Chapter 12

Summary and General Discussion

Summary

Alzheimer's disease (AD) is the predominant form of dementia in the aging population and its increasing incidence represents an important socio-economic and public health concern. The hallmarks of this disease, amyloid plaques and neurofibrillary tangles, are thought to develop early in the disease pathogenesis, up to decades before first clinical symptoms occur. However, these pathological hallmarks are still difficult to detect *in vivo*, and therefore a definitive diagnosis can only be made post-mortem. A clinical imaging technique or biomarker capable of visualizing and quantifying amyloid plaques and associated early changes thus may enable an earlier diagnosis, better understanding of the pathophysiology and eventually aid therapy development. The work presented in this thesis aimed to develop innovative diagnostic imaging techniques to detect the histological signatures of AD using emerging ultra-high field MRI technologies (**Part One**) and molecular imaging strategies (**Part Two**).

Detection of Aβ plaques by MRI has previously been demonstrated both *in vivo* in several transgenic AD mouse models and in *ex vivo* human AD brain tissue. The high magnetic field strengths needed to obtain these results only recently became available for *in vivo* human use. These high field human MRI systems (≥7 Tesla) offer new possibilities to specifically detect these neuropathological hallmarks, perhaps even at an earlier stadium than the traditional MRI biomarker of brain atrophy.

Part One of this thesis explores the correlation between intrinsic AD-induced MRI contrast changes and the underlying neuropathological changes, which is an essential step in the development of native contrast MRI techniques for diagnostic purposes.

After a brief general introduction into MRI (**Chapter 2**), we addressed the usability and limitations of post-mortem brain tissue for the development of novel MRI methods for AD (**Chapter 3**). Although post-mortem brain tissue is often exploited for this purpose, we strongly advise the use of formalin fixed brain tissue with fixation times less than two years, because prolonged fixation times induced destruction of normal brain tissue visible as hypointense imaging artifacts on MRI.

Correlating MRI contrast changes at high magnetic field strength to its underlying pathologic substrate is not straight-forward, due to the intrinsic differences in resolution between the techniques, and tissue deformation during processing. To solve these issues we designed a simple inductively-coupled microcoil setup that allowed us to obtain MRI images directly from a single 60 µm thick histological section that could be stained histologically afterwards, thereby allowing a direct comparison between the MRI and histological data (**Chapter 4**). The design can easily be integrated into any MRI system using existing commercial hardware while improving the signal-to-noise ratio. Initial application on human AD brain tissue demonstrated that human Aβ deposits indeed correspond to signal hypo-intensities on T_2*-weighted MRI.

Previous studies in the literature had suggested that Aβ deposits, irrespective of associated iron, might induce detectable MRI contrast. The direct MR microscopy of histological sections offered a valuable tool to investigate the MR properties of the different forms of human cerebral Aβ deposits (**Chapter 5**). We demonstrated that only when Aβ is deposited in its fibrillar amyloid conformation, a decreased signal can be observed on T_2* and T_2-weighted MRI. In addition, direct visualization of cerebral amyloid angiopathy (CAA) is possible, although signal attenuation

was not observed in all CAA-affected vessels. With regard to the cerebral presence of Aβ, this would imply that MRI would depict the cerebral fibrillar amyloid load rather than the amount of cerebral Aβ peptides.

In **Chapter 6** we examined changes in cortical appearance of AD patients using susceptibility weighted 7T MRI *in vivo*. Similar changes on MRI were found in post-mortem tissue of AD patients versus controls, which could be correlated to the histological changes found in the same tissue. On MRI, diffuse hypointense bands were frequently found in the cortex of the frontal lobes of AD patients (57%), but they were not observed in healthy age matched control subjects. Further histologic correlation revealed that the pattern of the susceptibility-weighted contrast in the cortex of AD patients does not primarily co-localize with amyloid plaques or neurofibrillary tangles, but with microglia- and myelin-associated iron accumulation and with an altered myelin cytoarchitecture. The current findings show great promise for the *in vivo* detection of the underlying pathological changes in AD. Nevertheless, future work should focus on the specificity of these findings for AD versus other neurodegenerative diseases.

Part Two is focused on the development of targeted contrast agents to allow the specific *in vivo* detection and visualization of different Aβ deposits. In general *in vivo* application of these molecular imaging strategies to study or diagnose neurodegenerative diseases, especially with regard to MRI, depends on the ability to enter the brain as well as the characteristics of the available imaging ligand, as is explained in **Chapter 7**.

Previously, we have selected heavy chain antibody fragments (V_HH) with high affinity specific for either cerebral amyloid angiopathy (CAA) or all types of human Aβ deposits. Derived from the Camelid heavy chain antibody repertoire, which completely lack light chains, their single N-terminal domain (V_HH) is fully capable of antigen binding with affinities comparable with those of conventional antibodies. The potential of V_HH as *in vivo* imaging agent to assess CAA and cerebral amyloid load hinges on their ability to enter the brain, and in **Chapter 8** we tested their ability to cross the blood-brain barrier (BBB) using an *in vitro* BBB system. V_HH ni3A showed the highest transmigration efficiency, which is to a large extent due to 3 different amino acid in its N-terminal domain, and occurred by means of active transport.

As a next step, in **Chapter 9** the *in vivo* properties of two V_HH, ni3A and pa2H, were assessed in transgenic APPswe/PS1delta9 mice. Following rapid renal clearance only ^{99m}Tc-pa2H showed a small yet significant higher cerebral uptake in the APP/PS1 animals one day post-injection. After circumventing the BBB, the *in vivo* specificity for Aβ was confirmed for both fluorescently labeled V_HH, where pa2H remained readily detectable for 24 hours or more after injection. Both V_HH showed affinity for parenchymal and vascular deposits, this in contrast to human tissue, where ni3A specifically targeted only vascular Aβ. Brain uptake as yet is too low for direct *in vivo* imaging, however this study provided evidence that V_HH detect Aβ deposits *in vivo* making them promising tools for further development.

A completely different approach is presented in **Chapter 10** in which we synthetized several novel fluorinated bis-styrylbenzenes to serve as potential ^{19}F MRI contrast agents and studied their fluorescent and Aβ binding characteristics. Ideally these small organic molecules carrying normal fluorine might allow direct MR imaging of the amyloid binding compound, without any endogenous background signal. Most compounds showed a high affinity for both murine and human amyloid. Interestingly, they bind to a different binding site than chrysamine G, one of

the parent compounds from which our bis-styrylbenzenes were derived. Despite their high logP values, many bis-styrylbenzenes were able to enter the brain and label murine amyloid *in vivo*. Initial post-mortem ^{19}F NMR studies using the most promising compound unfortunately showed that these compounds as yet do not warrant further MRI studies, mainly due to the severe reduction of the ^{19}F signal in the environment of the brain.

Chapter 11 presents a similar study in which we synthesized six bis-pyridylethenylbenzenes based on the assumption that the incorporation of nitrogen might lower the hydrophobicity of the previous compounds and perhaps thereby further improve their amyloid binding characteristics. These compounds did also not share a binding site with chrysamine G. With a logP value in between 3 and 5, most bis-pyridylethenylbenzenes were able to enter the brain and label murine amyloid *in vivo*. Based on its in and *ex vivo* binding characteristics and its solubility, bis(4-pyridylethenyl)benzene showed the most promise to serve as a novel amyloid-targeting backbone for amyloid imaging using fluorescence or for further development, e.g. as a PET tracer.

General discussion

The current view on Alzheimer's disease (AD) is that the neuropathological changes start two decades before the clinical symptoms occur.[1] The cognitive decline associated with AD, represents a late stage of the disease when neurodegeneration is extensive and therapeutic interventions may be too late. The search for new imaging methods is driven by the promise that earlier diagnosis, preferably before overt dementia is present, may help to identify patients early enough to benefit from future treatments.

Diagnostic biomarkers: Current status

Divided into two main categories, the most extensively characterized biomarkers for AD reflect either cerebral amyloid load or neuronal injury and degeneration. (**Table 12.1**) It is due to the increasing knowledge and understanding of the disease obtained by biomarker research that only recently, almost thirty years after its first publication, the clinical diagnostic criteria of AD as set by the NINDS-ADRDA have finally been revised. According to these new criteria a possible diagnosis of AD is no longer one of exclusion, solely based on clinical symptoms of dementia or mild cognitive impairment (MCI). The new criteria include the presence of one or more of the following five biomarkers: medial temporal lobe atrophy, abnormal cerebrospinal fluid levels of $A\beta_{1\text{-}42}$ or tau, or specific patterns of glucose metabolism or Aβ ligands as detected by PET neuroimaging. Furthermore, a new set of criteria has been added to stage pre-clinical AD only based on positive biomarkers, but without clinical symptoms.[2]

The revised criteria are based on the hypothetical dynamic biomarker model for AD, as initially proposed by Jack *et al*.[1] With new biomarker data continuously being gathered this model requires constant revision. According to this model the different biomarkers change in a specific temporal order and it is assumed that the accumulation of cerebral amyloid is one of the earliest and leading events. Many independent cross-sectional observations are in line with this model. However, the exact order in which biomarkers change over time and the relationship of these

changes with the clinical outcome is currently the main topic of many ongoing longitudinal studies. Recently, the initial results of one of the largest prospective biomarker studies, the Australian Imaging, Biomarkers & Lifestyle Study of Ageing (AIBL), again affirmed the model.[3] In this study, researchers retrospectively compared cerebral atrophy and amyloid load at baseline with respect to the revised clinical criteria for (pre)clinical AD. Based on amyloid imaging as a predictor for AD its outcome again suggested a disease onset of twenty years or more before noticeable clinical symptoms.

Meanwhile a recent revision of the model by Jack and his colleagues confirmed the typical sigmoid shape of the changes of each marker over time but also adjusted their temporal order. CSF-Aβ now is the first biomarker to change prior to amyloid detected by PET imaging. Subsequently, tau can be detected in CSF, and finally FDG PET changes and cerebral atrophy appear simultaneously.[4] (**Figure 12.1**) In addition, an initial attempt was taken to include cognitive or so-called brain reserve based on intelligence, education, occupation and leisure activities into the model to highlight the existing individual differences in clinical presentation with respect to the level of their biomarkers. Although Aβ is still considered to be the major hallmark as well as an important initiating factor in the progression of the disease, there is increasing evidence that other primary or interacting causes exist, e.g. the accumulation of tau or changes in myelin and iron metabolism.[5-7] As these mechanisms may act alongside Aβ accumulation or even further upstream in the disease process, it is important to continue improving Aβ biomarkers but also to investigate new possible biomarkers that may project brain reserve or any of the aforementioned alternative mechanisms of disease, such as tau-targeting PET agents or novel MRI techniques.

In this thesis, we aimed at developing improved imaging biomarkers for AD following two approaches: development of novel techniques to detect AD pathology using MRI (**Part One: Chapter 3 - 6**), and development of new Aβ- or amyloid-binding imaging probes (**Part Two**) based on either llama antibody fragments (**Chapter 8 and 9**) or small amyloid binding molecules (**Chapter 10** and **11**).

MRI-based biomarker research

Table 12.1 Classification of AD biomarkers *(adapted from Jack et al.[16])*

Biomarker	Type
Amyloid accumulation	
CSF $A\beta_{1-42}$	**CSF Analysis**
Amyloid PET	**Imaging**
Neurodegeneration	
CSF tau (total and phosphorylated)	**CSF Analysis**
Structural MRI	**Imaging**
FDG PET	**Imaging**

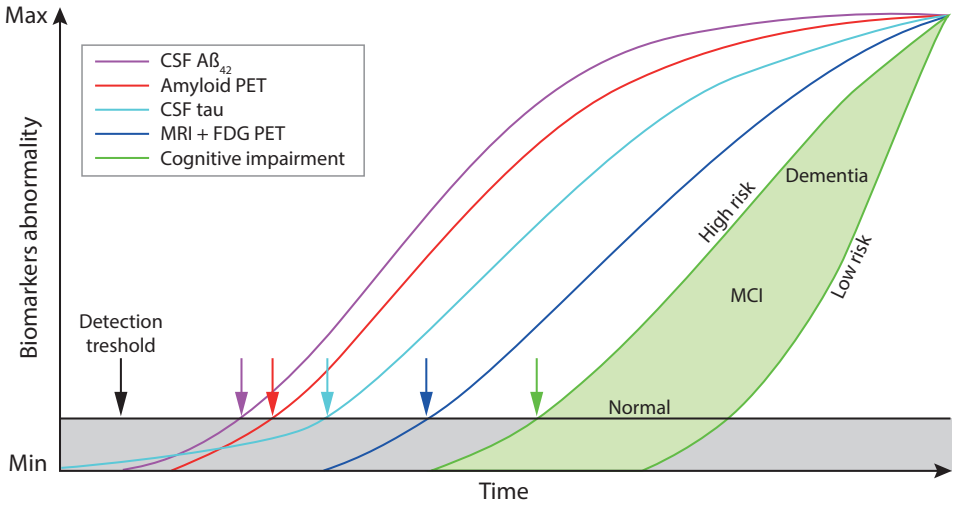

Figure 12.1 The revised dynamic biomarker model *(reproduced from Jack et al.[4])*
Pathophysiological changes that are below the detection threshold are visualized by the grey area. At a subthreshold level tau pathology is thought to precede Aβ deposition. Based on the current diagnostic techniques the detection of Aβ rises above the threshold first (*purple and red arrows*). As this accelerates tauopathy then comes CSF tau (*light blue arrow*). Later still, functional and metabolic biomarkers appear abnormal as seen by FDG PET and MRI (*dark blue arrow*). At last, a wide range of cognitive impairment becomes evident (*green arrow*) depending on the individual's risk profile (*light green-filled area*).

In AD patients structural MRI studies revealed specific patterns of regional cerebral atrophy.[8] Severity of atrophy was observed to correlate well with loss of cognitive function as well as with post-mortem Braak staging. Of all putative biomarkers, volumetric measures of hippocampal and medial temporal lobe atrophy showed the best correlation with the severity of clinical symptoms, and has therefore been incorporated as one of the five biomarkers in the revised diagnostic criteria. According to the dynamic marker model, however, brain atrophy is a late stage event, and therefore structural MRI may not be the method of choice for early or pre-symptomatic diagnosis.

As for other neuropathological hallmarks of AD, the development of MRI-based biomarkers has mainly focused on the detection of cerebral amyloid. Initial pre-clinical studies were able to detect individual amyloid plaques, as they induce a detectable decrease in T_2* or SWI signal due to the accumulation of iron or the protein aggregate itself.[9-11] The work in this thesis not only replicated these findings, but also showed that only the functionally relevant fibrillar amyloid induces detectable changes in MRI signal, and not just the presence of Aβ peptide. A good understanding of the neuropathological substrate of changes on MRI is important for further development and interpretation of novel MRI biomarkers for AD. However, despite the arrival of novel high magnetic field (≥7 Tesla) human MRI systems, it is not to be expected that direct detection of individual amyloid plaques by MRI is feasible in the near future. This is due to imaging artifacts based on physiological movements of patients, limited scan time, and the resulting limited resolution that can be obtained when scanning patients *in vivo*. As discussed in **Chapter 5**, although for the time being individual plaques may remain beyond *in vivo* detection the early and subtle changes in MRI signal due to cerebral amyloid accumulation might be

exploited by quantitative MRI methods. However, quantitative parameters reflecting bulk T_2 and T_2* signal in a region of interest will not only reflect cerebral amyloid, but also other tissue changes. In addition to amyloid, brain iron not directly associated with plaques and myelin also generate local variations in magnetic susceptibility, especially at high magnetic field strengths.[12] High-field (≥ 7 Tesla) human MRI systems therefore show great promise in visualizing new iron- and myelin-driven contrasts in the human brain.

The findings in **Chapter 6** provide convincing evidence that myelin structure and iron distribution in the cortex are disturbed in AD patients. Furthermore, our findings suggest that these changes, detected on SWI images at 7 Tesla, may allow distinguishing "probable AD" subjects and healthy age-matched controls. Interestingly, changes in iron metabolism and myelin breakdown have both been mentioned as potential upstream mechanisms for AD.[5,13] The ability to visualize these changes *in vivo* by MRI thus creates a putative novel biomarker with the potential to reflect a yet underappreciated change that may accompany the progression of AD. As discussed previously, further studies are required, comprising larger patient series as well as patients with different types of dementia. And also several additional questions should be answered, such as whether the presence of cortical changes is associated with cognitive dysfunction, whether the changes appear early in the course of AD, and whether the frontal lobes are the best location to detect these changes.

An important but still poorly understood mechanism that also requires further investigation is the influence of the normal ageing process on SWI images at 7 Tesla. Previous histopathological studies already pointed out that the cortical distribution of myelin not only differed between cortical regions but it was also subject to changes during life.[5] Whether a similar relationship exists between cortical iron and age has not yet been shown, but given its effect on MRI signal it has become essential to gain a better understanding of the role of cortical iron accumulation in normal brain aging. Full characteristics of cortical myelin and iron distribution over the lifespan starting from as early as the mid-twenties serves as a solid basis for the comparison between normal brain ageing or alterations of cortical myelin and iron due to AD or other neurodegenerative diseases.

Besides its novel *in vivo* finding, our work described in **Chapter 6** also demonstrates a new approach to study neurodegenerative diseases using post-mortem material. Thus far most neuropathological MRI studies have attempted to investigate the histopathological substrate corresponding the observed MRI contrast using MR images with almost microscopic resolution to allow direct and detailed comparison of the histology, similar to the work described in **Chapters 3 - 5**. Although full understanding of this relationship is of crucial importance for further development of clinical techniques, the direct translation of these microscopic findings to clinical MR images that are acquired at a much lower resolution remains however difficult and understudied.

In **Chapter 6** we started with a comparison of the post-mortem MR images acquired at a low or almost clinical resolution alongside their histological sections on a mesoscopic scale rather than directly focusing on the microscopic MRI-histology correlation using the high resolution images. Based on visual inspection we aimed to examine whether any of the applied stainings expressed a pattern or distribution similar to that observed on the clinical and low resolution MRI. In contrary to normal pathological examination, this analysis of the histological data

required a helicopter view to allow a more mesoscopic pattern recognition before diving into the exact underlying microscopic details. This approach provided crucial information that otherwise might have been lost in the details. It for instance revealed the difference in cortical distribution of the myelin-related iron observed throughout the neuropil, which by normal microscopic evaluation would have been difficult to discriminate.

Molecular imaging strategies

Since the mid-1990s, molecular imaging has gained interest as it attempts to detect non-invasively cellular and molecular signatures of disease processes by means of imaging. Crucial to molecular imaging is the availability of an imaging probe that targets a specific disease process, ideally allowing for an early and specific diagnosis.[14,15] For AD, thus far two molecular imaging strategies are used in the revised criteria: the detection of glucose hypometabolism using ^{18}FDG-PET, and visualization of cerebral amyloid load by radioactive PET ligands. (**Table 1**) The cerebral pattern of glucose hypometabolism is considered to be an indicator of synaptic loss that accompanies neurodegeneration, and has demonstrated to be a sensitive and specific indicator for conversion of MCI to AD.[16] However, currently, the most sensitive methods for early detection of AD are based on detection of cerebral amyloid.[3,4] According to the dynamic biomarker model, amyloid plaque accumulation is the first change to be present, and thereby constitutes a well-defined specific target for the development of *in vivo* molecular imaging strategies as highlighted in Part Two. It is important, however, to realize that the presence of cerebral amyloid might allow an early diagnosis, but it does not allow to follow disease progression due to its early plateau level.[3,4]

Based on preclinical research on the modification of existing amyloid-binding dyes like Congo Red and Thioflavin into imaging agents, the initial *in vivo* breakthrough came with the development of Pittsburgh Compound B, a neutral ^{11}C derivative of Thioflavin.[17] Despite many *in vivo* human studies its broad applicability is hampered by its short radioactive half-life (<20min), requiring the availability of a cyclotron, and therefore it has been mainly used for research purposes. Recently Amyvid, a novel amyloid-binding PET probe, has been the first probe to receive approval for clinical use to image amyloid plaque burden by the American Food and Drug Administration (FDA).[18] This ^{18}F-containing stilbene derivative does not need the presence of a cyclotron in the vicinity due to its long half-life of 109 minutes. When this first FDA approved amyloid imaging probe will prove both its clinical and commercial value this may push the development of different or more specific AD imaging probes, like those presented in this thesis.

For molecular imaging tracers based on small molecular compounds, such as Amyvid and our bis-pyridylethenyl- and fluorinated bis-styrylbenzenes thus far PET, and not MRI, remains the imaging modality of choice due to its higher sensitivity.[19] If possible MR-based Aβ targeting imaging agents are still desirable despite all the difficulties for the design of MR-based AD imaging probes as described in **Chapter 7**. Such agents could be used in one scan session comprising a comprehensive structural and functional scan protocol as well as a scan to detect the molecular imaging tracers, whereas one PET examination only provides one biomarker. Furthermore, the availability of MRI tracers would lower the threshold for performing amyloid scans given the wider availability of MRI systems as compared to PET systems, the lack of ionizing

irradiation and the lower costs involved in performing MRI as compared to PET. However, although existing PET-based amyloid tracers can be modified for MRI, e.g. by. addition of ^{19}F, current clinical MRI setups require an administration of such a high dose that it might create potential toxicity issues. Novel advances in brain delivery systems combined with AD targeting backbones, like V_HH, may perhaps overcome these hurdles in the future. Additionally, the recently introduced hybrid PET-MRI systems may be instrumental since they allow for acquiring PET and MRI data in one diagnostic imaging session.

Theragnostics

The integration of diagnostic and therapeutic components into a single compound or so-called theragnostic agent holds great promise for future drug development. The incorporation of an imaging moiety to a possible drug or drug delivery system enables the precise monitoring of *in vivo* temporal and spatial bioavailability and target engagement of the drug, and also treatment outcome.[20] During the development of the drug, crucial information can be obtained regarding the possible pharmacological challenges that have to be addressed to further enhance its efficacy, such as biodistribution, pharmacokinetics, fast metabolism, limited specificity and severe adverse effects.

With regard to AD, amyloid plaques have not only been an obvious target for diagnostics but the break-down or inhibition of these Aβ deposits has also attracted attention as a possible target for therapeutic intervention. Compounds that were initially probed for diagnostic purposes may also have a therapeutic effect. The development of anti-Aβ antibodies for therapeutic purposes initially faced the same restrictions that are met in the development of diagnostic agents. Despite almost no cerebral uptake, however, both passive and active immunization studies using animal models showed a decrease in cerebral amyloid and improved cognitive performance.[21] Their effect was partly attributed to the so-called "peripheral sink" mechanism that assumes that the removal of Aβ from the bloodstream leads to a lower concentration of Aβ in the circulation which then leads to the efflux of the peptide from the brain. Unfortunately translation of these findings into the clinic was less successful as therapeutic human anti-Aβ studies were stopped either due to unwanted side effects or because no significant clinical improvements were detected.[21] Besides their inability to gain sufficient entry into the brain, the general consensus on the reason for the therapeutic failure of these antibodies is that the trials started too late in the course of the disease. Several new trials, such as DIAN and A4, are starting this year with groups of pre-symptomatic, but amyloid-positive patients.[22] These drug development trials are only possible because of the progress that has been made over the last years in new diagnostic biomarkers, particularly PiB-PET.

Although initially designed for diagnostic purposes the llama antibody fragments or V_HH that we studied have many unique characteristics that make them especially interesting for theragnostics, as pointed out in **Chapter 9**. Several V_HH have already shown their potential therapeutic value *in vitro*, preventing aggregation of amyloid fibrils or oligomeric forms of Aβ and thus inhibiting formation of or disentangling amyloid plaques.[23] Compared to the conventional monoclonal antibodies or antibody fragments (Fab') used for clinical trials, like those described above, V_HH express a similar high level of specificity and affinity, but because of their single domain production and modification is relatively easy and cost-efficient.[24]

Possible immunologic reactions are easily avoided by humanization. Their selective binding to different Aβ species, like CAA or oligomeric Aβ, could shift Aβ brain efflux in the favored direction, which could be used to tailor anti-Aβ therapy, thereby further reducing therapy induced complications.
Although in this thesis a proof of principle is given regarding the specific brain uptake of V_HH, their full therapeutic effect may be hampered by a limited ability to enter the brain. Similar to the adaptations needed to improve their value as a diagnostic marker, novel strategies will have to be addressed to improve their therapeutic value.

Concluding remarks

In the next decade biomarker studies will play a crucial role in developing strategies for early detection and treatment of AD. It remains doubtful whether eventually one single biomarker will be able to give a final diagnosis, but the incorporation of biomarkers in the revised NINDS-ADRDA criteria mean an important step forward, and confirm the importance of AD biomarker research in general. Further evaluation and standardization of the included biomarkers may provide more insight on the yet unknown pathogenesis of AD and contribute to the development of effective treatment.
Finally, it should be noted that due to the current absence of an effective treatment the impetus to develop methods for early diagnosis for AD, particularly for the asymptomatic individual, is limited and creates a Catch 22 situation. However, as discussed earlier in this thesis, the ability to detect the early phases of the disease is expected to provide improve our understanding of the pathophysiology of the disease, which then sets the stage for developing more effective treatment. Once more effective treatment will be available, the same tools for early disease detection will become relevant to individual patients by opening the therapeutic window to the early phases of the disease they are suffering from.

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