012; Nielsen et al., 2009). Figure 8 provides an illustrated summary of the different functions that microglia serve in the healthy brain.

Genetic analyses show that microglia are involved in the development of AD (Kunkle et al., 2019; Nott et al., 2019). As the synergy of the different functions of microglia in healthy brains, is still not completely understood, their implications for AD remain ambiguous. Today's knowledge suggests neuroprotective as well as detrimental roles for microglia in AD and has been reviewed in detail by Hansen et al. (2018). In vitro experiments demonstrated that microglia can phagocyte Aβ and several receptors, such as scavenger receptors or CD14, have been associated with this process (Y. Liu et al., 2005; Paresce et al., 1996). However, it has been difficult to confirm Aβ clearance by microglia in vivo (Sierra et al., 2013). Although Meyer-Luehmann et al. (2008) observed in transgenic mice that microglia are recruited to a newly formed Aβ plaque within two days, they could not find evidence for phagocytosis of the plaque material.

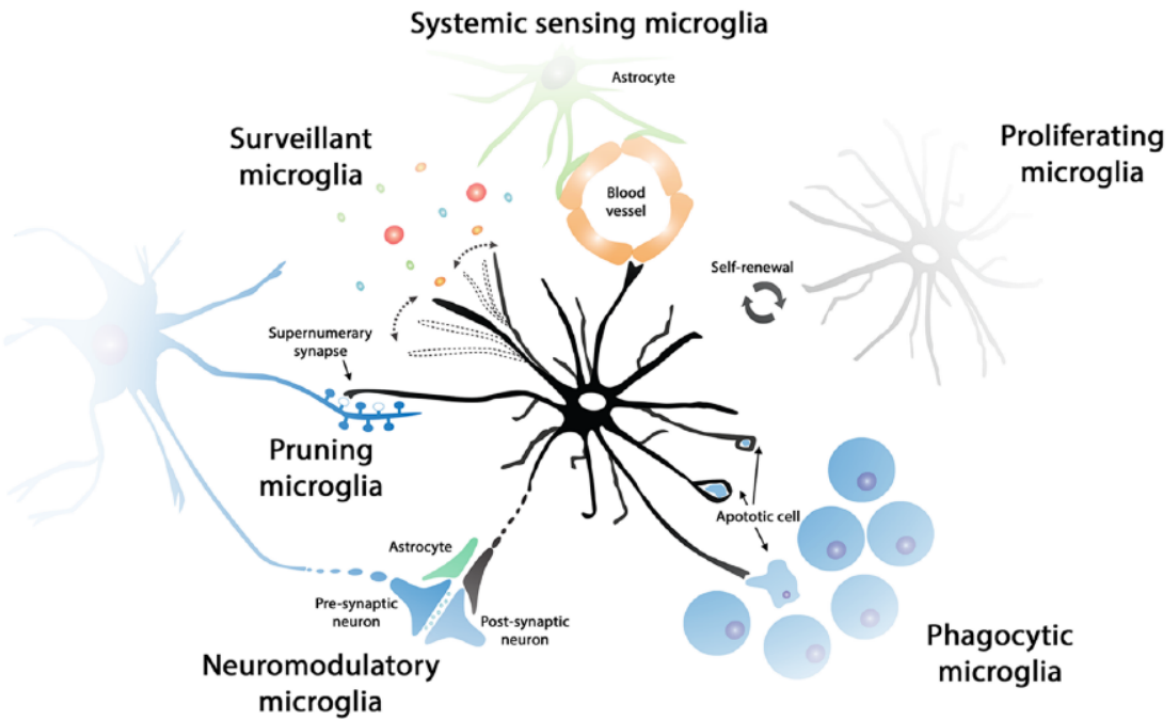


Figure 8: Microglia functions in the healthy brain

Proliferation of new microglia is independent of bone marrow-derived progenitors, as the population regenerates through self-renewal. **Systemic sensing microglia** are in close contact with the blood-brain barrier to detect pro-inflammatory mediators and other substances in the blood. Microglia are constantly monitoring the homeostasis of their microenvironment as **surveillant microglia**. They are also important for the correct function of neurons, as they **prune synapses** during brain development and uphold **neuromodulatory functions** for the neurons. As the resident **phagocytic cells** of the brain, microglia remove damaged or apoptotic cells, axonal debris, and pathogens within the brain. As previously described, astrocytes also hold important functions for brain homeostasis and are pictured here in close contact with blood vessels and synapses. Figure adapted from Gomez-Nicola & Perry, 2015.

In further experiments, Aβ has also been found to activate the clustering of microglia around plaques (Huang et al., 2009). Some authors proposed that microglia are connected to the deposit of Aβ rather than its clearance, but Scott et al. (1993) did not detect β-APP messenger ribonucleic acid (mRNA) in microglia that were clustered around plaques, which suggests that microglia are not producing Aβ (Frackowiak et al., 1992; Scott et al., 1993; Wegiel et al., 2004).

However, microglial activation has also been shown to increase inflammation and have detrimental effects on neurons. An animal study demonstrated that TNFα induces microglia to chronically produce inflammatory factors, which resulted in neuronal loss (Qin et al., 2007). In cell culture, Aβ stimulates microglia to activate various proinflammatory processes and produce neurotoxic substances (Combs et al., 1999; Halle et al., 2008; Y. Liu et al., 2020; Urrutia et al., 2017; Z.-F. Wang et al., 2020; Zu et al., 2020). It has been proposed that microglia activation in early AD stages promotes neuroprotective effects and removal of Aβ plaques, while the cells' proinflammatory activities in late AD mainly have detrimental effects (Fan et al., 2017; Prokop et al., 2013). Figure 9 provides an overview of this theory on the possible beneficial and harmful functions of microglia for the progression of AD.

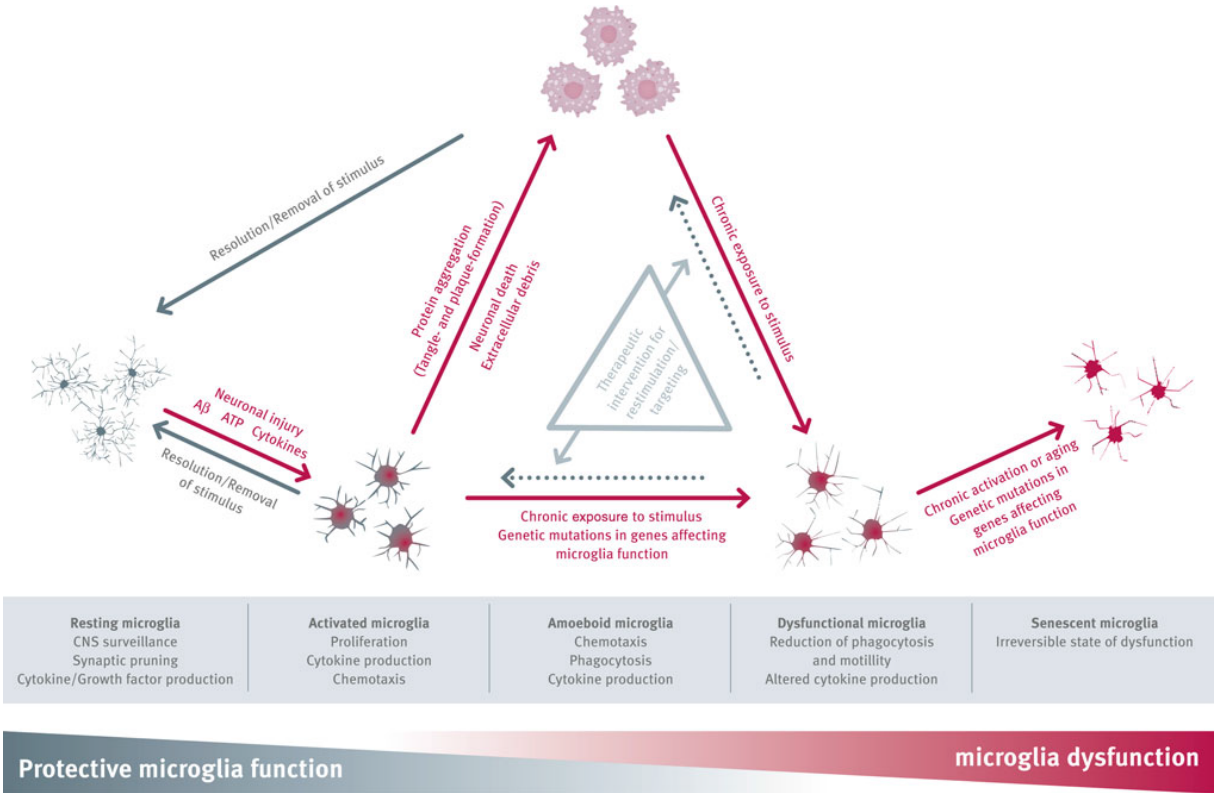


Figure 9: Microglia functions in Alzheimer's disease

The various functions of microglia can have both beneficial and detrimental effects on the pathology of Alzheimer's disease. **Resting microglia** are responsible for surveillance, pruning, and cytokine production while contributing to a healthy brain environment. They can be **activated** by neuronal damage, factors like adenosine triphosphate (ATP) and cytokines, or extracellular amyloid beta (Aβ), resulting in accumulation around the trigger through chemotaxis. Activation results in a transformation into **amoeboid microglia** that can phagocyte Aβ and damaged cells. After removal or resolution of the triggering stimulus, the activated microglia are able to return to their resting state. Chronic exposure or genetic mutations can result in a **dysfunctional** cell status, in which the microglia exhibit reduced phagocytic capacities, disabled motility, and altered cytokine production. In reaction to prolonged persisting stress, irreversible cell dysfunction is possible, a status described as **senescence**. Figure adapted from Prokop et al., 2013.

5.3.4 Other types of cells

Two further glial cell types are present in the CNS, namely ependymal cells and oligodendrocytes. Ependymal cells are lining the ventricular system of the CNS and are producing CSF. So far, no relevant connection of this glia cell type to AD has been described. Regarding the impact of oligodendrocytes, the cells that form the myelin of the CNS, on AD, a theory has been established in which Aβ induces oligodendrocyte dysfunction and myelin breakdown, which was reviewed in depth by Zhan et al. in 2018. As this issue is not relevant in the context of this thesis, I will not further describe it.

In summary, neurons, microglia, and astrocytes seem to be the central cellular factors in the context of AD pathology. Regarding neurons, early cell loss and the detection of intracellular Aβ are noteworthy (D'andrea et al., 2001; Giannakopoulos et al., 2003). Questions remain whether internal Aβ deposits are primarily signs of Aβ production by neurons, or are also contributing to the observed neuronal loss (D'andrea et al., 2001; Yu et al., 2010; Y. Zhang et

al., 2002). Astrogliosis in reaction to extracellular Aβ has been observed in cell culture as well as human tissue (DeWitt et al., 1998; Kashon et al., 2004). A potential function of astroglia in the removal of extracellular Aβ has been proposed, but these are opposed by indications that astrocytes may contribute to the local plaque load (Nagele et al., 2004; Wyss-Coray et al., 2003). Microglia seem to serve a protective role against AD by phagocyting Aβ and damaged neurons but can also have adverse effects on the disease progressing by increasing inflammation and neurotoxicity (Prokop et al., 2013).

5.4 Knowledge on Alzheimer's disease from genetic studies

The pathological hallmarks of AD have long been known and intensely studied, but the exact aetiology of the disease is still not known. However, the heritability of all AD forms taken together was estimated to be approximately 60 to 80 % (Gatz et al., 2006). The most common form of genetic variation in the human genome is single nucleotide polymorphism (SNP), which can be associated with an increased risk for a specific disease (Collins et al., 1997). Genome-wide association studies (GWAS) compare the genetic information of AD patients with age-matched cognitive healthy controls and have identified more than 40 gene loci with association to AD (Novikova et al., 2021). Identifying risk genes for AD can help to find new evidence on the underlying pathomechanisms of the disease.

Apolipoprotein E (APOE) ε4 is considered the strongest genetic risk factor for AD. APOE is an important protein for cholesterol and lipid metabolisms and shows a genetic polymorphism. With APOEε2, APOEε3, and APOEε4, three major alleles are present in the human population (Corbo & Scacchi, 1999). The allele APOEε4 has a frequency of 13.7 % and is strongly associated with an increased risk for AD (Corder et al., 1993; Farrer et al., 1997; Gharbi-Meliani et al., 2021). Of all known AD risk genes, APOEε4 had the strongest association with amyloid deposition measured by florbetapir PET scan (Apostolova et al., 2018).

Several studies found that the gene for the receptor adenosine triphosphate–binding cassette subfamily A member 7 (ABCA7) has a high association with AD risk (Bellenguez et al., 2017; Hollingworth et al., 2011; Kunkle et al., 2019; Lambert et al., 2013; Reitz et al., 2013; Schwartzentruber et al., 2021). Furthermore, GWAS showed that ABCA7 has a strong association with Aβ plaque burden (Apostolova et al., 2018; Hughes et al., 2014; Shulman et al., 2013; Q.-F. Zhao et al., 2016). Further evidence from genetic studies regarding ABCA7 will be described more in-depth below.

In multiple GWAS, TREM2 has shown a strong association to AD risk and brain amyloidosis (Apostolova et al., 2018; Bellenguez et al., 2017; Guerreiro et al., 2012). The protein binds to

lipoproteins such as APOE and plays an important role in phagocytotic processes of the brain (Kleinberger et al., 2014; Takahashi et al., 2005; F. L. Yeh et al., 2016). The majority of publications suggest that TREM2 is expressed by microglia, but these studies mainly used cell culture or mouse brain tissue for the experiments (Fassler et al., 2021; Joshi et al., 2021; Kleinberger et al., 2014; Schmid et al., 2002; Sessa et al., 2004; F. L. Yeh et al., 2016). However, several studies utilizing IHC did not find any TREM2 expression in microglia in the human brain (Fahrenhold et al., 2018; J. I. Satoh et al., 2013; J. ichi Satoh et al., 2011). Fahrenhold et al. (2018) proposed TREM2 as a marker of monocyte recruitment from the bloodstream instead. In contrast to these studies, TREM2 protein expression could be detected in human brains via IHC when using a different antibody (Lue et al., 2015). These discrepancies might result from post-translational modifications that affect the antigen-binding site of the two different antibodies, as Hashioka et al. (2020) suggest in a review. The TREM2 expression in monocytes highlights the possible role of peripheral immune processes in the development of AD (Fahrenhold et al., 2018; Hashioka et al., 2020). Even though the implications of this discrepancy are not yet clear, they illustrate the importance of IHC experiments on human tissue to further understand the function of AD risk genes in the human brain.

GWAS were able to identify a multitude of other genes with clear association to brain amyloidosis, including Desmoglein 2 (DSG2), Sortilin Related Receptor 1 (SORL1), clusterin (CLU), complement C3b/C4b receptor 1 (CR1), and phosphatidylinositol binding clathrin assembly protein (PICALM) (Apostolova et al., 2018; Chibnik et al., 2011; Shulman et al., 2013). Evidence from such genetic studies can be used to find new focuses for experimental studies on the pathomechanisms behind AD.

5.5 ATP-binding cassette subfamily A member 7 (ABCA7)

5.5.1 Genetics, structure and expression

GWAS identified ABCA7 as one of the top genetic risk loci for AD (Apostolova et al., 2018). The ABCA7 gene is located on chromosome 19p13.3 and consists of 47 exons (Broccardo et al., 2001; Kaminski et al., 2000). ABCA7 was first described by Kaminski et al. in 2000. The protein belongs to the full-size ATP-binding cassette transporters, a family of active transmembrane transporters for a variety of molecules (Kaminski et al., 2000). The structure of ABCA7, which is shown in Figure 10, is typical for ATP-binding cassette transporters and has the closest homology with ABCA1 (Kaminski et al., 2000). The protein is made of 2146 amino acids and has a molecular weight of about 220 kDa (Kaminski et al., 2000).

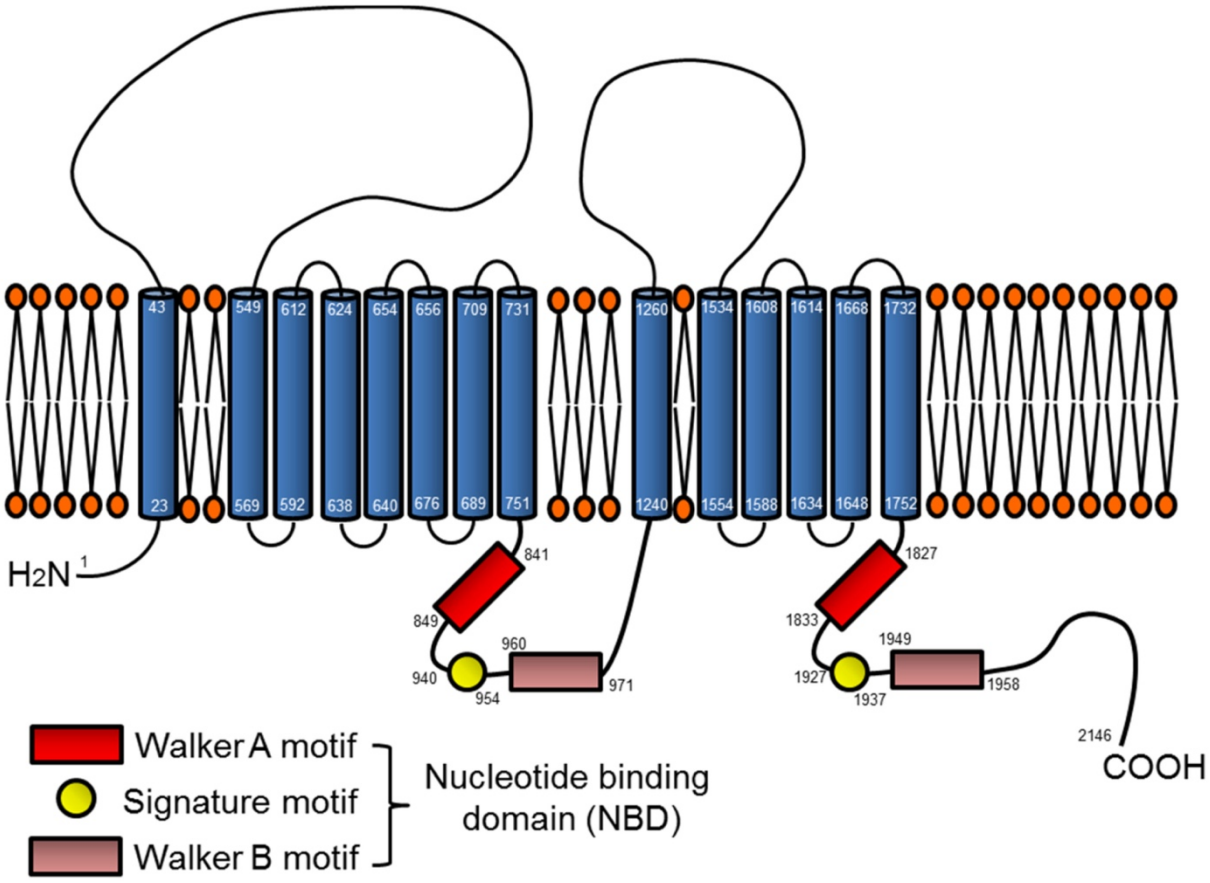


Figure 10: Topological structure of ATP-binding cassette subfamily A member 7

ATP-binding Cassette Subfamily A Member 7 (ABCA7) has two transmembrane domains, which determine the specificity of the transported molecule. Two large loops act as extracellular substrate-binding domains. The protein possesses two nucleotide-binding domains, each consisting of a Walker A, Walker B and signature motif. The transmembrane lipid transport is dependent on the binding of adenosine triphosphate (ATP) to the nucleotide-binding domains. (Dean & Allikmets, 1995; Kaminski et al., 2000) Figure adapted from Aikawa et al., 2018.

Due to alternative splicing, two isoforms of ABCA7 are found in humans. Type I ABCA7, which is produced from full-length cDNA, is found in the plasma membrane, and is predominantly expressed in the human brain and bone marrow (Y. Ikeda et al., 2003). The splicing variant type II is localized in the ER and seems to be more abundant in lymph nodes, spleen, thymus, and trachea (Y. Ikeda et al., 2003). Kaminski et al. (2000) found high expression of ABCA7 mRNA in human myelo-lymphatic tissue, such as thymus, spleen, lymph nodes, and bone marrow. In this first study on ABCA7, Kaminski et al. (2000) did not detect ABCA7 in brain tissue, but a study using real-time polymerase chain reaction (PCR) for mRNA quantification on human brain cells could detect high ABCA7 gene expression in microglia (Kim et al., 2006). Additionally, that study found ABCA7 expression in lower levels in oligodendrocytes, astrocytes and neurons (Kim et al., 2006). In mice, ABCA7 gene expression was detected in various tissues from adult animals and embryos and had the highest levels in spleen and hematopoietic tissue (Broccardo et al., 2001).

The localisation of ABCA7 protein expression seems to vary in different species. Sasaki et al. studied ABCA7 protein expression in rats and found the highest expression of the protein in platelets (Sasaki et al., 2003). Interestingly, Ikeda et al. (2003) did not detect ABCA7 protein in human peripheral blood cells. In mice, ABCA7 protein was widely expressed in various tissues, with the highest protein levels in the brain, lung, spleen, and adrenal gland (N. Wang et al., 2003). In wild-type mice, ABCA7 protein expression was detected in primary neurons and glial cells in comparable levels (K. Satoh et al., 2015).

5.5.2 Function of the protein

Similar to the closely related ABCA1, ABCA7 seems to play an important role in lipid metabolism. When analysing plasma lipid profiles of AD and control patients, the ABCA7 gene showed a differential association with the majority of diglycerides, which indicates a link between ABCA7 and lipid metabolism in the context of AD (Y. Liu et al., 2021). Several publications describe that ABCA7, as well as ABCA1, are responsible for the transport of phospholipids, and probably also cholesterol, out of the cell (Abe-Dohmae et al., 2006; M. Hayashi et al., 2005; Y. Ikeda et al., 2003; Picataggi et al., 2022; Quazi & Molday, 2013; N. Wang et al., 2003). It was shown in several studies using HEK293 cells that ApoA1 stimulates a dose-dependent lipid release from the cell (Abe-Dohmae et al., 2006; M. Hayashi et al., 2005; C. Wu et al., 2013). Ikeda et al. (2003) found that only type I ABCA7, which is localized in the plasma membrane, promotes ApoA1 mediated lipid release, but not type II from the ER. Using HEK293 cell clones, Wu et al. (2013) showed that ABCA7 expression is downregulated by 25-hydroxycholesterol, while an opposite regulating effect was shown for ABCA1. Contrarily to these findings, Kim et al. (2005) reported that cholesterol and phospholipid efflux did not change in ABCA7-deficient mice and therefore suggested that the previously described activities are not central to the proteins' physiological function. Interestingly, a recent study examined lipid subclasses in ABCA7 knockout mice and found gender-specific differences which were altered in comparison to wild type mice (Fu et al., 2022). The authors argue that these sex-specific differences in ABCA7 function might contribute to the previously described gender-differences in AD incidence. In conclusion, it appears that ABCA7 influences lipid homeostasis by mediating lipid release from the cell, but its exact impact on cellular functions remains uncertain.

In human brain cells, ABCA7 is predominantly expressed in microglia (Kim et al., 2006). Therefore, it is not surprising that the protein seems to have a function for phagocytic mechanisms. Downregulation of ABCA7 in cell culture and mouse models resulted in a significantly decreased phagocytic function (Iwamoto et al., 2006; Jehle et al., 2006; Tanaka et al., 2010).

ABCA7 has a high sequence similarity to the Caenorhabditis elegans gene CED-7, which is known to mediate phagocytosis of apoptotic cells in the nematode species (Jehle et al., 2006). Therefore, Jehle et al. (2006) investigated the role of ABCA7 in the phagocytosis of apoptotic cells. The signalling mechanisms behind the phagocytosis of apoptotic cells are only partially understood. Phagocytosis can be initiated by Fc receptors that recognize the Fc fragment of IgG antibodies (Aderem & Underhill, 1999). Activation of the serine/threonine kinase extracellular signal-regulated kinases (ERK) was as well described in several phagocytic cells (García-García et al., 2002; Hazan-Halevy et al., 2000; Karimi & Lennartz, 1998; Raeder et al., 1999). It was shown by Jehle et al. (2006) that ABCA7 is integral for phagocytosis of apoptotic cells via ERK phosphorylation, but does not seem to be involved in FcR-mediated processes of phagocytosis. Another study found that the levels of ERK were significantly elevated in ABCA7-deficient mice, which further validates that ABCA7 regulates ERK pathways in the brain (Sakae et al., 2016). It was further hypothesised that ABCA7 might be implicated in phospholipid translocation to the outer leaflet of the plasma membrane, possibly an important step in the phagocytic process (Jehle et al., 2006). The receptor LRP1 mediates, among others, endocytosis and has been hypothesised to modulate APP processing (S. L. Chan et al., 2008; De Strooper & Annaert, 2000; Knauer et al., 1996). Jehle et al. (2006) found that during phagocytosis, ABCA7 and LRP1 are trafficked together to the phagocytic cup where they then colocalise.

5.5.3 ABCA7 in the context of Alzheimer's disease

The first evidence for the association of ABCA7 and AD was published by Hollingworth et al. (2011), who identified the common SNP variant rs3764650 as a susceptibility locus for AD. Since then, several studies proved that ABCA7 expression is increased in cases with cognitive decline and AD (Humphries et al., 2015; Karch et al., 2012; Vasquez et al., 2013). Karch et al. (2012) identified an association of the minor allele of rs3764650 with later age at onset and shorter duration of AD. Vasquez et al. (2013) observed that this minor allele was associated with an increased ABCA7 expression and suggested that an upregulated ABCA7 expression through SNP variants reduces the AD risk. However, the same study detected an increased ABCA7 expression in individuals with AD which might be due to an inadequate upregulation in AD brains (Vasquez et al., 2013). Additionally, the low-frequency ABCA7 variant rs72973581 was found to provide statistically significant protection against AD (Cuyvers et al., 2015; Sassi et al., 2016).

Consistent with this, several studies observed that loss-of-function mutations in the ABCA7 gene increase the AD risk (Campbell et al., 2022; Cuyvers et al., 2015; Steinberg et al., 2015; Vardarajan et al., 2015). For example, a study in Belgian AD and control cohorts showed that

premature termination codon mutations in the ABCA7 gene, which lead to a loss of function, were more frequent in AD patients (Bossaerts et al., 2021).

By whole genome sequencing, May et al. (2018) identified two rare ABCA7 variants in families with a pattern of autosomal dominant AD disease and concluded that these might contribute to the development of FAD in these families.

Several GWAS found an association of ABCA7 variants with Aβ deposition in the brain (Apostolova et al., 2018; Hughes et al., 2014; Shulman et al., 2013; Q.-F. Zhao et al., 2016). Furthermore, Apostolova et al. (2018) included the ABCA7 variants rs3764650 and rs3752246 in the top 20 AD risk variants and reported that, behind APOE ε4, ABCA7 had the second-highest association to brain amyloidosis.

Taking the results of the various GWAS together, it is clear that ABCA7 plays an important role in the development of AD. Today, it is seen as one of the most important genes associated with AD risk, even though the exact mechanisms behind this connection still need to be further explored with experimental work (Apostolova et al., 2018; Berg et al., 2019; De Roeck et al., 2019).

Kim et al. (2013) crossed ABCA7-deficient mice with J20 mice, a common AD mouse model with overexpressed APP, and found that the ABCA7 loss led to a stark increase of insoluble Aβ and thioflavin S-positive plaques. Increased levels of soluble Aβ in ABCA7-deficient mice were also observed in other studies, but soluble Aβ in the interstitial fluid did not seem to be affected (Sakae et al., 2016; K. Satoh et al., 2015). Microglia and bone marrow-derived macrophages from ABCA7-deficient mice were found to have a reduced capacity for phagocytosis of oligomeric Aβ (Fu et al., 2016; Kim et al., 2013). Interestingly, the total number of Iba1 positive microglia and the number of microglia around Aβ plaques were not reduced in ABCA7-deficient mice (Kim et al., 2013; Sakae et al., 2016). The mentioned studies provide evidence that ABCA7 function is closely connected to Aβ deposition and microglia function, but the mechanisms behind this association still need to be further explored.

Chan et al. (2008) proved that ABCA7 regulates APP processing and inhibits Aβ production. Their experiments showed that transfecting ABCA7 cDNA into Chinese hamster ovary cells that constantly express human APP reduces the levels of secreted sAPPα, sAPPβ, and Aβ significantly. The authors stated that the observed decrease of Aβ was not connected with an increase in sAPPα secretion and was therefore not caused by increased activity of the α-secretase pathway. Further experiments using fluorogenic substrates on the same cell line proved that ABCA7 has no impact on the activity of α-, β-, or γ-secretases in cell culture (S. L.

Chan et al., 2008). Additionally, K. Satoh et al. (2015) reported that ABCA7-deficiency increased levels of Aβ and APPβ in mice, indicating that ABCA7 loss directly impacts APP processing. Post-mortem neuropathological examination of patients with loss-of-function and missense mutations showed typical AD and CAA pathology (Bossaerts et al., 2022; Campbell et al., 2022).

Several studies have shown that ABCA7 deficiency alters cognitive function in mice. Logge et al. (2012) found that even though short-term spatial memory was unchanged in five months old ABCA7-deficient mice, the male mice in this experiment showed significantly impaired novel object recognition memory. Intriguingly, another study found that ABCA7-deficient 20 months old mice showed spatial memory deficits in comparison with wild-type mice of the same age (Sakae et al., 2016). These contradictory results suggest an age-dependent cognitive decline associated with ABCA7 deficiency (Sakae et al., 2016). When male 17-month-old amyloidogenic J20 mice were compared to age-matched combined J20 and ABCA7-deficient mice, both groups showed memory deficits in comparison to wild-type mice but did not show any significant differences between each other (Kim et al., 2013). Using 5-Bromo-2-deoxyuridine staining to assess cell proliferation in mouse brains, Li et al. (2015) found that ABCA7 does not affect cell proliferation or neurogenesis in adult mice. Further experiments are needed to explain the effects of ABCA7 on cognitive function, with and without corresponding Aβ pathology.

A recent publication by Lyssenko & Praticò (2021) proposes a new hypothesis in which neurons produce a neurodegenerative lipid that is removed from the cell by ABCA7. Using western blot analyses of human brains, the authors discovered that individuals with low ABCA7 protein levels developed AD earlier in life and individuals with high ABCA7 levels developed AD very late. The study found that ABCA7 protein levels decline in normal ageing and proposes a dose-dependent protective role for ABCA7 in AD (Lyssenko & Praticò, 2021).

In conclusion, it can be said that ABCA7 possibly affects the development of AD by affecting lipid homeostasis, modulating Aβ production and altering the phagocytic function of microglia and macrophages, which is summarized in Figure 11 (Kim et al., 2013; Lyssenko & Praticò, 2021; Sakae et al., 2016; K. Satoh et al., 2015).

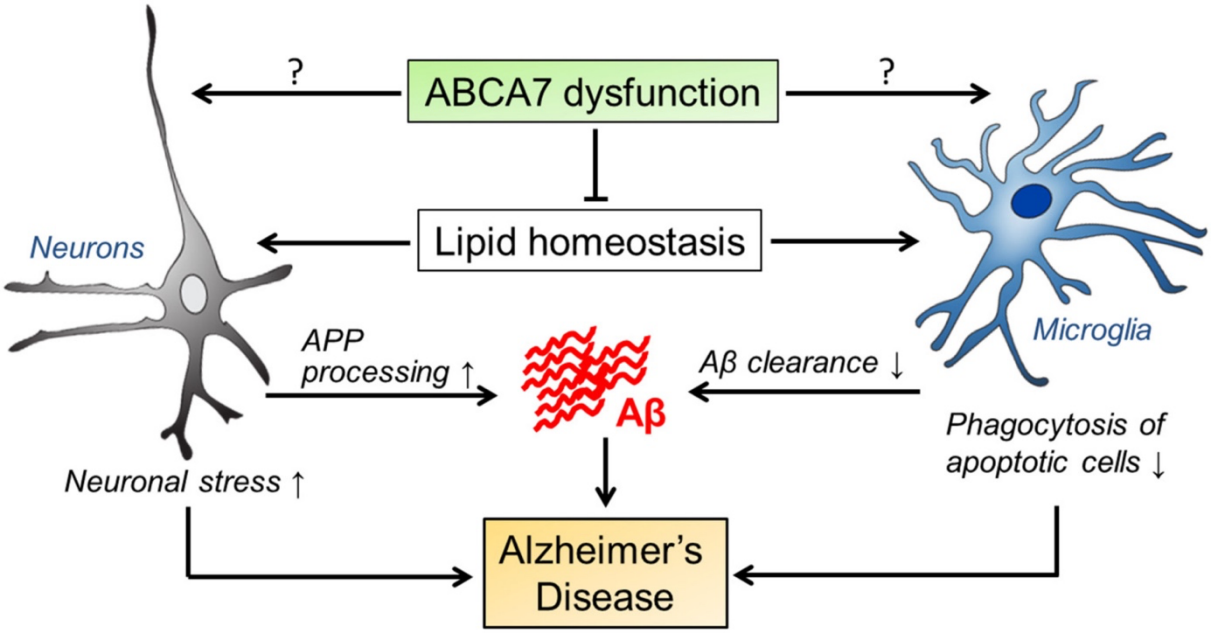


Figure 11: Possible pathways for the association of ABCA7 and Alzheimer's disease

Genome-wide association studies and animal experiments linked reduced expression of ABCA7 with increased amyloid beta (Aβ) accumulation. This figure visualises possible mechanisms behind the protein's impact on Aβ deposits and Alzheimer's disease. A possible explanation is **altered lipid homeostasis** in the brain, but direct mechanisms of influence on neurons and microglia are still unknown. ABCA7 could influence the disease progression through amyloid precursor protein (**APP) processing** and **phagocytosis of Aβ and apoptotic neurons**. Figure adapted from Aikawa et al., 2018.

Being one of the most important risk genes for AD, strong evidence from genetic studies shows the significance of understanding the functions of ABCA7 and its relation to this neurodegenerative disease. Table 1 lists the major discoveries about ABCA7 since its first description in 2000. Similar to other ATP-binding cassette transporters, ABCA7 seems to be involved in lipid metabolism. Genetic studies, as well as experiments with mouse models, show a clear association of ABCA7 expression and Aβ deposits. Different mechanisms involving phagocytosis and APP processing have been proposed as an explanation, but there is still little known about the function of the protein.

Table 2 provides a visualisation of the experimental materials and the level of expression that were examined by the most significant studies on ABCA7. It is notable that the current knowledge on the topic primarily comes from either genetic analyses or experiments using mouse models or cell culture. However, mouse models and in vitro work can not accurately represent AD pathology or the exact physiological processes in the human brain. Further research examining the protein expression of ABCA7 in human tissue needs to be conducted to gain more insight into the association of ABCA7 and AD pathology, which was established by genome-wide association studies.

Table 1: Overview of the current knowledge on ABCA7

Structure & Expression	
Kaminski et al. (2000)	The first description of the novel full-size ATP-binding cassette transporter ABCA7.
Ikeda et al. (2003)	Alternative splicing produces two types of ABCA7 mRNA: Type I is expressed in the plasma membrane. Type II is localised in the ER.
Kim et al. (2006)	In human cell culture, ABCA7 mRNA is most expressed in microglia.
Vasquez et al. (2013)	ABCA7 gene expression is increased in AD brains.
Lyssenko & Praticò (2021)	Comparable ABCA7 protein levels were found in the human hippocampus and parietal cortex. ABCA7 protein levels in the human brain decrease in normal ageing. Patients with high ABCA7 levels developed AD later than those with low ABCA7 levels.
Genetic variants with AD association*	
Hollingworth et al. (2011)	Novel SNP variant rs3764650 is associated with AD.
Karch et al. (2012)	ABCA7 gene expression is increased in dementia cases. The minor allele of rs3764650 is associated with age at onset and disease duration.
Vasquez (2013)	The rs3764650 allele is associated with increased ABCA7 gene expression and decreases the AD risk.
Shulman et al. (2013) **Hughes et al. (2014)** **Zhao et al. (2016)** **Apostolova et al. (2018)**	Association of multiple ABCA7 gene variants with brain amyloidosis.
Cuyvers et al. (2015) **Steinberg et al. (2015)** **Vardarajan et al. (2015)** **Campbell et al. (2022)**	Loss-of -function mutation of the ABCA7 gene increases AD risk in Caucasian populations.
Cuyvers et al. (2015) **Sassi et al. (2016)**	ABCA7 gene variant rs72973581 is protective against AD.
Lipid Metabolism	
Wang et al. (2003) **Hayashi et al. (2005)** **Quazi & Molday (2013)**	ABCA7 is involved in the transport of phospholipids and cholesterol out of the cell.
Kim et al. (2005)	Cholesterol and phospholipid efflux is not changed in ABCA7-deficient mice.
Wu et al. (2013)	ABCA7 expression is downregulated by 25-hydroxycholesterol.
APP Processing, Phagocytosis & Aβ Homeostasis	
Jehle et al. (2006)	ABCA7 has a high sequence similarity to CED-7. ABCA7 colocalises with LRP1. ABCA7 is involved in the phagocytosis of apoptotic cells via ERK phosphorylation.
Chan et al. (2008)	ABCA7 regulates APP processing and inhibits Aβ production in Chinese hamster ovary cells.
Kim et al. (2013) **K. Satoh et al. (2015)** **Sakae et al. (2016)**	ABCA7-deficiency leads to an increase in insoluble Aβ and plaques in mice. Soluble Aβ levels are not affected.
Kim et al. (2013) **Sakae et al. (2016)**	The numbers of microglia around plaques and total microglia are not reduced in ABCA7-deficient mice.
Kim et al. (2013) **Fu et al. (2016)**	ABCA7-deficiency decreases the phagocytosis capacity of microglia and macrophages from mice.
K. Satoh et al. (2015)	ABCA7-deficiency increases Aβ and APPβ levels in mice.
Effects on Neurobehavior in Mice	
Logge et al. (2012)	Male adult ABCA7-deficient mice show impaired novel object recognition memory. Five months old ABCA7-deficient mice show no change in short-term spatial memory.
Li et al. (2015)	ABCA7 does not affect cell proliferation or neurogenesis in adult mice.
Sakae et al. (2016)	ABCA7-deficient 20 months old mice show spatial memory deficits. * Multiple sources are stated if different publications had similar findings. Publications with more than one finding can be listed several times.

Table 2: Overview of study designs of notable publications on ABCA7

	DNA	RNA	PROTEIN
HUMAN TISSUE	**Hollingworth et al. (2011)** **Karch et al. (2012)** **Shulman et al. (2013)** **Vasquez et al. (2013)** **Hughes et al. (2014)** **Steinberg et al. (2015)** **Vardarajan et al. (2015)** **Sassi et al. (2016)** **Zhao et al. (2016)** **Apostolova et al. (2018)** **May et al. (2018)** **Bossaerts et al. (2022)** **Campbell et al. (2022)**	**Cuyvers et al. (2015)**	**Lyssenko & Praticò (2021)**
MOUSE MODELS	Studies on ABCA7 knockout mice: **Logge et al. (2012)** **Kim et al. (2013)** **K. Satoh et al. (2015)** **Sakae et al. (2016)**		**Kim et al. (2005)** **Jehle et al. (2006)**
CELL CULTURE		**Kaminski et al. (2000)** **Ikeda et al. (2003)** **Kim et al. (2006)** **Wu et al. (2013)**	**Ikeda et al. (2003)** **Wang et al. (2003)** **Hayashi et al. (2005)** **Jehle et al. (2006)** **Chan et al. (2008)** **Quazi & Molday (2013)** **Wu et al. (2013)** **Bossaerts et al. (2022)**

5.6 Aim of this study

The aim of this study is to investigate the protein expression of ABCA7 in brain samples of human AD cases. The neurodegenerative disease AD is the most common form of dementia and affects the lives of more than 1 million patients in Germany. The two main histopathological hallmarks, Aβ deposits and aggregates of tau protein, have been studied intensively, but the underlying pathological processes causing the disease are still not known today. GWAS have identified several genes with a high impact on AD risk and ABCA7 has been identified as one of the top AD risk gene loci. The majority of previous studies on ABCA7 have investigated genetic variants or examined the protein expression in cell culture or animal models. This study will be one of the first investigations of ABCA7 protein expression in the human brain and thus help to understand the role of ABCA7 in the context of AD. The objectives of this study are:

- to establish an IHC double staining protocol to visualize ABCA7 expression and its relation to other protein markers in human brain samples.
- to use this double staining method to classify the types of cells that are expressing ABCA7 in brains of human AD cases.
- to analyse the relation of ABCA7-positive cells to Aβ plaques to bring new insight into the protein's affiliation to AD pathology.

6. Material

Table 3: Instruments

Product	Supplier
Blue Cooler cooling plate	RWW Medizintechnik, Hallerndorf, Germany
Heating chamber	Heraeus Instruments, Hanau, Germany
Laboratory glassware	Schott, Mainz, Germany
Magnet stirrer	Heidolph Instruments, Schwabach, Germany
Microtome HistoCore Multicut	Leica Biosystems, Wetzlar, Germany
Microscope Zeiss Imager.Z2	Carl Zeiss, Göttingen, Germany
Pipettes P2, P20, P100, P200, P1000	Brand, Wertheim, Germany
pH meter	Knick, Berlin, Germany
Scale CP 4202 S	Sartorius, Göttingen, Germany
Steam cooker HD 9140	Philips, Amsterdam, Netherlands
Slide scanner Zeiss Axioscan.Z1	Carl Zeiss, Göttingen, Germany
Tissue-Tek® embedding console system	Sakura, Tokyo, Japan
Tissue processor HistoCore Pearl	Leica Biosystems, Wetzlar, Germany
Vortex shaker Reax top	Heidolph Instruments, Schwabach, Germany
Water bath GFL 1052	GFL, Burgwedel, Germany

Table 4: Experimental materials

Product	Supplier
Coverslips (24x40 mm)	R. Langenbrinck, Emmerdingen, Germany
Folded filters (150 mm)	Sartorius, Göttingen, Germany
Pipette tips (2 µl, 20 µl, 200 µl, 1000 µl)	Sarstedt, Nümbrecht, Germany
Light duty tissue wipers	VWR International, Radnor, USA
Microscope glass slides (76 x 26 mm)	R. Langenbrinck, Emmerdingen, Germany
Reagent tube (0.5 ml, 1.5 ml, 2 ml)	Eppendorf, Hamburg, Germany
Reagent tube (50 ml)	Sarstedt, Nümbrecht, Germany
Shandon plastic Coverplate™	Thermo Fisher Scientific, Waltham, USA

Table 5: Chemicals and reagents

Product	Supplier
Antibody diluent	Dako Agilent, Santa Clara, USA
Aquatex® aqueous mounting agent	Merck Millipore, Darmstadt, Germany
Casein pure	Serva, Heidelberg, Germany
Citric acid	Serva, Heidelberg, Germany
3,3'Diaminobenzidine (DAB)	Dako Agilent, Santa Clara, USA
DMSO	Serva, Heidelberg, Germany
Entellan® Neu	Merck Millipore, Darmstadt, Germany
Ethylenediamine tetraacetic acid	Serva, Heidelberg, Germany
Ethanol 99 %	SAV Liquid Production, Flintsbach am Inn, Germany
Formalin	Carl Roth, Karlsruhe, Germany
Formic acid	Otto Fishar, Saarbrücken, Germany
HistoChoice® clearing agent	Sigma-Aldrich, St. Louis, USA
Hydrogen chloride	Thermo Fisher Scientific, Waltham, USA
Hydrogen peroxide (30 %)	Otto Fishar, Saarbrücken, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Levamisole	Sigma-Aldrich, St. Louis, USA
Methoxy-X04	Bio-Techne, Minneapolis, USA
Meyer's hemalum solution	Merck Millipore, Darmstadt, Germany
Naphthol AS-BI-phosphate	Serva, Heidelberg, Germany
NaCl 0.9 %	Braun, Melsungen, Germany
New fuchsin	Thermo Fisher Scientific, Waltham, USA
N,N-Dimethylformamide	Merck Millipore, Darmstadt, Germany
Paraffin wax	Sigma-Aldrich, St. Louis, USA
Sodium hydroxide	Carl Roth, Karlsruhe, Germany
Sodium nitrite	Carl Roth, Karlsruhe, Germany
Tris(hydroxymethyl)aminomethane (TRIS)	Carl Roth, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich, St. Louis, USA
Tween 20	Thermo Fisher Scientific, Waltham, USA
VectaMount® permanent mounting medium	Vector Laboratories, Burlingame, USA
Xylene	Otto Fishar, Saarbrücken, Germany

Table 6: Primary antibodies and peptides

Name, catalogue number	Host, class	Dilution	Supplier
6E10 Aβ (Z932002-Y)	Mouse, monoclonal	1:1000	Zytomed Systems, Bargteheide, Germany
ABCA7 (25339-I-AP)	Rabbit, polyclonal	1:250	Proteintech, Rosemont, USA
ABCA7 peptide	immunogen of ABCA7 antibody		Proteintech, Rosemont, USA
AT8 pTau (MN1020)	Mouse, monoclonal	1:1000	Thermo Fisher Scientific, Waltham, USA
CD68 (M0876)	Mouse, monoclonal	1:100	Dako Agilent, Santa Clara, USA
Iba1 (5076)	Goat, polyclonal	1:500	Abcam, Cambridge, UK

Table 7: Prefabricated kits

Name of Kit	Supplier
EnVision® detection system peroxidase/DAB, rabbit/mouse	Dako Agilent, Santa Clara, USA
ImmPRESS®-AP horse anti-mouse IgG polymer kit	Vector Laboratories, Burlingame, USA
ImmPRESS®-AP horse anti-goat IgG polymer kit	Vector Laboratories, Burlingame, USA
Vector® Blue substrate kit, AP	Vector Laboratories, Burlingame, USA

Table 8: Buffer solutions

Buffer	Material	
Blocking buffer (0,2 % Casein)	Casein	100 mg
	Tween 20 (0.1 %)	1 ml
	Triton X-100 (0.01 %)	10 µl
	PBS	100 ml
Citrate buffer pH 6.0 (10x, 10 mM)	Citric acid	2.014 g
	H_2O (reagent quality)	1 l
Phosphate-buffered saline (PBS) pH 7.4 (10x)	NaCl	400 g
	KCl	10 g

	Na_2HPO_4	71 g
	NaH_2PO_4	69 g
	H_2O (reagent quality)	5 l
1x PBS	10x PBS	100 ml
	H_2O (reagent quality)	900 ml
TRIS-buffered saline (10x TBS)	NaCl	425 g
pH 7.4	TRIS	302.5 g
	H_2O (reagent quality)	5 l
1x TBS	10x TBS	100 ml
	H_2O (reagent quality)	900 ml
TRIS/EDTA buffer pH 9.0	TRIS-Base	0.65 g
	EDTA	0.18 g
	H_2O (reagent quality)	500 ml
	Tween 20	0.25 ml
TRIS/HCl buffer pH 8.5	TRIS-Base	9 g
	H_2O (reagent quality)	500 ml
	HCl	titration for pH 8.5

7. Methods

7.1 Case selection and ethical approval

A total of seven cases were selected from the tissue bank of the Institute of Neuropathology of the Saarland University. The tissue bank has obtained the donors' consent to use the tissue for scientific purposes. The cases were selected to represent varying intensities of AD pathology, which were evaluated using the CERAD classification according to Mirra et al. (1991). Ethical approval for the project was granted by the ethical commission of the Ärztekammer Saarland (file number 320/20).

7.2 Tissue processing and sectioning

After post-mortem macroscopic evaluation by an experienced clinical neuropathologist, the human brain was dissected and cut into blocks. Afterwards, the tissue was subjected to the standardised tissue treatment according to the protocol of the neuropathological department. Accordingly, the tissue was fixated for 24 hours in 10 % neutral buffered formalin. It was then dehydrated and paraffin impregnated overnight using the tissue processor HistoCore Pearl. This automated processing system dehydrated the brain tissue in increasing concentrations (50 %, 70 %, three times 90 %, three times 100 %) of isopropanol and then used xylene as an intermediate medium. Afterwards, the tissue was automatically immersed three times for one hour in 65 °C paraffin. The Tissue-Tek® Embedding Console System was used to manually embed the brain tissue in paraffin blocks. For this, the tissue was placed onto a plastic cassette and then cast with melted paraffin using steel base moulds. After the paraffin block had hardened, it was released from the mould. The tissue was sectioned with a microtome with a thickness of 1 µm. To facilitate clean cutting, the paraffin block was placed on a cooler beforehand. The thin tissue was then cautiously transferred into a warm water bath using a brush. This step extended the tissue and eliminated folds. From the water bath, the tissue was placed on silanated glass slides, a standard procedure previously described (Tourtellotte et al., 1987). The sections were afterwards dried at 37 °C overnight and stored at RT.

7.3 Immunohistochemistry

7.3.1 Principles of double staining

The goal of this investigation was, to study the expression of ABCA7 in relation to other structures found in the pathology of AD. Establishing a double staining protocol, allowed the staining of two different antigens within one section. Using primary antibodies from different species, in this case rabbit and mouse, made it possible to stain the antigens with two distinct colours. Two prefabricated kits were used as secondary antibodies. The EnVision® detection

system by Dako recognized primary antibodies raised in mice and rabbits and developed brown staining using HRP and DAB. By using polymeric conjugates of secondary antibodies and peroxidase enzymes, it ensured a high specificity of the staining (Sabattini et al., 1998). The ImmPRESS®-AP horse anti-mouse IgG polymer kit from Vector Laboratories used alkaline phosphatase enzyme micropolymers attached to anti-mouse IgG secondary antibodies. When using new fuchsin as a chromogen, the enzyme produced a red-violet colour.

In the first part of the double staining, a primary antibody derived from rabbit was used in combination with the EnVision® system and DAB. The second primary antibody was incubated on the sections after the development of the permanent DAB staining. It had mouse as a host and was combined with the ImmPRESS® kit and new fuchsin as a chromogen. In this order, the EnVision® system only recognized the rabbit primary antibodies and the ImmPRESS® kit stained the primary mouse antibody with new fuchsin.

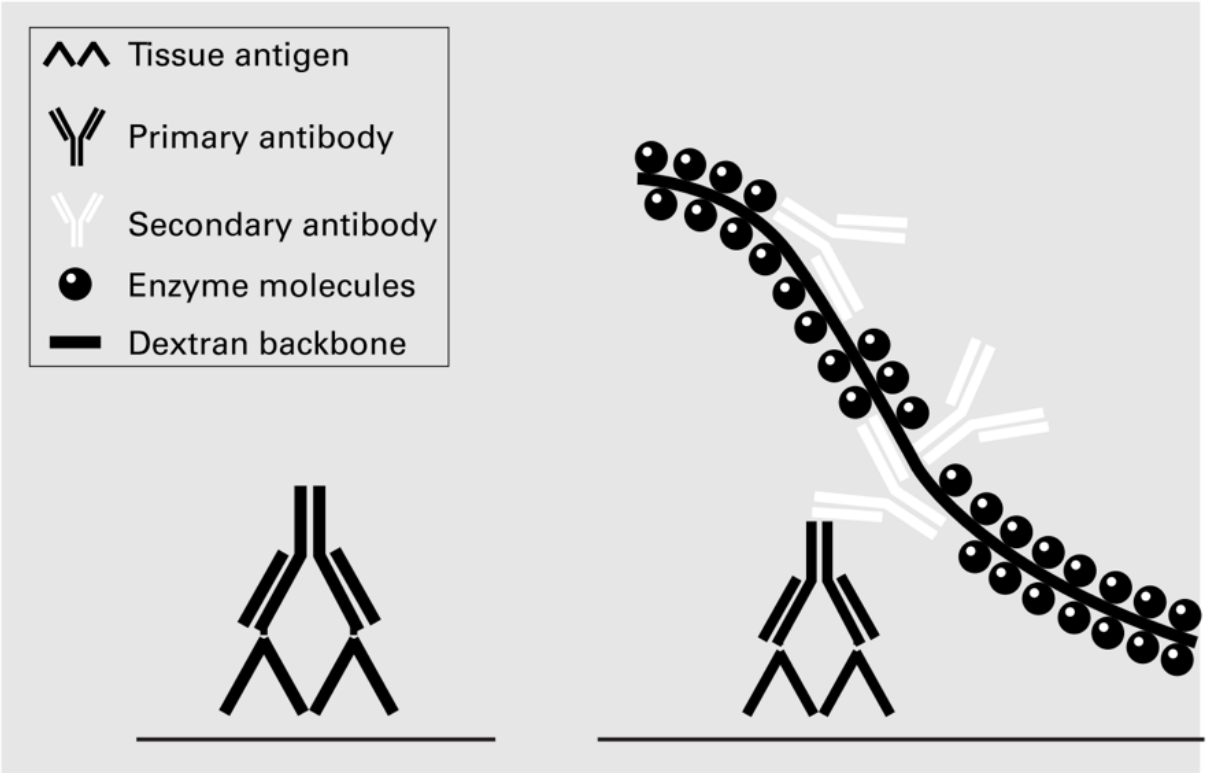


Figure 12: Two systems for polymer-based reagents

In the depicted EnVision® system the polymer is conjugated with secondary antibodies and labelled with horseradish peroxidase. The ImmPRESS® system uses a secondary antibody that is conjugated with an alkaline phosphatase enzyme polymer. It contains a similar polymer structure to the EnVision® system, but is not separately illustrated here. Figure adapted from Sabbattini et al. (1998).

7.3.2 Confirmation of antibody specificity

The specificity of each staining in the double staining was ensured by using control sections, on which one of the primary antibodies was omitted. To further test the specificity of the antibody against ABCA7, a blocking peptide was used. This synthesized peptide, which is the immunogen of the ABCA7 antibody, was ordered from the manufacturer of the primary antibody. The peptide's molecular weight was calculated according to its amino acid sequence. In three separate vials, the blocking peptide was added to the antibody at a ratio of 1:1, 1:5 and 1:25 by molecular weight of antibody to blocking peptide and diluted in antibody diluent. The blocking peptide was incubated with the antibody for 2 hours at RT. An equivalent amount of antibody diluent was added to the anti-ABCA7 antibody in a second vial and incubated parallelly. The staining protocol for ABCA7 IHC, as described below, was performed on sections from the same paraffin block. As a primary antibody, either the mixture of blocking peptide and antibody in varying concentrations or antibody diluted without the peptide was used. Staining that was not visible when using the blocking peptide was considered specific.

7.3.3 Double staining for ABCA7 and 6E10 amyloid beta

The tissue was deparaffinized with a decreasing alcohol gradient. This was started with an immersion of the sections twice for five minutes in xylene, then two times for five minutes in 100 % ethanol and one minute each in 96 % ethanol and 70 % ethanol. Which was followed by a washing step in running reagent-grade water. After this, the tissues' endogenous peroxidase was quenched with an immersion in 0.9 % H_2O_2 in reagent-grade water for ten minutes at RT and then washed three times with reagent-grade water. Antigen unmasking was performed with a TRIS/EDTA buffer with pH 9 using a pre-heated steam cooker for 15 minutes. The sections were then left in the pH 9 buffer to cool down for additional 30 minutes and washed with reagent quality water. As the use of the 6E10 clone as a primary antibody against Aβ requires further antigen unmasking, the sections were additionally treated with formic acid for 30 minutes at RT.

After another washing step with reagent-grade water, a blocking buffer containing 0.2 % casein was pipetted on the sections and left there for 15 minutes at RT. The slides were then brought onto plastic coverplates, mounted into a fitting deck, and washed once with TBS.

The anti-ABCA7 primary antibody was diluted with a concentration of 1:250 into a ready-to-use antibody diluent. Then, 100 µl of this freshly prepared solution were pipetted into each coverplate and left to incubate for 45 minutes at RT. The slides were then washed three times with TBS. While still mounted onto the coverplates, the sections were each incubated with 100

µl of the EnVision® solution for 30 minutes at RT and then washed three times with TBS. During this washing process, the DAB-substrate was freshly prepared with 25 µl DAB chromogen and 1 ml DAB-substrate solution, both from the Dako product range, providing an ideal outcome working together with the EnVision® secondary antibody system. The incubation time was set by evaluating the intensity of the staining by eye and under the microscope. While experience showed that an incubation time of 8 to 20 minutes is appropriate for most antibodies, the best results were seen with an incubation in DAB for 10 minutes at RT. After achieving the desired intensity of DAB-staining, the sections were washed with reagent-grade water.

After obtaining a permanent brown DAB staining of ABCA7-positive structures, the second part of the double staining protocol aimed to stain Aβ plaques with a distinguishable magenta colour. For this, the AP ImmPRESS® polymer detection kit was used in combination with new fuchsin as a chromogen. The ImmPRESS® system uses a secondary antibody made in horse. Therefore, an additional blocking step with 2.5 % horse serum, which was included in the ImmPRESS® polymer detection kit, was performed. After incubating the sections with 2.5 % horse serum for 20 minutes at RT, the slides were again mounted onto coverplates and washed once with washing buffer. The monoclonal primary antibody 6E10 against Aβ was diluted with a concentration of 1:1000 into antibody diluent and then pipetted into the coverplates. As in the first part of the double staining, 100 µl were used per section and the incubation time was 45 minutes at RT. Thereafter, the sections were washed three times and then incubated at RT for 30 minutes with the ImmPRESS®-AP polymer reagent.

While incubating the sections with the polymer reagent, three solutions needed for the new fuchsin chromogen were freshly prepared. TBS buffer was titrated with 1N sodium hydroxide to reach a pH of 8.8. To 50 ml of this TBS buffer with a pH of 8.8, 20 mg levamisole was added. In a different reagent tube, 14 mg naphthol AS-BI-phosphate was dissolved in 300 µl N,N-dimethylformamide. For the third solution, 10 mg sodium nitrite was dissolved into 250 µl reagent-grade water. Then, 100 µl new fuchsin solution, made from 5 g new fuchsin, 30 ml hydrogen chloride, and 100 ml reagent quality water, was added. The mixture of naphthol AS-BI-phosphate and N,N-dimethylformamide was then given into the TBS buffer with levamisole while stirring with a magnet stirrer. The pH of the new solution was raised to 8.9 with 1N sodium hydroxide and the already prepared new fuchsin and sodium nitrite solution was added to this. The prepared chromogen solution was filtered into a glass cuvette. While regularly checking the intensity of the staining by eye, the sections were immersed for 12 minutes in this chromogen solution and then gently rinsed with reagent-grade water.

Counterstaining was performed using hematoxylin and eosin (HE) with a staining time of five seconds and a five minutes development under warm running tap water. After washing once with reagent-grade water, the sections were dried with tissue paper and mounted with the aqueous mounting medium Aquatex®. To ensure timely drying of the mounting medium, the slides were kept for 30 minutes at 80 °C in a heating chamber. After this, the slides were stored in the dark at RT until they were assessed.

7.3.4 Single staining for ABCA7

For ABCA7 single staining, the previously described double staining protocol was followed until incubation with DAB, but with omittance of the incubation with formic acid. Counterstaining with HE was then directly performed. The sections were dehydrated with an ascending ethanol series, followed by immersion in xylene and mounted with Entellan® Neu.

7.3.5 Double staining for ABCA7 and AT8 tau protein

Double staining using primary antibodies against ABCA7 and pTau was performed by following the previously described protocol, but the treatment with formic acid was omitted. The anti-ABCA7 antibody was diluted with a concentration of 1:250 and the monoclonal antibody AT8 against pTau with a concentration of 1:1000. The time of development was 12 minutes in DAB and 14 minutes in new fuchsin. Counterstaining with HE was performed and the sections were mounted with Aquatex®.

7.3.6 Double staining for ABCA7 and CD68

When staining for ABCA7 and CD68, the previously described double staining protocol was used. The pre-treatment was performed with hydrogen peroxide, TRIS/EDTA buffer pH 9, and casein. The anti-ABCA7 antibody was used with a concentration of 1:250 and the anti-CD68 with a concentration of 1:100. DAB was developed for 10 minutes and new fuchsin for 12 minutes. HE counterstaining was performed as previously described and the sections were mounted with Aquatex®.

7.3.7 Sequential staining for ionized calcium-binding adapter molecule 1 and ABCA7

To achieve staining with antibodies against Iba1 and ABCA7 within the same structures in one section, a sequential double staining method was used. Antigen retrieval was performed for 30 minutes in citrate buffer pH 6 using a steamer. Afterwards, the sections were left in the buffer for further 30 minutes to cool down to RT. Non-specific binding was blocked by incubation with 2.5 % normal horse serum for 20 minutes. The sections were then incubated

with the anti-Iba1 antibody for one hour and diligently washed with TBS afterwards. The ImmPRESS®-AP horse anti-goat IgG polymer kit was used as a secondary antibody and incubated on the sections for 30 minutes. The substrate working solution from the Vector® Blue substrate kit for AP was freshly prepared in 100 mM TRIS-HCl solution according to the manufacturer's instructions. The sections were incubated in this substrate solution for 20 minutes and then washed in TBS and dehydrated using the xylene-free clearing agent HistoChoice®. The sections were finally mounted with VectaMount and stored at 4 °C. The staining was digitally scanned. To retrieve the Vector® Blue staining, the coverslip was first removed after a short incubation in xylene and the sections were then further immersed for two hours in xylene. As Vector® Blue is soluble in xylene this step completely cleared the previous staining and allowed a re-staining of the same section with the antibody against ABCA7. The staining for ABCA7 was performed according to the previously described protocol. The ABCA7 staining on the section was scanned again, which allowed me to simultaneously view and compare the stainings for Iba1 and ABCA7 on a computer.

7.3.8 Methoxy-X04

As an alternative to specific antibodies targeted at Aβ, the congo red derivative methoxy-X04 was also used to stain amyloid deposits. The chromogen is visible by fluorescence microscopy. This method of staining was added to a section after it had already been stained by IHC to offer further insight regarding the association of stained structures to Aβ deposits. After a standard dewaxing process, the sections were washed with PBS. In a reagent tube, 500 µl DMSO and 500 µl 0.9 % NaCl were mixed and 1 µl NaOH 1M was then added. Right before usage, 1 µl methoxy-X04 were added into the tube and mixed well with a vortex shaker. This solution was incubated on the sections for 15 minutes and then washed off with PBS. After a standard dehydration process followed by immersion in xylene, the slides were mounted with xylene-based Entellan® Neu. The slides were stored at 4 °C to avoid fainting of the fluorescent signal.

7.4 Image analysis and statistics

A first evaluation of the staining was done using a Zeiss Imager.Z2 microscope. The slides were then digitalized with a Zeiss Axioscan.Z1 slide scanner. For viewing and analysing the digitalized staining, the open-source software QuPath version 0.2.3 was used. The digital files were opened in a .czi format and viewed with the highest given quality. Brightness and contrast were adjusted to gain the best contrast between the DAB and new fuchsin staining. Stained microglia were identified and classified as associated with either a dense or diffuse Aβ plaque

or as microglia with no plaque association. The software's manual counting tool was used for quantification.

While the standard immunohistochemistry was viewed and scanned by brightfield microscopy, methoxy-X04 was only visible using fluorescence filters. The methoxy-X04 staining had the best visibility with the 430 nm filter. The two separate scans from the sequential double staining with methoxy-X04 and IHC were exported from the viewing software in the file format BigTIFF. Afterwards, the two files were digitally overlayed with the open-source graphic software GIMP 2.10.0, which enabled an uncomplicated analysis of the two files together.

Data analyses and graph creation were performed using GraphPad Prism 6. To determine the correlation of two variables, the Pearson correlation was used.

8. Results

8.1 ABCA7 expression in microglia of the human brain

Using IHC, ABCA7 expressing cells were detected in the frontal cortex of human brains with AD pathology. These cells had a ramified shape, which resembled the morphology of microglia. A double staining protocol for IHC staining of ABCA7 and CD68 was established and carried out on sections from the white and grey matter of an advanced AD case. The findings from this double staining are presented in Figure 13. In the grey matter, 77.1 % of all the cells expressing ABCA7 or CD68 were positive for both markers. In the same tissue area, 22.9 % of cells showed immunoreactivity for CD68 but not for ABCA7. In the white matter, 23.8 % of stained cells exhibited expression of both markers, while 76.2 % expressed CD68 alone. No cells with an expression of ABCA7 without CD68 were observed in either white or grey matter.

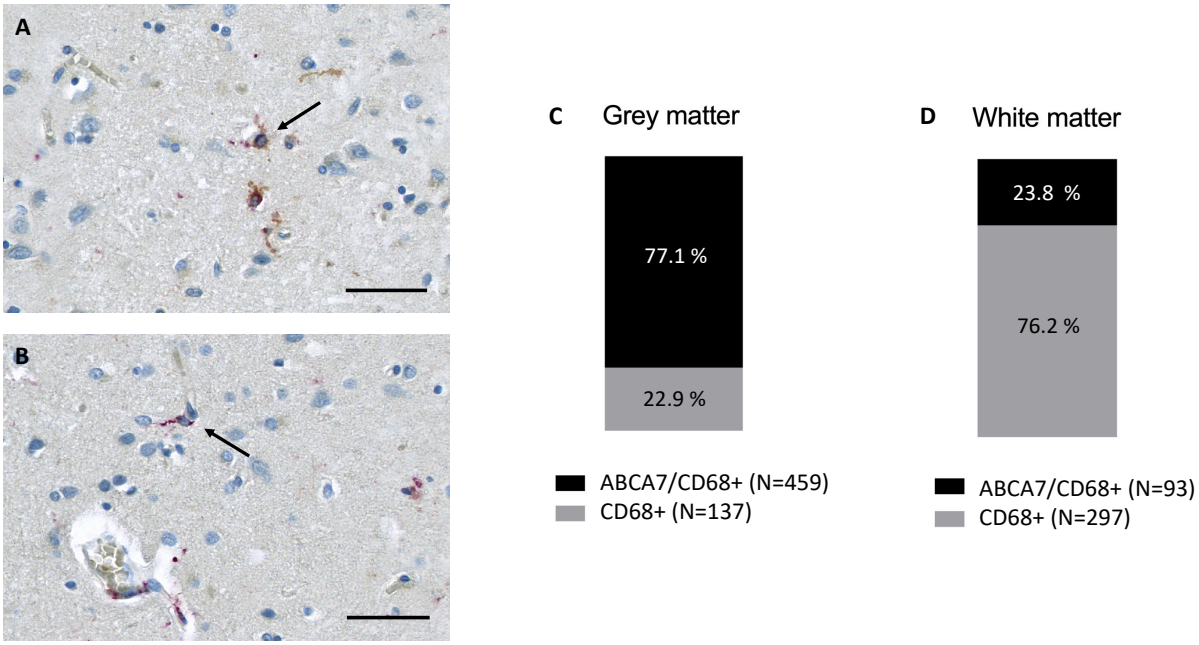


Figure 13: ABCA7 is expressed in a subset of CD68-positive cells

Immunohistochemistry with double labelling of ABCA7 (brown) and CD68 (red) was performed in the grey matter of the frontal cortex of an AD case classified as CERAD C. Hematoxylin and eosin counterstaining (blue). **A** The arrow marks a cell that is co-expressing ABCA7 and CD68. **B** The cell marked with an arrow is expressing CD68, but not ABCA7. **C** In the grey matter, the majority of labelled cells co-express ABCA7 and CD68, while some cells only express CD68. **D** In the white matter of the same case, the majority of labelled cells express only CD68, while some cells showed expression of both markers. No cells were found that express ABCA7 without CD68-expression. Scale bar=50 µm.

Sequential double staining for Iba1 and ABCA7, which is pictured in Figure 14, proved immunoreactivity for both markers in the same cells. All of the labelled cells were positive for Iba1 and ABCA7 but expressed the markers in varied intensity. The labelled cells had the morphology of microglial cells.

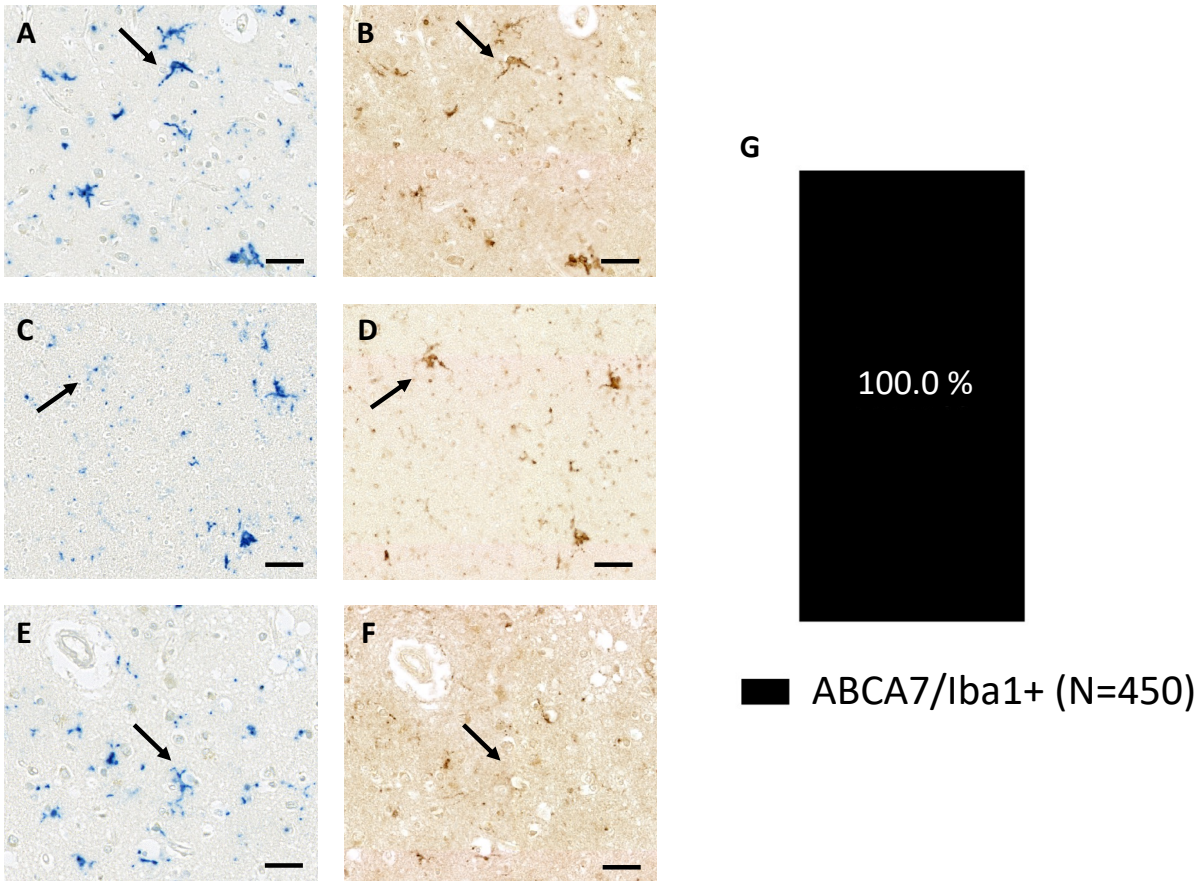


Figure 14: ABCA7 and ionized calcium-binding adapter molecule 1 are co-expressed in microglia in varying intensity

Immunohistochemistry with labelling of ABCA7 (brown) and the microglial marker Iba1 (blue) was sequentially performed in the grey matter of the frontal cortex of a CERAD C AD case. **A, B** Iba1 and ABCA7 are expressed in the same cells (arrow). Most of the labelled cells express Iba1 and ABCA7 in a comparable intensity. **C**, **D** A subtype of the labelled cells expresses ABCA7 in higher intensity than Iba1 (arrow). **E**, **F** Another subtype of cells expresses Iba1 in a higher intensity than ABCA7 (arrow). **G** All of the labelled cells express Iba1 and ABCA7. No cells expressing ABCA7 or Iba1 alone were found. Scale bar=50 µm.

The ABCA7-positive microglia were further categorised according to their morphology. In the examined brain sections, microglia of all stages of activation showed ABCA7 immunoreactivity. With the IHC labelling of ABCA7, resting microglia, microglia in early and late activation, and amoeboid forms could be differentiated. Figure 15 illustrates the distinct microglial stages that were detected in this staining.

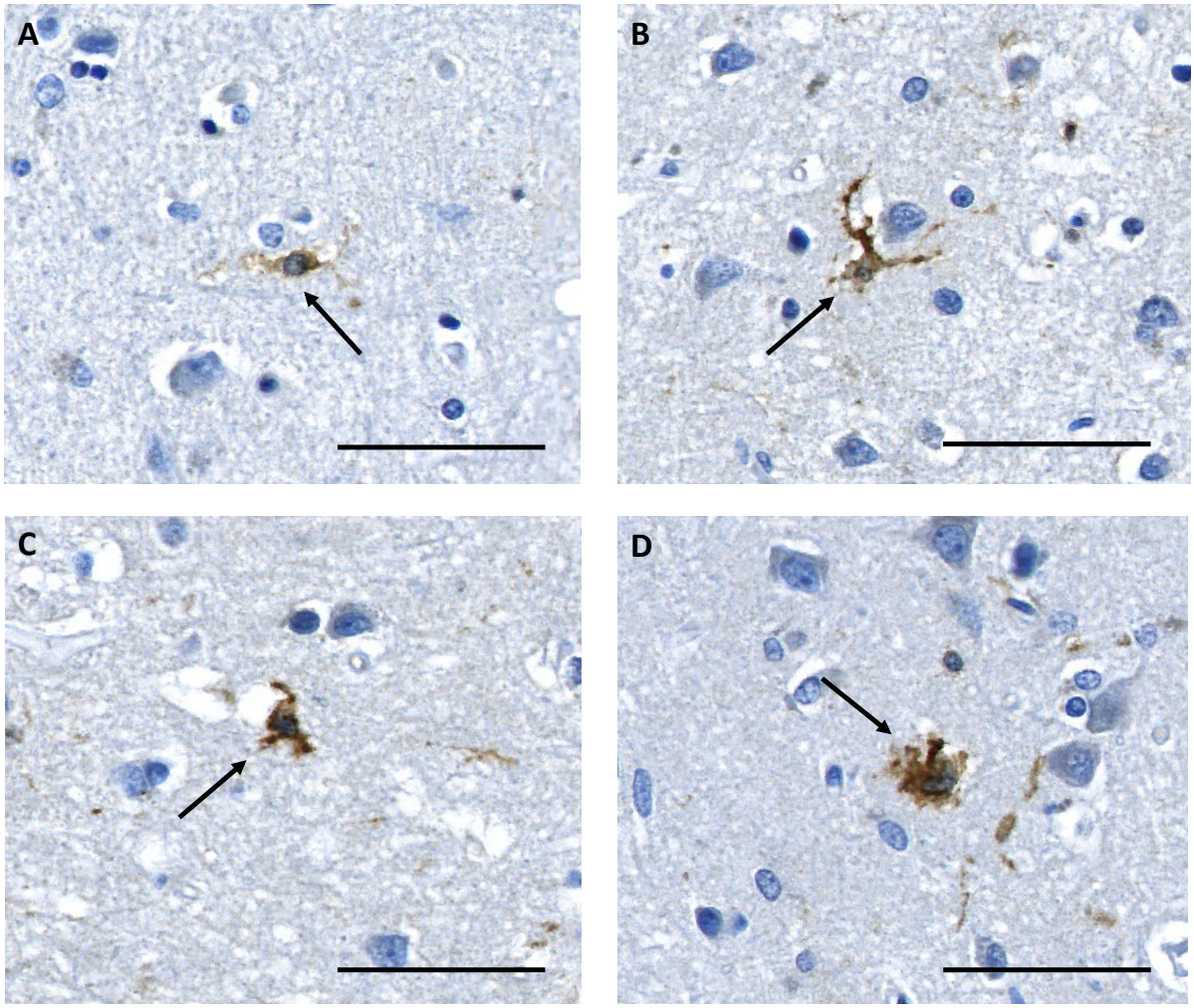


Figure 15: ABCA7 is expressed in microglia of all stages of activation

The expression of ABCA7 (brown) was studied by immunohistochemistry in the frontal cortex of several cases with varying CERAD classification. Hematoxylin and eosin counterstaining (blue). The provided images are taken from a CERAD C AD case. ABCA7 expression was found in cells of distinct morphology, which can be classified as varying stages of microglial activation. **A** Cells with ramified thin processes (arrow) were classified as resting microglia. **B** Hypertrophied cells with thick processes (arrow) were classified as microglia in the early stages of activation. **C** A subtype of cells showed a retraction of processes (arrow) and was classified as reactive microglia of later activational stages. **D** Cells with few or no processes (arrow) were classified as an amoeboid form of microglia. Scale bar=50 µm.

To summarize, ABCA7 was expressed in cells within the frontal cortex. ABCA7-positive cells were morphologically analogous to microglia of distinct activation stages. Partial co-expression of ABCA7 and CD68 in ramified cells of the human cortex was found. Double labelling of ABCA7 and Iba1 demonstrated a complete overlap of the two markers in cells with a microglial morphology.

8.2 Establishing a double staining protocol for ABCA7 and amyloid beta

Using a sequential staining protocol, two methods for the detection of Aβ plaques were compared on one tissue section of an advanced AD case. DAB immunostaining with the monoclonal 6E10 antibody against Aβ and the fluorescent marker methoxy-X04 were evaluated for their ability to reliably label Aβ plaques on human tissue sections. For this study, a simplified nomenclature for Aβ plaque types was used. Depending on their perimeter and shape, plaques were categorized as either dense or diffuse. Both methods clearly visualized Aβ expression and made a classification of plaques into dense or diffuse possible. Figure 16 depicts the findings from this experiment. In the sequential staining, both methods reliably labelled the Aβ plaques at the same location, but the diameter of the visualized dense plaques appeared smaller in the methoxy-X04 staining. The contrast between stained diffuse plaques and areas of the tissue without Aβ staining was lower in the methoxy-X04 staining than in 6E10 Aβ IHC. Immunolabelling for Aβ detected 1304 plaques per 30 mm^2. Of these, 73.1 % of plaques were categorized as dense and 26.9 % as diffuse plaques. In the same area, 999 Aβ plaques were detected by fluorescent labelling with methoxy-X04. Dense plaques made up 75.0 % of the total plaques labelled by methoxy-X04, while 25.0 % were categorized as diffuse. The 6E10 Aβ IHC was utilized for further experiments in this study.

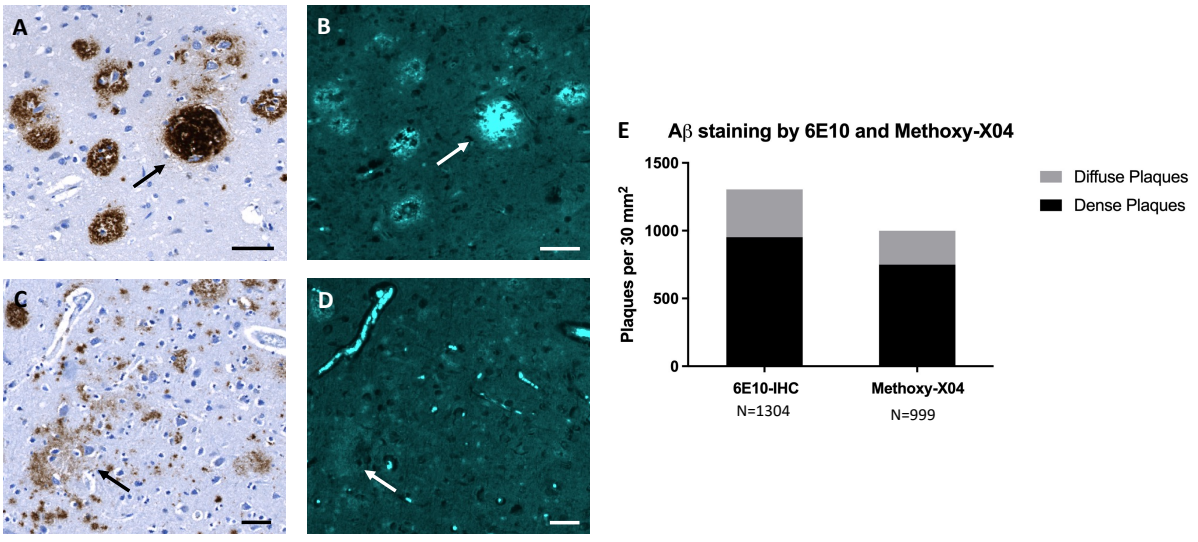


Figure 16: Comparison of amyloid beta plaque staining by immunohistochemistry with 6E10 antibody and methoxy-X04

An AD case classified as CERAD C was stained for amyloid beta with IHC using a 6E10 antibody (brown in **A**, **C**) and fluorescence labelling with methoxy-X04 (fluorescent blue in **B**, **C**). Hematoxylin and eosin counterstaining (blue) in **A**, **B**. To compare both methods, sequential staining on the same section was performed. **A, B** The dense plaque (arrow) is detected with both methods. **C, D** The diffuse plaque (arrow) is detected with both methods. **E** The plaques were quantified using both methods independently. More plaques were labelled with 6E10-IHC than with methoxy-X04. Both staining methods showed a comparable proportion of dense and diffuse plaques. Scale bar=50 µm.

For this project, I established a method for double immunolabelling of ABCA7 and Aβ in human tissue. Frontal cortex tissue sections from seven AD cases were used to compare this double labelling method with a standard IHC staining for ABCA7 alone. Both staining procedures produced clear immunoreactivity for ABCA7 in ramified cells with microglial morphology, as seen in Figure 17. The number of ABCA7-positive cells had a high variability between different cases, which ranged from 47 to 1543 cells per 50 mm^2. With a Pearson's correlation coefficient of 0.953, a strong correlation was found between the number of cells detected with the single and double staining method on sections from the same case.

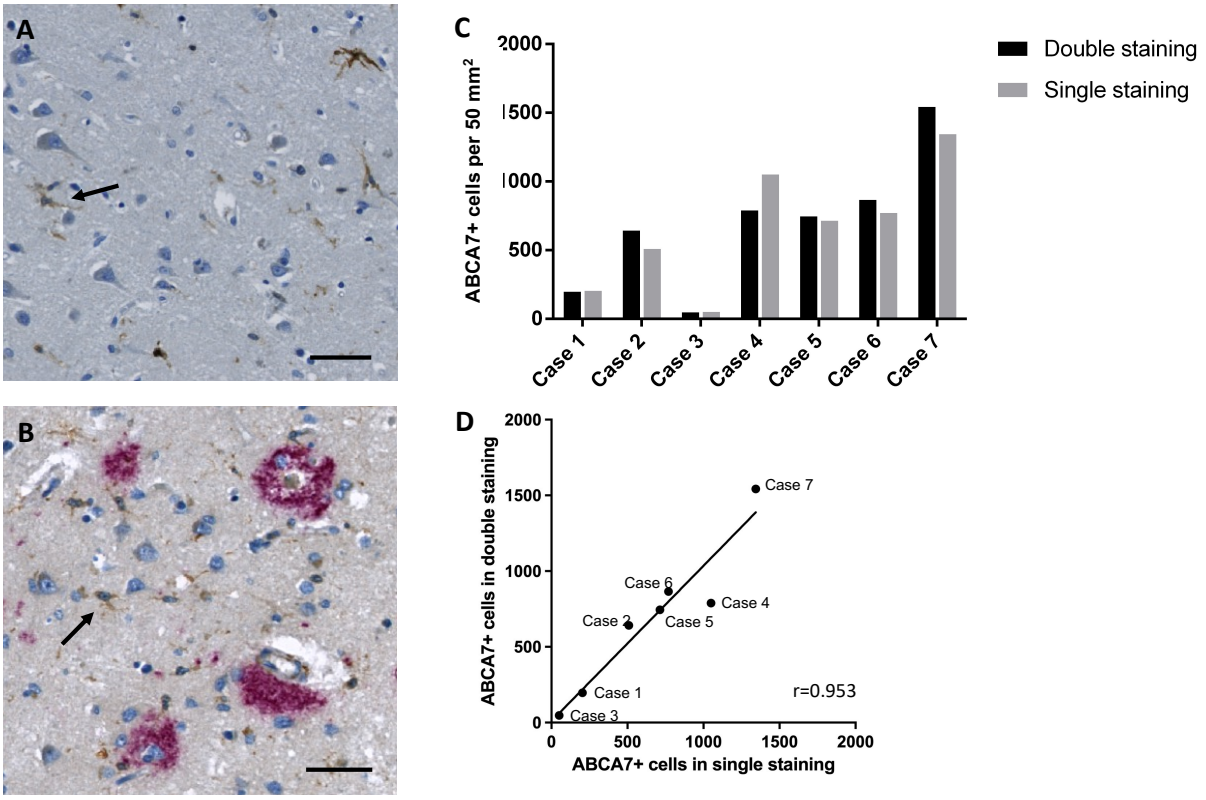


Figure 17: Repeatability of ABCA7-immunohistochemistry with double staining

Comparison of standard ABCA7 staining (**A**) and double staining (**B**) for ABCA7 (brown) and amyloid beta by 6E10 antibody (red). Hematoxylin and eosin counterstaining (blue). Both staining methods were performed on sections from the frontal cortex of seven cases with varying CERAD classification. **A**, **B** The provided images are taken from a CERAD C Alzheimer's disease case. The distribution of ABCA7 labelling is similar in the single and double staining, with clear expression in the processes of the ABCA7-positive cells (arrows). The double staining method provides clear labelling of ABCA7 (brown) and 6E10 (red) **C** ABCA7-positive cells were quantified in the same region in all sections. **D** Pearson's correlation between the number of ABCA7-positive cells labelled in the single and double staining. The number of ABCA7 labelled cells did not differ significantly in the single and double staining. Scale bar=50 µm.

The previously presented staining experiments present a new method for labelling ABCA7 and Aβ in human brain tissue. The protocol makes it possible to assess the two markers and their spatial and quantitative relation on a single tissue section. This method was used for further IHC experiments in this study.

8.3 Correlation of ABCA7-positive microglia and amyloid plaques in the cortex

The previously established IHC double staining protocol was carried out on seven cases with AD typical pathology of differing CERAD stages. In each case, a tissue section from the frontal cortex was stained and the superior frontal sulcus was analysed. Figure 18 provides detailed results from this experiment. Immunolabelling of Aβ with a 6E10 antibody and ABCA7 proved a strong correlation between the number of ABCA7-positive cells and total Aβ plaques detected by the 6E10 antibody in the grey matter. For this correlation, the Pearson's coefficient was 0.89. When dense and diffuse Aβ plaques were separately analysed, the strongest correlation was found between ABCA7-positive cells and dense plaques. The Pearson's correlation coefficient was 0.93 between the number of ABCA7-positive cells and dense plaques. The Pearson's correlation coefficient for ABCA7-positive cells and diffuse plaques was 0.74.

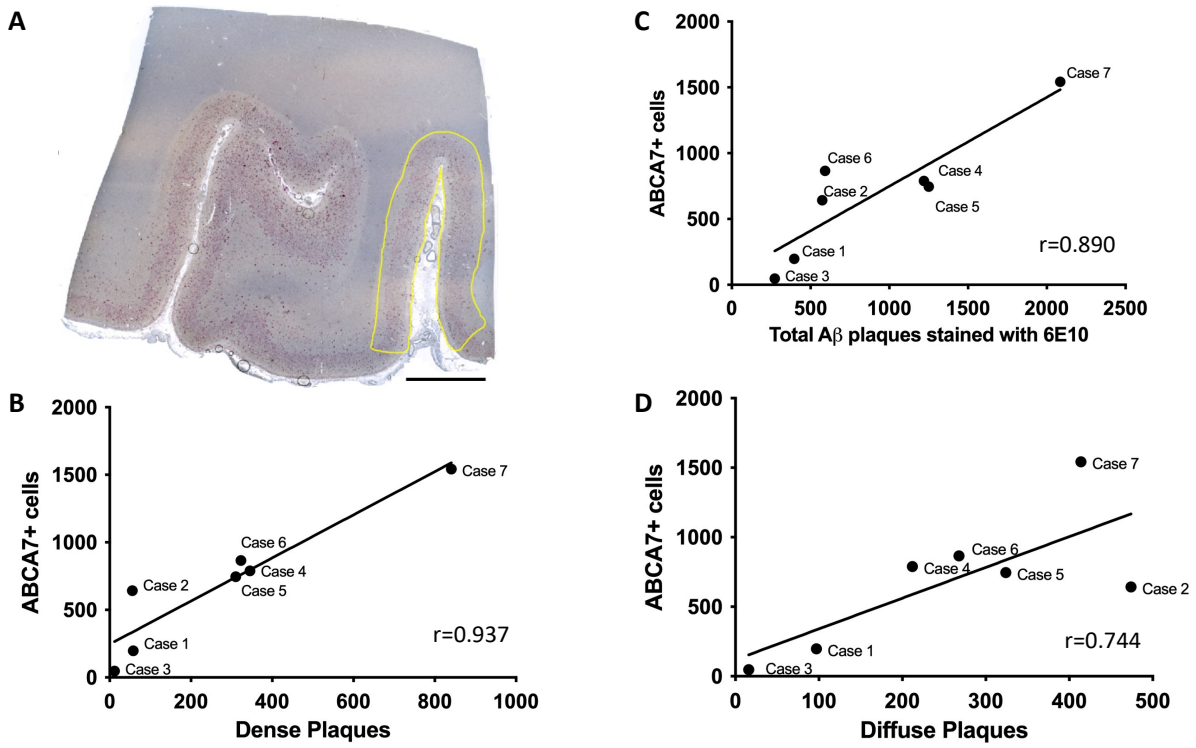


Figure 18: Number of ABCA7-positive cells correlates with amyloid beta plaque load in the grey matter of the frontal cortex

Immunohistochemistry with double labelling of amyloid beta (red) by 6E10 antibody and ABCA7 (brown) was performed in the grey matter of the frontal cortex of 7 cases of varying CERAD classification. Hematoxylin and eosin counterstaining (blue). **A** In each case, an area of 50 mm^2 in the superior frontal sulcus (outlined in yellow) was studied. In this area, the number of ABCA7-positive cells, dense plaques and diffuse plaques was quantified. **C** Pearson's correlation between the number of ABCA7-positive cells and total plaques stained by 6E10. **B** Pearson's correlation between the number of ABCA7-positive cells and dense plaques stained by 6E10. **D** Pearson's correlation between the number of ABCA7-positive cells and diffuse plaques stained by 6E10. Scale bar=5 mm.

All cells with ABCA7 immunoreactivity were evaluated for their association to 6E10 labelled Aβ plaques, as seen in Figure 19. Cells with plaque association had visible contact with their cell body or processes to a stained plaque. The evaluated seven cases had a mean percentage of 75.08 % of ABCA7-expressing cells that showed association with Aβ plaques. The average ratio of ABCA7-positive cells that were associated with a dense plaque was 34.38 %. An average of 40.70 % showed an association with diffuse plaques. An average of 24.78 % of the evaluated cells had no association with any plaque. Figure 19 D gives an overview of the plaque association detected in all evaluated cases. Case 7, which was classified as CERAD C, had the most ABCA7-positive cells of all evaluated cases. In this case, 54.50 % of the cells were associated with a dense plaque, while 26.83 % of cells were associated with diffuse plaques. In this AD case, 18.66 % of ABCA7-expressing cells were not associated with any Aβ plaque. With a total of 47 ABCA7-positive cells per 50mm^2, Case 3 had the lowest number of ABCA7-positive cells of all evaluated cases. In this case, 40.42 % of cells showed no plaque association, while 25.53 % were associated with dense Aβ plaques and 34.04 % to diffuse plaques. In case 2, which was diagnosed with CAA, 73.83 % of ABCA7-positive cells showed an association with diffuse plaques, while 8.72 % were associated with dense plaques.

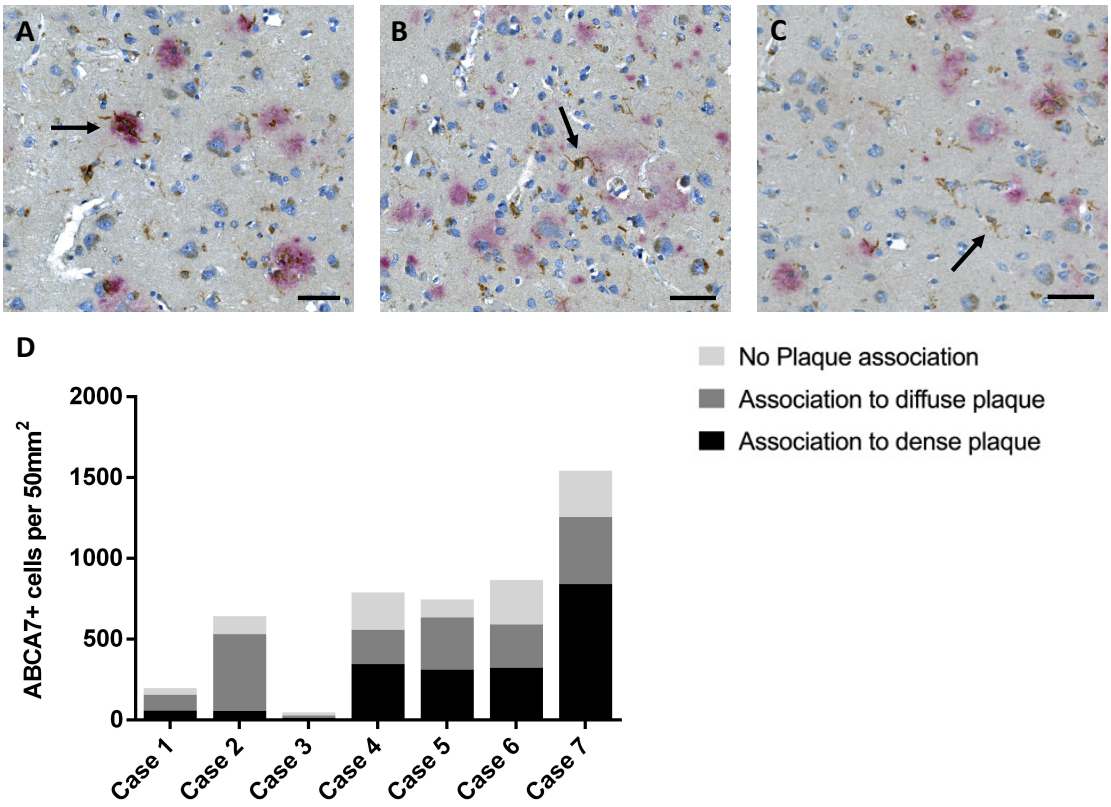


Figure 19: The majority of ABCA7-positive cells show association with amyloid beta plaques in the grey matter of the frontal cortex

Double labelling of ABCA7 (brown) and 6E10 (red) was performed in the frontal cortex of cases of varying CERAD classification. Hematoxylin and eosin counterstaining (blue). The provided images were taken from a CERAD C case. **A** The arrow marks an ABCA7-positive cell that shows association with a dense plaque. **B** The arrow marks an ABCA7-positive cell that shows association with a diffuse plaque. **C** The arrow marks an ABCA7-positive cell that is not associated with any amyloid beta plaque. **D** Association of ABCA7-positive cells to dense and diffuse plaques was quantified in all examined cases. Scale bar=50 µm.

The IHC double staining method that was utilized to assess the relation of ABCA7-positive cells and Aβ plaques was further validated using a second staining method, as presented in Figure 20. Sequential staining with ABCA7 immunolabelling and methoxy-X04 staining of Aβ was also able to visualize the colocalisation of ABCA7-positive cells and plaques. Both methods were applied on sections from the same tissue block and ABCA7-positive cells were categorized according to their plaque association. In the double staining that visualised Aβ with the fluorescent methoxy-X04, 60.00 % of ABCA7-positive cells were visibly associated with dense plaques and 29.34 % with diffuse plaques. In this staining, 10.65 % of ABCA7-positive cells showed no association with Aβ plaques that were labelled with methoxy-x04. With the IHC double labelling technique that used a 6E10 antibody against Aβ, 54.45 % of ABCA7-positive cells were identified as associated with dense plaques. In the same section, 26.91 % of cells were associated with a diffuse plaque, while 18.63 % of cells had no plaque association at all.

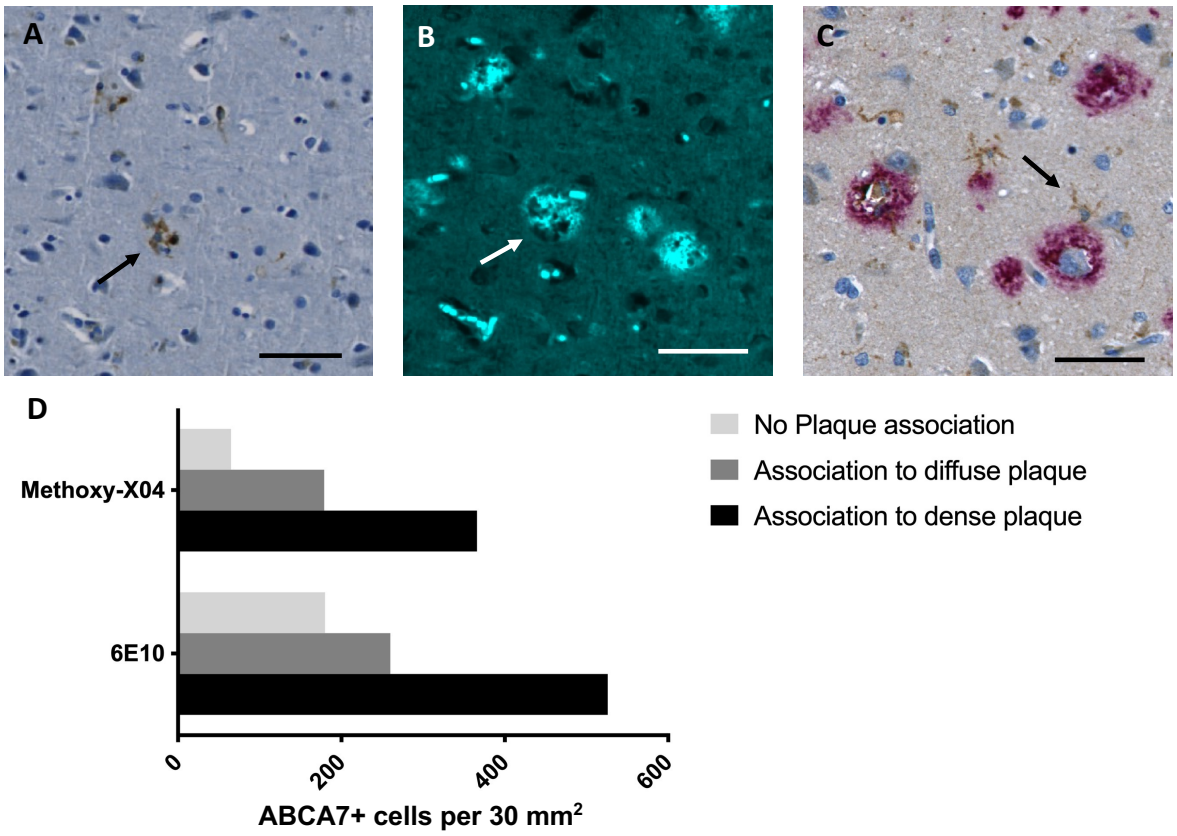


Figure 20: Association of ABCA7-positive cells to amyloid beta plaques confirmed by methoxy-X04 staining

Double labelling of ABCA7 and amyloid beta was performed with two different methods on sections of the same CERAD C case. **A, B** The section was stained with ABCA7 (brown) by immunohistochemistry (**A**) and with the fluorescent marker methoxy-X04 (blue in **B**). Images of the same area viewed with brightfield and fluorescence microscopy are provided. The arrows mark the same region in both images. **C** Immunohistochemistry double labelling of ABCA7 (brown) and amyloid beta by 6E10 antibody (red), were performed on a different section of the same case. The arrow marks an ABCA7-positive cell that is associated with an amyloid plaque. Hematoxylin and eosin counterstaining (blue). **D** ABCA7-positive cells and their association to dense and diffuse amyloid beta plaques were quantified in the same area in both double staining procedures. Scale bar=50 µm.

Additionally, double staining of ABCA7 and pTau, labelled by AT8 antibody, was performed in sections from the frontal cortex. The staining revealed pTau pathology in form of NFT and neuropil threads in all AD cases. However, no spatial association of ABCA7-positive cells to these pTau deposits could be detected.

In summary, a correlation between the number of ABCA7-positive cells and Aβ plaque load was found in cases with AD-typical pathologies. Using the previously established double staining method, a spatial association of ABCA7-positive microglia with Aβ plaques in the frontal cortex was demonstrated. This association was subsequently confirmed with a second staining method.

8.4 ABCA7 expression in the hippocampus

Following the studies on tissue from the frontal cortex, ABCA7-positive cells were also quantified in the hippocampal region. The findings from this experiment are presented in Figure 21. In the six analysed cases, the number of ABCA7-positive cells varied between 25 and 236 cells per 7 mm^2 in the hippocampus. A positive correlation between the number of ABCA7-positive cells in the frontal cortex and hippocampus was found. The Pearson's correlation coefficient between those two parameters in the examined six cases was 0.841. In the hippocampal area, the morphology of the ABCA7-expressing cells was typical for microglia. The cells predominantly presented with a ramified morphology, which was similar to the immunolabelled cells of the frontal cortex.

Double immunolabelling for ABCA7 and 6E10 was performed on tissue from the hippocampus of six cases with varying CERAD classification, as seen in Figure 22. For this experiment, the CA1 region of the hippocampus was analysed. It did not show an association of ABCA7-positive cells with Aβ plaques in the human hippocampus. The majority of labelled cells did not have close contact with a plaque via their cell processes. In the six examined cases, the mean share of ABCA7-positive cells with plaque association was 15.46 %. The Aβ plaques that were labelled with the 6E10 antibody in the hippocampus did not present with the classical morphology of the cortical plaques. The labelled Aβ plaques were of varying size and shape and no cored plaques were observed. A categorization into dense and diffuse plaques was therefore not possible.

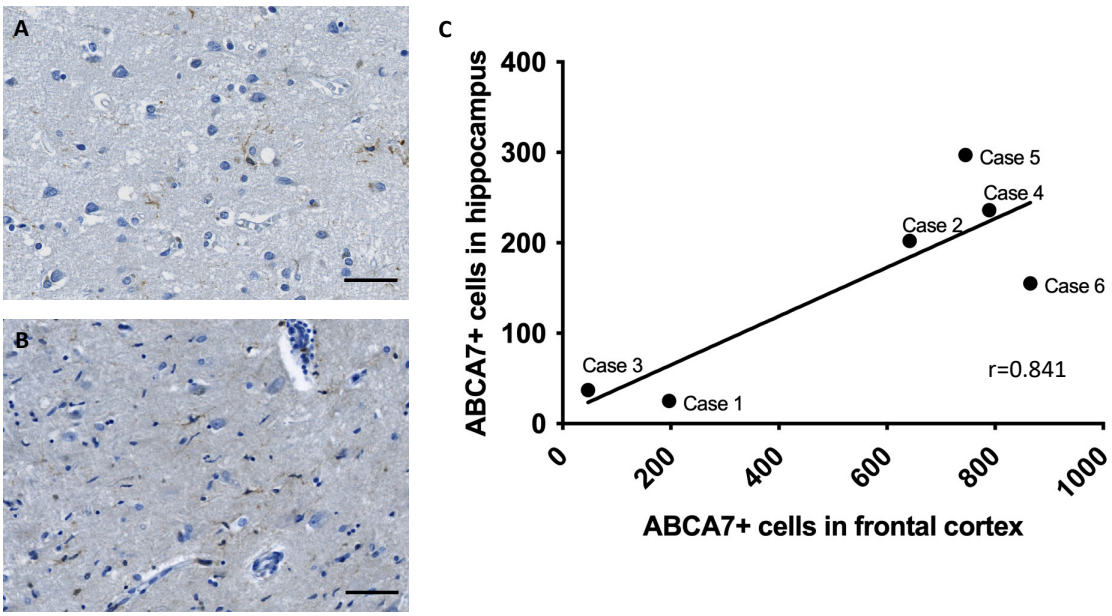


Figure 21: Positive correlation of the number of ABCA7-positive cells in the frontal cortex and hippocampus
By immunohistochemistry, ABCA7 was stained in the frontal cortex (**A**) and hippocampus (**B**) of AD cases of varying CERAD classification. Hematoxylin and eosin counterstaining (blue). The provided images are taken from an Alzheimer's disease case classified as CERAD C. **C** Pearson's correlation between the number of ABCA7-positive cells in the frontal cortex and hippocampus. Scale bar=50 µm.

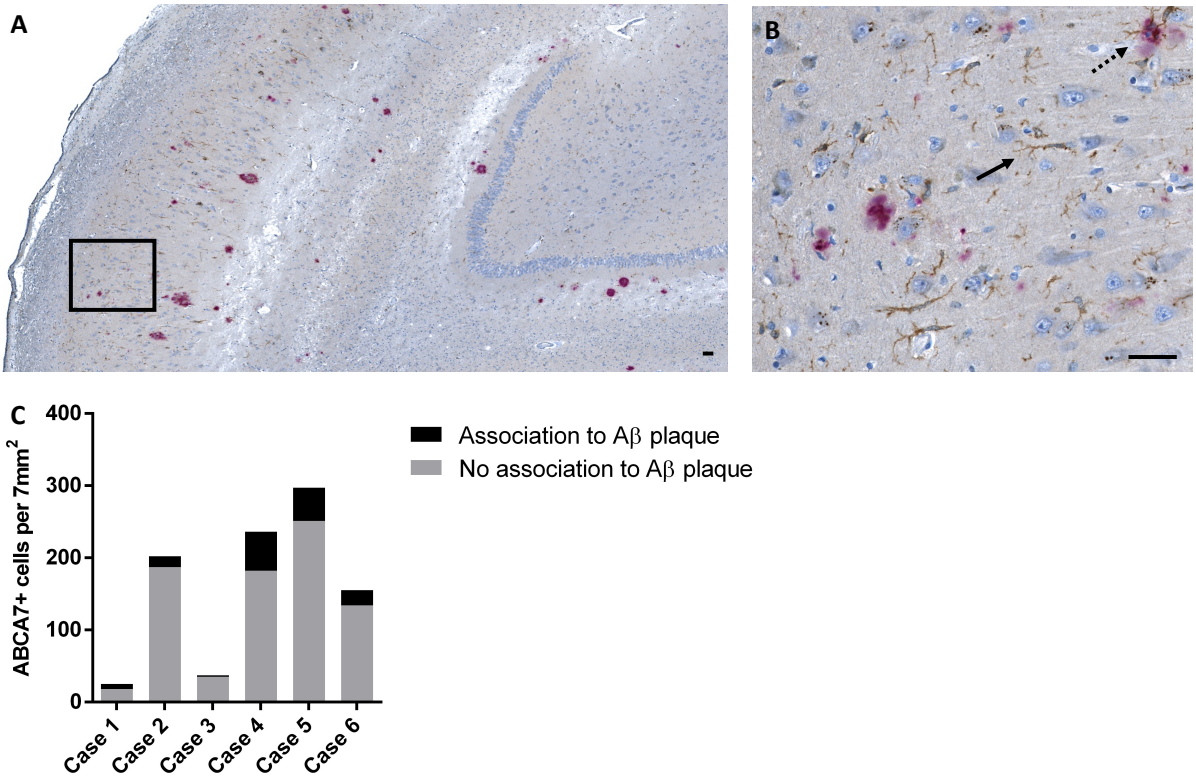


Figure 22: No association of ABCA7-positive cells and amyloid beta plaques in the hippocampus

Double labelling of ABCA7 and 6E10 was performed in the hippocampus of different cases with varying CERAD classification. Hematoxylin and eosin counterstaining (blue). **A** Overview of the CA1 area in the hippocampus of a case classified as CERAD C. **B** Detail image of the same region (as outlined in **A**). ABCA7-positive cells with (arrow with dashed line) and without (arrow with continuous line) association to plaques can be detected. **C** Quantification of ABCA7-positive cells and their association to plaques. Scale bar=50 µm.

In addition to the widely distributed ABCA7 expression in microglia, I observed intracellular staining for ABCA7 in neurons in the hippocampus of two cases with AD pathology classified as CERAD C. This intraneuronal staining occurred only in a few cells in the hippocampus of these two cases and was not detected in tissue from the frontal cortex. Exemplary pictures from one of these cases are provided in Figure 23. The ABCA7 signal was visible in form of several distinct round spots with DAB staining within the soma of cells with the typical morphology of neurons. The described ABCA7 signal in neurons did not show a spatial association to Aβ deposits.

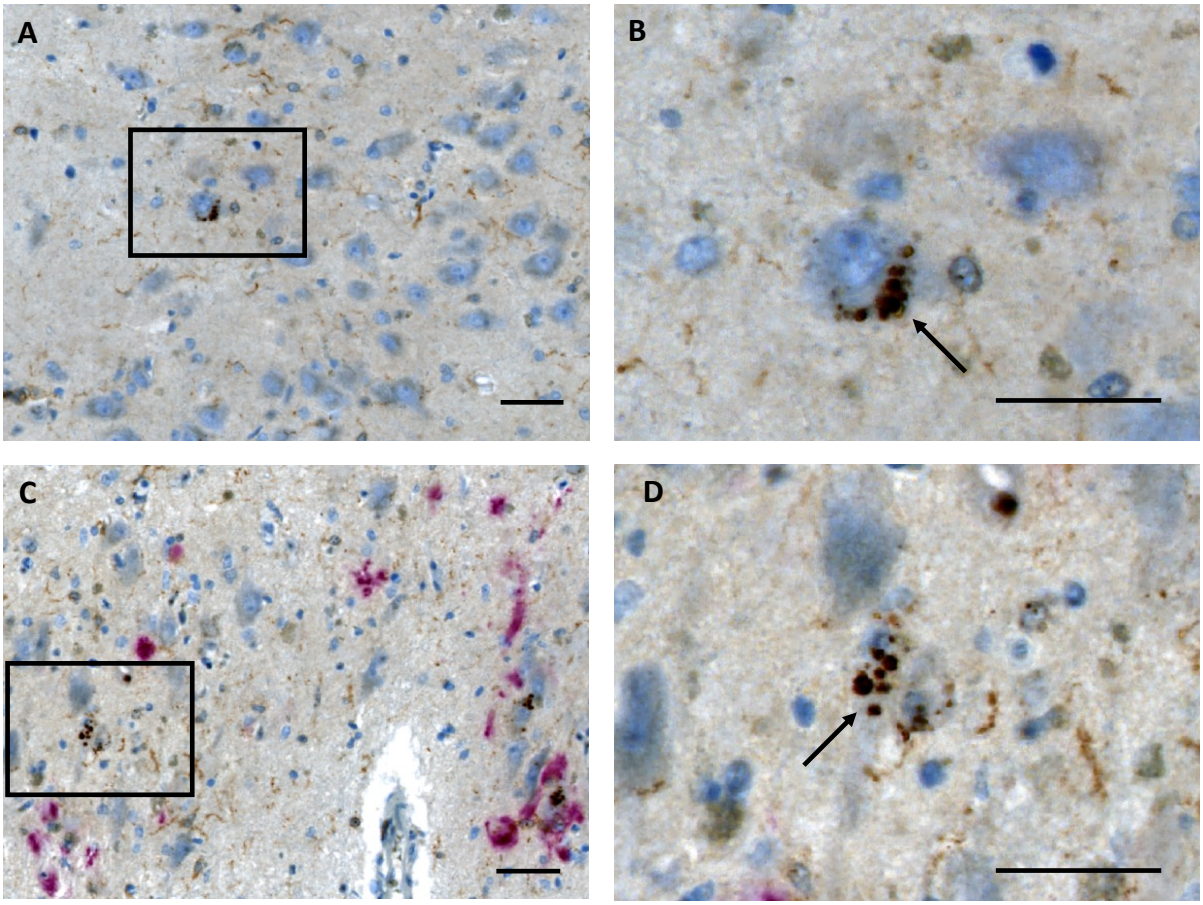


Figure 23: Intracellular ABCA7 signal in neurons

Immunohistochemistry with double labelling of ABCA7 (brown) and 6E10 (red) was performed in the hippocampus of an AD case classified as CERAD C. Hematoxylin and eosin counterstaining (blue). **A** Intracellular staining of ABCA7 in a neuron (cell surrounded by black box). The cell is surrounded by ABCA7-positive ramified microglia, no amyloid beta plaques are visible in this area. **B** Detailed image of the ABCA7-positive neuron (arrow). **C** Hippocampal area with frequent ABCA7-positive microglia and amyloid beta plaques. Neuron with intracellular ABCA7 staining (surrounded by black box). **D** Detailed image of neuron with intracellular ABCA7 staining (arrow). Scale bar=50 µm.

In conclusion, a correlation between the quantity of ABCA7-positive cells in the frontal cortex and hippocampus was demonstrated by this study. However, double labelling of ABCA7 and Aβ by IHC did not demonstrate an association of ABCA7 expressing cells to plaques in the hippocampus.

8.5 Confirmation of ABCA7 antibody specificity

The specificity of the ABCA7 antibody was confirmed with a specific blocking peptide from the same manufacturer. When the antibody was incubated with the blocking peptide before antibody treatment of the tissue, no staining was visible after the established IHC protocol. This experiment, which is presented in Figure 24, was carried out with varying concentrations of the blocking peptide. With a concentration of 1:5 of antibody to blocking peptide, no DAB staining of ABCA7 was visible. There was no difference in the staining when using concentrations of 1:5 or 1:25 of antibody to blocking peptide on sections from the same case. A control section without treatment of blocking peptide proved a high number of ABCA7 expressing cells in the used tissue. This control section without blocking peptide treatment had a higher background staining than the sections that were treated with the blocking peptide. These results confirmed the specific binding of the antibody against ABCA7 that was used in this study.

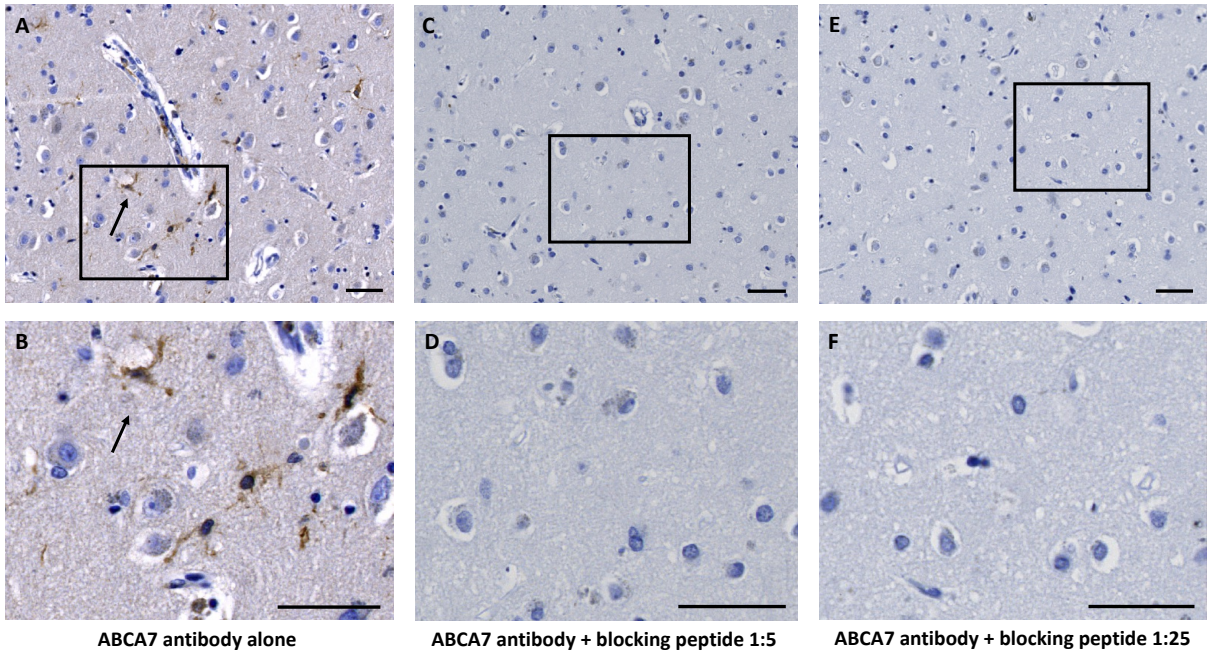


Figure 24: Confirmation of the specificity of the ABCA7 antibody using a blocking peptide

Immunohistochemistry was performed on three sections in the same area of the frontal cortex of a CERAD C case. **A, B** ABCA7-positive cells (brown) are clearly visible when staining with the ABCA7 antibody without a blocking peptide. **C, D** The ABCA7 antibody was incubated with its immunogen blocking peptide at a ratio of 1:5 before treating the section with it. ABCA7 staining is absent, confirming the antibody's specificity. **E, F** The ABCA7 staining is also absent when incubating the ABCA7 antibody with the blocking peptide at a ratio of 1:25. Hematoxylin and eosin counterstaining (blue). Scale bar=50 µm.

9. Discussion

9.1 Methodical aspects

The aim of this study was to investigate the protein expression of ABCA7 in the context of AD in the human brain. The histological analyses were performed on post-mortem brain tissue from seven patients. Tissue sections were taken from the frontal cortex and CA1 area of the hippocampus. The frontal cortex is affected early by neuropathological changes and is routinely examined for the histopathological classification of AD (Braak & Braak, 1991; Mirra et al., 1991; Montine et al., 2012; Thal et al., 2002). The hippocampal CA1 region was chosen because extensive and early Aβ deposition has been described in this region (Furcila et al., 2018; Thal et al., 2002).

As described in the introduction, genetic studies have identified ABCA7 as a major AD risk gene (Apostolova et al., 2018; Karch et al., 2012; Shulman et al., 2013). So far, the function of ABCA7 has predominantly been investigated using mouse models or cell culture. However, studies on human brain tissue are essential for understanding the actual mechanisms behind AD pathology in the human brain. In a recent review, Sierksma et al. (2020) question to what extent observations from mouse models can be translated into the pathological processes within the human brain. With this study, I aim to close this knowledge gap by investigating the distribution of ABCA7 within the human brain and describing its expression in correlation to AD pathology. This step is essential for translating knowledge from genetic studies into real insight into human AD pathology.

The main limitation of this study is the small size of the study cohort. I included and evaluated cases with varying intensity of AD pathology. The cohort included cases with sparse, moderate and frequent plaques according to the CERAD classification (Mirra et al., 1991). Studies on human AD brains are frequently published with similar case numbers (Lopes et al., 2008; Parachikova et al., 2007; Yang et al., 2022). For example, a recent study examined tissue from the hippocampus and cortex of 9 AD cases and 8 controls without clinical dementia to investigate vascular changes in AD brains (Yang et al., 2022). Because the present study was developed to investigate ABCA7 in the context of AD pathology, I did not include a control group without any AD pathology. I did not provide the age and gender of the cases in this work, because due to the small sample size of this study no further statistical analyses using this information are possible.

At the beginning of this study, I established a reliable protocol for ABCA7 immunolabelling on paraffin-embedded human brain tissue. For this, I used a polyclonal anti-ABCA7 antibody

raised in rabbit, which was manufactured by Proteintech. In one prior publication, this antibody has been successfully used for IHC on human ovarian and ovarian cancer tissue (X. Liu et al., 2018). As no previous publication described the application of this anti-ABCA7 antibody for IHC on human brain tissue, I tested the antibody thoroughly with different conditions. The clearest DAB staining without excessive background staining was achieved with an antibody concentration of 1:250. The best results were attained with a pre-treatment with TRIS/EDTA buffer at pH 9 in a steam cooker. As a secondary antibody, the Envision® kit, consisting of an HRP-conjugated polymer, was used in combination with a matching DAB reagent. The usage of a standardized kit ensured a consistent staining quality with low background signal. The Envision® kit had successfully been used on sections from the human brain and other tissue in prior publications (Carmona-Fonseca & Cardona-Arias, 2022; Röhr et al., 2020).

One previous study used two monoclonal anti-ABCA7 antibodies for IHC in human brain sections from AD patients and persons without AD (Tomioka et al., 2017). For this study, I chose a polyclonal antibody, because they can bind to multiple epitopes, are more stable to pH changes, and are therefore preferred for usage with fixed tissue (Lipman et al., 2005).

The specificity of this antibody against ABCA7 was tested by preincubation of the antibody with a specific blocking peptide. I adapted this method from a publication by Koper et al. (2020). A concentration ratio of at least 1:2 of antibody to blocking peptide was recommended in that publication and by Daneshtalab et al. (2010). In the presented study, the blocking peptide was used with a concentration of 1:5 and 1:25 and inhibited all signal, while no unspecific binding of the antibody could be detected. A publication on the specificity of antibodies against cannabinoid receptor type 2, explains that a blocking peptide experiment can not definitely prove antibody specificity, as it does not rule out binding to inappropriate antigen sites (Cécyre et al., 2014). However, the low amount of unspecific background staining together with the successful blocking peptide test indicate a high specificity of the used antibody against ABCA7.

Unfortunately, a validation of the findings of this study by using a second anti-ABCA7 antibody from a different manufacturer was not possible. When using the established IHC protocol with an anti-ABCA7 antibody manufactured by Abcam (ab247027 rabbit polyclonal), no staining of ABCA7 was feasible. Several trials with changes to the protocol did not lead to a reliable signal. The manufacturer describes a species reactivity with human tissue and specifically markets the antibody for usage with standard IHC protocols (Abcam, 2019). While the recommended primary antibody concentration is 1/200 to 1/500, no signal could be attained with antibody concentrations ranging from 1/200 to 1/800. The manufacturer recommends heat mediated antigen retrieval with citrate buffer pH 6. However, no positive results could be reached with

antigen retrieval with citrate buffer pH 6 or TRIS-EDTA buffer pH 9 by either steamer or microwave. There are currently no publications available that utilise this antibody for IHC. Possible explanations for the staining problems with the additional antibody could be quality issues with the antibody in general or the specific production lot we were sent. Another possible rationale is an interference of the tissue fixation with antibody binding. I abandoned the repetitional experiment with the additional ABCA7 antibody after several unsuccessful trials with various staining conditions.

The tested conditions for ABCA7 IHC with the well working ABCA7 antibody were then applied to establish a method for double labelling of ABCA7 together with other markers. Fluorescence double staining techniques are routinely used in many laboratories to study various cell markers in the context of AD in human brains (Griciuc et al., 2013; Hendrickx et al., 2017; Jung et al., 2015; Serrano-Pozo et al., 2013). However, fewer publications have utilized double staining protocols for non-fluorescence IHC. Satoh et al. (2016) studied TMEM119 and its co-expression with other microglial markers in human brains and used non-fluorescence double labelling. In this study, an HRP-conjugated secondary antibody with DAB and an AP-conjugated antibody with Warp Red chromogen were used for the visualization of different markers. This method, which uses HRP- and AP-conjugated secondary antibodies, is similar to the double staining technique used in the present study. Another publication studied TREM-2 co-expression with major histocompatibility complex class 2 (MHCII), GFAP, pTau, and Aβ, detected by a 6E10 antibody (Lue et al., 2015). In that study, standard DAB and the dark-blue nickel enhanced DAB were used for a clear distinction of the two protein markers in the double staining.

The double staining protocol that was established for this study labelled ABCA7 with brown DAB, while other markers were visualized with chromogens that could be easily differentiated by colour. Trials to use the ABCA7 antibody with fluorescence did not lead to satisfying results because of the strong autofluorescence of human brain tissue. Furthermore, the non-fluorescent IHC method that was used in this study provided a better understanding of the tissue structure of the brain. Due to the high stability of most IHC chromogens, stained sections can be indefinitely stored at room temperature and repeatedly viewed under the microscope without fading.

In a double staining with ABCA7, an anti-CD68 antibody was applied at a concentration of 1:100 and later visualized using an AP enzyme and the red new fuchsin chromogen. Previous studies on post-mortem AD brains have used CD68 as a phagocytic marker (Hendrickx et al., 2017; Toomey et al., 2020).

Double staining of ABCA7 and Iba1 was attained with a sequential staining protocol. Iba1 was visualized with a prefabricated kit utilizing AP and later dyed with Vector® Blue. Iba1 is used in many studies as a microglial marker in IHC on human tissue (Fahrenhold et al., 2018; Sakae et al., 2016; Satoh et al., 2016; Serrano-Pozo et al., 2016; Streit et al., 2018; Toomey et al., 2020).

Aβ plaques were marked with a 6E10 antibody in a double labelling with ABCA7. This antibody is commonly used in routine neuropathological investigations as well as for various scientific studies (Dinkel et al., 2020; Jin et al., 2017; Kageyama et al., 2021; Metaxas et al., 2019). For the present study, Aβ was visualized via an AP-conjugated polymer with the red chromogen new fuchsin. The stained plaques were later categorized as diffuse or dense according to their morphology. In this process, several publications with detailed descriptions and depictions of the different plaque types were instrumental (Boon et al., 2020; Röhr et al., 2020; Thal et al., 2006). The category of dense plaques included compact plaques as well as classic cored plaques. Both of these plaque types are fibrillar plaques that contain β-pleated amyloid sheets and are associated with clinical AD (Boon et al., 2020; Delaere et al., 1991; Thal et al., 2006). Diffuse plaques on the other hand contain comparatively low levels of β-pleated amyloid and were identified by their less compact structure and irregular shape (Röhr et al., 2020).

In this study, methoxy-X04 was used as an alternative method to label Aβ. Methoxy-X04 is a lipophilic derivative of Congo red that binds to fibrillar Aβ and is visible by fluorescence (Klunk et al., 2002). The fluorescent marker was first used by Klunk et al. (2002) to stain Aβ in post-mortem human brain tissue. Subsequently, it has been used in various publications for in vivo experiments on anaesthetized mice (Jung et al., 2015; Klunk et al., 2002; Meyer-Luehmann et al., 2008). In one study, methoxy-X04 was utilized for double and triple labelling of post-mortem brain tissue from AD patients by fluorescence alone (Furcila et al., 2018). For the present study, I developed a method to use fluorescence labelling by methoxy-X04 together with standard non-fluorescence IHC in human brain tissue. I compared the methoxy-X04 method to Aβ IHC by 6E10 antibody in single and double stains. Double labelling of Aβ with methoxy-X04 together with ABCA7 by IHC required two separate scanning steps using the Axioscan. Subsequently, the digitalized images needed to be viewed separately or required a digital overlay of the two files, while the results from the double labelling technique with IHC were visible in one virtual slide. Furthermore, protection against photobleaching of the fluorescent signal was needed before and during the scanning process. Therefore, I used the IHC double labelling method for the majority of my experiments. Methoxy-X04 staining was primarily used for confirmation of the results from the IHC experiments with a second method.

The stained tissue sections were digitalized using the slide scanner Axioscan. The digital image acquisition of the whole slide made an uncomplicated quantification of cells possible. The virtual slides have a high image resolution and can be indefinitely stored on a computer for later analyses. Therefore, the image acquisition with a slide scanner has clear advantages over standard microscopy and was chosen for this study.

9.2 ABCA7 expression by microglia

Double labelling of ABCA7 with Iba1 and CD68 was carried out to characterise the ABCA7 expressing cells, for further details see Figures 11 and 12. These experiments showed that 77.1 % of CD68-positive cells also expressed ABCA7 and proved a co-expression of ABCA7 and Iba1 in all labelled cells. The morphology of ABCA7-positive cells resembled the classical ramified cell structure of microglial cells. These results strongly indicate that ABCA7 is expressed by microglia.

Most publications agree that Iba1 is expressed in microglia of all stages, while CD68 mainly stains activated phagocytic cells (Hopperton et al., 2018; Streit et al., 2009; Toomey et al., 2020). It has been suggested by Hendrickx et al. (2017) that Iba1 is rather a marker of early microglial activation and its expression by microglia is variable in different types of brain pathology and brain regions. However, the study by Hendrickx et al. found that Iba1 is frequently expressed in ramified microglia in the grey matter. The present study found a complete overlap of expression of ABCA7 and Iba1 in that subtype of cells, which further confirms that ABCA7 is expressed by microglia in the grey matter of brains with AD pathology.

In the present study, ABCA7 expression was observed only in a part of the CD68-positive cells. This confirms an ABCA7 expression in the brain that is predominantly restricted to microglia, as CD68 is a phagocytic marker that is expressed in microglia, monocytes and macrophages (Boche et al., 2013; Hopperton et al., 2018; Toomey et al., 2020).

With these results, it can not definitely be proved that ABCA7 is expressed by all microglia, as there is still some uncertainty in the current literature if Iba1 is indeed expressed by all microglial stages (Boche et al., 2013; Hendrickx et al., 2017). There is a possibility that ABCA7 and Iba1 are co-expressed in the same subtype of cells, while a certain subtype of microglia might express none of the two markers. As presented in Figure 15, this study found ABCA7 expression in cells with variable morphology that resembled the cell structure of microglia of all stages of activation. A sole ABCA7 protein expression in only one microglial activation stage can therefore be ruled out.

Additionally, some intracellular ABCA7 staining in neurons was found in the hippocampus of two AD cases. The intraneuronal ABCA7 signal, which is pictured in Figure 23, was observed in form of several distinctly stained vacuoles within cells with the typical morphology of neurons. The intraneuronal staining had morphological similarities to cell processes that involve the formation of intracellular vacuoles. Macro-autophagy is a pathway for the elimination of damaged cell organelles that involves the formation of vacuolar autophagosomes (Feng et al., 2014). In AD brains, pathological membrane-bound vacuoles within neurons are frequently observed as a result of macro-autophagy (Koper et al., 2020). These neuropathological changes are called granulovacuolar degeneration and appear within the hippocampus in early AD stages (Hondius et al., 2021). As the described intraneuronal ABCA7 staining has been observed in the hippocampus, it could very well originate from granulovacuolar degeneration.

Furthermore, a special form of programmed cell death called necroptosis has been connected to neuronal loss in AD (Caccamo et al., 2017; Dhuriya & Sharma, 2018; Koper et al., 2020). A study by Caccamo et al. (2017) found that the levels of necroptotic markers positively correlated with the Braak stage in human AD brains. Interestingly, the study found that a decreased activation of necroptosis reduced cell loss in an AD mouse model. The observed intraneuronal ABCA7 staining in the present study was morphologically similar to previously published IHC images of necroptosis (Koper et al., 2020). Necroptotic markers have been found in granulovacuolar degenerative lesions and might play a role in neuronal death in AD brains (Koper et al., 2020). However, further experiments are needed to understand the origin of the described intraneuronal ABCA7 signal.

This study located ABCA7 protein expression in the human brain mainly in microglial cells and found a neuronal expression only in a few cells in two hippocampal sections. This is consistent with the results of real-time PCR assays that found high ABCA7 gene expression in human microglia and only low levels in neurons (Kim et al., 2006). However, ABCA7 staining in microglia and neurons has been described in a study using monoclonal antibodies against ABCA7 for IHC in the frontal cortex of human brains with and without AD (Tomioka et al., 2017). The IHC images provided in that publication show intraneuronal ABCA7 signal that is faint in comparison to the ABCA7 staining that I observed in microglia with a polyclonal antibody. It looks similar to unspecific background staining that was also present in early stages of my study, when the IHC protocol was not fully established. It is therefore possible that the intraneuronal ABCA7 signal observed by Tomioka et al. is due to unspecific antibody binding. Interestingly, Lipman et al. (2005) state in a publication that compares polyclonal and monoclonal antibodies that polyclonal antibodies often present with a better specificity.

Two previous studies on mouse models indicate that ABCA7 influences APP processing within neurons, which does not correspond with my finding of a strong ABCA7 expression that is restricted to microglia (Sakae et al., 2016; K. Satoh et al., 2015). Contrarily to my results, K. Satoh et al. (2015) found identical ABCA7 protein levels in neurons and glial cells of mouse brains using western blots. They propose that ABCA7 depletion results in increased production of Aβ and APP proteolysis (K. Satoh et al., 2015). The authors of a second study on ABCA7 deficient mice argue that ABCA7 deficiency increases Aβ production by influencing APP processing and activates the ERK pathway in neurons (Sakae et al., 2016). My results do not support this hypothesis that ABCA7 influences Aβ production in neurons, as I did not find widespread ABCA7 signal in neurons. These discrepancies could be explained by interspecies differences between human and mouse brains.

In summary, this study found ABCA7 expression by microglia of all activation stages. The relevance of the ABCA7 IHC signal in a few neurons in the hippocampus of individual cases needs to be examined in further experiments.

9.3 ABCA7 in correlation to amyloid beta plaques

In this study, double labelling of ABCA7 and Aβ by IHC revealed a correlation between the number of ABCA7-positive cells and Aβ plaques in the grey matter of brains with AD pathology. This validates the findings of Vasquez et al. (2013) who found an increased ABCA7 gene expression in AD cases and hypothesised that ABCA7 expression increases in response to AD pathology. Contrarily to this, Tomioka et al. (2017) detected more ABCA7-positive cells by IHC in brains without AD than in AD brains. They did, however, not find a statistical difference of ABCA7 protein levels by western blot between those two groups (Tomioka et al., 2017).

In a recent study, Lyssenko and Praticò (2021) found that lower ABCA7 protein levels in the brain are associated with early AD development, while patients with high ABCA7 levels developed AD later in life. In that publication, the authors develop a hypothesis in which ABCA7 has a temporary and dose-dependent protective function against AD. The previously mentioned study examined the connection between ABCA7 protein levels and the AD Braak stage in the human brain. Cases with no AD pathology or Braak stage I had significantly higher ABCA7 levels than cases with Braak stage II or higher. However, that study could not find significant differences in ABCA7 levels between unaffected controls and AD cases with Braak stages I to V (Lyssenko & Praticò, 2021). The Braak classification assesses the AD stage according to the distribution of NFT in different brain regions to define the AD stage (Braak & Braak, 1991). In the present study, the AD cases were instead assessed according to their Aβ

plaque load, which is considered in the CERAD classification that was first published by Mirra et al. in 1991.

The positive correlation between the number of ABCA7-positive cells and Aβ plaques which I detected in this study seemingly contradict the previously described findings of Lyssenko and Praticò (2021) and Tomioka et al. (2017). However, these discrepancies could be explained by a hypothesis that was first proposed by Vasquez et al. in 2013. In that publication, the authors suggest that continuingly increased ABCA7 levels, for example, due to an SNP, offer protection from AD, while upregulation of ABCA7 expression in response to AD pathology can ultimately not sufficiently inhibit the progression of the disease (Vasquez et al., 2013). This hypothesis would explain the dose-dependent protection from AD that Lyssenko and Praticò (2021) found as well as the elevated number of ABCA7-positive cells in cases with a high plaque load I observed in the present study. It is further supported by evidence from genetic studies in which loss-of-function mutations in the ABCA7 gene have been associated with increased AD risk (Campbell et al., 2022; Steinberg et al., 2015).

My double staining experiments further showed that the majority of ABCA7-positive microglia in the cortex of AD brains are in close contact with Aβ plaques via their processes. These results were verified with a second double labelling method utilizing the ABCA7 antibody and fluorescent staining with methoxy-X04. This intimate contact of microglia with Aβ fibrils has previously been described (Itagaki et al., 1989; Prokop et al., 2013). A study that examined ABCA7 knockout mice could find a colocalisation of Iba1 and Aβ plaques in ABCA7-deficient and wild type mice (Kim et al., 2013). This result shows that the plaque association of microglia that was observed in the present study is not solely dependent on ABCA7 function.

The described correlation and colocalisation of microglia with Aβ deposits were more prominent in connection to dense plaques than to diffuse plaques. A study with ABCA7 knockout mice found that the total area of dense plaques was higher in mice without ABCA7 than in wild-type mice, but no differences in diffuse plaques were detected (K. Satoh et al., 2015). These results confirm the link between ABCA7 expression and the occurrence of dense plaques that was found in the present study.

This study identified ABCA7 expression in microglia with close association to Aβ plaques, which supports two previous publications that suggest that ABCA7 influences AD pathology through microglial Aβ clearance (Aikawa et al., 2019; Kim et al., 2013). Kim et al. (2013) found increased Aβ plaque numbers and insoluble Aβ levels in the brains of ABCA7 knockout mice.

In another study, it was shown that ABCA7 haplodeficiency increases the Aβ accumulation within microglia in mice (Aikawa et al., 2019).

The AD risk genes ApoE, TREM2 and CD33 have already been connected to microglial clearance of Aβ, which confirms the important role of microglia in the occurrence of AD pathology (Griciuc et al., 2013; C. Y. D. Lee et al., 2012; Lue et al., 2015).

My findings, evaluated within the context of the previously discussed publications, support the following hypothesis that is adapted from Vasquez et al. (2013): ABCA7 expression in microglia contributes to microglial clearance of Aβ and therefore protects unaffected brains against AD. This is supported by my findings of a close colocalisation of ABCA7-positive microglia and Aβ plaques and results from genetic studies in which loss-of -function mutations of ABCA7 were associated with increased AD risk (Campbell et al., 2022; Steinberg et al., 2015). Following the onset of AD, ABCA7 expression is upregulated (Vasquez et al., 2013). In these pathological circumstances, however, the increased ABCA7 expression can not sufficiently counteract the ongoing AD pathology. This leads to increased numbers of ABCA7-positive cells and a high plaque burden, which I observed in brains with advanced AD pathology.

9.4 ABCA7 in the hippocampus

This study found a correlation between the number of ABCA7-positive cells in the frontal cortex and hippocampus. The total number of ABCA7-positive cells per area was higher in the cortex than in the hippocampus. A previous analysis of ABCA7 levels in non-demented and AD patients by western blot did not find a significant difference in ABCA7 protein levels between the hippocampus and parietal cortex in any of the analysed groups (Lyssenko & Praticò, 2021). It is important to note that the ABCA7 protein levels in the previously mentioned study were detected by considering the ratio of ABCA7 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the tissue. This constitutes a semi-quantitative method to detect protein expression levels that can be distorted by changes in GAPDH levels within the tissue. Interestingly, GAPDH has been shown to be influenced by AD pathology (Sultana & Butterfield, 2009). In the present study, I did not evaluate protein levels, but quantified the number of ABCA7-positive cells. One further needs to consider that the western blot analyses published by Lyssenko and Practicò (2021) were carried out on lysates from frozen tissue, while the IHC of this study was performed on paraffin-embedded tissue sections. Hence, the two studies used very different methods to evaluate ABCA7 protein expression in the human brain.

In the present study, I did not detect a correlation between ABCA7-positive cells and the Aβ plaque load in the CA1 area of the hippocampus. Furthermore, ABCA7 expressing microglia

did not colocalise with Aβ plaques in this area of the brain. Marlatt et al. (2014) studied Iba1-expression in the hippocampus using triple IHC staining with Aβ and the proliferation marker proliferating cell nuclear antigen. Even though the authors observed Iba1-positive microglia in close association with Aβ in AD brains, Iba1 expression was not especially associated with areas with a high burden of Aβ deposition. That study did not find a statistical increase of Iba1-positive cells in AD cases in comparison to demented patients with low Braak scores and non-demented controls (Marlatt et al., 2014). This shows that even though microglia that express Iba1 have been observed in close proximity to Aβ plaques in a prior study, their numbers do not show a clear statistical correlation to the plaque load in the hippocampus. According to Thal et al. (2002), Aβ deposition occurs later in the hippocampus than in the neocortex. It is possible that the plaques that I observed in the hippocampal area are early plaques that have not yet attracted microglia, while microglia in the frontal cortex have already been activated by the plaques.

Interestingly, the plaques I observed in the hippocampal area were morphologically distinct from the plaques of the frontal cortex. In the hippocampus, no division into dense and diffuse plaques was possible. In the previously described IHC study, Marlatt et al. (2014) were able to differentiate between primitive, diffuse and dense cored plaques in the hippocampus. The Aβ plaques that I observed in the present study are morphologically similar to plaques labelled as primitive by Marlatt et al. (2014). It is possible that the hippocampal plaques that I observed in this study are newer than the dense plaques found in the frontal cortex and therefore present with a distinct morphology.

Furcila et al. (2018) analysed plaque morphology in the CA1 hippocampal region of AD patients and found that only 13 % were cored plaques, while 87 % of plaques were labelled as non-cored plaques. This supports my observation that few plaques in that region present with the classical morphology described as dense plaques in this study. However, the varying nomenclature for the different types of Aβ plaques makes it difficult to compare studies on plaque morphology.

In summary, the clear association of ABCA7-positive microglia with Aβ plaques that was observed in the frontal cortex could not be replicated in the hippocampus of the same AD cases. Even though there was a positive correlation between the plaque load in the frontal cortex and hippocampus in each case, the hippocampal Aβ deposits were morphologically different from the plaques of the frontal cortex. These differences between the frontal cortex and hippocampus could be due to the sequence of Aβ deposition in different brain areas.

9.5 Outlook

In this study, I established reliable IHC protocols for single and double labelling of ABCA7 in human brain sections. This technique can be adapted in many ways to further examine the expression of ABCA7 in AD brains.

Expression of Iba1 and ABCA7 was observed in varying intensities in microglia. Further double staining experiments using different microglial activation markers would be helpful to understand which subtypes of microglia express ABCA7 in the highest levels. In a review on the use of microglial markers in IHC, Hopperton et al. (2018) suggested the MHCII cell surface receptor Human Leukocyte Antigen DR isotype (HLA-DR) and TREM2 as suitable markers for microglial activation. Furthermore, it would be interesting to examine the microglial co-expression of CD33 and ABCA7 in AD brains. CD33 is a genetic risk factor for AD that is expressed by microglia and inhibits Aβ clearance in microglial cell culture (Griciuc et al., 2013).

New insight about cell morphology and spatial distribution of the labelled cells could be gained when combining stereological analyses with IHC for ABCA7. Stereology provides information on the three-dimensional structure of cells while using the two-dimensional images that are available from brightfield microscopy of tissue sections. With this method, more accurate cell quantification and classification into microglial stages are possible. By utilising specific software, cell volume and length of the stained microglia can be quantified. For example, this method has been used to compare microglial changes in human brains in normal ageing and neurodegenerative diseases (Shahidehpour et al., 2021). The IHC protocol that was established in the present study would probably need to be modified to enable better penetration of the antibody through the thicker tissue sections that are required for this method (Hou et al., 2012).

This study quantified ABCA7 expression by counting positive cells in digitalized scans of the IHC staining. When measuring the intensity of protein expression in the brain tissue, protein levels of ABCA7 and other microglial markers can be compared. One technical solution for this is to digitally measure the DAB stained pixels with suitable software. Another possible method is the paraffin-embedded tissue (PET) blot, which enables quantification of the protein while also providing information on tissue distribution. Schulz-Schaeffer et al. first presented this method in 2000 and detailed technical information for implementation has been published by Moh et al. (2005). This method has, for example, been successfully used to examine the local distribution, expression patterns and expression density of prion proteins in formalin-fixed paraffin-embedded brain sections (Jürgens-Wemheuer et al., 2021).

In this study, I observed an intraneuronal ABCA7 signal in the hippocampus of several AD cases. As described above, the signal had morphological similarities to cellular processes like granulovacuolar degeneration or necroptosis. Further IHC analyses using specific antibodies for the detection of these cellular processes could define the origin of the observed intraneuronal ABCA7 signal. Possible IHC markers for further examination of these processes in connection with ABCA7 expression are casein kinase 1δ, which is expressed in granulovacuolar degeneration, and necroptosis markers receptor-interacting serine/threonine-protein kinase (RIPK) 1, RIPK3 and Mixed lineage kinase domain-like protein (MLKL) (Caccamo et al., 2017; Hondius et al., 2021; Koper et al., 2020).

By expanding the cohort of this study with more cases of each CERAD stage, the results could be analysed with additional statistical tests. This could, for example, deepen our understanding of the differences in ABCA7 expression between the AD stages. The present study did not include any cases with known ABCA7 risk variants. Examining the brains of carriers of such genetic variants can bring further insight into the protein's function. Changes in ABCA7 expression and their association to Aβ deposits can be evaluated with the double staining procedure that was used in this study.

This study and previous publications on ABCA7 are aimed to explore the function of the protein and its role in AD. Once we have gained more knowledge on ABCA7, the present basic research needs to evolve into clinical application. Current research on TREM2 proves that it is possible to translate the information on AD risk genes that has been gained from GWAS into studies with a clinical focus. In 2020, an experimental study on mice proved that the anti-human TREM2 antibody AL002 lowers the production of filamentous plaques, reduces neuronal dystrophy and increases microglial phagocytosis (S. Wang et al., 2020). At the moment, an ongoing phase II trial is evaluating the efficacy and safety of the AL002 antibody in patients with early AD (National Library of Medicine, 2022). As of February 2023, no results from this study have been published. As our understanding of ABCA7 grows with the ongoing basic research, the AD research community should look into its possibilities as an AD drug target eventually.

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12. Publications

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13. Curriculum vitae

Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht.

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