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Preclinical PET imaging and therapy of Alzheimer's disease

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Abstract

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The main histopathological hallmarks of Alzheimer's disease are extracellular amyloid-beta (A β) plaques and intracellular neurofibrillary tangles, containing tau protein. Because of misfolded and aggregated proteins, activated microglia and astroglia react with a neuroinflammatory response, which may contribute to disease progression and severity. To date, there is no treatment available that stops the underlying mechanisms of the disease, but several new drug candidates entered clinical trials every year during the last decade. New treatments, aiming to clear A β from the brain parenchyma or to reduce A β production, are dependent on diagnostic tools to follow changes in brain A β pathology *in vivo*. The presence of brain amyloid, verified with positron emission tomography (PET), is a regularly used criterion for enrolling patients in clinical trials. However, current amyloid radioligands such as [11C]Pittsburgh Compound B ([11C]PiB) have some disadvantages, e.g. early saturation during disease progression and reduced binding to diffuse A β pathology. Currently available radioligands for imaging of neuroinflammation are also suboptimal.

In this thesis, we investigated the potential of a brain-penetrating, bispecific A β antibody as a PET ligand to detect effects of treatment. In paper I and II, we demonstrated that this ligand can follow A β disease progression and that A β reduction due to treatment with a BACE-1 inhibitor can be quantified in a mouse model of AD. In paper II we also compared antibody-PET with [11C]PiB-PET and showed that the two ligands provided differing read-outs.

In paper III we created and investigated an antibody-based radioligand against the triggering receptor expressed on myeloid cells 2. Compared to wild type mice, transgenic animals displayed higher total *in vivo* exposure, calculated as the area under the concentration curve based on PET at 24 h, 48 h and 72 h post injection. However, differences were not evident in single time point PET images.

In paper IV we investigated brain delivery of a nanobody against GFAP with and without active transcytosis over the blood-brain barrier *in vivo*. Brain uptake with active transcytosis was two times higher. However, brain retention after 8 h, 24 h or 48 h did not differ between transgenic and wild type mice.

In paper V we studied the potential of a hexavalent and bispecific antibody construct against soluble A β aggregates for PET or immunotherapy *in vivo*. Its brain retention increased with age when applied at tracer doses in genetically modified mice. However, when applied at therapeutic dose, it had no or very low impact on A β levels measured in brain homogenates.

Keywords: Alzheimer's disease, amyloid-beta, positron emission tomography (PET), bispecific antibody, BACE-1, TREM2, GFAP

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List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I **Meier SR**, Syvänen S, Hultqvist G, Fang XT, Roshanbin S, Lannfelt L, Neumann U, Sehlin D. (2018) Antibody-based in vivo PET imaging detects amyloid- β reduction in Alzheimer transgenic mice after BACE-1 inhibition. *Journal of Nuclear Medicine*. 59:1885–1891.
- II **Meier SR**, Sehlin D, Roshanbin S, Lim Falk V, Saito T, Saido T, Neumann U, Rokka J, Eriksson J, Syvänen S. (2021) ^{11}C -PiB and ^{124}I -antibody PET provide differing estimates of brain amyloid-beta after therapeutic intervention. *Submitted to Journal of Nuclear Medicine*.
- III **Meier SR**, Sehlin D, Hultqvist G, Syvänen S. (2021) Pinpointing brain TREM2 levels in two mouse models of Alzheimer's disease. *Molecular Imaging and Biology*. *In press*.
- IV **Meier SR**, Sehlin D, Syvänen S. (2021) Passive and receptor mediated brain delivery of an anti-GFAP nanobody. *Manuscript*.
- V **Meier SR***, Rofo F*, Metzendorf N, Sehlin D, Syvänen S, Hultqvist G. Therapeutic and diagnostic potential of a multivalent antibody against soluble A β aggregates. *Manuscript*.
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List of non-thesis publications

- I Syvänen S, Fang XT, Hultqvist G, **Meier SR**, Lannfelt L, Sehlin D. (2017) A bispecific Tribody PET radioligand for visualization of amyloid-beta protofibrils - a new concept for neuroimaging. *Neuroimage*. 1;148:55-63
- II Sehlin D, Fang XT, **Meier SR**, Jansson M, Syvänen S. (2017) Pharmacokinetics, biodistribution and brain retention of a bispecific antibody-based PET radioligand for imaging of amyloid- β . *Scientific Reports*. 8;7(1):17254
- III Fang XT, Hultqvist G, **Meier SR**, Gunnar A, Sehlin D, Syvänen S. (2019) High detection sensitivity with antibody-based PET radioligand for amyloid beta in brain. *Neuroimage*. 1;184:881-888
- IV Michno W, Wehrli P, **Meier SR**, Sehlin D, Syvänen S, Zetterberg H, Blennow K, Hanrieder J. (2020) Chemical Imaging of Evolving Amyloid Plaque Pathology and Associated A β Peptide Aggregation in a Transgenic Mouse Model of Alzheimer's Disease. *Journal of Neurochemistry*. 152(5):602-616

Contents

List of papers	3
Introduction.....	9
Amyloid beta.....	10
Amyloid beta aggregation	11
Tau.....	11
Cerebrospinal fluid biomarkers	12
Clinical PET in AD	13
Blood biomarkers	14
Blood-brain barrier	15
Antibody-based brain PET	16
Immunotherapy	17
Secretase inhibition	19
Neuroinflammation in AD	21
TREM2.....	22
Methods	24
Animal models	25
PET imaging.....	26
SPECT imaging.....	27
Recombinant expression of antibodies.....	28
Antibody conjugation.....	29
Iodine-124/125 labeling	30
Brain samples centrifugation.....	30
ELISA	31
Immunohistochemistry.....	32
Autoradiography.....	33
Nuclear track emulsion.....	33
Aim	35
Results and discussion	36
Paper I	36
Paper II.....	37
Paper III.....	39
Paper IV	40
Paper V.....	41

Conclusion and future perspectives	42
Popular science summary	44
Acknowledgements.....	46
References.....	49

Abbreviations

^{11}C	Carbon-11
^{124}I	Iodine-124
^{125}I	Iodine-125
^{18}F	Fluorine-18
AD	Alzheimer's disease
Arc	Arctic amyloid-beta precursor protein mutation
A β	Amyloid-beta
A β PP	Amyloid-beta precursor protein
BACE-1	Beta-secretase
BBB	Blood-brain-barrier
BW	Body weight
CNS	Central nervous system
CSF	Cerebrospinal fluid
ELISA	Enzyme-linked immunosorbent assay
FDG	Fluorodeoxyglucose
HRP	Horseradish peroxidase
ID	Injected dose
IHC	Immunohistochemistry
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
NF-L	Neurofilament light
NFT	Neurofibrillary tangle
NHS	N-Hydroxysuccinimide
PET	Positron emission tomography
PiB	Pittsburg compound B
p-tau	Phosphorylated tau
ROI	Region of interest
ScFv	Single-chain variable fragment
SPECT	Single-photon emission computer tomography
sTREM2	Soluble triggering receptor expressed on cells 2
SUV	Standard uptake value
Swe	Swedish A β PP mutation
TCO	Trans-cyclooctene
TfR	Transferrin receptor
TREM2	Triggering receptor expressed on myeloid cells 2
t-tau	Total tau

Introduction

In 2020, 50 million people worldwide were living with dementia. With increasing life expectancy, this number is expected to rise to 152 million by 2050 [1]. The most common form of dementia is Alzheimer's disease (AD), representing 60-70% of all dementia cases.

Alois Alzheimer was first to describe the disease, after carefully studying one of his dementia patients, Auguste Deter, regarding symptoms and disease progression. Auguste Deter was hospitalized in 1901 in Frankfurt with symptoms such as reduced comprehension and memory, unpredictable behavior, aphasia, disorientation, hallucinations, and psychosocial impairment. After her death in 1906, Alzheimer investigated the postmortem brain tissue regarding histological abnormalities. Besides general atrophy of the brain, he described extracellular plaques containing assemblies of aggregated protein and intracellular neurofibrillary tangles (NFT) [2]. With today's state of knowledge, these extracellular protein deposits contained amyloid beta ($A\beta$). The NFTs were composed of tau protein. Today we also know that as a consequence of misfolded and aggregated proteins, activated microglia and astroglia react with a neuroinflammatory response, which may contribute to disease progression and severity.

The most prominent biological risk factor of AD is aging. Patients can be classified into two age groups. Early onset patients, about 10% of all, present their first symptoms of the age of 35-65 years, while the late onset group defines patients above 65 years [3]. The link between AD and genetic factors is not completely understood. It has been estimated that 70% of AD cases are attributable to genetic factors. Familial, autosomal dominant AD is rare (<1%) and usually reported in individuals with early onset AD [4].

Diagnosis of AD faces several fundamental challenges. Disease progression is slow from the beginning and neuropathological changes are on-going for decades before cognitive symptoms occur. Neuropsychological diagnosis relies on cognitive tests, cerebrospinal fluid (CSF) analysis and brain imaging using magnetic resonance imaging (MRI) or positron emission tomography (PET). To date, no treatment is available that stops the underlying causes of the disease. Acetylcholinesterase inhibitors or NMDA receptor antagonists can intervene on a symptomatic level, but the effect is limited.

PET can provide insights into *in vivo* mechanisms and changes related to the disease. In 2004, the development of Pittsburgh compound B (PiB) enabled for the first time the visualization of AD related pathology in the brain [5].

Since then, the carbon-11 (^{11}C) labelled molecule binding to $\text{A}\beta$ plaques has been the gold standard for PET in AD. However, the radioligand has limitations. The signal saturates early during disease progression. Further, the plaque load may be an undynamic measure of disease stage, especially after treatment intervention, since these densely packed protein structures dissolve slowly. The development of new treatments against AD requires sensitive and dynamic imaging compounds to follow changes in pathophysiology. This work explores new concepts of $\text{A}\beta$ and neuroinflammation PET imaging using antibody-based radioligands. The work especially focuses on the use of antibody-based PET for quantification and visualization of effects on AD brain pathology after treatment with anti- $\text{A}\beta$ drugs.

Amyloid beta

$\text{A}\beta$ is a 38-43 amino acid long peptide which is the outcome of the amyloid beta precursor protein ($\text{A}\beta\text{PP}$) sequential cleavage (Figure 1) [6]. $\text{A}\beta\text{PP}$, a transmembrane protein, is encoded on chromosome 21. Three major isoforms $\text{A}\beta\text{PP695}$, $\text{A}\beta\text{PP751}$ and $\text{A}\beta\text{PP770}$ are expressed in the endoplasmic reticulum and transported to the membrane through the secretory pathway [7,8]. So far, the physiological functions of $\text{A}\beta\text{PP}$ are not fully understood. Proposed functions include neuronal growth and migration, cell adhesion and receptor signaling. Missense mutations in $\text{A}\beta\text{PP}$ and the subgroup of the gamma-secretase complex (presenilin 1 and 2) lead to familial AD [9].

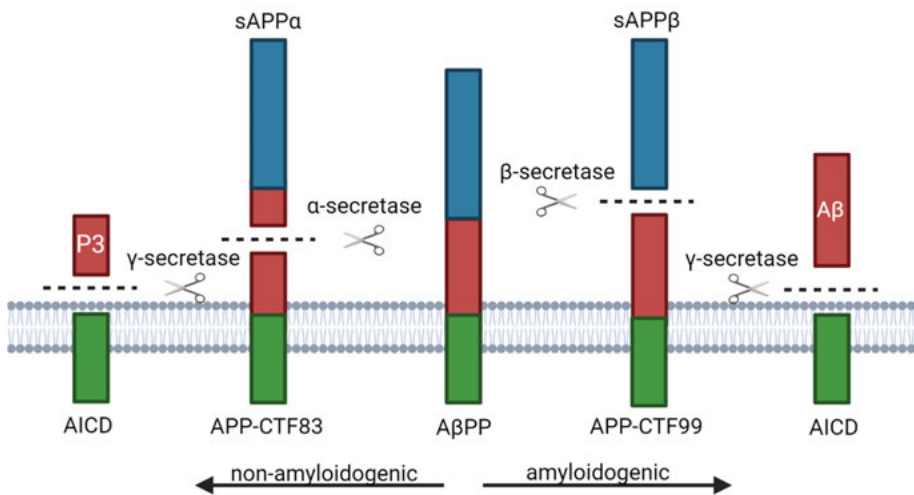


Figure 1. Cleavage pathways of $\text{A}\beta\text{PP}$. In the amyloidogenic pathway $\text{A}\beta\text{PP}$ is processed by beta-secretase cleavage followed by a gamma-secretase cleavage leading to the release of the $\text{A}\beta$ peptide. Alternatively, $\text{A}\beta\text{PP}$ is processed by alpha-secretase cleavage before gamma secretase processing. No $\text{A}\beta$ peptide is generated.

Amyloid beta aggregation

The most abundant species of A β in the brain are A β 40 and A β 42. Released monomeric peptides can undergo conformational change enhancing propensity to self-assembly. Overproduction or inefficient clearance enable the formation of oligomers and larger protofibrils leading to insoluble fibril structures which deposit as plaques (Figure 2) [10,11]. A β 42 is more prone to aggregate due to two additional hydrophobic amino acids at the C-terminal. The large and insoluble deposits were first considered to be toxic for neurons and potentially the onset of the disease. However, focus has increasingly shifted towards the earlier and still soluble forms of A β in the aggregation cascade. Hence, A β plaques are a histological sign of AD but correlate poorly with the progression of the disease [12]. Soluble A β aggregates seem to correlate better with synaptic loss and are suspected to be more neurotoxic[13].

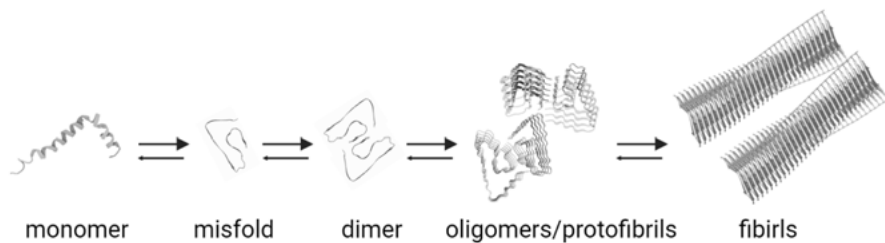


Figure 2. Aggregation pathway of A β . Misfolding causes aggregation of monomers into dimeric structures. These form larger aggregates such as oligomers and protofibrils. Further aggregation into insoluble fibrils forms plaque depositions.

Tau

The second histological hallmark reported by Alois Alzheimer was abnormal intracellular tangles. About 80 years later, it was discovered that these structures consist of tau, a microtubule-associated protein [14,15]. As a result of different splicing, six major isoforms of tau can be found in the brain which are between 352 and 441 amino acids long. They are mainly expressed in neurons but can also be found in glial cells [16,17]. The main function of tau is the stabilization and modulation of axonal microtubules. The protein has also been described to be involved in many other processes including neuronal development, cellular signaling or apoptosis [18].

Tau is natively unfolded and soluble but has a tendency for aggregation [19,20]. Monomers can pair to dimers and further into elongated oligomers that form β -sheet structures called paired helical filaments. These are the basic building blocks for NFTs [21,22]. Aggregated tau in NFT deposits have been reported to be highly phosphorylated on up to 85 known phosphorylation sites

[22]. A strong connection between abnormal phosphorylation and self-aggregation of tau into oligomers and larger aggregates has been reported [23]. However, a complete pathological phosphorylation pattern has not been identified yet. Aggregated tau can serve as seeds for further misfolding and aggregation. Pathological aggregates can be transmitted between cells, affecting previously healthy brain areas [22].

Cerebrospinal fluid biomarkers

CSF is a clear and colorless fluid derived from arterial blood plasma in the choroid plexus. Its main distinction compared to blood plasma is low protein levels. In humans, approximately 500 ml is generated daily while about 125 ml continuously circulates through the ventricles, the central canal of the spinal cord and the subarachnoid space. CSF is reabsorbed into the vascular system by entering the dural venous sinuses or draining into lymphatic vessels [24,25]. CSF has several mechanical functions such as buoyancy for the brain tissue, protection from mechanical injury and intercranial pressure regulation. The CSF allows waste clearing from the brain tissue since the interstitial fluid of the extracellular space is secreted into the CSF. Therefore, biochemical changes in the central nervous system (CNS) are likely to be reflected in the CSF. CSF can be collected by lumbar puncture and analyzed for biochemical, viral, or immunological parameters.

In AD, analysis of CSF biomarkers is a widely used for diagnosis after the discovery of the A β peptide in CSF samples [26]. Most notably A β 42 was reported to be decreased in AD patients compared to healthy controls [27]. The reduced CSF levels are likely a consequence of increased aggregation and deposition in plaques in the brain. A correlation has been reported between CSF levels and plaque load in neocortex and hippocampus of AD patients [28,29]. However, A β 40 levels are not affected but the A β 42/A β 40 ratio has better diagnostic performance than A β 42 alone [30–32].

Total tau (t-tau) and phosphorylated tau (p-tau) levels are increased in CSF sampled from AD patients. T-tau has also been reported to reflect the intensity of neuronal and axonal degeneration in disorders with rapid neuronal loss such as Creutzfeldt-Jakob [33]. Further, a correlation between CSF t-tau and NFT load has been reported [34]. P-tau levels are not increased to the same extent in other forms of dementia as in AD. Therefore, the possibility to detect increased levels of p-tau is of importance for a differential diagnosis of AD [35]. A β 42, t-tau and p-tau are well-established CSF biomarkers for AD, and their utility markedly improves when measured in combination [36,37].

Several additional fluid biomarkers are currently under intense research to achieve faster and earlier diagnostics. Proteins related to inflammatory processes or synaptic dysfunction are giving a more complete picture of disease progression. Soluble levels of the triggering receptor expressed on myeloid

cells 2 (sTREM2) or chitinase-3-like protein 1 are increased in AD and measurement of these proteins has the potential to give insights of on-going inflammation processes [35,38–40]. Neurogranin is a dendritic protein which is involved in post synaptic signaling pathways. It has been proposed as a biomarker since levels are increased in AD and mild cognitive impairment (MCI) subjects compared to controls [41]. Neurofilament is an axonal protein and its subunit, neurofilament light (NF-L), has been reported to be increased in AD patients [42]. However, NF-L levels correlate with brain atrophy and do not seem to be specific for AD [43,44].

Clinical PET in AD

Besides CSF sampling, PET is the second most used biomarker-based diagnostic method for AD. Hypermetabolism due to neurodegeneration can be detected by fluorodeoxyglucose-PET (^{18}F FDG-PET). The glucose consumption of the brain tissue is directly linked to astrocyte and glutamatergic synaptic activity [45,46]. This facilitates the assessment of neuronal dysfunction in affected brain regions. ^{18}F -FDG-PET is mainly applied to patients with mild cognitive impairment with suspected underlying AD. It can predict the development of AD earlier than structural analysis with MRI [47,48]. In combination with CSF or MRI, ^{18}F -FDG-PET has shown an added value with short-term progression prognosis which is crucial for future clinical monitoring and adaption of therapeutical interventions [49].

The first PET radioligand binding to fibrillar A β deposits enabled imaging of a core feature of AD. The carbon-11 (^{11}C) labeled Pittsburgh compound B, an analog of thioflavin T, has a high sensitivity and specificity for detection of extracellular amyloid plaques and vascular deposits without cross reactivity to NFT or Lewy bodies [5,50–54]. A second generation of amyloid tracers, labelled with ^{18}F , has been developed and approved by the FDA. Florbetapir, Flormetamol and Florbetaben have been shown to be comparable to PiB in clinical populations, although they show more nonspecific white matter binding [55,56]. Labelling with ^{18}F simplifies clinical use due to the longer half-life of ^{18}F compared to ^{11}C , and enables radioligand use in hospitals without on-site cyclotrons [57]. These radioligands have greatly improved AD diagnostics and are commonly used in the clinic.

Meta-analysis of amyloid-PET studies in AD patients have found a very high specificity for the radioligand (around 88%) [58]. However, a “positive” amyloid-PET scan is not a clear indication of AD since other forms of dementia, may be associated with amyloid pathology. Furthermore, cognitively normal or non-AD demented individuals can be PET positive, especially when carrying the APOE4 allele or when older than 60 years [58]. Furthermore, the amyloid radioligands have limitations regarding imaging of diffuse plaque pa-

thology [59]. Still, research using PET radioligands visualizing A β has contributed to improved models of disease pathogenesis and provided evidence for a preclinical disease phase that can last over decades. In addition, amyloid-PET frequently assists subject selection for clinical trials or serves as a secondary outcome measure for clinical trials aimed at delaying or preventing AD. For example, anti-A β antibodies bapineuzumab, solanezumab, gantenerumab and lecanemab have been evaluated using amyloid-PET [60–66].

Blood biomarkers

Screening patients for AD with PET imaging or CSF sampling is expensive, time consuming and when it comes to CSF, rather invasive. This makes it difficult to identify the right individuals for clinical studies and prolongs the enrollment processes. Further, the capacity for large scale PET and CSF sampling as general health monitoring in the population is limited in current health care systems. Fast screening possibilities for AD will be crucial to identify patients for upcoming new treatments. Blood-based biomarkers could serve as a solution and are currently under intense research. However, they face fundamental challenges and are not standardized yet. The BBB prevents the passage of molecules between the CNS and blood, which leads to low concentrations of brain-derived biomarkers in blood. AD related biomarkers, such as A β , are often also expressed in non-cerebral tissue which can potentially confound assay results. Further, peptides and proteins of interest can undergo proteolytic degradation by proteases in plasma [67].

A decrease of the A β 42/A β 40 ratio in plasma, similar to what is observed in the CSF, has been reported with ultrasensitive single molecule array. Although the separation between AD and controls was smaller compared to CSF analysis. Immunoprecipitation mass spectrometry confirmed these results with a diagnostic accuracy of about 90% [68,69]. These findings are promising but do not solve the confounding problem of peripheral A β expression. [67]

Recently, several breakthroughs have been reported for plasma tau quantification. Levels of p-tau181 seem to be increased in AD compared to controls and correlate well with CSF p-tau, amyloid-PET and tau-PET [70–72]. Changes of p-tau levels in plasma become significant even before amyloid-PET [72]. Recent validation studies showed similar results suggesting plasma p-tau as a biomarker for AD in the clinic [73–75]. Recently plasma p-tau217 was suggested to be an even earlier marker than p-tau181, and more strongly associated with AD [76,77]. However, further comparisons between the markers are needed [78].

Blood-brain barrier

The central nervous system requires a stable microenvironment for reliable neural signaling. The supply of essential molecules for the brain tissue is controlled by a physical barrier of endothelial cells that separates the blood from the brain, i.e. the blood-brain barrier (BBB). These endothelial cells are connected to each other by tight junctions that restrict the diffusion between the cells. Together they form the walls of the cerebral capillaries. This layer is surrounded by pericytes and astrocytes that give support, regulation, maintenance and sometimes act as a second barrier.

The BBB is the backbone for a limited and controlled transport of endogenous and exogenous molecules between the blood and the brain tissue. Nevertheless, there are routes of transport across the BBB (Figure 3). Lipophilic molecules <400-500 Da with a polar surface area <80 Å² and <6 hydrogen formation points may travel through the BBB by passive diffusion [79–81]. Essential nutrients such as glucose or amino acids passively enter the brain via solute-carrier transport proteins [82]. Transcytosis via mechanisms of endocytosis is the main route for macromolecules with large molecular weight such as peptides or proteins. Transferrin, insulin, or leptin bind to specific receptors that initiate transcytosis. Under pathological conditions even monocytes and macrophages can be recruited to the CNS and migrate through the cytoplasm of the endothelial cells or by opening of the tight junctions [83].

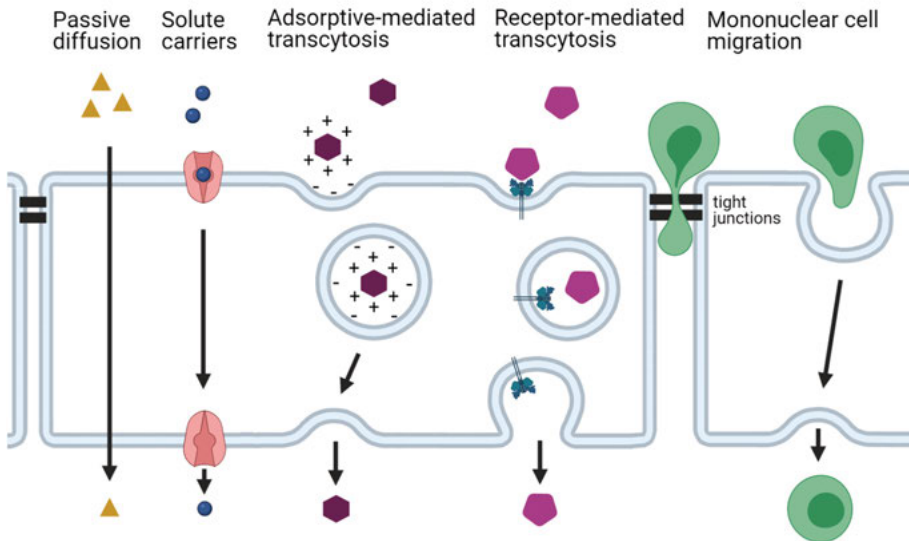


Figure 3. Transport routes across the blood-brain barrier.

However, antibodies or antibody-based constructs as used in this work are unlikely to be transported across the BBB due to their size of >150 kDa. Intravenous application of IgG antibodies results in a maximal IgG brain concentration of 0.1% (or less) of injected dose [84]. To increase the brain uptake of large molecules, the use of an active transcytosis system was suggested many years ago. *Friden* and *Pardrige* demonstrated such a Trojan horse strategy already in 1991 by conjugating methotrexate to the transferrin receptor (TfR) antibody Ox26 [85]. Increased brain uptake, compared to normal IgG, was also reported with the rat anti-mouse TfR antibody 8D3 in mice [86,87]. Both antibodies were initially developed for immunohistochemical staining of the brain capillaries and their high brain uptake *in vivo* was discovered later. Since then, TfR binding at the BBB has been intensively studied. Low or moderate binding to TfR seems to be beneficial to promote transcytosis, while high affinity probably leads to lysosomal degradation [88]. Other studies found that monovalent binding resulted in slower transcytosis, it was more efficient than bivalent binding to carry antibodies across the BBB, as it was suggested that bivalent binding may lead to increased lysosomal degradation [89]. In addition to binding affinity and avidity, pH dependent antibody binding to TfR may play an important role. It has been demonstrated that less efficient binding to the TfR at lower pH leads to increased transport through the endothelial cell layer *in vitro*, suggesting a dissociation of the TfR-antibody complex in the late endosome when the pH drops. On the other hand, pH independent binders remained trapped in the cells [90,91]. However, moving the drug delivery concept via TfR mediated transcytosis to the clinic seems rather difficult. Antibodies with species cross-reactivity are lacking because of inter-species differences in the amino acid sequence of the TfR. Furthermore, in addition to the brain endothelial cells, the TfR is expressed by many other cells which is a potential concern for side effects. Intense research and clinical trials are currently ongoing evaluating new human TfR binders [92–94]

Antibody-based brain PET

Antibodies are highly specific and often display high binding affinity for their targets. The number of epitopes they can be directed to is almost unlimited. Further they have advantages compared to small molecules recognizing quaternary protein structures such as aggregated fibril structures. Besides immunotherapy, antibodies have recently been applied as a new class of PET radioligands in the field of cancer [95]. It has even been demonstrated that intrabrain PET imaging with antibodies can be used to visualize gliomas when the BBB is disrupted due to the cancerogenic environment [96,97]. Antibodies can also be used as brain PET radioligands when modified for active brain uptake. Several constructs using TfR mediated transcytosis have been described by our group [98–102]. In these studies, antibodies or their fragments

against A β were linked to the TfR binding antibody 8D3 [87] to generate bispecific protein constructs. As a consequence of TfR binding and subsequent transcytosis across the BBB, the constructs display increased brain uptake and distribution compared to unmodified antibodies [103,104]. The modification allows for visualization of A β pathology *in vivo* in transgenic mice. In this work we explored this approach as a method to follow A β pathology progression and A β reduction after treatment. Further, we studied the ability of antibody-based PET for imaging of new targets other than A β .

Immunotherapy

Since the description of the *amyloid cascade hypothesis* by Hardy and Higgins in the 1990s [105], the idea of eliminating A β from the brain parenchyma has been regarded as a key for slowing down or stopping the course of the disease. However, there is still a dissension in the field about which aggregation state of A β is most suitable as a target for treatment (monomers, dimers, oligomers, fibrils, or senile plaques). The majority of recent studies indicate that therapy directed towards larger aggregates rather than monomers is more successful [106].

Beside the removal of toxic aggregates, the influence on the aggregation process itself, including inhibition of secondary nucleation, could play a major role in treatment with antibodies [107,108]. However, cerebral immunotherapy faces problems such as BBB penetration, the complexities of immunology, adequate dosing, systemic toxicity and side effects [109]. Sufficient target engagement has not been demonstrated in clinical trials to date.

Bapineuzumab

Bapineuzumab is the humanized version of the mouse monoclonal antibody 3D6, which binds to the N-terminus of A β . It was one of the first antibodies studied in human AD subjects. A phase III study showed a tendency towards an improved cognitive score, unfortunately with the occurrence of neurovascular complications [110]. Different trials investigated the relation between dose level and occurrence of amyloid-related imaging abnormalities (ARIA), showing that the frequency of ARIA was correlated with higher dose [111,112]. When excluding APOE4 carriers in another study, no clear benefits in efficacy were seen [113]. Consequently, the studies were discontinued.

Solanezumab

Solanezumab recognizes the mid region of A β (A β 16-24) [114]. It is the humanized version of the monoclonal antibody m266 that binds monomeric, soluble A β rather than fibrillar structures [115]. In two phase III studies, 2052 patients with mild to moderate AD were treated for 18 months [116]. None of the studies showed a significant improvement in primary outcome measures,

i.e. in the in AD assessment scale and Alzheimer's Cooperative Study-Activities of Daily Living Scale (ADAS-cog [109] and ADCS-ADL [117]) [113].

Gantenerumab

Roche's Gantenerumab binds to N-terminal and central epitopes of A β and has been reported to disassemble and degrade A β plaques by recruiting and activating phagocytosis of microglia [118]. Gantenerumab was investigated in a double-blinded, placebo-controlled phase III study over two years with a dosing of 105 mg or 225 mg per month. The study was discontinued due to futility, suggesting higher dosing to achieve a clinical effect [119]. A high-dose study was launched with a projected finish in 2022. A two year follow up study reported reduction of amyloid in a small group of patients investigated with PET [64]. In the third year of the study, PET showed that 80% of the patients had reduced amyloid levels below the positivity threshold [65]. Roche has further launched clinical trials with a new version of Gantenerumab linked to a human TfR binding moiety enabling active transcytosis into the brain parenchyma. After the completion of a first safety, tolerability, immunogenicity and pharmacokinetics study, the enrollment for a trial with multiple dosing in patients with prodromal or moderate AD will start in March 2021 [92,93].

Aducanumab

Aducanumab has been widely regarded as a very promising monoclonal IgG1 antibody. It targets soluble and insoluble A β aggregates, but not monomers. In a phase Ib study, it appeared to reduce A β in the human brain in a dose dependent manner [110]. These data were supported by a reduction of A β also in tg2576 mice [120]. A phase Ib clinical trial reported a significant reduction of A β with PET imaging [121]. It further seemed to slow down the rate of cognitive decline after 54 weeks of treatment [122]. Consequently, it entered clinical phase III studies. These were later halted as it seemed like the treatment was unlikely to reach the primary endpoint of the study, i.e. the slowing down of cognitive decline [123]. However, re-analysis of the clinical data concluded that Aducanumab's trial data was positive regarding efficacy in one of the two studies, i.e. the ENGAGE study [124]. A decision about Biogen's licensing application to the FDA is expected in May 2021.

Lecanemab

Lecanemab (BAN2401) is the humanized version of mAb158, which was derived from immunization with protofibrils with the Arctic A β PP mutation E22G [125]. It displays a higher affinity for protofibrils compared to monomers and fibrils [98,126]. In mild to moderate AD, safety and tolerability of BAN2401 was acceptable [127]. The outcome of a phase II study showed a slowdown of disease progression measured with ADAS-Cog and mini mental state examination (MMSE) after 18 months of treatment. Amyloid reduction

was also demonstrated with PET [128]. Lecanemab has now proceeded to phase III.

Donanemab

Lilly's Donanemab binds to an enzymatically modified form of A β . A β (p3-42) has a pyroglutamate at its N-terminus and is only found within A β plaques [129]. The antibody showed plaque removal capacities in a phase Ib trial and was continued in a phase II trial in early symptomatic AD patients. Donanemab met the primary endpoint of slowing cognitive and functional decline compared to placebo. Amyloid-imaging indicated a reduction of amyloid plaques after 76 weeks of treatment [66].

Secretase inhibition

A β is generated by cleavage of the A β PP protein (Figure1). Beta secretase (BACE-1) and gamma secretase are involved in the amyloidogenic pathway and lead to the release of the A β peptide. Since their discovery, blocking of their action has been the focus for a potential treatment strategy to reduce A β production. However, the development of such inhibitors quickly turned out to be a big challenge. Inhibitors must be able to cross the BBB for reaching the target. The inhibition should ideally be selective for the A β PP cleavage and should not affect the cleavage of the up to 40 other targets. To avoid further side effects, a potential therapeutic compound needs to be selective for its specific protease and should not bind to related enzymes.

The following selection of secretase inhibitors have reached clinical trial phase III:

Semagacestat

Semagacestat is a gamma-secretase inhibitor. The precise inhibition mechanism is unclear but it is suggested that the molecule blocks the function of the enzyme complex rather than a competitive inhibition of the active site [130]. The inhibitor has been reported to reduce A β in brain and CSF of transgenic PDAPP mice harboring the human APP V717F mutation [130]. In a clinical phase I study, A β 42 plasma levels were reduced [131]. A small phase II cohort study consisting of subjects with moderate AD reported a decrease of A β 42 in plasma, but A β 42 levels in CSF did not change [132]. The compound was taken into clinical phase III and was tested in 1537 subjects. Subjects were randomized into three groups with either 100 mg or 140 mg dose daily or placebo. Primary outcomes were cognition score tests (ADAS-cog and ADCS-ADL). All groups scored worse after treatment and both Semagacestat groups were significantly worse compared to placebo ($p < 0.001$). Furthermore,

side effects such as weight loss, skin cancer and laboratory test abnormalities became significantly more frequent compared to placebo [130].

Since then, the trials and related studies have been criticized for a lack of intense preclinical testing and characterization [133,134].

Lanabecestat

The selective BACE-1 inhibitor Lanabecestat (LY3314814 or AZD3293) reduced plasma, CSF, cerebral A β 40, A β 42, and sA β PP in rodents and a dog model [135]. Side effects such as macroscopic and microscopic hypopigmentation of the skin, hair and mucosa were reported [136]. The compound seemed to be well tolerated in a phase I study and showed a dose-dependent reduction of CSF A β [120]. The compound was studied in a double blind, placebo controlled, three-year phase III study. However, the study was prematurely terminated due to a lack of improvement on the ADAS-cog scale [137].

Verubecestat

Verubecestat (MK-8931) was discovered through screening and structure based rational design and interacts with the catalytic site of BACE-1 [138,139]. *In vitro*, the compound seemed to reduce A β 40, A β 42 and sA β PP by inhibiting mouse BACE-1 as well as human BACE-2 [122]. In rat and cynomolgus monkey, once daily oral administration reduced CSF A β 40, A β 42 and sA β PP and cortical A β 40 and sA β PP. The compound was well tolerated in animal models [140].

In a clinical phase III study with 1958 mild AD patients, subjects were randomized into 3 groups which received either 12 mg or 40 mg Verubecestat or placebo. An improvement of cognition was not reached in any of the treated groups compared to placebo. Adverse events such as rash, falls, sleep disturbance, weight loss, suicidal ideation and hair color change were increased in the Verubecestat group [141].

NB-360

The BACE-1 inhibitor NB-360 was developed by Novartis. The substance showed good pharmacokinetic properties such as high rate and extent of transport across the BBB, low recognition by the P-glycoprotein transport system and binding affinity to BACE-1 in the low nanomolar range [142]. The compound binds several thousand-times less to similar proteases such as cathepsin D, cathepsin E and pepsin. It inhibits the BACE-2 [143] equally well as BACE-1 [144]. Due to concerns regarding side effects, it was not selected as a clinical compound. However, it turned out to be a valuable pharmacological tool to study the effects of BACE-1 inhibition in A β PP transgenic mice. A structurally related compound, CNP520, was studied in clinical trials.

Umibecestat (CNP520)

Umibecestat was developed in parallel to NB-360 and is an oral, long acting, and selective BACE-1 inhibitor. It was designed for subjects in an asymptomatic stage at risk for AD. In transgenic mice and dogs, Umibecestat reduced soluble and insoluble A β in brain in a dose-dependent manner. No hair depigmentation has been reported when dosed chronically to mice [145]. In a phase I study the inhibitor was well tolerated and after multiple dosing, a reduction of A β 40 levels in CSF was observed. Safety, tolerability, pharmacokinetics and pharmacodynamics were investigated in a phase II double-blind, placebo-controlled, dose ranging study for 13 weeks in 125 elderly subjects [145]. Due to the positive outcome regarding tolerability and dose dependent A β reduction in CSF, two phase II/III studies in cognitively normal, homozygote or heterozygote APOE4 carriers were launched. The studies were discontinued because a cognitive worsening in treated subjects was observed. In addition, brain atrophy and weight loss were increased in treated patients compared to untreated controls.

Neuroinflammation in AD

It has become evident that neuroinflammation plays a key role in the pathophysiology of AD. Increased glial cell activation is widely reported and genome wide association studies have revealed genes associated with immunity and inflammation with increased risk of AD [146]. Triggered microglia and astrocytes engulf misfolded proteins and release inflammatory mediators. It is currently discussed if neuroinflammation is supportive or contributes to disease progression and severity [146].

Microglia are the resident immune cells of the CNS and play a key role in brain surveillance and homeostasis [147]. Microglia are neuroprotective and constantly monitor the brain for pathogens and cellular debris. The cells provide factors to support tissue maintenance and contribute to the remodeling of synapses and their protection [148,149]. These actions can be mediated by trophic factors which can contribute among other things to memory formation [150]. Activated by pathological triggers such as pathogenic proteins or neuronal death, the cells are able to migrate to lesions where they initiate an immune response.

Astrocytes are glial cells in the CNS performing many functions, e.g. nutrition supply to the nervous tissue, maintenance of ion balance, regulation of blood flow or support of endothelial cells forming the BBB or synaptic regulation and remodeling [151]. As a response to CNS injury or disease such as infection, trauma, stroke or neurodegenerative diseases, astrocytes change their molecular expression and morphology [152,153]. Under these conditions the glial fibrillary acidic protein (GFAP) is overexpressed in the cells. The

intracellularly expressed protein plays a role in mitosis and has many functions, e.g. in cell-to-cell communication, structural and functional support for cell cytoskeleton maintenance [154]. Due to its upregulation, GFAP is one of the most extensively used markers for reactive astrocytes *ex vivo*.

TREM2

Structure and expression

TREM2 is expressed on the membrane by microglia and myeloid cells. The receptor is part of the immunoglobulin superfamily and consists of a transmembrane domain, a small cytoplasmic tail and an extracellular Ig-like domain [155]. Human TREM2 is genetically located on chromosome 6p21.1 [156,157]. TREM2 interacts with TYROBP triggering pathways that are involved in cell activation and phagocytosis [156,158]. The extracellular domain of TREM2 can undergo proteolytic cleavage generating sTREM2 by the metalloprotease ADAM10 or ADAM17 [159]. sTREM2 diffuses into CSF where levels can be detected [38].

TREM2 function

TREM2 is active in the innate immune response [155]. Signaling pathways can be activated by binding to lipopolysaccharides, phospholipids, HDL and LDL, APOE, apoptotic neurons and A β [160–166]. The functions of TREM2 are currently under intense research. An established function is the impact on the regulation of cell proliferation [163,167,168]. Further a knockdown of TREM2 decreases the immune response in primary microglia in case of traumatic brain injury, AD pathogenesis and aging in general [167–169]. TREM2 deficiency decreases the migration of microglia towards apoptotic neurons [170].

TREM2 mutations are related to neurodegeneration

Biallelic mutations in TREM2 in the transmembrane domain e.g. D134G, K186N, W198X or in the ectodomain e.g. Y38C, T66M, T96K can lead to a loss of function causing Nasu-Hakola disease or frontotemporal dementia [155,156,171–173].

Several point mutations in the TREM2 gene have been identified as risk factors for AD. The R47H mutation was independently detected in a cohort in Europe and North America, and in Iceland [174,175]. The R62H mutation increases the risk of late onset AD [176]. Nasu-Hakola disease variants impact the protein stability and lead to a decreased expression on the cell surface [177]. The AD related variants R47H and R62H impact ligand binding and lead to a loss of function [178]. The mechanism proposed in case of A β pathology, suggests a recruitment of microglia around plaques. The recruited

microglia form a physical barrier that encapsulates A β and restricts plaque growth [179,180]. AD patients with deficiency or the heterozygous variants R47H or R62H have fewer plaque associated microglia [181].

In an early study, sTREM2 was reduced in CSF in AD patients [158]. However, the opposite has been suggested by emerging evidence. sTREM2 seems to be increased in CSF of AD patients and correlated to tau but not to A β 42 levels [39,182–184]. The levels of sTREM2 in CSF are significantly increased in subjects with the R47H mutation, while the T96K and W198X mutation carriers have lower levels [183]. Data from recent meta-analysis report increased sTREM2 levels in early course of AD, suggesting sTREM2 as a potential biomarker in early AD and for disease progression [38].

Methods

This work included several *in vitro* and *in vivo* techniques, spanning from protein design and expression to *in vivo* studies of drug effects analyzed by PET imaging and postmortem tissue analysis (Figure 4).

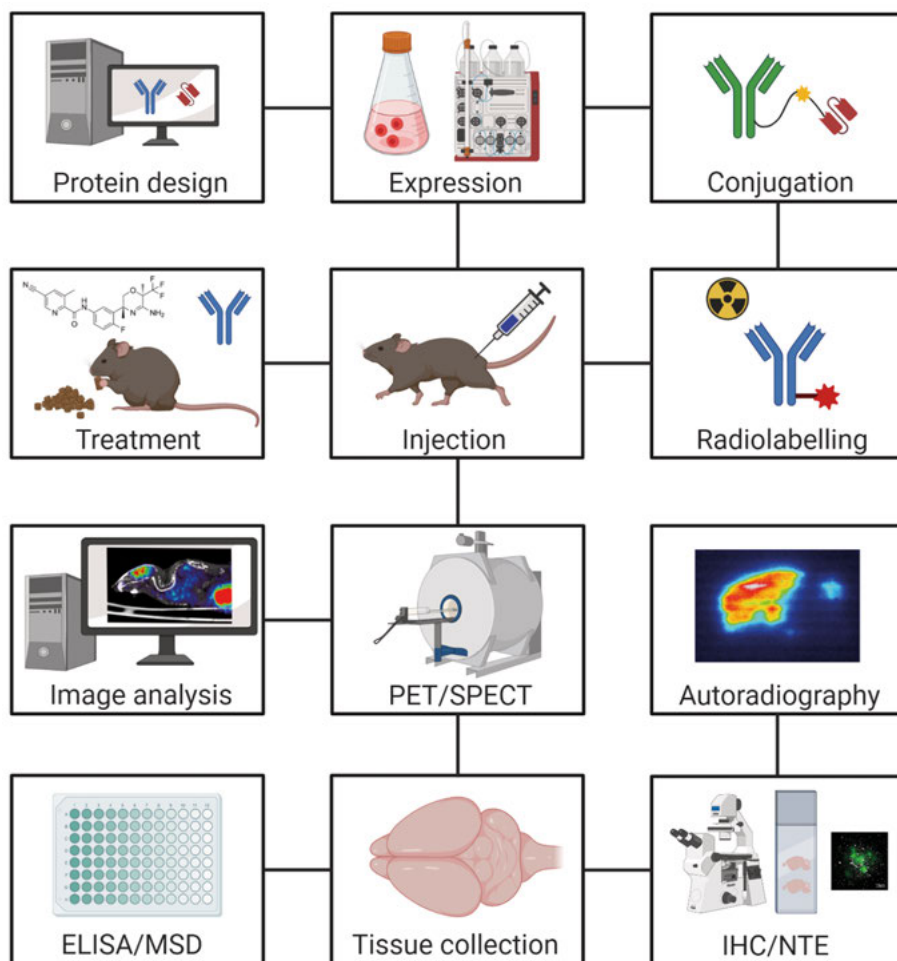


Figure 4. Methodological work steps and analyses that were performed in this work.




Animal models

Animal models such as genetically modified mice or rats are commonly used in biomedical research. Pathological processes and mechanisms of diseases are highly complex and diverse. When *ex vivo* cell studies or the analysis of human tissue reach their limits, animal models are a possibility to study disease mechanisms that can relate to human situations. Here, we used three different mouse models; two transgenic models overexpressing human A β PP and a second-generation knock-in model where the mouse wild-type A β PP gene sequence was genetically modified to human A β PP with mutations (Table 1).

The Swe mouse line is based on the C57BL/6 genetic background and harbors the human A β PP with the Swedish mutation (Swe). The mutation was first reported in two Swedish families [174]. The Swe mutation is a double mutation located at the β -secretase cleavage site in the APP (AP-PKM670/671NL) leading to increased A β PP cleavage. In Swe mice, intraneuronal A β aggregation pathology starts at the age of six months [185] and extracellular plaque formation starts at the age of 12 months in cerebral cortex, hippocampus and thalamus [186].

The ArcSwe mouse line harbors the Swedish and the Arctic APP (Arc) mutation (E693G) [125]. The Arc mutation was identified in a family from northern Sweden where individuals were affected by early onset AD [125]. The Arc mutation leads to an earlier onset of intraneuronal A β aggregation at about one month of age [185,186] and extracellular plaque formation at around five to six months of age. Plaque pathology starts in the cerebral cortex, hippocampus and thalamus [187] and spreads to other brain parts such as cerebellum with increasing age [102]. In contrast to the Swe line, TBS soluble A β levels such as protofibrils are strongly elevated and increase with age. Therefore, the ArcSwe model is a suitable model to study the effects and mechanisms of soluble oligomers and protofibrils. A β deposits are SDS insoluble and resemble plaques found in humans that are highly insoluble. Mice show behavioral deficiencies such as declined spatial learning and memory retention that correlate with protofibril levels but not with the total A β load in the tissue [188]. In both transgenic mouse models, microgliosis and astrogliosis follows the amyloidosis and is dominantly located around A β depositions.

Table 1. Mouse models used in this work.

			
Name:	Swe	ArcSwe	<i>App^{NL-G-F}</i>
Model:	Transgenic	Transgenic	Knock-in
A β PP mutations:	Swe	Arc, Swe	Arc, Swe, Iberian
Start A β deposition:	12 months	5-6 months	2 months
Used in study:	Paper III	Paper I-IV	Paper II and V

The *App^{NL-G-F}* mouse model is a knock-in model. The humanized A β PP is expressed at wild-type level which avoids potential artefacts caused by an over-expression. The levels of pathogenic A β are elevated, caused by three mutations that are linked with familial AD. Besides the Swe (NL) and Arc (G) mutations, the model harbors the Iberian mutation (F). The amino acid change of I45F affects the gamma-secretase specificity and elevates the A β 42/A β 40 ratio [189]. A β accumulation in insoluble deposits starts at the age of two months and progresses fast until near saturation at the age of seven months. Plaques are widespread over the whole brain tissue and smaller in size compared to ArcSwe animals. Microgliosis, astrogliosis and synaptic loss come along with amyloidosis. An age-associated cognitive impairment shown by the Y maze test starts at six months of age [190].

PET imaging

PET is a tomographic imaging technique for the visualization of biological processes and structures *in vivo*. The method is based on radiolabeled compounds, such as small molecules or proteins that bind to or accumulate in biological structures. Due to the labeling with beta decaying radioactive isotopes, the compounds called radioligands can be localized in the tissue. In the decay process, an antimatter particle, a positron, is released and collides with an electron in the tissue. Travelling distance of the positron varies depending on its energy and can be around a tenth of a millimeter to a few millimeters. The annihilation of the positron and electron simultaneously emits two gamma photons with identical energies (511 keV) in around 180-degrees opposing direction. Near-simultaneous detection of two photons by a ring detector placed around the tissue allows to localize their origin. The detectors record the photon events and “stamp” them with the event time with a precision of about 1 nanosecond. This data is further processed by matching the “time

stamps” of opposite detectors. If a pair of events are recorded in a time window of about 6-10 nanoseconds, they are considered as a coincident. The acquired data can further be mapped and displayed as either one single static image or as a dynamic set of images showing the change of the radioactivity over time.

In this work we mainly performed PET scans with radiolabeled antibodies (**paper I-III**). Compared to small molecules, antibodies have a longer half-life in blood. Therefore, the scans were performed 1-4 days after injection when the majority of the radioligand was eliminated from the blood compartment. Study subjects were compared to each other with respect to radioligand concentration by normalizing the ligand concentration (c_{img}) in the region of interest (ROI) for the injected dose ($\%ID/g_tissue$) (Eq.1) or by the injected dose per body weight, yielding the standard uptake value (SUV) (Eq.2). SUVs of disease related ROIs can be compared to a reference ROI in the tissue which is assumed to have similar uptake properties in all animals regardless of age, pathology, or genotypes. Such a ratio (SUVR) is often generated using the cerebellum as the reference region. However, SUVs were not applied in this work as an outcome measure because pathology was spread in the whole brain tissue of the used mouse models. The potential reference regions, e.g. the cerebellum or parts of cerebellum such as periaqueductal grey [191], were affected by disease progression and by treatment and could therefore not be used for normalizing radioactivity in the regions regarded as high pathology ROIs.

$$\%ID/g_tissue = \frac{c_{img} \times 100}{ID} \quad \text{Eq.1}$$

$$SUV = \frac{c_{img}}{ID/BW} \quad \text{Eq.2}$$

SPECT imaging

Single-photon emission computer tomography (SPECT) is an imaging technique, similar to PET, for visualization of the distribution of a radioligand in biological tissue. The radioligands are labeled with a gamma ray emitting radionuclide before injection. Gamma rays are detected by a set of collimated scintillation detectors located around the patient or animal. During the scan, the detector is repositioned to acquire data from different views. Detected events can be mapped to 3D images visualizing radioligand concentrations in the tissue. SPECT imaging is less expensive than PET but has lower resolution than PET imaging.

Recombinant expression of antibodies

Recombinant proteins were expressed mainly according to the protocol of Fang et. al 2017 [192] (Figure 5). The sequence encoding the constructs was cloned into a pcDNA3.4 vector. For later purification, a histidine-tag was attached to proteins lacking an Fc domain. The plasmid DNA was expressed in *E.coli* (Top10) cells and purified from endotoxins.

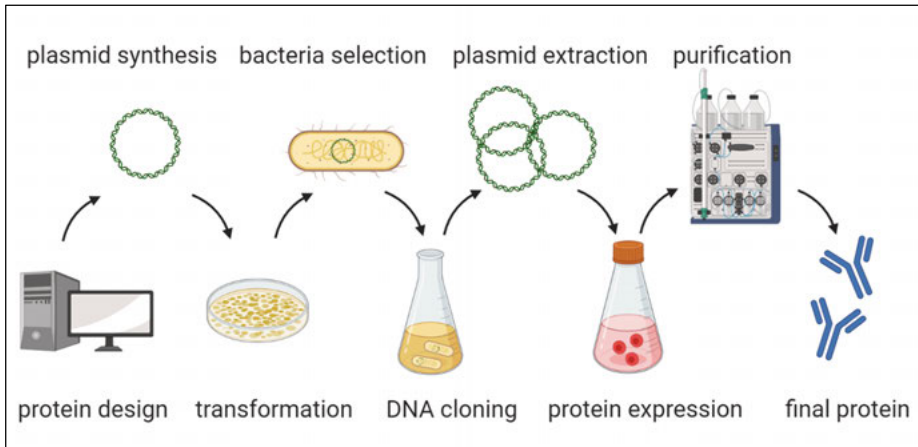


Figure 5. Steps for recombinant protein production.

The protein expression was performed in human Expi293 cells. The cells were transfected with the plasmids followed by 7-12 days incubation. Cell media containing the proteins were run over affinity columns to harvest the desired protein constructs. Proteins containing an Fc domain bind to Protein G in the column and can be eluted by a pH change. Histidine tags bind to nickel ion columns and can be eluted by high concentration of imidazole, that competes for the binding to the nickel. After concentration and buffer exchange, the proteins were ready to use (Figure 6).

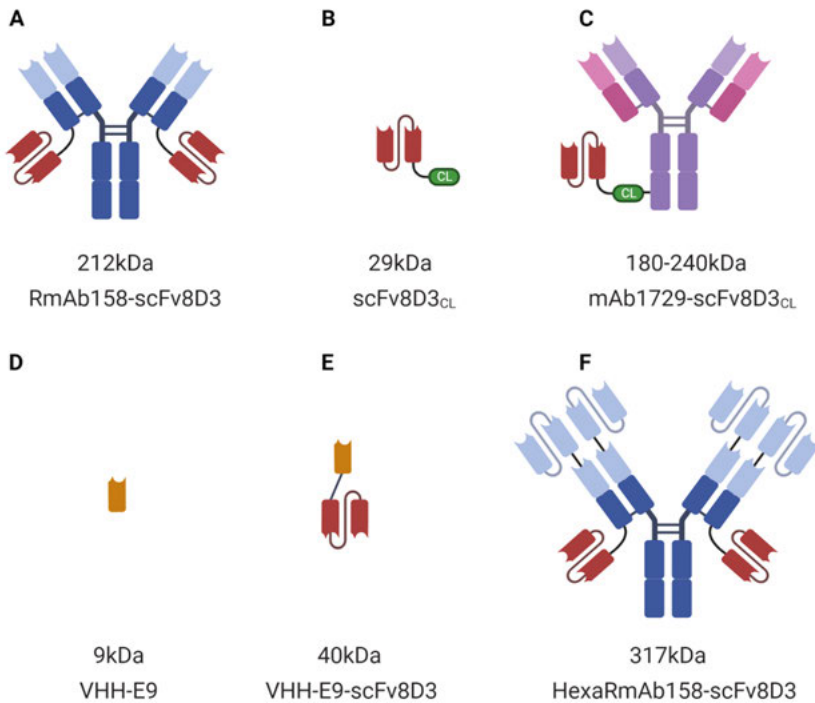


Figure 6. Expressed antibody/protein ligands in this work. **A:** The bispecific antibody based on mAb158 with scFv8D3 binding moieties for TfR binding (RmAb158-scFv8D3) was used as a radioligand in paper I, paper II and paper V. **B:** The scFv8D3_{CL} was expressed for conjugation to TREM2 antibody mAb1729 and applied in paper III. **C:** Chemically conjugated anti-TREM2 protein used in paper III. **D:** The anti GFAP nanobody VHH-E9 was explored as a radioligand in paper IV. **E:** VHH-E9 linked to the TfR binding moiety scFv8D3 was applied in paper IV for uptake and distribution in comparison to VHH-E9. **F:** HexaRmAb158-scFv8D3 was investigated as a radioligand, and in higher dosing, for treatment purposes. The protein was based on mAb158 but was expressed with additional 4 scFv moieties binding to A β .

Antibody conjugation

The amino acid sequence of commercially available antibodies is usually not accessible to the user due to economic interests of the supplier. Without knowledge of the sequence, recombinant expression of such antibodies for bispecific formats is not possible. An alternative is chemical conjugation of the different fragments. In **paper III**, a bispecific radioligand was synthesized from the commercially available anti-TREM2 antibody mAb1729 and the single-chain variable fragment (scFv) 8D3 (scFv8D3_{CL}) binding to TfR using a *trans*-cyclooctene (TCO) - tetrazine click-reaction. The click reagents were

linked to the proteins by N-Hydroxysuccinimid (NHS) ester binding to amines in amino acid residues. The scFv8D3 was expressed with an enriched lysine tail on its C-terminal end to increase site specific conjugation (double flag tag, 2x DYKDDDK). After the conjugation of the two fragments, the bispecific ligand was separated from free scFv8D3_{CL} by gel filtration.

Iodine-124/125 labeling

The bispecific proteins were labelled with radionuclides to quantify their concentrations in different tissues and compartments *in vivo*. To perform PET imaging, the constructs were labelled with iodine-124 (¹²⁴I) which has a half-life of 110.3h (4.18 days). The beta decay modes of ¹²⁴I are 74.4% electron capture and 25.6% positron emission. It is a suitable isotope for preclinical experiments involving PET, but due to a relatively long half-life and thyroid accumulation it is not appropriate for clinical use. Iodine-125 (¹²⁵I) decays by electron capture only and has a half-life of 1427.8 h (59.49 days). Due to its long half-life and low energy emission, it is a preferred isotope for radioimmunoassays. In our studies we primarily used it for the quantification of radioligand retention *ex vivo* in pilot experiments or to study pharmacokinetics (**paper I, III, IV and V**). ¹²⁵I was also used for SPECT imaging in **paper II**. Iodine labeling of proteins was performed by direct iodination of the phenolic ring of tyrosine residues [193]. The iodination is unspecific and can occur theoretically at any tyrosine residue including the antigen binding sites of proteins. Therefore, binding capacity of the labelled radioligands was compared to unlabeled constructs with *indirect* enzyme linked immunosorbent assay (ELISA).

Brain samples centrifugation

Regardless of *in vivo* scanning or *ex vivo* experiments, mice were transcardially perfused and the brains were removed at the end of the experiment. The brain tissue was divided into right and left hemisphere. The right hemisphere was immediately frozen on dry ice and saved for sectioning and further histological analysis. The left hemisphere was divided into different brain parts such as cerebellum and cerebrum (**paper I-IV**) or additionally into cortex and hippocampus (**paper V**). Radioactivity was measured in a gamma-counter. The brain parts from the left hemisphere were homogenized and extracted for quantitative analysis of disease related proteins with ELISA or MSD (Figure 7).

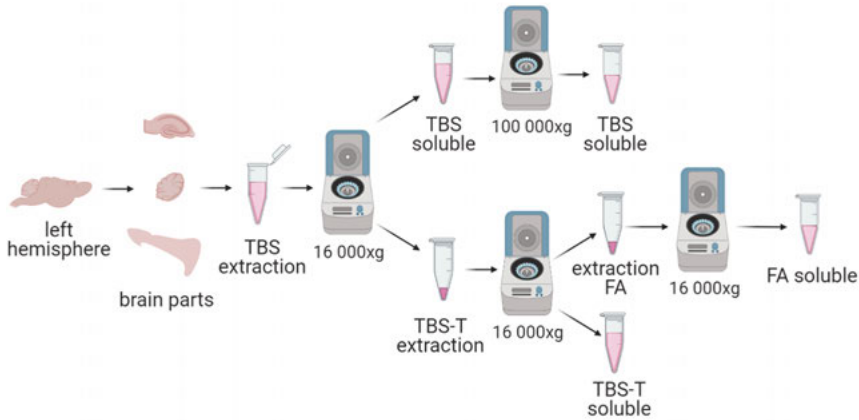


Figure 7. The left hemisphere was divided into the different brain parts e.g., cortex, hippocampus, cerebellum, followed by homogenization. TBS soluble A β was separated by centrifugation at 16 000xg. Smaller A β aggregates were separated in another centrifugation step at 100 000xg. The pellet from the first extraction was dissolved with TBS-tritonX100 to extract membrane bound and more diffuse A β . The separation was done by centrifugation at 16 000xg. The pellet was dissolved in 70% formic acid followed by another centrifugation step. This last fraction contained A β aggregated in plaques.

ELISA

ELISA is an assay that relies on antibodies binding to an antigen. A detection antibody is linked to a horseradish peroxidase (HRP) enzyme that catalyzes a color reaction which can be spectrophotometrically detected. The absorbance values, which are relative to the amount of bound antibody, can be determined and used for the quantification or estimation of binding affinities.

In this work, mainly *indirect* and *sandwich ELISA* was performed (Figure 8). *Indirect ELISA* was used to verify the binding capacity of bispecific antibodies after radiolabeling. Both binding sites of bispecific antibodies were investigated regarding the binding affinity to their antigens. Labeled and unlabeled constructs were compared to distinguish the influence of radiolabeling on binding affinity.

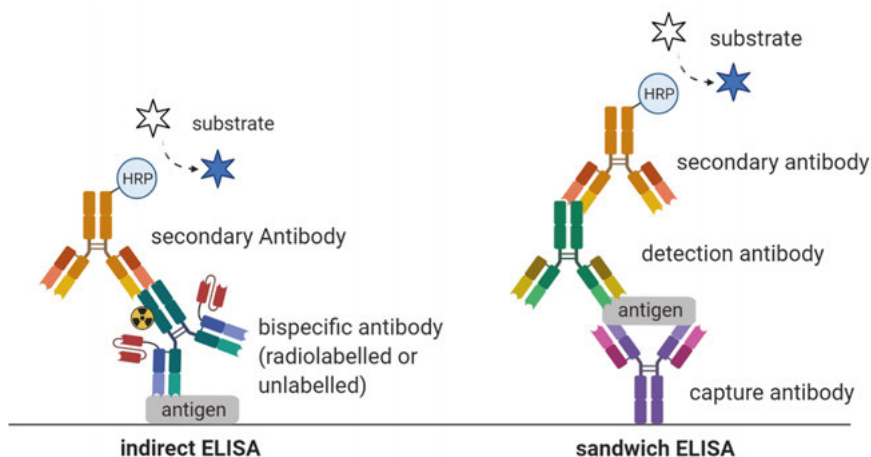


Figure 8. ELISA setups used in this work. Affinity of antibody-based ligands was compared before and after labeling with *indirect* ELISA. Protein levels in biological samples were analyzed with *sandwich* ELISA.

Sandwich ELISA was used to quantify the levels of specific proteins in biological brain samples. A 96-well plate was first coated with a capture antibody, which bound to the desired antigen in a sample. A detection antibody “sandwiches” the antigen by another binding. The secondary antibody is either coupled to a HRP directly or labeled with biotin that can be linked to streptavidin-HRP in an additional step. The color reaction with HRP was performed with TMB (3,3',5,5'-Tetramethylbenzidine) as a substrate. Soluble A β aggregates were quantified using homogenous sandwich ELISA. The same monoclonal antibody was used as a capture and detection antibody. The binding to the same epitope avoided the detection of monomers.

Immunohistochemistry

Immunohistochemistry (IHC) is an imaging technique to identify antigens, mostly proteins, in cell samples or on tissue sections (Figure 9). In this work, IHC was applied on brain tissue to identify A β , astrocytic or microglial markers. Paraffinized or fresh frozen tissue was sectioned and fixated on glass slides followed by antigen retrieval treatment steps. Primary antibodies against the antigen in the tissue were applied. Secondary antibodies linked to HRP or a fluorophore were then used to detect the primary antibodies. The signals were developed by a color reaction, if HRP was involved, and visualized by light microscopy. Fluorescent signals were visualized by confocal laser scanning microscopy. IHC was used for qualitative analysis in this work but can also be applied quantitatively.

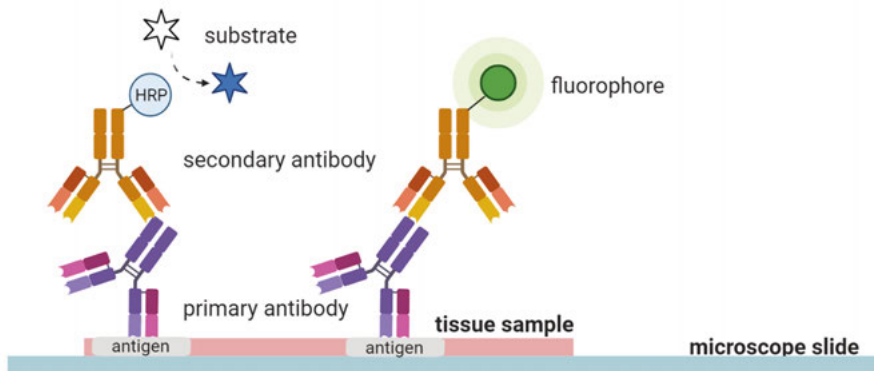


Figure 9. IHC setup on brain tissue samples applied in this work.

Autoradiography

Autoradiography is a visualization technique of a chemical component or protein labeled with radionuclides. Tissue sections with bound radioactive ligands were exposed to a phosphor screen in an x-ray cassette. The screen obtains a radiogram that can be digitized by a phosphor imaging system. In this work we investigated the distribution of the radioligands in the brain tissue at the terminal time point. Mice were intracardially perfused with isotonic NaCl solution to eliminate the radioactive signal from remaining blood. Brain sections with ^{124}I labeled ligands were exposed for around two half-times and ^{125}I labeled ligands were exposed for 2-3 weeks (**paper I-IV**). Autoradiography can also be applied *in vitro*, when tissue sections are dipped into solutions containing a radioligand (**paper IV**).

Nuclear track emulsion

Bound radioligand in the postmortem tissue can be visualized by nuclear track emulsion analysis (NTE). Compared to autoradiography, NTE has a higher resolution and is visualized by microscopy. The tissue can be stained with IHC to mark underlying structures. Radioligand binding and biological target structures can ideally be associated with each other (Figure 10). The chemical principal of NTE is fundamentally the same as general photographic emulsion. If silver halide crystals absorb light or ionizing radiation, clusters of silver ions are formed. Reducing these ion clusters to metallic silver, also called development, makes the exposure visible. A final fixation step removes the residual silver halide by leaving the metallic silver to form the image.

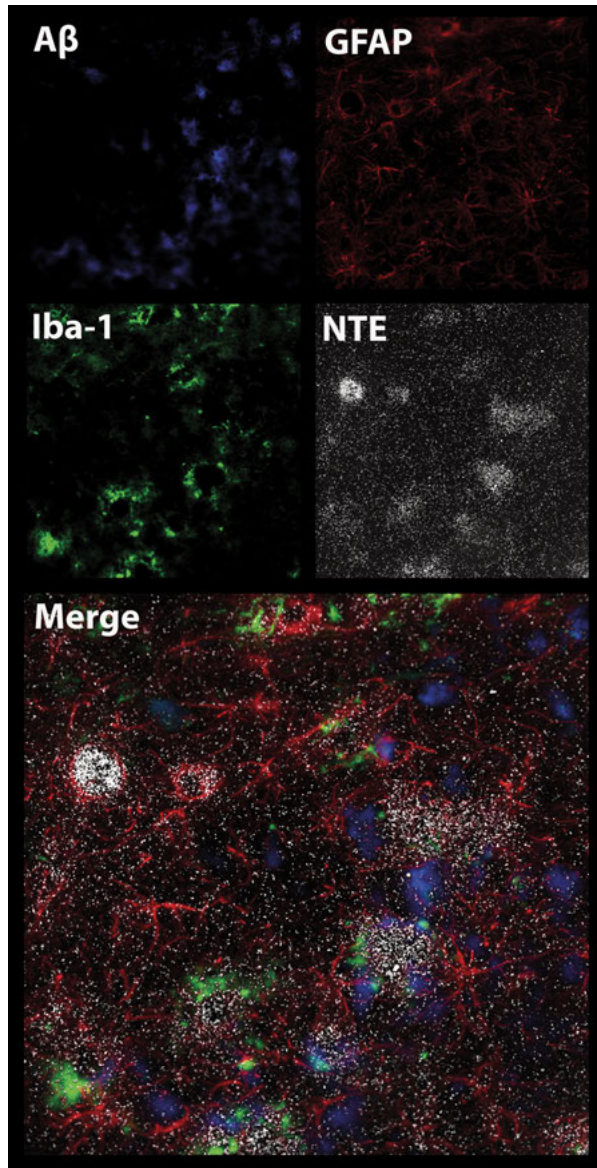


Figure 10. NTE shows the retention of a radioligand in the brain tissue of an AD mouse model. The tissue has been stained for A β (blue), GFAP (red) and Iba-1 (green) prior to NTE. White dots indicate radioligand binding in the tissue.

Aim

The aim of this thesis was to evaluate antibody-based PET imaging of soluble A β aggregates as a dynamic biomarker for AD focusing on quantification of effects of A β reducing treatments. In addition, we aimed to investigate the potential of novel antibody-based radioligands for imaging of AD associated neuroinflammation.

Paper I

To investigate whether the PET radioligand [124 I]RmAb158-scFv8D3 can be used to detect changes in brain A β levels during disease progression and after treatment with a BACE-1 inhibitor in ArcSwe mice with modest pathology resembling an early disease stage.

Paper II

To examine the potential of the antibody-based radioligand [124 I]RmAb158-scFv8D3 to detect changes in brain A β levels after treatment with a BACE-1 inhibitor in ArcSwe and the *App*^{NL-G-F} mice with established A β pathology resembling the clinical stage at which patients are diagnosed and enrolled into clinical trials. Further, the study aimed at comparing the antibody-PET read-out to the current clinical gold standard, [11 C]PiB-PET.

Paper III

To study sTREM2 brain levels in the Swe and ArcSwe mouse models. To investigate the ability to measure sTREM2 levels *in vivo* using a bispecific antibody as a radioligand.

Paper IV

To explore the potential of the anti-GFAP nanobody VHH-E9, without and with fusion to a TfR binder moiety. To investigate the *in vivo* brain delivery of the two proteins and their potentials as PET radioligands.

Paper V

To investigate the potential of the hexavalent, bispecific antibody construct hexaRmAb158-scFv8D3 as a radioligand and to study its eligibility for immunotherapy.

Results and discussion

Paper I

In **paper I** we investigated if antibody-based ligands against soluble A β can be used to detect decreasing A β levels caused by A β reducing treatments. As a model compound to achieve A β reduction we used the BACE-1 inhibitor NB-360 from Novartis. The compound has been reported to reduce A β in APP23 mice [194].

The study was divided in two experimental parts. In a first set, ArcSwe mice were investigated to follow disease progression *in vivo*. Animals between 7 and 16 months were injected with the radioligand [124 I]RmAb158-scFv8D3 and PET-scanned after 4 days. The brain tissue was analyzed after the scan with regards to brain A β levels. *Ex vivo* autoradiography of brain sections showed the spatial distribution of the radioligand in comparison to pathology visualized with IHC. The second part of the study focused on the A β reducing treatment. ArcSwe mice at the age of 10 months were treated for three months with food that was supplemented with BACE-1 inhibitor NB-360. The NB-360 group was compared to a vehicle group that received standard food without inhibitor. These groups were PET-scanned at the end of the treatment period. A third group was PET-scanned at the age of 10 months as a baseline group. A β levels in brain homogenates were determined and compared to PET results.

The concentration of [124 I]RmAb158-scFv8D3 in cortex and hippocampus increased with age in the 7 to 16 month old mice investigated with PET. The PET signal corresponded well with *ex vivo* autoradiography images, which showed the remaining radioligand in the tissue without the blood signal. A β 40 plaque staining was performed to visualize regions of A β 40 pathology. An overlay of the autoradiography and the A β 40 staining showed a high co-localization of A β pathology and [124 I]RmAb158-scFv8D3.

The brain concentration of [124 I]RmAb158-scFv8D3 in the NB-360 treated group was similar to the baseline group. A slight reduction was measured in the hippocampus of treated animals. However, the radioligand concentration was increased in cortex and hippocampus of vehicle animals compared to baseline and NB-360 treated animals. Postmortem brain tissue analysis of A β

in homogenates was in line with PET. Animals of the vehicle group had higher A β protofibril levels than those treated with NB-360.

In conclusion, antibody-based PET imaging of soluble A β protofibrils is a sensitive tool for following progression of brain A β pathology in ArcSwe mice. Further, we were able to use the novel antibody-based PET radioligand to visualize and quantify a reduction in brain A β achieved by BACE-1 inhibition.

Paper II

Paper II was a continuation of the studies carried out in **paper I**. Clinical studies with compounds aiming for a reduction of A β , such as BACE-1 inhibitors or immunotherapy, focus on an early stage of the disease to halt pathological processes before mature damage occurs. In **paper I** and other preclinical studies, the treatment intervention with NB-360 was initiated before major plaque formation started, probably mimicking a pre-symptomatic stage in humans [145,195,196]. However, an efficient and cheap screening for AD in the pre-symptomatic stage is not available today. Most AD patients are diagnosed at a rather late stage when also treatments, when available, will be applied. In this preclinical study we therefore focused on the detection of BACE-1 inhibition treatment effects at an advanced pathology stage.

Based on the pathology progression, studied in **paper I**, we selected ArcSwe mice at the age of 16 months for the start of BACE-1 inhibition treatment. At this age ArcSwe mice display abundant plaque pathology including large and dense-cored plaques. The study was expanded to include a second mouse model, the *App^{NL-G-F}*. Knock-in models lack the massive overproduction of A β PP and are claimed to closer mimic human pathology. Plaque formation starts at earlier age but is more diffuse and deposits are smaller in size compared to ArcSwe. *App^{NL-G-F}* animals were treated for 2 months between 8 and 10 months of age. Increased plaque load allowed to compare the antibody-based approach to the current gold standard for clinical A β imaging, [¹¹C]PiB. As in **paper I**, the study followed a cross-sectional design with three groups. NB-360 treated mice were compared to a vehicle and a baseline group. Each mouse was scanned with [¹¹C]PiB and within a week, by an antibody-scan with [¹²⁴I]RmAb158-scFv8D3 (ArcSwe) or [¹²⁵I]RmAb158-scFv8D3 (*App^{NL-G-F}*).

The main findings were that NB-360 treatment was effective in reducing TBS soluble A β aggregates in brain homogenates of ArcSwe mice. Especially levels of smaller soluble aggregates separated with 100 000xg were drastically decreased while the main component of the plaques, formic acid soluble

A β 40, was not significantly affected by the treatment. *In vivo* PET imaging with [124 I]RmAb158-scFv8D3 was able to quantify the decreased levels of soluble A β aggregates in hippocampus and thalamus. The A β changes were not quantifiable with [11 C]PiB. [11 C]PiB binds to the inner core of the plaques, which were not affected by the treatment according to ELISA measurement in the formic acid extractions.

App^{NL-G-F} mice had a similar reduction of soluble TBS A β aggregates separated with 100 000xg after BACE-1 inhibition as ArcSwe mice. TBS soluble A β aggregates separated with 16 000xg were not affected. However, plaque associated, formic acid soluble A β was somewhat decreased in the NB-360 group. SPECT imaging with [125 I]RmAb158-scFv8D3 was able to quantify a reduction of the radioligand concentration in treated mice in hippocampus, cortex and cerebellum compared to vehicle treated mice. This stands in contradiction to soluble A β aggregate levels in brain homogenates, that were not decreased as measured by ELISA, and that should be the main target of the radioligand. Although RmAb158 displays 10 times lower affinity towards fibrils than to soluble aggregated A β , we probably imaged insoluble A β to a certain extent. Insoluble A β is more frequent in comparison to TBS soluble A β . In **paper V** we investigated the retention of HexaRmAb158-scFv8D3 in the different fractions of brain homogenates and results suggested that a substantial fraction of the radioligand bound to insoluble A β three days after injection.

Quantifications with [11 C]PiB imaging did not show a difference in treated compared to vehicle animals. Low activity in A β associated brain regions assessed with autoradiography suggested low specific binding of [11 C]PiB to A β in the *APP*^{NL-G-F} mouse model.

In conclusion we were able to demonstrate that antibody-based PET imaging can detect the effects of BACE-1 inhibition treatment in ArcSwe as well as in the *App*^{NL-G-F} mice with established A β pathology. The read-out of [11 C]PiB was different and did not reflect the biochemical changes in A β levels due to treatment.

Paper III

The concept of PET imaging based on antibodies in combinations with TfR-mediated transport over the BBB has been investigated intensively by our group since 2015 [98–100,104,196]. However, the successful approach focused on A β as a target in brain tissue as in **paper I-II**. In this study we explored a ligand against TREM2.

Levels of TREM2 in the ArcSwe and Swe mouse brain indicated an upregulation of the receptor in the transgenes compared to wild type. We found that TREM2 levels increased with age in ArcSwe animals and correlated with soluble A β aggregates, similar to levels reported in PS2APP mice [197]. Swe animals at the age of 18 months had increased levels in comparison to age-matched wild type but lower levels than age-matched ArcSwe.

In a next step we investigated the ability to follow those upregulated levels *in vivo*. We created a bispecific radioligand composed of the TREM2 binding antibody mAb1729 and the TfR binder scFv8D3 by chemical conjugation. The resulting ligand, mAb1729-scFv8D3_{CL}, was radiolabeled with ¹²⁵I and administered to transgenic and wild type mice. The brain uptake was studied after 2 h in wild type mice and was in the range of other chemically conjugated bispecific constructs with 8D3 [98]. Brain retention assessed *ex vivo* after 24 h was increased in both transgenic mouse models compared to wild type, but not after 72 h. Autoradiography confirmed binding in cortex, thalamus, and hippocampus where activated microglia is located. NTE in combination with IHC showed that the binding of the ligand was located close to activated microglia in A β plaque associated areas. Brain concentrations of mAb1729-8D3_{CL} were investigated with *in vivo* PET imaging. ArcSwe animals, 18 months old, were compared to wild type at 24 h, 48 h and 72 h post injection of [¹²⁴I]mAb1729-8D3_{CL}. PET was not able to quantify a difference between ArcSwe and wild type at a single timepoint, i.e. at 24 h, 48 h or 72 h, but the total brain exposure, calculated as the area under the concentration curve (AUC) based on all three PET scans was higher in ArcSwe compared to wild type. We speculate that an antibody with higher affinity towards TREM2 could improve the possibility to distinguish wild type and ArcSwe also with a single PET scan.

Paper IV

In **paper IV** we investigated a nanobody against GFAP for PET imaging of astrogliosis. Nanobodies have pharmacokinetic advantages due to their size. Kidney clearance allows for faster removal from the blood compartment compared to full sized antibodies that show limited renal clearance. Therefore, the time between injection and imaging can be reduced. GFAP is a widely used marker for reactive astrocytes *ex vivo* in connection with amyloidosis. However, GFAP is an intracellular protein and a potential radioligand must cross the cell wall of astrocytes to reach the target. This is an additional hurdle besides the BBB. The anti-GFAP nanobody VHH-E9, obtained from alpacas, has been reported to cross the BBB due to its basic isoelectric point (PI). Further, cell membrane penetrating properties have also been reported for the nanobody. The construct has also been suggested for imaging purposes [198–200]. However, the previous studies performed in mice used very high dosing. The VHH was injected in the carotid artery (4–25 mg/mouse) or the tail vein (2 mg/mouse). In **paper IV** we investigated the potential of VHH-E9 with reasonable dosing (5–10 $\mu\text{g}/\text{mouse}$) for imaging and compared its uptake when linked to scFv8D3 for active transcytosis.

We recombinantly expressed three constructs: VHH-E9, VHH-E9-scFv8D3 and scFv8D3. The brain uptake of VHH-E9 was around 0.17% of the injected dose (%ID/g) at 2 h post injection, which is higher than conventional IgG antibodies. Nevertheless, VHH-E9-scFv8D3 and scFv8D3 had a two times higher uptake at 2 h. Brain retention of VHH-E9 and VHH-E9-scFv8D3 was studied at 8 h, 24 h and 48 h after injection in ArcSwe mice with progressed gliosis. Neither of the constructs displayed increased brain concentration compared to wild type. Different reasons may apply for the negative outcome. The fraction entering the astrocytes may be small and followed by a fast degeneration mechanism. As a non-residualizing radionuclide, ^{125}I will be eliminated from the cell when cleaved off from the antibody.

The study showed that brain uptake of nanobodies is increased compared to IgG antibodies and that significantly higher brain concentrations can be achieved if combined with active transcytosis, potentially high enough for imaging. However, the study concluded that intracellular targets may be difficult to image to a sufficient extent with antibody-based ligands.

Paper V

HexaRmAb158-scFv8D3 is a newly designed bispecific protein based on the protofibril specific mAb158 and scFv8D3 for TfR mediated brain uptake (Figure 6F). The construct has four additional binding sites for A β promoting increased binding avidity. *In vitro*, strong binding to oligomers and protofibrils has been reported[201]. Furthermore, the smaller distance between the binding sites enables binding to smaller sized oligomers compared to conventional mAb158 [201]. In **paper V**, we investigated the potential of the construct for PET imaging and immunotherapy *in vivo*. Low (0.05 mg/kg) and high dosing (2 mg/kg) were compared to RmAb158-scFv8D3 for acute brain uptake in wild type and long-term retention in *App*^{NL-G-F} mice. At low dosing, we found that the brain uptake of [¹²⁵I]HexaRmAb158-scFv8D3 after 2 h was around 1.1%ID/g, and thus, slightly lower than RmAb158-scFv8D3 with 1.5 %ID/g. RmAb158-scFv8D3 also showed greater uptake when administered at higher dosing. These results are in line with results obtained with *in vitro* cell based transcytosis assay. We suggest that decreased HexaRmAb158-scFv8D3 uptake is caused by the bigger size of the construct and probably a steric hindrance of the TfR binding site by the additional A β binding sites.

Probably because of greater uptake, RmAb158-scFv8D3 had higher brain retention after three days compared to HexaRmAb158-scFv8D3, when injected at tracer dosing. Levels of RmAb158-scFv8D3 were increased in all age groups (2.5 months, 5 months, and 9 months) of *App*^{NL-G-F} mice.

Therapeutic potential of both ligands was evaluated in 5-month-old *App*^{NL-G-F} mice. Neither RmAb158-scFv8D3 nor HexaRmAb158-scFv8D3 showed a reduction of soluble or insoluble A β species in brain homogenates. Negative outcome can be connected to dosing since the administration of 5 mg/kg has previously shown a substantial reduction of soluble A β in ArcSwe mice [202]. Further, aggregation processes may be different in *App*^{NL-G-F} compared to ArcSwe mice.

Conclusion and future perspectives

In vivo PET imaging in AD has come a long way since the development and first use of [¹¹C]PiB for imaging of A β pathology in the early 2000s. The traditionally used brain radioligands based on small molecules are lipophilic and therefore ideal for entering the brain and reaching their target. However, sometimes these radioligands have low specific binding, which can lead to a nonspecific PET signal. When it comes to AD, established radioligands such as [¹¹C]PiB do not recognize all forms of A β .

Antibodies are highly specific for their antigen and have previously shown great potential for imaging of A β in the brain [104]. In this work, we demonstrate that this concept can be used in animal models of AD to follow disease progression and amyloid reduction following BACE-1 inhibition at both early and advanced stages of the disease (**paper I-II**). Further we investigated the potential of A β imaging and immunotherapy with an antibody-based construct with increased binding avidity (**paper V**).

The principle of bispecific antibodies as radioligands for PET may allow for *in vivo* monitoring of other specific targets and disease mechanisms in the brain besides A β . Neuroinflammation as a consequence of AD pathology is intensively investigated and discussed in the field. In **paper III** we investigated the potential of TREM2 as an imaging target for activated microglia with PET. We were able to quantify differences in radioligand concentrations *ex vivo*. However, the differences were too small for quantification with antibody PET. This is probably due to the fast cleavage of the receptor from the cell surface. Another hurdle for protein-based radioligands may be cell membranes which deny access to intracellular targets as shown in **paper IV**. Radioligands must be able to cross such barriers to a relevant extent for imaging of intracellular targets. Further, the uptake into cells may be followed by a fast degeneration mechanism.

Antibody delivery to the brain for diagnostic or therapeutic purposes faces the challenge of crossing the BBB. In this thesis we have demonstrated that the use of facilitated delivery, such as TfR mediated transcytosis, can enable antibody-based brain PET imaging. However, it should be kept in mind that the successful concepts demonstrated in the preclinic cannot be directly translated to humans since the human TfR differs somewhat from the murine TfR.

Intense research is ongoing regarding this limitation. Roche and Denali Therapeutics are currently developing human TfR binders for brain delivery [93,94,203,204].

In addition to development of human TfR binders, the pharmacokinetic properties of antibody-based radioligands should be improved to enable their use in a clinical environment. Antibody-based radioligands developed so far need a circulation and elimination time of at least 8-12 h before PET can be performed. Currently studied pre-targeting strategies could be an alternative approach for antibody-based ligands.

Finally, the radioisotope ^{124}I , as used in this work, is not suitable for humans since it accumulates in the thyroid. The long half-life of four days would further lead to high exposure of radioactivity to the human body. More clinically compatible radiolabeling methods for brain penetrating antibodies, such as ^{18}F labelling or the use of radiometals, are under development [205].

Popular science summary

The most common form of dementia is Alzheimer's disease (AD). With increasing life expectancy, it is expected that in the year 2050, 152 million people will be affected by the disease. Neurodegeneration leads to reduced comprehension and memory, unpredictable behavior, aphasia, disorientation, hallucinations, and psychosocial impairment. Besides general atrophy of the brain, extracellular A β plaques and intracellular neurofibrillary tangles are the main histological hallmarks. As a consequence of misfolded and aggregated proteins, activated microglia and astroglia react with a neuroinflammatory response, which may contribute to disease progression and severity.

Disease progression is slow and neuropathological changes are on-going for decades before cognitive symptoms occur. Neuropsychological diagnosis relies on cognitive tests, cerebrospinal fluid analysis and brain imaging using magnetic resonance imaging or positron emission tomography. Unfortunately, there is no treatment available that stops the underlying causes of the disease. The development of new treatments has been focused on preventing the overproduction or removing of A β from the brain. Potential treatments, such as secretase inhibitors or immunotherapy, have so far not been effective or suffered from side effects. However, new treatments are under development and many compounds enter clinical trials every year. A major challenge for drug development is to prove efficacy of drug candidates since measurable cognitive changes are slow.

PET can provide insights into *in vivo* mechanisms and changes related to the disease. The development of [^{11}C]PiB enabled for the first time the visualization of AD related pathology in the brain. Still, the radioligand has limitations. The signal saturates early during disease progression since A β plaques do. Moreover, the plaque load is a rather undynamic out-come measure after treatment intervention. The development of new treatments against AD requires sensitive and dynamic imaging compounds to follow changes in pathophysiology.

This work explores new concepts of A β PET imaging using antibody-based radioligands. The advantages of antibodies compared to smaller molecules are that they usually are very specific for their targets, and that they bind with high affinity to proteins such as for example soluble A β aggregates. The capillary walls of blood vessels in the brain are impermeable to many substances, such as antibodies, to protect the brain tissue from harmful molecules. Supply of essential nutrients over this blood-brain barrier is therefore often provided via

selective active uptake. In our antibody-based PET approach, we used a Trojan horse strategy to infiltrate the brain by taking advantage of the receptor normally transporting iron into the brain, i.e. the transferrin receptor.

This thesis shows the potential for such radioligands at a preclinical stage. We showed that antibody-based ligands transported into the brain with the transferrin receptor are very sensitive and have the capacity to follow progression of A β pathology and quantify treatment effects. We were able to detect a difference between treated and untreated subjects when the treatment was applied at an early stage, when A β plaques, the hallmark of AD, just started to form, but also at a late stage, when A β plaque pathology was abundant.

Furthermore, we investigated whether antibody-based PET imaging can potentially be applied to follow inflammation in the brain. We showed that TREM2, a marker for inflammation, was abundant in brains of mice with AD-like pathology and that TREM2 levels were detectable with a radioligand in isolated brain tissue, but not in the living brain. Development of antibody-based radioligands for other targets than abundant protein deposits such as A β is challenging, probably due to short half-lives of the targets in the brain and restricted access to them.

In the last years, preclinical research using antibodies with active uptake into the brain has shown great potential for imaging and immunotherapy. However, further steps are needed to translate the approach for use in humans, e.g. the development of a protein that can transport antibodies across the human blood-brain barrier and radiolabeling with clinically appropriate radioisotopes.

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I came to the Molger Lab in January in 2015 for my Master's thesis project. We had an introduction meeting in the first days with my supervisors Stina and Dag about the scientific background and the design of the project. I vaguely remember a discussion about A β PP fragments that may influence the notch pathway. Dag stated that he doesn't know and this is not really known in general. Coming from 3.5 years of pharmacy studies basically just learning stuff by heart or performing designed lab experiments for students, my first thought was: What, how do you mean unknown? How can you not know? In the following days and months, I learned that everything was about not knowing, and finding out. This fascinated me and maybe for the first time in my studies I was challenged, excited, and had fun at the same time. In the following years I learned a lot about research, scientific thinking and about failure and success. I am grateful that I got the opportunity to do this work abroad and would like to thank everyone that has been involved in the amazing journey of the last 4.5 years.

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