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ADVANCED DIAGNOSTICS AND THERAPEUTICS FOR ALZHEIMER'S DISEASE

Jo Armand Albert STEVENS

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ADVANCED DIAGNOSTICS AND THERAPEUTICS FOR ALZHEIMER'S DISEASE

PROEFSCHRIFT

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1

INTRODUCTION

1.1. CLINICAL AND PATHOLOGICAL FEATURES OF ALZHEIMER’S DISEASE

Alzheimer’s disease (AD) is a major neurocognitive disorder that accounts for 60-80% of all dementia cases. AD is a progressive disease affecting one-third of the people over 85 years [1]. In mild stages people have significant impairments in memory and learning abilities, but in general they are still able to live on their own. As the disease progresses to a moderate stage, people need more and more supervision and personality changes start to occur. Finally, in the severe stage, people lose language abilities and need help with basic motor functions such as swallowing. The majority of AD patients die from aspiration pneumonia caused by swallowing problems [2].

Recently, new classification and criteria have been devised based on disease-specific biomarkers present in cerebrospinal fluid (CSF), positron-emission tomography (PET) and magnetic resonance imaging (MRI) [3] (see Table 1.1). A distinction is now made between prodromal AD, where mild cognitive deficits and AD specific biomarkers are present, and mild cognitive impairment (MCI), where these same deficits are present, but AD specific biomarkers are absent [3].

	AD Diagnosis	Presence of impairment of tests	Evidence of biomarkers in vivo	Additional requirements
Prodromal AD	Yes	Required	Required	Absence of dementia
AD Dementia	Yes	Required	Required	Presence of dementia
Asymptomatic at risk	No	Not present	Required	Absence of AD symptoms
Presymptomatic	No	Not present	Not required	Absence of AD symptoms; presence of AD mutation
Mild Cognitive Impairment	No	Not required	Not required	Absence of AD symptoms or biomarkers

Table 1.1: New criteria for the diagnosis of AD (modified from [3]). Although cognitive tests and biomarkers are not specifically defined, they usually include a test such as the ADASCog. Biomarkers could be A β ₄₂ or tau levels in CSF or amyloid PET scan.

A distinction is also made between asymptomatic at-risk patients, who have positive biomarkers but no cognitive impairments, and pre-symptomatic patients, where people have autosomal dominant mutations that will (most likely) result in AD, but do not show any symptoms yet. Especially from a therapeutic point of view, these groups might be the easier to treat, since they can receive preventive treatment.

Diagnosis and classification can also be done based on neuropathological hallmarks

[4]. Hallmarks of AD consist of extracellular plaques mainly consisting out of amyloid β ($A\beta$) and intraneuronal neurofibrillary tangles (NFT) composed of hyperphosphorylated tau. This classification can only be done post-mortem, and until recently, this was the only method to have a certain diagnosis of AD. Other common causes of dementia are vascular dementia, dementia with Lewy bodies, and fronto-temporal lobe dementia [5].

1.2. PATHOLOGY AND TREATMENT OPTIONS

AD has a complicated etiology with several identified risk factors, but the exact cause remains unknown for the majority (90%) of AD patients (sporadic AD, SAD). In a minority of cases, the cause can be reduced to mutations in a gene in the amyloid precursor protein (APP) processing pathway resulting in familial AD (FAD) [6]. To date, a number of mutations are known in the APP gene itself, or in presenilin-1 (PSEN1) or presenilin-2 (PSEN2) that form part of the gamma-secretase complex, responsible for the cleavage of APP to $A\beta$. These mutations result in a total increase of $A\beta$ production, or in a shift from $A\beta_{40}$ to the more aggregation-prone $A\beta_{42}$ (see Chapter 2, Figure 1).

Besides mutations in APP, PSEN1 or PSEN2, which will result in certain development of AD, there are a large number of factors that increase the risk to develop AD. The most important risk factor is age, although it is not clear how this factor contributes to the disease process [7]. It has been shown that amyloid depositions increase with age even in healthy persons without AD [8]. Additionally, old age results in a state of chronic low-grade inflammation, which might also influence the disease development [9].

Family history suggests that there is a hereditary component to AD, and in recent years, a large number of genes have been implicated in AD etiology [10]. Most of these genes play a role in lipid metabolism, the proteasome system or inflammatory processes. The biggest genetic risk factor for AD is the APOE gene, which is involved in cholesterol metabolism. Homozygous carriers of the APOE $\epsilon 4$ allele, run almost 15 times more risk to develop AD [11]. The APOE $\epsilon 2$ allele on the other hand, confers a protective effect to AD. While the exact role of APOE in the AD process is not completely known, APOE4 has been shown to lead to an impairment in $A\beta$ clearance and possible increased aggregation [12].

Apart from age, there are also a number of other non-genetic risk factors associated with AD. One important factor associated with AD is a history of head trauma or traumatic brain injury (TBI) [13]. $A\beta$ has been shown to be increased after TBI in the short and long term [14, 15]. Additionally, head trauma also results in chronic inflammation, further exacerbating the risk for AD [16].

Vascular factors have also been associated with AD [17]. Hypertension, diabetes, smoking and heart disease have all been shown to play a role. The role of these factors is further complicated by the fact that they are also related to old age. In addition, it is not clear if these associations arose because of misdiagnosis of AD with vascular dementia [17]. Finally, lifestyle factors also influence AD risk. A Mediterranean-style diet and increased physical activity have been shown to reduce AD risk [18].

Several theories have been offered as to how AD starts and progresses to dementia [19]. At the moment, the leading hypothesis is the amyloid cascade hypothesis. This hypothesis states that an increase in $A\beta$ production or a decrease in $A\beta$ clearance leads to range of downstream processes eventually leading to neuronal death [20] (see Figure

1). It has been shown that initially $A\beta$ will increase neuroinflammation as characterized by increased pro-inflammatory cytokines and gliosis [20]. Neuroinflammation and amyloid aggregation will cause mitochondrial damage and production of reactive oxygen species (ROS), resulting in a vicious circle where inflammation is causing further damage and ROS production causing more $A\beta$ production (see Figure 1) [21]. $A\beta$ and ROS subsequently cause hyperphosphorylation of tau, eventually leading to the formation of NFTs [22]. At this point, neurons start dying and the first clinical symptoms occur. The cholinergic system is affected first, with neuronal degeneration starting in the locus coeruleus, and causing memory loss [23]. NFT load also correlates better with clinical symptoms than $A\beta$ plaque load [24]. At later stages also the other monoamine systems, such as the glutamatergic system is affected, resulting in further cognitive complications.

Figure 1.1: An increase in Aβ production (caused by risk factors such as head trauma), or a decrease in clearance (caused by eg. APOE4) lead to Aβ aggregation. This process can be accelerated under the influence of metals. Once Aβ aggregates, a complex process of several interacting factors leads eventually to plaque formation, microglial dysfunction and increased inflammation. This inflammatory status will eventually lead to mitochondrial dysfunction and increased ROS production. All these factors interact to increase tau phosphorylation, leading to neuronal death and dysfunction of several neuronal neurotransmitter systems. In green are drugs counteracting AD pathology currently on the market. In red are drugs targeting various components of these pathway currently in clinical trials. The focus of this thesis (Aβ immunotherapy) is emphasized in blue.

At the moment, no disease modifying therapies are available. Currently available symptomatic therapies include acetylcholine esterase inhibitors which are effective in mild AD, and memantine, a NMDA-antagonist, which can be used for treatment of moderate AD [25]. These drugs compensate the neuronal loss caused by AD (see Figure 1). However, they do not modify the processes leading up to this neuronal loss, and when this loss becomes too severe, these drugs become ineffective.

A large number of potential therapeutic drugs are currently being tested in clinical trials. They target various mechanisms involved in the disease process [26]. Whereas the large majority are designed to decrease amyloid production or aggregation, others are aimed at preventing tau phosphorylation or aggregation. To date all clinical trials conducted have yielded negative results (see Figure 1) [27].

1.3. DIAGNOSIS OF AD

Up until recently, diagnosis of AD was made post-mortem using immunohistochemical analysis of brain tissue sections. While post-mortem pathological evaluation is still considered the gold standard, new diagnostic tools have become available for *in vivo* diagnosis of AD. Before the diagnosis of AD is made, cognitive performance is usually evaluated by means of one or more neuropsychological tests.

A short screening test, such as the mini mental state exam (MMSE) can be performed for fast initial assessment [28]. However, a longer cognitive assessment test specifically developed for assessing dementia is preferred. Two commonly used tests are the Alzheimer's Disease Assessment Scale – Cognitive section (ADASCog) and the Clinical Dementia Rating (CDR), which are also often used as inclusion criteria or endpoints in clinical trials [28]. One issue associated with these tests, is that they suffer from ceiling effects and are less sensitive for accurate diagnosis in early disease stages [29]. For this reason, effort is currently being invested in improving these scales.

Advances in technology and understanding of the disease have resulted in the development of novel biomarkers. At the moment, most biomarker-based diagnostics can be divided into two groups: CSF-based and imaging based. A large research effort has been spent in investigating and validating a large number of possible CSF biomarkers for AD [30]. However, in practice the most commonly used and best validated measurements are $A\beta_{1-42}$, total tau (t-tau) and phosphorylated tau (p-tau), or combinations and ratios of these measurements [31]. These three measurements have now also been adopted into a consensus by the Alzheimer's biomarkers standardization initiative [32]. CSF $A\beta_{1-42}$ shows a negative correlation with disease progression, presumably because it is deposited into plaques, while tau shows a positive correlation with AD progression. These measures provide a high sensitivity (~92%) and specificity (~82%) [33]. Additionally, they are also useful in predicting which MCI patients will progress to full-blown AD [34]. One concern about CSF values is the high intra- and inter-laboratory differences between the tests, which can result in up to 26% of individuals receiving a wrong diagnosis [35]. It is hoped that standardization of these tests should solve this problem [31].

Besides CSF biomarkers, imaging based markers have found widespread use in AD diagnosis. For imaging based diagnosis either structural MRI or PET based diagnostics are used. A structural MRI is most commonly used as a biomarker for diagnostic

purposes [36]. A MRI scan can reveal medial temporal lobe atrophy, which can be used as a diagnostic marker for mild AD [37]. Visual rating scales of hippocampal atrophy provide a sensitivity and specificity of about 80-85%, however, other measures are still needed to exclude other forms of dementia, since MRI measures alone do not provide enough specificity, as e.g. in the case of frontotemporal lobe dementia [38]. As a consequence, other MRI based measurements such as voxel based approaches to increase sensitivity and specificity are under investigation [39].

Another imaging approach is to use PET tracers to detect AD specific alterations such as reduced brain metabolism, amyloid deposits or tau tangles. Fluorodeoxyglucose-PET (FDG-PET) is the most commonly used and best established PET tracer for AD diagnosis [40]. FDG-PET can be used to measure glucose metabolism in the brain. In AD patients, glucose metabolism decreases over time, and this assessed on an FDG-PET scan. While FDG-PET shows a good sensitivity (~90%) to detect AD pathology, the specificity to differentiate from other dementias is lower (~70%) [40]. FDG-PET correlates well with functional assessments such as the ADAS-Cog and can be used to be predict future cognitive decline [41].

Recent advances in the development of amyloid binding PET tracers have enabled *in vivo* imaging of plaques. The first amyloid specific PET tracer, ^{11}C Pittsburgh compound B (^{11}C -PiB), was tested for the first time more than 10 years ago [42]. ^{11}C -PiB is a thioflavin based analogue which binds to fibrillar amyloid deposits. In AD patients ^{11}C -PiB retention is mainly observed in the cortex and is consistent with post-mortem studies. In addition, it has a good sensitivity (~90%) and specificity (~83%) [43]. The main drawback of ^{11}C -PiB is the short half-life of only 20 minutes, limiting its use to PET centers that have access to an onsite cyclotron. Because of this problem, several ^{18}F -based tracers have been developed.

^{18}F -based tracers offer the advantage of a longer half-life (110 min) and this allows their routine use in all PET centers. The ^{18}F -based tracers are all similar to ^{11}C -PiB and thioflavin in that they all bind to fibrillary amyloid. At the moment, the tracers florbetapir, flutemetamol and florbetaben have all gained FDA approval and are in routine clinical use [44]. A comparison of the different tracers is presented in Table 1.2.

Name	Trade Name	Manufacturer	Sensitivity	Specificity
^{11}C -PiB	-	University of Pittsburgh	90%	83%
Florbetapir	Amyvid	Eli Lilly	~87%	95%
Florbetaben	Neuraceq	Piramal	97%	78%
Flutametamol	Vizamyl	GE Healthcare	90%	86%

Table 1.2: Comparison of the different commonly used amyloid PET tracers available (modified from [40])

Recently, also a PET ligand for tau has been developed. Although still in an early stage, this tracer is expected to provide a great deal of new information about AD progression and pathogenesis. ^{18}F -AV-1451 binds specifically to paired helical filaments (PHF) [45] and first reports suggest that this tracer allows *in vivo* imaging of tau in patients [46].

One important advance of all these diagnostic techniques is that they have greatly improved our understanding about AD pathology. It is now clear that A β deposition start already a long time before the onset of the disease, even up to 10-15 years before the first clinical symptoms are present [47]. This highlights the fact that possible therapies will need to take into account that plaques are already present when patients present themselves with memory problems.

1.4. AIMS AND OUTLINE

The aim of this thesis was to investigate the use of antibodies for advanced diagnostic and therapeutic purposes.

Chapter 2 reviews the current literature about the role of inflammation and anti-A β immunotherapy in AD. The current leading AD hypothesis, the amyloid cascade hypothesis is discussed and recent evidence of other factors in AD are reviewed. Next, the importance of neuroinflammation, more specifically the role of microglia and the innate immune system is highlighted. Finally, we review effector mechanisms of A β immunotherapy and provide a detailed overview of current clinical trials using immunotherapy.

In **Chapter 3** an immunotherapy experiment is described that aims to bring further understanding in the role of effector mechanisms of A β immunotherapy. To this end, a recombinant mouse-human chimeric antibody (3D6) was produced *in vitro*. The human variant of this antibody (bapineuzumab) was also used in clinical trials, but with disappointing results. Two different isotypes of this antibody were produced: a mouse IgG1 (mIgG1) with anti-inflammatory properties, and a mouse IgG2a (mIgG2a) with inflammatory properties. Next, these antibodies were administered in both a preventive fashion (before onset of behavioral deficits) and therapeutic fashion (after onset of behavioral deficit) in a mouse model of AD.

As a model, we choose for the 5xFAD mouse which is transgenic for both human APP and PSEN1, with 3 familial AD mutations present in APP and 2 mutations present in PSEN1 [48]. While no tau tangles are present in this model, it shows a very fast onset of AD-like symptoms, with the first plaques appearing at 2-3 months of age. At 5 months of age, the first cognitive deficits are present. The 5xFAD model is one of the few mouse models which shows neuronal loss. Next, we performed behavioral testing of these animals to evaluate the effects of immunotherapy. On a molecular level, A β load was evaluated and effects on gliosis were studied through immunofluorescence.

In **Chapter 4**, the focus is shifted to testing the use of an A β antibody for diagnostic purposes. To this end, a human anti-A β antibody (bapineuzumab) was labeled with a long-lived positron emitter (^{89}Zr) and its performance as an *in vivo* PET tracer was evaluated in the 5xFAD mouse model at various ages as a proof-of-concept. An isotype control and blocking experiment were also performed in the same animal model. After *in vivo* PET scans, bio-distribution studies were performed on macro-dissected brain and organs. Results were further supported by the use of autoradiography and immunofluorescence and histochemistry to prove specific binding of administered antibody in the brain.

Having established the technique of immuno-PET for amyloid imaging, we next compared the *in vivo* binding of different A β antibodies in **Chapter 5**. We used three

different antibodies targeting monomeric A β (solanezumab), oligomers (20C2) and plaques (bapineuzumab). Antibodies were labeled again with ^{89}Zr and administered to 5xFAD animals at different ages. *In vivo* PET scans were performed and afterwards bio-distribution was performed. Immunohistochemistry and fluorescence was also performed to gain more insight in the different behavior of the antibodies.

Finally, we provide a summary of the key results and conclusions in **Chapter 6**. In addition, we also discuss the main limitations of the studies performed and offer some insights about future developments.

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INFLAMMATION AND ALZHEIMER'S DISEASE: LESSONS FROM IMMUNOTHERAPY

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ABSTRACT

Alzheimer's disease (AD), the most common cause of dementia, poses an enormous health burden and at the moment no disease-modifying treatment is available. One of the hallmarks of the disease is the buildup of amyloid- β ($A\beta$) plaques in the brain. In the last 20 years, over a 100 clinical trials using $A\beta$ immunotherapy have been conducted using approximately 15 different antibodies and 10 different $A\beta$ vaccinations involving over 25 000 patients. In this review, we provide a current overview of the involvement of the immune system in AD, with a special focus on the antibody effector mechanisms of amyloid immunotherapy, and lessons that can be learned. In addition, we provide an up-to-date overview of finished and ongoing trials using immunotherapy. While therapies have been unable to halt the disease so far, twelve different $A\beta$ immunotherapies are still being tested in patients, with some of them showing promising preliminary results.

Alzheimer's disease (AD) is the most common cause of dementia worldwide and no cure is available at the moment. AD poses and will pose an enormous burden for society, it is expected that by 2050 over 130 million people will suffer from it [1]. The disease is characterized by the build-up of extracellular plaques composed of amyloid- β ($A\beta$), and of intracellular tangles composed of hyperphosphorylated tau protein. At the moment, several clinical trials are ongoing or have been completed using immunotherapy against $A\beta$. $A\beta$ immunotherapy aims to alter $A\beta$ deposition by inducing a humoral immune response to $A\beta$ (active) or administering anti- $A\beta$ antibodies (passive). Here, we focus on the crucial role of the immune system in the neuropathophysiology of AD, the relationship to $A\beta$ immunotherapy and lessons learned from immunotherapy trials.

2.1. THE AMYLOID CASCADE HYPOTHESIS

About 1-5% of AD patients suffer from early-onset familial AD (EOFAD), which is characterized by a mendelian inheritance pattern and an early-onset (before <65) of the disease [2]. EOFAD is caused by mutations in amyloid precursor protein (APP), presenilin-1 (PSEN1) or presenilin-2 (PSEN2), two proteins which are part of the γ -secretase complex, which is responsible for the generation of $A\beta$ [3]. Of these, mutation in PSEN1 are the most common [4]. However, the majority of AD patients suffer from sporadic AD (SAD), of which the cause is unknown, and which usually has a late onset (> 65). The heritability of SAD patients has been estimated to be between 60-80%, so the environment also plays a significant role [5]. While clinically there are some differences between EOFAD and SAD in the onset of the disease, in that EOFAD usually presents with worse symptoms, histologically both groups have the same hallmarks, i.e. $A\beta$ plaques and tau tangles.

The combined fact that patients suffering from EOFAD always have mutations in proteins involved in the APP processing pathway and that $A\beta$ plaques are always present in the brain of AD patients, led to the postulation of the amyloid cascade hypothesis.

Initially, the hypothesis suggested that $A\beta$ is toxic, and the buildup of $A\beta$ in the plaques gives rise to AD by causing neurons to stop functioning and eventually dying off [6].

To understand the amyloid cascade hypothesis, it is useful to take a closer look at the APP processing pathway. APP is a 110-135 kDa membrane bound protein, of which the exact physiological function is unknown [7]. Data from knock-out models and studies of the APP promoter suggest that APP plays a role in neuronal development, but the function in later life remains unclear [8]. APP can be cleaved by α -secretase, of which ADAM10 is the most abundant in the brain [9]. Cleavage of APP by α -secretase, results in two fragments called soluble APP α (sAPP α) and the membrane bound C-Terminal Fragment- α (CTF α). Subsequently, CTF α can be cleaved by γ -secretase resulting in the p3 fragment and the cytosolic Amyloid Intracellular Cyttoplasmic Domain (AICD) (see Figure 2.1). The p3 fragment is in fact a truncated form of $A\beta$, but is thought to be pathologically irrelevant [10]. Cleavage by α -secretase puts APP to the so-called non-amyloidogenic arm, since no $A\beta$ is generated.

APP can also be cleaved by a β -secretase, of which BACE1 is the pathologically relevant secretase for AD. Cleavage by BACE1 will result in an sAPP β fragment and a

CTF β fragment. Subsequent cleavage of CTF β by γ -secretase will eventually result in generation of A β and the AICD fragment (see Figure 2.1) [11]. This is called the amyloidogenic pathway.

Figure 2.1: APP can be cleaved by α -secretase resulting in the generation of sAPP α and CTF α . This will shift APP processing to the non-amyloidogenic pathway, since subsequent processing by γ -secretase (yellow scissors) will result in the non-toxic p3 fragment and the AICD fragment. However, if APP is cleaved by β -secretase (red scissors), APP processing will shift to the amyloidogenic pathway, resulting in generation of A β by subsequent processing of γ -secretase. Next, soluble A β monomers can start to aggregate to oligomers and protofibrils. Finally, protofibrils will aggregate into non-soluble fibrils and be deposited into amyloid plaques.

Depending on the exact cleavage of γ -secretase, different sizes of A β are formed which can range from 37-49 amino acids [11]. From these different proteins, A β 40 is viewed as the normal cleavage product, while A β 42 has been suggested to be the pathogenic form eventually resulting in AD [12]. Patient data also shows that they produce more A β 42 than healthy individuals [13]. Furthermore, A β 42 has been shown to have a higher tendency to aggregate [14] and mutations in APP, PSEN1 or PSEN2 result in generation of more A β 42, or in a shift in processing towards the amyloidogenic pathway [15].

This generation of more A β 42 resulting in plaques has long been considered the central event in the amyloid cascade hypothesis. In recent years however, the hypothesis has been refined due to conflicting evidence about A β 42 [16]. Monomeric A β 42 aggregates into oligomers, protofibrils, fibrils and eventually gets deposited into plaques (see Figure 2.1). Contradictory data exists about which form is more toxic, but recent research suggests that it is the oligomeric form, and not the fibrillar form which is more toxic [17]. Additionally, oligomeric A β in CSF has been shown to correlate better with clinical progression of AD, while tau or A β 42 levels did not [18]. Other research

suggests that other forms of $A\beta$, such as N-truncated $A\beta$, might be the more toxic forms and the actual initiator of the disease [19].

2.2. OTHER FACTORS IN AD

AD is a multifactorial disease and there are different theories which may explain its etiology and progression. Additionally, the clinical picture is complicated due to the fact that 60 % of AD patients have at least three comorbidities [20].

Besides $A\beta$, tau tangles are the most important hallmark of the disease brain. Tau is an intracellular, highly soluble protein whose main function is to stabilize axon microtubuli. Under physiological conditions it is mainly contained to the axonal compartment, while under pathological conditions it gets missorted to the somadentrical compartment. Not only in AD, but also in other tauopathies, tau gets hyperphosphorylated, missorted, starts forming intracellular tangles, and eventually causes neuronal dysfunction [21]. Data from animal models suggests that there is at least a synergy between the two pathological hallmarks of the disease, tau and $A\beta$. Some studies show that tau pathology is worsened with the presence of $A\beta$ [22]. Other studies, where tau knockout mice are crossed with AD mouse models with $A\beta$ plaques, show that without tau, $A\beta$ does not have toxic effects on the brain, or there is a reduced plaque burden [23, 24]. Interestingly, cognitive decline seems to correlate better with tau pathology, suggesting that tau might be the driving force in clinical AD progression [25].

The strongest genetic risk factor for AD is the APOE $\epsilon 4$ allele. APOE is the most common lipoprotein in the brain and consists of three isoforms. The $\epsilon 4$ allele occurs in about 15% of the normal population, but is present more in the AD population with a percentage of 40%. Homozygous carriers with $\epsilon 4/\epsilon 4$ even run 15 times more risk to develop AD [26]. Carriers with the $\epsilon 2$ allele on the other hand, seem to be protected for AD. Despite the fact that APOE4 is such a strong risk factor, it is not yet clear whether the increased risk is caused by a toxic gain-of-function, a neuroprotective loss-of-function, or both [16]. APOE4 has been suggested to have an effect on $A\beta$ aggregation and clearance (for a review see: Liu et al [27]). Interestingly, the $\epsilon 4$ allele also has significant effects on neuroinflammation. In $\epsilon 3/\epsilon 4$ carriers, the innate immune response after LPS administration is stronger than in $\epsilon 3/\epsilon 3$ carriers [28]. Furthermore, post-mortem analysis has shown increased microglial activation in the brain of APOE4 AD patients [29] (for a review: Tai et al [30]).

2.3. THE IMMUNE SYSTEM AND AD

Recently, the role of neuroinflammation in AD has become more important. Already in his original descriptions of the disease, Alois Alzheimer mentioned that plaques were surrounded by microglia, the macrophages of the brain, but it was believed for many years that the immune system played only a secondary role in the disease [31]. Other clues about the importance of the immune system in AD came from epidemiological studies showing that anti-inflammatory drugs might be protective against AD [32, 33]. However, clinical trials using non-steroid anti-inflammatory drugs (NSAIDs) failed to show any therapeutic effect [34, 35].

While both the innate and the adaptive immune system play a role in AD, current knowledge points to a much greater involvement of the innate immune system [36]. In the next sections, we will highlight more in depth the various components of the innate immune system that play a role in AD, namely microglia, the complement system, and the inflammasome.

2.3.1. THE ROLE OF MICROGLIA IN AD

Microglia can detect danger and pathogen associated molecular patterns (DAMPs and PAMPs) through receptors. It has been known for many years that microglia can be activated by binding of fibrillary A β to CD36 [37]. Upon binding of A β , CD36 will recruit Toll-like receptors and will form a complex with TLR4 and TLR6, resulting in activation of microglia and secretion of IL-1 β [38]. This sustained pro-inflammatory response will eventually lead to a downregulation of critical A β degrading enzymes, resulting in an impairment of microglial A β clearance [39]. Additionally, it has been shown that tau can also activate microglia resulting in the release of IL-6 [40]. While there have been ample studies, the role of microglia activation in the disease process remains unclear [41]. Current evidence points to a protective role of the cells in early stages of AD, while prolonged activation might be detrimental [42].

As stated before, microglia can recognize A β through means of CD36, after which phagocytosis can follow and subsequent degradation by the proteases neprilysin and insulin-degrading enzyme [43]. In AD patients, microglial clearance has been shown to be impaired [44], which is caused by the sustained pro-inflammatory environment of the AD brain [43].

In the last decade several findings have further established the important role of microglia in the disease. First, genome wide association studies (GWAS) revealed that a number of immune related genes are associated with sporadic AD, of which CD33 is an important gene related to microglia [45]. CD33 is a type I transmembrane protein which is predominantly expressed on microglia cells. In patients, CD33 expression is increased and it has been shown that CD33 can be activated by binding of sialic acid residues on glycolipids and proteins present in amyloid plaques leading to inhibition of microglial A β internalization [46].

GWAS further brought the immune system to the forefront of AD research with the discovery that rare variant mutations in TREM2 increase the risk to develop AD with a factor similar to APOE4 [47], a finding which has been confirmed by a number of other studies [48]. TREM2 is a receptor of the innate immune system expressed on a variety of cells such as monocytes, macrophages and microglia. TREM2 is involved in microglia and macrophages activation, and can be stimulated by negatively charged lipids such as LPS and phospholipids [48]. Of these lipids, sphingomyelin has been shown to be one of the most potent activators [49]. TREM2 has also been shown to have an anti-inflammatory function, but its exact role in the pathogenesis of AD remains elusive [50]. Studies in AD mouse models and TREM2 knock-outs report conflicting results from beneficial to detrimental [51]. The exact cause for this discrepancy is not clear, but could be due to different mouse models used, different ages of the mice, or the different type of knockout (knockout of the full-protein or knockout of the extracellular part of TREM2).

2.3.2. COMPLEMENT AND THE INFLAMMASOME IN AD

The complement system plays an important role in AD progression. Already many years ago, it was shown that A β can directly activate the classical complement pathway by binding to C1q [52]. Activation of C1q occurs through binding of A β to the collagen-like domain of C1q [53]. However, in mice models of AD, it has been shown that complement activation has been weaker compared to human complement activation, which might be due to the different structure of C1q in mice [54, 55].

Interestingly, the ceramide transporter (CERT), which is abundant in brain and the only human protein known to transfer ceramide between membranes, can also activate C1q via the classical pathway and is associated with plaques [56]. Ceramide is the backbone of all complex sphingolipids which play both a structural role in membranes as well as act as second messengers balancing death and survival signals in the cell [57]. This provides an additional link between lipid metabolism and AD [58, 59].

Perhaps counterintuitively, complement can also have beneficial and protective effects in the CNS. This is evidenced by the fact that C1q is also responsible for synaptic pruning, which plays an important role during development [60]. C1q has also been shown to have neuroprotective effects, but only in the absence of other downstream complement factors [61]. In AD, the role of complement seems to be more complicated. Deficiency of C1q has been shown to ameliorate neuropathology in an AD mouse model, which is probably due to a reduction in inflammation [62]. A comparative genome-wide expression analysis in different AD mouse models, also pinpointed C1q as a central hub in both A β and tau mouse models, further implicating complement in AD pathogenesis [63].

In healthy wild type animals, C3 deficiency seems to be protective for age-related memory loss, which is in line with C1q studies [64]. Additionally, recent results also point out that C3 by itself impairs phagocytic abilities of microglia, further suggesting that C3 plays a detrimental role in AD [65]. Two studies have shown that C3 might have a beneficial role in ameliorating AD by increasing phagocytosis [66, 67]. These studies are limited however in that they make use of C3 knock-out models, and therefore do not have prolonged activation and deposition of C3 as observed in patients or other mouse models [68].

The importance of complement also became clear with recent GWAS results showing that Complement Receptor 1 (CR1) is a potential risk factor for AD [69]. CR1 acts as an inhibitor of the complement pathway by binding and subsequent clearing of C3b or C4b, two factors of the complement cascade [70]. So far, studies investigating the described mutation in CR1 have been unable to pinpoint its exact functional contribution to AD [71]. A possible reason for this is that mice do not express CR1 on erythrocytes, while it has been shown that A β 42 can be cleared by binding of C3-opsonized A β to CR1 on human erythrocytes [72].

The inflammasome is a protein complex where several signaling pathways from the innate immune system, such as those initiated by TLRs, converge and result in the production of cytokines by activation of caspase-1 [73]. Several inflammasome complexes have been discovered and in AD Nod-like receptor pyrin domain inflammasomes (NLRP) have been implicated in the disease process. In particular, NLRP3, which is a cytosolic inflammasome sensor, has been shown to be activated by

$A\beta$ [74]. The activation of the NLRP3 inflammasome results in the production of IL-1 β , which has been reported to be elevated in AD patients [75], just as the levels of NLRP3 inflammasome itself [76]. Finally, studies with AD mouse models have shown that disabling the NLRP3 inflammasome pathway, restores memory and improves $A\beta$ clearance [77].

2.4. EFFECTOR MECHANISMS OF $A\beta$ IMMUNOTHERAPY

Two different approaches of anti- $A\beta$ immunotherapy can be employed: active or passive immunization. In active immunization, partial or full-length $A\beta$ is injected into the patient together with an adjuvant (vaccination). The patient will produce anti- $A\beta$ antibodies which will clear-away the plaques. Classes of adjuvants are (1) mineral compounds such as alum, (2) bacterial products such as lipopolysaccharide (LPS), (3) emulsion-based adjuvants such as Freund's, and (4) immunestimulatory compounds such as cytokines [78]. Adjuvants are necessary to, amongst other things, enable a more rapid immune response, increase antibody titers or overcome immune senescence which is common in elderly patients [79]. They exert their action by acting like a depot that releases antigen over a prolonged period of time (eg. emulsion based adjuvants), or by stimulating the immune system by increasing cytokine production, recruit immune cells, or activation of inflammasomes (eg. alum) [80].

In passive immunization, (recombinant) antibodies are administered to the patient for as long as necessary, in other words the patient does not need to make its own anti- $A\beta$ antibodies. While passive immunization offers the benefit that the therapy can be more controlled (e.g. immune response) and the target can be better chosen, it also has significant cost disadvantages, since antibody production in these quantities is costly and time consuming. Additionally, there is the risk that patients will develop neutralizing antibodies, which might also lead to serious consequences such as anaphylactic shock [81]. With active immunization on the other hand, there is the risk that the patients might not generate an antibody titer at all, because of self-tolerance against $A\beta$ or because of immune system senescence at old age [82].

While some effector mechanisms of immunotherapy have been studied on post-mortem brain tissue of patients, most of the details have been elucidated by immunotherapy studies in preclinical mouse models. Three possible mechanisms have been proposed: (1) Fc-Receptor mediated phagocytosis; (2) peripheral sink action; and (3) catalytic dissolution (see Figure 2.2). The three mechanisms are not mutually exclusive and may act in concert. Surprisingly, a possible role of the complement system has not been investigated in immunotherapy. We will discuss all three mechanisms in more detail, and review the evidence for them.

Figure 2.2: Effector mechanisms of anti-A β immunotherapy. Several mechanisms for immunotherapy have been proposed. First, antibodies have catalytic activity by themselves and are able to break down plaques into smaller peptides, or allow soluble A β to be removed from the brain by means of the LRP1 receptor. In a second mechanism, soluble A β in the blood could be bound to antibodies which could prevent it from re-entering the brain by means of the RAGE receptor. This would essentially lead to a net efflux of A β from the brain. Finally, antibody bound to A β plaques could activate microglia and by means of Fc-receptor mediated phagocytosis, the plaque would be engulfed and removed.

2.4.1. FcR MEDIATED PHAGOCYTOSIS

A first effector mechanism of A β clearance by immunotherapy is Fc-receptor mediated phagocytosis of plaques by microglia. As mentioned earlier, it is not clear if microglia play a beneficial or detrimental role in AD. This controversy regarding the role of microglia in AD is also found back in research investigating their function during immunotherapy in AD. Microglia have been shown to be able to phagocytose A β via various receptors such as TLR4 [83] and the scavenger receptor [84]. It is hypothesized that antibodies opsonize plaques and increase the phagocytosis of plaques by microglia, thereby decreasing total A β burden in the brain.

Schenk et al. suggested already in the first active immunization study in mice that A β clearance might be mediated by microglial phagocytosis and offered some support by showing cells from their immunized mice with a microglial phenotype that contained A β [85]. A later study performed by Brazil et al. showed that *in vitro* microglial uptake of A β is indeed enhanced after coating plaques with IgG or C1q, but degradation rates in lysosomes remains the same [86]. Backsai et al. subsequently showed by means of *in vivo* 2-photon imaging that after application of anti-A β antibodies more microglia were associated with plaques [87]. Later on, Bard et al.

observed that phagocytosis of A β is dependent on isotype and epitope specificity of the antibody by showing in an *ex vivo* assay that only antibodies directed against N-terminal epitopes of A β are able to clear plaques via FcR-mediated phagocytosis [88]. They also reported that murine IgG2a antibodies, which have a higher affinity for the murine FcR compared to murine IgG1, are better at reducing plaque load compared to IgG1 antibodies [88]. An overview of the effector functions of the different mouse and human isotypes is presented in Table 2.1. Wilcock et al. showed at the same time that clearance of A β in the mouse brain through intracranial injection of an (IgG1) antibody resulted in three different phases of clearance: a first phase which lasts approximately 24 hours and is microglial independent, a second, longer phase between 24 and 72 hours which is marked by an increase in microglial activation as assessed by CD45 and MHC-II staining, and a third phase which shows a normalization to baseline microglial activation [89]. Interestingly, their results are corroborated by data from the AN-1792 trial, in which post-mortem material of participants shows an initial activation phase, followed by a resolution of activation [90].

In a different study, Wilcock et al. also showed that FcR activation seems to be necessary for efficient removal of compact plaques, but not for diffuse plaques. They found that Fab-fragments, which do not bind the FcR, were less efficient in plaque clearance than whole IgG. The same result was found when microglial activation was inhibited with dexamethasone. No difference was found for diffuse plaques clearance, which seems to be mediated in an FcR independent way [91]. Furthermore, in a follow-up study using systemic injection of antibody, they confirmed that microglia cells show more FcR expression after 1 month of antibody administration, and more CD45-expression after 2 months of administration, while after 3 months these markers returned to normal levels together with a significant reduction of A β load in the brain [92].

All in all, these experiments demonstrate that microglia phagocytosis has an important contribution to plaque clearance [88].

2.4.2. PERIPHERAL SINK

The peripheral sink hypothesis states that immunotherapy changes the A β influx/efflux equilibrium to the brain. Antibodies bound to A β in the periphery disturb their influx back into the brain, resulting in a net increase of A β efflux. The overall result is less A β in the brain. The peripheral sink hypothesis was first coined by DeMattos et al. while studying the m266 antibody for immunotherapy (now in trial as solanezumab) [93, 94]. M266, binds to the mid-region of A β (AA 13-28) and has a high affinity for soluble monomers [95]. In these studies, DeMattos showed that administration of m266 in PDAPP mice caused a rapid increase in plasma A β levels followed by a decrease in brain A β load [93]. Since transgenic mice only express human A β in the brain, this result could only be explained by the fact that peripheral administration of the antibody caused an increase in brain efflux [93]. Furthermore, in a follow-up experiment they also show that there is a correlation between plasma A β levels and total A β brain burden after administration of m266, suggesting that plasma A β levels after immunotherapy could be used as a serum-based biomarker to assess amyloid status [94].

It should be noted that the results from DeMattos et al. have failed to be replicated

by a different group. Seubert et al. find no reduction in brain A β load after administration of m266 antibody [96]. They do find elevated plasma levels of A β after m266 administration, which might have been caused by the fact that m266 increases the serum half-life of A β . Combined with the high-turnover rate of A β , this might explain the increase in serum A β . A possible explanation for these conflicting results might be found in the animal model, which shows variable plaque accumulation between animals, a fact also noted by DeMattos [96]. The fact remains that the rapid elevation in plasma A β levels might indeed be due to an increase in serum-half-life of A β as this has also been reported by other groups [97]. Interestingly, Dodart et al. report improvement in cognitive performance of animals without any reduction in brain A β load after administration of m266 in an acute treatment model, where animals were tested one day after antibody administration [98].

The peripheral sink effect is probably mediated through the Receptor for Advanced Glycation End-products (RAGE). RAGE has A β affinity in the nanomolar range and blockage of RAGE suppresses A β influx into the brain [99]. Recently, it has been shown that a peripheral sink action of an antibody through RAGE is also epitope specific [100]. Bachmeier et al. showed using an *in vitro* BBB model expressing RAGE that N-terminal antibodies are better at preventing A β influx into the brain across the RAGE receptor. C-terminal directed antibodies had no effect at all, while mid-terminal antibodies had a mild effect [100]. It would be interesting to see how Solanezumab performs in this experiment, or whether there is some indication that the peripheral sink effect is dependent on other mechanisms besides RAGE transport.

While these studies support the peripheral sink mechanism, a recent study sheds some doubt about this hypothesis. By utilizing neprilysin to degrade A β in the periphery, Henderson et al. showed that in monkeys, rats, and non-human primates, A β in the periphery is degraded without any effect on A β levels in the CNS [101]. An alternative explanation might be that antibodies targeting soluble forms of A β , prevent A β from aggregating into more toxic oligomers by binding to it and increase the efflux of A β from the brain [102]. This would also explain the plasma spike of A β seen after immunotherapy with some antibodies. A possible mechanism by which antibodies increase A β efflux from the brain could be by transport of A β bound to antibodies by means of the neonatal Fc-receptor (FcRn) [103].

2.4.3. CATALYTIC DISSOLUTION

One of the simplest putative effects of immunotherapy is the catalytic dissolution of plaques or the prevention of fibril formation. The reason why this mechanism is simple, is because it is a direct effect of the antibody and not dependent on other processes such as microglial activation. Several experiments showed that anti-A β antibodies could prevent fibrillogenesis of A β *in vitro* [104, 105, 106, 107, 108, 109] and application of Fab fragments *in vivo* support this hypothesis [110]. Furthermore, the ability of an antibody to prevent fibril formation also seems to be dependent on the affinity of the antibody [105] and the recognition site in A β (epitope). In a direct comparison study between the 3D6 (N-terminal) and m266 (mid-terminal) antibody, both antibodies performed equally well in a Thioflavin T binding assay, showing that both antibodies prevented beta-sheet structure formation. Analysis of this effect by atomic force

microscopy revealed that m266 was more effective in preventing fibril formation [111]. Besides full antibodies, also scFv fragments, have been shown to prevent fibril formation and prevent toxicity [112].

Antibodies might also catalyze the direct dissolution of plaques or fibrils. The strongest example of this process has been shown with light chains that directly cleave A β , thereby preventing the formation of cytotoxic plaques [113]. Specifically, it has been shown that naturally occurring IgM antibodies exert proteolytic activity toward A β and that catalytic activity of autoantibodies increases with age [114, 115]. Anti-A β antibodies can be isolated from IVIG which is a cocktail of human IgG isolated from a large amount of healthy donors, and usually applied in autoimmune diseases because of its anti-inflammatory properties. These anti-A β antibodies from IVIG have been shown to not only prevent fibril formation, but also dissolve existing plaques [104].

2.5. BLOOD BRAIN BARRIER

One of the main issues with targeting the brain is delivery of pharmaceuticals across the BBB. While extensive efforts have been directed towards the delivery of small molecules in the brain, there has been surprisingly little work done on how antibodies can exert their therapeutic effects in the brain of Alzheimer patients [116].

The BBB is a network of astrocytes surrounding the blood vessels of the brain combined with tight-junction of vessel endothelial cells that restrict the entry of molecules into the brain. Especially passage of large molecules is limited, unless there is a particular transporter system in place for these molecules. Research has shown that antibodies cross the BBB by means of the extracellular pathway [117]. The extracellular pathway is a collection of leaky vessels mostly located around the circumventricular organs [118]. This influx of antibodies is offset by an active transport mechanism present at the blood brain barrier that transports IgG back to the periphery.

There is conflicting evidence about the role of the neonatal Fc receptor (FcRn) regarding IgG efflux of the brain. Deane et al. showed that the FcRn is necessary for anti-A β IgG mediated efflux from the brain, using a combination of AD mice and FcRn knockouts [103]. Another study, using human monoclonal antibodies with different affinities for the FcRn also showed involvement of the FcRn in rat [119]. Other studies, also using FcRn and Fc-gamma receptor knockouts, showed no involvement of the FcRn in antibody efflux from the brain [120, 121]. Possible explanations for these discrepancies might be due to different administration methods (systemic and intracranially) or the reduced antibody half-life in FcRn knockout mice.

An additional factor that influences the penetration of antibodies into the brain of AD patients is the fact that the BBB in AD patients is more leaky [122]. This increased permeability is likely caused by factors such as hypoxia, inflammation in the AD brain and amyloid itself [123]. Furthermore, experimental evidence in animal models show that this altered permeability results in decreased A β efflux and increased A β influx, possibly resulting in further progression of the disease [124]. An increased permeability might make it easier for therapeutic antibodies to reach the brain. One animal study shows that BBB integrity was restored after active immunization, suggesting that once the amyloid pathology has been resolved, BBB permeability is restored to normal levels [125]. Nevertheless, it is important to keep in mind that even when the BBB is intact,

about 1% of circulating antibodies are present in the brain [126].

2.6. CLINICAL RESULTS OF ANTI-A β IMMUNOTHERAPY

Besides the extensive research performed in several AD animal models, at least 25 immunotherapies against A β with a total of over 25 000 patients have progressed to clinical trials (see Table 2.2 and Table 2.3). So far the results from the first wave of these trials have shown little benefits for the patients due to either too advanced disease status of the patients, heterogeneity of the patient cohort or unsuitable effector functions of the therapeutic antibodies, or a combination of the aforementioned. Nevertheless, very valuable information has been obtained from them and modified, improved strategies are currently ongoing. In the following sections, results from human clinical trials are summarized. Active immunization trials that have been conducted can be found in Table 2.2 and are discussed below. Subsequently, in Table 2.3 ongoing and completed passive immunization studies are presented.

2.6.1. ACTIVE IMMUNIZATION

A first prove of concept that immunization might be a viable treatment strategy was provided by Schenk et al. over 15 years ago in their seminal paper on active immunization with A β 42 in the PDAPP mouse model, overexpressing mutated human APP [85]. This vaccination was tested in patients, after successfully completing a phase I trial, but the drug was halted in a phase IIa trial (called AN-1792) after 6% of the patients developed meningoencephalitis [127]. The active immunization protocol consisted of repeated vaccination with full-length A β 42 administered with the QS-21 adjuvant to boost the immune response. QS-21 is a emulsion based adjuvant that increases antibody response, but also T-cell response [128].

Although an initial study reported slowing of cognitive decline in responders [129], later studies showed that even though A β plaques were removed, cognitive deterioration was not halted [130]. A long-term follow-up study of AN-1792 participants, performed almost 5 years after vaccination, did show significant slowing of decline on some cognitive and functional scales, suggesting there might be some long-term benefits to vaccination [131].

From a biological point of view, much was learned from this vaccination study. The majority of the antibodies generated were directed against the N-terminus of A β [132]. This might pose an issue since it is known that up to 60% of A β species in patients has a modified or truncated N-terminus and this might therefore offer an explanation for the absence of a strong effect [133]. Additionally, it was also observed that immediately following immunization, A β is solubilized and transported from the parenchyma to the white matter and the vasculature [134, 135]. Interestingly, A β 42 levels in the brain were lowered in immunized patients as assessed by immunohistochemistry [134], but ELISA measurements in two patients showed no differences in A β load [135]. Whether this reduction is a beneficial or harmful effect is currently unknown. Besides a reduction in A β 42 in the brain, also a reduction in phospho-tau was observed, although this reduction was confined to neuronal processes and not to the cell body [136].

Initial imaging studies shortly after the vaccination showed an increase in

ventricular volume [137], which has been attributed to faster loss of already damaged neurons due to increased microglial activity [138]. This finding would also be consistent with the fact that long-term follow-up studies show no increased cerebral atrophy in the immunized patients compared to controls [131]. Another study from the group also shows that immediately following vaccination inflammation is increased, while long-term follow-up shows that inflammatory markers are reduced beyond observed levels at similar AD stages [90].

Sterile meningoencephalitis observed in 6% of the cases has been attributed to possible activation of cytotoxic T-lymphocytes, a finding which later has also been confirmed in one case study [139]. This aberrant activation has been attributed to the use of the QS-21 adjuvant which can also stimulates T-cell activation [140].

At the moment, new active vaccination therapies that make use of different adjuvants are under investigation, with as goal in mind to generate a sufficiently strong immune response without causing aberrant T-cell activation, thereby making them more safe to use [82]. One of these, CAD106, developed by Novartis, has been tested extensively in patients and immunizes with $A\beta_{1-6}$ [141]. So far, the trials are continuing without any cases of meningoencephalitis or safety problems. Preliminary analysis from Phase IIa trials in 45 patients shows no cognitive benefits as assessed by the ADAScog (Alzheimer's disease assessment scale-cognitive subscale) [142]. It is important to note that these trials were underpowered to detect any possible cognitive effects, and were solely conducted to assess safety and tolerability.

Results from a Phase IIa trials with the active vaccine ACC-001 showed similar results. ACC-001 is an active vaccine making use of $A\beta_{1-7}$ conjugated to a carrier protein and also makes use of the QS-21 adjuvant used in the AN-1792 trial. So far, no safety issues have been reported and patients showed a robust immune response, but exploratory cognitive evaluations also showed no effect of the therapy [143]. This study was also underpowered to detect cognitive benefits, and additionally, no biomarker analysis was done before inclusion of patients, so it is possible that dementia patients without AD were also included.

Affiris has developed a therapy called Affitope where a peptide resembling $A\beta$ is used to elicit anti- $A\beta$ antibodies. Because of the fact that no self-peptide is used, alum can be used as an adjuvant [144]. Surprisingly, in a Phase II clinical trial testing the Affitope vaccine, patients in the placebo group (AD04, the vaccine without the affitope), performed better than patients in the experimental group (AD02, the vaccine with the affitope) on various cognitive outcome measures such as the ADAScog [145].

ACI-24 is an active liposome based vaccine and results in the generation of antibodies targeting conformational epitopes present on oligomeric $A\beta$ [146]. This vaccine is currently being tested in Phase I/II clinical trial. Although no clinical results have been reported yet, the vaccine is noteworthy because it will be the first immunotherapy which will be tested in patients with Down syndrome.

2.6.2. PASSIVE IMMUNIZATION

After the failure of the AN-1792 trial, passive immunotherapy was brought forth as a safe alternative, and in the last decade several passive immunotherapy trials have been conducted. A first trial was conducted with bapineuzumab. Bapineuzumab (IgG1

isotype) is the humanized form of the murine 3D6 anti-A β antibody, which binds the N-terminal of A β and has a high affinity for plaques [147]. 3D6 had previously been shown to be effective in reducing plaque load in the PDAPP mouse model [148, 149]. The phase I trial of bapineuzumab was characterized by the occurrence of Amyloid-Related Imaging Abnormalities (ARIA) [150]. ARIA, sometimes also described as micro-bleeds, is transient in nature and can be detected on a MRI scan. Two types of ARIA are distinguished: ARIA-E, which is thought to represent vasogenic edema and which can be detected by a FLAIR MRI; and ARIA-H, which is related to microhemorrhages and hemosiderosis and can be detected by a T2* weighted MRI scan [151]. What the clinical consequences of ARIA are or even if it poses a health risk for the patient is unknown [152].

In a phase II trial, primary endpoints on cognitive benefits were not met [153]. However, exploratory analysis did show a potential benefit for APOE4 non-carriers on various outcomes such as the ADAScog and other cognitive scales [153]. In addition, amyloid PET scans showed a reduction of 8.5% in fibrillary amyloid in bapineuzumab treated patients, while there was a concomitant increase of 16.9% in placebo controlled patients [154].

Analysis showed that risk factors for ARIA were APOE4 status and dose, with more occurrences in the high dose group (2 mg/kg) [151]. Because of these reasons, it was decided to only administer the lowest dose of 0.5 mg/kg in two phase III trials, with one trial only including APOE4 carriers, and the other trial only including non-carriers [153].

In 2012, the two phase III trials were concluded. Unfortunately, both failed to meet their primary endpoints, and no evidence of clinical benefit was present on any of the applied scales [155]. Suggestions were made that the bapineuzumab dose might have been too low, treatment started too late, or the wrong A β species (fibrillary A β) was targeted [155]. Amyloid PET, using 11C-PiB, did show a reduction of fibrillary A β [156]. Furthermore, similar to reports from the AN-1792 trial, increases in ventricular volume were reported compared to placebo [157]. Clinical trials using a modified version of bapineuzumab with reduced Fc effector function, called AAB-003, were completed in 2014, but to date, no results are reported [158, 159]. This modified version of bapineuzumab uses an IgG4 backbone and does not activate complement or binds to Fc-receptors (see Table 2.1) [160].

At approximately the same time as the bapineuzumab clinical phase I trial a solanezumab phase I trial started in parallel. Solanezumab is a humanized IgG1 antibody, (the original mouse is called m266, see section Peripheral sink). Interestingly, the original mouse isotype used in the pre-clinical studies was a mouse IgG1 isotype [93], of which the effector functions would be more comparable to the human IgG4 isotype [161] (see Table 2.1). In contrast to bapineuzumab, solanezumab seems to be better tolerated with lower incidences of ARIA even though the doses used were higher [162].

Table 2.1: A comparison of effector functions of the different human and mouse isotypes. The human Fc γ RIIB and the mouse Fc γ RIIB are inhibitory Fc γ -receptors, while the other Fc γ -receptors are activating. The Fc γ Rn (neonatal) is involved in antibody half-life and transport. (Table adopted from [201] and [161])

Human	Isotype	IgG1	IgG2	IgG3	IgG4
	Complement activation	+++	+	++	-
	FcγRI	++	-	++	++
	FcγRIIA	+	+-	+	+
	FcγRIIB	+	+-	+	+
	FcγRIIC	+	+-	+	+
	FcγRIIIA	+	+-	+	+
	FcγRIIIB	+	-	+	+
	FcγRn	++	++	+	++
Mouse	Isotype	IgG1	IgG2a	IgG2b	IgG3
	Complement activation	+	++	+++	++
	FcγRI	-	++	+	+-
	FcγRIIB	+	+	+	-
	FcγRIII	+	+	+	-
	FcγRIV	-	++	++	-
	FcγRn	++	++	++	++

Shortly after the bapineuzumab results were announced, the results from two phase III trials with solanezumab were also published. Results showed that again primary endpoints as assessed by the ADAS-cog were not reached [163]. However, patients with mild AD (Mini Mental State Exam (MMSE) score between 20 and 26) showed a small, but significant benefit (34% reduction in decline) on the ADAScog and the MMSE [164].

For the first time in the AD field, a delayed-start trial is being performed on patients that participated in the phase III solanezumab trials. In the delayed-start trial, all patients that participated in the study, including those from the placebo group, will receive solanezumab. Patients and physicians are still blinded to their original condition however. If, at the end of the trial, the difference in cognitive deterioration between the original placebo group and experimental group remains, there is a true treatment effect of the drug. The midterm evaluation from this trial shows that for mild AD patients, the early treatment benefit remains and thus it seems likely that solanezumab has true disease modifying effects, while also lending some evidence that treatments need to start early in the disease process [165]. At the moment, Solanezumab is being investigated in a phase III study with only mild AD patients and in a parallel preventive trial organized by the Dominantly Inherited Alzheimer Network (DIAN) where it will be administered to patients with familial AD before the onset of symptoms [166, 167].

Gantenerumab is an IgG1 anti-amyloid antibody [168]. Contrary to bapineuzumab and solanezumab, it is a fully human antibody generated by phage display. It binds N-terminally to A β with a high affinity for amyloid fibrils, and therefore has similar binding characteristics as bapineuzumab [168]. In a phase II trial, it has been shown to show dose-dependent reduction of A β as assessed by PiB PET scans [169]. Like bapineuzumab,

it also shows problems with ARIA [169]. Gantenerumab was tested in two trials: a phase III trial and a prevention trial (DIAN). In winter 2014, the phase III trial was unexpectedly halted after interim analysis showed no cognitive benefit [170]. The antibody is now being continued in a phase III trial using only patients with mild AD [171].

Another antibody being tested is crenezumab from Genentech. In contrast to all other antibodies tested, crenezumab is a humanized antibody with an IgG4 backbone with reduced effector functions and it might have less side-effects such as ARIA [172]. Interestingly, it also binds soluble A β monomers similar to solanezumab (also mid-terminal) and structural analysis has shown it has almost identical binding properties as solanezumab [173]. In principle, isotype effects could now be compared and this might yield valuable information about the role of inflammation in AD. Furthermore, like solanezumab in the DIAN trial, crenezumab is being tested in a preventive trial called the Alzheimer's Prevention Initiative (API), a trial conducted in the Columbia cohort [174]. In 2014, Genentech announced that the antibody failed to meet its primary endpoints in a phase II trial, but the planned API trial will continue [175].

One promising therapeutic antibody is aducanumab. While no reports have been published yet, some promising results have been publically shared. Aducanumab is a fully human IgG1 antibody targeting fibrils and A β oligomers and binds to epitope A β ₃₋₆ [176, 177]. It has been isolated from a healthy centenarian by NeuroImmune and is now being trialed by Biogen Idec. In a phase I trial, the antibody showed a dose dependent slowing of cognitive decline on several cognitive assessment scales. While this group was small, the results looked very promising. One possible caveat was the high incidence of ARIA-E with the antibody. The antibody is now progressing directly to a phase III trial [178].

Another trial, BAN2401, uses a humanized monoclonal antibody targeting A β protofibrils. Protofibrils occur after oligomerization of A β and have been shown to be possibly more neurotoxic than fibrils [179]. Phase I results from this antibody showed a good safety profile and the therapy is currently under further development [180]. Interestingly, the Phase II study will make use of an innovative trial design based on Bayesian statistics [181]. This design involves frequent interim analyses and changing the randomization procedure based on the outcome of this analysis during the trial. This should allow for both a reduction in the size and the duration of the trial, because group sizes of eg. placebo or certain doses are decreased or increased based on the interim analysis.

An interesting alternative for the use of monoclonal antibodies is the use of intravenous immunoglobulin (IVIG). The mechanism of action for treatment of AD is presumed to depend on the occurrence of natural antibodies present in healthy people [182] including natural anti- β antibodies [104]. Trials using IVIG have been modest in the numbers of patients enrolled. Four phase I and II trials enrolling between 6 and 24 patients showed very good results, with an improvement on various outcome measures or a stabilization of cognitive decline [183]. However, a larger phase II trial with 55 patients and a phase III trial with 390 patients provided no proof for cognitive benefit [183]. At the moment, IVIG is being tested in various clinical trials in patients with mild AD or prodromal stages. An exploratory study in a small group of mild cognitive impairment (MCI) patients suggests that IVIG can delay the onset of AD by at least 1

year [184].

2.7. ADDITIONAL SAFETY CONCERNS IN IMMUNOTHERAPY

Besides the meningoencephalitis cases observed in the AN1792 trial, there are a number of other concerns related to amyloid immunotherapy. The most observed safety concern in immunotherapy is of course ARIA. As described earlier, there are two types of ARIA: ARIA-E and ARIA-H (see section “Passive immunization”). Little is known about the long-term effects of ARIA, but they are detected in A β immunotherapy studies in both humans and rodents. Mouse studies already suggested that the occurrence of ARIA is dependent on the A β form being recognized by the antibodies (soluble versus insoluble) [185], and this is confirmed in the human clinical trials, where none to low incidences of ARIA were reported in the solanezumab and crenezumab trials, which both targeted soluble forms of A β [175, 164]. The exact cause of ARIA is unknown, but it is probably triggered by removal of vascular A β , causing a temporary increase in vascular permeability [186]. This is further supported by the fact that antithrombotic agents increase the risk for ARIA-H [187]. Additionally, mouse studies show that A β immunotherapy can result in increased matrix metalloproteinase activation, occasioning degradation of the extracellular matrix [188].

An often overlooked point is that plaque dissolution might be detrimental to AD progression in patients. Although still no consensus has been reached on the most toxic form of A β , there is a general tendency to attribute the strongest toxic effects to the (proto-)fibrillar and oligomeric forms of A β [189]. In this view, plaques are considered a neutralized form of A β that is actually protective since they clear away (accumulate) the toxic forms. By dissolving the plaques, immunotherapy might actually increase neurotoxicity.

One study found that while patients from the AN1792 trial had decreased A β plaques, soluble A β in the form of dimers was increased compared to patients receiving placebo [190]. Furthermore, autoantibodies against A β isolated from patients may promote aggregation and increase neurotoxicity [191], and anti-oligomeric antibodies (but not monomeric) increase microglial mediated inflammation and subsequent neurotoxicity [192].

Additionally, immunotherapy might also increase neuroinflammation. Results from a previous study show that TNF- α , a pro-inflammatory cytokine, is increased in brain after active immunization [190]. Studies in mouse models seem to suggest that active immunotherapy results in a biphasic response, showing increased expression of pro-inflammatory cytokines in early phases after vaccination, while inflammation is dampened in later phases [193].

More worrying are the results from a recently published mouse study failing to show any cognitive improvement using the murine variant of solanezumab (m266) and an oligomer specific antibody (1C22) [194]. Surprisingly, they observe an increase in 20% mortality rate in antibody treated AD mice, which might be related to increased excitatory neuronal activity caused by binding of A β to the NMDA receptor resulting in deadly convulsions [195].

2.8. CONCLUSIONS AND FUTURE PERSPECTIVES

So far, most clinical trials with anti-A β antibodies have reported negative results. However, 12 different therapies are still ongoing. Of these, solanezumab showed some promise in mild AD patients and also of aducanumab impressive results were reported on conferences.

The discrepancy between results obtained in AD animal models and patients, suggests that our current AD models are not sufficiently similar to the pathogenesis of human AD. One big difference between AD mouse models and human AD patients, is that many while many models have A β pathology, very few have tau pathology.

One study using direct intrathecal injection of anti-A β antibodies showed that targeting A β , when plaques are already developed, results in reduction of tau, but not of hyperphosphorylated tau aggregates in the 3xTg mouse model, which expresses both hAPP and mutant tau, resulting in plaques and tangles [196]. Only when the therapy was initiated before hyperphosphorylated tau was present, could tau tangles be prevented. The same result was confirmed in an active vaccination study in the same model using an amyloid oligomer vaccine [197]. These results are in concordance with those from clinical trials, showing that immunotherapy reduces plaques, but only has a limited effect on tau tangles [136].

A lesson learned is that (A β) immunotherapy should start earlier. Since clinical symptoms of AD correlate better with tau tangles [198], the question remains whether it will ever be possible to start treatment early enough in sporadic AD patients. If a patient has already memory problems, plaques and tangles are by now present, and probably it is at this time too late for anti-A β immunotherapy to have an effect, since so far only limited effects on tau have been shown. These questions will soon be addressed when the various prevention trials are finished. Maybe a combination trial, with antibodies targeting both A β and tau might provide a solution for this conundrum.

Another important aspect to consider is that passive immunotherapy is economically costly. The likelihood that immunotherapy only works in very early stages, means that patients will most likely need to receive antibodies for several years on a regular basis, increasing the financial burden. An additional problem is that patients might generate neutralizing the antibodies against the treatment they receive, thereby rendering the therapy less effective [81].

The anti-amyloid therapy road has broadened our knowledge about A β and its pathogenesis. Despite the fact that various trials have ended in negative results, some strategies still show promise as a potential AD treatment. These trials have taught us that it is necessary to define the target group better (e.g. individuals at risk, MCI, familiar forms of AD), as well as to consider the antibody effector functions and specificity for the future. New immunotherapy targets are already in progress, with various anti-tau clinical trials currently started. Finally, pre-clinical research might also offer some other targets, such as anti-APOE4 therapy specifically for APOE4 carriers or anti-IL-23 therapy [199, 200].

Table 2.2: Active immunotherapy studies listed in www.clinicaltrials.gov. When a clinical trial is still ongoing, the expected end date is listed. When a clinical trial has been prematurely ended, the term terminated is used. The following abbreviations are used: **Clinical trial Phase:** Obs-observational **Imaging measures:** vMRI-volumetric magnetic resonance imaging; ARIA-amyloid related imaging abnormalities; PiB PET-Pittsburgh Compound B PET (amyloid PET); FDG-PET-Fludeoxyglucose PET (glucose metabolism) **Safety related:** ECG-Electrocardiogram; PK-pharmacokinetic; ATA-Anti-therapy antibodies **Cognition/functional tests:** CANTAB-Cambridge Neuropsychological Test Automated Battery; ADCS-PACC-Alzheimer's Disease Cooperative Study-Preclinical Alzheimer's Cognitive Composite; CDCS-CGIC-Clinician Global Impression of Change; CDR-Clinical Dementia Rating; CDR-GS;Clinical Dementia Rating-global score; C-SSRS-Columbia Suicide Severity Rating Scale; DAD-disability assessment for dementia; DIAN-TU-Dominantly Inherited Alzheimer Network Trial; EQ-5D-EuroQol Five Dimensions Questionnaire; FCSRT-Free and Cued Selective Reminding Test; GDS-Geriatric Depression Scale; QoL-AD-Quality of Life-AD; RUD-Lite-Resource Utilization in Dementia; WAIS-R-Wechsler Adult Intelligence Scale; ZCI-AD-Zarit Caregiver Interview for AD

Therapy	Company	Adjuvant	Antigen	Trial Listing	Phase	Status	Primary endpoints	Secondary Endpoints	Patients	Inclusion
AN1792	Janssen/Pfizer	QS21	A β 1-42	NCT00021723	2	Terminated	Not provided	Not provided	375	
CAD106	Novartis	Q β bacteriophage	A β 1-6	NCT00411580	1	Completed	Safety, antibody titers	Immune response, cognitive tests	58	Mild to moderate AD (MMSE 16-26)
				NCT01097096	2	Completed	Safety, AE	Anti-A β IgG, CSF tau/A β	177	Mild AD (MMSE 20-26)
				NCT00956410	2	Completed	Safety	Immune response	21	Open label extension
				NCT01023685	2	Completed	Safety	AE, immune response	24	Open label extension
				NCT00795418	2	Completed	Safety	Immune response, cognitive tests	31	Mild AD (MMSE 20-26)
				NCT00733863	2	Completed	Safety	Immune response	27	Mild AD (MMSE 20-26)

Therapy	Company	Adjuvant	Antigen	Trial Listing	Phase	Status	Primary endpoints	Secondary Endpoints	Patients	Inclusion
AD01	Affiris	Alum	Affitope	NCT02565511	2/3	Aug 2023	Time to MCI, APCC	Immune response, CDR-SB, Safety, RBANS, Ecog, CSF tau/A β , amyloid PET, tau PET	1340	MMSE > 24, APOE4
				NCT00711139	1	Completed	Safety	Immunological and clinical efficacy	22	Mild to moderate AD (MMSE 16-26)
				NCT00495417	1	Completed	Safety		24	Follow up extension
				NCT01225809	Obs	Completed	Safety		17	
				NCT01093664	1	Completed	Safety	Immunological and clinical efficacy	20	Booster immunization
				NCT00633841	1	Completed	safety	Immunological and clinical efficacy	24	Mild to moderate AD (MMSE 16-26)
AD02	Affiris	Alum	Affitope	NCT00711321	Obs	Completed	safety	Immunological and clinical efficacy	23	Follow up extension
				NCT01357629	Obs	Completed	safety		11	

Therapy	Company	Adjuvant	Antigen	Trial Listing	Phase	Status	Primary endpoints	Secondary Endpoints	Patients	Inclusion
AD03	Affiris	Alum	Affitope	NCT01117818	2	Completed	ADASCog and ASCS-ADL	Cognitive battery, CDR-SB, NPI, vMRI	335	Early AD (MMSE >20)
				NCT02008513	2	Completed	Safety	Composite cognitive	194	Follow up observational
				NCT01309763	1	Completed	ADASCog		28	Mild AD (MMSE 20-26)
				NCT01568086	Obs	Terminated	Safety	Immunological and clinical efficacy	16	Follow-up
ACC-001	Janssen	QS21	A β 1-7	NCT01284387	2	Completed	Efficacy	Safety, biomarkers, cognitive scales	126	AD and positive amyloid PET
				NCT00960531	2	Terminated	AE, safety	Anti-A β IgG/IgM	160	Long term extension
				NCT00955409	2	Completed	AE, safety	Anti -A β IgG/IgM	50	Long term extension
				NCT00752232	2	Completed	AE, safety	Anti -A β IgG/IgM	40	AD (MMSE 16-26)
				NCT00498602	2	Completed	AE, safety	Anti -A β IgG/IgM	160	AD (MMSE 16-26)
				NCT01227564	2	Completed	Amyloid PET	Biomarkers, immunogenicity	63	Mild AD (MMSE $\geq$ 25), amyloid PET
				NCT01238991	2	Terminated	Safety, vMRI, AE	Biomarkers, immunogenicity, cognitive outcomes	53	AD (MMSE >10)
				NCT00479557	2	Completed	Safety, vMRI, AE	Anti -A β IgG/IgM	86	AD (MMSE 16-26)

Therapy	Company	Adjuvant	Antigen	Trial Listing	Phase	Status	Primary endpoints	Secondary Endpoints	Patients	Inclusion
				NCT00959192	2	Completed	Safety, vMRI, AE	Anti -A β IgG/IgM CANTAB, RBANS, Vineland, NPI,	32	AD (MMSE 16-26)
ACI-24	AC Immune	Liposome	A β 1-15 (oligomer)	NCT02738450	1	Aug 2019	Safety, antibody titers	Amyloid PET, Plasma A β , CSF tau/A β , vMRI, ARIA	24	Down syndrome
UB-311	United Neuroscience Ltd	CpG+Alum	A β 1-14	NCT00965588	1	Completed	Safety	Immunogenicity	19	Mild moderate AD (MMSE15-25)
				NCT01189084	Obs	Completed	immunogenicity, safety	Efficacy	14	Follow-up
				NCT02551809	2	Dec 2017	Safety	ADASCog, ADCS-ADL, MMSE, CDR-SB, NPI	45	Mild AD (MMSE 20-26)
Lu AF20513	H. Lundbeck A/S	Unknown		NCT02388152	1	May 2017	Safety, antibody titers		35	AD
V950	Merck Sharp & Dohme Corp.	ISCOMATR IX+Alum		NCT00464334	1	Completed	Safety, antibody titers		86	Mild moderate AD

Table 2.3: Passive immunotherapy studies listed in www.clinicaltrials.gov. When a clinical trial is still ongoing, the expected end date is listed. When a clinical trial has been prematurely ended, the term terminated is used. The following abbreviations are used: **Imaging measures:** vMRI-volumetric magnetic resonance imaging; ARIA-amyloid related imaging abnormalities; PiB PET-Pittsburgh Compound B PET (amyloid PET); FDG-PET-Fludeoxyglucose PET (glucose metabolism) **Administration methods:** IV-intravenous; SC-subcutaneous; IM-intramuscular **Safety related:** ECG-Electrocardiogram; PK-pharmacokinetic; ATA-Anti-therapy antibodies **Cognition/functional tests:** CANTAB-Cambridge Neuropsychological Test Automated Battery; ADCS-PACC-Alzheimer's Disease Cooperative Study-Preclinical Alzheimer's Cognitive Composite; CDCS-CGIC-Clinician Global Impression of Change; CDR-Clinical Dementia Rating; CDR-GS;Clinical Dementia Rating-global score; C-SSRS-Columbia Suicide Severity Rating Scale; DAD-disability assessment for dementia; DIAN-TU-Dominantly Inherited Alzheimer Network Trial; EQ-5D-EuroQol Five Dimensions Questionnaire; FCSRT-Free and Cued Selective Reminding Test; GDS-Geriatric Depression Scale; QoL-AD-Quality of Life-AD; RUD-Lite-Resource Utilization in Dementia; WAIS-R-Wechsler Adult Intelligence Scale; ZCI-AD-Zarit Caregiver Interview for AD

Antibody	Company	Target	Isotype	Trial number	Phase	Status	Primary endpoints	Secondary endpoints	Patients	Admin	Inclusion
Gantenerumab (RO4909832)	Roche	Plaques	IgG1	NCT00531804	1	Completed	AE, safety, PK	CSF biomarkers, clinical efficacy	60	IV	
				NCT02133937	1	Completed	PK	AE Safety,	31	SC	Healthy
				NCT01636531	1	Completed	Bioavailability	dose-exposure relationship plasma A β , CSF tau, MMSE, ADAScog	120	SC	
				NCT01656525	1	Completed	AE, PK, CSF/plasma	CSF tau/A β ₄₂ vMRI, CDR-SB, MMSE, safety	28	SC	
				NCT02051608	3	July 2018	Florbetapir, ADAS-cog, ADCS-ADL		389		Preventive trial in patients with familial AD Prodromal AD (MMSE>24 and amyloid PET positive)
				NCT01760005	2/3	Dec 2019	DIAN-TU	PiB-PET, CSF tau/A β ₄₂ , vMRI, FDG-PET	210	SC/IV	

Antibody	Company	Target	Isotype	Trial number	Phase	Status	Primary endpoints	Secondary endpoints	Patients	Admin	Inclusion
Solanezumab (LY2062430)	Eli Lilly	Monomers	IgG1	NCT01224106	3	Nov 2021	CDR-SB, amyloid PET	ADASCog, safety, PK	770	SC	
				NCT00329082	2	Completed	AE	PK, plasma A β	25	IV	Dose finding in AD and healthy
				NCT01148498	2	Completed	Plasma A β	plasma A β	55	IV	
				NCT00749216	2	Completed	AE	ADASCog, PK	33	IV	Dose finding in AD
								CDR-SB, NPI, vMRI, MMSE,			
				NCT00904683	3	Completed	ADASCog, ADCS-ADL	RUD-Lite, EQ-5D, QoL-AD, plasma A β	1040	IV	
								CDR-SB, NPI, MRI, MMSE,			
				NCT00905372	3	Completed	ADASCog, ADCS-ADL	RUD-Lite, EQ-5D, QoL-AD, plasma A β	1000	IV	
								ADASCog, ADCS-ADL, CDR-SB,			
				NCT01127633	3	Nov 2018	vital signs, ECG	NPI, RUD-Lite, QoL-AD, MMSE, plasma A β , vMRI, amyloid PET	1275	IV	Extension from first trials

Antibody	Company	Target	Isotype	Trial number	Phase	Status	Primary endpoints	Secondary endpoints	Patients	Admin	Inclusion
Aducanumab (BIIB037)	Biogen Inc.	Plaques	IgG1	NCT01900665	3	Oct 2016	ADASCog, ADCS-ADL	MMSE, CDR, plasma Aβ, MRI, PK, amyloid PET, CSF Aβ, NPI, RUD-Lite, QoL-AD	2100	IV	MMSE 20-26, PET or CSF positive
				NCT02614131	1	July 2017	AE	PK, plasma Aβ	130	IV/SC	Dose finding in MCI, mild AD and healthy At risk for memory loss
				NCT02008357	3	April 2020	ADCS-PACC	CFI, ADCS-ADL, Aβ PET, CSF tau/Aβ, vMRI	1150	IV	(MMSE 25-30, CDR 0, amyloid PET positive)
				NCT01397539	1	Completed	AE, vital signs, ECG, ARIA, neurological	PK	53	IV	Safety in AD
				NCT01677572	1	April 2016	AE, vital signs, ECG, ARIA (MRI), neurological	Aβ PET, PK, ATA	197	IV	dose finding in prodromal/ mild AD (MMSE 20-30, CDR 0.5-1), amyloid positive
				NCT02434718	1	Nov 2016	AE, vital signs, ECG, ARIA (MRI), neurological	PK, ATA	20	IV	Safety in AD

Antibody	Company	Target	Isotype	Trial number	Phase	Status	Primary endpoints	Secondary endpoints	Patients	Admin	Inclusion
Crenezumab/ MABT5102A	Roche Genentech AC Immune	Monomers	IgG4	NCT02484547	3	February 2020	CDR-SB	MMSE, ADASCog, ADCS-ADL-MCI	1350	IV	MCI or Mild AD (CDR-0.5, MMSE24-40, positive amyloid)
				NCT02477800	3	February 2020	CDR-SB	MMSE, ADASCog, ADCS-ADL-MCI	1350	IV	MCI or Mild AD (CDR-0.5, MMSE24-40, positive amyloid)
				NCT02427243	1	Completed	PK	AE, ATA	60	SC	Safety and dose in healthy
				NCT00997919	1	Completed	Safety	PK	22	IV/SC	Healthy
				NCT00736775	1	Completed	Safety	PK, ATA	56	IV	Safety and PK in AD
				NCT02427243	1	Completed	PK	AE, ATA	60	SC	Healthy
				NCT01397578	2	Completed	Amyloid PET	CSF biomarkers, FDG-PET, ADAS-cog	91	IV/SC	Evaluate brain amyloid
				NCT01343966	2	Completed	ADAS-cog, CDR-SOB	ADCS-ADL	488	IV/SC	Efficacy and safety in AD
				NCT02353598	1	May 2017	ATA, C-SSRS, ECG, vital signs, ARIA, AE	PK	72	IV	Safety and dose in AD
				NCT01723826	2	Feb 2017	AE, vital signs, ATA, ARIA		360		Follow up of ABE4869g or ABE4955g

Antibody	Company	Target	Isotype	Trial number	Phase	Status	Primary endpoints	Secondary endpoints	Patients	Admin	Inclusion
Bapineuzumab	Janssen Pfizer	Plaques	IgG1	NCT01998841	2	Sep 2020	APCC	AE, progression to AD, CDR, CDR-SB, FCSRT, RBANS, amyloid PET, FDG-PET, vMRI, CSF tau, ATA ADASCog, MMSE, ADL, CDR-GS, ADCS-ADL, NPI, QoL-AD, ZCI-AD, EQ-5D ATA, ADASCog, DAD, CDR-SB, MMSE, CSF tau/A β , Plasma A β PK, ATA, plasma A β	300	SC	PSEN1 E280A carriers, MMSE $\geq$ 24, does not meet criteria for dementia or MCI
				NCT02670083	3	Aug 2020	CDR-SB	ADCS-ADL, NPI, QoL-AD, ZCI-AD, EQ-5D ATA, ADASCog, DAD, CDR-SB, MMSE, CSF tau/A β , Plasma A β PK, ATA, plasma A β	750	IV	Prodromal and mild AD (MMSE $\geq$ 22, CDR-GS 0.5-1) CSF or amyloid PET
				NCT01193608	1	Completed	C-SSRS, vMRI, safety	DAD, CDR-SB, MMSE, CSF tau/A β , Plasma A β PK, ATA, plasma A β	88	IV	Safety and PK in AD
				NCT00397891	1	Completed	AE, safety, MMSE	PK, ATA, plasma A β	80	IV	Safety, PK in AD
				NCT01254773	2	Completed	Cerebral amyloid	Safety	146	SC	AD
				NCT00663026	2	Completed	AE, safety	PK	79	SC	Probable AD
				NCT00916617	2	Terminated	AE, safety, vMRI	PK	66	SC	Probable AD
				NCT00112073	2	Completed	AE, safety	PK	234	IV	Safety and PK in AD
				NCT00575055	3	Completed	Cognitive and functional	Amyloid imaging, CSF biomarkers	1121	IV	APOE4 carriers

Antibody	Company	Target	Isotype	Trial number	Phase	Status	Primary endpoints	Secondary endpoints	Patients	Admin	Inclusion
				NCT00574132	3	Completed	Cognitive and functional	Amyloid imaging, CSF biomarkers	1331	IV	Apoe4 non-carriers
				NCT00996918	3	Terminated	AE, safety	Adas-Cog, DAD, NPI, MMSE	198	IV	Phase 3 extension
				NCT00667810	3	Terminated	ADASCog, DAD	Amyloid PET, CSF tau, vMRI, ADASCog, DAD, CDR-SOB	901	IV	In Apoe4 non-carriers
				NCT00606476	2	Terminated	AE, safety	ADASCog, RUDLite, DAD, MMSE	194	IV	Open-label, long-term extension
				NCT00676143	3	Terminated	ADASCog, DAD	Amyloid PET, CSF tau, vMRI, CDR-SB	1099	IV	APOE4 carriers
				NCT00998764	3	Terminated	AE, safety, vMRI	ADASCog, DAD, NPI, MMSE	494	IV	Open-label, long-term extension of APOE4 carriers
				NCT00937352	3	Terminated	AE, safety, vMRI	Efficacy	896	IV	Open-label, long-term extension
				NCT01658722	3	Terminated	Dependence Scale	-	169	-	Follow up of patients who discontinued treatment

Antibody	Company	Target	Isotype	Trial number	Phase	Status	Primary endpoints	Secondary endpoints	Patients	Admin	Inclusion
AAB-003 (PF-05236812)	Janssen/Pfizer	Plaques	IgG4	NCT01369225	1	Completed	C-SSRS, vMRI, safety	ATA, ADASCog, DAD, CDR-SB, MMSE, CSF tau/A β , Plasma A β	52	IV	Open label extension
N3pG-A β (LY3002813)	Eli Lilly	A β _{p3-42}	Unknown	NCT01837641	1	Sep 2016	AE, safety	PK	100	IV/SC	Safety and PK in healthy, MCI and AD
				NCT02624778	1	Aug 2018	amyloid PET	PK, ATA	150	IV	Effect on brain amyloid in AD
MEDI1814	AstraZeneca	Oligomers	Unknown	NCT02036645	1	Sep 2016	AE, safety	PK, ATA	121	IV/SC	Safety and PK in AD
BAN2401	Eisai Inc	Protofibrils	IgG1	NCT02094729	1	Completed	AE, safety	PK, ATA, CSF tau/A β	26	IV	Safety in MCI and mild AD patients (MMSE 22-30 and CDR 0.5)
				NCT01230853	1	Completed	Safety	-	80	IV	Safety and dose finding in AD
				NCT01767311	2	Jun 2018	ADCOMS, AE	vMRI, amyloid PET	800	IV	Clinical efficacy and dose response
GSK933776	GlaxoSmithKline	Plaques	IgG1	NCT01424436	1	Completed	CSF A β	CSF tau/A β , Safety	19	IV	MCI or mild AD (MMSE 20-26)
				NCT02033668	1	Completed	PK	AE, Safety, ATA	36	IV/SC/IM	Healthy
				NCT00459550	1	Completed	AE	PK, CSF, Amyloid PET, ATA	50	IV	Mild AD (MMSE 18-26)
				NCT01342926	2	April 2016	Geographic atrophy	visual acuity, PK	184	IV	Age-related macular degeneration

Antibody	Company	Target	Isotype	Trial number	Phase	Status	Primary endpoints	Secondary endpoints	Patients	Admin	Inclusion
SAR228810	Sanofi	Protofibrils	IgG4	NCT01485302	1	Completed	AE	PK	48	IV/SC	PK and safety in AD
Ponezumab (PF-04360365)	Pfizer	A β 1-40	IgG2a	NCT00733642	1	Completed	Safety	PK	15	IV	PK and safety in AD
				NCT01125631	1	Completed	Safety, PK	Plasma A β	8	IV	PK and safety in AD
				NCT00607308	1	Completed	Safety	PK	20	IV	PK and safety in AD
				NCT01005862	1	Completed	CSF A β	AE	17	IV	Healthy and AD patients
				NCT00455000	1	Completed	Safety, PK	PK, ATA	37	IV	PK and safety in AD
				NCT00945672	2	Completed	Safety, PK, brain amyloid, CSF A β	ADASCog, DAD, plasma A β , CSF tau/A β , ATA	36	IV	Safety in AD
				NCT01821118	2	Completed	Cerebrovascular reactivity	plasma A β	57	IV	Safety in CAA
				NCT00722046	2	Completed	Safety, PK	ADASCog, DAD, plasma A β , CSF tau/A β , ATA	198	IV	Safety and PK in AD

Antibody	Company	Target	Isotype	Trial number	Phase	Status	Primary endpoints	Secondary endpoints	Patients	Admin	Inclusion
Octagam	Octapharma		IVIG	NCT00812565	2	Completed	plasma A β	Anti-A β antibody, CSF tau, ADASCog, CDR-SOB ADCS-ADL, NPI, GDS, QOL,	58	IV	Probable AD
Gammagard	Weill Medical College of Cornell University		IVIG	NCT00299988	2	Completed	ADASCog, CDCS-CGIC	ADCS, Plasma anti-A β titers, plasma A β , FDG-PET, Amyloid PET, AD	24	IV	
Gammagard	Baxalta US Inc.		IVIG	NCT00818662	3	Completed	ADASCog, ADCS-ADL	ADASCog, ADCS-ADL, ASCS-CGIC, MMSE, QOL-AD, WAIS-R, AE	390	IV	AD
Flebogamma	Grifols Biologicals Inc.		Albumin and IVIG	NCT01561053	2/3	Dec 2016	ADASCog, ADCS-ADL	Cognitive, CSF tau/A β , FDG-PET, vMRI	350	IV	AD
NewGam	Sutter Health		IVIG	NCT01300728	2	Unknown	vMRI, CDR-SB, CSF A β , cognitive performance	-	52	IV	MCI (MMSE 24-30, CDR 0.5)
Gammagard	Baxalta US Inc.		IVIG	NCT01736579	3	Terminated	AE	Cognition	6	IV	Open label extension

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3

ISOTYPE DEPENDENT EFFECTOR MECHANISMS OF IMMUNOTHERAPY IN AD

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ABSTRACT

The anti-amyloid antibody bapineuzumab was recently tested in large clinical trial as a potential treatment for Alzheimer's disease (AD). Unfortunately, the trial did not show a therapeutic effect. In this study, the effect of acute (therapeutic) and preventive treatment of a mouse chimeric version of bapineuzumab (3D6) was investigated in the 5xFAD mouse model of AD. In addition, to assess the contribution of the inflammatory response, we compared an anti-inflammatory mouse IgG1 (mIgG1) variant of 3D6 with the inflammatory IgG2a (mIgG2a) isotype.

Our results show that treatment with chimeric 3D6 in 5xFAD mice results in passive systemic anaphylaxis (PSA) caused by generation of neutralizing antibodies against the administered 3D6. Furthermore, we show that preventive treatment with both isotypes is able to reduce anxiety-like effects in 5xFAD animals. In addition, mIgG1 worsens memory problems as assessed by the fear conditioning test. Preventive treatment is more efficient in reducing plaques, but causes a shift of A β from the formic acid soluble fraction to the detergent soluble fraction. Therapeutic treatment on the other hand, solely causes a reduction of detergent soluble A β . No treatment regimen had an effect on water soluble A β . Finally, we show that treatment with mIgG2a 3D6 significantly reduced astrogliosis.

These results show that caution is needed when utilizing immunotherapy for the treatment of AD. In addition, our results show that using isotype specific effects are important to take into consideration and warrant further investigation to further improve immunotherapy for AD.

3.1. INTRODUCTION

Alzheimer's disease (AD) is the main cause of dementia. The best known hallmarks of AD are the buildup of extracellular plaques consisting mainly out of amyloid- β ($A\beta$), intracellular tau tangles, neuronal loss and gliosis. In the last decade, several clinical trials utilizing anti- $A\beta$ antibodies have been concluded with largely disappointing results [1].

Only few studies so far have addressed the role of antibody effector mechanisms of immunotherapy in AD. Antibody effector mechanisms are determined by the isotype of the antibody and can involve activation of complement (complement dependent cytotoxicity or CDC), antibody-dependent cellular cytotoxicity (ADCC), which is usually mediated by NK cells, or Fc γ -receptor mediated phagocytosis. For anti- $A\beta$ therapy, CDC and Fc γ -receptor mediated phagocytosis are thought to play the most important role [2]. A third isotype-independent mechanism, called the peripheral sink, is also thought to play a role in $A\beta$ immunotherapy. In this mechanism, anti- $A\beta$ antibodies sequester $A\beta$ in the periphery and prevent them from re-entering the brain, causing a net efflux of $A\beta$ [3].

In this study, isotype dependent effects of the anti-amyloid antibody 3D6 were investigated by recombinant production of mouse IgG1 (mIgG1) and mouse IgG2a (mIgG2a) 3D6. 3D6 is the murine precursor of the humanized antibody bapineuzumab, which has been tested in two large Phase III clinical trials and now has been discontinued because of negative results [4]. Bapineuzumab is recombinantly produced as a human IgG1 (hIgG1) isotype, while the original hybridoma cell-line produces 3D6 as a mouse IgG2a (mIgG2a) isotype. Both isotypes have similar capacities to activate complement and bind Fc-receptors [4]. In humans, IgG4 (hIgG4) antibodies have been shown to lack complement activation capabilities, while also having reduced affinity for Fc γ -receptors. The mIgG1 isotype is comparable to hIgG4, in the regard that it can only weakly activate complement and has reduced affinity for Fc-receptors [4]. At the moment, an IgG4 variant of bapineuzumab is also being tested in patients with mild to moderate AD [5].

One reason anti-amyloid trials have been negative so far, might be because treatment started too late [6]. Cross-sectional amyloid imaging studies in patients with familial forms of AD, have shown that $A\beta$ accumulation starts already up to 15 years before the onset of clinical symptoms [7]. For this reason, several anti-amyloid immunotherapies are now being tested in preventive trials in patients with hereditary forms of AD [8].

In this study, we investigated the effects of immunotherapy with 3D6 using two different isotypes (mIgG1 and mIgG2a). Additionally, efficacy was investigated in a preventive and therapeutic regimen. To this end, we utilized the 5xFAD mouse model of AD. The 5xFAD model contains human APP and PSEN1 with three mutations in APP (APPSwedish, APPFlorida, APPLondon) and 2 in PSEN1 (PSEN1*M146L*L286V), with the first plaques appearing at ~3 months of age, and cognitive deficits first apparent at 5 months of age.

3.2. METHODS

3.2.1. ANIMAL BREEDING

A breeding colony was established of B6/SJL (WT) and 5xFAD animals that were obtained from Jackson Laboratories (stock 6554). Mice were bred hemizygotously as described, except that F3 B6/SJL mice were used for generating experimental groups. Additionally, the *Pde6b* gene was absent in our colony. All mice were female, housed socially on a reversed 12 hour day-night cycle and had *ad libitum* access to food and water. This experiment was approved by the Animal Welfare Committee at Maastricht University (DEC2013-006) and followed the laws and regulations of the Netherlands.

3.2.2. ANTIBODY PRODUCTION

Bapineuzumab variable heavy (VH) and variable light (VL) sequences (humanized sequences) were synthesized by GeneArt (Invitrogen). The CH1, CH2 and CH3 sequences of the constant heavy chain of mIgG1, mIgG2a and CL mouse kappa light chain sequences were cloned from pFuse plasmids (Invivogen) into a custom designed vector containing a CMV5 promotor and a WPRE element. Additionally, the mIgG1 sequence was mutated in the Fc γ -receptor binding site with E233P and in the C1q binding site with E318A, K320A, and R322A to completely eliminate binding to the Fc γ -receptor and complement activation [9, 10]. Clones were verified by sequencing (GATC). Plasmids were produced large scale in a BioFlo 110 fermentor (New Brunswick). Transfected *E. coli* was grown to high density and plasmid growth was temperature induced. After 2 days, bacteria were pelleted by centrifugation and pellets were frozen at -80 °C until further processing.

For purification of plasmids, typically around 8 grams of bacterial pellet was lysed using 150 ml of each buffer of the alkaline lysis method [11]. RNA was precipitated by addition of CaCl₂ to a final concentration of 1.4 M. Plasmids were subsequently purified on an AKTA explorer system using a CIM DEAE column (BIA Separations).

Antibodies were produced by transient transfection of HEK293F cells. HEK293F cells were grown to confluence in 4-layer cell factories (Nunc) in Freestyle 293 medium. Cells were transfected with 22 kDa linear PEI (Polysciences) with a DNA:PEI ratio of 1:4. Heavy to light chain ratio was 1.5:2. Four hours after transfection, valproic acid was added to a final concentration of 4 mM. Cultures were typically maintained for 10-12 days after which the supernatant was further processed.

Supernatant was filtered first through glass wool, then through a glass fiber membrane of 0.8 μ m and finally through a 0.45 μ m sterile filter (Pall). Next, supernatant was concentrated on an artificial kidney (Fresenius Medical Care) and purified using a MabSelect protein A column (GE Healthcare) (for mIgG2a) or a custom packed protein G column (for mIgG1) (GE Healthcare) on an AKTA explorer system (GE Healthcare). Eluate was immediately neutralized by means of a buffer exchange to PBS using a column packed with Sephadex G50. Antibody purity was confirmed by electrophoresis and Coomassie blue staining. After purification, antibodies were concentrated using a spin column (Millipore) to a final concentration of 1 mg/ml, sterile filtered by a 0.22 μ m syringe filter, and stored at 4 °C until further use.

3.2.3. EXPERIMENTAL SETUP AND TISSUE PROCESSING

The complete experimental setup consisted of 6 groups, of 24 animals each. The experimental setup is depicted in Figure 1. Animals in the preventive regimen received weekly 100 µg of mIgG1 3D6 or mIgG2a 3D6 starting at 4 months of age. After 4 weeks, the antibody dose was lowered to 50 µg. From this point forward, they also received 1 ml of saline injections subcutaneously in addition to the antibody to reduce side effects caused by antibody administration. All the other groups received weekly PBS injections starting at 4 months of age. At 5 months of age, the animals in the therapeutic groups received 100 µg of mIgG1 3D6 or mIgG2a 3D6 per week instead of PBS. During the last two weeks of therapy, animals underwent behavioral testing.

After the behavioral procedures, animals were anaesthetized and perfused. Serum was taken before perfusion and stored at -80 °C until further use. One group of animals was perfused using ice-cold Tyrode solution, and brains were snap-frozen with liquid nitrogen. Another group of animals was perfused with ice-cold Tyrode solution followed by fixation with 4% paraformaldehyde. Brains were post fixed overnight in 4% paraformaldehyde, after which they were cryoprotected using 20% sucrose and frozen using CO₂. Sagittal brain section with a thickness of 16 µm were cut on a cryostat (Leica CM3050S).

For amyloid beta measurements using ELISA, non-fixed frozen brain hemispheres were crushed using a pestle with liquid nitrogen and divided into 10 mg aliquots. For extraction of amyloid, 15 times (w/v) of TBS containing 1x Complete protease inhibitors (Roche) and phosSTOP (Roche) was added. Suspensions were homogenized using a micropestle. Following sonication, suspensions were centrifuged for 30 minutes at 30000 g. Supernatant was stored at -80 °C (soluble fraction) until further use and an equal volume of TBS containing 1% Triton was added to the pellets. Pellets were resuspended using a micropestle, sonicated and centrifuged again for 30 minutes at 30 000 g. Supernatant was stored at -80 °C (insoluble fraction) and pellet was further resuspended to a concentration of 150 mg/ml in 70% formic acid. Samples were incubated under gentle agitation for 2 hours at room temperature and finally centrifuged at 30 000 g for 30 minutes. Supernatant was neutralized by addition of 20 times 1 M Tris.

3.2.4. BEHAVIORAL PROCEDURES

OPEN FIELD TEST

The open field test was used to assess anxiety and locomotor changes. The open field consisted of a 50x50 cm square PVC arena divided into 4 equal fields of 25x25 cm to test 4 mice in parallel. The arenas were surrounded by grey PVC walls of 25 cm high. Mice were placed centrally in an arena for 20 minutes and tracked using an overhead attached camera. After each testing round, the arena was thoroughly cleaned with 70% ethanol. For assessment of locomotor activity, distance moved and velocity were calculated of the entire 20 minutes using Ethovision XT10 (Noldus). As a measure for anxiety, corners and walls were defined as the area up until 8 cm of distance from the wall, and the time spent in the central area during the first five minutes was measured using Ethovision XT10.

ELEVATED ZERO MAZE

To measure anxiety related behavior, the elevated zero maze (EZM) task was used. The EZM consisted of a circular runway with a diameter of 50 cm and a walking path of 5 cm wide. It was located 70 cm above the floor and divided equally into two opposing closed and open parts. The closed parts were surrounded by 50 cm high walls, while there was a 5 mm high rim around the walking path of the open part to prevent the mouse from falling off. The mouse was placed centrally into an open arm facing one the closed arms. The EZM was performed in low-light conditions using only red light and animals were tracked using an overhead infrared camera. The EZM was cleaned with 70% ethanol between tests. For analysis, latency to the first closed arm entry, total distance, and the percentage of time spend in the closed area were measured using Ethovision XT10 (Noldus). The percentage of time spend in the closed area was corrected for the latency until entry into the first closed arm. Two animals were excluded from the analysis because the latency to the first closed arm entry was more than one minute.

ALTERNATING Y-MAZE

The spontaneous alternating Y-Maze was used to assess spatial working memory. The Y-maze used consisted of 3 acrylic arms arranged at an 120°angle. The arms were 40 cm long, 17 cm long, and with a bottom of 4 cm wide and an open top of 13 cm wide. Visual cues were attached to the walls next to maze. Start location was randomized and mice were placed at the end of one of the arms. Mice were left to explore the maze freely for 5 minutes. The maze was thoroughly cleaned with 70% ethanol between tests. Mice were tracked using an overhead camera and distance moved and percent triads were analyzed automatically using Ethovision XT as described [12]. A triad was defined as a visit through all three arms consecutively. The percentage of alternations was calculated as the number of triads divided by the maximum possible of triads (total number of arm entries minus 2).

CONTEXTUAL AND CUED FEAR CONDITIONING

Contextual and cued fear conditioning (FC) were used to measure hippocampal and amygdala functioning. The conditioning chamber consisted of a transparent acrylic chamber with metal grid floors. A sound generator was attached directly to the side of the chamber. For cued FC recall, a triangular black plastic wall was placed in the chamber and the floor was covered by a white plastic plate. Between tests of mice, the chamber was cleaned with 70% ethanol. For conditioning, mice were allowed to acclimatize for 2 minutes after which 4 trials of paired stimuli spaced 1 minute apart were presented. Each trial started with the presentation of a cued conditioning stimulus (CS) consisting of a 70 dB tone presented for 20 seconds. During the last 2 seconds a foot shock unconditioned stimulus (US) of 0.75 mA was given. Twenty-four hours after training, contextual freezing behavior was measured in half of the conditioned mice. Mice were recorded continuously with an overhead camera for three minutes in exactly the original chamber and freezing behavior was measured automatically with Ethovision XT10. For the other half of the mice cued freezing behavior was measured. For cued recall, mice were placed in the modified chamber and allowed to acclimatize for 2 minutes. After this period, the CS was presented 4 times for 20 seconds every other minute. Freezing behavior was measured automatically during these 20 seconds using

Ethovision XT. Forty-eight hours after conditioning, contextual recall was performed for the mice that underwent cued recall the day before and vice versa. Animals that did not show any physical reaction to shocks during the conditioning phase were excluded from the analysis.

3.2.5. IMMUNOFLOUORESCENCE AND HISTOCHEMISTRY

A triple immunofluorescent staining for microglia, plaques and cell nuclei was performed. Sections were incubated with rabbit anti-Iba-1 (Wako, 1:1000) and human anti-amyloid 20C2 (1:4000) (recombinant anti-amyloid antibody) overnight at 4 °C. Sections were washed with Tris-buffered saline (TBS), TBS with 0.3% Triton X-100 (TBS-T) and TBS. Next, sections were incubated with mouse anti-human Alexa 594 (Jackson Immuno, 1:500) or donkey anti-rabbit 488 (1:300, Invitrogen). Sections were subsequently washed with TBS, TBS-T, and TBS. Sections were incubated with Hoechst 33342 (Sigma-Aldrich, 1:500) for 15 minutes and washed with three times with TBS. Finally, sections were dried at 50 °C for one hour, cleared in xylene and cover slipped using DPX (Klinipath). For GFAP staining, the same procedure was followed, except that rabbit anti-GFAP (Dako, 1:1000) was used.

Microhemorrhages were assessed by Prussian blue staining of hemosiderin deposits. Sections were washed in distilled water followed by a 30-minute incubation in 5% potassium ferrocyanide (Sigma) with 5% HCl. Sections were washed 5 times in distilled water, dehydrated, cleared in xylene, and mounted with DPX.

Quantification of GFAP, Iba1 and plaques was performed as follows. Two to three midsagittal sections spaced 160 µm apart were used per animal. Whole section photomicrographs were acquired using µManager software [13] on a BX51WI spinning disk confocal fluorescence microscope (Olympus) with an EM-CCD C9100 camera (Hamamatsu). Images were background corrected and analyzed using Fiji [14]. For analysis, regions-of-interest (ROIs) were manually delineated. ROIs comprised cortex, subiculum, hippocampus, and midbrain combined with striatum. Next, a median filter was applied, and images were thresholded and converted to binary images. Plaques, microglia, and astrocytes density were measured using the particle analyzer tool. The density was averaged over all sections and this measure was used for statistical analysis. A measure of plaque-associated microglia and astrocytes was obtained by measuring the density of microglia or plaques in a 50 µm² radius around the plaques. Densities were averaged per animal and this measure was used for statistical analysis. The same settings were used for all images.

For quantification of microhemorrhages, per animal, 3-4 sections (spaced 160 µm) were analyzed on a BX51 microscope (Olympus). Sections were manually inspected by two investigators who were blind to experimental groups. Incidences were counted per section and averaged per animal.

3.2.6. ELISA

For determination of immune-complexes, high-binding ELISA plates (Greiner) were coated with fully human 3D6 or human IgG (Sigma). Plates were blocked with 0.4% non-fat dry milk (Biorad), followed by incubation with mouse serum (1:10-10 000 diluted). Plates were washed with PBS containing 0.1% Tween (PBST) and incubated with HRP

conjugated anti-mouse IgG (Organon, 1:10 000). After washing, plates were developed by incubation with TMB (Sigma) and stopped with 2 M H₂SO₄. Possible anti-3D6 mouse IgE was assessed using the same ELISA setup, but using biotinylated anti-mouse IgE (clone RME-1, Biolegend, 1:1000) as detection antibody, followed by incubation with streptavidin-HRP (Jackson Immuno, 1:4000).

ELISA plates were coated with human 266 overnight at room temperature. After washing, plates were blocked with 0.4 % non-fat dry milk and incubated with brain homogenates. Next, plates were washed, incubated with biotinylated 20C2 and washed again. Finally, plates were incubated with streptavidin-HRP (Jackson Immuno) and developed using TMB.

3.2.7. STATISTICAL ANALYSES

Statistical analysis was performed using SPSS 23 (IBM) and Graphpad Prism 6. One-way ANOVA was conducted with a combination of planned contrasts and post-hoc corrections. LSD was used as correction for multiple comparisons. Contrasts were designed to specifically test the following hypotheses: is there a difference between treatment and prevention groups and is there a difference between mIgG1 and mIgG2a groups.

3.3. RESULTS

3.3.1. IMMUNOTHERAPY WITH IgG1 AND IgG2a 3D6 ANTIBODIES CAUSES INCREASED MORTALITY OF 5xFAD MICE

Figure 3.1: A Kaplan-Meier plot shows that antibody treatment significantly affected survival of animals in experimental groups ($p=0.04$). 5xFAD animals were either treated in a preventive or therapeutic fashion with a weekly dose of 100 µg of 3D6 per week. The antibody dose in the prevention group was lowered to 50 µg/week after 4 weeks because of lethal effects. Control groups (untreated 5xFAD and WT animals) received weekly injections with PBS ($n=24$ per group).

5xFAD animals were treated with mIgG1 and mIgG2a 3D6 in either a preventive or therapeutic regimen in an effort to resolve AD symptoms and pathology. Unexpectedly, antibody treatment significantly affected survival of the animals ($p=0.04$). Nine percent dropout was observed in both antibody groups under the therapeutic regimen. In the preventive mIgG2a group 5 out of 24 animals died (~20 %), while in the preventive mIgG1 group almost 30% of animals was lost (7 out of 24) (see Figure 3.1). This difference was not statistically significant ($p=0.5$). Compared to the therapeutic group, significantly more animals were lost in the preventive group ($p=0.03$). Deaths started occurring after 3 administrations of antibody and always occurred within several hours after injection; in one case, death was observed within half an hour after administration. Symptoms observed after antibody administration were consistent with those of passive systemic anaphylaxis (PSA). Antibody dose in the preventive groups was halved after 4 weeks, which reduced the rate of mice dropping out (see Figure 1)

To exclude that microhemorrhages might have been the cause of death, incidences were counted in brains sections using Prussian blue staining. No significant differences were found between any of the groups ($p=0.75$), but average number of microhemorrhages was increased by about 20% in preventive treated animals compared

to WT animals (data not shown).

Since the administered antibodies were chimeric antibodies (Fv from human origin and constant region from mouse) a possible immune response against our treatment was assessed in the mouse serum. To this end, fully human 3D6 (bapineuzumab) was coated on Elisa plates and mouse IgG was measured. Additionally, we also measured a possible antibody titer against human IgG.

Figure 3.2A shows that most animals in the immunotherapy groups have a possible anti-3D6 response. Five (19%) untreated 5xFAD animals also were positive in the anti-3D6 ELISA. A possible explanation might be that these animals have natural antibodies against amyloid or against human IgG. In the therapeutic groups 73% (mIgG1) and 75% (mIgG2a) of animals were positive, while the percentages in the mIgG1 (78%) and mIgG2a (82%) preventive groups were slightly higher. No animals had IgE antibodies against bapineuzumab (data not shown).

Figure 3.2: A) An ELISA was performed to detect mouse IgG against fully human 3D6 (Bapineuzumab). In groups that received 3D6 ~80% of animals had possible antibodies against 3D6. B) Mouse antibodies against human IgG were detected by coating with polyclonal human IgG. Preventive treatment with mIgG2a resulted in 70% of animals developing anti-human IgG antibodies. ($n_{WT}=21$, $n_{5xFAD}=21$, $n_{mIgG1Ther}=15$, $n_{mIgG2aTher}=16$, $n_{mIgG1Prev}=14$, $n_{mIgG2aPrev}=17$)

To further confirm that these mice developed antibodies against the therapeutic antibody, a mouse anti-human IgG assay was performed. In this assay, 3 untreated 5xFAD animals (10%) showed a natural cross-reaction to human IgG, while no WT animals were found positive. The therapeutic mIgG1 (20%), therapeutic mIgG2a (12%) and preventive mIgG1 group (21%) all had a low number of positive animals, while in the preventive mIgG2a group 70% of all animals had anti-human IgG antibodies (see Figure 3.2C).

3.3.2. mIgG1 WORSENE COGNITIVE DEFICITS IN THE CONTEXTUAL FEAR CONDITIONING TEST

After antibody treatment, locomotion, anxiety, and memory were assessed using a test battery consisting of the open field test, the alternating Y-maze, the EZM, and the contextual and cued FC test. The open field was used mainly to test locomotion deficits

in the mice, although anxiety was also assessed by measuring the percentage of time spent in the center of the open field during the first 5 minutes. No difference in the percentage spent in the center could be found between any of the groups ($p=0.87$) (see Figure 3.3). However, mice treated therapeutically with mIgG2a showed reduced velocity compared to untreated 5xFAD mice or mice treated in a preventive fashion with mIgG1 ($p<0.01$) (see Figure 3.3). Additionally, mice treated with mIgG2a had reduced velocity compared to mice treated with mIgG1 ($p=0.03$) and also a therapeutic regimen resulted in reduced speed compared to a preventive regimen ($p=0.049$).

Figure 3.3: A) Average velocity in the open field was measured to assess possible locomotor deficits. Velocity was significantly affected by antibody isotype and treatment duration (regimen). Animals treated with mIgG1 animals moved more than mIgG2a and therapeutic animals moving less than preventive animals (B) The time spent in center of the open field was used as a measure for anxiety. No differences were found in the amount of time spent in center ($n_{WT}=23$, $n_{5xFAD}=24$, $n_{mIgG1Ther}=18$, $n_{mIgG2aTher}=21$, $n_{mIgG1Prev}=16$, $n_{mIgG2aPrev}=18$) C-D) The alternating Y-maze was utilized as a test for working memory. (C) No differences were found in the percentage of alternations. (D) An isotype specific effect was present in the number of arms entered, with mIgG2a animals entering less arms than mIgG1 animals ($n_{WT}=23$, $n_{5xFAD}=25$, $n_{mIgG1Ther}=20$, $n_{mIgG2aTher}=22$, $n_{mIgG1Prev}=16$, $n_{mIgG2aPrev}=18$) Error bars indicate the SEM in all graphs.

Next, working memory was assessed in the alternating Y-maze. With this test, the working memory of 5-month-old 5xFAD mice was shown to be impaired [15]. We did not observe differences of working memory between the groups ($p = 0.29$).

Additionally, when comparing each group to the chance level of 50%, no working memory impairments were found in 5xFAD mice. The mIgG2a prevention group had significantly less arm entries compared to the three other treatment groups (see Figure 3.3). Additionally, both mIgG2a groups had less arm entries compared to the mIgG1 groups ($p < 0.01$).

The EZM was used to assess anxiety related behavior, which is normally decreased in AD-like animals. Two measures for anxiety were assessed: (1) the latency to enter the closed arm and (2) the percentage of time spent in the closed arms. WT animals entered significantly faster in the closed arm than untreated 5xFAD animals ($p = 0.04$), or animals in both therapeutic groups ($p < 0.01$ for both mIgG1 and mIgG2a groups). Animals in the prevention groups performed at a similar level as the WT group and the prevention group performed significantly better than the therapeutic group ($p < 0.01$). No differences were found between any of the groups in the percentage spent in the closed arms.

Figure 3.4: A-B) The elevated zero maze (EZM) was used as a measure for anxiety-like behavior A) The latency to enter to closed arm was restored to WT levels in the preventive groups. (B) No differences were found in the time spent in the closed arms ($n_{WT}=22$, $n_{5xFAD}=24$, $n_{mIgG1Ther}=16$, $n_{mIgG2aTher}=22$, $n_{mIgG1Prev}=16$, $n_{mIgG2aPrev}=17$) C-D) The contextual and cued fear conditioning test were used to assess amygdala and hippocampal dependent memory. (C) No differences were present in the cued fear conditioning test. In the contextual fear conditioning test, animals treated with mIgG1 performed worse than untreated animals or animals treated with mIgG2a (D) ($n_{WT}=20$, $n_{5xFAD}=19$, $n_{mIgG1Ther}=20$, $n_{mIgG2aTher}=21$, $n_{mIgG1Prev}=13$, $n_{mIgG2aPrev}=17$)

While the cued FC test mainly assessed the integrity of the amygdala, the contextual FC test is dependent on integrity of the hippocampus and frontal cortex [16]. In the cued FC test, no difference between any of the groups was observed ($p=0.69$). However, in the contextual FC test, animals treated with mIgG1 in the therapeutic as well as in the prevention group performed worse than untreated 5xFAD animals ($p=0.01$ and $p=0.02$ for the prevention and therapeutic group respectively). In addition, prevention animals treated with mIgG1 performed worse than WT animals ($p=0.03$) and prevention animals treated with mIgG2a ($p=0.02$). Overall, there was an effect of antibody isotype, with animals treated with mIgG1 showing less freezing behavior compared to mIgG2a treated animals ($p<0.01$).

Figure 3.5: Sections were stained for A β and quantified using ImageJ in the brain (cortex, hippocampus, basal ganglia, thalamus) A) Preventive treatment resulted in a significant reduction of plaque density compared to untreated 5xFAD animals. Therapeutic treatment did not result in a significant reduction of plaque load. B- C) Small and medium plaques were reduced by half in the preventive groups compared to untreated 5xFAD animals, while no differences were present with the therapeutic groups. D) Large plaques were reduced five-fold in the preventive groups compared to untreated 5xFAD animals ($n_{\text{WT}}=5$, $n_{\text{5xFAD}}=8$, $n_{\text{mIgG1Ther}}=7$, $n_{\text{mIgG2aTher}}=10$, $n_{\text{mIgG1Prev}}=6$, $n_{\text{mIgG2aPrev}}=6$) Error bars denote SEM.

3.3.3. PREVENTIVE IMMUNOTHERAPY REGIMENS DECREASED A β PLAQUE LOAD

Next, the effect of regimen and isotype was investigated on the plaque load and plaque size using immunohistochemistry. Immunotherapy significantly reduced plaque load compared to untreated 5xFAD animals, but only with long term treatment in both prevention groups ($p=0.01$). Overall, the preventive mIgG1 group had the strongest reduction in plaque load, followed by the preventive mIgG2a group (see Figure 3.5A). No significant reduction was found in the therapeutic groups when compared to untreated 5xFAD. The preventive mIgG1 group showed a significant lower plaque load compared to the therapeutic mIgG1 group ($p=0.01$), but not with the therapeutic mIgG2a group ($p=0.12$). There was no significant difference between untreated 5xFAD animals and therapeutically treated mIgG1 animals ($p=0.75$), while a small, but non-significant decrease was observed in the therapeutic mIgG2a group compared to untreated animals ($p=0.12$). No isotype specific effects were found ($p=0.72$).

The effects of immunotherapy on total plaque load was investigated further by measuring the size of individual plaques. Preventive IgG administration reduced small and medium sized plaques by half compared to untreated animals ($p=0.02$ and $p=0.01$), while large sized plaques were reduced almost ten-fold ($p=0.03$) (see Figure 3.5B-D). Again, only the preventive immunotherapy regimens reduced plaque numbers of different sizes, compared to untreated 5xFAD mice.

Figure 3.6: A β in different fraction (TBS, Triton, Formic acid (FA)) was determined by ELISA in total brain homogenates. A) Immunotherapy did not result in a reduction of soluble A β in any of the groups. B) Therapeutic treatment with 3D6 resulted in a significant reduction of triton soluble A β compared to untreated 5xFAD animals. In prevention groups, triton soluble A β was increased compared to untreated 5xFAD animals. C) The FA soluble fraction was unaffected by therapeutic treatment. Preventive treatment with mIgG1 resulted in a significant reduction of FA soluble A β compared to untreated 5xFAD animals.

Total A β in TBS soluble, triton soluble and formic acid soluble fractions was also measured using ELISA. In the soluble fraction, no difference between any of the groups could be detected ($p=0.052$) (see Figure 3.6A). Unexpectedly, A β was increased in the triton fraction of both prevention groups compared to untreated 5xFAD animals ($p<0.01$), while triton soluble A β was decreased in both therapeutic groups compared to untreated 5xFAD animals ($p<0.01$) (see Figure 3.6B). Interestingly, preventive treatment with mIgG1 caused a reduction in formic acid soluble A β ($p=0.035$), suggesting that antibody treatment might shift A β between fractions. mIgG2a also caused a (non-significant) reduction in the formic acid fraction of A β , but this difference was lower,

which was consistent with the results from immunohistochemistry (see Figure 3.5).

3.3.4. REDUCTION IN ASTROGLIOSIS IS ISOTYPE DEPENDENT

AD is also characterized by increased gliosis. To investigate the effects on gliosis microglial and astrocyte activation were assessed using immunofluorescence with the markers Iba1 (microglia) and GFAP (astrocytes). Additionally, the amount of plaque associated microglia and astrocytes was assessed.

No significant differences could be found in microglial density between any of the groups, although microglial activation was increased in the untreated animals and animals therapeutically treated with mIgG1 compared to other groups (see Figure 3.7A). There were also no differences present in plaque associated microglia (see Figure 3.7D). Analysis of specific subregions of the brain also showed no significant differences in microgliosis between groups (data not shown).

Untreated 5xFAD animals showed strong astrogliosis compared to WT animals ($p < 0.01$). Animals treated with mIgG2a had a significant reduction in astrogliosis in whole brain compared to untreated animals. In the hippocampus, treatment with mIgG2a resulted in less astrocyte activation compared to treatment with mIgG1 ($p = 0.02$) (see Figure 3.7D). In the whole brain, no significant differences were present in plaque associated astrocytes between any of the groups ($p = 0.132$). However, in the hippocampus, both therapeutic and preventive treatment with mIgG2a significantly reduced plaque-associated astrocytes compared to untreated 5xFAD animals ($p = 0.02$) (see Figure 3.7). mIgG1 had no effect on astrogliosis.

Figure 3.7: Gliosis was assessed by measuring reactivity against Iba1 (microglia) and GFAP (astrocytes) in different brain regions using immunofluorescence. Plaque associated astrocytes and microglia were assessed by double labeling of A β and Iba1 or GFAP. A) Representative images of subiculum show a significant reduction in astrocytes (green) present in animals treated with mIgG2a. In addition, less astrocytes are present near plaques (red) in mIgG2a treated animals. B) No significant differences were present in microglia density in the brain ($n_{WT}=3$, $n_{5xFAD}=8$, $n_{mIgG1Ther}=7$, $n_{mIgG2aTher}=9$, $n_{mIgG1Prev}=4$, $n_{mIgG2aPrev}=6$). C-D) Astrocyte density was significantly lowered by treatment with mIgG2a 3D6, but not with mIgG1 3D6 ($n_{WT}=4$, $n_{5xFAD}=6$, $n_{mIgG1Ther}=4$, $n_{mIgG2aTher}=8$, $n_{mIgG1Prev}=4$, $n_{mIgG2aPrev}=6$). E) There were no differences in the amount of plaque associated microglia ($n_{5xFAD}=8$, $n_{mIgG1Ther}=7$, $n_{mIgG2aTher}=9$, $n_{mIgG1Prev}=4$, $n_{mIgG2aPrev}=6$). F-G) In the whole brain there was no difference in plaque associated astrocytes, but in the hippocampus mIgG2a treatment caused a significant reduction in plaque associated astrocytes. ($n_{WT}=4$, $n_{5xFAD}=6$, $n_{mIgG1Ther}=4$, $n_{mIgG2aTher}=8$, $n_{mIgG1Prev}=4$, $n_{mIgG2aPrev}=6$) Error bars denote SEM.

3.4. DISCUSSION

In this study, the isotype dependent effects of mAb 3D6 were investigated in the 5xFAD mouse model. Additionally, a preventive and therapeutic regimen were compared. Our results show that preventive treatment reduced anxiety as assessed by the EZM. Additionally, preventive immunotherapy also had stronger plaque reducing effects, as assessed by total density and by plaque size. Interestingly, triton soluble A β was increased in the preventive immunotherapy groups, while formic acid soluble A β was decreased by preventive treatment with mIgG1. Therapeutic treatment on the other hand reduced triton soluble A β , while it had no effect the formic acid fraction. We also found that treatment with mIgG2a 3D6 reduced astrogliosis.

Surprisingly we find that anti-inflammatory immunotherapy with the mIgG1 isotype results in a worsened memory as assessed by contextual fear conditioning. Finally, as also reported in a recent publication, immunotherapy can be lethal, raising concerns for passive immunotherapy in human trials [17].

Previous studies using F(ab')₂ 3D6 showed that non-Fc mediated mechanisms are involved in clearing amyloid plaques in a different mouse model of AD, and we confirm that mIgG1 is equally effective in clearing plaques than mIgG2a [18]. Another study that compared different anti-amyloid antibodies showed that mIgG1 antibodies were less effective in clearing plaques, but their results might have been confounded by the fact that different antibodies were used, and the epitope and affinity might play a crucial role in addition to the isotype [19].

For the moment, human immunotherapy trials have been unsuccessful in halting AD. Our results support the notion that preventive immunotherapy trials are potentially more effective than therapeutic trials. Only when treatment was started early in the disease process, were we able to reduce latency to enter the closed arm back to baseline levels in the EZM. These results offer hope for current ongoing preventive trials in humans.

Surprisingly, we found that treatment with mIgG1 3D6 increased memory problems in the 5xFAD animal model. A possible explanation could be found in the dust-raising effect previously reported for N-terminal antibodies [20]. The dust-raising effect states that A β oligomers are more toxic than fibrils, and transforming A β from its less neurotoxic fibrillary form in plaque deposits into its more neurotoxic oligomeric form might actually worsen the disease. Treatment with mIgG2a 3D6 would possibly cause oligomeric A β to be removed by Fc-mediated phagocytosis, while this would not be the case when treating with mIgG1 3D6, causing the freed oligomeric A β to exert its more neurotoxic effects. Additionally, anti-inflammatory treatment using ibuprofen has also been reported to worsen behavioral outcomes in this mouse model [21].

Interestingly, our results show that mIgG2a 3D6, but not mIgG1 3D6, dampens astrogliosis, while the effect on microgliosis is less pronounced. While it has been shown that astrocytes express Fc γ -receptor [22, 23], their role in neurodegenerative disorders and especially in immunotherapy has not been studied in detail. However, given the active role that astrocytes play in the maintenance of the blood brain barrier (BBB), it is not surprising that they might play an active role in immunotherapy.

Similar to a recent preclinical immunotherapy study we also report increased mortality in animals receiving therapy with 3D6 [17]. While in the study of Mably et

al. the cause of the mortality was not determined, we have found that most animals developed neutralizing antibodies against our administered antibody. Given the rapid onset of death and the observed symptoms, it is likely that animals subsequently died from passive systemic anaphylaxis (PSA), although we could not confirm this by for instance temperature measurements. We further showed that the PSA is IgG-, and not IgE-mediated, and one study indicated that the severity of the reaction is related to the amount of antigen present [24]. Possibly, this reaction was triggered by the use of our chimeric antibody.

A limitation of our study is that the mice only showed a weak phenotype in our behavioral paradigm. Only in 1 out of 4 tests, a difference was observed between WT and untreated 5xFAD animals. One possible reason for this could be the employed breeding scheme we utilized. Instead of using WT B6/SJL F1 hybrids [15], WT B6/SJL F3 mice were used to generate offspring. Additionally, the Pde6b^{rd1} was bred out of our colony. Both factors might have resulted in a weaker phenotype, as is also reported by Jackson Laboratories for the 5xFAD strain on congenic C57/B6 background.

Another limitation is that our results might have been confounded by that fact that immunotherapy caused PSA. This acute state of systemic inflammation might have influenced the inflammatory state in the CNS, which might explain why there were no differences in microglial activation. Additionally, neutralizing antibodies against the administered 3D6 antibodies could have lowered the effective dose reaching the brain, resulting in less efficacy.

In conclusion, our study confirms that preventive immunotherapy treatment might be more effective than therapeutic treatment. In addition, isotype dependent effects of immunotherapy should be further investigated, since our results suggest that the isotype affects the behavioral phenotype, independent of plaque load. This is especially important keeping in mind the disappointing results of the hIgG4 (mIgG1 equivalent) antibody crenezumab in its phase II trial [25]. Additionally, it has been shown that monoclonal antibody treatment can cause PSA in up to 70% of the patients [24], and our results show that this could potentially occur in passive amyloid immunotherapies.

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4

IN VIVO PET IMAGING IN AN AD MODEL

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ABSTRACT

A hallmark of Alzheimer's disease (AD) is the accumulation of amyloid beta ($A\beta$) peptide in the brain. Several $A\beta$ antibodies are currently tested in clinical trials as a possible treatment for AD. We investigated the possibility to use a radiolabeled $A\beta$ antibody (bapineuzumab) for *in vivo* imaging of $A\beta$ pathology in the brain of an AD mouse model (5xFAD).

Recombinant bapineuzumab and an isotype control antibody were labeled with 89-zirconium and administered to 3-, 6- and 9-month old female 5xFAD or wild type (WT) control mice. Already at 3 months of age, when plaque pathology was still limited, specific uptake of bapineuzumab in 5xFAD animals could be detected *in vivo* using PET scans. In addition, gamma counting showed an age-related accumulation of bapineuzumab in the brain of 5xFAD animals, which correlated with plaque load. No difference between 5xFAD and WT animals was found when using an isotype control immunoglobulin, or when blocking labelled bapineuzumab *in vivo* with an excess of unlabeled bapineuzumab. Results were validated by both autoradiography and immunofluorescence, with the latter showing co-localization of bapineuzumab in $A\beta$ plaques.

This study demonstrates that uptake of 89-zirconium labelled bapineuzumab in the brain can be specifically imaged *in vivo* using PET.

4.1. INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia and characterized by a buildup of extracellular plaques, containing aggregates of the protein amyloid-beta ($A\beta$), and intracellular tangles, containing tau protein. In the last years, the amyloid cascade hypothesis, which states that the buildup of $A\beta$ is the cause of AD, has been refined to postulate that other forms of $A\beta$, such as pyroglutamate or oligomeric $A\beta$, are the more neurotoxic species. *In vivo* positron emission tomography (PET) tracers, such as ^{18}F -florbetapir, ^{18}F -florbetaben and ^{18}F -flutemetamol (Vizamyl), can only detect fibrillary $A\beta$ deposits, while these other $A\beta$ assemblies can only be detected post-mortem using specific antibodies.

Immuno-PET combines the high specificity of antibodies with the sensitivity of PET. Furthermore, by using a long-lived isotope such as zirconium-89, the *in vivo* distribution of an antibody can be monitored over a period of several days [1, 2]. While immuno-PET has been widely used in the field of oncology, applications in the central nervous system (CNS) are still not common. This is probably due to a common misconception that antibodies do not cross the blood brain barrier (BBB) [3]. IgG concentrations in cerebrospinal fluid (CSF) are about 500 times lower than in serum, but they still cross the BBB with a rate of 0.018 mg/min, have a turnover of 0.0036 mg/min (which is comparable to the CSF turnover, ~4 times per day), and IgG comprises about 9.8% of total CSF protein [4, 5]. Thus while the brain penetration of antibodies is not high compared to traditional PET tracers, highly specific and high affinity antigen-antibody interactions can potentially be utilized to valuably complement the diagnostic potential of other tracers.

In this study, we evaluated the potential application of immuno-PET in an animal model for AD. We used the anti- $A\beta$ antibody bapineuzumab, which binds to the N-terminal part of $A\beta$ and recognizes monomers, oligomers and fibril, although it has a higher affinity for fibrils. Bapineuzumab has been tested in several clinical trials [6, 7, 8]: while therapeutically not effective to prevent progression of AD, it has been found safe for humans at moderate doses [8]. Two possible reasons for its ineffectiveness might be the fact that up to 20% of included patients might not have had AD or that the treatment started too late [9, 10].

We used the 5xFAD mouse model for AD, which has five familial AD mutations (APPSwedish, APPFlorida, APPLondon and PSEN1*M146L*L286V) and is characterized by early-onset plaque pathology starting at 2-3 months of age [11]. ^{89}Zr -bapineuzumab was administered at various disease stages followed by *in vivo* microPET imaging, biodistribution, autoradiography and immunohistochemistry analysis. We observed that as early as 3 months of age, when $A\beta$ pathology was still modest, there was significantly more ^{89}Zr -bapineuzumab uptake in the brain of AD mice. Our results show great promise of ^{89}Zr -immuno-PET for imaging of specific antibody binding to $A\beta$ pathology in the brain.

4.2. MATERIAL AND METHODS

4.2.1. ANIMALS

B6/SJL wild type (WT) and 5xFAD animals were obtained from Jackson laboratory (stock 6554) and bred hemizygotously [11] using F3 WT mice. Female mice were used at the ages of 3, 6 and 9 months (numbers are listed in Table 4.1). Mice were housed socially on a 12 hour day-night cycle and had *ad libitum* access to food and water. All experiments were approved by the Animal Welfare Committee of Maastricht University and followed the laws, rules and guidelines of the Netherlands.

Table 4.1: Number of animals per experimental group

Age (months)	^{89}Zr -mAb 637	^{89}Zr -bapineuzumab	^{89}Zr -bapineuzumab/ bapineuzumab (1/10)
	WT	5xFAD	WT
3	5	5	5
6	5	5	5
9	5	4	4

4.2.2. ANTIBODY LABELING

Antibodies were produced recombinantly by transient transfection of HEK cells. Human IgG (IgG1) was chosen to facilitate *ex vivo* detection of injected antibody. The light and heavy chain coding sequences of bapineuzumab [12] were synthesized *de novo* (Invitrogen-Geneart) and cloned in pFuse IgG expression vectors (Invivogen). Plasmids were produced at large scale in *E. coli* grown in a Bioflo 110 fermentor (New Brunswick) and purified using a CIM DEAE column (BIA Separations) according to manufacturer instructions. For production of antibody, HEK293-F cells (Invitrogen) were grown to confluence in a 4-layer Nunc cell factory (Thermo Scientific) in Freestyle 293 medium (Invitrogen). Cells were subsequently transfected using 25 kDa linear PEI (Polysciences) dissolved in purified water. DNA-PEI complexes were prepared using a 1:4 DNA:PEI ratio. Four hours after transfection, valproic acid (Sigma-Aldrich) was added to a concentration of 4 mM and supernatant was harvested after 9-10 days of incubation. Antibody was subsequently purified on an AKTA explorer (GE Healthcare Life Sciences) with a MabSelect Protein A column (GE Healthcare Life Sciences) immediately followed by a buffer exchange using a Sephadex column (GE Healthcare Life Sciences). Antibody purity was confirmed by Coomassie blue staining and concentration was measured using a Lowry protein assay (Biorad). The recombinant antibody mAb 637 (monoclonal human IgG1, directed against human muscle AChR $\alpha 1$ subunit) was used as an isotype control; cloning and production are described elsewhere [13]. MAb 637 was tested for immuno-reactivity in mouse muscle and brain, and was found to be non-reactive.

Affinity of labeled bapineuzumab was determined by ELISA. Briefly, 250 pg/well recombinant $A\beta_{1-42}$ (rPeptide) was coated overnight at 4 °C on a HiBind 96-well plate (Greiner Bio-One). The plate was washed, blocked with 4% non-fat dry milk (Biorad) and subsequently incubated with labeled or unlabeled bapineuzumab. After washing,

the plate was incubated using anti-human IgG HRP (Jackson ImmunoResearch) and developed using tetramethylbenzidine (TMB). The OD was measured on a Victor plate reader (Perkin Elmer) at 450 nm, and EC50 (apparent affinity) value was calculated using Graphpad Prism 5. This showed that both labeled and unlabeled bapineuzumab bound immobilized amyloid beta with a similar apparent affinity (see Fig. S1C). Antibody specificity before and after labelling was also confirmed using immunohistochemistry on APP/PSEN1 mouse brain slices: labeled bapineuzumab retained binding on APP/PSEN1 sections (not shown).

Antibodies were labeled with ^{89}Zr using described methods [14] with an average overall labeling yield of 78.6% (81.2% for bapineuzumab and 76% for mAb 637), a radiochemical purity always higher than 99% and a specific activity typically in the range of ~ 37 MBq/mg.

A total of 100 μg of antibody (0.5 $\mu\text{g}/\mu\text{L}$, dissolved in saline) was injected intraperitoneally (i.p.) while animals were under isoflurane anesthesia. Average injected activity was 3.56 ± 0.24 MBq. For blocking experiments, animals were injected with 1 mg of unlabeled bapineuzumab, together with 100 μg of ^{89}Zr -bapineuzumab.

4.2.3. IMAGING AND DATA ANALYSIS

PET images were acquired using a Siemens MicroPET Focus 120 (Siemens Healthcare) with an axial field of view (FOV) of 7.6 cm and a resolution of about 1.3 mm. Animals were anesthetized using isoflurane (2.5% in O_2), and body temperature was maintained using an electrical heating pad controlled by an anal temperature probe. The animal was positioned supine and data was acquired for 30 minutes. Acquired data were corrected for dead time, random coincidences and decay. Images were reconstructed using OSEM2D. Volumes of interest were analyzed with Carimas 2.9 [15, 16].

The head was first coregistered using normalized cross correlation to a CT template scan that was acquired as described [17]. Manual realignment was performed if the automatic coregistration algorithm failed. Next, a volume of interest (VOI) was selected in the CT template and mean Bq/mL was calculated of the VOI. The VOI had a volume of 25 μL and was placed centrally in the brain in order to minimize partial volume effects caused by high activity in the surrounding tissues (see Fig. 1); especially bone (and thus skull) have been shown to have high uptake of ^{89}Zr [18]. The standardized uptake value (SUV) was calculated by correcting the mean activity of the VOI (in Bq/ml) for body weight (in g), injected dose (Bq) and decay.

4.2.4. BIODISTRIBUTION

Animals were sacrificed by cervical dislocation under isoflurane anesthesia after the last scan. The brain was removed and one hemisphere was rapidly frozen by liquid nitrogen. The other hemisphere was macrodissected into hindbrain (cerebellum and brain stem), bulbus, hippocampus and cortex. 50-100 μL of blood was obtained from the heart. For analysis of antibody uptake in other tissues, the following organs were dissected: salivary glands, cervical lymph nodes, skin, eyes, spleen, intestines, liver, stomach, bladder, kidneys, lungs and heart. The body was further divided into limbs, head and trunk. Radioactivity content in all these body parts was measured using a Wizard² gamma counter (Perkin Elmer) for 2 minutes per sample. Results were corrected

for radioactive decay and expressed as percentage of total injected dose (in Bq) per gram of tissue (%ID/g).

4.2.5. AUTORADIOGRAPHY, IMMUNOFLUORESCENCE AND MICROSCOPY

The contralateral frozen hemisphere was cut on a cryostat at -17 °C. For autoradiography, 50 µm sections were incubated on a phosphor film for about 2 weeks. The film was read out by a Typhoon FLA 7000 (Perkin Elmer).

For immunohistochemistry, 16 µm thick sections were stained with biotinylated goat anti-human IgG (Brunschwig, 1:500). Plaque load was determined by incubation with biotinylated bapineuzumab (1:2000). For co-localization studies, a double staining was performed with mouse-anti human IgG Alexa 594 (Jackson ImmunoResearch, 209-585-082, 1:250) and biotinylated human anti-Aβ mAb 20C2 (recombinantly produced [19], 1:1000).

Photomicrographs were acquired using µManager software [20] on a BX51WI spinning disk confocal fluorescence microscope (Olympus) with a EM-CCD C9100 digital camera (Hamamatsu) and a QIcam color camera (QImaging). Images were background corrected and stitched using a plug-in [21] of ImageJ software (www.imagej.nih.gov/ij/).

For quantification of plaques two midsagittal sections per animal were used, spaced 160 µm apart. Images were analyzed using ImageJ 1.49. First, regions-of-interest (ROIs) were manually delineated. These ROIs comprised the cortex, subiculum, hippocampus, cerebellum, bulbus, brainstem and the midbrain combined with the striatum. Next, a median filter was applied, and by using the threshold function, images were converted to binary images. Using the particle analyzer tool, plaque area was determined and divided by the total ROI area. For colocalization analysis, average fluorescence intensity of bapineuzumab and 20C2 was measured in the plaque areas (defined by 20C2 immunoreactivity). The same settings were used for all images.

4.2.6. STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS Statistics 22 software (IBM). PET and biodistribution data were analyzed using a 3-way ANOVA, with animal age, genotype, and antibody as independent factors. If interaction was present, data were analyzed by a pair-wise comparison of estimated marginal means using Bonferroni correction. Analysis of the optimal imaging time point after injection was performed with a repeated measures model. A p-value of <0.05 was considered significant.

4.3. RESULTS

4.3.1. SPECIFIC ACCUMULATION OF ⁸⁹Zr-BAPINEUZUMAB IN THE 5xFAD BRAIN *IN VIVO*

Figure 4.1: Quantification of bapineuzumab labeled plaques in the 5xFAD animal model and affinity determination of ⁸⁹Zr-bapineuzumab. (A) Immunohistochemical staining with bapineuzumab using 5xFAD brain sections at different ages. (B) Plaque density was measured in different brain regions using ImageJ. At all ages, the subiculum has the highest plaque density, followed by plaque density in the cortex. (C) A direct ELISA with A β ₄₂ shows that the apparent affinity of unlabeled and labeled bapineuzumab are similar.

As a reference for PET experiments, the A β plaque load in brain sections of 3, 6, and 9 months old 5xFAD mice was analyzed by immunohistochemistry with bapineuzumab (Figure 4.1). A β pathology started in the subiculum and the cortex and increased with age, in agreement with published data [11]. For PET imaging of A β pathology, ^{89}Zr -bapineuzumab was administered to 9 month old mice and antibody accumulation was measured over various days. Three days after injection, there was a ~2-fold difference between the 5xFAD and WT groups ($p=0.021$), and uptake in 5xFAD animals further increased in the following days (day 5: $p=0.007$, day 7: $p<0.001$ and day 9: $p<0.001$) (see Fig. 4.1).

In 9-month old animals, a blocking experiment was performed to test the specificity of ^{89}Zr -bapineuzumab by co-administration of a 10-fold excess of unlabeled bapineuzumab. In the blocking group, uptake of ^{89}Zr -bapineuzumab was reduced to baseline values (Figure 4.2) ($p=0.908$) and significantly reduced compared to 5xFAD animals treated with only ^{89}Zr -bapineuzumab ($p<0.001$).

Isotype control ^{89}Zr -mAb 637 uptake was not statistically different between genotypes ($p=0.386$, Figure 4.2), while ^{89}Zr -bapineuzumab uptake was increased in 5xFAD animals compared to ^{89}Zr -mAb 637 ($p<0.001$), further proving the specificity of ^{89}Zr -bapineuzumab uptake.

Figure 4.2: Quantification of brain PET scans demonstrated specific uptake of ^{89}Zr -bapineuzumab in 5xFAD animals. (A) In 9-month-old 5xFAD and WT animals ($n=2$ per group) increased accumulation of ^{89}Zr -bapineuzumab in 5xFAD animals was observed 3 days after injection. ^{89}Zr -bapineuzumab further accumulated in the brain during the following days. (B) Animals of 6 and 9 months were scanned 5 days after injection, and 3-month-old animals were scanned 8 days after injection. As soon as 3 months of age, increased uptake of ^{89}Zr -bapineuzumab was observed in 5xFAD animals injected with ^{89}Zr -bapineuzumab and this difference with WT animals remained present at older ages. In animals injected with the isotype control (^{89}Zr -mAb 637) this difference was not observed. When blocking ^{89}Zr -bapineuzumab with a 10-fold excess of unlabeled bapineuzumab, values were reduced to baseline. Error bars correspond to the SEM. Detailed animal numbers are listed in Table 4.1.

4.3.2. EARLY DETECTION OF ^{89}Zr -BAPINEUZUMAB IN YOUNG 5xFAD MICE

To assess the sensitivity of immuno-PET using ^{89}Zr -bapineuzumab, animals were imaged at 3, 6 and 9 months of age using ^{89}Zr -bapineuzumab and isotype control ^{89}Zr -

mAb 637. As early as 3 months of age, when A β load was still low, a specific increase in accumulation of ^{89}Zr -bapineuzumab was present in the brain; this increase was significant for all ages studied (Figure 4.2, $p < 0.001$), although we did not observe an age-related increase in ^{89}Zr -bapineuzumab uptake. No difference was detected between WT and 5xFAD animals treated with the isotype control ^{89}Zr -mAb 637 ($p = 0.582$) at any age.

Figure 4.3: Overview of biodistribution data from all analyzed organs. The conditions are explained in Fig. 3 and the Biodistribution section in the Material and Methods. In the bulbous a significant difference between WT and 5x FAD animals was found only in 9 month old animals injected with ^{89}Zr -bapineuzumab. Furthermore, increased activity in the limbs for both ^{89}Zr -bapineuzumab and ^{89}Zr -mAb 637 was found in 5x FAD animals.

4.3.3. BIODISTRIBUTION ANALYSIS CONFIRMED EARLY AGE-SPECIFIC UPTAKE OF ^{89}Zr -BAPINEUZUMAB

Figure 4.4: Biodistribution showed an age dependent increase of ^{89}Zr -bapineuzumab uptake in some anatomical regions of the 5xFAD brain. Biodistribution of ^{89}Zr -bapineuzumab and isotype control (^{89}Zr -mAb-637) was analyzed in total brain (A), cortex (B), hindbrain (cerebellum and brain stem) (C), hippocampus (D), cervical lymph nodes (E) and the correlation between plaque load and radioactivity (F) (other organs are shown in supplemental Figure S2). In 9-months-old animals, ^{89}Zr -bapineuzumab was blocked with a 10 times excess of unlabeled bapineuzumab (^{89}Zr -bapineuzumab/bapineuzumab (1/10)). (A-C) ^{89}Zr -bapineuzumab uptake increased in the brain of aging 5xFAD animals following the reported A β build up in this model. (D) In the hippocampus no significant differences were found between the experimental groups. (E) In cervical lymph nodes, an increased uptake of ^{89}Zr -bapineuzumab was detected in 5xFAD compared to WT mice at all ages. (F) Total brain radioactivity had a strong correlation to the quantified plaque load ($r=0.832$). All values were normalized for tissue weight, radioactive decay and injected dose (%ID/g). Error bars correspond to SEM and animal numbers are listed in Table 4.1.

Five days after antibody administration, uptake was studied in the brain and other organs. In total brain, the uptake (Figure 4.4) closely paralleled the results obtained by immuno-PET (Figure 4.2). In concordance with analysis of the PET scans, ^{89}Zr -bapineuzumab brain uptake was significantly higher at all ages for 5xFAD mice compared to age matched WT mice ($p=0.02$ at 3 months, and $p<0.001$ at 6 and 9 months). Additionally, and in contrast to the *in vivo* PET scans, we found that ^{89}Zr -bapineuzumab accumulation in brain tissue increased with age in 5xFAD animals ($p<0.001$) (Figure 4.4). A correlation analysis between plaque density and radioactivity in the brain showed a strong correlation ($r=0.832$, $p<0.001$, Fig 4.4). There was no difference at any age in the isotype control groups or when ^{89}Zr -bapineuzumab was blocked with cold bapineuzumab, further proving that ^{89}Zr -bapineuzumab uptake was specific to A β pathology.

Because A β load in the 5xFAD model differs between brain regions [11] (Figure 4.1), macro dissection of the brain was performed (cortex, hindbrain, hippocampus, and bulbus) and radioactivity in each region was measured. For the cortex, there was a significant difference in ^{89}Zr -bapineuzumab uptake between WT and 5xFAD at all analyzed ages ($p<0.001$; Figure 2B). Blocking ^{89}Zr -bapineuzumab uptake with an excess of unlabeled bapineuzumab showed a decreased radioactivity compared to 5xFAD animals treated with only ^{89}Zr -bapineuzumab ($p<0.001$), in line with the results of whole brain. However, when comparing 5xFAD and WT animals in the blocking group, there was still a small but significant increase of ^{89}Zr -bapineuzumab uptake ($p=0.019$), indicating incomplete blockage of bapineuzumab binding in the cortex.

Results from the hindbrain (Figure 4.4) showed similar results as the cortex, except that uptake of ^{89}Zr -bapineuzumab in the 5xFAD mice could be completely blocked. At 3 months of age, there was already a 3-fold increase of ^{89}Zr -bapineuzumab uptake in the hindbrain of 5xFAD animals compared to WT ($p=0.005$). A further increased uptake was observed at 6 and 9 months of age of respectively 3.4 and 4.4 fold ($p<0.001$ for both). For the bulbus, increased uptake of bapineuzumab in 5xFAD animals was observed only at 9 months of age ($p=0.001$, Figure 4.3). No significant differences in uptake were found for the hippocampus (Figure 4.4).

Besides the brain, specific increased uptake of ^{89}Zr -bapineuzumab was observed in cervical lymph nodes of 5xFAD mice ($p=0.001$, Figure 4.4), suggesting the presence of A β in draining lymph nodes of the brain. This was not observed when the isotype control antibody was injected or when ^{89}Zr -bapineuzumab uptake was blocked with cold bapineuzumab. Non-specific uptake of ^{89}Zr -bapineuzumab and ^{89}Zr -mAb 637 was increased in the limbs of 5xFAD (Figure 4.3), but only at 9 months of age. This difference was probably related to different weight distribution in the 5xFAD mice at this age. In general, the biodistribution of ^{89}Zr -mAb 637 was different from ^{89}Zr -bapineuzumab (Figure 4.3). However, the clearance rate of both antibodies was the same (Figure 4.3).

Figure 4.5: Autoradiography showed increased uptake of ^{89}Zr -bapineuzumab in the hindbrain. The strongest activity was observed in the hindbrain of 5xFAD animals injected with ^{89}Zr -bapineuzumab. This activity was much lower in all animals injected with ^{89}Zr -mAb 637 (isotype control). Blocking bapineuzumab in 5xFAD mice reduced activity to similar levels as observed in WT animals. Sections of 6-month-old cannot be compared to 9-month-old animals because of inter-assay variations. The hindbrain is orientated to the right in all panels.

4.3.4. ^{89}Zr -BAPINEUZUMAB CO-LOCALIZED WITH $\text{A}\beta$ PLAQUES IN THE BRAIN

The antibody distribution in the brain was investigated by autoradiography on the brain of 6 and 9 month old animals (Figure 4.5). A clear difference in distribution of radioactivity was observed between the bapineuzumab-injected 5xFAD animals and the control groups: for the 5xFAD animals, most of the activity was observed in the hindbrain, and near the hippocampus region (Figure 4.5).

To investigate the specificity of the *in vivo* binding of ^{89}Zr -labeled antibodies, injected human IgG was immunohistochemically stained in sagittal brain sections. Figure 4.6A and 4.6B show that plaques stained with ^{89}Zr -bapineuzumab, but not with the isotype control ^{89}Zr -mAb 637.

Specificity was further shown by a double immunofluorescent staining for human IgG and $\text{A}\beta$ plaques. ^{89}Zr -bapineuzumab colocalized with $\text{A}\beta$ plaques (Figure 4.6C). While antibody 20C2, which has a preference for oligomeric $\text{A}\beta$, mostly stained the plaque periphery, bapineuzumab was found in the plaque core.

Figure 4.6: *In vivo* injected ^{89}Zr -bapineuzumab is present in A β plaques. (A) Immunohistochemistry for human IgG (anti-human IgG) with a methyl green counterstain on brains of 9-month old mice showed a diffuse staining predominantly in the mid- and hindbrain. In 5xFAD animals injected with ^{89}Zr -bapineuzumab, also plaques were clearly stained. In all groups, there is clearly less human IgG present in the hippocampus. (B) High power photomicrographs of the 5xFAD brain stem showed that plaques were immunoreactive for human IgG in animals treated with ^{89}Zr -bapineuzumab. This immunoreactivity could not be found in 5xFAD animals treated with mAb 637. (C) Double labeling of plaques in the brain stem with anti-A β 20C2 (green) and anti-human IgG (red) showed colocalization. Cell nuclei were stained with Hoechst (blue). ^{89}Zr -bapineuzumab was detected in the plaque core (arrows), while 20C2 staining was predominantly in the plaque periphery. More intense staining of plaques was observed when bapineuzumab was blocked with excess unlabeled bapineuzumab. The isotype control did not show colocalization with plaques (arrow head).

4.4. DISCUSSION

This study shows that immuno-PET with ^{89}Zr -bapineuzumab can be used for specific and sensitive detection of $\text{A}\beta$ pathology in the brain. As early as at 3 months of age, brain uptake of ^{89}Zr -bapineuzumab was increased in 5xFAD mice. The observed age-related increase of ^{89}Zr -bapineuzumab accumulation in the brain in the biodistribution analysis clearly followed the documented $\text{A}\beta$ build-up with age in this animal model [11]. Interestingly, ^{89}Zr -bapineuzumab also showed specific uptake in the cervical lymph nodes of 5xFAD. Since much of the CSF drains through this route, $\text{A}\beta$ or IgG - $\text{A}\beta$ complexes are likely to accumulate in these lymph nodes [22].

The specificity of the signal was demonstrated by using a radiolabeled isotype control mAb, which showed no difference in brain accumulation between 5xFAD and WT mice. This suggests that ^{89}Zr -bapineuzumab accumulation is specific to $\text{A}\beta$ pathology and not due to an increased permeability of the BBB. In this context it is important to mention that the BBB allows controlled entry of IgG in the brain under physiological conditions [5]. Even though the amount of penetrated antibody is low, the good signal-to-noise ratio still makes it possible to detect a specific signal. Blocking with cold bapineuzumab reduced brain uptake of ^{89}Zr -bapineuzumab to baseline values, providing further evidence for the specificity of immuno-PET using ^{89}Zr -bapineuzumab.

The long half-life of ^{89}Zr allowed us to assess antibody kinetics over a period of 9 days. Three days after ^{89}Zr -bapineuzumab injection, significant uptake of the antibody was detected in the brain. This uptake increased further during the subsequent days, showing accumulation of antibody over time.

Recently, similar results were obtained by Sehlin and colleagues., where an anti- $\text{A}\beta$ antibody against protofibrils was used for *in vivo* PET imaging [23]. In contrast to our study, Sehlin et al. made use of a bispecific antibody targeting the transferrin-receptor in order to increase antibody uptake in the brain. We show that it possible to use antibodies for CNS imaging without modifications besides the radiolabel. Both our results prove that antibodies allow for specific *in vivo* imaging in the CNS.

Rojas et al. compared current amyloid PET tracers in 5xFAD and WT animals. They were also able to discriminate between 5xFAD and WT animals with a difference of 14.5% and 21% in activity between 11-months-old 5xFAD and WT animals using florbetapir and PiB respectively. Here we show that immuno-PET with ^{89}Zr -bapineuzumab offers a significant leap in sensitivity. Over a 50% difference of ^{89}Zr -bapineuzumab uptake was present between 9-month-old 5xFAD and WT animals [24]. These results show that despite the lower penetration of bapineuzumab compared to traditional amyloid tracers, a higher sensitivity can be reached, likely because of very high-affinity binding of bapineuzumab to $\text{A}\beta$.

A limitation of this study is the difference in biodistribution of the isotype control ^{89}Zr -mAb 637 and ^{89}Zr -bapineuzumab. However, the clearance rate of both antibodies from the blood was the same. We confirmed the age-related increase of plaque load in the 5xFAD model by immunohistochemistry with bapineuzumab. This increase was also observed in the biodistribution analysis with ^{89}Zr -bapineuzumab. However, no age-related increase of ^{89}Zr -bapineuzumab uptake was observed in the corresponding *in vivo* PET scans. This might be related to partial volume effects in the PET scans, caused by accumulation of ^{89}Zr in the skull, making quantification of the brain more difficult.

This seems to be especially a problem with rodents, and less of an issue when imaging with ^{89}Zr in humans [25]. Additionally, while a relatively high dose of radioactivity was used in our mice, new advances in clinical PET scanners allow for the use of a significant lower dose in humans [26].

Our results show that it is possible to use an antibody to detect $\text{A}\beta$ pathology in the CNS *in vivo*. With ^{89}Zr -immuno-PET it is possible to image specific antibody binding to $\text{A}\beta$, even in the early disease stage, when $\text{A}\beta$ load is low. Anti- $\text{A}\beta$ immuno-PET could potentially be useful as an inclusion criterion for passive anti- $\text{A}\beta$ immunotherapy trials. It may also be a powerful tool for the selection of the optimal antibody format (e.g. intact antibody or antibody fragment), and for optimization of the antibody dosing schedule in anti- $\text{A}\beta$ immunotherapy studies. Furthermore, if new therapeutic antibodies become available [27], this technique will allow such antibodies to be used as a theranostic tool to screen patients at baseline and during treatment, although attention has to be paid to radiation safety when multiple scans are undertaken. Finally, antibodies might be useful to develop new tracers for other neurodegenerative disorders by targeting for example α -synuclein in Parkinson's disease, or tau tangles in AD or other tauopathies.

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5

A COMPARISON OF THREE ANTI-AMYLOID ANTIBODIES WITH IMMUNO-PET

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ABSTRACT

The amyloid cascade hypothesis is the leading hypothesis to explain Alzheimer's disease (AD) pathogenesis. It states that there are different forms of A β , of which the oligomeric form is the most toxic. In the last years, several immunotherapies have been developed to remove A β from the brain, but so far, results have been largely disappointing. In an effort to better understand the behavior of these A β antibodies *in vivo*, three anti-A β antibodies targeting monomers (solanezumab), oligomers (20C2), and fibrils (bapineuzumab) were radiolabeled with ^{89}Zr , a long-lived positron emitter. Labeled antibodies were administered to 5xFAD mice of different ages and *in vivo* PET scans were performed. In addition, a detailed biodistribution study was performed. Results obtained from these assays were compared with *ex vivo* A β quantification. Our results showed that ^{89}Zr -bapineuzumab accumulated in an age-related manner in the brain, correlating with plaque load. While oligomers accumulated as animals age *ex vivo*, *in vivo* ^{89}Zr -20C2 only accumulated in AD animals at later ages. In the early disease stage, ^{89}Zr -20C2 was actually removed faster from AD animals. Monomeric A β remained constant as animals age, and there was also no ^{89}Zr -solanezumab accumulation *in vivo*. Interestingly, ^{89}Zr -solanezumab accumulated in adipose tissue in 5xFAD animals and this correlated with monomeric plaque load in the brain. These results prove that it is important to further study *in vivo* antibody properties in order to further improve and refine antibody targets.

5.1. INTRODUCTION

Amyloid- β ($A\beta$) is one of the defining hallmarks of Alzheimer's disease (AD) and has been one of the main targets of new experimental AD therapies. Most of these therapies focus on lowering $A\beta$ production (eg. gamma- and beta-secretase inhibitors) or on increasing $A\beta$ clearance from the brain [1]. The latter has been attempted by means of active and passive immunization against $A\beta$.

Several passive immunization strategies, where $A\beta$ antibodies are administered to the patient, have been or are being conducted. The first passive immunotherapy phase II clinical trial, conducted by Johnson and Johnson, made use of the antibody bapineuzumab. Bapineuzumab, the humanized variant of the original mouse antibody 3D6, targets the N-terminus region of $A\beta$ (amino acids 1-5) [2]. Bapineuzumab has been shown to bind both soluble and fibrillary amyloid [3]. While the phase II trial did not meet clinical end-points, the antibody was progressed to phase III based on a subset analysis showing some possible benefit for APOE4 non-carriers [4]. The phase III trial was concluded with negative primary endpoints in the summer of 2013 [5]. Recent results did indicate that bapineuzumab lowered fibrillary $A\beta$ load as assessed by ^{11}C -PiB-PET imaging [6].

Several reasons have been postulated for this failure. A first reason is that during the trial, dosage had to be lowered 2-fold because patients experienced microbleeds because of treatment [6]. It might therefore be the case that antibody dosing was too low to be effective. Secondly, it is estimated that up to 20% of the patients were misdiagnosed and did not suffer from AD at all [7]. The net result being that the power of the trial is severely lowered and a possible beneficial effect could not be picked up. Third, it has been suggested that treatment started too late and patients need to start treatment when the disease is still in its beginning stages [8]. A final hypothesis might be that bapineuzumab targeted the wrong $A\beta$ species, and another species such as monomeric or oligomeric $A\beta$ might have showed more efficacy [6].

Solanezumab is another antibody that has been tested in large phase III clinical trials. Solanezumab, mouse equivalent antibody m266, binds to the midregion of $A\beta$ (amino acids 16-26) [9]. Furthermore, it has been shown to have a high affinity for monomeric amyloid, presumable because the epitope is masked in fibrillary or oligomeric assemblies of $A\beta$ [9]. Primary endpoints of this antibody in the phase III trial were not met. There was a small effect in patients with mild AD, and the antibody is now being continued in a prevention trial [10]. Compared to bapineuzumab, this antibody did not seem to cause microbleeds in patients [11]. Another difference is its presumed mechanism of action. Solanezumab is assumed to not enter the brain, but instead lowers $A\beta$ in the brain by sequestering free $A\beta$ in the blood stream, and preventing this $A\beta$ from reentering in the brain, thereby causing a net efflux of amyloid. This mechanism is also known as the peripheral sink hypothesis [12].

While the clinical trials of these antibodies have yielded disappointing results, it is still important to further characterize these antibodies so more can be understood as to why these antibodies failed clinical trials. Recently, we proved that immuno-PET can be a valuable tool to gain more understanding about $A\beta$ antibody binding and distribution *in vivo*. In that study, bapineuzumab was conjugated to the long-lived positron emitter 89-zirconium (^{89}Zr) and injected in an AD mouse model (5xFAD) at various disease

stadia. The 5xFAD model is rapid disease onset mouse model containing five familial AD mutations (3 in APP and 2 PSEN1) [13]. Immuno-PET with bapineuzumab allowed detection of AD pathology at the young age of 3 months [14]. At this age, the first plaques appear in the 5xFAD model [13].

In this paper, we expand on these results by comparing bapineuzumab against solanezumab using immuno-PET in the 5xFAD mouse model. Additionally, an anti-A β antibody targeting oligomeric forms of A β was also investigated. The amyloid cascade hypothesis posits amyloid accumulation as the central cause of AD [15]. Over the years, the hypothesis has been updated and fine-tuned, and it is now believed that oligomeric assemblies of A β are the most cytotoxic and the main cause of AD [16]. Surprisingly, no immunotherapies focusing on targeting oligomeric forms of A β have been tested yet in pre-clinical or clinical trials. Antibody 20C2 is an antibody that targets soluble oligomers of A β [17]. It has been extensively characterized and binds to a conformational epitope [18]. To gain further insight into the targeting mechanisms of these three different antibodies, we compared them in the 5xFAD model at different ages. Since monomeric and oligomeric forms of A β originate first, we hypothesized that solanezumab and 20C2 would allow for more sensitive and early detection than bapineuzumab, which binds mostly to plaques. Our hypothesis was not confirmed, due to the fact that antibodies binding soluble forms of A β also get cleared faster. Furthermore, the anti-oligomeric antibody 20C2 exhibited different distributional patterns in different stages of the disease. These results might have important implications for ongoing and planned clinical trials.

5.2. METHODS

5.2.1. ANIMALS

BS/SJL wild type (WT) and 5xFAD animals were purchased from Jackson Laboratories (stock 6554). Mice were bred hemizygotously using F3 and F4 WT mice. Mice were housed socially with *ad libitum* access to food and water on a 12-hour day-night cycle. For all experiments, female mice were used of the ages 3, 6, or 9 months. All experiments were approved by the Animal Welfare Committee of Maastricht University and followed the law and guidelines of the Netherlands.

Table 5.1: Number of animals per experimental group

Age (months)	⁸⁹ Zr-bapineuzumab	⁸⁹ Zr-solanezumab	⁸⁹ Zr-20C2
	WT	5xFAD	WT
3	5	5	5
6	5	5	7
9	4	4	4

5.2.2. ANTIBODY PRODUCTION AND LABELING

Antibodies were cloned and produced as described [14]. Briefly, antibody sequences of bapineuzumab [19], solanezumab [20] and 20C2 [21] were synthesized *de novo* by

GeneArt (Invitrogen) and cloned into pFuse expression vectors (Invivogen). Plasmids were produced at large scale in a New Brunswick Bioflo 110 bioreactor and purified on an AKTA explorer system equipped with a CIM DEAE column.

HEK-293 F cells were transfected with linear 22 kDa PEI (Polysciences) in Nunc cell factories in Freestyle 293 medium. Four hours after transfection valproic acid (Sigma Aldrich) was added to a concentration of 4 mM. After 10 days, supernatant was filtered through a 0.45 μm membrane and concentrated using an artificial kidney (Fresenius Medical Care). Antibody was subsequently purified using a protein A column (GE Healthcare) followed by a buffer exchange to PBS using a Sephadex column (GE Healthcare).

Labeling with zirconium-89 was carried out as described.

A total of 100 μg antibody dissolved in 200 μL saline was injected intraperitoneally in animals under isoflurane anesthesia. Average injected activity was 3.47 MBq. Animals numbers per group are listed in Table 5.1.

5.2.3. IMAGING

In vivo PET images were acquired as described [14]. Animals were placed supine in the center field of view (FOV) of a Siemens MicroPET Focus 120 (Siemens Healthcare) with an axial FOV of 7.6 cm and a resolution of 1.3 mm. Scans were acquired for a total of 30 minutes while animals were anesthetized using isoflurane (2.5% in O_2). Body temperature was maintained using an electrical heating pad. Data were acquired with correction for dead time, random coincidences and decay. Images were reconstructed using OSEM2D and PET data were analyzed using Carimas 2.9 [22]. For analysis, the head was first coregistered using normalized cross correlation to a CT template scan that was acquired as described [23]. Manual realignment was performed if the automatic coregistration algorithm failed. Next, a volume of interest (VOI) of 25 μL was selected in the CT template and mean Bq/mL was calculated of the VOI (see Fig. 1). The VOI was chosen small to minimize partial volume effects caused by high activity of the surrounding tissues such as the bone [24]. The standardized uptake value (SUV) was calculated by correcting the mean activity of the VOI (in Bq/mL) for body weight (in g), injected dose (Bq) and decay.

5.2.4. BIODISTRIBUTION

After sacrificing animals by cervical dislocation, biodistribution was performed as described [14]. Briefly, one brain hemisphere was frozen with liquid nitrogen and stored at -80. The other hemisphere was macrodissected into hindbrain (with cerebellum and brain stem), bulbus, hippocampus and cortex. The following tissues and organs were dissected, and radioactivity was measured: blood, salivary glands, cervical lymph nodes, skin, abdominal adipose tissue, eyes, spleen, intestines, liver, stomach, bladder, kidneys, lungs and heart. Remaining body parts were divided into limbs, head, and trunk. Radioactivity was measured using a 2470 Wizard² gamma counter (Perkin Elmer) for a total time of 2 minutes. All results were corrected for decay and expressed as a percentage of total injected dose (Bq) per gram of tissue (%ID/g).

5.2.5. IMMUNOSTAINING AND MICROSCOPY

Frozen hemispheres were cut sagittal into 16 μm sections on a Leica cryostat at -17 °C. Sections were post-fixed with 4% paraformaldehyde. For analysis of plaque load, sections were incubated overnight with biotinylated bapineuzumab (1:4000), solanezumab (1:2000) or 20C2 (1:4000). Sections were washed and incubated with ABC Kit (Vectastain). After washing, sections were incubated with horseradish peroxidase, dehydrated and coverslipped with DPX (Klinipath).

Photomicrographs were acquired using μ Manager software [25] on a IX-81 inverted microscope (Olympus). Images were background corrected and stitched using a plug-in [26] of ImageJ 1.49 software (www.imagej.nih.gov/ij/). Next, regions-of-interest (ROIs) were delineated consisting of cortex, subiculum, hippocampus, midbrain, thalamus and striatum. A median filter was applied to all images, and a threshold was determined. Images were binarized and plaque load was analyzed using the particle analyzer tool in ImageJ. All processing steps were identical for each antibody staining.

5.2.6. STATISTICAL ANALYZES

Statistical analyzes were performed with SPSS 23 (IBM). *In vivo* PET data and biodistribution was calculated with a 3-way ANOVA with age, antibody and genotype as factors. The radioactivity of level of the fat tissue was compared using a 2-way ANOVA with age and genotype as factor for each antibody, because no data was available for bapineuzumab for 9 month old animals. For correlations, Pearson correlation was calculated. A p-value of 0.05 was considered significant.

5.3. RESULTS

Figure 5.1: A qualitative and quantitative comparison of bapineuzumab, solanezumab and 20C2. A) Representative images of 9 months old 5xFAD animals show that staining with bapineuzumab and 20C2 gives comparable patterns.

Solanezumab staining is characterized by an increased background, diffuse staining pattern. In addition, plaques are much smaller in comparison to stained plaques with bapineuzumab and 20C2. B) Plaque load was assessed by measuring the density in the brain at three different ages. While initially, at the age of 9 months, bapineuzumab and 20C2 showed a significantly increased plaque density compared to solanezumab ($p<0.001$). Plaque density as measured with solanezumab remained constant over time.

C) Staining with solanezumab shows significant more detection of small-sized plaques than staining with bapineuzumab or 20C2 ($p<0.01$). In addition, while bapineuzumab and 20C2 show an increase in amount of plaques over time, this was not the case with solanezumab. D) Almost no medium sized plaques are labeled with solanezumab. Bapineuzumab and 20C2 show an age-related increase in medium-sized plaque numbers. No differences were present between bapineuzumab and 20C2. E) Almost no large sized plaques are labeled with solanezumab. Bapineuzumab and 20C2 show an age-related increase, and 20C2 showed a trend to label larger sized plaques at 9 months of age ($p=0.065$). (n=2-7 per group, error bars denote SEM).

5.3.1. SOLUBLE A β DOES NOT INCREASE IN BRAIN OVER TIME

Three anti-A β antibodies, targeting soluble A β , oligomers, and fibrils, were compared *in vivo* and *ex vivo* on their binding properties in the 5xFAD mouse brain at different ages. First, plaque load and size was assessed at the ages of 3, 6, and 9 months. An immunohistochemical staining was performed using solanezumab (soluble A β), 20C2 (A β oligomers), and bapineuzumab (A β fibrils). Qualitative comparison showed that staining with solanezumab gave a very diffuse staining, and only lightly labeled plaques (see Figure 5.1A). Plaques looked also much smaller when compared to the other antibodies. 20C2 and bapineuzumab gave a very comparable staining pattern. All three antibodies showed the most abundant plaque load in the subiculum and the cortex.

Quantitatively, we could detect an age-related increase in plaque load with bapineuzumab and 20C2. Solanezumab was different in this regard, in that it showed no increase at all ($p < 0.01$). The difference between these three antibodies became further apparent when we compared the amount of plaques that they detected at the different ages. Solanezumab detected mostly small plaques ($< 150 \mu\text{m}^2$), and the amount of plaques detected remained constant over time ($p = 0.847$). In addition, solanezumab detected very few to none medium sized ($150\text{--}1000 \mu\text{m}^2$) and large plaques ($> 1000 \mu\text{m}^2$) (see Figure 5.1). No significant differences were found between 20C2 and bapineuzumab on plaque load or plaque sizes (see Figure 5.1), although 20C2 did show a trend to label more large-sized plaques than bapineuzumab ($p = 0.065$).

5.3.2. ONLY ^{89}Zr -BAPINEUZUMAB ALLOWS FOR *IN VIVO* DIAGNOSIS

To further compare the binding differences between these antibodies *in vivo* experiments were performed. Previously, we have shown that immuno-PET is a suitable technique for investigating antibody binding *in vivo* [14]. For immuno-PET experiments, antibodies were first labeled with ^{89}Zr . Next, antibodies were administered to 5xFAD animals and WT controls at different ages. Five days after administration, *in vivo* PET scans were performed followed by biodistribution.

As previously reported, quantification of PET scans showed that there was increased ^{89}Zr -bapineuzumab uptake in 5xFAD animals at all ages ($p < 0.01$), but uptake did not increase as animals aged. In contrast to ^{89}Zr -bapineuzumab uptake, retention of ^{89}Zr -solanezumab ($p = 0.99$) or ^{89}Zr -20C2 ($p = 0.39$) was not increased in 5xFAD animals compared to WT animals at any age (see Figure 5.2A). Uptake of ^{89}Zr -20C2 in both 5xFAD and WT animals was comparable to ^{89}Zr -bapineuzumab uptake in WT animals. ^{89}Zr -solanezumab uptake however was significantly lower in both WT and 5xFAD animals at every age when compared to the other two antibodies ($p < 0.01$).

Figure 5.2: A) Animals were scanned five days after antibody administration. Increased uptake of ^{89}Zr -bapineuzumab could be detected at all ages in 5xFAD animals. Both ^{89}Zr -20C2 and ^{89}Zr -solanezumab did not show any increased accumulation in 5xFAD animals compared to WT animals. B) Biodistribution analysis of total brain showed an age-related accumulation of ^{89}Zr -bapineuzumab at all ages in 5xFAD animals. ^{89}Zr -20C2 also showed an age-related increase, but difference with WT animals only became apparent at the age of 6 months. ^{89}Zr -solanezumab accumulated very little in the brain. C) ^{89}Zr -bapineuzumab and ^{89}Zr -solanezumab showed similar patterns in the cortex as in the total brain. However, at 3 months of age, ^{89}Zr -20C2 uptake was reduced in 5xFAD animals compared to WT animals. D) Hind brain results of all antibodies showed the same pattern as total brain accumulation, although the difference between WT and 5xFAD animals was bigger. E) In the hippocampus only ^{89}Zr -20C2 showed increased accumulation in 5xFAD animals, and this only at the late age of 9 months. (n=2-7 per group, error bars denote SEM).

5.3.3. BIODISTRIBUTION SHOWS AGE-DEPENDENT DIFFERENCE IN DISTRIBUTION

After *in vivo* μPET scans, the brain was dissected and measured in a gamma-counter. These results confirmed that ^{89}Zr -solanezumab does not accumulate in the brain, since radioactivity was on average five-fold lower compared to ^{89}Zr -20C2 and ^{89}Zr -bapineuzumab in both WT and 5xFAD animals ($p < 0.01$) (see Figure 5.2B).

^{89}Zr -bapineuzumab uptake was increased in total brain in 5xFAD animals at the early age of 3 months ($p = 0.03$) and at later ages ($p < 0.01$). At 3 months of age, there was no difference in ^{89}Zr -20C2 uptake in WT and 5xFAD animals ($p = 0.25$). However, at 6 months of age, more ^{89}Zr -20C2 antibody was present in the total brain of 5xFAD ($p = 0.04$) and this increase was also present at 9 months of age ($p < 0.01$) (see Figure 5.2B). ^{89}Zr -solanezumab did not show any specific accumulation at any age ($p = 0.73$).

In the hind brain the uptake pattern was similar to that in total brain (see Figure 5.2D). ^{89}Zr -bapineuzumab uptake was significantly higher at all ages in 5xFAD animals ($p < 0.01$), while for ^{89}Zr -20C2 only at 6 and 9 months of age an increased uptake in 5xFAD animals was present ($p = 0.02$ and $p = 0.04$ respectively). There was no difference in uptake of ^{89}Zr -solanezumab in any of the animals or at the ages.

Unexpectedly, ^{89}Zr -20C2 uptake in the cortex was lower at 3 months of age in 5xFAD animals compared to WT animals ($p = 0.04$). At 6 and 9 months of age, the difference was

reversed, with a higher ^{89}Zr -20C2 uptake in 5xFAD animals than in WT animals ($p=0.02$ and $p<0.01$ respectively). ^{89}Zr -solanezumab showed no difference in uptake at any age. ^{89}Zr -bapineuzumab meanwhile, showed significant increased uptake at all ages ($p<0.01$) as reported previously.

In the hippocampus, only ^{89}Zr -20C2 showed a significant increased accumulation in 5xFAD animals, and this only at 9 months of age ($p=0.01$) (see Figure 5.2E). Both ^{89}Zr -solanezumab and ^{89}Zr -bapineuzumab showed no significant accumulation in the hippocampus of 5xFAD animals at any of the ages, but there was trend for ^{89}Zr -bapineuzumab accumulation in the hippocampus of 5xFAD animals at 6 and 9 months of age ($p=0.06$ in both cases).

Figure 5.3: Biodistribution analysis of other organs revealed significant, specific antibody uptake in a number of organs. A) In cervical lymphnodes, increased ^{89}Zr -bapineuzumab accumulation was found in 5xFAD animals at all ages. Other antibodies did not show this accumulation. B) In visceral adipose tissue, we found a significant increase in ^{89}Zr -solanezumab in 5xFAD animals. No increased accumulation of other antibodies was present. C) ^{89}Zr -20C2 shows increased clearance from blood in 5xFAD animals at 3 months of age. At other ages, no difference in clearance was present between genotypes. Other antibodies also did not show any AD specific differences in clearance at any age. ($n=2-7$ per group, error bars denote SEM).

5.3.4. ^{89}Zr -SOLANEZUMAB SHOWS INCREASED UPTAKE IN ADIPOSE TISSUE

Biodistribution analysis also yielded important information about distributional differences between the three different antibodies. As previously reported, ^{89}Zr -bapineuzumab showed increased uptake in cervical lymphnodes of 5xFAD animals ($p<0.01$), while this accumulation was absent with ^{89}Zr -solanezumab and ^{89}Zr -20C2 (see Figure 5.3A).

Surprisingly, there was increased accumulation of ^{89}Zr -solanezumab in abdominal adipose tissue in 5xFAD animals at all ages ($p=0.04$). ^{89}Zr -20C2 and ^{89}Zr -bapineuzumab did not show accumulation in this tissue, suggesting that only monomeric $\text{A}\beta$ accumulates in adipose tissue (see Figure 5.2B). In addition, there was a strong correlation ($r=0.70$) between ^{89}Zr -solanezumab uptake in adipose tissue and $\text{A}\beta$ load in the brain ($p=0.01$).

Biodistribution analysis revealed that ^{89}Zr -20C2 was cleared from the blood faster in 3 months old 5xFAD animals compared to WT animals ($p<0.01$). At later ages, this difference in clearance between genotypes was not present. Furthermore, the other two antibodies did not show a significant difference in antibody clearance between

genotypes (see Figure 5.3C). These results correlated ($r=0.66$) with the results found in the cortex for ^{89}Zr -20C2 ($p<0.01$), which showed lower cortical accumulation in 5xFAD mice at 3 months of age. In ^{89}Zr -20C2 treated 5xFAD animals, total activity was also lower at 3 months of age compared to WT animals, showing that the antibody actually gets eliminated from the body ($p<0.01$). At 9 months of age, this pattern was reversed, with increased total accumulation of ^{89}Zr -20C2 in 5xFAD animals ($p=0.01$) (see Figure 5.4).

Figure 5.4: Overview of biodistribution data from all analyzed organs. The increased clearance of ^{89}Zr -20C2 in 3 months old 5xFAD animals also affected radioactivity levels in other organs such as the bulbus, head (without eyes and brain), heart, limbs, lungs, trunk and total body radioactivity. In addition, a significant increase of ^{89}Zr -bapineuzumab was present in the bulbus of 9 months old 5xFAD animals. (n=2-7 per group, error bars denote SEM).

5.4. DISCUSSION

This study compares the *in vivo* and *ex vivo* binding properties of three different antibodies targeting different A β conformational forms (monomers, oligomers, fibrils) using immuno-PET. First, our *ex vivo* results showed that monomeric A β load does not increase as animals age. In contrast, oligomers and fibrillary A β (plaques), did show an age-dependent increase in both plaque load and in the amount of plaques of different size.

In vivo analysis using immuno-PET showed that only ^{89}Zr -bapineuzumab accumulates in the brain at all ages. When measuring radioactivity in the different brain regions using biodistribution, which has a better signal-to-noise ratio, we found that also ^{89}Zr -20C2 (oligomers) showed an age-related accumulation in 5xFAD animals at 6 and 9 months of age.

Surprisingly, there was less ^{89}Zr -20C2 present in the cortex in 3 months old 5xFAD animals compared to WT animal. Furthermore, there was also less ^{89}Zr -20C2 present in blood and in the total animal, showing that ^{89}Zr -20C2 was eliminated at a higher rate at this young age in 5xFAD animals. A possible explanation for these results is that while oligomers are still soluble at a young age, ^{89}Zr -20C2 can actually remove them from a brain. At later ages, oligomers might get converted to insoluble forms and incorporated in A β plaques, which is also supported by the *ex vivo* plaque load analysis.

In accordance with the *ex vivo* A β quantification, we did not find any age-related increase of ^{89}Zr -solanezumab in 5xFAD animals. However, there was also no increased binding compared to WT animals. The fact that ^{89}Zr -solanezumab did not show any accumulation in the 5xFAD brain at any age, confirms previous reports that solanezumab does not accumulate in the brain, but rather acts as a peripheral A β sink [12].

Interestingly, there was specific uptake of [^{89}Zr]-solanezumab in visceral adipose tissue in 5xFAD mice. While it has been reported that solanezumab can also bind non-specifically to serum proteins [27], we did not find this increase in WT animals, showing that this accumulation was AD specific. In the 5xFAD animal model, APP is expressed under the Thy1 promoter [13]. A possible explanation for this accumulation could be that this promoter is also active in adipose tissue. However, research on Thy1-YFP reporter mice shows that the Thy1 promoter is not active in adipose tissue [28]. In addition, we also found a strong correlation with monomeric plaque load in the brain. This accumulation warrants further investigation, since there is a strong link between metabolic syndrome and AD [29]. Furthermore, this insight might provide some clue as to possible therapeutic mechanisms of solanezumab.

These results also provide a hint regarding the working mechanisms of immunotherapy for AD. From the human clinical trials, we know that bapineuzumab did not show any beneficial effect [5], while solanezumab showed a small beneficial effect, but only in patients with mild AD [10]. This study proves that both antibodies work in completely different ways, with solanezumab not accumulating in the brain, while bapineuzumab does accumulate, and already at a young age. It would therefore be interesting to test ^{89}Zr -20C2 in an intervention study and see whether it works in a prevention or therapeutic regimen, since the binding properties of this antibody change as animals age.

One limitation of this study is that these antibodies were only compared in one animal model. While the 5xFAD animal model is extensively used, several other animal models are available which might provide different insights into plaque formation and age-related deposition [30].

In conclusion, we show the binding properties of different A β antibodies *in vivo*. By making use of ^{89}Zr -labeled antibodies, we were able to show that oligomers get removed by anti-amyloid administration only at a young age. In addition, we show accumulation of A β monomers in adipose tissue, a finding which warrants further investigation.

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6

DISCUSSION

This thesis investigates the potential use of amyloid- β ($A\beta$) antibodies for both the treatment and possible diagnosis of Alzheimer's disease (AD). While enormous research efforts are directed towards a cure for AD, no disease modifying treatment has been achieved in the last 15 years. The most important hallmarks of AD are $A\beta$ plaques and tau tangles [1]. We investigated the role of anti- $A\beta$ antibodies in AD as possible therapies, to learn more about antibody effector mechanisms and their potential for *in vivo* diagnostics in the CNS.

6.1. SUMMARY OF KEY FINDINGS

Chapter 2 presents an up-to-date literature review about the role of neuroinflammation in AD and the current state of the art of anti- $A\beta$ immunotherapy. In the last years, neuroinflammation has emerged as one of the key players of AD pathology [2]. While neuroinflammation is a complex interplay of several factors, the two most important factors in AD are microglia and complement. Besides the microglial changes observed in patients, the microglial association with AD has become prominent with the emergence of CD33 and TREM2 as risk factors for AD [3]. In addition to microglia, the complement system plays an important role in AD [4]. Genetic evidence points to Complement Receptor 1 (CR1) playing a crucial role in AD development, while evidence from patients and mouse models points to a further important role of complement factors and the inflammasome in AD pathogenesis.

Chapter 2 also further elaborates on the past and current immunotherapy efforts targeting $A\beta$ as a possible treatment for AD. From animal studies, several possible effector mechanisms have been proposed and investigated, although our literature search showed that there is a lack of attention to the role that complement might play in immunotherapy. In human clinical trials, we find that already 25 different immunotherapies have been or are being tried in over 25 000 patients. So far most therapies have ended with negative results, although some results suggest that immunotherapy might hold a benefit in the early disease stages [5].

In **Chapter 3**, we investigated the effector mechanisms of $A\beta$ immunotherapy in a therapeutic and preventive fashion in the 5xFAD mouse model. To this end, an anti-inflammatory (a modified mouse IgG1) and an inflammatory (mouse IgG2a) anti- $A\beta$ antibody (3D6) were administered to 5xFAD mice before (preventive) and after (therapeutic) the onset of behavioral symptoms and overt plaque pathology. Surprisingly, we found that antibody administration resulted in IgG mediated passive systemic anaphylaxis (PSA) caused by generation of anti-therapeutic antibodies (ATA). Preventive treatment was successful in reducing anxiety-like effects in 5xFAD animals. Our results also showed that mIgG 3D6 worsens memory problems in the contextual fear-conditioning test.

On a molecular level, we discovered that preventive treatment resulted in a stronger reduction on plaque load than therapeutic treatment. We also investigated the effects of immunotherapy on the different $A\beta$ fractions in brain homogenates (water soluble, detergent soluble and formic acid soluble $A\beta$). While therapeutic treatment lowered $A\beta$ in the detergent soluble fraction, preventive treatment on the other hand increased $A\beta$ in the detergent soluble fraction, caused by a shift from the formic acid soluble fraction to the detergent soluble fraction. No effects on microgliosis were observed, but our

data shows that treatment with inflammatory antibodies (mIgG2a) significantly reduced astrogliosis. These results suggest an important role for astrocytes in the mediation of neuroinflammatory processes.

Given the fact that mIgG1 3D6 (anti-inflammatory) induced an isotype specific worsening of memory, while mIgG2a 3D6 (inflammatory) reduced astrogliosis, it is likely that isotype specific effects on inflammation need to be taken into account when designing immunotherapies for AD. Furthermore, our results suggest that a more pro-inflammatory therapy might be more beneficial for AD treatment.

Chapter 4 shows a proof-of-concept for the use of A β antibodies for *in vivo* imaging of amyloid. In this experiment, an A β specific antibody (bapineuzumab) was radiolabeled with a long-lived positron emitter (^{89}Zr) and compared with an isotype control (mAb 637) in the 5xFAD mouse model at several disease stages. This experiment showed that specific *in vivo* accumulation of antibodies can be detected using PET imaging. In addition, biodistribution experiments proved an age-related increase of ^{89}Zr -bapineuzumab uptake in the brain. To further prove specificity, we could show that competition with unlabeled bapineuzumab reduced ^{89}Zr -bapineuzumab uptake to background levels. No uptake of the isotype control could be found. Specific accumulation of administered antibody was further proved by autoradiography and by colocalisation of administered ^{89}Zr -bapineuzumab with plaques in the brain.

The previous experiment formed the basis for a follow-up study comparing different A β antibodies in **Chapter 5**. Using again 5xFAD mice of different ages, we now compared the *in vivo* binding properties of three A β antibodies targeting monomers (solanezumab), oligomers (20C2), and plaques (bapineuzumab). To our surprise, only ^{89}Zr -bapineuzumab allows for *in vivo* detection of plaques. With ^{89}Zr -solanezumab and ^{89}Zr -20C2 no increased *in vivo* binding could be found.

Biodistribution showed, as previously published, that ^{89}Zr -solanezumab does not seem to enter or remain in the brain in large concentrations. However, at 6 months of age, there was a significant accumulation of ^{89}Zr -20C2 in 5xFAD animals, which binds oligomers, in the brain. More interestingly, we found that at 3 months of age, there is a reduction of ^{89}Zr -20C2 binding in the cortex of 5xFAD animals compared to WT animals, a situation that was reversed at the later ages of 6 and 9 months. Analysis of antibody concentration in the blood also shows that there ^{89}Zr -20C2 is cleared more rapidly at 3 months of age in 5xFAD animals than in other conditions, a situation we did not observe with the other antibodies.

While ^{89}Zr -solanezumab was cleared very rapidly from the animals (resulting in low concentrations in both 5xFAD and WT animals), we did find a specific accumulation of ^{89}Zr -solanezumab in adipose tissue. Since only ^{89}Zr -solanezumab binding was increased in adipose tissue in 5xFAD animals, it is likely that monomeric, soluble A β accumulates in fat deposits in the body.

Finally, we also showed that ^{89}Zr -bapineuzumab accumulated in cervical lymphnodes of 5xFAD animals. This might be due to draining amyloid present in these lymphnodes, although it is more likely that there is an antibody specific mechanism, since the other antibodies fail to show any accumulation in the lymphnodes.

6.2. LIMITATIONS

While this thesis delivers significant contributions to the understanding of AD, and more specifically to the use of A β antibodies in treatment and diagnosis, there are also some limitations to our studies. One significant limitation that all our studies share is the use of the same AD mouse model. Because AD is such a complex disease, animal models are widely used to encompass as much as possible of the different disease processes when investigating drugs. While spontaneously models such as certain species of dogs, cats, or nonhuman primates exist, the vast majority of the current disease models are either pharmacologically induced or based on transgenic rodents [6].

With regards to the mouse model used in our study (5xFAD), some remarks about possible translatability have to be made. First, in contrast to the majority of AD patients, 5xFAD mice start to develop AD pathology at a very young age (2-3 months) with behavioral deficits showing at about 5-6 months of age, which would translate to a human in its early thirties [7]. At this young age, results about the immune system in AD have to be carefully interpreted, since the immune system at old age is less active than at young age, because of a process called immuno-senescence [8].

In addition, as most transgenic AD mouse models, the 5xFAD model overexpresses human APP and PSEN1. A comparative study showed that they express about 6-fold more APP than humans, while the numerous mutations in APP also cause an increased A β ₄₂/A β ₄₀ ratio [9]. This increased A β in the brain of 5xFAD mice compared to humans might limit the translation of our immuno-PET imaging results, since less A β will be available for the antibodies to bind to in humans (**Chapter 4 and Chapter 5**). On the other hand, PET imaging in humans is more sensitive than in mice and likely to result in a better signal-to-noise ratio, potentially offsetting the disadvantage lower amyloid load in the human brain [10]. Furthermore, the fact that we can also detect *in vivo* differences at a very early age in the 5xFAD mice, suggest that it should be possible to translate this technique to clinic.

Another limitation of Chapter 4 and Chapter 5 is the fact that no comparison could be made to existing amyloid PET tracers. Several amyloid PET tracers are currently being used in patients [11]. To get a good comparison about the sensitivity of immuno-PET imaging with existing amyloid PET tracers, one or more of these tracers would have preferentially been used in our animal model to enable a direct comparison.

Another limitation related to the use of the 5xFAD animal model relates to the unstable behavioral phenotype. While initial pilot studies showed a very strong phenotype (unpublished data), this could not be replicated in subsequent studies in immunotherapy (**Chapter 3**), resulting in a failure to find a difference between WT and untreated 5xFAD mice. A possible explanation for this might be the difference in breeding of our cohort compared to the original description of the 5xFAD model, where offspring is generated by breeding 5xFAD mice with B6/SJL F1 mice [7]. We on the other hand bred 5xFAD mice with B6/SJL F3 mice. In addition, the Pde6b gene, which can cause retinal degeneration, was bred out of our colony.

While investigating the effects of A β immunotherapy using recombinant produced antibodies (**Chapter 3**), 5xFAD animals developed anti-treatment antibodies (ATA), resulting in IgG mediated passive anaphylactic shock. The development of ATA could have affected our results, because therapeutic antibodies could be neutralized and not

reach the brain. In addition, systemic inflammatory reaction caused by the anaphylactic shock, could also have influenced the inflammatory processes in the brain.

6.3. PERSPECTIVES

This thesis provides important insights into the use of antibodies for the treatment and diagnosis of AD. Contrary to our expectations, results show that a more pro-inflammatory antibody might be more beneficial than an anti-inflammatory antibody, despite a stronger reduction in plaque load. The antibodies solanezumab and crenezumab both bind the same A β epitope, while solanezumab has an inflammatory isotype (human IgG1) and crenezumab has an anti-inflammatory isotype (human IgG4). At the moment, solanezumab has completed phase 3 clinical trials with overall negative results, but possibly showing a small effect in patients with mild AD [12]. Interim reports of a phase 2 trial of crenezumab have likewise delivered disappointing reports [13]. One difference with our pre-clinical experiment is that these antibodies target soluble, monomeric A β .

Finally, our immuno-PET studies offer an interesting tool to investigate A β antibody distribution *in vivo*. PET imaging using ^{89}Zr -labeled antibodies are routinely used in humans and several A β antibodies have already been tested in clinical trials in humans. It should therefore be only a relatively small step to conduct human trials using ^{89}Zr -labeled A β antibodies. This might yield important new information about disease progression and immunotherapy for AD. In addition, this technique could maybe also be used to image other proteinopathies such as Parkinson's disease.

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7

VALORISATION

Alzheimer's disease (AD) is the leading cause of dementia worldwide. Over 46 million people suffer from dementia, with AD accounting for 60-80% of the cases and this number is expected to double every twenty years [1]. This means that about every 3 seconds, a new case of dementia will be diagnosed worldwide. By 2050, over 130 million people are expected to suffer from dementia, of which around 90 million will have AD.

The societal burden of AD is especially high, since not only the patient who has the dementia is affected, but also family and friends, and society as a whole by providing care and loss of productivity. In general, dementia patients require more intensive care than patients from other diseases, and the costs for dementia are higher than the combined costs for cancer, heart disease and stroke [2]. In addition, cognitive impairment, a symptom of dementia, is the strongest prediction for transition of elderly people to a care home [3].

In terms of economic value, the global cost of dementia exceeds 800 billion US\$, which accounts for over 1% of global gross domestic product (GDP). A minority of these costs (20%) is attributable to direct medical care costs, possible because very few treatment options are available [1]. Direct social care (home, nursing) and informal care costs each attribute 40% to the costs. Informal care costs are costs made by unpaid informal care givers (such as relatives) who are for example unable to work during this time.

Despite the fact that the societal cost of AD is substantial higher than those of stroke, heart disease and cancer, research funding into AD is less than in these disease [4]. It is therefore imperative that both fundamental and applied research funding is increased to prevent an economical disaster in the coming decades. The problem is further aggravated by the fact that there is no cure for the disease available. In addition, the exact disease process remains vague and also diagnosis of AD is not straightforward [5].

The thesis contains both a therapeutic and a diagnostic component that offers direct possibilities for valorization. First we will discuss possible valorization options from a therapeutic point of view, while subsequently we will discuss diagnostic options suggested in this thesis.

7.1. THERAPEUTIC OPPORTUNITIES

At the moment, 5 different drugs are approved for AD. Every year, about 1 billion dollars are spent on these drugs in the USA alone [6]. While these drugs provide some symptomatic relief for patients, they do not tackle the underlying cause of AD, and as such, they lose their effectiveness over time.

In this thesis, immunotherapy against amyloid was investigated as a possible treatment option. Anti-A β treatment has been tested in over 25 000 patients in various clinical trials (Chapter 2). Although cost estimates are not available, these trials are likely to represent a huge investment on the part of pharmaceutical companies. Furthermore, there is a large societal costs and effort required both on the patient and caregiver side. Knowledge obtained in this thesis helps to improve future immunotherapy trials and possible increase the success rate of anti-A β strategies, if these strategies are further pursued in the future.

Despite the fact that immunotherapy trials have failed so far, we show that preventive treatment might be a viable option for the treatment of AD. One key question remains

of course whether it will be possible to identify patients early enough. A big problem is that amyloid pathology starts about 15-20 years before the onset of cognitive problems [7]. It remains thus to be seen how it would be possible to select patients for possible treatment, or if treatment starting in the state of Mild Cognitive Impairment (MCI) is sufficient to obtain a disease modifying effect.

Our results show that there might be valorization opportunities in custom antibody formats and effector mechanisms. In this thesis, an antibody with full effector mechanisms (Fc γ -receptor binding and complement activation) was compared with an antibody with no effector mechanisms. A possible valorization opportunity would be to investigate antibodies with partial effector mechanisms (eg. only complement activation or only Fc γ -receptor binding) and see which mechanisms is the most important for an efficient and safe therapy. This specific modification could then be patented. Additionally, it might also be worthwhile to investigate different, more exotic antibody formats, such as bispecific antibodies to increase brain penetration and increase the effective dose in the brain.

7.2. DIAGNOSTIC OPPORTUNITIES

While AD can only be diagnosed with certainty post-mortem, a number of options are currently available for early diagnosis of the disease. As discussed in Chapter 1 of these thesis, diagnostic tools currently in common use are cognitive tests and CSF measurements. A β and tau measurements in CSF are now routinely being used as part of the neurological examination in patients with cognitive impairments. However, besides these measures, also imaging biomarkers are being routinely used. One advantage over imaging techniques is that they also provide additional spatial information as to where pathology is spreading and about the severity of the pathology. While structural Magnetic Resonance Imaging (MRI) is routinely used for diagnosis, the use of Positron Emission Tomography (PET) to image amyloid is now finding routine use.

One of the disadvantages of current amyloid PET tracers is that they are only able to image fibrillar A β deposits (plaques). However, recent experimental data suggests that it is actually the oligomeric A β species which are more toxic. It would therefore be of great diagnostic and scientific value if different A β species could be imaged. This thesis proves in pre-clinical studies that this might be feasible.

The use of monoclonal antibodies radiolabeled with ^{89}Zr is routinely applied in the clinic for imaging of tumors [8]. Despite the fact that radiation dose is always a concern with PET imaging, the technology has proven safe in humans. We, and very recently also others, have shown that antibodies can be used for imaging amyloid in the brain *in vivo* [9, 10]. This opens opportunities to try this technology in humans. Since both the use of ^{89}Zr and some of the antibodies we tested have already passed clinical trials, and proven safe, amyloid immuno-PET could be tried very fast in humans. This could provide valuable new insights in amyloid pathology in patients. *In vivo* imaging using this technique in mice suffers from several drawbacks, such as the high bone uptake of ^{89}Zr [11]. In humans, this problem would not occur, and the technique would therefore benefit from a higher signal-to-noise ratio.

Besides applications for diagnosis and fundamental science, immuno-PET also offers the possibility to use monoclonal antibodies as a theranostic tool. Since several

antibodies are still in clinical trial, this would provide a valuable addition to current clinical trials. A theranostic is a drug that is a diagnostic tool and a therapeutic drug at the same time. By using the same antibody that is used for therapy for imaging pathology, target engagement can be shown on an individual patient basis. This will allow to screen patients before inclusion in clinical trials, possibly increasing success of the trial and preventing people with non-AD dementia from being included in the trial. Furthermore, therapy effects could be monitored over time.

Finally, the fact that immuno-PET can be used for $A\beta$ imaging, as we show in this thesis, might also mean that immuno-PET can be used to target other proteins in the CNS. Applications for immuno-PET might therefore include also other proteinopathies such as Parkinson's disease, for which no tracers are available for the moment. Monoclonal antibodies against α -synuclein could be radiolabeled and used to confirm Parkinson's disease. Other possible disease could be for instance Prion disease, but also in AD there are still possible targets left. Besides $A\beta$, also tau might prove a valuable target to image with immuno-PET, and currently there are only few tau tracers available, and these are still in early development. A possible caveat is that tau is intracellular, and it remains an open question whether antibodies will be able to bind then. However, results from a study using tau antibodies for *in vivo* imaging system (IVIS) imaging, shows that this might not be such a big issue [12].

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8

SUMMARY

This thesis investigates the use of antibodies for the treatment and diagnosis of Alzheimer's disease (AD).

Chapter 1 gives a short historical perspective on the etiology and clinical features of AD, while also providing an overview of the current diagnostic options that are now available.

In **Chapter 2** we present an up-to-date literature review about the role of neuroinflammation in AD and the current state of the art of anti-A β immunotherapy. We describe the interplay between microglia and complement, which together lead to an altered inflammatory status in AD, and how they might play a role in the disease process. Next, we describe past and foreseeable future therapies making use of both passive and active immunization. Potential effector mechanisms are discussed summarizing findings from pre-clinical and clinical studies. A complete overview of all completed and ongoing human clinical trials is presented, showing that despite more than 25 000 participating AD patients, still no therapy has reached clinical practice. Currently, most therapies are refocusing their target group to patients in the early stages of the disease, with the aim of preventing disease progression.

In **Chapter 3**, we investigated the effector mechanisms of A β immunotherapy in a therapeutic and preventive fashion in the 5xFAD mouse model. To this end, an anti-inflammatory (a modified mouse IgG1) and an inflammatory (mouse IgG2a) anti-A β antibody (3D6) were administered to 5xFAD mice, either before (preventive treatment) or after (therapeutic treatment) the onset of behavioral symptoms and overt plaque pathology. Surprisingly, we found that antibody administration results in IgG mediated passive systemic anaphylaxis (PSA) caused by generation of anti-therapeutic antibodies (ATA). Preventive treatment was successful in reducing anxiety-like effects in 5xFAD animals. Our results also showed that mIgG 3D6 worsened memory problems in the contextual fear-conditioning test.

On a molecular level, we discovered that preventive treatment resulted in a stronger reduction on plaque load than therapeutic treatment. We also investigated the effects of immunotherapy on the different A β fractions in brain homogenates (water soluble, detergent soluble and formic acid soluble A β). While therapeutic treatment lowered A β in the detergent soluble fraction, preventive treatment on the other hand increased A β in the detergent soluble fraction, caused by a shift from the formic acid soluble fraction to the detergent soluble fraction. No effects on microgliosis were observed, but our data showed that treatment with inflammatory antibodies (mIgG2a) significantly reduced astrogliosis. These results suggest an important role for astrocytes in the mediation of neuroinflammatory processes.

Given the observation that mIgG1 3D6 (anti-inflammatory) induced an isotype specific worsening of memory, while mIgG2a 3D6 (inflammatory) reduced astrogliosis, we suggest that isotype specific effects on inflammation need to be taken into account when designing immunotherapies for AD. Furthermore, our results suggest that a more pro-inflammatory therapy might be more beneficial for AD treatment.

Chapter 4 shows a proof-of-concept for the use of A β antibodies for *in vivo* imaging of amyloid. For these experiments, an A β specific antibody (bapineuzumab) was radiolabeled with a long-lived positron emitter (^{89}Zr) and compared with an isotype control (mAb 637) in the 5xFAD mouse model at several disease stages. The results

showed that specific *in vivo* accumulation of antibodies can be detected using PET imaging. In addition, biodistribution experiments proved an age-related increase of ^{89}Zr -bapineuzumab uptake in the brain. To further prove specificity, we could show that competition with unlabeled bapineuzumab reduced ^{89}Zr -bapineuzumab uptake in the brain to background levels. No uptake of the isotype control could be found. Specific accumulation of administered antibody was further demonstrated by autoradiography and by colocalisation of administered ^{89}Zr -bapineuzumab with plaques in the brain.

In **Chapter 5** we compared different antibodies for their potential to use for *in vivo* PET imaging. Using 5xFAD mice of different ages, we now compared the *in vivo* binding properties of three A β antibodies targeting monomers (solanezumab), oligomers (20C2), and plaques (bapineuzumab). Only ^{89}Zr -bapineuzumab allowed for *in vivo* detection of plaques. With ^{89}Zr -solanezumab and ^{89}Zr -20C2 no increased *in vivo* binding could be found. Biodistribution revealed that, as previously published, ^{89}Zr -solanezumab does not seem to enter or remain in the brain in large concentrations. However, at 6 months of age, there was a significant accumulation of ^{89}Zr -20C2 in 5xFAD animals, which binds oligomers, in the brain. More interestingly, we found that at 3 months of age, there is a reduction of ^{89}Zr -20C2 binding in the cortex of 5xFAD animals compared to WT animals, a situation that is reversed at the later ages of 6 and 9 months. Analysis of antibody concentration in the blood also showed that there ^{89}Zr -20C2 was cleared more rapidly at 3 months of age in 5xFAD animals than in other conditions, a situation we did not observe with the other antibodies.

While ^{89}Zr -solanezumab was cleared very rapidly from the animals (resulting in low concentrations in both 5xFAD and WT animals), we did find a specific accumulation of ^{89}Zr -solanezumab in adipose tissue. Since only ^{89}Zr -solanezumab binding is increased in adipose tissue in 5xFAD animals, it is likely that monomeric, soluble A β accumulates in fat deposits in the body.

Finally, we also showed that ^{89}Zr -bapineuzumab accumulated in cervical lymphnodes of 5xFAD animals. This might be due to draining amyloid present in these lymphnodes, although it is more likely that there is an antibody specific mechanism, since the other antibodies did not show any accumulation in the lymphnodes.

In this work I present several advances using antibodies for both the diagnostic and therapy for AD. From a therapeutic point of view, I discovered that the effector mechanisms of an antibody need to be carefully considered to achieve a therapeutic effect. Furthermore, I showed that earlier treatment might be more beneficial than late treatment. In subsequent work, I explored the use of antibodies for imaging. While better techniques are available to image general amyloid load in the brain, we showed that using immunoPET imaging, we can obtain valuable information about antibody behavior *in vivo* and potentially also learn more about the AD disease process.

9

SAMENVATTING

In deze thesis wordt het gebruik van antilichamen voor de behandeling en de diagnose van de ziekte van Alzheimer (AD) onderzocht.

In **Hoofdstuk 1** wordt een kort historisch perspectief geboden over de etiologie en de klinische symptomen van AD. Daarnaast wordt ook een overzicht gegeven van de huidige diagnostische technieken die ter beschikking staan.

In **Hoofdstuk 2** wordt aan de hand van een literatuuronderzoek de rol van neuroinflammatie in AD en de laatste stand van zaken met betrekking tot anti-amyloide immunotherapie beschreven. De interactie tussen microglia en het complement systeem, dat ervoor zorgt dat patiënten een verandering in de inflammatoire toestand ondergaan, werd uitgebreid beschreven. Daarnaast wordt dieper ingegaan op reeds ontwikkelde en toekomstige passieve en actieve immunotherapieën. Mogelijke werkingsmechanismen worden besproken aan de hand van pre-klinische en klinische studies. Verder wordt een volledig overzicht gegeven van alle klinische studies uitgevoerd bij patiënten tot nu toe. Hieruit blijkt dat ondanks het feit dat reeds meer dan 25 000 patiënten deelgenomen hebben aan deze studies, er nog steeds geen therapie voor algemeen gebruik beschikbaar is. Op dit moment verleggen veel therapieën de focus naar patiënten in een vroeg stadium van de ziekte.

In **Hoofdstuk 3** worden de resultaten beschreven van een onderzoek waarbij de werkingsmechanismen van A β immunotherapie in een acuut en preventieve manier onderzocht zijn in het 5xFAD muismodel. Hiervoor werden een anti-inflammatoire (een gewijzigd muis IgG1) en een pro-inflammatoir (muis IgG2a) anti-A β antilichaam (3D6) toegediend voor zowel een *preventieve* als ook een *curatieve* behandeling. Verassend genoeg vonden we dat toediening van antistof kan leiden tot anafylactische schok bij muizen, dat mogelijk veroorzaakt werd door het ontstaan van neutraliserende antistoffen. Preventieve behandeling was succesvol in het reduceren van angstig gedrag in 5xFAD muizen. Onze resultaten lieten ook zien dat geheugenproblemen erger werden in muizen behandeld met anti-inflammatoir antistof in de *fear conditioning* test. Op moleculair gebied, zagen we dat *preventieve* behandeling leidde tot een sterkere reductie van plaques dan de *curatieve* behandeling. We hebben ook het effect van immunotherapie op verschillende A β fracties onderzocht in breinhomogenaten. Hierbij zagen we dat, afhankelijk van de behandeling, er een verschuiving van A β plaatsvond. Daarnaast vonden we dat het inflammatoir antilichaam astroglisis sterk kon reduceren.

In **Hoofdstuk 4** lieten we zien dat het mogelijk was A β antilichamen te gebruiken voor *in vivo* beeldvorming. Hiervoor werd bapineuzumab gelabeld met een langlevende positron emitter (^{89}Zr) en vergeleken met een isotype controle in verschillende stadia bij de 5xFAD muis. Dit experiment liet zien dat *in vivo* accumulatie van antilichaam in het brein gedetecteerd kon worden met behulp van een PET scan. Daarnaast liet de biodistributie zien dat er een leeftijd gerelateerde verhoging van opname is ^{89}Zr -bapineuzumab. Dit was niet het geval bij de isotype controle. Specificiteit van de techniek werd verder aangetoond met autoradiografie en colocalisatie studies.

In **Hoofdstuk 5** werden drie verschillende A β antilichamen vergeleken met *in vivo* PET imaging. Deze drie antistoffen waren gericht tegen monomeren (solanezumab), oligomeren (20C2), en plaques (bapineuzumab). Alleen ^{89}Zr -bapineuzumab kon *in vivo* plaques detecteren. ^{89}Zr -solanezumab and ^{89}Zr -20C2 lieten geen specifieke brein opname *in vivo* zien. Zoals andere studies ook lieten zien kwam ^{89}Zr -solanezumab niet

in het brein terecht. ^{89}Zr -20C2 liet een verhoogde opname zien vanaf 6 maanden in AD dieren, maar verassend was er een verlaagd opname van ^{89}Zr -20C2 bij 3 maanden in AD dieren. Dit kan te wijten zijn aan het kortere half-leven van dit antilichaam bij 3 maanden. Interessant genoeg vonden we wel verhoogde concentraties van ^{89}Zr -solanezumab in vetweefsel bij AD muizen, wat suggereert dat monomeer $\text{A}\beta$ in vetweefsel accumuleert. Tenslotte lieten we ook zien dat ^{89}Zr -bapineuzumab in de lymfknoten van de 5xFAD muis accumuleerde, wat erop kan wijzen dat $\text{A}\beta$ van het brein hierin terecht komt.

Samenvattend presenteer ik verschillende nieuwe inzichten voor het gebruik van antistof in therapie en diagnose voor de ziekte van Alzheimer. Vanuit een therapeutisch perspectief, ontdekte ik dat de werkingsmechanismen van antilichamen beter onderzocht moeten worden om een therapeutisch effect te bereiken. Daarnaast liet ik zien dat een preventieve behandeling beter werkt dan een curatieve behandeling. Ook de mogelijke toepassing van antilichaam voor diagnose werden onderzocht. Hoewel het waarschijnlijk niet haalbaar is om deze techniek routinematig bij patiënten toe te passen, liet ik zien dat immunoPET nuttige inzichten kan opleveren over de werking van antistoffen in het lichaam en we zo ook meer kunnen leren over de ziekte van Alzheimer.

10

CURRICULUM VITAE

Jo Stevens was born on the 24th of May 1983 in Tongeren, Belgium. He obtained his high school degree in 2001 from the Onze-Lieve-Vrouwhumaniora in Tongeren. After this, he moved to Maastricht, Netherlands to study Knowledge Engineering. During this time he also was active in the faculty council, and followed a summer school at Baylor University, Waco, Texas. For his master thesis he developed a robotic goalkeeper under the supervision of Dr. Nico Roos. He obtained his Master degree from Maastricht University in 2006, with a major in Artificial Intelligence. Subsequently, he started studying Psychology and Molecular Life Sciences at Maastricht University in 2006. While studying these two programs, he was also active in the faculty council of the Faculty of Psychology and Neuroscience, and later as a board member from the same faculty. He also fulfilled various functions in the board of study association SV Helix. Meanwhile, he also worked as a software developer for UM Career Services from 2006 onwards. In 2008, he also received an appointment as lecturer at the Department of Knowledge Engineering for the teaching of the course Cognitive Psychology and Knowledge Presentation, which he taught for five years. In addition to this, he also served as a tutor for various courses such as programming and statistics at the Faculty of Psychology and Neuroscience. He received his Bachelor in Psychology in 2009. After this, he went on to study the Research Master Cognitive and Clinical Neuroscience, with a major in Fundamental Neuroscience in 2009. In 2009 he received an appointment as a research assistant at the School for Mental Health and Neuroscience. He was also active in the educational board of the Research Master. For his master thesis, he investigated the role of Dok7 in Myasthenia Gravis and the role of CERT in Alzheimer's disease. In 2011, he received a Bachelor in Molecular Life Sciences and a Master in Cognitive and Clinical Neuroscience. He subsequently got a job as a PhD student under the supervision of Prof. Dr. Marc de Baets, Dr. Mario Losen and Dr. Pilar Martinez-Martinez. During his PhD, he was also active as PhD representative for PhD students of Division 3 in the Department of Neuroscience. The topic of his PhD was the investigation of antibodies for the therapy and diagnostics of AD, the result of which is described in this thesis. He now work as a post-doc for Roche Diagnostics GmbH in München, Germany where he develops novel technologies for antibody generation.

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LIST OF PUBLICATIONS

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A. M. Gomez, **J.A.A. Stevens**, P. Molenaar, H. Duimel, F. Verheyen, J. Cossins, D. Beeson, M.H. De Baets, M. Losen, P. Martinez-Martinez, *Silencing of Dok-7 in adult rat muscle increases susceptibility to passive transfer myasthenia gravis*, American Journal of Pathology, In press.

P. Martinez-Martinez, P.C. Molenaar, M. Losen, C. Hoffmann, **J.A.A. Stevens**, L.D. de Witte, T. van Amelsvoort, J. van Os, B.P.F. Rutten, *Auto-immune disorders as a possible cause of neuropsychiatric syndromes*, Tijdschrift voor Psychiatrie, 2015. [Dutch]

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Martinez-Martinez P, Molenaar PC, Losen M, **Stevens JAA**, Baets MH, Szoke A, Honnorat J, Tamouza R, Leboyer M, Os JV, Rutten BP, *Autoantibodies to neurotransmitter receptors and ion channels: from neuromuscular to neuropsychiatric disorders*, Frontiers in Genetics, 2013

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J.A.A. Stevens, P. Martinez-Martinez, L. Dubois, D.J. Vugts, T.E. Nell, S.M. Crivelli, W.A. Buurman, P.C. Molenaar, B. Brans, P. Jelle Visser, M. de Baets, G.A. van Dongen, M. Losen. *In vivo PET imaging using an anti-amyloid antibody in an Alzheimer's disease model*.

J.A.A. Stevens, A. Franssen, S.M. Crivelli, M. de Baets, P. Martinez-Martinez, M. Losen. *Isotype dependent effector mechanisms of immunotherapy in Alzheimer's disease*.

J.A.A. Stevens, M. Losen, M. de Baets, P. Martinez-Martinez. *Inflammation and Alzheimer's disease: lessons from immunotherapy*.

I. Koneczny, **J.A.A. Stevens**, A. de Rosa, S. Huda, M. G. Huijbers, A. Saxena, M. Maestri, K. Lazaridis, P. Zisimopoulou, S. Tzartos, J. Verschuuren, S.M. van der Maarel, P. van Damme, A. Vincent, M.H. de Baets, P.C. Molenaar, R. Ricciardi, P. Martinez-Martinez, M. Losen. *IgG4 autoantibodies against muscle-specific kinase undergo Fab-arm exchange in myasthenia gravis patients*.

A. Saxena, **J.A.A. Stevens**, H. Cetin, I. Koneczny, R. Webster, K. Lazaridis, S. Tzartos, K. Vrolix, G. Nogales Gadea, P.C. Molenaar, J. Damoiseaux, M. H. De Baets, A. Marx, A. Vincent, M. Losen and P. Martinez-Martinez. *Characterization of an anti-fetal AChR monoclonal antibody isolated from a myasthenia gravis patient*.

IN PREPARATION

J.A.A. Stevens, P. Martinez-Martinez, L. Dubois, D.J. Vugts, T.E. Nell, S.M. Crivelli, W.A. Buurman, M. de Baets, G.A. van Dongen, M. Losen. *Anti-amyloid antibody*

characterization with immuno-PET.

C. Hoffmann, **J.A.A. Stevens**, M. Losen, P. Molenaar, M. de Baets, B. Rutten, J. van Os, P. Martinez-Martinez. *Analysis of auto-antibodies targeting the alpha7 acetylcholine receptor in schizophrenia.*

R.J.G. Gentier, **J.A.A. Stevens**, L.M. Verheijen, C.D. van 't Hekke, D. van den Hove, D.H.J.P. Hermes, H.W.M. Steinbusch, J.J. Cheng, M.O. Grimm, V. J. Haupenthal, W. Annaert, T. Hartmann and F.W. van Leeuwen, *Paradoxical effects of mutant ubiquitin and γ -secretase during modelled Alzheimer's disease neuropathogenesis.*

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