CHAPTER 1

Introduction
General introduction and outline
Alzheimer’s disease

Alzheimer’s disease is the most common form of dementia, affecting a total of 36 million people worldwide. The major risk factor for Alzheimer’s disease is age, and with the increasing age of the world population, the number of patients is estimated to increase to almost 66 million by 2030 (WHO and Alzheimer’s Disease International, 2012). Alzheimer’s disease is characterized by progressive cognitive deterioration, which interferes with daily living\(^1\). The disease is invariably fatal and patients live, on average, another ten years after diagnosis. The diagnosis of possible or probable Alzheimer’s disease can be made when a person has memory deficits and cognitive disability in at least one other cognitive domain. Following the established NINCDS-ADRDA guidelines\(^1\), Alzheimer’s disease diagnosis is based on clinical symptoms with no absolute confirmation on the underlying pathology. Hence, a definite diagnosis can only be made post-mortem, after a pathologist has examined the diseased patients’ brain for the main pathological hallmarks of Alzheimer’s disease: the amyloid plaques and neurofibrillary tangles shown in Figure 1.

Early diagnosis of Alzheimer’s disease is, in my opinion, important for two main reasons. First, patients that visit the memory clinic suffer from (subjective) cognitive decline are desperate for an accurate diagnosis and prognosis of their clinical symptoms. Even without the availability of medication. Secondly, early diagnosis can facilitate future interventions studies and medical trials. Since Alzheimer’s disease pathology starts decades before clinical symptoms\(^3\), an inhibitor of the disease could prevent the irreversible loss of cognitive abilities and should, in order to enhance the chance of success, be administered in the earliest stages. Therefore it is of importance to accurately

![Image of Alzheimer cells with amyloid plaques and neurofibrillary tangles](http://www.alz.org/brainimages/plaques_tangles.jpg)

**Figure 1.** Amyloid plaques and neurofibrillary tangles in Alzheimer’s disease.

identify persons that will develop Alzheimer’s disease. A potential third benefit of early and accurate biochemical markers of disease pathology is that they may be used to monitor the efficacy of drugs and treatments.

**APOE genotype**
The major genetic risk factor for sporadic Alzheimer’s disease is the APOEɛ4 allele. The APOE gene consists of mainly three isoforms, which result in three structurally distinct proteins, called Apolipoprotein E, which only differ in two amino acids. Carrying the APOEɛ4 allele gives a dose-dependent increase in AD-risk in non-demented individuals, where carrying a single allele increases the risk of AD three times, and double APOEɛ4 carriers are fifteen times more likely to get Alzheimer’s disease. Although many studies provide possible functional explanations for the effect of APOEɛ4, the molecular pathways that cause such a remarkable increase in the risk of AD in APOEɛ4 carriers in humans is not well understood.

**Pathological hallmarks**

*Amyloid beta plaques*
The amyloid plaques are extracellular depositions of the amyloid beta (Aβ) peptide and are found in the brain parenchyma as well as in the cerebral vasculature (CAA). The Aβ peptide is a breakdown product of the enigmatic amyloid precursor protein (APP), which resides mainly in the membrane of neurons. After consequent cleavage of this protein by gamma-secretase and beta-secretase, the hydrophobic Aβ peptide is released. Production of Aβ is a physiological process, present in both health and disease, though the amount of Aβ increases in the brain of Alzheimer’s disease patients. Also, there is a shift towards the 42 (Aβ_{42}) over the 40 amino acid long isoform (Aβ_{40}), of which the 42 subtype is more prone to aggregate and a potent neurotoxin.

The accumulation of Aβ in Alzheimer’s disease may be due to a chronic overproduction of the Aβ peptide and/or a decrease in breakdown or transport from the brain. Either way, the accumulation of Aβ induces self-aggregation and ultimately leads to the formation of insoluble amyloid fibrils and amyloid plaques.

Amyloid accumulation is hypothesized to be the trigger of Alzheimer’s disease pathology for mainly three reasons. First, all the genetic mutations that cause familial forms of Alzheimer’s disease are found in APP or either of the cleavage enzymes that produce Aβ. Secondly, amyloid plaques are found in brains of non-demented people without neurofibrillary tangles, but tangles are rarely present without amyloid plaques. It must be noted, however, that the amyloid burden (plaque-load) itself poorly reflects the severity of clinical symptoms, and other factors likely determine whether an individual is protected against or vulnerable for cognitive decline. Thirdly, in Downs’ syndrome there is a third copy of chromosome 21, which encodes the APP protein. Consequent overproduction of APP leads to chronic overproduction of Aβ which initiates the formation of amyloid plaques, classical complement activation and formation of neurofibrillary tangles at relatively young age.
Chapter 1

Neurofibrillary tangles

Tau is a microtubule-associated protein, used to stabilize the internal infrastructure of the cell. Through phosphorylation and de-phosphorylation of Tau, the cell regulates the stability of microtubules, which are essential for energy, waste and nutritional distribution within the cells. Tau-pathology is directly involved in neuronal cell-death, as hyperphosphorylation of tau and the formation of paired helical filaments of tau (tangles) suffocate the neurons and ultimately cause cell-death. The amount of cognitive disability, in contrast to the amyloid deposits, is reflected by the amount of neurofibrillary tangles. The biochemical understanding of how extracellular Aβ accumulation can lead to intraneuronal tau aggregation is limited. Some proteins or pathways could hypothetically connect these disparate pathological events, although Aβ aggregations have also been shown to affect tau degradation and phosphorylation directly.

Amyloid associated proteins

A wide variety of proteins are found co-localized with the amyloid plaques, many of which were shown to directly influence Aβ aggregation and toxicity. Complement activation products, clusterin and apolipoprotein E, for example, are found in early diffuse plaques and are capable of directly binding the Aβ peptide. Moreover, they influence the toxicity and regulate the cellular uptake of Aβ aggregates in cell culture and mouse models of AD. Although the strong association of APOE genetic polymorphisms and AD has been well established, also genetic variations of the clusterin (CLU-) gene were described in two initial independent genome-wide association studies to be strongly related to the risk of AD. Due to their intimate involvement with the aggregation, toxicity and clearance of Aβ, the amyloid associated proteins are crucial in the etiology of Alzheimer’s disease.

Established Alzheimer-biomarkers

Biochemical markers of Alzheimer’s disease pathology have been discovered, and laboratory tests are now routinely used to quantify the amount of Aβ42, Tau and pTau in the cerebrospinal fluid. Cerebrospinal fluid is in close contact with the brain and reflects the brains’ biochemical processes. The concentration of Aβ42 in cerebrospinal fluid is decreased, possibly due to its accumulation in the brain parenchyma. Tau is released upon neuronal cell-death, and is found in increased levels in cerebrospinal fluid from Alzheimer’s disease patients. By quantification of these biomarkers in cerebrospinal fluid, one can discriminate Alzheimer’s disease patients from controls, as visualized in Figure 2. The traditional Alzheimer-biomarkers (Aβ42, Tau and pTau) in cerebrospinal fluid are usually detected by conventional enzyme-linked immunosorbent assays (ELISA), although recently also multiplex assays such as Luminex xMAP and Mesoscale discovery are used. Such multiplex platforms have the advantage of reducing hands-on time, possibly reducing human errors and could be more cost-effective. Also, less cerebrospinal fluid is required for the analysis. Validation on whether the Alzheimer-biomarkers are detected with equal sensitivity and selectivity, and have at least similar clinical value when detected by the different platforms, is crucial.
Potential early biochemical markers of AD pathology

The $A_\beta_{42}$ and Tau levels in cerebrospinal fluid are indicators of AD, yet they poorly predict disease progression in an early phase of the disease. A marker that would be able to discriminate different stages of the disease (early, intermediate, late) or that would be able to detect AD in non-demented individuals, is much needed in the clinic. Such a marker or combination of markers is feasible, as Alzheimer-pathology starts decades before the onset of clinical symptoms.

Clusterin

Clusterin is a multifunctional protein that is involved in a wide variety of physiological mechanisms, ranging from a regulator of cell-death\textsuperscript{25,26} and complement activation,\textsuperscript{27} to a molecular chaperone\textsuperscript{28}. Interestingly, clusterin is found co-localized with diffuse or "early" amyloid plaques even before clinical onset of dementia\textsuperscript{17,18}. Clusterin can directly interact with the $A_\beta$ peptide and influence its aggregation and toxicity\textsuperscript{29,30}. Moreover, clusterin affects the cellular uptake of $A_\beta$\textsuperscript{31} and is a potent transporter of $A_\beta$ across the blood-brain barrier through receptor-mediated transport\textsuperscript{32}. Because of clusterins’ involvement and local up regulation in early AD-pathology, fluctuations in clusterin levels in cerebrospinal fluid or blood may be indicative of pathological alterations in the earliest phases of AD.

Clusterin levels in blood correlate with pathology and prevalence of Alzheimer’s disease\textsuperscript{33,34}, but are not increased in pre-symptomatic patients\textsuperscript{35}. Also, clusterin levels are related to the amount of brain atrophy, and reflect the amount of pathology in brain regions that are vulnerable for AD. Furthermore, clusterin plasma levels were associated with changes in cognitive performance in patients with mild cognitive impairment\textsuperscript{36}. Two studies that examined clusterin levels in cerebrospinal fluid as a biomarker for AD reported elevated levels of CSF clusterin in Alzheimer’s disease\textsuperscript{37,38}. Overall, the involvement and up regulation of clusterin levels in advanced stages of Alzheimer’s disease seems well established. However, mostly due to insufficient longitudinal data, the pathophysiological role of clusterin in early stages of Alzheimer’s disease has not been adequately addressed.
**Oligomers**

Small, soluble aggregates of the Aβ peptide, called Aβ-oligomers, have gained substantial scientific interest due to their direct toxic effects at the synapse level and their early involvement in Alzheimer pathogenesis. Aβ-oligomers arise during Aβ aggregation, but may be formed off-route of Aβ aggregation into amyloid fibrils and plaques (Figure 3). Studies of these potent neurotoxins have been plentiful, and the concentration of Aβ-oligomers was associated with plaque-load and disease severity.

Publications in which Aβ-oligomers levels in CSF were successfully quantified and used as a potential biomarker for AD have reported promising findings. Although these cross-sectional studies focus on the direct diagnostic potential of Aβ-oligomers, longitudinal changes in Aβ-oligomer levels may herald additional personalized diagnostic or pathophysiological insight in AD. Quantification of Aβ-oligomers in cerebrospinal fluid is challenging, and the many different platforms of detection may detect different subtypes that, in turn, may be involved in different pathological pathways. Therefore, characterization of the molecular targets of Aβ-oligomers assays are crucial for comparison of results. Interestingly, because of the cross-sectional nature of previous studies and often a lack of target characterization, it is unknown Aβ-oligomer levels can predict who will develop Alzheimer’s disease in the future, or which subspecies are best to be used as a biomarker.

![Figure 3. Overview of the dynamic aggregation of Aβ into fibrillar aggregates.](image-url)
Aims and outline

The main aim of this thesis was to investigate established and novel biomarkers of neurodegenerative disease, especially for the early detection of disease. To this end, the following aims were formulated:

- Determine the usefulness of established AD-biomarkers to diagnose and predict Alzheimer’s disease with different ways of detection.
- Study the usefulness of clusterin and ApoE as a diagnostic and prognostic biomarker for neurodegenerative disease, and their role in the etiology of Alzheimer’s disease.
- Quantify $A\beta$-oligomers, and to assess their clinical value in CSF for the diagnosis of Alzheimer’s disease.

In Chapter 2 we compared two methods to quantify Alzheimer-biomarkers $A\beta_{42}$, Tau and $p$Tau in cerebrospinal fluid. Results obtained with both assays in their ability to diagnose and predict Alzheimer’s disease were compared.

In Chapters 3 and 4 we describe the development of assays to quantify potential biomarkers: clusterin and $A\beta$-oligomers. In addition, we measured these markers, and also apolipoproteins E, in clinically well-characterized human samples in order to assess their added value for the (early) detection of Alzheimer’s disease. We discuss the role of these key pathological entities in the pathophysiology and etiology of Alzheimer’s disease.

In Chapter 5 our main findings are summarized and discussed, followed by recommendations for future research.
REFERENCES


CHAPTER 2

The usefulness of established AD-biomarkers with different ways of detection
Discriminatory and predictive capabilities of enzyme-linked immunosorbent assay and multiplex platforms in a longitudinal Alzheimer’s disease study

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ABSTRACT

Background
Multiplex assays such as xMAP have been proposed for the assessment of Alzheimer’s disease (AD) biomarkers amyloid β 42 (Aβ42), tau (Tau), and phosphorylated tau (pTau) in cerebrospinal fluid (CSF). Here, we compared the traditional enzyme-linked immunosorbent assay (ELISA) and xMAP with respect to their: (1) absolute biomarker concentration, (2) ability to distinguish AD from nondemented subjects, (3) ability to monitor AD longitudinally, and (4) ability to predict progression from mild cognitive impairment (MCI) to AD.

Methods
We selected 68 AD, 62 MCI, and 24 nondemented subjects, performed clinical examinations, and obtained CSF at baseline and 2 years later. Aβ42, Tau, and pTau were measured with both ELISA and xMAP.

Results
Biomarker levels differed considerably between the two assays, and the differences were concentration dependent. No differences were observed in ability to distinguish nondemented subjects from AD patients between ELISA (area under curve of 0.84 for Aβ42, 0.79 for Tau, and 0.75 for pTau) and xMAP (area under curve of 0.82 for Aβ42, 0.75 for Tau, and 0.73 for pTau), all P < .05. Increased Aβ42 levels of AD patients at follow-up compared with baseline were detected with ELISA, whereas increased Tau levels for nondemented subjects and MCI patients were only detected with xMAP. The hazard ratios for progression from MCI to AD did not differ between the assays.

Conclusion
Both ELISA and multiplex assays can be used to measure AD biomarker levels in CSF to support clinical diagnosis and predict progression from MCI to AD with similar accuracy. Importantly, the assays’ output in absolute biomarker concentrations is remarkably different, and this discrepancy cannot be reconciled with simple correction factors.
Introduction

Alzheimer’s disease (AD) is the major cause of dementia, which currently accounts for more than 35 million patients worldwide (World Alzheimer Report 2010 - The global Economic Impact of Dementia. Alzheimer’s Disease International 2010). AD is a neurodegenerative disease, pathologically characterized by extracellular amyloid plaques and intracellular neurofibrillary tangles. Amyloid plaques contain, among other components, a 42 amino acid long isoform of amyloid beta (Aβ42). Neurofibrillary tangles consist mainly of hyperphosphorylated forms of the microtubule-associated protein tau (Tau).

The analysis of cerebrospinal fluid (CSF) levels of the predominant proteins involved in the formation of amyloid plaques and neurofibrillary tangles has gained wide acceptance as a supportive measure in the differential and early diagnosis of AD. CSF levels of Tau and Tau phosphorylated at threonine 181 (pTau) are generally elevated in AD-patients, whereas Aβ42 levels are typically decreased. Quantification of these biomarkers in CSF is traditionally accomplished through enzyme-linked immunosorbent assays (ELISA). Multiplexed quantitative assay such as xMAP have become available to determine CSF levels of Aβ42, Tau and pTau simultaneously. xMAP technology performs bioassays on the surface of color-coded beads, using a dedicated read-out apparatus, following the same fundamental principles as used in the traditional ELISA, but requires less hands-on time and a smaller CSF sample volume. As a consequence of the multiplex approach, misreading one of the biomarkers in xMAP requires re-analysis of all three biomarkers in a separate run. Compared to ELISA, xMAP was found to have lower variability when determining CSF levels of Aβ42, Tau and pTau.

Multicenter studies are required to obtain sufficient subjects to study risk factors for AD and to investigate the effects of potential therapeutics. In such multicenter studies, accurate diagnostic tests are necessary and variations in test outcomes of Aβ42, Tau and pTau assays between centers should be minimized by the use of uniform protocols and the use of quality control samples. Quality control studies have already reduced the intercenter variations for ELISA. Multicenter studies, however, often use compiled data sets obtained by both ELISA and xMAP technology. In order to know if both types of testing result in comparable data, or that datasets obtained with one assay type need to be corrected before compilation with data sets obtained with the other assay, thorough comparison of analytical and clinical performance of ELISA and xMAP is crucial.

The aim of the current study was therefore to directly compare ELISA and xMAP with respect to 1) absolute biomarker concentration, 2) their ability to distinguish AD from non-demented subjects 3) monitor AD longitudinally and 4) predict progression from Mild Cognitive Impairment (MCI) to AD.
MATERIALS AND METHODS

Participants
For this study, participants were selected at the Alzheimer Center at the VU University Medical Center. At baseline all patients underwent standard dementia screening including physical and neurological examination, laboratory tests, electroencephalogram (EEG), and magnetic resonance imaging (MRI). Cognitive screening included an MMSE, but usually involved comprehensive neuropsychological testing. Additionally, all patients were offered a lumbar puncture, with a total response rate of approximately 75%\(^\text{17}\). Diagnosis of Mild Cognitive Impairment (MCI) (n=62) was made according to the criteria set by Petersen et al\(^\text{18}\), and the diagnosis of AD (n=68) was made following the National Institute of Neurological and Communicative Disease and Stroke/AD and Related Disorders Association (NINCDS-ADRDA) guidelines\(^\text{19}\). In addition, we included a group of non-demented subjects (n=24), which consisted of 20 patients with subjective memory complaints, two patients with a psychiatric disorder and two patients with temporal epilepsy. Clinical diagnoses were made by consensus of a multidisciplinary team and were independent of the biomarker levels in CSF. Demographic data of the study sample can be found in the Supplementary Data.

Thirty-four of the 62 MCI patients had progressed to AD at follow-up, three to frontotemporal lobar degeneration\(^\text{20}\), two to vascular dementia\(^\text{21}\), one to dementia with Lewy bodies 22 and one was diagnosed with normal pressure hydrocephalus. Six non-demented subjects had developed MCI, two developed AD, and one was diagnosed with vascular dementia at follow-up.

The VU University Medical Center ethics committee approved the study and all participants gave written informed consent.

CSF analysis
CSF was obtained through lumbar puncture between the L3/L4 and L4/L5 intervertebral space, collected in 12 mL polypropylene tubes. A fraction of the CSF was stored at -20°C for ELISA and routine analysis including total cell counts (for leucocytes and erythrocytes), total protein and glucose determination. Samples were centrifuged at 1800 x g for 10 minutes at 4°C within two hours after lumbar puncture. CSF was aliquoted into 0.5 or 1 mL polypropylene tubes and stored at -80°C. Staff involved in CSF analyses was blinded to the clinical diagnosis.

ELISA and xMAP assays were performed in duplicate on all patient samples. ELISA measurements took place within four weeks after each lumbar puncture. xMAP was applied to stored baseline and follow-up samples simultaneously.

ELISA
A\(_{\beta_42}\), Tau and pTau concentrations were determined using the Innogenetics sandwich ELISA (Innotest \(\beta\)-amyloid\(_{1-42}\), htau Ag and Phospo-tau (181P) Innogenetics, Ghent, Belgium), according to manufacturer’s instructions. The A\(_{\beta_42}\) assay uses the 21F12 monoclonal antibody for capture (primary) and the biotinylated 3D6 monoclonal antibody for
ELISA VERSUS XMAP

detection (secondary). Tau was captured by AT120 monoclonal antibody and detected by using two biotinylated monoclonal antibodies: HT7 and BT2. The capture antibody for pTau was the HT7 monoclonal, and was detected using biotinylated AT270 monoclonal antibodies. For an overview of antibodies used in ELISA, see Table 1.

CV of duplicates = 100 * Standard Deviation / Average. The intra-assay CV for ELISA was calculated as the average CV of all samples. Inter-assay CV was determined by using aliquots from batches of pooled surplus CSF specimens, which were stored at -80°C, that were included in each run (Table 1). For a detailed report on the intra- and inter-assay CV’s of our ELISA, we refer to\textsuperscript{23,24}. It should be noted that multiple batches of capture and detection antibodies were used during the course of this study.

xMAP

Quantification of A\textsubscript{β}42, Tau and pTau with xMAP was accomplished using the INNO-BIA AlzBio3 kit (Innogenetics, Ghent, Belgium), following manufacturer’s instructions, which simultaneously measures A\textsubscript{β}42, Tau and pTau\textsuperscript{13}. The AlzBio3 kit uses the same capture and detection monoclonal antibodies as ELISA, with the exception of the 4D7A3 antibody, which replaces the 21F12 antibody for capturing A\textsubscript{β}42. Tau is detected without the use of the BT2 antibody. All samples were handled according to the protocol provided by the manufacturer and measured using the Bioplex 200 system (Bio-Rad Laboratories b.v., Veenendaal, Netherlands). For an overview of antibodies used in xMAP, see Table 1.

For determination of the inter-assay CV we included pooled surplus CSF samples as quality controls, of which the concentrations were 55 and 133 pg/mL for A\textsubscript{β}42, 75 and 215 pg/mL for Tau and 30 and 47 pg/mL for pTau. CV of duplicates = 100 * Standard Deviation / Average. The intra-assay CV for xMAP was calculated as the average CV of all samples. For intra- and inter-assay CV we refer to Table 1.

Statistical analysis

One-way analysis of variance (ANOVA) was used to study differences in biomarker concentrations between diagnostic groups, with post-hoc Bonferroni correction for multiple comparisons. Receiver Operator Characteristics (ROC) - curves were extrapolated to determine the biomarker concentrations that provided the highest cumulative sensitivity and specificity. The comparison of ROC-curves was accomplished with the Hanly and McNeil method\textsuperscript{25} and the calculation of the area under the curve (AUC) was performed using MedCalc® Version 7.5.0.0. for Windows. Paired T-tests were used to compare baseline and follow-up values for each biomarker, group and type of assay to assess longitudinal changes in biomarker concentrations. To analyze the comparability of data from ELISA and xMAP, Bland-Altman plots were constructed\textsuperscript{25}. Pearson’s correlation was used to determine the correlation between total biomarker levels and the ratio of ELISA and xMAP data.

Comparison of ELISA and xMAP in their ability to predict AD was accomplished by comparison of hazards ratio’s and their 95% confidence intervals for progression to AD within the group of MCI patients, obtained from Cox proportional hazards models. For the Cox proportional hazards models, we used biomarker value (dichotomized – based
on median values) as independent variable, conversion to AD as dependent variable and
time from baseline lumbar puncture to date of last diagnosis as time variable. Age and
gender were entered as covariates. A\textsubscript{β} values below median and Tau and pTau values
above median were considered AD-positive biomarker profiles. The seven MCI patients
that had progressed to other neurological disorders at follow-up were excluded in the
Cox proportional hazards analysis.

For performing statistical analyses and generation of graphs, we used SPSS 18.0 (IBM,
New York, USA) for Mac OS X Snow Leopard.
P-values \leq 0.05 were considered significant.

Table 1. Analytical properties of ELISA and xMAP for A\textsubscript{β}42, Tau and pTau in CSF

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>xMAP</th>
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<tbody>
<tr>
<td>Primary antibody</td>
<td>A\textsubscript{β}42 21F12</td>
<td>4D7A3</td>
</tr>
<tr>
<td></td>
<td>Tau AT120</td>
<td>AT120</td>
</tr>
<tr>
<td></td>
<td>pTau HT7</td>
<td>AT270</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>A\textsubscript{β}42 3D6</td>
<td>3D6</td>
</tr>
<tr>
<td></td>
<td>Tau HT7 and BT2</td>
<td>HT7</td>
</tr>
<tr>
<td></td>
<td>pTau AT270</td>
<td>HT7</td>
</tr>
<tr>
<td>Dynamic range (min-max)</td>
<td>A\textsubscript{β}42 125 - 2000 pg/mL</td>
<td>47 - 1693 pg/mL</td>
</tr>
<tr>
<td></td>
<td>Tau 75 - 1200 pg/mL</td>
<td>22 - 1563 pg/mL</td>
</tr>
<tr>
<td></td>
<td>pTau 15.6 - 500 pg/mL</td>
<td>13 - 209 pg/mL</td>
</tr>
<tr>
<td>Intra-assay CV (%)</td>
<td>A\textsubscript{β}42 2.8</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>Tau 7.3</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>pTau 9.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Inter-assay CV (%) (low - high control)*</td>
<td>A\textsubscript{β}42 10.3 - 8.4</td>
<td>5.8 - 5.6</td>
</tr>
<tr>
<td></td>
<td>Tau 9.9 - 8.9</td>
<td>6.0 - 6.4</td>
</tr>
<tr>
<td></td>
<td>pTau 8.5 - 10.6</td>
<td>4.4 - 3.7</td>
</tr>
<tr>
<td>Lot# changes in this study</td>
<td>A\textsubscript{β}42 &gt;6</td>
<td>Single kit</td>
</tr>
<tr>
<td></td>
<td>Tau &gt;6</td>
<td>Single kit</td>
</tr>
<tr>
<td></td>
<td>pTau &gt;6</td>
<td>Single kit</td>
</tr>
</tbody>
</table>

Abbreviations: ELISA, enzyme-linked immunosorbent assay; CSF, cerebrospinal fluid; CV, coefficient of variation.
*Concentrations were 55 and 133 pg/mL for A\textsubscript{β}42, 75 and 215 pg/mL for Tau, and 30 and 47 pg/mL for pTau.
RESULTS

Analytical performance
The dynamic range of both assays differed substantially, with xMAP consistently reporting lower biomarker levels than ELISA, as can be seen in Table 2. Bland-Altman plots show that the difference between both assays depended on biomarker concentrations (Figure 1). While the differences in Aβ42 and Tau levels determined by ELISA and xMAP became larger with increasing biomarker concentrations, the opposite was observed for pTau.

Clinical performance
AD versus controls at baseline
ANOVA showed similar discriminatory performance of ELISA and xMAP at baseline (Table 2). To compare the sensitivity and specificity of biomarkers for AD diagnosis of both assays, we calculated the area under the curve (AUC) of the ROC-curves for each AD-biomarker. Table 3 represents the ability of both assays to distinguish AD patients (n=68) from non-demented subjects (n=24) based on Aβ42, Tau and pTau. No differences were observed between ELISA and xMAP, as judged from the 95% confidence intervals for the AUC.

Table 2. CSF biomarker concentrations as determined by ELISA and xMAP for nondemented subjects, AD and MCI patients at baseline and follow-up

<table>
<thead>
<tr>
<th></th>
<th>Nondemented</th>
<th>MCI</th>
<th>AD</th>
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<tbody>
<tr>
<td>Aβ42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>Baseline</td>
<td>675 (497-1006)*</td>
<td>467 (371-648)*</td>
</tr>
<tr>
<td></td>
<td>Follow-up</td>
<td>621 (485-926)</td>
<td>503 (404-669)</td>
</tr>
<tr>
<td>xMAP</td>
<td>Baseline</td>
<td>405 (286-514)*</td>
<td>270 (212-386)</td>
</tr>
<tr>
<td></td>
<td>Follow-up</td>
<td>337 (300-514)</td>
<td>288 (214-407)</td>
</tr>
<tr>
<td>Tau</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>Baseline</td>
<td>334 (213-502)*</td>
<td>539 (280-845)**</td>
</tr>
<tr>
<td></td>
<td>Follow-up</td>
<td>337 (250-591)</td>
<td>489 (292-877)</td>
</tr>
<tr>
<td>xMAP</td>
<td>Baseline</td>
<td>81 (62-125)*</td>
<td>134 (72-214)</td>
</tr>
<tr>
<td></td>
<td>Follow-up</td>
<td>91 (70-183)</td>
<td>143 (86-192)</td>
</tr>
<tr>
<td>pTau</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>Baseline</td>
<td>49 (37-76)*</td>
<td>72 (45-101)</td>
</tr>
<tr>
<td></td>
<td>Follow-up</td>
<td>57 (42-89)</td>
<td>72 (47-109)</td>
</tr>
<tr>
<td>xMAP</td>
<td>Baseline</td>
<td>26 (17-45)**</td>
<td>36 (22-48)</td>
</tr>
<tr>
<td></td>
<td>Follow-up</td>
<td>34 (21-39)</td>
<td>38 (22-56)</td>
</tr>
</tbody>
</table>

Abbreviations: AD, Alzheimer’s disease; MCI, mild cognitive impairment.
NOTE. Biomarker concentrations are in pg/mL and depicted as median (minimum–maximum).
Compared with AD at baseline *P < 0.01 **P < 0.05.
* Missing data on one AD patient.
* Missing data on three AD patients and one MCI patient.
* No follow-up comparison because of disease progression.
Figure 1. Bland-Altman plots for Aβ42, Tau and pTau, depicting the effect of average biomarker concentrations (x-axis) on the ratio between ELISA and xMAP values (y-axis). A concentration-dependent effect is observed for all three biomarkers.
It has to be noted that $A\beta_{42}$ measured in ELISA resulted in lower sensitivity than the 85% desired in the clinic\textsuperscript{26}. Also, the EOC for pTau using xMAP failed to reach this clinical threshold.

*Change over time*

To compare ELISA and xMAP in their ability to monitor disease progression over time, we compared $A\beta_{42}$, Tau and pTau concentrations at baseline and at an average two-year follow-up using paired t-tests in each diagnostic group. With ELISA, we observed a significant increase in $A\beta_{42}$ levels within the group of AD patients ($p <0.005$) over-time, which was not detected with xMAP. Conversely, only using xMAP we were able to detect an increase in Tau levels in the group of MCI patients ($p \leq 0.000$) and non-demented subjects ($p <0.05$) at follow-up. There was no difference in longitudinal Tau levels between MCI patients that progressed to AD and stable MCI patients. All other biomarker levels were stable over time for both ELISA and xMAP.

**Table 3.** EOC when distinguishing AD patients and non-demented subjects

<table>
<thead>
<tr>
<th></th>
<th>EOC (pg/mL)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>AUC (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A\beta_{42}$</td>
<td>ELISA 494</td>
<td>78</td>
<td>79</td>
<td>0.84 (0.75 - 0.91)</td>
</tr>
<tr>
<td></td>
<td>xMAP* 389</td>
<td>95</td>
<td>58</td>
<td>0.82 (0.73 - 0.90)</td>
</tr>
<tr>
<td>Tau</td>
<td>ELISA 362</td>
<td>88</td>
<td>67</td>
<td>0.79 (0.69 - 0.87)</td>
</tr>
<tr>
<td></td>
<td>xMAP* 98</td>
<td>90</td>
<td>63</td>
<td>0.75 (0.65 - 0.83)</td>
</tr>
<tr>
<td>pTau</td>
<td>ELISA 56</td>
<td>85</td>
<td>67</td>
<td>0.75 (0.65 - 0.84)</td>
</tr>
<tr>
<td></td>
<td>xMAP* 35</td>
<td>75</td>
<td>71</td>
<td>0.73 (0.63 - 0.82)</td>
</tr>
</tbody>
</table>

Abbreviations: EOC, estimated optimal cutoff values; AUC, area under the curve; ROC, receiver operating characteristic. NOTE. EOC when distinguishing 68 AD patients and 24 non-demented subjects based on ROC curve analyses.

*Values on three AD patients missing.

**Table 4.** Hazard ratios for progression to AD of MCI patients with AD-positive biomarker results

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>xMAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A\beta_{42}$</td>
<td>3.9 (1.7–9.2)</td>
<td>2.8 (1.2–6.1)</td>
</tr>
<tr>
<td>Adjusted for age and sex</td>
<td>4.0 (1.7–9.3)</td>
<td>2.8 (1.2–6.3)</td>
</tr>
<tr>
<td>Tau</td>
<td>4.1 (1.8–9.2)</td>
<td>4.0 (1.7–9.0)</td>
</tr>
<tr>
<td>Adjusted for age and sex</td>
<td>4.3 (1.8–9.8)</td>
<td>4.5 (1.9–10.3)</td>
</tr>
<tr>
<td>pTau</td>
<td>2.6 (1.2–5.5)</td>
<td>3.2 (1.5–6.7)</td>
</tr>
<tr>
<td>Adjusted for age and sex</td>
<td>2.6 (1.2–5.7)</td>
<td>3.1 (1.5–6.7)</td>
</tr>
</tbody>
</table>

NOTE. For the Cox proportional hazards models, we used biomarker value (dichotomized, based on median values) as independent variable, conversion to AD as dependent variable, and time from baseline lumbar puncture to date of last diagnosis as time variable. Age and gender were entered as covariates. $A\beta_{42}$ values below median and Tau and pTau values above median were considered AD-positive biomarker profiles.
Figure 2. Kaplan-Meier curves for Aβ_{42}, Tau and pTau for the conversion to AD within the group of MCI patients. Time (years) represents the time between the first lumbar puncture and follow-up diagnosis. Aβ_{42} levels below and Tau and pTau levels above the median are considered AD-positive.
Progression of MCI patients to AD
A total of 34 patients with MCI progressed to AD during an average of two years. Both ELISA and xMAP could distinguish MCI-converters from MCI-non converters based on Aβ42, Tau and pTau levels at baseline (p < 0.05 for each biomarker). For visual exploration of the predictive capabilities of both assays for progression from MCI to AD in time, we constructed Kaplan-Meier plots. Survival analysis based on data acquired by ELISA and xMAP show substantial similarity, as can be appreciated from Figure 2. Cox proportional hazards models showed comparable risks for progression from MCI to AD based on Aβ42, Tau and pTau for ELISA and xMAP, as judged from the 95% confidentiality interval of the hazard ratio’s (Table 4).

DISCUSSION
Our main finding concerning the analytical performance of the assays was that we consistently measured higher biomarker concentrations with ELISA compared to xMAP. In contrast to the finding of Olsson et al.13, we found a concentration-dependent change in the ELISA to xMAP ratio. In accordance with an earlier study by Reijn et al.27, the non-linearity between averaged concentrations as determined by ELISA and xMAP, and the ELISA to xMAP ratio was substantial for both Aβ42, Tau and pTau levels. A single correction factor for comparing data from ELISA with xMAP data will therefore not be sufficient. Our findings on the concentration-dependent differences in absolute biomarker levels are in accordance with previously described studies14,27,28. Considering our assays were both provided by Innogenetics, and basically use the same antibodies for detection, it is hard to formulate an explanation for the observed differences in absolute biomarker concentrations. Possibly the difference is caused by different interactions of the assays with the biomarkers in CSF, as the primary antibodies are immobilized in ELISA and in solution in the xMAP assay.

For multicenter studies, the implementation of single correction factors for direct comparison of xMAP and ELISA data may have detrimental consequences. For both Aβ42 and Tau, the lower-range concentrations measured with xMAP will be overcorrected, leading to lower lows, and the higher-range concentrations under corrected, leading to higher highs as compared to ELISA. pTau values will be affected in the opposite direction, correcting all xMAP data towards mid-range values. Such corrections can lead to overestimated differences between patient groups for Aβ42 and Tau, and a decreased distinction based on pTau values. Either way, implementation of a single correction factors leads to erroneous representation of the analytical data.

Our second objective was to compare the ability of both assays to detect AD. Therefore we estimated optimal cut-off values, which were the values that provided the maximum cumulative specificity and sensitivity for each biomarker when distinguishing AD patient from non-demented subjects. Neither Aβ42 when measured with ELISA, nor pTau quantified with xMAP reached the 85% sensitivity threshold for detecting AD, as was advocated in the Reagan consensus report26. It should be noted, however, that this study

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was not designed to assess the diagnostic accuracy of these markers per se, and that the derived cut-off values are meant for direct comparison purposes only. AUC’s for the biomarkers quantified by xMAP were similar to those determined by ELISA, indicating comparable usefulness in a diagnostic setting.

The study design was such, that for each subject CSF samples were analyzed that had been collected at baseline as well as at follow-up clinical examinations. For the group of non-demented subjects, this probably led to an increased inclusion of subjects that progressed to AD, since these subjects had returned to the Alzheimer Center due to persistent cognitive complaints. Indeed, a total of six out of 24 non-demented subjects had progressed to MCI, two to AD and one to vascular dementia. Moreover, the high conversion rate may have led to an underestimation of the power of both ELISA and xMAP to discriminate between patient groups. Indeed, we found lower diagnostic accuracy for all three biomarkers measured with ELISA compared to our earlier findings.

To the best of our knowledge, we are the first to report a direct comparison of the disease-monitoring capabilities of ELISA and xMAP. The unique design of this study allowed us to compare baseline and follow-up biomarker levels for each individual subject in both assays. Our analysis showed three differences in biomarker concentrations over time between ELISA and xMAP. ELISA, but not xMAP data showed an increase in A\text{\textsubscript{\textbeta}}\text{\textsubscript{42}} levels within the group of AD patients at follow-up. xMAP, but not ELISA detected an increase in Tau levels within the groups of MCI patients and non-demented subjects at follow-up. Discrepancies in longitudinal biomarker levels, can possibly be attributed to the different analytical precisions of the assays. ELISA has a lower intra-assay CV for A\text{\textsubscript{\textbeta}}\text{\textsubscript{42}}, and xMAP for Tau and pTau. High analytical precision may well be a prerequisite for the detection of minor changes in biomarker levels over a two-year period.

The overall assay performances presented in this study could be affected by a number of variables. Firstly, samples analyzed with the xMAP assay were processed and stored shortly after each lumbar puncture, and baseline and follow-up samples were quantified simultaneously. Therefore, a single batch of primary and secondary antibodies was used in the xMAP assay. A different procedure was applied to the samples analyzed by ELISA, as biomarker concentrations were determined within four weeks after each lumbar puncture. In contrast, and as a consequence of the time frame in which our samples were acquired, the ELISA’s were performed using different batches of reagents, which probably affected the assay’s performance. Secondly, the increased storage time for all samples analyzed with xMAP may have affected the biomarker concentrations and, thus, the discriminatory power of the xMAP assay. It should be noted that all our samples were consistently stored at -80°C, which in an earlier study was shown not to affect the levels of A\text{\textbeta}, Tau or pTau.

Finally, the ability of both assays to predict progression to AD within the group of MCI patients based on baseline biomarker levels was compared. No differences were observed in hazard ratios for progression of MCI to AD between biomarker levels determined by either ELISA or xMAP. This indicates that both assays have a similar prognostic potential. We conclude that the xMAP assay can be implemented as a supportive measure for
clinical diagnosis and possibly prognosis of AD without loss of power, as compared to ELISA. We do advice a reevaluation of the optimal cut-off values for optimal support of AD diagnosis for values determined by xMAP, since the cut-off values for ELISA cannot be directly extrapolated. Although ELISA and xMAP both have their analytical and financial advantages, our results do not argue towards the use of either assay.

Acknowledgments
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CHAPTER 2

REFERENCES