Summary and Discussion
In this thesis we investigated the early pathophysiology of AD by comparing different markers for amyloid aggregation in cognitively normal individuals and investigating the association of amyloid aggregation with proteomic changes in cerebrospinal fluid (CSF) and cognitive function. The main findings of this thesis are (Figure 1):

I. Assessment of amyloid aggregation in cognitively normal individuals
   - Amyloid-PET visual assessment of amyloid aggregation on parametric \([^{18}F]\)flutemetamol BP\textsubscript{ND} images is more accurate than visual assessment of amyloid aggregation on SUV images.
   - CSF ratio amyloid-\(\beta\) 42/40 and \([^{18}F]\)flutemetamol PET BP\textsubscript{ND} seem to measure amyloid aggregation in a similar way.

II. Pathophysiology of amyloid aggregation
   - Increased amyloid production may be involved in AD pathophysiology in cognitively normal elderly.
   - The onset of amyloid aggregation in cognitively normal elderly is under substantial influence of unique environmental factors.
   - BACE1 may play a central role in pre-amyloid stages of AD.
   - CSF proteomic signatures associated with amyloid aggregation are dependent on APOE \(\varepsilon_4\) genotype. APOE \(\varepsilon_4\) carriers show changes in proteins associated with inflammation followed by changes in proteins associated with synapse function while this order was the other way around in individuals without the APOE \(\varepsilon_4\) allele.
   - Amyloid aggregation is associated with visual memory performance but not with cognitive complaints in community dwelling cognitively normal elderly.

Next, we will discuss these findings in more detail and comment on methodological issues regarding the studies in this thesis. We will conclude with implications of these results and future perspectives.
Summary and Discussion | Chapter 7

I. PRECLINAD COHORT

AD starts in cognitively normal individuals but there are relatively few cohorts that have tested amyloid pathology in these individuals. To study early pathophysiological mechanisms associated with amyloid pathology we initiated the EMIF-AD PreclinAD study in 2014.

In chapter 2 we describe the PreclinAD study population, consisting of 285 cognitively normal elderly, recruited from two ongoing cohorts. At Manchester University 81 subjects were recruited from the Manchester and Newcastle Age and Cognitive Performance Research Cohort in the United Kingdom. At the VU University medical center in Amsterdam 204 subjects (including 99 monozygotic twin pairs) were selected from the Netherlands Twin Register (NTR). All subjects had data available for neuropsychological testing and questionnaires, medical history and medication use, physical measures, such as height, weight, waist measurement and resting blood pressure, ultrasound of the carotid artery, dynamic [\(^{18}\)F]flutemetamol amyloid-PET scanning, and MRI. In the NTR sample CSF collection, Magneto-encephalography (MEG), and retinal imaging was performed as well (Figure 2). Participants were on average 74.8 (SD=9.7) years old, 64% female, and 30% APOE ε4 carrier. Manchester participants were older (85.7 vs 70.8, p<0.001), more often female (78 vs 58%, p<0.01), and had less often a positive family history for AD (19 vs 45%, p<0.001).
Fifty-eight participants (22%) had an abnormal amyloid-PET scan, as visually read from static images. Participants from Manchester had more often an abnormal amyloid-PET scan (34 vs 16% p<0.01), probably because they were older. Amyloid abnormality increased with age, with 12% of subjects aged 60-70 years having an abnormal amyloid-PET scan, 16% of the subjects between 70-80 years and 36% of the subjects 80 years and older. These findings resemble earlier findings for amyloid pathology in the cognitively healthy elderly population [1].

II. ASSESSMENT OF AMYLOID PATHOLOGY

In chapter 3 we investigated which of two methods to classify $[^{18}F]$flutemetamol PET images, SUVr or BP$_{ND}$, was best for visual assessment of amyloid pathology on PET in cognitively normal elderly. Visual rating is typically performed on summed late images or SUV images obtained from static PET acquisition [2-4], however SUVr might overestimate amyloid load compared to quantitative BP$_{ND}$ which is derived from dynamic PET acquisition [5]. As such, quantitative images may be more reliable, also for visual interpretation. We found a better inter-reader agreement for the visual assessment of the $[^{18}F]$flutemetamol dynamic images (BP$_{ND}$) compared to the static images (SUVr), and our analysis provided evidence that static images indeed overestimated actual amyloid load. When adding (semi) quantification to the visual assessment, the number of false-positive individuals decreased in the assessment of static images and decreased in the assessment of dynamic images to zero. A disadvantage of acquiring BP$_{ND}$ images is that dynamic scanning is required from the moment of tracer injection (in our study 0-30 minutes after injection) in addition to the scanning in the plateau phase (90-110 minutes after injection). This may lead to a higher burden for participants, but for cognitively normal subjects this burden may be acceptable. The benefits of reducing false-positive diagnoses may outweigh the extra burden of an additional 30-minute scan, in particular if subjects are selected for an anti-amyloid trial.

In chapter 4 and 6 we used CSF ratio Aβ$_{42/40}$ and PET BP$_{ND}$ values to assess the relation between these amyloid aggregation markers and their relation with memory performance. In chapter 4 we found a moderately strong association between CSF ratio Aβ$_{42/40}$ and PET BP$_{ND}$. In addition, the cross-twin cross-trait correlations between CSF ratio Aβ$_{42/40}$ and PET BP$_{ND}$ were...
BPND was statistically significant, as well, supporting the notion that these markers measure largely, but not entirely, the same biological construct of amyloid aggregation. The moderate strength of the association is possibly due to a low variability in PET BPND values as 86% of the individuals had a normal amyloid-PET. It is also possible that CSF is an earlier marker for amyloid pathology, as suggested in previous studies using SUV PET images [6]. However, using the twin discordance design as an amyloid disease stage model, we found the same dose effect for CSF ratio Aβ42/40 and PET BPND values (Figure 3). In chapter 6 we found a relation between amyloid pathology and memory performance on the Rey Complex figure recall. We found a relation between Rey Figure recall and CSF ratio Aβ42/40, and with amyloid-PET status as visually read on BPND images. Additionally, worse performance on the Rey complex figure recall was related to increased amyloid aggregation, measured with both CSF and PET. Nevertheless differences in amyloid aggregation marker performance between CSF and PET were found: PET BPND data were skewed with low variability and PET BPND as a continuous measure was not associated with Rey complex figure performance. This might suggest that PET BPND values are a bit less sensitive compared to CSF ratio Aβ42/40. Overall, we found no strong evidence of CSF being an earlier marker for amyloid aggregation compared to PET in the earliest stage of AD.

Figure 3. Twins discordance model as a hypothetical disease stage model
Boxplots for ratio Aβ42/40 (left) and [18F]flutemetamol BPND (right) showing amyloid load according to twin discordance status. Hypothetical model for amyloid aggregation severity: twins of a pair who have both a normal PET scan (concordant normal, Control), twins from a discordant pair with normal amyloid (discordant normal, Stage I), twins from a discordant pair with abnormal amyloid positive subjects (discordant abnormal, Stage II), and twin pairs who both have an abnormal PET scan (concordant abnormal, Stage III). From left to right with gradually increasing amyloid load from control group to stage I, II and III.
III. PATHOPHYSIOLOGY OF AMYLOID AGGREGATION

Relation between amyloid production and aggregation
In chapter 4 we investigated whether there is a relation between amyloid production and aggregation in the preclinical stage of AD. For this, we used three markers to assess amyloid production (CSF BACE1, Aβ40, Aβ38) and two measures to assess amyloid aggregation (CSF ratio Aβ42/40 and [18F]flutemetamol BP ND). We found a negative association between BACE1 and the CSF ratio Aβ42/40 in the total group and in addition a cross-twin cross-trait correlation between BACE1 and the CSF ratio Aβ42/40 at trend level, possibly suggesting a shared biological background for this relation. Since CSF ratio Aβ42/40 contains in part amyloid production (i.e. Aβ40), it could be suggested that amyloid production is driving the association between BACE1 and CSF ratio Aβ42/40. However, using the twin discordance approach, based on visual amyloid-PET rating, we also found higher levels of BACE1 in both twins of discordant twin pairs (i.e. the twin with normal amyloid-PET and the co-twin twin abnormal PET), compared to concordant normal twins. The higher levels of BACE1 present in the non-affected twin of the discordant pairs suggest that BACE1 increases just before amyloid aggregation becomes detectable on visual read. Together these findings provide evidence for a role of increased amyloid production in very early sporadic AD. However, follow-up data are required to determine the temporal ordering of events, whether it is the case that BACE1 increases leading to amyloid aggregation, or the other way around. The relatively weak relation between amyloid production and aggregation suggests that there are other causes for amyloid aggregation, such as clearance problems or vascular damage causing amyloid to aggregate in sporadic AD [7].

Environmental influence on amyloid aggregation and production
In chapter 4 we found that, of the 94 monozygotic twin pairs of which both twins had amyloid-PET data available, 14 (15%) were discordant for amyloid-PET, which indicates a substantial influence of unique environmental factors to amyloid pathology. This is supported by the lower twin-pair correlations (0.52-0.54) for amyloid aggregation markers compared to those for amyloid production markers (0.79-0.86). So unlike amyloid production, amyloid aggregation is considerably influenced by unique environmental, and therefore possibly modifiable, factors. Discovering these factors may lead to new prevention strategies for AD.

Effect of APOE ε4 on protein expression in CSF in AD subjects
In chapter 5 we found that APOE ε4 carriers with amyloid aggregation showed altered concentrations of proteins involved in the complement pathway and glycolysis when cognition was normal and lower concentrations of proteins involved in synapse structure and function when cognitive impairment was moderately severe. APOE ε4 non-carriers with AD showed lower expression of proteins involved in synapse structure and function
when cognition was normal, and lower concentrations of proteins that were associated with complement and other inflammatory processes when cognitive impairment was mild. These results imply that AD pathophysiology depends on APOE genotype and that treatment for AD may need to be tailored according to APOE genotype and severity of the cognitive impairment. Cognitively normal subjects without amyloid but in possession of the APOE ε4 showed a subtle increase in BACE1 levels in CSF. The use of proteomics in CSF is a promising novel method for in vivo measurement of biological processes in the brain. Our findings are comparable to earlier studies, post-mortem and mice [8, 9], confirming our findings to be robust.

**Memory performance in preclinical AD**

In chapter 6 we found amyloid aggregation is associated with visual memory performance in cognitively normal elderly. Twin discordance analysis, used as a disease stage model for amyloid pathology (Figure 3), showed visual memory and face-name associative memory to be among the types of memory sensitive to be influenced by early stage AD. This supports the notion that amyloid aggregation leads to subtle memory dysfunction in very early stages of AD. We found two different patterns of memory performance; for Rey complex figure subjects showed worse performance in the more advanced stage (concordant abnormal amyloid-PET, stage III), while for the FNAME subjects in the earliest stage (discordant with normal amyloid-PET, stage I) already tended to show lower scores. This might suggest that the FNAME can function as a screening tool for increased risk of amyloid pathology in the general population, however further studies in large samples are necessary to validate this hypothesis. There was no association between amyloid aggregation and SCD nor between SCD and memory. The inclusion and exclusion criteria applied in our study were very strict, resulting in a very healthy elderly cohort, reducing the range of amyloid aggregation, memory performance and complaint scores, which therefore limited the ability to detect associations. Hence, this might not be the right sample to investigate the effect of cognitive complaints in early AD.

**IV. METHODOLOGICAL CONSIDERATIONS**

**Samples**

In this thesis we analyzed data from two cohorts, one newly collected data set of cognitively normal elderly (PreclinAD study) and one existing dataset consisting of cognitively normal elderly and patients with AD across the disease spectrum (ADNI study). Both cohorts have a rich set of biomarkers available, facilitating us to thoroughly investigate AD pathophysiology, however some limitations must be acknowledged.
Chapter 7 | Summary and Discussion

PreclinAD
The preclinAD cohort is a two-site study, with less than 30% of the subjects included in Manchester. Subjects from Manchester were older compared to Amsterdam subjects and showed amyloid pathology more often. One explanation for the younger age in Amsterdam is that we included complete monozygotic twin pairs. Older twin-pairs are relatively scarce, since when one of them is cognitively impaired or fulfilled other exclusion criteria, both twins of a pair were excluded from the study. Furthermore, Amsterdam subjects were all monozygotic twins. While this offers the possibility of exploring genetic and environmental influences on amyloid pathology, it decreases power in group-wise analysis as twins from a pair are not independent. Although scanning protocols were aligned at both sites, different PET scanners (HRRT in Manchester vs PET-MRI in Amsterdam) were used. While we did not perform analysis in the combined sample yet, the differences in design and data acquisition need to be taken into account in future analysis.

ADNI
The commonly used ADNI cohort consists of a selected population recruited mainly by advertisements. Together with the observation that ADNI participants are highly educated, this may limit the generalizability of the findings from this cohort to other settings.

Use of cross-sectional data
For all analysis performed in this thesis we used cross-sectional data. This has the disadvantage that we cannot be certain that observed findings are the result of different stage of disease or simply the natural variation of a trait. Furthermore, it is not possible to infer conclusions about causality in the observed associations. It will therefore be important to validate our findings in longitudinal studies with repeated biomarker and cognitive assessments.

Twin methodology
By design, we did not include dizygotic twin pairs in our twin sample. Although the classic twin design, to calculate heritability, requires besides monozygotic also dizygotic twins, for the aim of our study, assessing whether there is an actual biological background for a relation between two traits (using a monozygotic difference approach), and to assess the unique environmental influence on certain traits, a monozygotic twin design is the strongest approach [10]. However, studies applying a classic twin design with mono- and dizygotic twins, showed that for brain aging and cognition measures common environment does not play a role [11, 12], and that monozygotic twin-pair correlations may therefore be interpreted as the amount of variance within these traits explained by genetic variation (heritability).
An advantage of the monozygotic twin difference design is the possibility to support or reject causality of a relation between two traits, although for definite conclusions follow-up data is also needed [13]. We used the discordant twin design as a disease stage model for amyloid aggregation severity (Figure 2). However, AD-type dementia is ‘only’ 80% heritable, and with this amyloid disease stage model we assumed that both twins of a pair will get amyloid pathology even though they do not always both become demented. Our assumption was driven by earlier findings from Scheinin et al. who reported that twins that were discordant for a clinical diagnosis of AD-type dementia were concordant for amyloid aggregation on amyloid-PET imaging, while this was not the case for dizygotic twins [14]. However, AD pathology is also characterized by neuronal injury, therefore we must acquire follow-up data to establish to what extent preclinical AD in twins resembles AD-type dementia later on. This is underlined by twin-pair correlations for amyloid aggregation being substantially lower than twin concordance for AD-type dementia, however these dementia diagnoses were not biomarker confirmed.

CSF proteomics
The use of proteomics is approaching a data driven manner of investigating biological processes. However, our proteomic panels included up-to 300 pre-selected proteins based on earlier findings from other neurodegenerative diseases. Since in CSF thousands of proteins are present we may therefore have missed proteomic pathways. Another challenge of CSF proteomics, is interpretation of the data. Protein expression can be lower or higher, however, the consequences for different pathways are not straightforward. For example lower expression of a certain protein might indicate decreased activity, since there is less available of that protein in CSF (as with tau), or it may suggest increased activity of this protein, as it is used and therefore ‘out of stock’. Pathway analysis used to interpret the findings of these proteomic expression profiles is a way to perform data-reduction, but these pathways are based on previous observations and may not cover novel pathophysiological mechanisms.

V. IMPLICATIONS

Early detection of amyloid pathology
Although it is common practice to use static amyloid-PET images for visual read [4], for the selection of subjects with preclinical AD, dynamic amyloid-PET images should be considered to avoid inclusion of false-positives in clinical trials. Both the twin and CSF analysis suggests CSF BACE1 upregulation to be the earliest sign of the start of amyloid aggregation, however this needs to be established further. When validated, BACE1 levels in CSF can be used as a selection criterion for inclusion in BACE1 inhibitor trials in the future. Since we show CSF ratio
Aβ42/40 and PET BPND to measure the same biological construct, these measures both seem to be applicable as exchangeable continuous amyloid aggregation markers in preclinical AD [15].

Pathophysiology
CSF proteomic analysis is a useful tool to measure parallel processes in vivo in humans, it might therefore be applied in longitudinal studies to study evolvement of biological processes underlying AD. BACE1 may play a central role in pre-amyloid stages of AD. We found that cognitively normal subjects without amyloid pathology but genetically at risk, either through a monozygotic co-twin already showing abnormal amyloid-PET or by possession of the APOE ε4 allele, to show a subtle increase in BACE1 levels in CSF, compared to controls, either twins with both a normal PET or subjects without an APOE ε4 allele.

Treatment
Since we only found a weak relation between amyloid production and aggregation, clinical trials might want to focus more strongly on clearance problems, instead of inhibition of amyloid production solely. The involvement of different proteins in amyloid pathology depending on APOE ε4 genotype, suggesting specific biological processes underlying AD pathology within these groups, suggests treatment might need to be tailored to APOE ε4 genotype. The substantial influence of environmental factors (around 50%), either directly or via epigenetic changes, on amyloid pathology in cognitively normal elderly shows that identification of these factors might lead to novel AD prevention targets.

Endpoints in trials
Our findings that specific biological processes underlie AD pathology dependent on APOE ε4 genotype and disease stage indicates that trials may need to select outcome measures for trial that differ for disease stage and/or APOE ε4 genotype.

VI. FUTURE PERSPECTIVES
For defining novel targets for anti-AD targets for lifestyle or medication, we next should try to identify the environmental factors, and/or epigenetic changes, that are influencing early AD pathophysiology. Therefore differences within twin-pairs discordant for amyloid-PET should be accurately investigated to identify factors for life style advice and/or medication targets. By studying epigenetic differences within twin-pairs discordant for amyloid aggregation novel targets for drug development might be identified. Furthermore, follow-up studies are needed to study the outcome of twin discordance in order to discover which twin-pairs become concordant abnormal (genetic influence) or stay discordant.
(environmental influence). The collection of longitudinal data for twin analysis is also needed to assess possible relations between amyloid aggregation and actual cognitive decline in these healthy subjects. As injury markers in CSF are more strongly related to cognitive performance than markers of amyloid aggregation [16], these markers should be obtained and possible relations with cognitive decline investigated. Follow up data for proteomic CSF analysis will be essential, as for now it is not clear which proteins actually reflect disease cause or consequence. Finally, since monozygotic twins share 100% of their genes, CSF proteomics in this population might even shed light on gene expression difference in brain tissue. As a gold standard for amyloid pathology, and reflection of other biological processes in the brain, post-mortem pathological evaluation of brain tissue is important, therefore participating twins are currently asked to subscribe to the Netherlands Brain Bank, to enable studying their brain tissue after they are deceased.
REFERENCES