Abstract

Background: Alzheimer's disease (AD) is a severe neurodegenerative disease and its prevalence is rising globally. Evidence suggests that the pathological lesion of AD in the brain starts 10-15 years before the onset of clinical symptoms. Due to the absence of clinical features, it is difficult to diagnose the disease early in the pre-clinical stage. Once cognitive impairment sets in, it is currently impossible to reverse the changes that have occurred in the brain. However, identification of biomarkers might allow detection of AD in the pre-symptomatic stage. Three core biomarkers (Aβ42, total-tau and phosphorylated-tau) have been well-studied and show high diagnostic accuracy in the discrimination of AD patients from healthy elderly individuals. However, low specificity of these biomarkers in differentiating AD from other forms of dementia, as well as technical variability in measuring levels, e.g. in Cerebrospinal Fluid (CSF), calls for continuing the search for additional candidate biomarkers. Aβ43 has recently emerged as a potential biomarker of AD after the finding that it is more neurotoxic than other amyloid peptides. However, few studies have been conducted on Aβ43 levels in CSF, and no longitudinal study has been reported so far.

Objective: This study was designed to quantify the concentration of Aβ43 in the CSF of subjects with amnestic type of Mild Cognitive Impairment (MCI) who remained stable (sMCI) or progressed to AD (pMCI) during two years of study and early AD patients, compared to healthy control individuals. Diagnostic accuracy for patients was determined for serial levels at baseline (T0) and follow-up after 12 and 24 months respectively (T12 and T24).

Method: 94 subjects (62 patients and 32 controls) were followed up clinically for 2 years and serial CSF samples were collected. ELISA kits were used to measure the levels of CSF biomarkers and the data were analyzed using SPSS version 21.

Results: The data indicated a significantly reduced concentration of CSF Aβ43 in patients compared to controls. Longitudinally, marked reductions were seen in follow up levels (T12, T24) compared to baseline (T0). In addition, a high diagnostic potential was demonstrated for distinguishing the patient group progressing to AD in two years and early AD group from controls.

Conclusions: Lowered levels of Aβ43 in patients as compared to cognitively normal individuals suggest that Aβ43 may be a potential biomarker with high diagnostic performance for differentiating MCI individuals who progress to AD from healthy elderly subjects. In addition, the lowered concentration of CSF Aβ43 in serial measurements of prodromal individuals may be an indicator of progression to AD.

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Though only my name appears on the cover of this dissertation, a great many people have contributed to its production. I owe my gratitude to all those people who have made this dissertation possible and because of whom my master's experience has been one that I will cherish forever. First of all I would like to thank Professor Linda R. White for having faith in me and giving me the opportunity to work under her supervision. Her wide spectrum of knowledge, logical ways of thinking and personal attention have been of great value to me. Her understanding, encouraging and personal guidance provided a good basis for my thesis. The thesis would not have been completed without her immense support, advices and constructive feedback during the writing process. I am deeply grateful to her for the long discussions that helped me sort out the technical details of my work. I am also thankful to her for encouraging the use of correct grammar in my writings and for carefully reading and commenting on countless revisions of this thesis.

My special thanks goes to my co-supervisor, Dr. Sigrid Botne Sando for providing the CSF samples used in the project. I benefited immensely from her practical knowledge and the discussions I had with her. I would also like to extend my thanks to Phd student Camilla Lauridsen for motivating me and always being available to answer my queries patiently. She was also very helpful in bringing the thesis to its current form. Guro Berge also deserves my sincere gratitude for the stimulating discussions in spite of having a very busy schedule. Thanks also to Ina Møller for the technical help in the lab.

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Most importantly, none of this would have been possible without the love and patience of my family especially, when the times got tough due to my fathers demise who was a continuous source of encouragement and inspiration for me. Finally, my sincere thanks to my dear husband Adil for encouraging and believing in me.

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Abbreviations

Aβ Amyloid beta

ACE Angiotensin Converting Enzyme

AD Alzheimer's disease

APOE Apolipoprotein E gene

ApoE Apolipoprotein E

APP Amyloid precurser protein

AUC Area under the curve

BACE1 β - site APP cleaving enzyme-1

CSF Cerebrospinal fluid

ELISA Enzyme linked immunosorbent assay

FDG 2-(^{18}F) fluoro-2-deoxy-D-glucose

FDG-PET Fluoro-2-deoxy-D-glucose positron emission tomography

fMRI functional Magnetic Resonance Imaging

HRP Horse Radish Peroxidase

IQR Interquartile range

IWG International Working Group

LOAD Late-onset Alzheimer's disease

MCI Mild cognitive impairment

MMSE Mini Mental Score Examination

MRI Magnetic Resonance Imaging

mRNA Messenger Ribo Nucleic Acid

NFT Neurofibrillary tangles

NIA-AA National Institute on Aging and the Alzheimer Association

NINCDS-ADRDA National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer Disease and Related Disorders Association

PET Positron Emission Tomography

PiB ^{11}C-Pittsburgh Compound B

PiB-PET Pittsburgh compound B - Positron Emission Tomography

pMCI progressive Mild Cognitive Impairment

PSEN Presenilin gene

p-tau Phosphorylated Tau

P-tau181 Tau Phosphorylated at Threonine 181

P-tau199 Tau Phosphorylated at Serine 199

P-tau231 Tau Phosphorylated at Threonine 231

ROC Receiver Operating Characteristic

sMCI stable Mild Cognitive Impairment

sMRI structural Magnetic Resonance Imaging

Tau-PET Tau Positron Emission Tomography

TBI Traumatic Brain Injury

t-tau Total Tau

1 INTRODUCTION

1.1 What is Alzheimer's disease ?

Alzheimer's disease (AD) is a fatal and slowly progressing neurodegenerative disorder and the leading cause of senile-dementia. It is clinically manifested initially by insidious onset of progressive impairment of memory along with decline in other cognitive functions including deterioration of judgement, language skills and attention, personality changes and behavioural symptoms. Later, as the disease progresses, these symptoms become more severe, ultimately interfering with social function and performance of daily activities of life.

The main histopathological findings in the brain of AD patients are aggregation of extracellular senile plaques composed of amyloid β (Aβ) peptides, and intracellular neurofibrillary tangles (NFT) made up of abnormally phosphorylated tau proteins and are considered as the hallmark of the disease (Blennow et al. (2006), Hyman et al. (2012). These changes are often associated with diffuse cortical atrophy, synaptic loss and neuronal degeneration, as well as deposition of amyloid in blood vessels (Dubois et al. (2010), Perrin et al. (2009)).

On the basis of age of onset, AD is categorised into early-onset AD and late-onset AD (LOAD) (Kim et al. (2007)). Early onset AD is rare with mean age of onset below 65 years and is generally caused by autosomal dominant mutations. LOAD accounts for more than 95 % of all cases, is mostly sporadic and appears at or after 65 years. The cause of LOAD pathology is unknown.

Worldwide prevalence of AD is approximately 24 million and is expected to increase four fold by the year 2050 (Reitz and Mayeux (2014)). The occurrence of new AD cases is rising as a result of increase in number of elderly people due to improved life expectancy (Hebert et al. (2003)).

AD is a rapidly growing disease with no known treatment presently. According to the current National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer Disease and Related Disorders Association (NINCDS-ADRDA criteria, 1984), AD cannot be diagnosed unless it has progressed to the stage of dementia. However, according to the new revised research criteria proposed in 2011, pathology of the disease starts much earlier, before the patient develops clinically identifiable signs and symptoms. Since the individual is able to function normally in spite of the pathological changes in the brain during the early stages, it is difficult to make a definitive diagnosis with the prevailing diagnostic criteria, but may be indicated by biomarkers.

As the prevalence rate is rising, there is a pressing need to identify biomarkers in order to allow diagnosis of the disease at an early stage, and facilitate therapeutic interventions to prevent or slow down progression from preclinical stages to AD. Mild Cognitive Impairment or MCI is a prodromal stage of AD with mild impairment of memory but without overt dementia (Blennow et al. (2006)). Biomarkers demonstrated topographically or in CSF can be used to differentiate MCI due to AD and other diseases. It has been well established that Aβ peptide and tau protein are currently the core biomarkers for the diagnosis of AD. However, newer biomarkers are needed to enhance diagnostic accuracy of early AD and prevent progression to the stage of overt dementia.

1.2 Neuro-pathological findings in the brain of AD Patients

1.2.1 Amyloid plaques

Amyloid plaques (Figure 1) are insoluble material found in the brain of AD patients which contain a central core of amyloid fibrils surrounded by a circular band of dystrophic nerve cell processes, activated microglial cells and reactive astrocytes (Masters et al. (2006)). These plaques have been found in the brain of nondemented elderly people which suggests that the process of amyloid plaque build-up starts before the onset of cognitive decline (Serrano-Pozo et al. (2011)). The main component of amyloid plaque is Aβ peptide, which ranges mainly from 37 to 43 amino acid residues in length (Masters et al. (2006), Holtzman (2011)). It is generated by stepwise proteolytic cleavage of amyloid precursor protein (APP) producing mainly Aβ40 and Aβ42 with a small amount of other Aβ peptides. APP is a transmembrane glycoprotein with a large amino-terminal and a short carboxy-terminal. APP undergoes proteolytic processing by two distinct pathways: "non-amyloidogenic" (α) pathway and "amyloidogenic" (β) pathway, Figure 2 (Dong et al. (2012)). Under normal conditions, APP processing occurs first by α-secretase releasing soluble APP-α (sAPP-α) extracellularly, and subsequently by γ-secretase resulting in release of p3, a non-pathogenic form of Aβ. This pathway is considered as non-amyloidogenic as pathological Aβ is not generated. Under pathological conditions, APP is cleaved first by β-secretase enzyme (βsite APP cleaving enzyme1/BACE1) liberating soluble APP-β (sAPP-β). The carboxy-terminal splits further by γ-secretase releasing different isoforms of Aβ peptides which accumulate in the senile plaques and vessel walls (Selkoe (1996), Selkoe (2001)). Aβ40 and Aβ42 (40 & 42 amino acids long respectively) constitute the most common form of amyloid β-peptides (Selkoe (2001)). The shorter one, Aβ40 monomers are more abundantly produced comprising nearly 90% of all Aβ peptide in diffuse plaques (Zou et al. (2013)). Aβ40 was also considered as pathogenic earlier, but some evidence shows that Aβ40 is not pathogenic and in fact plays a protective role by inhibiting the aggregation

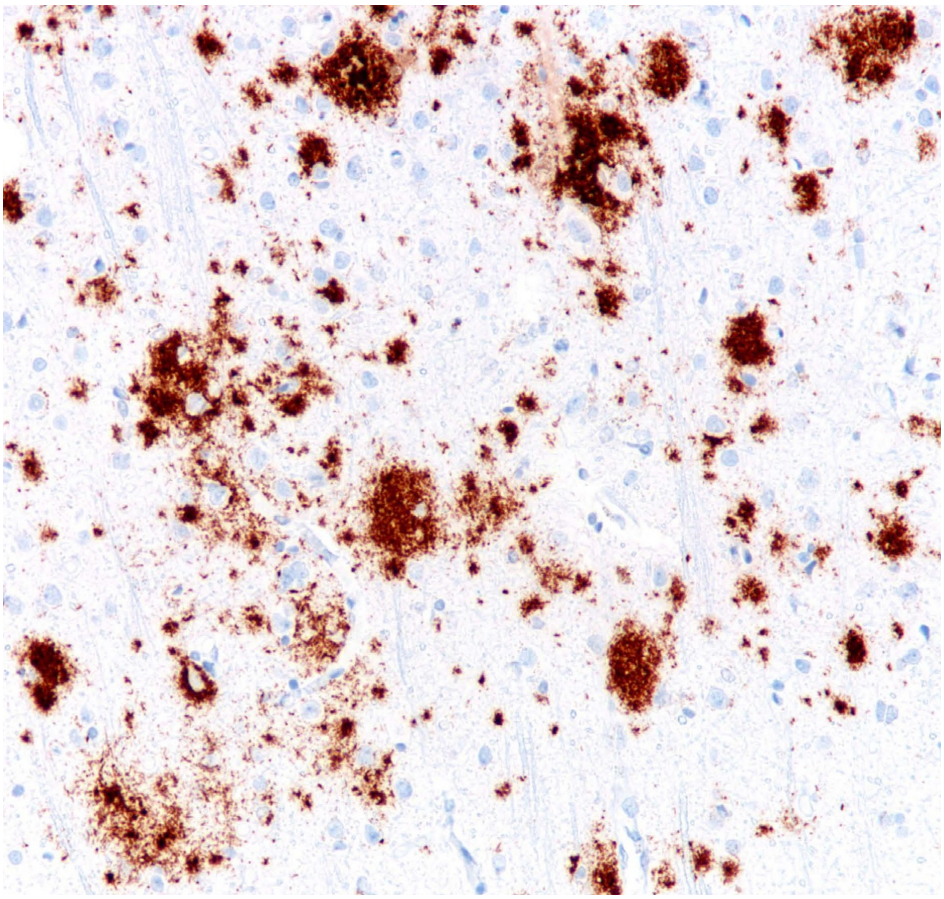


Figure 1: Amyloid plaques in the cerebral cortex seen on immunohistochemistry Castellani et al. (2010)

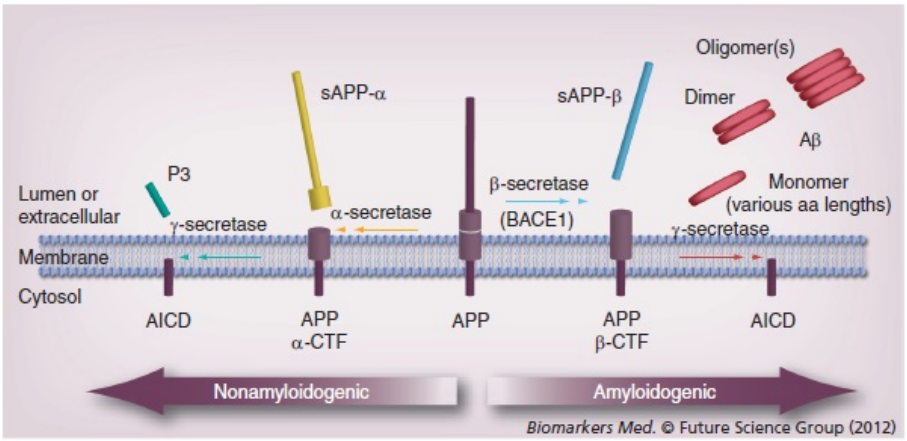


Figure 2: Two pathways of APP processing with two outputs. Non amyloidogenic (left) leading to sAPP-α and p3 by α-secretase and γ-secretase. Amyloidogenic (right) producing sAPP-β and Aβ by β-secretase and γ-secretase respectively (Fagan and Perrin (2012))

of Aβ42 monomers (Yan and Wang (2007); Kim et al. (2007)). Even though Aβ42 is a minor component of amyloid plaque (10%), it indeed appear early and predomi-

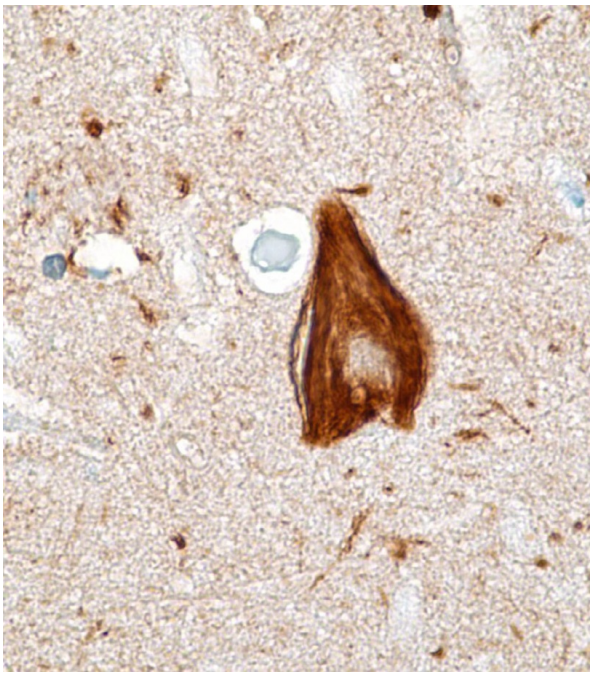


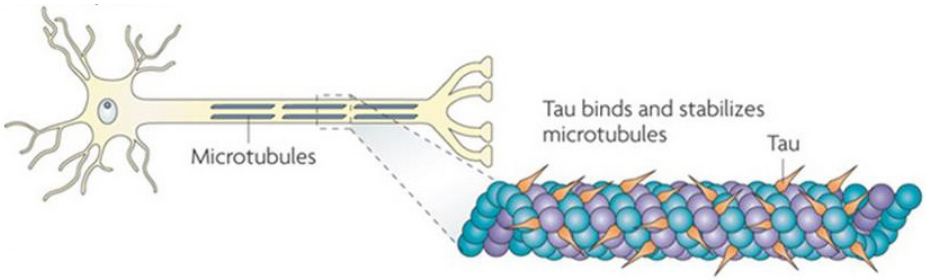
Figure 3: Immunohistochemimcal staining showing Neurofibrillary Tangles Castellani et al. (2010)

nates in senile plaques (Iwatsubo et al. (1994)). This is due to its hydrophobic nature, which makes it more prone to undergo oligomerization and aggregation rapidly to form dimers, oligomers, protofibrils, fibrils and fibrillar aggregates (Blennow et al. (2006)).

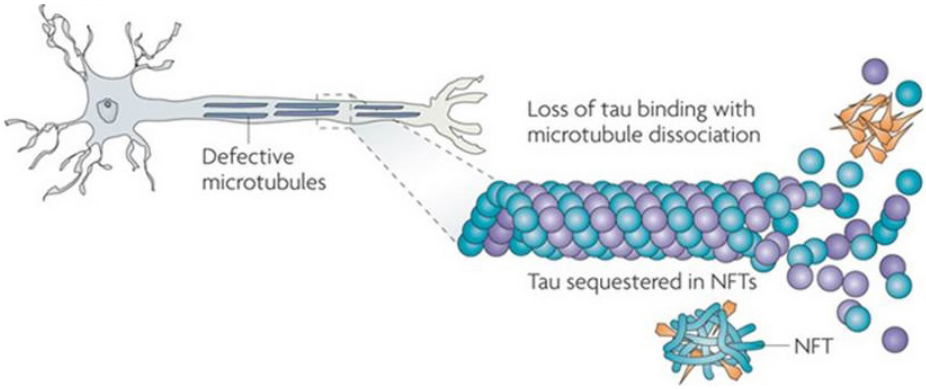
1.2.2 Neurofibrillary tangles

Neurofibrillary tangles (NFT) (Figure 3) are composed of abnormal filaments made up of hyperphosphorylated forms of tau (Brion (1998)). Tau is a normal microtubule-associated protein found mainly in axons. The most important function of tau protein is to bind with microtubules and promote their stabilization, thereby regulating axonal transport of organelles (Figure 4(a)). In AD, tau protein undergoes abnormal hyperphosphorylation and loses its ability to bind to the microtubules (Figure 4(b)). So, microtubules become unstable and collapse. This culminates in impaired axonal transport ultimately leading to progressive synaptic loss, degeneration and death of neurons (Grundke-Iqbal et al. (1986). Unbound tau undergoes misfolding and assembles as oligomers and fibrils, and eventually forms NFTs. Tau-mediated degeneration of neurons develops as a result of the combined effect of loss-of-function of tau protein, with gain-of-function of hyperphosphorylated tau aggregates (Ballatore et al. (2007)).

Six different isoforms of tau protein are expressed in the adult human brain derived from alternative splicing of tau mRNA (Goedert et al. (1989); Neve et al. (1986)). In vitro studies have shown that kinase enzymes are responsible for phosphorylation of tau, but this has not been proven yet *in vivo* (Buee et al. (2000)). In addition,



(a) Healthy neurons



(b) Diseased neurons

Figure 4: Normally tau protein binds to microtubules and stabilizes it. In Alzheimer's disease tau gets phosohorylated and looses its affinity to microtubules resulting in its accumulation as neurofibrillary tangles. Brunden et al. (2009)

many phosphatase enzymes critical for the reverse reaction have also been identified, but their exact role is not completely understood (Tian and Wang (2002)). It can therefore be considered that aberrant phosphorylation occurs as a result of increased functioning of kinases or decreased functioning of phosphatases. Increasing evidence suggests that the number and extent of NFT accumulation correlates with the degree of cognitive decline and severity of dementia (Arriagada et al. (1992)).

In 1991, Braak presented a model of the progression of the disease by categorising it into six stages (Braak and Braak (1991)). The NFT first appear in the entorhinal cortex, followed by involvement of the limbic regions (hippocampus and amygdala), and finally spreading to the neocortical regions as well.

1.2.3 Other associated changes

Apart from the two neuropathological hallmarks discussed above, the brain of AD cases is associated with other changes. Macroscopically, there is gross atrophy of the cortex (Masters et al. (2006)). On microscopic examination, there is extensive degeneration and loss of neurons. It has been found that neuronal loss selectively affects mainly layer II of entorhinal cortex, olfactory bulb, amygdala, and nucleus basalis of Meynert (Gan and Patel (2013); Tapiola et al. (2009)). The amount of neuronal loss correlates

with amyloid and tangle deposition. Synaptic loss has been documented at an early stage of the disease and correlates with cognitive decline (Burns et al. (1997)). It is considered that loss of synaptic function is an important factor in the deterioration of cognitive abilities. In addition, inflammatory reactions and gliosis occur secondary to deposition of amyloid plaques, and involve activation of microglia and astrocytes (McGeer et al. (2000)).

1.3 Etiology and Risk factors

Although the exact cause of AD is still not clearly understood, some risk factors have been found to be associated with the disease. Advancing age represents by far the greatest risk factor for the development of AD (Allsop and Mayes (2014)). Other potential risk factors include genetic and environmental factors.

1.3.1 Genetic factors

Individuals with a family history of AD are more likely to develop the disease than people who do not have a first degree relative with AD (reviewed in Thies (2013). Mutations in three genes have been identified as accounting for the majority of familial cases of AD (Goate et al. (1991)). Mutation of the amyloid precursor protein (*APP*) gene located on chromosome 21 was the first to be identified for familial cases of AD (Allsop and Mayes (2014)). Research studies suggests that both genomic duplication in the *APP* locus and mutations in the *APP* gene play a role in the pathogenesis of AD (Dong et al. (2012)). These mutations result in the substitution of isoleucine for valine at codon 717 which induces changes in the steps involved in APP processing, with subsequent increased production of the total amount of Aβ, especially Aβ42 (Golde et al. (2000), Masters et al. (2006)). Specific mutations in the genes for presenilin 1 (*PSEN* 1) on chromosome 14 and presenilin 2 (*PSEN* 2) on chromosome 1 are the most common genetic cause of familial form of AD, accelerating the rate of production and deposition of the toxic Aβ peptide variant, Aβ42, leading to neurodegeneration in AD. *PSEN* genes are also involved in regulating degradation of Aβ. More research is needed to fully understand the role of these genes in the pathogenesis of familial AD.

The presence of *APOE* ε4 allele on chromosome 19 is considered to be the major genetic risk-factor for the development of sporadic or LOAD (Voller et al. (1978)).

1.3.2 Role of Apolipoprotein E in AD pathology

Apolipoprotein E (ApoE) is the principal lipoprotein expressed in the central nervous system. It is expressed by various tissues with highest expression in liver, followed by brain. In the brain, it is primarily synthesized and secreted by astrocytes, and microglia. ApoE proteins are involved in many functions such as binding, transport

and distribution of lipids, particularly cholesterol; synapse formation, repair of neurons, inflammation, removal of amyloid proteins and tau phosphorylation (Allsop and Mayes (2014)).

APOE encodes three common types of alleles: *APOE* ε2, *APOE* ε3, and *APOE* ε4, translating as their corresponding proteins ApoE 2, ApoE 3 and ApoE 4. The resulting protein isoforms differ in the position of two of total 299 amino acids, E2 (Cys^{112}, Cys^{158}), E3 (Cys^{112} ,Arg^{158}), and E4(Arg^{112}, Arg^{158}) (Dong et al. (2012), Leoni (2011)). These differences between the alleles are responsible for the difference in the structure and function of the corresponding ApoE proteins. *APOE* ε3 is the most frequently occurring allele. The ε2 form of *APOE* is considered as neuroprotective by reducing the risk and delaying the age of onset of the disease. In a study conducted by Berge et al (Berge et al. (2014)), it was found that APOE ε2 delays the onset of AD in central Norway by four years.

It is well established that Aβ peptide is generated from APP. ApoE ε4 stimulates endocytic recycling of APP thereby increasing the total production of APP promoting more Aβ formation (Ye et al. (2005)). ApoE 4 promotes Aβ deposition and its aggregation into fibrils. However, ApoE 2 and ApoE 3 plays a protective role towards development of AD by promoting Aβ clearance (Holtzman et al. (1999)). Although *APOE* has been found to be linked with LOAD, genetic testing of APOE is not advised due to low sensitivity and specificity (Patterson et al. (2008))

1.3.3 Environmental and lifestyle factors

De Bruijn and Ikram summarised the association between AD and cardiovascular risk factors including stroke, atrial fibrillation, hypertension, hypercholesterolemia, coronary heart disease and heart failure (de Bruijn and Ikram (2014)). The mechanism underlying link between cardiac diseases and AD may be due to a compromised blood supply resulting in loss of neuronal tissue. Although the exact cerebrovascular factor underlying AD is unclear, it has been found that infarction, vasculopathies and white matter changes may enhance the risk of dementia (Reitz and Mayeux (2014)). Moreover, diabetes mellitus, smoking and obesity have been found to increase the risk of AD, but the exact mechanism is not clearly understood (Moreira (2012),Cataldo et al. (2010)). Epidemiological studies also suggest that traumatic brain injury (TBI) can be a predisposing factor for the development of AD (Van Den Heuvel et al. (2007)). Furthermore, inflammation, chronic kidney disease, and thyroid dysfunction are considered as emerging risk factors to be implicated in AD (de Bruijn and Ikram (2014)).

The Mediterranean diet which includes a high intake of fruit, vegetables, fish and unsaturated fatty acids and a lower intake of saturated fats and red meat in the food, is associated with a lower risk of MCI and AD (Singh et al. (2014)) . This may be due to a higher level of antioxidants and polyunsaturated fatty acids in these foods that

reduces neuronal damage. A higher level of physical activity has been linked to lower incidence of AD (Buchman et al. (2012)).

1.3.4 Cognitive reserve

Brain reserve, or cognitive reserve, is the ability of the brain to cope with changes due to ageing and pathological damage without showing any obvious clinical manifestation (Fratiglioni and Wang (2007)). Some people have a higher threshold to tolerate damage than others, and beyond that cognitive reserve finishes and cognitive decline sets in. Evidence from many studies has shown that individuals with a higher level of education have a lower risk of developing clinical evidence of neuropathology (Liberati et al. (2012)). Case-control studies show that individuals who are engaged in brain stimulating activities like playing games, reading and learning, have less chance of developing dementia.

1.4 Diagnostic criteria

1.4.1 Prevailing diagnostic standard

A set of clinical criteria was established by the NINCDS-ADRDA for the diagnosis of Alzheimer's disease in 1984 (McKhann et al. (1984)). According to these criteria, the diagnosis of AD can only be "probable" in life (except in some cases where brain-biopsy can be done). Moreover, a probabilistic diagnosis is made when the threshold of dementia is crossed and other systemic or brain diseases are excluded which may promote progressive memory impairment (Dubois et al. (2010)). However, a "definite" clinical diagnosis of AD can only be made post-mortem as it requires proof of the presence of amyloid plaques and neurofibrillary tangles on histopathological examination of brain tissue on autopsy.

1.4.2 Revision of the research criteria

There has been significant advance in the past three decades towards a better understanding of the pathogenic mechanism of the disease process. Dementia was an essential requirement of the 1984-criteria, so by the time the disease was diagnosed, it had already progressed to an advanced stage causing significant functional disability and interventions were impossible. Accurate diagnosis of AD is critical in the early stages to allow secondary prevention of the disease. Thus, many proposals have been made to revise the original NINCDS-ADRDA criteria. Varma et al. (1999) showed that the NINCDS-ADRDA criteria had a high sensitivity of 93% but low specificity of 23% in the diagnosis of AD in a group of patients with cortical dementia (Alzheimer disease

and fronto-temporal dementia) and the accuracy rate was significantly low ranging between 65% - 92% .

The need to update the current research criteria led to the presentation of two major sets of criteria; one published by the International Working Group (IWG) of dementia experts in 2007 and updated in 2010 (Dubois et al. (2010)), and the second proposed by the National Institute on Aging and the Alzheimer Association (NIA-AA). The IWG pointed out that AD can be identified on the basis of two features:

- clinical evidence of episodic memory impairment
- *in vivo* biological evidence of AD pathology by studying specific and reliable molecular and neuroimaging biomarkers.

The newly proposed algorithm allows extension of the diagnosis of AD with a high level of accuracy, even at the stage of the earliest clinical manifestation, the prodromal stage of the disease (before the occurrence of full-blown dementia with functional disability) (Dubois et al. (2010)).

The novel diagnostic framework supported a major change in the concept of AD and aroused debate about the definition of AD and related conditions (Dubois et al. (2010)). Only typical AD with amnestic presentation and biomarker positivity was focussed and other variants of the disease were not considered. Annual meetings were convened by IWG to further advance the new research criteria and a new lexicon was proposed to redefine AD in 2010. This culminated in the IWG and NIA-AA jointly proposing recommendations for harmonized clinical diagnostic criteria for AD (Morris et al. (2014)). According to these criteria, AD represents a brain disorder regardless of clinical status, and the clinically-expressed stage from mild cognitive loss to severe dementia should be considered as "symptomatic AD". In addition, it was also recommended to incorporate the use of biomarkers in the diagnostic algorithm as soon as sample collection and processing can be standardized internationally. Furthermore, it was proposed to allow non-amnestic, atypical presentations to be included as symptomatic AD, particularly when supporting biomarker evidence is present. This group proposed that AD is a continuous process of cognitive and functional decline. Later stages (MCI/prodromal AD and AD dementia) can be diagnosed easily as "Symptomatic AD" on the basis of the presence of amnesia. Nonetheless, early stages with no signs of memory deficits can also be regarded as symptomatic on the basis of biomarker evidence.

1.5 Alzheimer's disease is a chronic disease

According to the revised criteria, AD is considered as a continuum comprising a long asymptomatic "preclinical" stage and a symptomatic stage including mild cognitive impairment / predementia AD and AD dementia (McKhann (2011)).

Table 1: Recommendations to harmonize clinical diagnostic criteria for Alzheimer's disease Morris et al. (2014).

Concept	*Alzheimer disease* refers to the brain disorder regardless of clinical status
Terminology	*symptomatic AD* refers to the clinically expressed disorder, from very mild (‚MCI due to AD and *prodromal AD*) to severe dementia
Biomarkers	Incorporation of molecular and degeneration biomarkers into the clinical diagnostic algorithm after completion of the standardization process, and until then they can be used to support the clinical diagnosis, especially in cases with atypical presentation
Memory	Typical AD presents with amnesia. Cases with non-amnestic presentation can also be diagnosed by the support of biomarkers.

1.5.1 Preclinical AD

The term preclinical AD is assigned to the long asymptomatic phase of AD continuum in which pathological changes in the brain begin to accumulate but cognitive ability is normal. Preclinical AD *in vivo* can be identified in cognitively normal individuals by the use of imaging and Cerebrospinal fluid (CSF) biomarkers. Clinically normal individuals with biomarker evidence of AD are at increased future risk of cognitive decline and progression to AD (Fagan et al. (2007)). However, this does not imply that all individuals showing evidence of early AD pathology will progress to the dementia phase (Sperling et al. (2011)). In the NIA-AA criteria, preclinical AD has been classified into three stages:

1. Stage 1 is characterised by abnormal amyloid markers,
2. stage 2 by abnormal amyloid and neuronal injury markers,
3. stage 3 includes features of stage 2 plus subtle cognitive changes (Sperling et al. (2011)).

Stage 0 was also proposed to include asymptomatic cases devoid of amyloid, neurodegeneration and cognitive changes. Jack et al. (2011) proposed another biomarker classification system to include asymptomatic individuals. Each individual is labelled as positive or negative for amyloid (A) and neurodegeneration (N). A^-N^- corresponds to stage 0 of NIA-AA, A^+N^- to stage 1 and A^+N^+ to stage 2 and 3. A^-N^+ was included corresponding to subjects suspected with non-AD pathology.

1.5.2 Mild Cognitive Impairment (MCI)

MCI is a term coined for the symptomatic predementia phase of AD (Albert et al. (2011)). Individuals in this stage show evidence of change in cognition compared to their past, but functional abilities are preserved. Cognitive functions include 5 domains:

1. memory (ability to learn new information)
2. language
3. attention
4. executive functions
5. visuo-spatial skills

For the diagnosis of MCI, a patient must show poor performance in one or more of these cognitive domains expected for the patient's age and educational background, but not severe enough to constitute dementia. The presence of an autosomal dominant mutation (*APP, PSEN1* and *PSEN2*) increases the chances of EOAD (Schellenberg (2006)). However, a carrier of one or two ε4 alleles of the *APOE* gene has increased risk of developing LOAD. Evidence suggests that an individual meeting the clinical and etiological criteria for MCI is more likely to progress to AD dementia in relation to cognitively normal subjects. Ganguli et al. (2004) found that 27 % of MCI patients progressed to the stage of dementia over the following 10 years.
Nonetheless, MCI is not always progressive; some cases re vert back to normal or remain stable (Mitchell and Shiri-Feshki (2009)), so there is a need for better methods to identify those MCI cases which progress to AD. Better definition of MCI criteria and use of biomarkers are required to improve case selection. Studies show that biomarkers can serve as tools for identification of MCI due to AD, and MCI due to causes other than AD (Petersen et al. (2014)).

1.5.3 Dementia

AD dementia refers to the clinical syndrome that arises secondary to the pathophysiology of AD (McKhann (2011)). The diagnosis relies on the presence of cognitive decline from previous levels sufficient to interfere with normal functional and social abilities. According to the new criteria, individuals with dementia due to AD can be classified into probable AD dementia and possible AD dementia. Probable AD cases can be further categorised into amnestic presentation (involvement of memory domain) or non-amnestic presentation (involvement of cognitive domains other than memory). A diagnosis of possible AD dementia is made in a case of atypical presentation (sudden onset of cognitive impairment) or mixed presentation (concomitant presence of other medical condition that could have an effect on cognition).

Table 2: Recommendations for general criteria for MCI Winblad et al. (2004)

General criteria for MCI
Not normal, not demented (Does not meet criteria (DSM IV, ICD 10) for a dementia syndrome)
Cognitive decline
Self and/or informant report and impairment on objective cognitive tasks
Evidence of decline over time on objective cognitive tasks
AND/OR
Preserved basic activities of daily living / minimal impairment in complex instrumental functions

1.6 Pathogenesis

Although the exact mechanism is still unclear, several theories have been suggested to explain the molecular mechanism leading to AD.

1.6.1 Cholinergic hypothesis

Degeneration of cholinergic projection neurons from the nucleus Basalis of Meynert to the cortex and hippocampus was reported as a major event in the pathogenesis of AD (Whitehouse et al. (1982)). The cholinergic hypothesis was the first theory to be proposed to explain the mechanism of AD, which states that loss of cholinergic neurons, and enzymes involved in acetylcholine synthesis and degradation contributes towards the development of cognitive decline in advanced age and AD (Bartus (2000)).

However, the exact mechanism involved in degeneration of cholinergic cells is not completely understood. While it is well documented that severe cholinergic dysfunction occurs at later stages of AD, it is debatable whether the cholinergic hypofunction is also present in the initial stages (Contestabile and Ciani (2008)). No reduction in the activity of cholinergic enzymes was found in MCI or mild AD stages. Indeed, the activity was found to be enhanced in frontal cortex and hippocampus during MCI (DeKosky et al. (2002)). Drugs aimed at correcting acetylcholine deficiency provide only symptomatic relief and fail to cure the disease (Smith et al. (2003)). These observations make it unclear whether cholinergic dysfunction is a primary event or secondary to another pathological process.

1.6.2 Amyloid cascade hypothesis

The amyloid cascade hypothesis (Figure 5) posits that the deposition of β-amyloid protein as fibrils and plaques is the central etiologic event in the pathogenesis of AD (Hardy and Selkoe (2002)). An imbalance between the production and clearance of $A\beta$

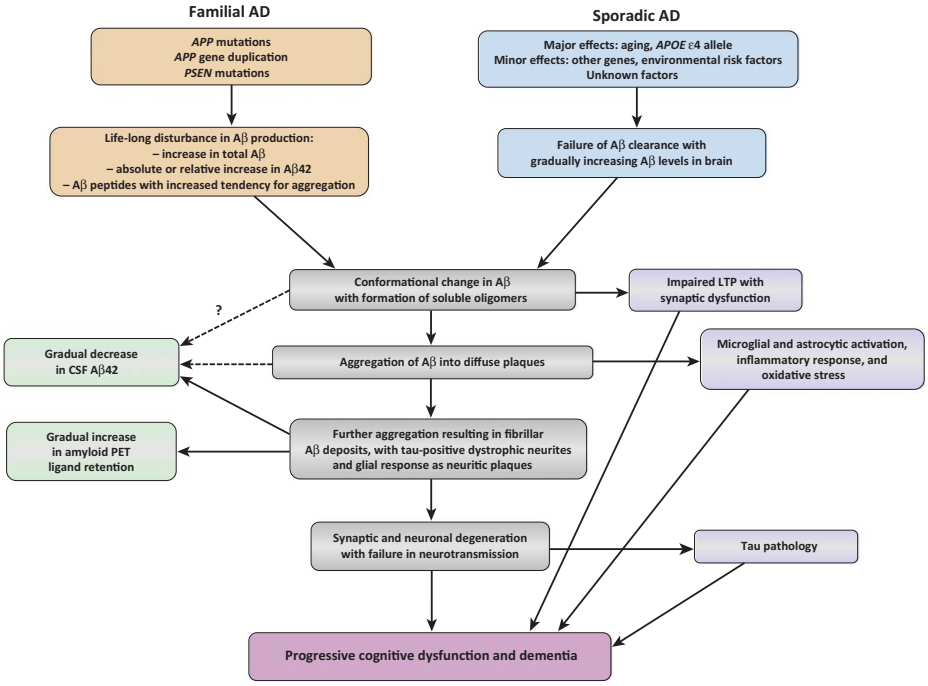


Figure 5: Amyloid cascade hypothesis. It states that the imbalance between Aβ production and clearance is the initial event in AD pathogenesis leading to its aggregation and accumulation in the form of oligomers and fibrils. Aβ oligomers inhibit potentiation and synaptic dysfunction in the hippocampal neurons as well as inflammatory response, oxidative stress and neuronal degeneration resulting in cognitive impairment. Tau pathology is considered as a downstream event contributing to cognitive decline. Blennow et al. (2015)

causes it to accumulate in the brain activating an array of reactions which results in abnormal tau aggregation, neuronal degeneration, synaptic dysfunction, brain shrinkage and ultimately dementia (Masters et al. (2006)). This theory is strongly supported by genetic studies (Hardy and Selkoe (2002)). The discovery that mutations in the genes encoding either the substrate (APP), or the enzymes (*PSEN 1* and *PSEN 2*) responsible for generation of Aβ are sufficient to cause all the symptoms of AD, provides the strongest evidence in favour of this hypothesis (Goate et al. (1991);Levy-Lahad et al. (1995); Sherrington et al. (1995)). Moreover, people with Down's syndrome (trisomy of chromosome 21) carrying three copies of the APP gene, possess amyloid plaque in the brain and show presenile cognitive decline. Thus the amyloid cascade hypothesis seems to be most suitable for FAD.

However, in the more common form, sporadic AD, the amyloid load does not correlate well with neuronal loss, cognitive dysfunction or disease progression (Schmitz et al. (2004); Ballatore et al. (2007)). Although the amyloid cascade hypothesis seems to explain some of the pathology of the disease process, the interaction between Aβpeptide and tau is not considered by this model. It is also still unclear how Aβ deposition promotes to AD. Despite the evidence in favor of the amyloid cascade hypothesis, it has

remained highly controversial due to poor correlation between the density of amyloid fibrils and neurological dysfunction (Terry et al. (1991)). There are thus some missing links to be revealed. More recent variations of the hypothesis focus on Aβ oligomers as the primary driver of the disease.

1.6.3 Oligomer hypothesis

Clinical trials have shown that soluble oligomeric forms of Aβ rather than insoluble Aβ fibrils are the fundamental molecular pathogens and initiator culprit which triggers AD (Klein et al. (2001)). This idea is supported by the finding that soluble Aβ levels correlate better with the degree of cognitive impairment in AD and extent of synaptic loss (Lue et al. (1999), McLean et al. (1999)). Aβ-derived oligomers were found to inhibit long term potentiation (Shankar et al. (2008)) and impair synaptic functions in mature neurons (Lambert et al. (1998), Walsh et al. (2002)). These findings led to the formulation of the "oligomer hypothesis" to explain the pathogenesis of AD. Memory loss starting early in the disease can be due to disruption of synaptic plasticity by the oligomers and later degeneration and death of neurons (Ferreira and Klein (2011)). Multiple forms of Aβ oligomers appear to exist as small as dimers and as large as protofibrils made up of many monomers (reviewed in Glabe (2008). However, it is still unclear which type of oligomer contributes mostly to the neurodegeneration and synaptic dysfunction. The studies mentioned above support the involvement of Aβ oligomers in disease causation. More studies are needed to establish a consensus.

1.6.4 Tau mediated neurodegeneration hypothesis

Although the amyloid cascade hypothesis provides the most convincing scientific explanation for the pathogenesis of AD, it now seems unlikely that tangles form as a consequence of amyloid deposition. The number of tangles correlates more closely to neuronal loss and degree of cognitive impairment than the number of amyloid plaques (Gomez-Isla et al. (1997)). In addition, a number of studies conducted on early AD cases demonstrated that tau pathology appears earlier than amyloid plaques (Braak and Del Tredici (2011); Schonheit et al. (2004)). Axonal swellings were found in an early AD stage in a transgenic mouse model in those regions of the brain lacking amyloid β deposition (Stokin et al. (2005)). Aβ accumulation has also been observed in areas of axonal damage after traumatic brain injury and cerebral ischemia (Smith et al. (2013); Jendroska et al. (1995)). These evidences shows that neurodegeneration acts as a substrate for accumulation of Aβ, and Aβ deposition occurs secondary to neurodegeneration.

Some researchers believe that Aβ-peptide and tau are independent events leading together to cognitive dysfunction in AD (Bloom (2014)). This is supported by the

fact that initial tangle formation and earliest amyloid plaque deposition are separate both temporally and spatially. Subsequently, they interact with each other; amyloid accelerates the process of taupathy, resulting in an increase in the number of tangles and the rate of tau deposition (Price and Morris (1999)).

The relationship between plaque and tangles still remains controversial. A number of modifications have been proposed but not accepted. However, it is to date unresolved whether Aβ protein is the cause or consequence of AD. Any finding clearly neither negates the amyloid cascade nor presents more convincing evidence to replace it.

1.7 Definition of a Biomarker

In general, a biomarker can be defined as a measurable clinical entity that provides an insight of a normal biological or pathogenic processes. It provides an objective means to improve differential diagnosis, estimate disease progression, and monitor pharmacological response to therapeutic interventions of the physiologic or pathological condition (Reitz and Mayeux (2014)). The diagnostic applicability of a biomarker is determined by its sensitivity, specificity and ease of use. An ideal biomarker should fulfil some criteria to be able to be used as a diagnostic biomarker. These have been listed in the box below.

- Reflect the physiological process
- Reflect major physiological changes in the brain
- Show the effect of any pharmacological intervention
- Highly sensitive to detect the disease
- Highly specific for the disease
- Allows repeated measurements
- Reproducible everywhere
- The test is non-invasive, easy, inexpensive, less time-consuming
- Samples stability allowing easy and cheap transport
- Data are published in peer reviewed journal
- Data are reproduced in at least two independent research studies

1.8 Currently used biomarkers of AD

Extensive research has been conducted to identify AD-specific biomarkers. Currently accepted biomarkers of AD include imaging studies of size and metabolic activity of specific areas of the brain and quantification of proteins related to amyloid or tau deposition in the brain (Wurtman (2015)).

1.8.1 Imaging biomarkers

As histopathological changes in the brain precede the clinical manifestations of AD, *in vivo* brain imaging has made possible the detection of individuals at risk for AD, and reveals the extent of pathology during pre-clinical stages. Both MRI and PiB-PET are non-invasive techniques for obtaining evidence of amyloid deposition in the brain. The main drawbacks of neuroimaging techniques are high cost and limited availability of those sophisticated instruments (Humpel and Hochstrasser (2011)).

1.8.1.1 Structural Magnetic Resonance Imaging (sMRI)

AD patients show global brain atrophy with early involvement of entorhinal cortex and hippocampus as shown in Figure 6 (Harper et al. (2014)). MRI studies can be used to differentiate AD subjects from normal ageing with a very high accuracy of 80-90 % (Jagust (2006)). It can also predict progression from MCI stage to clinical AD with high sensitivity and specificity (Ahmed et al. (2014)). Nonetheless, only a few studies on MRI have addressed the differentiation of AD from other dementia illnesses on the basis of hippocampal atrophy (Blennow et al. (2006)). As hippocampal and entorhinal atrophy are also present in other dementias, MRI studies are not specific to AD. The main limitation of MRI is that it is comparatively time and labour intensive (Barber (2010)).

1.8.1.2 Functional MRI (fMRI)

Functional MRI (fMRI) measures changes in cerebral blood flow and metabolism that provides information about neuronal activity in the brain. It can detect brain dysfunction related to AD and monitor response to treatment. AD Patients show reduced brain activity in hippocampus and temporal lobes (Johnson et al. (2012)). As fMRI requires considerable expertise, their use is limited.

1.8.1.3 FDG-PET

2-(^{18}F) fluoro-2-deoxy-D-glucose (FDG) is radiolabelled glucose used to assess cerebral glucose metabolism, and mainly reflects synaptic activity (Cedazo-Minguez and Winblad (2009)). AD patients show a specific pattern of decreased FDG-PET uptake

restricted to parietal, temporal, prefrontal and posterior cingulate cortices, indicating altered functioning of synapses in these regions as shown in Figure 6 (Pakrasi and O'Brien (2005)). FDG-PET can differentiate AD patients from cognitively intact elderly people with good sensitivity (75-80%) and specificity (55-70%) (Jagust (2006)). Moreover, FDG-PET can also differentiate AD from other dementia with a sensitivity of 93 % but low specificity of 73-78 % (Silverman et al. (2001)s).

1.8.1.4 PiB PET Imaging of Aβ burden

$[^{11}C]$-labelled Pittsburgh compound B is a radioactive tracer used extensively for in vivo identification of cerebral amyloid plaques (Klunk et al. (2004)). It binds specifically to the insoluble fibrillar form of Aβ protein (neurotoxic) in amyloid plaques (Klunk et al. (2004); Ikonomovic et al. (2008)). On PET imaging, AD patients demonstrate increased cerebral uptake of PiB (PiB-positive) as compared with controls as shown in Figure 6 (Fagan et al. (2006)). Positive PiB binding in AD patients suggests sequestration of Aβ peptide in the brain resulting in a lower level in the CSF. Moreover, PET imaging has been shown to detect individuals with MCI that are at increased risk for progression to AD dementia. The main limitation of this technique is the short half-life of ^{11}C, necessitating the search for compounds labelled with other tracers with a longer half-life, like ^{18}F (fluorine-18).

1.8.1.5 Tau-PET

It is well established that accumulation of tau protein consistently correlates with the degree of cognitive impairment and neurodegeneration associated with AD dementia. Tau-PET imaging provides a good source of *in vivo* study of the amount and distribution of tau protein in the human brain (Okamura et al. (2014)). PET radiotracers targeting tau protein may be helpful in early detection and differential diagnosis of AD, as well as in monitoring disease progression and severity. Recently, a number of potential tau radiotracers have been developed and show promising results (Shah and Catafau (2014)). Nevertheless, more studies are needed to check their reliability and validate their selectivity to tau proteins. In the future, PET imaging could be useful to monitor outcomes of anti-tau treatment.

1.8.2 Fluid Biomarkers

1.8.2.1 Plasma Biomarkers

Blood can be obtained and processed easily which allows routine screening with repeatable measurements. However, blood based biomarkers for AD have met little success. Several biomarkers have been evaluated in blood, yet the results showed only marginal

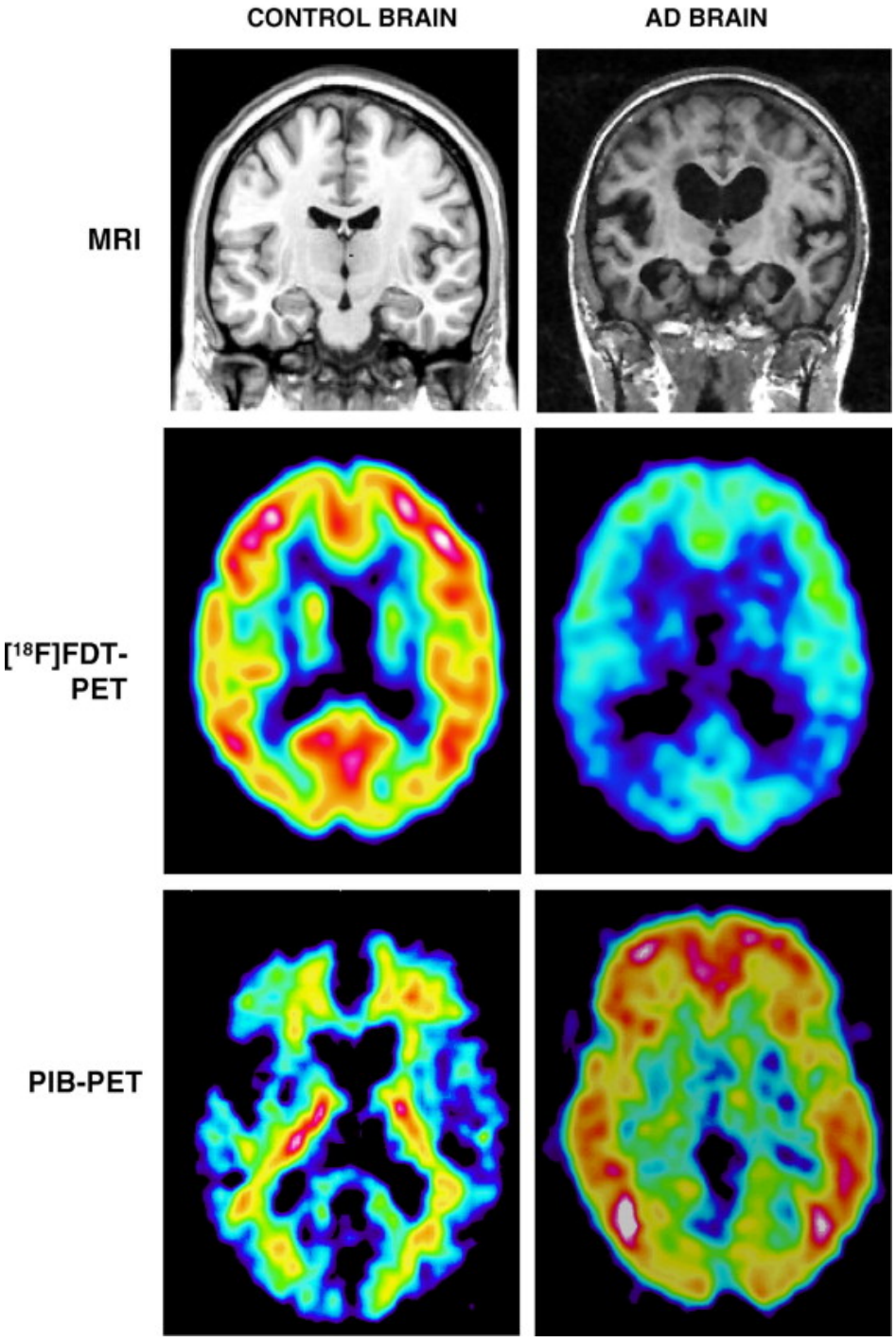


Figure 6: MRI and PET scan of Alzheimer patients and age matched control. MRI scan of AD brain shows symmetric atrophy of hippocampus and enlargement of lateral ventricles. FDG-PET pictures show reduced metabolism (mainly in parital lobes) and PiB-PET shows increased cortical Aβ burden (Cedazo-Minguez and Winblad (2009))

differences between AD patients and controls (Rosen et al. (2013)). The concentration of Aβ peptides in plasma is very low (100-fold) compared to the level in CSF (Lewczuk et al. (2004)), that can be due to the presence of the blood-brain barrier restricting the release of proteins into blood. Moreover Aβ peptides are also produced peripherally by platelets and thus the level in plasma does not reflect brain amyloid load. Furthermore these biomarkers are substantially diluted on entering large volume plasma, and are degraded by various enzymic reactions.

The findings from different studies of the classical biomarkers in plasma produced inconsistent results (Cedazo-Minguez and Winblad (2009)). Le Bastard et al. (2010) could not find any significant difference in the concentration of Aβ42 and Aβ40 between dementia (AD and non-AD type) patients and healthy controls. The amount of tau in the plasma is below the level of detection, so is not considered as an effective biomarker (Tang and Kumar (2008)). Some researchers suggest a diagnostic algorithm including assessing the levels of a combination of plasma biomarkers as the first step for the diagnosis of AD (Babic et al. (2014)), restricting analysis of CSF biomarkers and neuroimaging to only those patients who show an altered level of plasma biomarkers. Thus, plasma biomarkers might prove to be a useful screening tool for dementia in the future.

1.8.2.2 CSF Biomarkers

CSF is a translucent fluid that is in direct contact with the extracellular space of brain. Proteins and metabolites that may be reflective of metabolic processes in the brain and disease pathology are most likely to be diffused into CSF, making it an ideal source of potential biomarker (Raedler and Wiedemann (2006)). CSF can be obtained by lumbar puncture, which is an invasive and painful technique requiring skilled professionals for CSF tapping. However, postlumber puncture headache is usually mild and of short duration (Zetterberg et al. (2010)).

Although a multitude of CSF biomarkers have been proposed to study pathological changes in the brain of AD patients, so far the most important and intensively studied biological markers used for AD diagnosis are Aβ42, total tau protein and phosphorylated tau.

Aβ peptide: In AD patients, CSF Aβ42 shows a significant reduction to nearly 50% of the level in healthy controls, and this has been reported consistently in numerous studies (Blennow et al. (2006)). Although the exact mechanism is not clearly understood, several explanations have been offered for the low CSF Aβ42 levels. It is considered that the reduction is due to sequestration of Aβ42 in plaques with consequent less clearance in CSF (Fagan et al. (2006); Spies et al. (2012)). A strong correlation between reduced CSF Aβ42 and increased amyloid burden in the brain supports this explanation (Hampel et al. (2010)). Its presence in CSF makes it a promising candi-

date biological marker of AD. This reduction starts very early in the pre-clinical stage even prior to the onset of clinical features (dementia), and remains low during the entire disease process (Fagan and Holtzman (2010)). CSF Aβ42 can therefore aid AD diagnosis even in the prodromal (MCI) stage (Diniz et al. (2008)). It also shows a high sensitivity of 78-100% but low specificity of 47-81% for differentiating AD patients from healthy control individuals (Blennow and Hampel (2003)). However, lowering of CSF Aβ42 also occurs in other diseases, such as Lewy body dementia, vascular dementia and fronto-temporal dementia (Sjogren et al. (2001); Kanemaru et al. (2000)). Reduced CSFAβ42 is therefore not specific for AD. In addition, CSF Aβ42 does not correlate well with duration of the disease or its severity (Holtzman (2011)), and no correlation has been reported between plaque burden and the degree of dementia (Humpel and Hochstrasser (2011)). The concentration of CSF Aβ40 remains unchanged or slightly raised in AD patients as compared to controls (Holtzman (2011)). However, the ratio of Aβ42 and Aβ40 has been found to be lowered in AD subjects (Lewczuk et al. (2004)).

Total tau (t-tau): CSF t-tau levels has been found to be markedly raised in AD cases as compared to healthy controls probably as a result of axonal degeneration and neuronal death (Humpel (2011)). T-tau in the CSF shows a high sensitivity and specificity of 84 % and 91 % respectively (Blennow (2004)). However, the level of tau protein is also enhanced in other conditions such as stroke, vascular dementia and Creutzfeldt-Jakob disease (CJD) (Humpel and Hochstrasser (2011)). Moreover, the tau level remains nearly stable throughout the symptomatic period of AD and does not correlate well with the severity of the disease (Holtzman (2011)). So, total tau is not specific to AD pathophysiology, but reflects the amount of neuronal degeneration. MCI cases converting to AD showed high levels of CSF t-tau as compared with stable MCI cases, which make it possible to differentiate between these two groups with high sensitivity and specificity (Babic et al. (2014)).

Phosphorylated tau (p-tau): P-tau reflects the phosphorylation state of tau protein and tangle formation in the brain. Tau can be phosphorylated at many sites and this is regulated by kinases and phosphatases, resulting in reduced affinity to microtubules. Various p-tau epitopes have been measured, those phosphorylated at threonine 181, 231 (P-tau181, P-tau231) and at serine 199 (P-tau199) are the most studied. Like t-tau, the concentration of p-tau in CSF is considerably enhanced in AD patients compared to healthy controls Moreover, p-tau correlates more strongly with tangle pathology in AD (Buerger et al. (2006)). Increased p-tau in CSF yields a sensitivity of 80 % and a specificity of 92 % to discriminate AD from healthy controls (Humpel and Hochstrasser (2011)). It is also more specific in differentiating AD from other causes of dementia (Hampel et al. (2004)). So, it seems that p-tau is a more specific biomarker than t-tau for AD. Research is ongoing to analyse other forms of p-tau that might aid in enhanced sensitivity and specificity of AD.

1.8.3 Combination of biomarkers

A number of studies have shown that combining various biomarkers may increase the diagnostic accuracy of AD, such as combining imaging markers and CSF biomarkers (Lista et al. (2014)). Hansson et al. showed that the combination of low CSF Aβ42 and elevated t-tau can be used to differentiate individuals with progressive MCI and stable MCI with a sensitivity of 95% and specificity of 83% (Hansson et al. (2006)). Integrating ratios of Aβ42/Aβ38 or Aβ42/Aβ38/p-tau enhances the accuracy of AD diagnosis. Moreover, combining p-tau with Aβ42/Aβ38 is 94% sensitive for identification of AD and 85% specific to rule out other causes of dementia (Welge et al. (2009)). Assessing the ratio of Aβ42 and Aβ40 or Aβ42/Aβ38 instead of only Aβ42 increases the specificity to discriminate AD from non-AD (Welge et al. (2009)). The ratio of p-tau and Aβ42 was found to be markedly increased in patients with AD, with a high diagnostic accuracy for differentiating AD and other causes of dementia (Maddalena et al, 2003). Thus, a combination of two or three biomarkers results in enhanced diagnostic accuracy in comparison with one of them alone.

1.8.4 Biomarker models and in vivo staging of AD

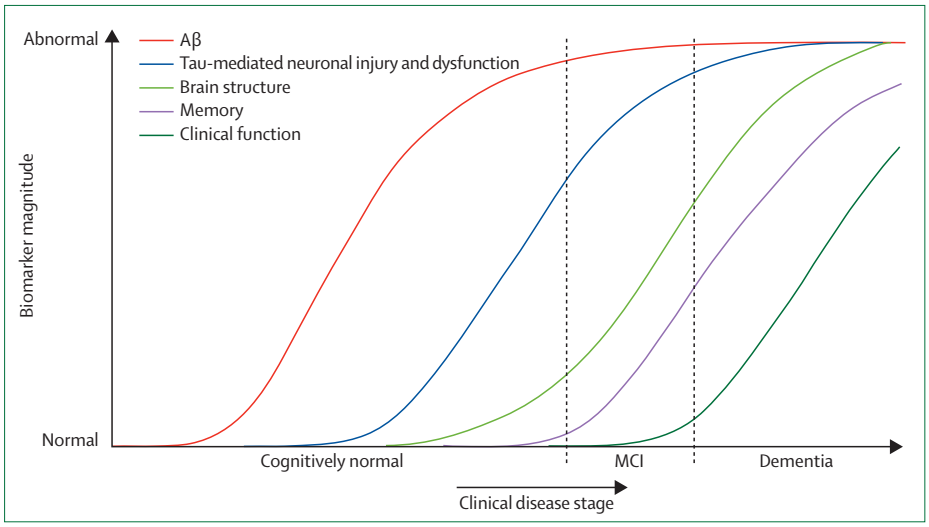


Figure 7: Changes in biomarkers of Alzheimer's disease in relation to clinical stages Jack et al. (2013)

Biochemical and imaging biomarkers indicate disease-associated specific pathological changes in the brain of AD patients. Changes in all the biomarkers do not occur at the same time, but rather in a temporally ordered manner (Jack et al. (2010)). Many biomarker-based AD models representing disease progression have been proposed (Jack et al. (2013)). CSF Aβ42 and amyloid PET correlate with deposition of Aβ fibrils, whereas t-tau, p-tau and FDG-PET show correlation with neurofibrillary

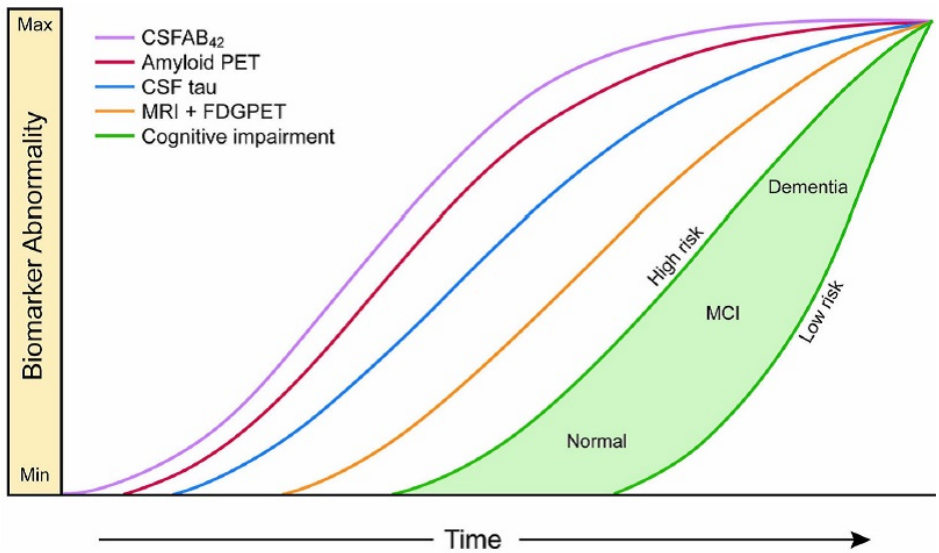


Figure 8: Revised Biomarker Model of AD (2012). Horizontal axis shows disease progression with time. Cognitive response is shown by the green area with right border (low risk) and left border (high risk). Jack et al. (2013)

tangle formation, and brain atrophy correlates with neuronal loss. Jack et al. presented a biomarker model of the AD pathological cascade in 2010 taking into account that biomarkers of amyloid deposition (reduced concentration of Aβ42 level in CSF and raised in amyloid PET) occur earliest, in the preclinical stage, followed by biomarkers of neurodegeneration (enhanced tau level in the CSF and lowered in FDG-PET), with subsequent neuronal loss (brain atrophy on MRI), and finally the development of clinical symptoms. These alterations follow a sigmoidal-shaped curve over time (Jack et al. (2010)), and biomarkers show an initial period of acceleration with subsequent deceleration to a plateau (Figure 7). Jack et al. (2013) proposed a revised model in 2013 which differed from the original model in some respects. In the newer model the horizontal axis was expressed as time and not stage of the disease. Individuals respond to AD pathophysiology differently; the cognitive response of those people at high risk of cognitive decline (due to the presence of more genetic risk alleles, low cognitive reserve and life-style promoting to AD) is shifted to the left, while people at low risk (protective genetic make-up, high cognitive reserve and disease delaying lifestyle) show a cognitive response shifted to right (Figure 8).

1.8.5 Need for novel biomarkers in the CSF

Apart from the three established markers (Aβ42, p-tau, t-tau), several other proteins which show a change between AD patients and controls are being studied to improve the diagnostic accuracy of AD (Craig-Schapiro et al, 2011). Some of these are summarized in a review by Babic et al. (2014), including sAPPα and sAPPβ, and isoforms of Aβ: Aβ37, Aβ38, Aβ39, Aβ14, Aβ15, and Aβ16. Aβ43 is a longer variant of Aβ peptide

which has been implicated in the pathogenesis of AD. It is generated from the cleavage of Aβ49 via Aβ46 and is further converted to Aβ40 or Aβ38 by γ-secretase enzyme (Okochi et al. (2013)).

Interest in Aβ43 has increased in recent years after immunohistochemistry showed that Aβ43 deposition occurs more often than Aβ40 in plaque cores of AD patients, not only in familial AD but also in sporadic cases (Welander et al. (2009), Keller et al. (2010)). Aβ43 is more hydrophobic than Aβ42, which can be attributed to the presence of an additional threonine amino acid at the carboxy terminal end (Saito et al. (2011)). It exhibits a higher propensity to aggregate rapidly than Aβ42 and also drives Aβ42 polymerization, making it more toxic to the brain in comparison to Aβ42. Moreover, Aβ43 deposition in plaques is more extensive and appears earlier than Aβ42 and Aβ40 (Zou et al. (2013)). This suggests that Aβ43 deposition occurs first, followed by Aβ42, and finally Aβ40. In light of this evidence, Aβ43 is suggested to play a pivotal role in the neuropathogenesis of AD. Deposition of Aβ43 has been found at an early stage of the AD brain (Parvathy et al. (2001)), and the amount of Aβ43 in amyloid plaques occurs in relation to cognitive impairment. Studies have demonstrated a reduced level of Aβ43 and Aβ43/Aβ40 ratio in CSF, in both MCI and AD patients as compared to controls (Kakuda et al. (2012), Zou et al. (2013)). However, the mechanism behind the altered activity of γ-secretase is not clearly understood. A lower level in CSF might be explained by increased activity of γ-secretase leading to enhanced conversion of Aβ43 to Aβ40 (Kakuda et al. (2012)), though the concentration of Aβ43 and ratio of Aβ43 to Aβ42 is raised in serum of AD cases (Zou et al. (2013)). Aβ43 may also be converted to Aβ40 by angiotensin converting enzyme (ACE) (Zou et al. (2013)). Drugs targeting inhibition of ACE might therefore prove useful against Aβ43 deposition, as has been shown for Aβ42 by master student Ingrid medben. More clinical studies are needed to elucidate the role of Aβ43 in AD pathophysiology. In the present study, Aβ43 was quantified in CSF from patients with amnestic mild cognitive impairment or early AD, as well as from elderly controls healthy for their age.

2 AIM OF THE STUDY

The increasing prevalence of AD and lack of effective treatment is making it an issue of great concern. Besides the classical biomarkers (Aβ42, t-tau and p-tau) of AD, additional CSF biomarkers are desired for detection of subjects in early stages of AD. Aβ42 has been extensively studied, but Aβ43 has hardly been studied.

2.1 Aim of the thesis

- Assess alteration in the concentration of Aβ43 in CSF samples of a group of patients comprising of MCI stable over the two years of study (sMCI), MCI progressing to AD in the two years study (pMCI), and AD patients compared to age matched elderly control individuals.
- Analyse the diagnostic performance of Aβ43 in distinguishing patients from controls.
- Compare longitudinal changes in biomarker levels of patients in CSF samples collected three times at an interval of six months over two years.

2.2 Hypothesis of the study

- The concentration of Aβ43 in CSF samples from patients with amnestic MCI or early AD will decrease in a manner similar to Aβ42, compared to healthy controls.
- CSF measurements of Aβ43 may serve as another valuable biomarker of AD.
- A combination of Aβ43 and Aβ42 might improve the discriminative diagnostic power between patients with amnestic MCI and AD.

3 MATERIALS AND METHODS

3.1 Study design

This was a cohort study, and participants were recruited as a part of SAMBA, an under project of the Trønderbrain study. SAMBA is a longitudinal project started in 2009 to find biomarkers for AD in CSF and blood. In the present work, only CSF samples were examined from 94 subjects; 62 patients and 32 controls. Since this is an ongoing project, some of the data in this material from the "core" biomarkers was obtained by other members of the research group.

3.2 Study population

3.2.1 Patients

Those enrolled in the study were 53-78 years old, and included individuals diagnosed with the amnestic type of MCI (on the basis of criteria proposed by Winblad et al. (2004)) or early AD (based on NINCDS-ADRDA criteria, McKhann et al. (1984)). They were followed up clinically every six months over two years, and CSF and blood samples obtained. Cognitive tests were also performed at each visit including the Mini mental score examination (MMSE). Patients were enrolled by a single neurologist. MMSE is a test to assess mental abilities and detect whether there is any objective evidence of cognitive dysfunction (Folstein et al. (1975)). It includes a series of questions and tests which is given a score 1 for each correct answer. The maximum score is 30 if all the answers are correct. Usually, a score of 27 or more is considered as normal and people with AD scores less.

3.2.2 Controls

The control population consisted of 32 age and gender matched healthy volunteers without symptoms of cognitive impairment ($MMSE \geqslant 27$) or psychiatric disease, and no first degree relatives suffering from dementia. They were either spouses of enrolled patients or recruited through societies for the elderly. CSF and blood samples were obtained, and cognitive tests performed, only once at the time of enrolment (baseline, T0).

3.3 Ethical aspects

All participants gave written, informed consent to participate in the SAMBA study. The study was approved by the Regional Committee for Medical Research Ethics.

3.4 Obtaining and storing CSF

Lumbar puncture was performed at level L4-L5 in the morning to avoid diurnal variation, with participants in lateral decubitus or sitting position. CSF samples were collected and aliquoted into polypropylene vials which reduce the chance of proteins sticking to the walls of the tube. The samples were marked as T0 (first sample), T6 (second sample after 6 months), T12 (third sample after 12 months), T18 (fourth sample after 18 months), or T24 (fifth sample after 24 months). Collected samples were placed directly into ice-water, frozen within 30 minutes of collection, and stored in the freezer at -80^oC until analysis. Usually, the CSF samples were not centrifuged except ones contaminated with blood.

3.5 Quantifying biomarker levels

The levels of biomarker protein in the CSF samples collected from patients and controls were determined by Sandwich Enzyme Linked Immunosorbent Assay (ELISA). ELISA is an immunobiochemical assay to detect and quantify the amount of an antigen in a given sample using specific antibodies and measuring an enzyme-driven colour change (Gan and Patel (2013)). Sandwich ELISA is a variant of ELISA that captures protein to be analyzed between two layers of antibodies, thus the name (Figure 9).

The CSF samples were analysed for Aβ43, Aβ42, Aβ40, t-tau and p-tau with ELISA kits obtained from Innogenetics (Aβ42, t-tau and p-tau) and IBL (Aβ40, Aβ43). The tests were performed according to manufacturer's instructions. The basic principles for all the tests were the same with slight variations (See Appendix). The analysis was performed blinded to the diagnosis of the participants. Samples from both controls and patients were included in the same plate to reduce any variance. Six standards and one blank were also included in the same plate. All the samples, standard and blanks were run in duplicate.

3.5.1 Procedure

CSF samples were thawed gently in thermal blocks immersed in ice-water. In this assay 96-well (12×8) microtiter plates provided by the manufacturer were used, where the inner surface of the wells were coated with a monoclonal antibody specific to the protein to be measured. Antigen-containing samples were added and then incubated

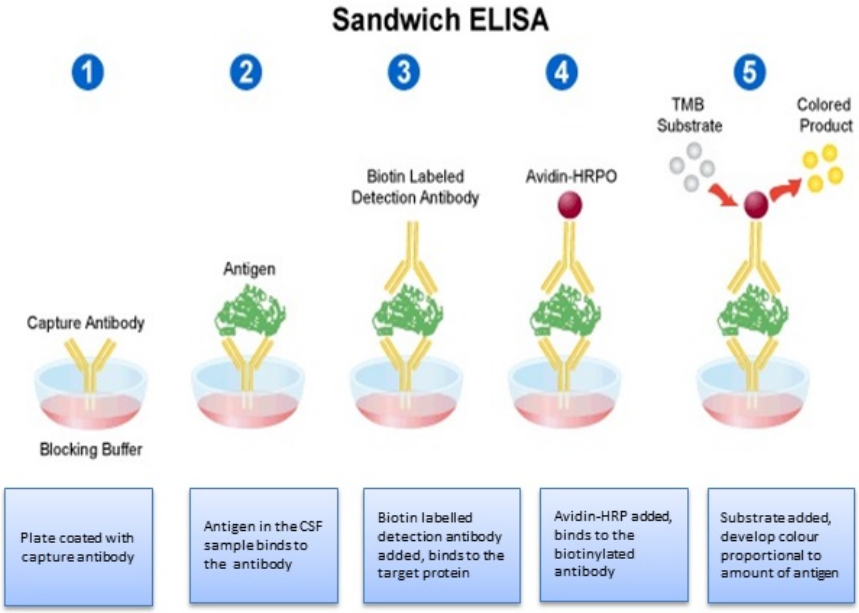


Figure 9: Principle of Sandwich ELISA. The wells were washed between all the steps shown above in the figure. (http://www.leinco.com/sandwich_elisa("ELISA Principle"))

so that the antigen could bind to the antibody. The plates were rinsed with wash solution (phosphate buffer diluted with distilled water) to remove any unbound material. Another biotinylated antibody was then added to the wells, and bound at another site of the antigen. In this way, the antigen to be analysed was "captured" and "immobilised" between the two antibodies. After a further washing step, peroxidase (Horse Radish Peroxidase, HRP)-labelled streptavidin was added and the plate was washed again. A colourless substrate (tetramethyl benzidine) was added, which is converted to a coloured product by the HRP proportional to the amount of antigen in the sample. The enzymic reaction was stopped by adding a stop solution (dilute sulphuric acid), after which the absorbance was read by spectrophotometer (Figure 10(b)) at a specific wavelength (Voller et al. (1978)). The absorbance is proportional to the amount of protein in the sample.

A standard curve was created for each protein on each plate by plotting optical density values obtained from reference samples of known concentration (standards). This curve was then used to calculate the concentrations of specific protein in the samples. The whole process is shown as a flowchart in Figure 11.

3.6 Determination of APOE alleles

APOE genotyping was performed for patients and healthy controls by PhD-student Guro Berge as described in Berge et al. (2014).

(a) ELISA kit for the analysis of Aβ43

(b) ELISA plate with CSF samples read on Spectrophotometer

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	S4	SAK-01	SA-02 T0	SA-03 T12	SA-07 T0	SA-09 T12	SA-10 T24	SA-12 T0	SA-13 T12	SA-14 T24	SA-16 T0
B	BLANK	S4	SAK-01	SA-02 T0	SA-03 T12	SA-07 T0	SA-09 T12	SA-10 T24	SA-12 T0	SA-13 T12	SA-14 T24	SA-16 T0
C	S7	S3	SAK-02	SA-02 T12	SA-06 T0	SA-07 T12	SA-09 T24	SA-11 T0	SA-12 T12	SA-13 T24	SA-15 T0	SA-16 T12
D	S7	S3	SAK-02	SA-02 T12	SA-06 T0	SA-07 T12	SA-09 T24	SA-11 T0	SA-12 T12	SA-13 T24	SA-15 T0	SA-16 T12
E	S6	S2	SAK-04	SA-02 T24	SA-06 T12	SA-07 T24	SA-10 T0	SA-11 T12	SA-12 T24	SA-14 T0	SA-15 T12	SA-16 T24
F	S6	S2	SAK-04	SA-02 T24	SA-06 T12	SA-07 T24	SA-10 T0	SA-11 T12	SA-12 T24	SA-14 T0	SA-15 T12	SA-16 T24
G	S5	S1	SAK-30	SA-03 T0	SA-06 T24	SA-09 T0	SA-10 T12	SA-11 T24	SA-13 T0	SA-14 T12	SA-15 T24	SA-34 T6
H	S5	S1	SAK-30	SA-03 T0	SA-06 T24	SA-09 T0	SA-10 T12	SA-11 T24	SA-13 T0	SA-14 T12	SA-15 T24	SA-34 T6

(c) Layout of the ELISA plate

Figure 10: Equipments

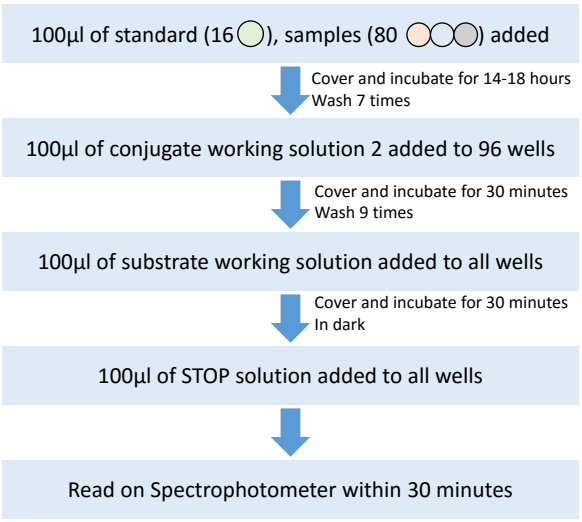


Figure 11: Flowchart showing the main steps of Sandwich ELISA technique used for the analysis of biomarkers in cerebrospinal fluid. 96-well microtitre plates were used in the procedure. The wells were washed with buffer solution. Refer to Figure 10(c) for well layout on the ELISA plate.

3.7 Statistical analysis

The results obtained from the experiments were statistically analyzed using SPSS version 21 from IBM® for Windows. Due to small sample size (62 patients and 32 controls), skewed distribution of the data and presence of outliers, non-parametric test were considered suitable for data analysis. The kruskal-Wallis test was used to identify differences between multiple groups followed by the Mann-Whitney U-test for comparison between two individual groups. The Friedman test was used to identify differences between levels of biomarker proteins at different time points. The Wilcoxon Signed Ranks test was used to compare within groups. The Chi-square test was used to detect significant differences between two categorical variables. A probability level (p-value) of less than 0.05 was considered as significant and $0.05 - 1.00$ as a trend.

3.7.1 Range

It can be defined as the difference between the maximum and minimum observed value of a variable in a data set.

$$Range = Max - Min \tag{1}$$

3.7.2 Correlation

It is a statistical method to determine the extent to which two continuous variables may be related. The Coefficient of correlation ranges from -1 through 0 to +1 where -1 indicates a negative correlation (value of one variable decreases as the other increases),

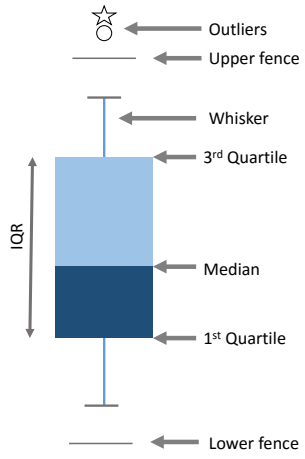


Figure 12: Sample box plot

0 shows no correlation and +1 indicates positive correlation between the two variables). Spearman's correlation coefficient (r_s) was calculated in this study to assess correlation amongst parameters. All significant correlations were plotted graphically as scatter plots to confirm linear relationships (see Appendix)

No corrections were made for multiple comparisons in the current work.

3.8 Graphs

Boxplots and scatterplots were used for graphical representation of the data set.

3.8.1 Boxplot

Also known as box and whisker diagram (Figure 12) is a standardized way to display the distribution of values in a data set. It consists of a box representing the middle 50% of the data, with the upper and lower edges representing respectively the 75% and 25% quartile. The line within the box represents the median of the data set. Two whiskers extend above and below from the edges of the box to the maximum value on one side and minimum value on the other, excluding outliers.

An outlier is a data value that is much larger or smaller than the other values in the data set. Mild outliers can be defined as a value equal to or more than 1.5 times the interquartile range (IQR) above the upper quartile, or a value equal to or less than 1.5 times the IQR below the lower quartile. It can be calculated by the equation below:

$$X > Q3 + 1.5 \times IQR \tag{2}$$

or

$$X < Q1 - 1.5 \times IQR \tag{3}$$

Extreme outliers represent values equal to three times the IQR above the upper quartile or equal to or more than three times the IQR below the lower quartile, as shown by the following equation

$$y > Q3 + 3 \times IQR \tag{4}$$

or

$$y < Q1 - 3 \times IQR \tag{5}$$

In a boxplot, outliers are represented by an unfilled circle, and extreme outliers by a star. Boxplots generated by SPSS shows a number attached to the outliers which displays the sample number in the database.

3.8.2 Scatterplot

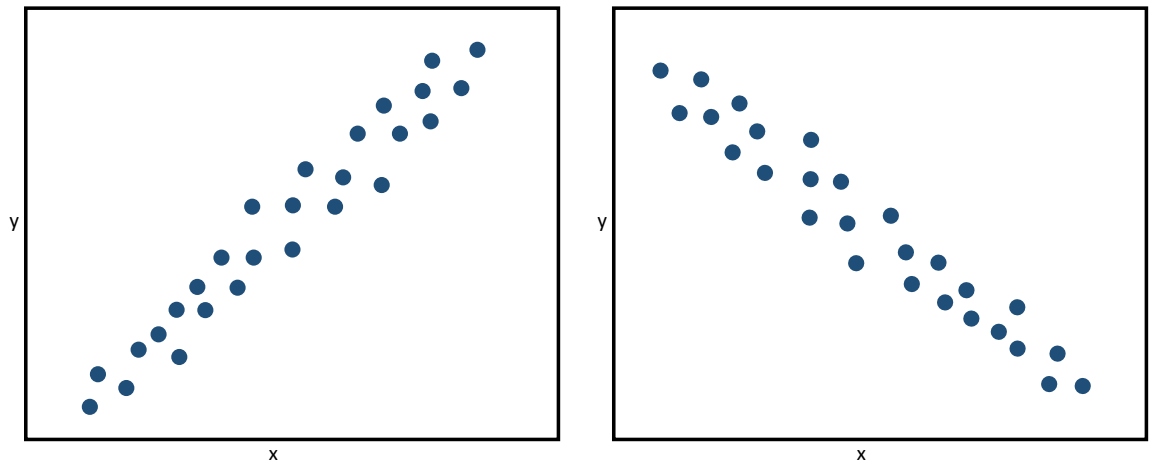
It displays a relationship (correlation) between two quantitative variables. It consists of an X-axis, a Y-axis and a series of dots. XY coordinates of a value is represented by a dot, and a line of best fit can be drawn through the dots. If the dots lie close to the line, the correlation is strong. However, widely spread dots show a weak correlation. A positive slope on a scatterplot indicates a positive correlation between two variables, and a negative slope means a negative correlation. If there is no trend, there is no relationship between the variables.

3.8.3 Receiver Operating Characteristic (ROC) curves

ROC curves were used to determine the diagnostic performance of the CSF proteins and thus their suitability as biomarkers. A ROC curve is a plot of test sensitivity on the y-axis versus (1- specificity) on the x-axis across all possible cut-off values of the diagnostic test. Sensitivity (true positive rate) can be defined as the proportion of cases that give positive test results and thus carry the disease. It is calculated as ratio of true positive cases and actual positive cases plus true negative cases. Specificity (true negative rate) is the proportion of cases that give negative test results and are free of the disease condition. It is calculated as the ratio of true negative cases and true negative plus false positive cases (Table). In this study, sensitivity and specificity values higher than 80% were considered as significant.

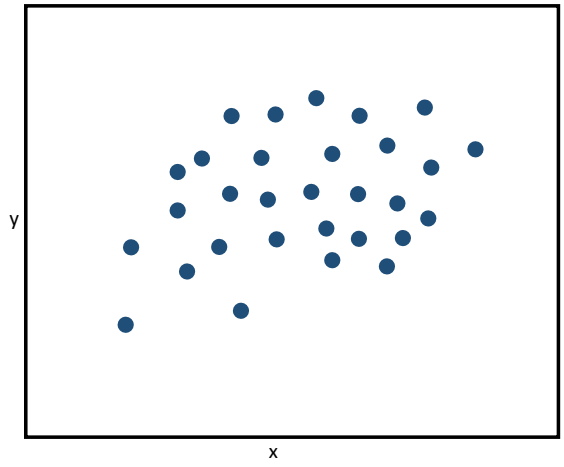
A diagnostic test is perfect where there are no false negative cases (sensitivity is 1) and no false positive cases (specificity of 1). The closer the curve is to the diagonal reference line, the less accurate the test.

Diagnostic performance of a test is measured by area under the ROC curve (AUC). It corresponds to the average value of sensitivity for all possible values of specificity. AUC reflects how good a test is to differentiate patients from controls. The value

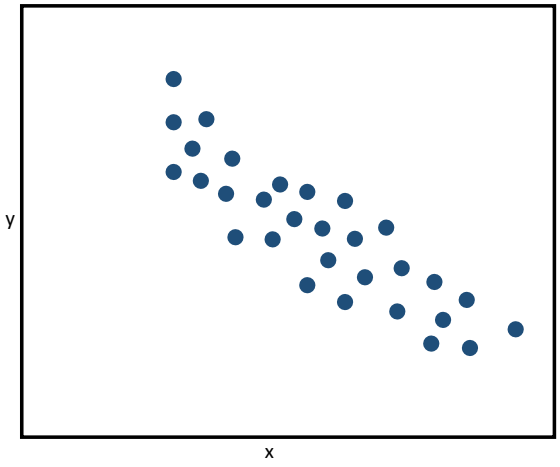


(a) Strong Positive

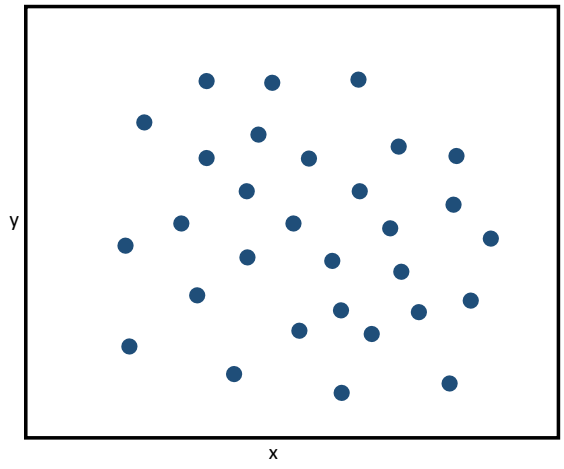
(b) Strong Negative



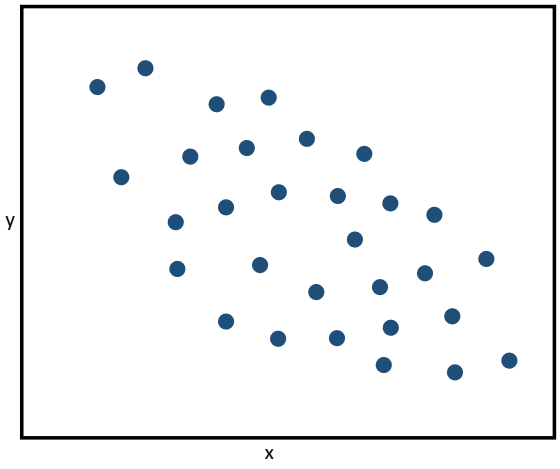
(c) Weak Positive



(d) Moderate Negative



(e) None



(f) Weak Negative

Figure 13: Types of correlation between two sets of variables plotted on X and Y axis

ranges from 0 to 1 and the closer the value of AUC is to 1, the better the diagnostic performance of the test.

The ROC curve was used to calculate optimum cut-off values, sensitivity, specificity, Youden's index and likelihood ratio.

The slope of the tangent at a cut-off point gives the likelihood ratio. A positive likelihood ratio is the ratio of proportion of subjects with the disease and also test positive, to the proportion of subjects without the disease that are test positive. The negative likelihood ratio is the ratio of proportion of subjects with the disease but test negative, to the proportion of subjects without the disease and also test negative.

4 RESULTS

4.1 Demographic Characteristics

Demographic data for the study participant age, gender, and *APOE* ε4 characteristics of the study population are shown in Table 3. Patients with amnestic MCI (aMCI) have been divided into subgroups designated sMCIor pMCI dependent on whether they progressed to AD or not over the two-year study period. They are therefore referred to as sMCI or pMCI groups in these results.The Kruskal-Wallis test was performed to compare age between the groups. It showed a strong trend in age at enrolment between the various groups (p=0.06). The χ^2 - test was used to check the distribution of males and females between the groups. No significant difference was seen in the distribution of males and females between the groups (p=0.2). The χ^2 - test showed

Table 3: Demographic data of the study population

	Control	**sMCI**	**pMCI**	AD
No. of individuals (n)	32	23	20	19
Age at enrolment in years, median (range)	67.0 (58-74)	64.0(53-77)	64.5(56-71)	64.0(57-78)
Gender male / female (n,%)	08/24 (25%/75%)	10/13 (43%/57%)	09/11 (45%/55%)	10/09 (53%/47%)
APO E ε4 carriers (n,%)	9 out of 29 (31%)	10 (43%)	16(80%)	16(84%)
Homozygous for *APOE* (ε4 / ε4) (n,%)	0	6 (26%)	9 (45%)	6 (32%)
Heterozygous for *APOE*(ε2 / ε4 or ε3 / ε4) (n, %)	9 (28%)	4 (17%)	7 (35%)	10 (53%)

a higher frequency of the *APOE* ε4 allele in the patient groups in comparison with the control group ($p < 0.0005$). No individual in the control group was found to be homozygous for *APOE* ε4, though 28.1 % were heterozygous carriers of one allele (*APOE* ε2/ε4 or ε3/ε4). In the patient groups, respectively 26%, 45% and 32% were homozygous for the *APOE* ε4 allele in the sMCI, pMCI and AD groups, the data for heterozygous individuals being 17%, 35%, and 53% for the *APOE* ε4 allele.

4.2 Biomarker levels in CSF

4.2.1 Aβ43

The concentration of Aβ43 in CSF in the various groups is shown as the median and range in Table 4. The Kruskal-Wallis test was performed first that showed a significant

difference in the level of Aβ43 between the groups ($p < 0.0005$). The Mann-Whitney U test was then performed to find the difference between individual groups. At baseline (T0), all the patient groups showed a significantly reduced concentration of Aβ43 in comparison with the control group ($p \leq 0.003$). The level was also found to be significantly reduced in the pMCI and AD groups compared to the sMCI group respectively ($p = 0.04, 0.01$) at T0, an effect that was also found at T12 ($p = 0.02, 0.005$) and T24 ($p = 0.05, 0.01$).

Table 4: Concentrations of CSF Aβ43 (pg/ml) in control and patient groups, presented as the median and range at T0, T12 and T24.

	Control	**sMCI**	**pMCI**	**AD**
T0	44.9 (16.9-80.9)	23.8 (7.2-100.8)	18.4 (10.1-64.6)	16.2 (11.1-42.9)
T12	N/A	27.8 (7.9-103.5)	16.2(8.8-41.0)	15 (8.8-37.0)
T24	N/A	30.8 (7.6-106.3)	17.05 (9.2-46.6)	15.5 (7.7-36.9)

The Friedman test for related samples was performed to find significant differences between CSF levels of Aβ43 at different time-points (T0, T12, and T24). A significant difference was found within the pMCI ($p = 0.04$) and AD ($p = 0.04$) groups. When paired groups were examined using Wilcoxon Signed Rank test, the concentration of Aβ43 was significantly lowered at T12 as compared to T0 in pMCI ($p = 0.03$) and AD ($p = 0.02$). A strong trend was seen in the AD group between T0 and T24 ($p = 0.06$).

Boxplots in Figure 14(a) illustrate the levels of CSF Aβ43 in controls at baseline (T0) and patient groups at T0, T12, and T24. The data indicated one outlier each in the control and sMCI groups, and two outliers in the pMCI group. These outliers were consistent at all time points. One consistent outlier was also seen at T0, T12 and T24 in AD, as well as a single weaker outlier at T12.

4.2.2 Aβ42

Results for the analysis of Aβ42 in CSF in various groups are shown as the median and range in Table 5. The Kruskal-Wallis test showed a significant difference between the level of Aβ42 in the various groups ($p < 0.0005$). The levels were compared between each group using the Mann-Whitney U test. At baseline (T0), all the groups showed a significantly reduced concentration of Aβ42 in comparison with controls ($p < 0.05$). However, trends were observed between sMCI & AD ($p = 0.07$) and between pMCI and AD ($p < 0.06$).

No significant difference was found test between the levels of Aβ42 at different time-points (T0, T12, and T24) by the Friedman test. Figure 14(b) shows the levels of CSF Aβ42 at T0, T12, and T24 for control and patient groups as boxplots. One mild outlier

Table 5: Concentration of CSF Aβ42 (pg/ml) in control and patient groups, presented as the median and range at T0, T12 and T24.

	Control	sMCI	pMCI	AD
T0	1078.9 (532.0-1674.0)	647.2 (173.0-1252.8)	539.0 (356.6-1059.8)	423.1 (211.6-813.3)
T12	NA	566.8 (177.0-1396.1)	517.72 (306.1-981.7)	415.6 (208.4-806.5)
T24	NA	591.3 (178.0-1518.3)	522.5 (357.4-1134.8)	437.6 (197.0-848.5)

was seen in the control group, and one consistent outlier at T0, T12, T24, plus a single outlier at T24 in pMCI. One consistent outlier and another at two time points were seen in AD.

4.2.3 T-tau

The level of CSF total tau (t-tau) in the various groups is shown as the median and range in Table 6. A significant difference was seen by the Kruskal-Wallis test between the levels of t-tau in the groups ($p < 0.02$). The Mann-Whitney U test was used to find the difference, between individual groups. At baseline (T0), the level of t-tau was significantly enhanced in the pMCI and AD groups compared to controls ($p < 0.0005$). A significant increase was also seen in the pMCI and AD groups compared to sMCI at T12 and T24 ($p < 0.05$).

Table 6: Concentration of total tau (pg/ml) in control and patient groups presented as the median and range at T0, T12 and T24.

	Control	sMCI	pMCI	AD
T0	259.4 (137.5-1314.1)	315.2 (98.5-1057.0)	550.8 (162.9-1580.1)	646.6 (221.9-2325.3)
T12	NA	330.0 (117.1-988.0)	589.6 (168.4-1756.3)	751.9 (199.3-2721.3)
T24	NA	305.8 (118.1-1586.0)	579.5 (164.8-2572.5)	753.7 (224.0-5364.0)

The longitudinal data were analyzed using the Friedman test. The test indicated a significant difference in the level of t-tau in the pMCI group ($p = 0.03$), and a trend was observed in sMCI. The Wilcoxon Signed Rank test was performed to compare pairs of samples at different time points (T0, T12, and T24). In both sMCI and pMCI, the level was significantly enhanced at T24 compared to T0 ($p = 0.01$).

Boxplots in Figure 14(c) illustrate the levels of CSF t-tau at T0, T12, and T24 for control and patient groups. One sample (67) showed an extreme value in the control group. Sample 20 in the sMCI group indicated a higher value at T12 and T24. Two samples (47 and 54) at T12 and one (54) at T24 showed higher CSF t-tau levels in the pMCI group. In the AD group, one sample (43) was consistently higher.

4.2.4 P-tau

Phosphorylated tau (p-tau) levels in the CSF of various groups is shown as the median and range in Table 7. The Kruskal-Wallis test showed a significant difference between the level of p-tau between the groups ($p < 0.02$). The Mann-Whitney U test was performed between pairs of groups. At T0, the level of p-tau was significantly increased in the pMCI and AD groups compared to controls ($p < 0.0005$), and the level was also significantly enhanced in pMCI and AD compared to sMCI ($p = 0.005$). At T12 & T24, the groups showed a significantly raised concentration of p-tau in both the pMCI and AD groups compared to sMCI ($p < 0.05$).

Table 7: Concentration of phosphorylated tau (pg/ml) in control and patient groups, presented as the median and range at T0, T12 and T24.

	Control	sMCI	pMCI	AD
T0	55.3 (32.8-135.3)	57.2 (15.9-131.0)	88.2 (37.3-156.8)	91.7 (35.9-168.8)
T12	NA	53.7 (16.1-139.5)	90.8 (36.6-151.4)	93.4 (37.3-180.7)
T24	NA	54.3 (12.9-176.8)	92.4 (38.3-156.7)	101.1 (39.9-188.1)

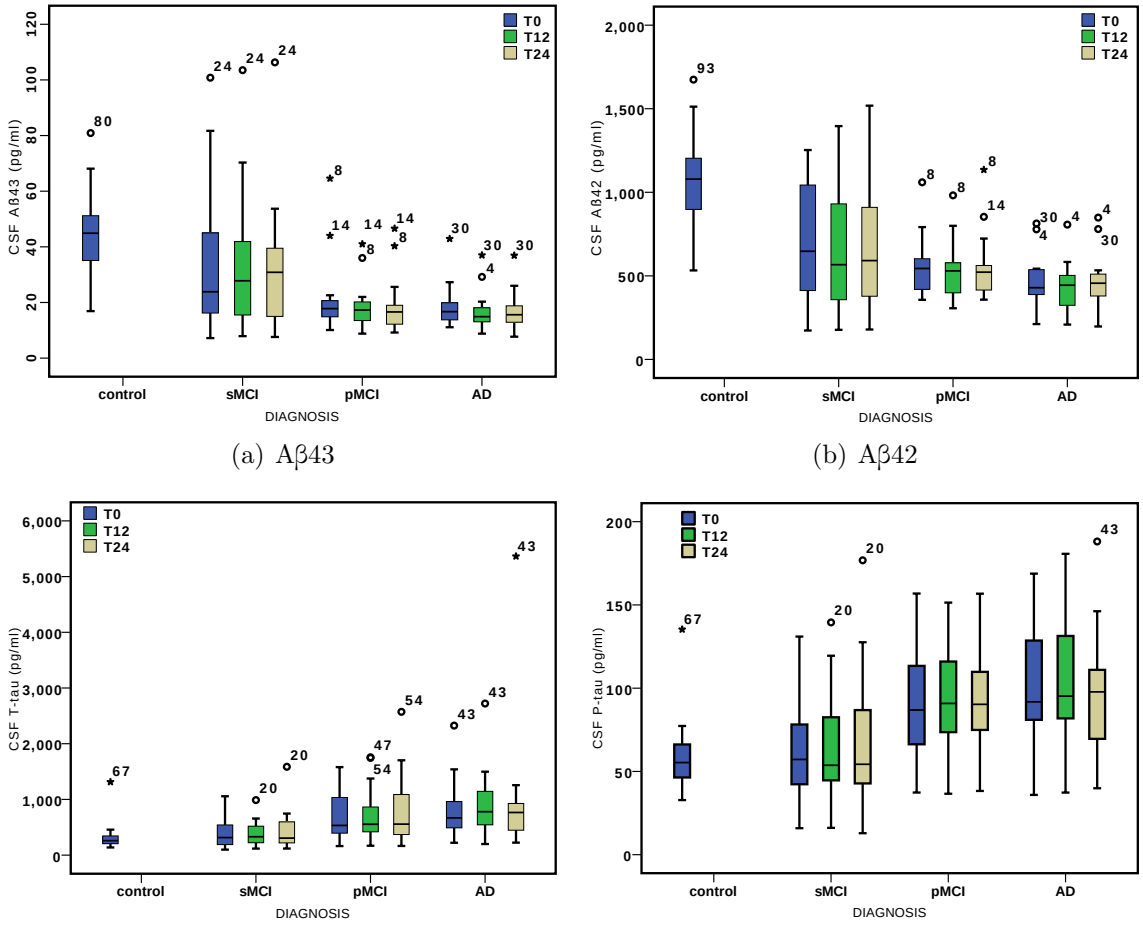
The Friedman test used for longitudinal analysis of the data, showed no significant difference in CSF p-tau levels between the groups at different time points.

Boxplots illustrating the concentration of CSF p-tau at T0, T12, and T24 for control and patient groups are shown in Figure 14(d). The data indicated that patient 67 in the control group, and 20 (T12 and T24) in sMCI, and 43 (T24) in AD tended to have a higher CSF p-tau concentration.

4.3 Correlation between biomarkers

Spearman's correlation coefficient (r_s) was calculated for correlations between biomarker levels in controls at baseline (T0) and patient groups at T0, T12 and T24.

In controls, a significant positive correlation was found between baseline CSF levels of Aβ43 and Aβ42 ($p < 0.005$), and between t-tau and p-tau ($p < 0.005$). A significant



(a) Aβ43 (b) Aβ42

(c) t-tau (d) p-tau

Figure 14: Boxplot showing the concentration of different biomarkers in CSF from controls (T0) and patient groups (T0, T12, T24). ○: mild outlier, ∗: extreme outlier.

positive correlation was also observed between Aβ43 and t-tau ($p = 0.03$). The values of r_s are shown in the Table 8.

Table 8: Spearman's correlation coefficient (r_s) values between CSF Aβ43, Aβ42, total tau and phosphorylated tau levels in controls at baseline (T0) ((* Correlation is significant at $p < 0.05$, ** Correlation is significant at $p < 0.01$))

	Aβ43	Aβ42	t-tau
Aβ42	0.665**		
t-tau	0.446*	0.264	
p-tau	0.227	0.214	0.896**

In the sMCI and pMCI groups, there was a significant positive correlation in the CSF levels of Aβ43 and Aβ42 ($p < 0.0005$), and between t-tau and p-tau ($p < 0.0005$) at all time-points T0, T12 and T24. The values of Spearman's correlation coefficient (r_s) for the sMCI and pMCI groups of patients are shown in Tables 9 and 10.

Table 9: Spearman's correlation coefficient (r_s) values for comparisons of CSF biomarkers in sMCI at T0, T12, and T24. ((* Correlation is significant at $p < 0.0005$.))

	T0			T12			T24		
	Aβ43	Aβ42	t-tau	Aβ43	Aβ42	t-tau	Aβ43	Aβ42	t-tau
Aβ42	0.878*			0.928*			0.850*		
t-tau	0.087	−0.068		0.121	−0.042		−0.059	−0.121	
p-tau	0.077	−0.077	0.939*	0.086	−0.018	0.928*	0.025	−0.068	0.855*

Table 10: Spearman's correlation coefficient (r_s) values between CSF biomarkers in pMCI at T0, T12, and T24. ((* Correlation is significant at $p < 0.0005$

	T0			T12			T24		
	Aβ43	Aβ42	t-tau	Aβ43	Aβ42	t-tau	Aβ43	Aβ42	t-tau
Aβ42	0.779*			0.797*			0.779*		
t-tau	−0.093	−0.118		−0.098	−0.115		−0.267	−0.189	
p-tau	−0.177	−0.074	0.898*	−0.069	−0.088	0.953*	−0.152	−0.129	0.924*

In the AD group, a significant positive correlation was observed between Aβ43 and Aβ42 ($p = 0.02$) and between t-tau and p-tau ($p < 0.0005$) at baseline (T0). At T12, a significant positive correlation was observed between Aβ43 and p-tau ($p = 0.04$) and between t-tau and p-tau ($p < 0.0005$). There was a significant positive correlation

between Aβ43 and Aβ42 ($p = 0.03$) and between t-tau and p-tau ($p < 0.0005$) at T24. The values of r_s are shown in Table 11.

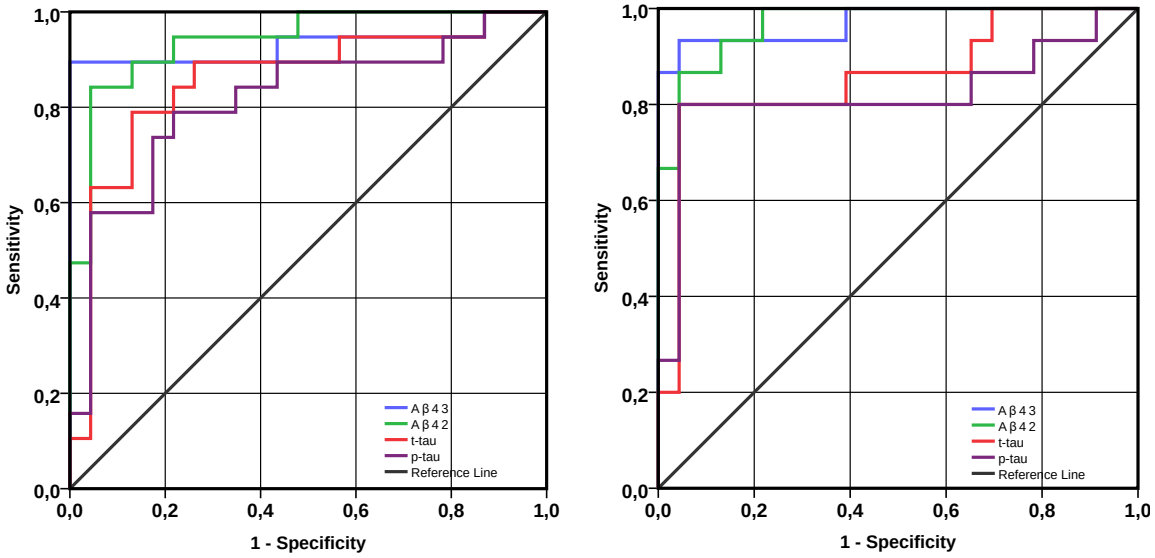
Table 11: Spearman's correlation coefficient (r_s) values between CSF biomarkers in AD at $T0$, $T12$ and $T24$. (Correlation is significant at $^{*}p < 0.05$, $^{**}p < 0.0005$)

	T0			T12			T24		
	Aβ43	Aβ42	t-tau	Aβ43	Aβ42	t-tau	Aβ43	Aβ42	t-tau
Aβ42	0.607*			0.375			0.615*		
t-tau	0.082	−0.206		0.336	−0.036		0.044	−0.118	
p-tau	0.239	−0.115 – 0.672	0.962**	0.539*	−0.046	0.932**	0.203	−0.161	0.929**

4.4 Diagnostic performance

ROC curves were generated to analyze the diagnostic performance of various CSF biomarkers. Curves showing an AUC close to average are shown in the appendix, and not described further. Only ROC curves with significant diagnostic performance are given here.

The ROC curves for Aβ43 in the pMCI and AD groups versus the controls are shown in Figure 15(a) and 15(b) respectively. ROC curves for other groups are in the appendix. The ROC curve for Aβ42, t-tau and p-tau has been added for comparison.



(a) pMCI vs controls

(b) AD vs control

Figure 15: Receiver Operating Characteristic (ROC) curves for Aβ43, Aβ42, t-tau and p-tau in differentiating patients from controls

Table 12 shows the results of the ROC curves for Aβ43 in the groups of patients and controls. Values of Youden's index (sensitivity + specificity-1) were calculated and the

maximum was used to select the cut-off values. The table shows AUC values between 0.9-1.0 for controls compared to pMCI, and for controls compared to AD. The highest value (AUC=0.96) was found for differentiating between AD patients and controls. It also indicates high sensitivity and specificity.

CSF Aβ43 had fairly good capability to distinguish between sMCI and the controls (AUC=0.72), between sMCI and pMCI (AUC=0.71), and between sMCI and AD (AUC=0.77).

Table 12: The area under the ROC curve (AUC), cut-off values, sensitivity and specificity levels, Youden's index, and likelihood ratios for the ability of CSF Aβ43 levels to differentiate between patients and controls.Youden's index: (sensitivity + specificity-1), likelihood ratio: (sensitivity/1-specificity).

	Control:sMCI	Control:pMCI	Control:AD	sMCI:pMCI	sMCI:AD	pMCI:AD
AUC	0.71	0.93	0.97	0.71	0.77	0.59
Cut-off (pg/ml)	< 26.1	< 24.8	< 27.7	< 21.1	< 20.9	< 18.8
Sensitivity	50	90	93	79	87	73
Specificity	100	100	96	70	70	53
Youden's index	0.50	0.90	0.89	0.49	0.57	0.25
Likelihood ratio			23.3	2.6	2.9	1.5

Table 13 presents the results of the ROC curves for Aβ42 in the groups of patients and controls. The maximum value of Youden's index was used to select cut-off values. The table shows that CSF Aβ42 discriminated well between patients with pMCI and controls (AUC=0.94) and between AD and healthy controls (AUC=0.97). The value for sMCI and controls (AUC=0.7) indicated fair capability for distinguishing these groups.

Table 13: Area under the ROC curve (AUC), cut-off values, sensitivity and specificity levels, Youden's index, and likelihood ratios for the ability of CSF Aβ42 levels to differentiate between patients and controls.Youden's index: (sensitivity + specificity-1), likelihood ratio: (sensitivity/1-specificity).

	Control:sMCI	Control:pMCI	Control:AD	sMCI:pMCI	sMCI:AD	pMCI:AD
AUC	0.79	0.94	0.97	0.57	0.65	0.67
Cut-off (pg/ml)	< 692.8	< 635.1	< 604.4	< 792.7	< 558.7	< 546.2
Sensitivity	60	84	87	95	87	87
Specificity	92	96	96	40	55	47
Youden's index	0.52	0.80	0.83	0.34	0.42	0.35
Likelihood ratio	7.5	21	21.75	1.58	1.93	1.64

Table 14 shows AUC values for CSF total tau between 0.8-0.9 for control versus

pMCI, control versus AD, and sMCI versus AD, indicating good potential to distinguish pMCI (AUC=0.86) and AD (AUC=0.87) from healthy controls as well as sMCI from AD (AUC=0.81). Specificity is quite high for these groups. It also differentiated fairly well between sMCI and pMCI (AUC 0.75); sensitivity was good, but specificity was very low.

Table 14: Area under the ROC curve (AUC), cut-off values, sensitivity and specificity levels, Youden index, and likelihood ratios for the ability of the CSF total tau levels for differentiating between patients and controls. Youden's index: (sensitivity +specifiity -1), likelihood ratio: (sensitivity/1-specificity).

	Control:sMCI	Control:pMCI	Control:AD	sMCI:pMCI	sMCI:AD	pMCI:AD
AUC	0.58	0.86	0.87	0.75	0.81	0.58
Cut-off (pg/ml)	$>$ 406.2	$>$ 392.8	$>$ 471.1	$>$ 328.0	$>$ 591.0	$>$ 481.3
Sensitivity	43	79	81	90	63	81
Specificity	88	98	96	52	90	42
Youden's index	0.30	0.66	0.77	0.42	0.53	0.23
Likelihood ratio	3.43	6.31	19.36	1.88	6.58	1.40

The values from the ROC curves for CSF phosphorylated tau in the groups of patients and controls are presented in Table 15. The AUC values for controls versus pMCI and controls versus AD lie between 0.8-0.9, indicating good capability to differentiate between patients with pMCI and AD from healthy controls.

Table 15: Area under the ROC curve (AUC), with cut-off values, sensitivity and specificity levels, Youden index, and likelihood ratios for the ability of the p-tau level in CSF to differentiate between patients and controls. Youden's index: (sensitivity +specifiity -1), likelihood ratio: (sensitivity/1-specificity).

	Control:sMCI	Control:pMCI	Control:AD	sMCI:pMCI	sMCI:AD	pMCI:AD
AUC	0.55	0.82	0.83	0.75	0.77	0.56
Cut-off (pg/ml)	$>$ 77.3	$>$ 65.2	$>$ 76.9	$>$ 58.1	$>$ 89.2	$>$ 74.1
Sensitivity	29	79	81	84	69	81
Specificity	96	79	96	57	86	42
Youden's index	0.24	0.58	0.77	0.41	0.54	0.23
Likelihood ratio	6.81	3.79	19.35	1.96	4.81	1.40

4.5 Ratios of Biomarkers

4.5.1 Aβ43/ Aβ42

The CSF Aβ43/ Aβ42 ratios were calculated for controls and patients at T0, T12 and T24 and are presented in Table 16. Significant difference was seen between the groups by the Kruskal-Wallis test ($p < 0.01$). Mann-Whitney U test was done to see the difference between individual groups. At baseline (T0), the Aβ43/ Aβ42 ratio was significantly reduced in pMCI ($p = 0.01$) compared to controls. A significant reduction was also observed between the subgroups of patients with MCI ($p = 0.004$), and a trend was observed between the group with AD compared to sMCI ($p = 0.07$). At T12 and T24, the ratio was found to be lowered in pMCI ($p < 0.005$ and $p = 0.001$ respectively) and in AD ($p = 0.013$, $p = 0.018$) as compared to sMCI.

The Friedman test showed no significant difference in the levels of Aβ43/ Aβ42 in the various groups at the different time-points.

Table 16: Ratios of CSF Aβ43/Aβ42 in patients and controls presented as the median and range at T0, T12 and T24.Actual values have been multiplied by 1000.

	Control	sMCI	pMCI	AD
T0	41.09 (26.76-86.42)	44.12 (28.71-86.29)	35.73 (22.47-60.95)	38.4 (26.56-78.92)
T12	NA	45.95 (29.48-74.70)	34.73(19.90-51.28)	36.71 (21.17-86.37)
T24	NA	47.66 (29.14-94.04)	34.59 (20.88-54.63)	35.48 (18.14-57.36)

Figure 16(a) illustrates boxplots of CSF Aβ43/ Aβ42 ratios in control and patient groups at T0, T12 and T24. There were outliers in all groups except sMCI, but not so consistent as seen above in groups showing Aβ43 or Aβ42 concentration levels.

4.5.2 Aβ43/ Aβ40

Result for the analysis of CSF Aβ43/ Aβ40 ratios are presented as the median and range in Table 17. The Kruskal-wallis test showed significant difference between the groups ($p < 0.01$). The Mann-Whitney U test indicated significant difference between the groups. At baseline (T0), the Aβ43/ Aβ40 ratio was significantly reduced in both pMCI and AD ($p < 0.0005$) compared to controls. At T0, T12 and T24, a significant reduction was found in pMCI and AD as compared to sMCI ($p < 0.05$).

The Friedman test was performed to check the level of CSF Aβ43/ Aβ40 at different time-points and showed no significant difference between the groups.

Figure 16(b) shows the level of Aβ43/ Aβ40 in the CSF as boxplots in control and patient groups at T0, T12 and T24. One subject (67) was an outlier in the control group. In pMCI, samples 8 and 14 were consistent outliers.

Table 17: Ratios of CSF $A\beta 43/A\beta 40$ in control and patient groups shown as the median and range at $T0$, $T12$ and $T24$.The values have been multiplied by 1000.

	Control	sMCI	pMCI	AD
T0	2.62 (0.77-3.97)	2.71 (0.62-4.13)	1.23 (0.56-5.36)	1.18 (0.57-2.79)
T12	NA	2.83 (0.48-5.20)	1.08 (0.44-4.54)	1.05 (0.48-1.56)
T24	NA	2.39 (0.39-4.56)	1.02 (0.46-3.72)	1.33 (0.57-2.52)

4.5.3 Aβ43/ t-tau

Table 18 shows the CSF Aβ43/ t-tau ratios as the median and range in patients and healthy controls at T0, T12 and T24. The Kruskal-Wallis test was performed that showed a significant difference in the level of Aβ43/ t-tau between the groups ($p < 0.002$). The Mann-Whitney U test was used to find the difference between individual groups. At baseline (T0), the Aβ43/ t-tau ratios were significantly reduced in pMCI and AD cases as compared to controls ($p < 0.0005$). At T0 and T12, the levels were significantly reduced in AD as compared to sMCI ($p < 0.0005$). At T24, the concentration of Aβ43/ t-tau was found to be reduced in AD in comparison with sMCI. At T0, T12 and T24, the level was lowered in pMCI in comparison with sMCI ($p < 0.05$).

Table 18: Ratios of CSF Aβ43/t-tau in groups of patients and controls shown as the median and range at T0, T12 and T24.The values have been multiplied by 1000

	Control	sMCI	pMCI	AD
T0	181.16 (23.89-275.50)	98.80 (16.08-243.34)	29.63 (6.52-396.44)	22.65 (6.79-182.17)
T12	NA	87.98 (16.47-323.63)	23.44 (5.04-213.75)	19.76 (5.44-76.27)
T24	NA	72.08 (12.11-277.09)	25.81 (3.58-244.54)	22.78 (2.91-133.99)

A significant difference was observed in the level of Aβ43/ t-tau by the Friedman test in sMCI ($p = 0.05$) and pMCI ($p = 0.02$). On using the Wilcoxon test, a significant reduction was indicated in sMCI at T24 in comparison with T0 ($p = 0.03$) and T12 ($p = 0.02$). In pMCI, a significant reduction was found in T12 in comparison with T0 and a trend was observed in T24 in comparison with T0 ($p = 0.05$).

Boxplots in Figure 16(c) illustrate the values of CSF Aβ43/ t-tau levels in control and patient groups at T0, T12 and T24. There were several outliers in the control group. Samples 8 and 14 were again outliers in the pMCI group, and number 25 was a consistent outlier in AD.

4.5.4 Aβ42/ t-tau

The Aβ42/ t-tau ratios in patients and healthy controls are presented as the median and range in Table 19 at T0, T12 and T24. Significant difference was found between CSF Aβ42/ t-tau ratios by the Kruskal-Wallis test ($p < 0.01$). The Mann-Whitney U test showed significant difference between individual groups. At T0, the Aβ42/ t-tau ratios were significantly lowered in sMCI ($p = 0.005$), pMCI ($p < 0.0005$) and AD cases ($p < 0.0005$) as compared to controls. A significant reduction was found at all time-points in both pMCI and AD groups compared to the sMCI group ($p < 0.05$).

Table 19: Ratios of CSF Aβ42/t-tau in patient groups and controls, given as the median and range at T0, T12 and T24.The values have been multiplied by 1000

	Control	sMCI	pMCI	AD
T0	4366.2 (405.22-6572.14)	2214.0 (234.63-8287.86)	1019.8 (265.30-6503.84)	635.2 (121.58-3304.88)
T12	NA	2107.91 (252.02-7583.04)	852.65 (253.38-5828.76)	537.30 (99.22-2534.87)
T24	NA	1675.53 (157.63-7174.30)	861.55 (167.31-6886.17)	608.74 (50.72-2832.61)

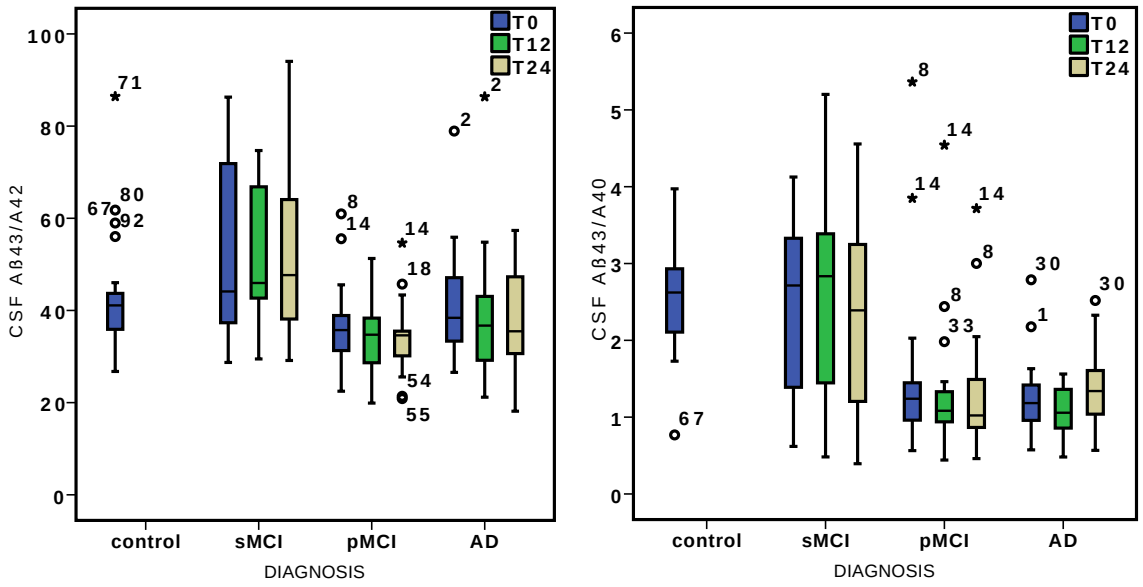
The Friedman test indicated a significant difference in Aβ42/ t-tau ratios in sMCI ($p = 0.014$) and pMCI ($p = 0.016$). The Wilcoxon test showed a significant reduction in sMCI at T12 ($p = 0.024$) and T24 ($p = 0.040$) in comparison with T0. In pMCI, the level was found to be reduced in T12 in comparison with T0 ($p = 0.035$). Figure 16(d) illustrates boxplots showing Aβ42/ t-tau ratios in the CSF of control and patient groups at T0, T12 and T24. In the sMCI group, there was a consistent outlier, numbers 8 and 14 were again outliers in pMCI, and number 25 was again an outlier in AD.

4.6 Diagnostic performance of ratios

ROC curves were plotted for the ratios of concentration of CSF Aβ43/Aβ42, Aβ43/Aβ40, Aβ43/total tau and Aβ42/total tau.

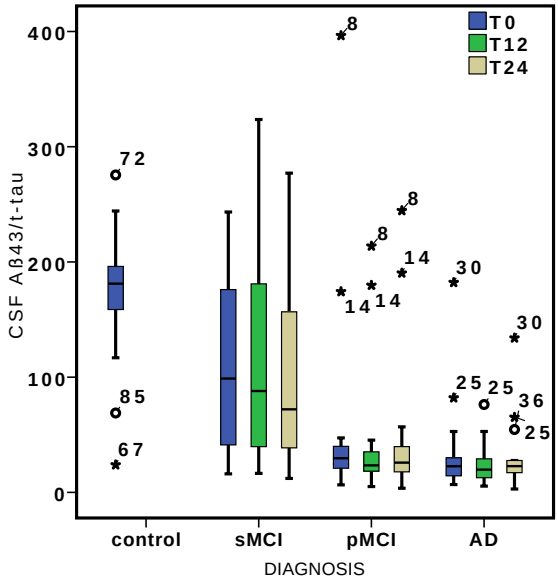
4.6.1 Aβ43/Aβ42

Table 20 presents the AUC, sensitivity and specificity values of CSF Aβ43/Aβ42 levels for distinguishing between patients and healthy controls. Youden's index and likelihood ratio were calculated for each group of patients. The values show many non-significant values of ($AUC < 0.7$), suggesting a poor test. CSF Aβ43/Aβ42 had a fair ability to distinguish pMCI from controls (AUC=0.72) and sMCI (AUC=0.77).

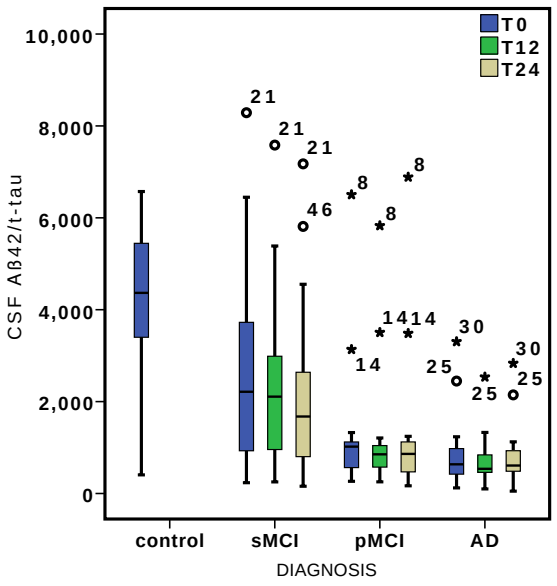


(a) $A\beta 43/A\beta 42$

(b) $A\beta 42/A\beta 40$



(c) $A\beta 43/t-tau$



(d) $A\beta 42/t-tau$

Figure 16: Boxplot showing ratios of CSF in controls ($T0$) and patient groups sMCI, pMCI and AD at T0, T12 and T24. ∘: mild outlier, ∗: extreme outlier. The values have been multiplied by 1000.

Table 20: Area under the ROC curve (AUC), cut-off values, sensitivity and specificity levels, Youden's index, and likelihood ratios of CSF Aβ43/Aβ42 ratio for differentiating between patients and controls. Youden's index: (sensitivity +specificity -1), Likelihood ratio: (sensitivity/1-specificity).

	Control:sMCI	Control:pMCI	Control:AD	sMCI:pMCI	sMCI:AD	pMCI:AD
AUC	0.36	0.72	0.58	0.77	0.68	0.39
Cut-off (pg/ml)	< 34.1	< 39.94	< 34	< 40.7	< 43.5	< 34.2
Sensitivity	20	84	47	84	73	47
Specificity	88	64	88	70	65	58
Youden's index	0.08	0.48	0.35	0.54	0.38	0.04
Likelihood ratio	1.7	2.3	3.9	2.8	2.1	1.1

4.6.2 Aβ43/Aβ40

The values generated from the ROC curves for Aβ43/Aβ40 are shown in Table 21. According to the values of AUC, CSF Aβ43/Aβ40 has excellent capability to distinguish AD patients from controls (AUC=0.90), and a good ability to distinguish between pMCI and healthy controls (AUC=0.86). There was fair differentiation between sMCI and pMCI (AUC=0.73) and AD (AUC=0.79).

Table 21: Area under the ROC curve (AUC), cut-off values, sensitivity and specificity levels, Youden's index, and likelihood ratio of CSF Aβ43/Aβ40 ratios for differentiating between patients and controls. Youden's index: (sensitivity +specificity -1), Likelihood ratio: (sensitivity/1-specificity).

	Control:sMCI	Control:pMCI	Control:AD	sMCI:pMCI	sMCI:AD	pMCI:AD
AUC	0.51	0.86	0.90	0.73	0.79	0.54
Cut-off (pg/ml)	< 1.59	< 1.62	< 1.68	< 1.71	< 2.29	< 1.23
Sensitivity	33	84	87	84	93	60
Specificity	96	96	96	67	61	38
Youden's index	0.29	0.80	0.82	0.51	0.54	0.18
Likelihood ratio	7.74	19.58	20.16	2.52	2.39	1.42

4.6.3 Aβ43/t-tau

Table 22 shows the AUC values of CSF Aβ43/t-tau generated from ROC curves. The values of AUC suggests that CSF Aβ43/t-tau has an excellent capability to differentiate pMCI and AD from controls (AUC=0.9) and a good ability to distinguish pMCI and AD from sMCI (AUC=0.8).

Table 22: Area under the ROC curve (AUC), cut-off values, sensitivity and specificity levels, Youden's index, and likelihood ratios of CSF Aβ43/t-tau ratios for differentiating between patients and controls. Youden's index: (sensitivity +specificity -1), Likelihood ratio: (sensitivity/1-specificity).

	Control:sMCI	Control:pMCI	Control:AD	sMCI:pMCI	sMCI:AD	pMCI:AD
AUC	0.72	0.91	0.95	0.80	0.85	0.62
Cut-off (pg/ml)	< 115.7	< 58.1	< 99.4	< 37.2	< 33.8	< 16.03
Sensitivity	55	90	93	74	80	40
Specificity	92	96	92	90	90	90
Youden's index	0.47	0.85	0.85	0.64	0.70	0.29
Likelihood ratio	6.62	21.3	11.24	7.37	8	3.8

4.6.4 Aβ42/t-tau

The AUC values of CSF Aβ42/t-tau are shown in Table 23. CSF Aβ42/t-tau has an excellent capability to differentiate pMCI and AD from controls (AUC=0.9). The ability of the CSF Aβ42/t-tau to discriminate sMCI patients from AD is good (AUC=0.8).

Table 23: Area under the ROC curve (AUC), cut-off values, sensitivity and specificity levels, Youden's index, and likelihood ratios of CSF Aβ42/ t-tau ratios for differentiating between patients and controls. Youden's index: (sensitivity +specificity -1), Likelihood ratio: (sensitivity/1-specificity).

	Control:sMCI	Control:pMCI	Control:AD	sMCI:pMCI	sMCI:AD	pMCI:AD
AUC	0.75	0.91	0.95	0.73	0.81	0.63
Cut-off (pg/ml)	< 3103.8	< 1512.7	< 1466.8	< 1617.1	< 883.4	< 870.2
Sensitivity	71	90	88	90	75	75
Specificity	87	96	96	62	81	58
Youden's index	0.58	0.85	0.83	0.51	0.56	0.33
Likelihood ratio	5.49	20.81	20.34	2.34	3.94	1.78

5 DISCUSSION

Several earlier studies have consistently reported a decline in the level of CSF Aβ42 in AD patients compared to control subjects and assessed its diagnostic accuracy in the identification of MCI subjects at higher risk of evolving to AD (Blennow et al. (2006), Diniz et al. (2008)). Not much is known about another long species of Aβ peptide, Aβ43 which has been found to possess higher propensity to undergo aggregation (Conciella and Fawzi (2014)) and is more neurotoxic to neurons than Aβ42. Zou et al. (2013), demonstrated that Aβ43 deposition as plaques starts even before Aβ42. Thus, Aβ43 seems to be an interesting candidate biomarker for AD and a better target for the development of anti-AD drugs. In the present study, CSF Aβ43 was quantified along with other biomarkers (Aβ42, Aβ40, t-tau and p-tau) in a group of patients comprising of MCI over 2 years (sMCI), MCI who progressed to AD in two years (pMCI) and AD patients compared with healthy controls. The data obtained were used to compare it with more extensively studied biomarker, CSF Aβ42. Levels and diagnostic performance of other core biomarkers (Aβ42, t-tau and p-tau) in the groups were also studied and showed values as expected from numerous studies (Humpel and Hochstrasser (2011)).

5.1 Cross-sectional analysis

One of the most important findings of the current study was a significant reduction in the concentration of CSF Aβ43 (approximately 50%) in all the patient groups as compared to age matched healthy controls, in agreement with prior studies (Kakuda et al. (2012)). This may reflect sequestration of Aβ43 in amyloid plaques in the brain, similar to the trapping of Aβ42, resulting in decreased clearance in the CSF and thus the lower level. Another explanation can be either lowered production of Aβ43 from APP (due to death of neurons that produce Aβ peptides) or increased degradation to Aβ40 or Aβ38 by altered activity of γ-secretase enzyme or Angiotensin Converting Enzyme (ACE) (Kakuda et al. (2012)). It is also possible that Aβ43 forms oligomers that could not be detected by the ELISA. Further studies using other methods for CSF Aβ43 level detection may be required. Imaging of the brain at all time-points to assess the brain amyloid load could have proved useful to find out the exact cause. MCI patients who progressed to AD (pMCI) as well as patients in the AD group also presented significantly lowered levels of Aβ43 than that of the group of patients who remained cognitively stable (sMCI). This indicates that CSF Aβ43 levels seems useful in the identification of patients with a worse prognosis (pMCI).

5.2 Longitudinal assessment

Longitudinal assessment of the biomarker levels over 2 years depicted significant decline from the baseline (T0) to T12 in both pMCI and AD groups. This suggests that AD is progressive in nature and the pathological process of amyloid deposition occurs continuously throughout the disease process. However, the reduction was marginal and may be due to the slow rate of accumulation of Aβ peptide over time. The slow rate of Aβ deposition might prove beneficial in a sense that it would provide a wide window for therapeutic interventions with anti-Aβ medications. In addition, the levels were increased during the follow up period in subjects categorized under sMCI. This can be explained by the heterogenous nature of the sMCI group, and the cause of dementia and lower CSF value at the baseline could be due to causes other than AD.

5.3 Correlation analysis

An important observation of the present study was the positive correlation between CSF Aβ43 and Aβ42 levels within all the groups of patients and controls, as well as longitudinally at T0, T12 and T24. This might reflect association between the two species of amyloid peptide and supports the assumption that Aβ43 deposition in senile plaques occurs via a related mechanism to Aβ42. These two peptides are generated via different pathways, Aβ43 is generated from Aβ49 →Aβ46 →Aβ43; while Aβ42 is produced from Aβ48 →Aβ45 →Aβ42. However, the enzyme involved in the mechanism is the same, γ-secretase. Hence, correlation between these two peptides supports that altered activity of γ-secretase might be the cause of Aβ43 production. CSF Aβ43 was also found to correlate significantly, although weakly with t-tau in control subjects which due to unknown reasons was lost in all the patient groups.

5.4 Diagnostic performance

Aβ43 showed good diagnostic accuracy (AUC=0.9) between pMCI versus control and AD versus control with high levels of sensitivity (90%, 93% respectively) and specificity (100%, 96% respectively). This indicates that CSF Aβ43 can differentiate well between patients in the pMCI and AD groups from control participants.

5.5 Comparision with Aβ42

CSF Aβ42 level decreased in all patient groups. A trend was seen between pMCI and sMCI, and between AD and sMCI. No significant differences were observed on longitudinal analysis. It seems that the concentration of Aβ42 reached a plateau phase at the time of enrollment of the candidates, and thus did not show any significant difference longitudinally. Thus, the finding of this study points in favor of Aβ43 as a

better prognostic biomarker. However, no other longitudinal study for Aβ43 has been reported so far, and more research is needed to confirm the findings. The diagnostic accuracy of Aβ42 was similar to Aβ43 (AUC=0.9). However, the sensitivity (84%, 87% respectively) and specificity (96%, 96% respectively) were lower for pMCI versus control and AD versus control. This lends further support to the hypothesis that Aβ43 analysis in the CSF in addition to the core biomarkers might be more informative than Aβ42 alone.

5.6 Ratios of biomarkers

The ratios of Aβ43 to Aβ42 and Aβ40 were significantly decreased in subjects who developed AD during the follow up, as compared to healthy controls as well as to clinically stable patients, and the difference was maintained at T12 and T24. The AUC values in the ROC analysis displayed that Aβ43/Aβ40 has better discriminative power to differentiate between various groups of patients and controls, with high sensitivity and specificity. This suggests that the CSF Aβ43/Aβ40 ratio is more informative than the Aβ43/Aβ42 ratio.

Significantly lowered levels of ratios of Aβ43 and Aβ42 to t-tau were observed in both AD and pMCI cases as compared to age matched healthy subjects and sMCI. On longitudinal analysis, the CSF Aβ43/t-tau ratio showed a significant reduction in both the sMCI and pMCI groups at T12 and T24. Similarly, in the sMCI group, the Aβ42/t-tau ratio demonstrated a reduction at follow up. Nevertheless, concerning the pMCI group, a significant lowering was seen in the levels of Aβ42/t-tau ratio at T12 only, and then it remained stable at T24. Taken together, follow-up of Aβ42 and the Aβ42/t-tau ratio indicates that CSF Aβ42 reaches a plateau phase earlier than Aβ43. The AUC values of Aβ43/t-tau and Aβ42/t-tau are approximately the same, with the latter being more sensitive while the former is more specific.

Overall, the results of this study suggest that in addition to the analysis of core biomarkers, CSF Aβ43 levels can provide more information in differentiating candidates with worse prognosis (pMCI) from clinically stable subjects (sMCI). It can further improve the diagnostic accuracy and seems to be a better predictor of AD.

5.7 Other aspects

As advanced age is the most important risk factor in Alzheimers disease, the individuals recruited in the study were controlled for age. A strong trend was seen in the age of subjects between the groups. It is important that candidates in the control and patient groups are as similar as possible to avoid any bias. No significant difference was seen in the distribution of gender among the patient and control groups. *APOE* ε4 allele is an established risk factor for AD. As expected, the occurrence of subjects carrying

APOE ε4 allele was significantly higher in patients compared to controls, and none of the controls were homozygous for *APOE* ε4. This difference indicates good quality of the subjects included in the study. This study includes lots of statistical comparisons, which means higher chances of Type I error. Bonferroni correction is the simplest way to reduce the likelihood of Type I error. Although Bonferroni correction lowers down the chances of false positives, it can however increase the risk of generating false negatives (Type II errors). So, no corrections were made in the current study.

5.8 Strengths

The main strength of this project was that all the subjects recruited for the study were diagnosed by a very skilled neurologist, who is specialized in dementia. This restricts the chances of variability between the groups. Further, all the CSF samples were collected in the morning to avoid any diurnal variation. Despite the low number of individuals, marked reduction was observed in Aβ43 and Aβ42 levels, while significant elevation was seen in total and hyperphosphorylated tau in the two years of study in MCI and AD patients in comparison with controls. The enhanced level of t-tau and p-tau in the patients as compared to controls is in agreement with previous studies and provides support to the quality of the material. This study is the first longitudinal study to be reported till date on CSF Aβ43 levels, and marked reductions were observed longitudinally in all the patient groups.

5.9 Limitations

A major limitation of this project is the small size of the cohort, with only 62 patients and 32 controls. So, the reliability of the results is questionable. Due to the small sample size and frequent presence of outliers, non-parametric statistical tests were used in this study. Non-parametric tests have low power than the corresponding parametric tests, especially in small number of samples. Low power results in a higher chance of Type II error. However, this pilot study would lead to further research in large cohorts. In large samples, even non-parametric tests have as much power as the parametric ones. As the diagnosis was based on clinical criteria without neuropathological confirmation, it cannot be excluded that misdiagnosis of some patients may have occurred.

5.10 Future perspective

Although this study showed differences in the concentration of Aβ43 longitudinally over a two year period, the gradient was less and it seems that the patients are approaching the plateau phase of the biomarker level. So, it would be more interesting to see early changes in Aβ43 levels during preclinical phase of AD. As this is a pilot study, the

results of this project requires further validation and further studies are needed with a larger number of individuals.

6 CONCLUSIONS

The results of this study demonstrated a significantly reduced level of CSF Aβ43 in all the patient groups compared to non-demented controls. Moreover, CSF Aβ43 also showed higher diagnostic accuracy than that of the core biomarkers i.e. for Aβ42, t-tau and p-tau for distinguishing amnestic MCI patients who progressed to AD in two years and early AD patients from age matched elderly controls. Therefore, this study supports the potential ability of Aβ43 as a potential candidate biomarker with improved diagnostic power for differentiating MCI individuals who progress to AD from healthy controls. Further, the concentrations of CSF Aβ43 fell longitudinally over 2 years of study. It can be concluded that the decreasing levels of CSF Aβ43 in serial measurements of pMCI groups may be an indicator of disease progression.

References

Ahmed, R. M., Paterson, R. W., Warren, J. D., Zetterberg, H., O'Brien, J. T., Fox, N. C., Halliday, G. M., and Schott, J. M. (2014). Biomarkers in dementia: clinical utility and new directions. *J Neurol Neurosurg Psychiatry*, 85(12):1426–34.

Albert, M. S., DeKosky, S. T., Dickson, D., Dubois, B., Feldman, H. H., Fox, N. C., Gamst, A., Holtzman, D. M., Jagust, W. J., Petersen, R. C., Snyder, P. J., Carrillo, M. C., Thies, B., and Phelps, C. H. (2011). The diagnosis of mild cognitive impairment due to alzheimer's disease: recommendations from the national institute on aging-alzheimer's association workgroups on diagnostic guidelines for alzheimer's disease. *Alzheimers Dement*, 7(3):270–9.

Allsop, D. and Mayes, J. (2014). Amyloid beta-peptide and alzheimer's disease. *Essays Biochem*, 56:99–110.

Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T., and Hyman, B. T. (1992). Neurofibrillary tangles but not senile plaques parallel duration and severity of alzheimer's disease. *Neurology*, 42(3 Pt 1):631–9.

Babic, M., Svob Strac, D., Muck-Seler, D., Pivac, N., Stanic, G., Hof, P. R., and Simic, G. (2014). Update on the core and developing cerebrospinal fluid biomarkers for alzheimer disease. *Croat Med J*, 55(4):347–65.

Ballatore, C., Lee, V. M., and Trojanowski, J. Q. (2007). Tau-mediated neurodegeneration in alzheimer's disease and related disorders. *Nat Rev Neurosci*, 8(9):663–72.

Barber, R. C. (2010). Biomarkers for early detection of alzheimer disease. *J Am Osteopath Assoc*, 110(9 Suppl 8):S10–5.

Bartus, R. T. (2000). On neurodegenerative diseases, models, and treatment strategies: lessons learned and lessons forgotten a generation following the cholinergic hypothesis. *Exp Neurol*, 163(2):495–529.

Berge, G., Sando, S. B., Rongve, A., Aarsland, D., and White, L. R. (2014). Apolipoprotein e epsilon2 genotype delays onset of dementia with lewy bodies in a norwegian cohort. *J Neurol Neurosurg Psychiatry*, 85(11):1227–31.

Blennow, K. (2004). Cerebrospinal fluid protein biomarkers for alzheimer's disease. *NeuroRx*, 1(2):213–25.

Blennow, K., de Leon, M. J., and Zetterberg, H. (2006). Alzheimer's disease. *Lancet*, 368(9533):387–403.

Blennow, K. and Hampel, H. (2003). Csf markers for incipient alzheimer's disease. *Lancet Neurol*, 2(10):605–13.

Blennow, K., Mattsson, N., Scholl, M., Hansson, O., and Zetterberg, H. (2015). Amyloid biomarkers in alzheimer's disease. *Trends Pharmacol Sci*, (25840462):783–793.

Bloom, G. S. (2014). Amyloid-beta and tau: the trigger and bullet in alzheimer disease pathogenesis. *JAMA Neurol*, 71(4):505–8.

Braak, H. and Braak, E. (1991). Neuropathological stageing of alzheimer-related changes. *Acta Neuropathol*, 82(4):239–59.

Braak, H. and Del Tredici, K. (2011). The pathological process underlying alzheimer's disease in individuals under thirty. *Acta Neuropathol*, 121(2):171–81.

Brion, J. P. (1998). Neurofibrillary tangles and alzheimer's disease. *European Neurology*, 40(3):130–140.

Brunden, K. R., Trojanowski, J. Q., and Lee, V. M. Y. (2009). Advances in tau-focused drug discovery for alzheimer's disease and related tauopathies. *Nature Publishing Group*, 8(10):783–793.

Buchman, A. S., Boyle, P. A., Yu, L., Shah, R. C., Wilson, R. S., and Bennett, D. A. (2012). Total daily physical activity and the risk of ad and cognitive decline in older adults. *Neurology*, 78(17):1323–9.

Buee, L., Bussiere, T., Buee-Scherrer, V., Delacourte, A., and Hof, P. R. (2000). Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res Brain Res Rev*, 33(1):95–130.

Buerger, K., Ewers, M., Pirttila, T., Zinkowski, R., Alafuzoff, I., Teipel, S. J., DeBernardis, J., Kerkman, D., McCulloch, C., Soininen, H., and Hampel, H. (2006). Csf phosphorylated tau protein correlates with neocortical neurofibrillary pathology in alzheimer's disease. *Brain*, 129(Pt 11):3035–41.

Burns, A., Whitehouse, P., Arendt, T., and Forsti, H. (1997). Alzheimer's disease in senile dementia: loss of neurones in the basal forebrain. whitehouse, p., price, d., struble, r., clarke, a., coyle, j. and delong, m. science (1982), 215, 1237-1239. *Int J Geriatr Psychiatry*, 12(1):7–10.

Burns, M. P., Noble, W. J., Olm, V., Gaynor, K., Casey, E., LaFrancois, J., Wang, L., and Duff, K. (2003). Co-localization of cholesterol, apolipoprotein e and fibrillar a in amyloid plaques. *Molecular Brain Research*, 110(1):119–125.

Castellani, R. J., Rolston, R. K., and Smith, M. A. (2010). Alzheimer disease. *Dis Mon*, 56(9):484–546.

Cataldo, J. K., Prochaska, J. J., and Glantz, S. A. (2010). Cigarette smoking is a risk factor for alzheimer's disease: an analysis controlling for tobacco industry affiliation. *J Alzheimers Dis*, 19(2):465–80.

Cedazo-Minguez, A. and Winblad, B. (2009). Biomarkers for alzheimer's disease and other forms of dementia: clinical needs, limitations and future aspects. *Exp Gerontol*, 45(1):5–14.

Conciella, A. and Fawzi, N. (2014). The c-terminal threonine of abeta43 nucleates toxic aggregation via structural and dynamical changes in monomers and. *Biochemistry*, 53(19):3095–3105.

Contestabile, A. and Ciani, E. (2008). The place of choline acetyltransferase activity measurement in the "cholinergic hypothesis" of neurodegenerative diseases. *Neurochem Res*, 33(2):318–27.

de Bruijn, R. F. and Ikram, M. A. (2014). Cardiovascular risk factors and future risk of alzheimer's disease. *BMC Med*, 12(1):130.

DeKosky, S. T., Ikonomovic, M. D., Styren, S. D., Beckett, L., Wisniewski, S., Bennett, D. A., Cochran, E. J., Kordower, J. H., and Mufson, E. J. (2002). Upregulation of choline acetyltransferase activity in hippocampus and frontal cortex of elderly subjects with mild cognitive impairment. *Ann Neurol*, 51(2):145–55.

Diniz, B. S., Pinto Junior, J. A., and Forlenza, O. V. (2008). Do csf total tau, phosphorylated tau, and beta-amyloid 42 help to predict progression of mild cognitive impairment to alzheimer's disease? a systematic review and meta-analysis of the literature. *World J Biol Psychiatry*, 9(3):172–82.

Dong, S., Duan, Y., Hu, Y., and Zhao, Z. (2012). Advances in the pathogenesis of alzheimer's disease: a re-evaluation of amyloid cascade hypothesis. *Transl Neurodegener*, 1(1):18.

Dubois, B., Feldman, H. H., Jacova, C., Cummings, J. L., Dekosky, S. T., Barberger-Gateau, P., Delacourte, A., Frisoni, G., Fox, N. C., Galasko, D., Gauthier, S., Hampel, H., Jicha, G. A., Meguro, K., O'Brien, J., Pasquier, F., Robert, P., Rossor, M., Salloway, S., Sarazin, M., de Souza, L. C., Stern, Y., Visser, P. J., and Scheltens, P. (2010). Revising the definition of alzheimer's disease: a new lexicon. *Lancet Neurol*, 9(11):1118–27.

Fagan, A. M. and Holtzman, D. M. (2010). Cerebrospinal fluid biomarkers of alzheimer's disease. *Biomark Med*, 4(1):51–63.

Fagan, A. M., Mintun, M. A., Mach, R. H., Lee, S. Y., Dence, C. S., Shah, A. R., LaRossa, G. N., Spinner, M. L., Klunk, W. E., Mathis, C. A., DeKosky, S. T., Morris, J. C., and Holtzman, D. M. (2006). Inverse relation between in vivo amyloid imaging load and cerebrospinal fluid abeta42 in humans. *Ann Neurol*, 59(3):512–9.

Fagan, A. M. and Perrin, R. J. (2012). Upcoming candidate cerebrospinal fluid biomarkers of alzheimer's disease. *Biomark Med*, 6(4):455–76.

Fagan, A. M., Roe, C. M., Xiong, C., Mintun, M. A., Morris, J. C., and Holtzman, D. M. (2007). Cerebrospinal fluid tau/beta-amyloid(42) ratio as a prediction of cognitive decline in nondemented older adults. *Arch Neurol*, 64(3):343–9.

Ferreira, S. T. and Klein, W. L. (2011). The abeta oligomer hypothesis for synapse failure and memory loss in alzheimer's disease. *Neurobiol Learn Mem*, 96(4):529–43.

Folstein, M. F., Folstein, S. E., and McHugh, P. R. (1975). Mini-mental state'. *Journal of Psychiatric Research*, 12(3):189–198.

Fratiglioni, L. and Wang, H. X. (2007). Brain reserve hypothesis in dementia. *J Alzheimers Dis*, 12(1):11–22.

Gan, S. D. and Patel, K. R. (2013). Enzyme immunoassay and enzyme-linked immunosorbent assay. *J Invest Dermatol*, 133(9):e12.

Ganguli, M., Dodge, H. H., Shen, C., and DeKosky, S. T. (2004). Mild cognitive impairment, amnestic type: an epidemiologic study. *Neurology*, 63(1):115–21.

Glabe, C. G. (2008). Structural classification of toxic amyloid oligomers. *J Biol Chem*, 283(44):29639–43.

Goate, A., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., and et al. (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial alzheimer's disease. *Nature*, 349(6311):704–6.

Goedert, M., Spillantini, M. G., Potier, M. C., Ulrich, J., and Crowther, R. A. (1989). Cloning and sequencing of the cdna encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mrnas in human brain. *EMBO J*, 8(2):393–9.

Golde, T. E., Eckman, C. B., and Younkin, S. G. (2000). Biochemical detection of abeta isoforms: implications for pathogenesis, diagnosis, and treatment of alzheimer's disease. *Biochim Biophys Acta*, 1502(1):172–87.

Gomez-Isla, T., Hollister, R., West, H., Mui, S., Growdon, J. H., Petersen, R. C., Parisi, J. E., and Hyman, B. T. (1997). Neuronal loss correlates with but exceeds neurofibrillary tangles in alzheimer's disease. *Ann Neurol*, 41(1):17–24.

Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M., and Binder, L. I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau (tau) in alzheimer cytoskeletal pathology. *Proc Natl Acad Sci U S A*, 83(13):4913–7.

Hampel, H., Buerger, K., Zinkowski, R., Teipel, S. J., Goernitz, A., Andreasen, N., Sjoegren, M., DeBernardis, J., Kerkman, D., Ishiguro, K., Ohno, H., Vanmechelen, E., Vanderstichele, H., McCulloch, C., Moller, H. J., Davies, P., and Blennow, K. (2004). Measurement of phosphorylated tau epitopes in the differential diagnosis of alzheimer disease: a comparative cerebrospinal fluid study. *Arch Gen Psychiatry*, 61(1):95–102.

Hampel, H., Shen, Y., Walsh, D., Aisen, P., Shaw, L., Zetterberg, H., Trojanowski, J., and Blennow, K. (2010). Biological markers of amyloid beta-related mechanisms in alzheimer's disease. *Exp. Neurol*, 223(2):334–346.

Hansson, O., Zetterberg, H., Buchhave, P., Londos, E., Blennow, K., and Minthon, L. (2006). Association between csf biomarkers and incipient alzheimer's disease in patients with mild cognitive impairment: a follow-up study. *Lancet Neurol*, 5(3):228–34.

Hardy, J. and Selkoe, D. J. (2002). The amyloid hypothesis of alzheimer's disease: progress and problems on the road to therapeutics. *Science*, 297(5580):353–6.

Harper, L., Barkhof, F., Scheltens, P., Schott, J. M., and Fox, N. C. (2014). An algorithmic approach to structural imaging in dementia. *J Neurol Neurosurg Psychiatry*, 85(6):692–8.

Härtig, W., Brckner, G., Schmidt, C., Brauer, K., Bodewitz, G., Turner, J. D., and Bigl, V. (1997). Co-localization of β-amyloid peptides, apolipoprotein e and glial markers in senile plaques in the prefrontal cortex of old rhesus monkeys. *Brain Research*, 751(2):315–322.

Hebert, L. E., Scherr, P. A., Bienias, J. L., Bennett, D. A., and Evans, D. A. (2003). Alzheimer disease in the us population: prevalence estimates using the 2000 census. *Arch Neurol*, 60(8):1119–22.

Holtzman, D. M. (2011). Csf biomarkers for alzheimer's disease: current utility and potential future use. *Neurobiol Aging*, 32 Suppl 1:S4–9.

Holtzman, D. M., Bales, K. R., Wu, S., Bhat, P., Parsadanian, M., Fagan, A. M., Chang, L. K., Sun, Y., and Paul, S. M. (1999). Expression of human apolipoprotein e reduces amyloid- deposition in a mouse model of alzheimer's disease. *The Journal of Clinical Investigation*, 103(6):R15–R21.

Humpel, C. (2011). Identifying and validating biomarkers for alzheimer's disease. *Trends Biotechnol*, 29(1):26–32.

Humpel, C. and Hochstrasser, T. (2011). Cerebrospinal fluid and blood biomarkers in alzheimer's disease. *World J Psychiatry*, 1(1):8–18.

Hyman, B. T., Phelps, C. H., Beach, T. G., Bigio, E. H., Cairns, N. J., Carrillo, M. C., Dickson, D. W., Duyckaerts, C., Frosch, M. P., Masliah, E., Mirra, S. S., Nelson, P. T., Schneider, J. A., Thal, D. R., Thies, B., Trojanowski, J. Q., Vinters, H. V., and Montine, T. J. (2012). National institute on aging-alzheimer's association guidelines for the neuropathologic assessment of alzheimer's disease. *Alzheimers Dement*, 8(1):1–13.

Ikonomovic, M. D., Klunk, W. E., Abrahamson, E. E., Mathis, C. A., Price, J. C., Tsopelas, N. D., Lopresti, B. J., Ziolko, S., Bi, W., Paljug, W. R., Debnath, M. L., Hope, C. E., Isanski, B. A., Hamilton, R. L., and DeKosky, S. T. (2008). Post-mortem correlates of in vivo pib-pet amyloid imaging in a typical case of alzheimer's disease. *Brain*, 131(Pt 6):1630–45.

Ingelsson, M., Fukumoto, H., Newell, K. L., Growdon, J. H., Hedley-Whyte, E. T., Frosch, M. P., Albert, M. S., Hyman, B. T., and Irizarry, M. C. (2004). Early abeta accumulation and progressive synaptic loss, gliosis, and tangle formation in ad brain. *Neurology*, 62(6):925–31.

Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N., and Ihara, Y. (1994). Visualization of a beta 42(43) and a beta 40 in senile plaques with end-specific a beta monoclonals: evidence that an initially deposited species is a beta 42(43). *Neuron*, 13(1):45–53.

Jack, C. R., J., Albert, M. S., Knopman, D. S., McKhann, G. M., Sperling, R. A., Carrillo, M. C., Thies, B., and Phelps, C. H. (2011). Introduction to the recommendations from the national institute on aging-alzheimer's association workgroups on diagnostic guidelines for alzheimer's disease. *Alzheimers Dement*, 7(3):257–62.

Jack, C. R., J., Knopman, D. S., Jagust, W. J., Petersen, R. C., Weiner, M. W., Aisen, P. S., Shaw, L. M., Vemuri, P., Wiste, H. J., Weigand, S. D., Lesnick, T. G., Pankratz, V. S., Donohue, M. C., and Trojanowski, J. Q. (2013). Tracking pathophysiological processes in alzheimer's disease: an updated hypothetical model of dynamic biomarkers. *Lancet Neurol*, 12(2):207–16.

Jack, C. R., J., Knopman, D. S., Jagust, W. J., Shaw, L. M., Aisen, P. S., Weiner, M. W., Petersen, R. C., and Trojanowski, J. Q. (2010). Hypothetical model of dynamic biomarkers of the alzheimer's pathological cascade. *Lancet Neurol*, 9(1):119–28.

Jagust, W. (2006). Positron emission tomography and magnetic resonance imaging in the diagnosis and prediction of dementia. *Alzheimers Dement*, 2(1):36–42.

Jendroska, K., Poewe, W., Daniel, S. E., Pluess, J., Iwerssen-Schmidt, H., Paulsen, J., Barthel, S., Schelosky, L., Cervos-Navarro, J., and DeArmond, S. J. (1995). Ischemic stress induces deposition of amyloid beta immunoreactivity in human brain. *Acta Neuropathol*, 90(5):461–6.

Johnson, K. A., Fox, N. C., Sperling, R. A., and Klunk, W. E. (2012). Brain imaging in alzheimer disease. *Cold Spring Harb Perspect Med*, 2(4).

Kakuda, N., Shoji, M., Arai, H., Furukawa, K., Ikeuchi, T., Akazawa, K., Takami, M., Hatsuta, H., Murayama, S., Hashimoto, Y., Miyajima, M., Nagashima, Y., Yamaguchi, H., Kuwano, R., Nagaike, K., and Ihara, Y. (2012). Altered gamma-secretase activity in mild cognitive impairment and alzheimer's disease. *EMBO Mol Med*, 4(4):344–52.

Kanemaru, K., Kameda, N., and Yamanouchi, H. (2000). Decreased csf amyloid beta42 and normal tau levels in dementia with lewy bodies. *Neurology*, 54(9):1875–6.

Keller, L., Welander, H., Chiang, H. H., Tjernberg, L. O., Nennesmo, I., Wallin, A. K., and Graff, C. (2010). The psen1 i143t mutation in a swedish family with alzheimer's disease: clinical report and quantification of abeta in different brain regions. *Eur J Hum Genet*, 18(11):1202–8.

Kim, J., Onstead, L., Randle, S., Price, R., Smithson, L., Zwizinski, C., Dickson, D. W., Golde, T., and McGowan, E. (2007). Abeta40 inhibits amyloid deposition in vivo. *J Neurosci*, 27(3):627–33.

Klein, W. L., Krafft, G. A., and Finch, C. E. (2001). Targeting small abeta oligomers: the solution to an alzheimer's disease conundrum? *Trends Neurosci*, 24(4):219–24.

Klunk, W. E., Engler, H., Nordberg, A., Wang, Y., Blomqvist, G., Holt, D. P., Bergstrom, M., Savitcheva, I., Huang, G. F., Estrada, S., Ausen, B., Debnath, M. L., Barletta, J., Price, J. C., Sandell, J., Lopresti, B. J., Wall, A., Koivisto, P., Antoni, G., Mathis, C. A., and Langstrom, B. (2004). Imaging brain amyloid in alzheimer's disease with pittsburgh compound-b. *Ann Neurol*, 55(3):306–19.

Knopman, D. S. and Petersen, R. C. (2013). Mild cognitive impairment and mild dementia: A clinical perspective. *Mayo Clinic Proceedings*, 89(10):1452–1459.

Lambert, M. P., Barlow, A. K., Chromy, B. A., Edwards, C., Freed, R., Liosatos, M., Morgan, T. E., Rozovsky, I., Trommer, B., Viola, K. L., Wals, P., Zhang, C., Finch, C. E., Krafft, G. A., and Klein, W. L. (1998). Diffusible, nonfibrillar ligands derived from abeta1-42 are potent central nervous system neurotoxins. *Proc Natl Acad Sci U S A*, 95(11):6448–53.

Le Bastard, N., Leurs, J., Blomme, W., De Deyn and, P. P., and Engelborghs, S. (2010). Plasma amyloid-β forms in alzheimer's disease and non-alzheimer's disease patients. *Journal of Alzheimer's Disease*, 21(1):291–301.

Leoni, V. (2011). The effect of apolipoprotein e (apoe) genotype on biomarkers of amyloidogenesis, tau pathology and neurodegeneration in alzheimer's disease. *Clin Chem Lab Med*, 49(3):375–83.

Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D. M., Oshima, J., Pettingell, W. H., Yu, C. E., Jondro, P. D., Schmidt, S. D., Wang, K., and et al. (1995). Candidate gene for the chromosome 1 familial alzheimer's disease locus. *Science*, 269(5226):973–7.

Lewczuk, P., Esselmann, H., Otto, M., Maler, J. M., Henkel, A. W., Henkel, M. K., Eikenberg, O., Antz, C., Krause, W. R., Reulbach, U., Kornhuber, J., and Wiltfang, J. (2004). Neurochemical diagnosis of alzheimer's dementia by csf abeta42, abeta42/abeta40 ratio and total tau. *Neurobiol Aging*, 25(3):273–81.

Liberati, G., Raffone, A., and Olivetti Belardinelli, M. (2012). Cognitive reserve and its implications for rehabilitation and alzheimer's disease. *Cogn Process*, 13(1):1–12.

Lista, S., Zetterberg, H., Dubois, B., Blennow, K., and Hampel, H. (2014). Cerebrospinal fluid analysis in alzheimer's disease: technical issues and future developments. *J Neurol*, 261(6):1234–43.

Lue, L. F., Kuo, Y. M., Roher, A. E., Brachova, L., Shen, Y., Sue, L., Beach, T., Kurth, J. H., Rydel, R. E., and Rogers, J. (1999). Soluble amyloid beta peptide concentration as a predictor of synaptic change in alzheimer's disease. *Am J Pathol*, 155(3):853–62.

Masters, C. L., Cappai, R., Barnham, K. J., and Villemagne, V. L. (2006). Molecular mechanisms for alzheimer's disease: implications for neuroimaging and therapeutics. *Journal of Neurochemistry*, 97(6):1700–1725.

McGeer, P. L., McGeer, E. G., and Yasojima, K. (2000). Alzheimer disease and neuroinflammation. *J Neural Transm Suppl*, 59:53–7.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., and Stadlan, E. M. (1984). Clinical diagnosis of alzheimer's disease: report of the nincds-adrda work group under the auspices of department of health and human services task force on alzheimer's disease. *Neurology*, 34(7):939–44.

McKhann, G. M. (2011). Changing concepts of alzheimer disease. *JAMA*, 305(23):2458–2459.

McLean, C. A., Cherny, R. A., Fraser, F. W., Fuller, S. J., Smith, M. J., Beyreuther, K., Bush, A. I., and Masters, C. L. (1999). Soluble pool of abeta amyloid as a determinant of severity of neurodegeneration in alzheimer's disease. *Ann Neurol*, 46(6):860–6.

Mitchell, A. J. and Shiri-Feshki, M. (2009). Rate of progression of mild cognitive impairment to dementia–meta-analysis of 41 robust inception cohort studies. *Acta Psychiatr Scand*, 119(4):252–65.

Moreira, P. I. (2012). Alzheimer's disease and diabetes: an integrative view of the role of mitochondria, oxidative stress, and insulin. *J Alzheimers Dis*, 30 Suppl 2:S199–215.

Morris, J. C., Blennow, K., Froelich, L., Nordberg, A., Soininen, H., Waldemar, G., Wahlund, L. O., and Dubois, B. (2014). Harmonized diagnostic criteria for alzheimer's disease: recommendations. *J Intern Med*, 275(3):204–13.

Nelson, P. T., Alafuzoff, I., Bigio, E. H., Bouras, C., Braak, H., Cairns, N. J., Castellani, R. J., Crain, B. J., Davies, P., Del Tredici, K., Duyckaerts, C., Frosch, M. P., Haroutunian, V., Hof, P. R., Hulette, C. M., Hyman, B. T., Iwatsubo, T., Jellinger, K. A., Jicha, G. A., Kovari, E., Kukull, W. A., Leverenz, J. B., Love, S., Mackenzie, I. R., Mann, D. M., Masliah, E., McKee, A. C., Montine, T. J., Morris, J. C., Schneider, J. A., Sonnen, J. A., Thal, D. R., Trojanowski, J. Q., Troncoso, J. C., Wisniewski, T., Woltjer, R. L., and Beach, T. G. (2012). Correlation of alzheimer disease neuropathologic changes with cognitive status: a review of the literature. *J Neuropathol Exp Neurol*, 71(5):362–81.

Neve, R. L., Harris, P., Kosik, K. S., Kurnit, D. M., and Donlon, T. A. (1986). Identification of cdna clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. *Brain Res*, 387(3):271–80.

Nordberg, A., Rinne, J. O., Kadir, A., and Langstrom, B. (2010). The use of pet in alzheimer disease. *Nat Rev Neurol*, 6(2):78–87.

Okamura, N., Harada, R., Furumoto, S., Arai, H., Yanai, K., and Kudo, Y. (2014). Tau pet imaging in alzheimer's disease. *Curr Neurol Neurosci Rep*, 14(11):014–0500.

Okochi, M., Tagami, S., Yanagida, K., Takami, M., Kodama, T. S., Mori, K., Nakayama, T., Ihara, Y., and Takeda, M. (2013). gamma-secretase modulators and presenilin 1 mutants act differently on presenilin/gamma-secretase function to cleave abeta42 and abeta43. *Cell Rep*, 3(1):42–51.

Pakrasi, S. and O'Brien, J. T. (2005). Emission tomography in dementia. *Nucl Med Commun*, 26(3):189–96.

Parvathy, S., Davies, P., Haroutunian, V., Purohit, D. P., Davis, K. L., Mohs, R. C., Park, H., Moran, T. M., Chan, J. Y., and Buxbaum, J. D. (2001). Correlation between abetax-40-, abetax-42-, and abetax-43-containing amyloid plaques and cognitive decline. *Arch Neurol*, 58(12):2025–32.

Patterson, C., Feightner, J. W., Garcia, A., Hsiung, G. Y., MacKnight, C., and Sadovnick, A. D. (2008). Diagnosis and treatment of dementia: 1. risk assessment and primary prevention of alzheimer disease. *CMAJ*, 178(5):548–56.

Perrin, R. J., Fagan, A. M., and Holtzman, D. M. (2009). Multimodal techniques for diagnosis and prognosis of alzheimer's disease. *Nature*, 461(7266):916–22.

Petersen, R. C., Caracciolo, B., Brayne, C., Gauthier, S., Jelic, V., and Fratiglioni, L. (2014). Mild cognitive impairment: a concept in evolution. *J Intern Med*, 275(3):214–28.

Petersen, R. C., Smith, G. E., Ivnik, R. J., Tangalos, E. G., Schaid, D. J., Thibodeau, S. N., Kokmen, E., Waring, S. C., and Kurland, L. T. (1995). Apolipoprotein e status as a predictor of the development of alzheimer's disease in memory-impaired individuals. *JAMA*, 273(16):1274–8.

Price, J. L. and Morris, J. C. (1999). Tangles and plaques in nondemented aging and "preclinical" alzheimer's disease. *Ann Neurol*, 45(3):358–68.

Raedler, T. J. and Wiedemann, K. (2006). Csf-studies in neuropsychiatric disorders. *Neuro Endocrinol Lett*, 27(3):297–305.

Reitz, C. and Mayeux, R. (2014). Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers. *Biochem Pharmacol*, 88(4):640–51.

Rosen, C., Hansson, O., Blennow, K., and Zetterberg, H. (2013). Fluid biomarkers in alzheimer's disease - current concepts. *Mol Neurodegener*, 8:20.

Saito, T., Suemoto, T., Brouwers, N., Sleegers, K., Funamoto, S., Mihira, N., Matsuba, Y., Yamada, K., Nilsson, P., Takano, J., Nishimura, M., Iwata, N., Van Broeckhoven, C., Ihara, Y., and Saido, T. C. (2011). Potent amyloidogenicity and pathogenicity of abeta43. *Nat Neurosci*, 14(8):1023–32.

Sando, S. B., Melquist, S., Cannon, A., Hutton, M. L., Sletvold, O., Saltvedt, I., White, L. R., Lydersen, S., and Aasly, J. O. (2008). Apoe epsilon 4 lowers age at onset and is a high risk factor for alzheimer's disease; a case control study from central norway. *BMC Neurol*, 8:9.

Schellenberg, G. D. (2006). Early alzheimer's disease genetics. *J Alzheimers Dis*, 9(3 Suppl):367–72.

Schmitz, C., Rutten, B. P., Pielen, A., Schafer, S., Wirths, O., Tremp, G., Czech, C., Blanchard, V., Multhaup, G., Rezaie, P., Korr, H., Steinbusch, H. W., Pradier, L., and Bayer, T. A. (2004). Hippocampal neuron loss exceeds amyloid plaque load in a transgenic mouse model of alzheimer's disease. *Am J Pathol*, 164(4):1495–502.

Schonheit, B., Zarski, R., and Ohm, T. G. (2004). Spatial and temporal relationships between plaques and tangles in alzheimer-pathology. *Neurobiol Aging*, 25(6):697–711.

Selkoe, D. J. (1996). Amyloid beta-protein and the genetics of alzheimer's disease. *J Biol Chem*, 271(31):18295–8.

Selkoe, D. J. (2001). Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev*, 81(2):741–66.

Serrano-Pozo, A., Frosch, M. P., Masliah, E., and Hyman, B. T. (2011). Neuropathological alterations in alzheimer disease. *Cold Spring Harb Perspect Med*, 1(1).

Shah, M. and Catafau, A. M. (2014). Molecular imaging insights into neurodegeneration: Focus on tau pet radiotracers. *J Nucl Med*, 55(6):871–874.

Shankar, G. M., Li, S., Mehta, T. H., Garcia-Munoz, A., Shepardson, N. E., Smith, I., Brett, F. M., Farrell, M. A., Rowan, M. J., Lemere, C. A., Regan, C. M., Walsh, D. M., Sabatini, B. L., and Selkoe, D. J. (2008). Amyloid-beta protein dimers isolated directly from alzheimer's brains impair synaptic plasticity and memory. *Nat Med*, 14(8):837–42.

Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J. F., Bruni, A. C., Montesi, M. P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R. J., Wasco, W., Da Silva, H. A., Haines, J. L., Perkicak-Vance, M. A., Tanzi, R. E., Roses, A. D., Fraser, P. E., Rommens, J. M., and St George-Hyslop, P. H. (1995). Cloning of a gene bearing missense mutations in early-onset familial alzheimer's disease. *Nature*, 375(6534):754–60.

Silverman, D. H., Small, G. W., Chang, C. Y., Lu, C. S., Kung De Aburto, M. A., Chen, W., Czernin, J., Rapoport, S. I., Pietrini, P., Alexander, G. E., Schapiro, M. B., Jagust, W. J., Hoffman, J. M., Welsh-Bohmer, K. A., Alavi, A., Clark, C. M., Salmon, E., de Leon, M. J., Mielke, R., Cummings, J. L., Kowell, A. P., Gambhir, S. S., Hoh, C. K., and Phelps, M. E. (2001). Positron emission tomography in evaluation of dementia: Regional brain metabolism and long-term outcome. *JAMA*, 286(17):2120–7.

Singh, B., Parsaik, A. K., Mielke, M. M., Erwin, P. J., Knopman, D. S., Petersen, R. C., and Roberts, R. O. (2014). Association of mediterranean diet with mild cognitive impairment and alzheimer's disease: a systematic review and meta-analysis. *J Alzheimers Dis*, 39(2):271–82.

Sjogren, M., Vanderstichele, H., Agren, H., Zachrisson, O., Edsbagge, M., Wikkelso, C., Skoog, I., Wallin, A., Wahlund, L. O., Marcusson, J., Nagga, K., Andreasen, N., Davidsson, P., Vanmechelen, E., and Blennow, K. (2001). Tau and abeta42 in cerebrospinal fluid from healthy adults 21-93 years of age: establishment of reference values. *Clin Chem*, 47(10):1776–81.

Smith, D. H., han Chen, X., Iwata, A., and Graham, D. I. (2003). Amyloid β accumulation in axons after traumatic brain injury in humans. *Journal of Neurosurgery*, 98(5):1072–1077.

Smith, D. H., Johnson, V. E., and Stewart, W. (2013). Chronic neuropathologies of single and repetitive tbi: substrates of dementia? *Nat Rev Neurol*, 9(4):211–21.

Sperling, R. A., Aisen, P. S., Beckett, L. A., Bennett, D. A., Craft, S., Fagan, A. M., Iwatsubo, T., Jack, C. R., J., Kaye, J., Montine, T. J., Park, D. C., Reiman, E. M., Rowe, C. C., Siemers, E., Stern, Y., Yaffe, K., Carrillo, M. C., Thies, B., Morrison-Bogorad, M., Wagster, M. V., and Phelps, C. H. (2011). Toward defining the preclinical stages of alzheimer's disease: recommendations from the national institute on aging-alzheimer's association workgroups on diagnostic guidelines for alzheimer's disease. *Alzheimers Dement*, 7(3):280–92.

Spies, P. E., Verbeek, M. M., van Groen, T., and Claassen, J. A. (2012). Reviewing reasons for the decreased csf abeta42 concentration in alzheimer disease. *Front Biosci (Landmark Ed)*, 17:2024–34.

Stokin, G. B., Lillo, C., Falzone, T. L., Brusch, R. G., Rockenstein, E., Mount, S. L., Raman, R., Davies, P., Masliah, E., Williams, D. S., and Goldstein, L. S. (2005). Axonopathy and transport deficits early in the pathogenesis of alzheimer's disease. *Science*, 307(5713):1282–8.

Tang, B. L. and Kumar, R. (2008). Biomarkers of mild cognitive impairment and alzheimer's disease. *Ann Acad Med Singapore*, 37(5):406–10.

Tapiola, T., Alafuzoff, I., Herukka, S. K., Parkkinen, L., Hartikainen, P., Soininen, H., and Pirttila, T. (2009). Cerebrospinal fluid beta-amyloid 42 and tau proteins as biomarkers of alzheimer-type pathologic changes in the brain. *Arch Neurol*, 66(3):382–9.

Terry, R. D., Masliah, E., Salmon, D. P., Butters, N., DeTeresa, R., Hill, R., Hansen, L. A., and Katzman, R. (1991). Physical basis of cognitive alterations in alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol*, 30(4):572–80.

Thies, W. (2013). 2013 alzheimer's disease facts and figures. *Alzheimers Dement*, 9(2):208–45.

Tian, Q. and Wang, J. (2002). Role of serine/threonine protein phosphatase in alzheimer's disease. *Neurosignals*, 11(5):262–9.

Van Den Heuvel, C., Thornton, E., and Vink, R. (2007). Traumatic brain injury and alzheimer's disease: a review. *Prog Brain Res*, 161:303–16.

Varma, A., Snowden, J., Lioyd, J., Talbot, P., Mann, D., and D., N. (1999). Evaluation of the nincds-adrda criteria in the differentiation of alzheimer's disease and frontotemporal dementia. *Journal of Neurology, Neurosurgery & Psychiatry*, 66(2):184–188.

Voller, A., Bartlett, A., and Bidwell, D. E. (1978). Enzyme immunoassays with special reference to elisa techniques. *J Clin Pathol*, 31(6):507–20.

Walsh, D. M., Klyubin, I., Fadeeva, J. V., Rowan, M. J., and Selkoe, D. J. (2002). Amyloid-beta oligomers: their production, toxicity and therapeutic inhibition. *Biochem Soc Trans*, 30(4):552–7.

Welander, H., Frnberg, J., Graff, C., Sundstrm, E., Winblad, B., and Tjernberg, L. O. (2009). $a\beta 43$ is more frequent than a40 in amyloid plaque cores from alzheimer disease brains. *Journal of Neurochemistry*, 110(2):697–706.

Welge, V., Fiege, O., Lewczuk, P., Mollenhauer, B., Esselmann, H., Klafki, H.-W., Wolf, S., Trenkwalder, C., Otto, M., Kornhuber, J., Wiltfang, J., and Bibl, M. (2009). Combined csf tau, p-tau181 and amyloid-β 38/40/42 for diagnosing alzheimers disease. *Journal of Neural Transmission*, 116(2):203–212.

Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T., and Delon, M. R. (1982). Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science*, 215(4537):1237–9.

Winblad, B., Palmer, K., Kivipelto, M., Jelic, V., Fratiglioni, L., Wahlund, L.-O., Nordberg, A., Bckman, L., Albert, M., Almkvist, O., Arai, H., Basun, H., Blennow, K., De Leon, M., DeCarli, C., Erkinjuntti, T., Giacobini, E., Graff, C., Hardy, J., Jack, C., Jorm, A., Ritchie, K., Van Duijn, C., Visser, P., and Petersen, R. (2004). Mild cognitive impairment beyond controversies, towards a consensus: report of the international working group on mild cognitive impairment. *Journal of Internal Medicine*, 256(3):240–246.

Wurtman, R. (2015). Biomarkers in the diagnosis and management of alzheimer's disease. *Metabolism*, 64(3 Suppl 1):S47–50.

Yan, Y. and Wang, C. (2007). Abeta40 protects non-toxic abeta42 monomer from aggregation. *J Mol Biol*, 369(4):909–16.

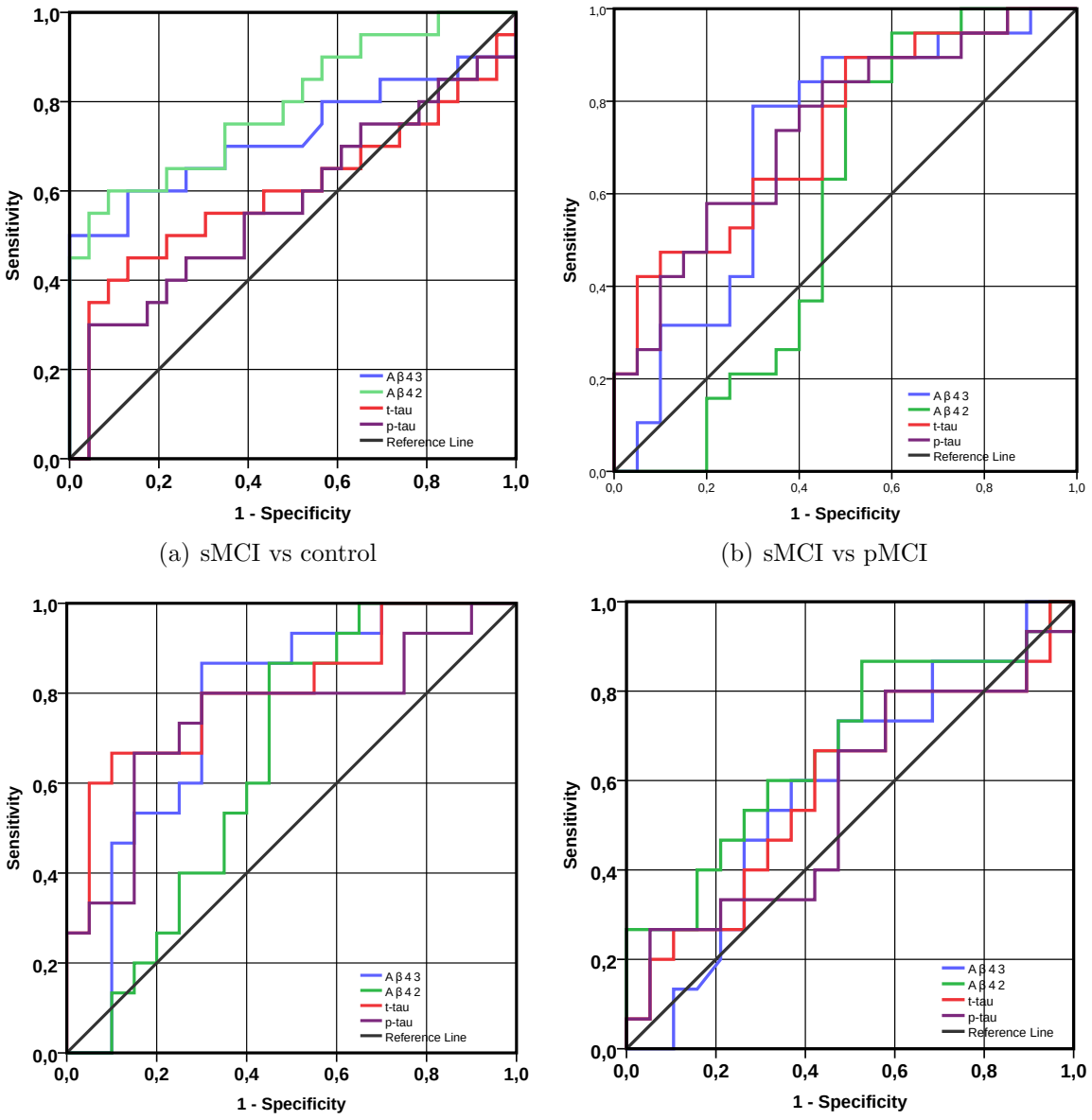
Ye, S., Huang, Y., Mllendorff, K., Dong, L., Giedt, G., Meng, E. C., Cohen, F. E., Kuntz, I. D., Weisgraber, K. H., and Mahley, R. W. (2005). Apolipoprotein (apo) e4 enhances amyloid peptide production in cultured neuronal cells: Apoe structure as a potential therapeutic target. *Proceedings of the National Academy of Sciences of the United States of America*, 102(51):18700–18705.

Zetterberg, H., Tullhog, K., Hansson, O., Minthon, L., Londos, E., and Blennow, K. (2010). Low incidence of post-lumbar puncture headache in 1,089 consecutive memory clinic patients. *Eur Neurol*, 63(6):326–30.

Zou, K., Liu, J., Watanabe, A., Hiraga, S., Liu, S., Tanabe, C., Maeda, T., Terayama, Y., Takahashi, S., Michikawa, M., and Komano, H. (2013). Abeta43 is the earliest-depositing abeta species in app transgenic mouse brain and is converted to abeta41 by two active domains of ace. *Am J Pathol*, 182(6):2322–31.

A Appendix

A.1 ROC curves



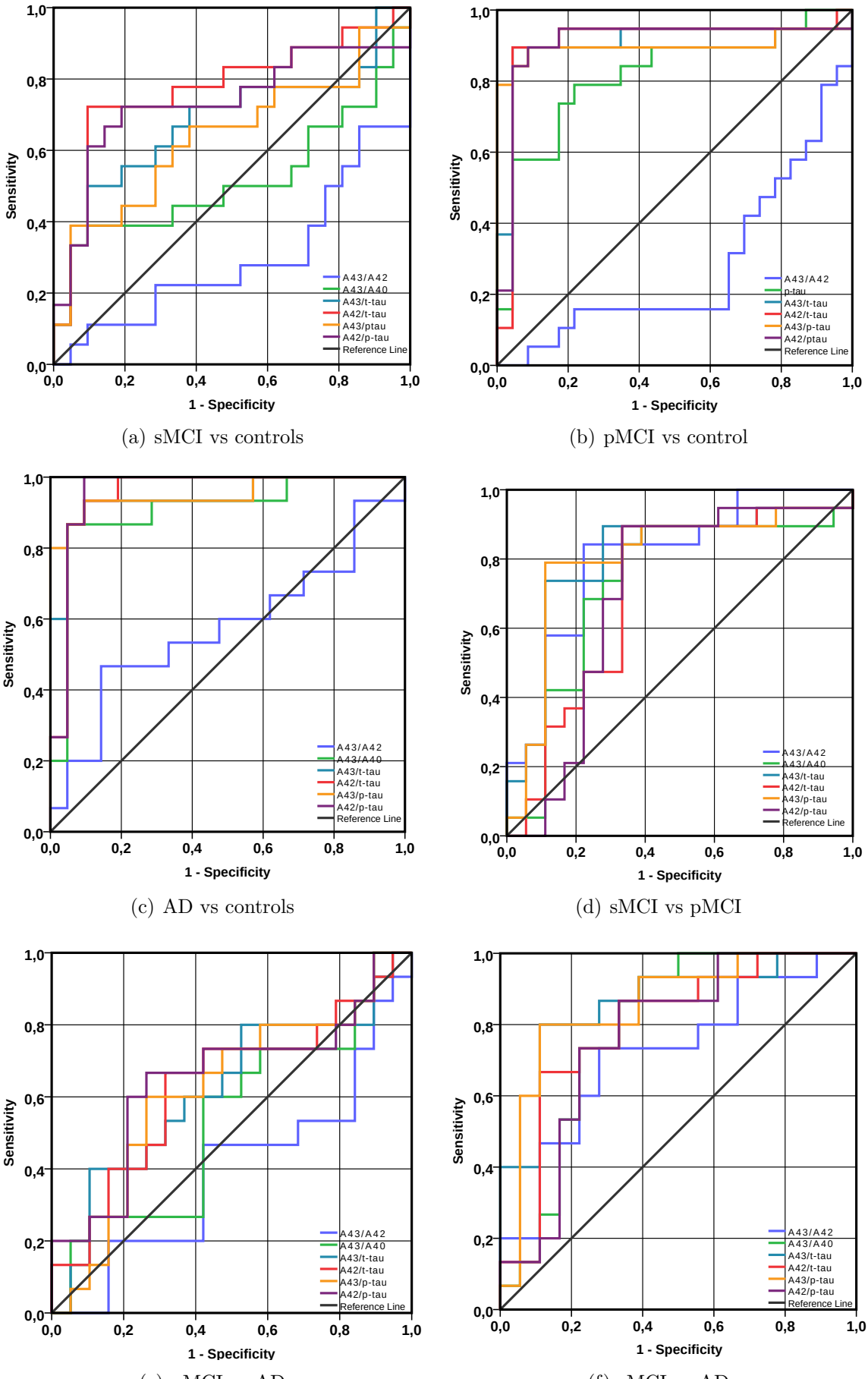
(a) sMCI vs control

(b) sMCI vs pMCI

(c) sMCI vs AD

(d) pMCI vs AD

Figure 17: Receiver Operating Characteristics (ROC) curve for the diagnostic performance of CSF Aβ43, Aβ42, t-tau and p-tau in differentiating different patients groups and controls.



(a) sMCI vs controls

(b) pMCI vs control

(c) AD vs controls

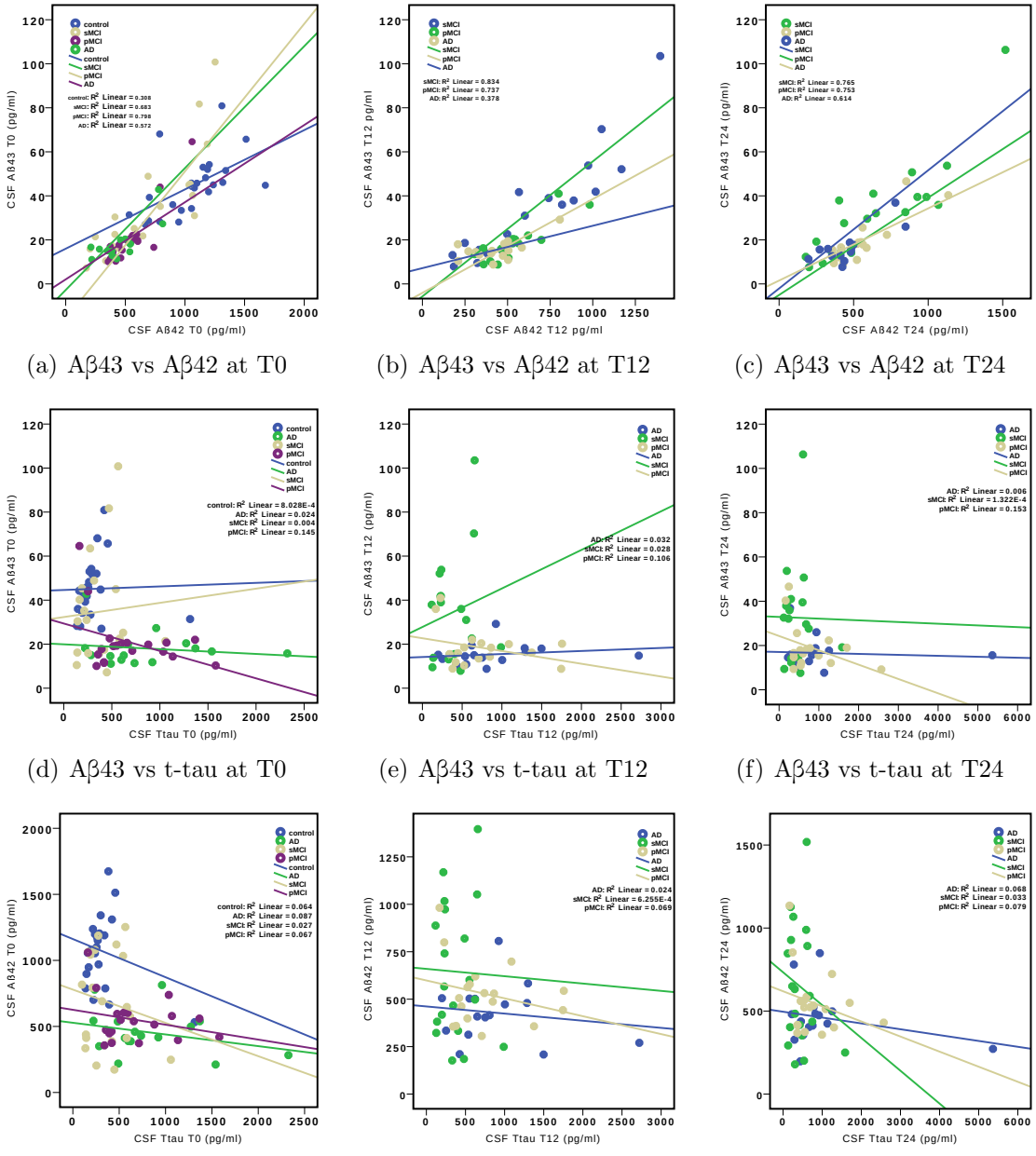
(d) sMCI vs pMCI

(e) pMCI vs AD

(f) sMCI vs AD

Figure 18: Receiver Operating Characteristic (ROC) curves for the diagnostic performance of CSF Aβ43/Aβ42, Aβ43/Aβ40, Aβ43/t-tau and Aβ42/t-tau, Aβ43/p-tau, Aβ42/p-tau in distinguishing between patients and controls.

A.2 Scatter plots to show correlations between biomarkers

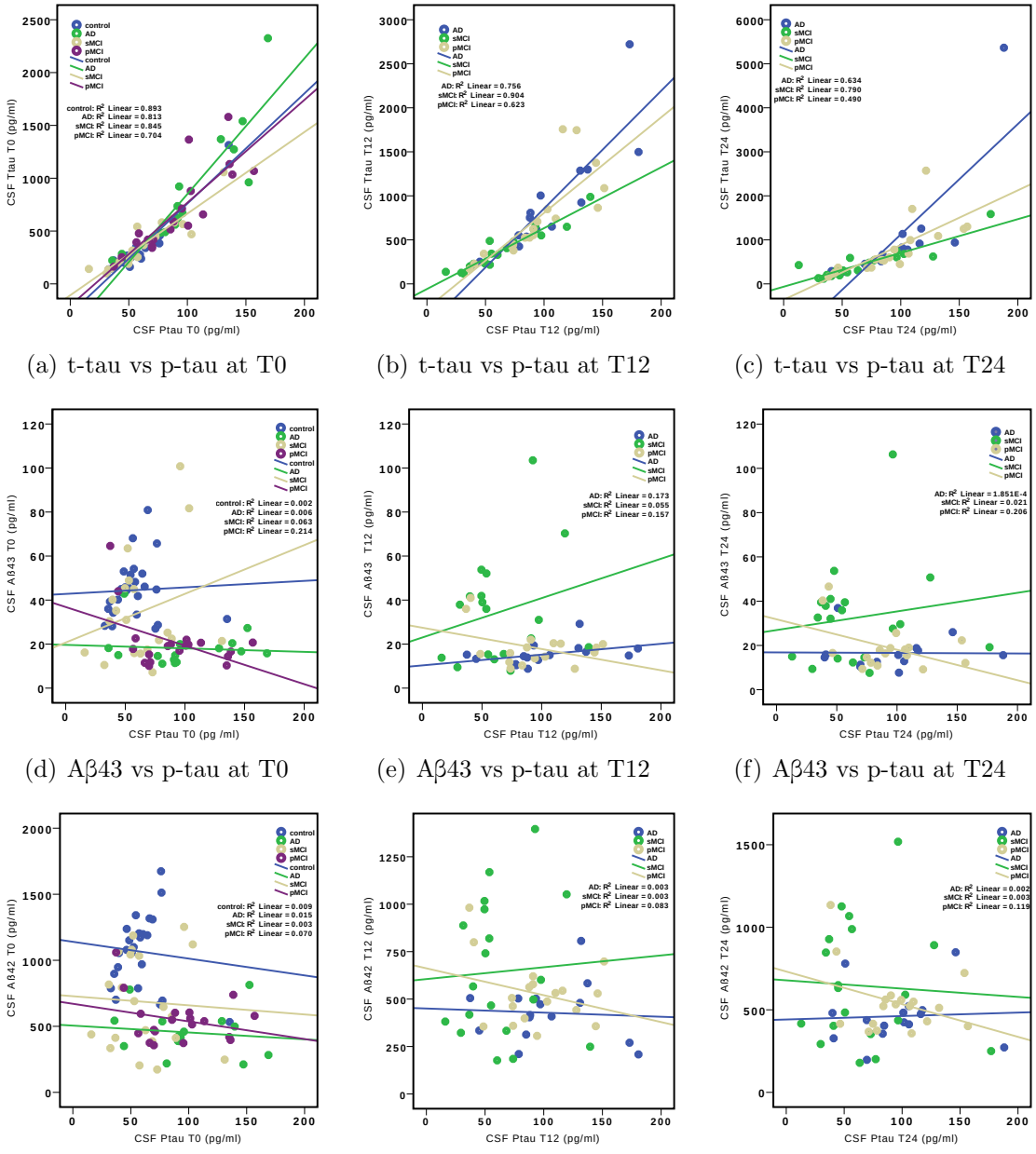


(a) Aβ43 vs Aβ42 at T0 (b) Aβ43 vs Aβ42 at T12 (c) Aβ43 vs Aβ42 at T24

(d) Aβ43 vs t-tau at T0 (e) Aβ43 vs t-tau at T12 (f) Aβ43 vs t-tau at T24

(g) Aβ42 vs t-tau at T0 (h) Aβ42 vs t-tau at T12 (i) Aβ42 vs t-tau at T24

Figure 19: Scatterplots showing relation between CSF levels of different biomarkers in participants at different times. A best-fit line has been added to the groups.



(a) t-tau vs p-tau at T0 (b) t-tau vs p-tau at T12 (c) t-tau vs p-tau at T24

(d) Aβ43 vs p-tau at T0 (e) Aβ43 vs p-tau at T12 (f) Aβ43 vs p-tau at T24

(g) Aβ42 vs p-tau at T0 (h) Aβ42 vs p-tau at T12 (i) Aβ42 vs p-tau at T24

Figure 20: Scatterplots showing relation between CSF levels of different biomarkers in patients and controls at different times. A best line of fit has been added to the groups.

A.3 ELISA procedure for the analysis of CSF Aβ43

Ref: IBL International, Amyloid-beta (1-43) High Sensitive ELISA (RE59711)

1. Determine wells for reagent blank. Put 100 µL each of 4, EIA buffer into the wells.

2. Determine wells for test sample blank, test sample and diluted standard. Then, put 100 µL each of test sample blank (tube-8), test sample and dilutions of standard (tube-1-7) into the appropriate wells.

3. Incubate the precoated plate overnight at 4^oC after covering it with plate lid.

4. Wash each well of the precoated plate vigorously with wash buffer using the washing bottle. Then, fill each well with wash buffer and leave the precoated plate laid for 15-30 seconds. Remove wash buffer completely from the precoated plate by snapping. This procedure must be repeated more than 7 times. Then, remove the remaining liquid from all wells completely by snapping the precoated plate onto paper towel. In case of using a plate washer, after 4 times washing with plate washer, washing with above washing bottle must be repeated 3 times.

5. Pipette 100 L of labeled antibody solution into the wells of test samples, diluted standard and test sample blank.

6. Incubate the precoated plate for 60 minutes at 4^oC after covering it with plate lid.

7. Wash the precoated plate 9 times in the same manner as 4).

8. Take the required quantity of 6, Chromogen into a disposable test tube. Then, pipette 100 µfrom the test tube into the wells. Please do not return the rest of the test tube to 6, Chromogen bottle to avoid contamination.

9. Incubate the precoated plate for 30 minutes at room temperature in the dark. The liquid will turn blue by addition of 6, Chromogen.

10. Pipette 100 µof 7, Stop solution into the wells. Mix the liquid by tapping the side of precoated plate. The liquid will turn yellow by addition of 7, Stop solution.

11. Remove any dirt or drop of water on the bottom of the precoated plate and confirm there is no bubble on the surface of the liquid. Then, run the plate reader and conduct measurement at 450 nm against a reagent blank. The measurement shall be done within 30 minutes after addition of 7, Stop solution.

A.4 ELISA procedure for the analysis of CSF Aβ42

Ref: 80324 INNOTEST® β-AMYLOID$_{(1-42)}$/256685 v19/ KEY-CODE: INX93594

1. Take the strip-holder with the required number of strips, taking into account that for each test run, 6 standards and one blank should be included. Standards, samples and blanks must be run in duplicate. Place any unused strip in the plastic minigrip bag with the silicagel desiccant.

2. Immediately after vortexing the CSF samples for 10s, add the CSF samples and standards and blank of Sample Diluent to the polypropylene plate provided in the kit. Add sufficient sample volume to the polypropylene plate so that 2 replicates of 25 µL each can be transferred to the antibody-coated plate.

3. Prepare conjugate working solution 1 and standards according to preparations for use.

4. Add 75 µL conjugate working solution 1 to each well of the antibody-coated plate.

5. Using a multi-channel pipette, transfer 25 µL from each well of the polypropylene plate to the corresponding well on the antibody-coated plate.

6. Make sure that standards and CSF samples are adequately mixed by carefully tapping the stripholder or by shaking 1 minute at 1000 rpm. Cover the strips with an adhesive sealer. Incubate for one hour at room temperature ($18 - 30^oC$).

7. Prepare conjugate working solution 2 just before the end of step 6.

8. Wash each well 5 times (see Directions for washing).

9. Add 100 L conjugate working solution 2 to each well. Cover the strips with a new adhesive sealer and incubate for 30 minutes at room temperature ($18 - 30^oC$).

10. Wash each well 5 times (see Directions for washing).

11. Prepare substrate working solution just before end of step 9.

text description Add 100 µL substrate working solution to each well. Incubate for 30 minutes at 18-30C in the dark.

12. To stop the reaction, add 50 µL Stop Solution to each well in the same sequence and at the same time intervals as the substrate solution. Tap the stripholder carefully to ensure optimal mixing.

13. Read (within 15 minutes after step 13) the absorbance at 450 nm (single wavelength). For dual wavelength analysis 620 nm can be used as the reference wavelength.

A.5 ELISA procedure for the analysis of CSF t-tau

Ref: 80323 INNOTEST® hTAU Ag /25684 v17/ KEY-CODE: INX95222

1. 1. Take the strip-holder with the required number of strips, taking into account that for each test run, 5 standards and one blank should be included. Standards, samples and blanks must be run in duplicate. Place any unused strip in the plastic minigrip bag with the silicagel desiccant.

2. Prepare conjugate working solution 1 and standards according to the preparations for use.

3. Add 75 µL conjugate working solution 1 to each well of the antibody-coated plate.

4. Add 25 µL of each standard (including the blank of 25 µL Sample Diluent) and the samples to duplicate wells of the antibody-coated plate.

5. Make sure that standards and CSF samples are adequately mixed by carefully tapping the stripholder or by shaking 1 minute at 1000 rpm. Cover the strips with an adhesive sealer. Incubate overnight (14-18hrs) in an incubator at 25 ± 2^oC.

6. Prepare conjugate working solution 2 just before the end of step 5.

7. Wash each well 4 times (see Directions for washing).

8. Add 100 µL conjugate working solution 2 to each well. Cover the strips with a new adhesive sealer and incubate for 30 3 minutes in an incubator at 25 ± 2^oC.

9. Prepare substrate working solution at the end of step 8.

10. Wash each well 4 times (see Directions for washing).

11. Add 100 L substrate working solution to each well. Incubate for 30 ± 3 minutes at room temperature $(18 - 30^oC)$ in the dark.

12. To stop the reaction, add 100 L of 2N sulfuric acid to each well (or use STOP solution of INNOTEST β-Amyloid or INNOTEST PHOSPHO-TAU(181P)), in the same sequence and the same time intervals as the substrate solution. Tap the stripholder carefully to ensure optimal mixing.

13. Read (within 15 minutes after step 12) the absorbance at 450 nm (single wavelength). For dual wavelength analysis, 620 nm can be used as the reference wavelength.

A.6 ELISA procedure for the analysis of CSF p-tau

Ref: 80317 INNOTEST® PHOSPHO-TAU$_{181p}$ /24898 v18/ KEY-CODE: INX41292

1. Take the strip-holder with the required number of strips, taking into account that for each test run, 6 standards and one blank should be included. Standards and blanks should be run in duplicate. Preferably the samples are also run in duplicate.

2. Add 25 µL of Conjugate working solution 1 to each test well. Add 75 µL sample or standard to each well. CSF samples should be vortexed before testing. Add 75 µL of Sample Diluent to each test well reserved as blank. Mix gently by tapping the side of the plate or by using a plate shaker (1000 rpm). Cover the strips with an adhesive sealer and incubate overnight 14-18 hr at $2 - 8^oC$.

3. Wash each well 5 times (see Directions for washing).

4. Add 100 µL Conjugate working solution 2 to each well. Cover the strips with a new adhesive sealer and incubate for 60 ± 5 minutes at room temperature $(18 - 30^oC)$.

5. Wash each well 5 times (see Directions for washing).

6. Add 100 µL of Substrate working solution to each well and incubate for 30 ± 3 minutes at room temperature $(18 - 30^oC)$ in the dark.

7. Add 50 µL of Stop Solution to each well, in the same sequence and at the same time intervals as the Substrate working solution. Tap the stripholder carefully to ensure thorough mixing.

8. Read (within 15 minutes after step 7) the absorbance at 450 nm (single wavelength).

A.7 MMSE documents

Aldring og helse
Nasjonal kompetansetjeneste

Helsedirektoratet

NORSK REVIDERT MINI MENTAL STATUS EVALUERING (MMSE-NR2)

Carsten Strobel & Knut Engedal, 2014

Pasient (PAS): _____ Fødselsdato/alder: _____

Nasjonalitet/morsmål/tolk: _____ Høyre-/venstrehendt: _____

Utdanning: _____ Antall år: _____ Yrke: _____

Hørsel/høreapparat: _____ Syn/briller: _____ Geriatrisk leseprøve: _____

Testleder (TL): _____ Dato: _____ Klokken: _____

Teststed/hjemmebesøk: _____ Er PAS testet med MMSE-NR samme sted tidligere? Ja ☐ Nei ☐

Hvis ja, når? _____ Når/hvor ble PAS sist testet med MMSE-NR (oppgavesett)? _____

MMSE-NR er ikke en demenstest, kun et grovt kognitivt funksjonsmål som supplerer annen utredning som somatisk undersøkelse (inkl. medikamentgjennomgang) og komparentintervju (inkl. forløp/varighet av kognitiv svikt og endret ADL-funksjon). Alle som administrerer MMSE-NR bør ha opplæring og god kjennskap til manual (lastes ned fra www.aldringoghelse.no). Følg standardisert instruksjon, ikke gi ledetråder, se retningslinjer for administrasjon, oppfølgende spørsmål og skåring på skjema og i manual. Ved lav norskspråklig kompetanse og annet morsmål enn norsk bruk fagutdannet tolk, ikke slektninger/bekjente. For oppgave 16 og 18, bruk standardiserte oversettelser og stimuliark der disse foreligger.

Instruksjon

Utfør testing en-til-en, uten pårørende til stede. Unngå at PAS ser skjema og skåring, bruk f.eks. skriveunderlag med klemme. Les fet skrift (**bold**) høyt, tydelig og langsomt. Pause (markert: [*pause*]) skal vare 1 sekund. Samtlige spørsmål skal stilles, også om PAS har besvart oppgaveledd under tidligere stilte spørsmål. Instruksjon kan gjentas, unntatt på oppgave 12 og 17 hvor det er svært viktig at instruksjon kun gis én gang. Skriv ordrett ned svar på hvert spørsmål. PAS kan korrigere svar underveis, gi derfor ikke tilbakemelding om svar er rett eller galt.

Ved retesting skift alltid oppgavesett som angitt på oppgave 11,12 og 13 for å redusere læringseffekt. Sett kryss i ruten for «0» ved feil svar og i ruten for «1» ved rett svar, gi aldri ½ poeng. Totalskåre regnes alltid fra 30 poeng: Er PAS ikke testbar på en oppgave pga. ikke-kognitive handikapp, angi hvorfor og sett ring rundt ruten for «0». Gir PAS utrykk for ikke å klare en oppgave, oppfordre likevel til å gjøre et forsøk. Er du usikker på hvordan et svar skåres etter å ha sjekket manual, rådfør deg med en erfaren kollega. Lavere alder og høyere utdanning gir ofte bedre skåre. Likeså testing på hjemmebesøk/vante omgivelser pga. stedsorienteringsledd. Lav motivasjon, dårlig dagsform, trettbarhet, afasi, lese- og skrivevansker, redusert syn og hørsel, depresjon, testangst, legemiddeleffekter (bivirkninger/interaksjoner), akutt somatisk sykdom, lav norskspråklig kompetanse, stress og liten testledererfaring kan påvirke resultat negativt. Totalskåre sier lite om spesifikke kognitive sviktområder som kan være diagnostisk og klinisk relevante, presisér derfor alltid utfall. Skåringsprofil og kvalitativ vurdering av utførelse kan også gi informasjon om kognitive restressurser og kompenserende mestringsstrategier som kan gi innspill til hvordan tilrettelegge aktivitet og samhandling. Bemerk påfallende forhold som lang tidsbruk, usikkerhet, mange korrigeringer, behov for gjentakelse av instruksjon, årsaker til testavbrudd e.l.

Skåring MMSE-NR2. Oppgavesett (ordsett/starttall oppgave 11, 12 og 13) administrert i dag: 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐

		KOMMENTARER TIL SPESIFIKKE OPPGAVELEDD:	
Tidsorientering	*(oppgave 1–5)*		**/5**
Stedsorientering	*(oppgave 6–10)*		**/5**
Umiddelbar gjenkalling	*(oppgave 11)*		**/3**
Hoderegning	*(oppgave 12)*		**/5**
Utsatt gjenkalling	*(oppgave 13)*		**/3**
Språk og praksis	*(oppgave 14–19)*		**/8**
Figurkopiering	*(oppgave 20)*		**/1**
Total poengskåre			**/30**

Versjon 2, 18.05.2014

Vurderer du som testleder (TL) at samarbeid/motivasjon/testinnsats var uten anmerkning? Ja ☐ Nei ☐ Usikker ☐

Vurderer du at oppmerksomhet/bevissthetsnivå/våkenhet var uten anmerkning? Ja ☐ Nei ☐ Usikker ☐

Vurderes ikke resultat som valid/gyldig, angi årsak(er): _____

Spesielt å bemerke (henvisningsgrunn, medikamenter som kan påvirke kognitiv funksjon, atferd, dagsform, stemningsleie, smerter, afasi, ikke-kognitive handikapp, bruk av ikke-dominant hånd f.eks. ved lammelse, tidsbruk, vansker på distraksjonsbetingelsen, glemt briller/høreapparat e.l.):

_____

_____

_____

Basert på: Folstein, M.F., Folstein, S.E., & McHugh, P.R. (1975). "Mini-Mental State": A practical method for grading the cognitive state of patients for the clinician. Journal of Psychiatric Research, 12, 189-198.
Engedal, K., Haugen, PK., Gilje, K., & Laake, P. (1988). Efficacy of short mental tests in the detection of mental impairment in old age. Compr Gerontol A, 2, 87-93.
Strobel, C., & Engedal, K. (2008). MMSE-NR. Norsk Revidert Mini Mental Status Evaluering. Revidert og utvidet manual. Oslo. Nasjonalt kompetansesenter for aldring og helse.
Palmquist, S., Terzis, B., Strobel, C., & Wallin, A. (2012). Mini Mental State Examination, Svensk Revidering (MMSE-SR). Svensk Förening för Kognitiva sjukdomar.

Start med introduksjonsspørsmålet: **Synes du hukommelsen har blitt dårligere siste år?** Ja ☐ Nei ☐ Usikker ☐
Jeg skal nå stille deg noen spørsmål vi bruker bl.a. for å undersøke hukommelsen. Svar så godt du kan.

TIDSORIENTERING

Det er TL sitt ansvar å forhindre at PAS kan ta i bruk ledetråder: Se ut av vindu (årstid, måned), bruke kalender, avis, innkallingsbrev (årstall, måned, ukedag, dato), sjekke dato på klokke, mobil e.l.

1. **Hvilket årstall har vi nå?** (Kun fullt årstall med 4 sifre gir poeng) _____ 0☐ 1☐
2. **Hvilken årstid har vi nå?** (Ta hensyn til vær og geografiske forhold) _____ 0☐ 1☐
3. **Hvilken måned har vi nå?** (Kun rett navn på måned gir poeng, ikke nummer på måned) _____ 0☐ 1☐
4. **Hvilken dag har vi i dag?** (Kun rett navn på ukedag gir poeng) _____ 0☐ 1☐
5. **Hvilken dato har vi i dag?** (Unngå følgefeil: Kun dagsledd må være rett, måned/år kan være feil) _____ 0☐ 1☐

STEDSORIENTERING

Bruk best egnet stedsord og spørsmålsstilling, sett ring rundt valgt alternativ. Landsdel* skal kun benyttes ved testing i Oslo.

6. **Hvilket land er vi i nå?** _____ 0☐ 1☐
7. **Hvilket (fylke/landsdel*) er vi i nå?** (For landsdel gi poeng for Østlandet og Sør-Norge) _____ 0☐ 1☐
8. **Hvilken (by/tettsted/kommune) er vi i nå?** _____ 0☐ 1☐
9. **Hva heter dette (stedet/sykehuset/sykehjemmet/legekontoret e.l.)?** Eller **Hvor er vi nå?** _____ 0☐ 1☐
10. **I hvilken etasje er vi nå?** (Still spørsmål selv der bygg kun har én etasje. Ta hensyn til språk/kultur) _____ 0☐ 1☐

Unngå at PAS kan se ut av vindu (sted, etasje). Avhengig av inngang vil bygg i skrånende terreng kunne oppfattes å ha ulik etasje-angivelse for samme etasje. Gi poeng om PAS i tråd med språk/kultur benevner norsk 1. etasje som grunnplan (f.eks. Erdgeschoss, ground floor, stuen) og norsk 2. etasje som 1. etasje (1. Stock/Etage, first floor, 1. sal). Ved testing på hjemmebesøk, se manual.

UMIDDELBAR GJENKALLING

Bruk alltid nytt ordsett som angitt ved retesting for å hindre læringseffekt fra tidligere administrasjon. Sett ring rundt dagens ordsett. Ved 1. adm. bruk oppgavesett 1, ved 2. adm. bruk sett 2 osv., ved 6. adm. bruk sett 1, ved 7. adm. bruk sett 2 osv.

11. **Hør godt etter. Jeg vil si 3 ord som du skal gjenta etter meg. Disse skal du også prøve å huske, for jeg kommer til å spørre deg om dem litt senere. Er du klar?**
Nå kommer ordene: ..... [*pause*], **.....** [*pause*], **.....** [*pause*]**. Nå kan du gjenta disse ordene.**
Gjentar ikke PAS alle 3 ord, repeteres hele ordsettet inntil alle 3 ord gjengis i samme forsøk, opptil 3 presentasjoner. Gi *kun* poeng for riktige ord etter 1. presentasjon, rekkefølge PAS sier ordene er uten betydning. Antall presentasjoner: _____ stk.

Oppgavesett:	1	2	3	4	5		
Nå kommer ordene: ...	**HUS**	**STOL**	**SAFT**	**KATT**	**FLY**	_____	0☐ 1☐
	KANIN	**BANAN**	**LAMPE**	**AVIS**	**EPLE**	_____	0☐ 1☐
	TOG	**NÅL**	**BÅT**	**LØK**	**SKO**	_____	0☐ 1☐

Etter 3 gjenkalte ord eller 3 presentasjoner, si: **Husk disse ordene, for jeg vil spørre deg om hvilke de er litt senere.**

HODEREGNING (Bruk alltid obligatorisk distraksjonsbetingelse i tillegg)

Bruk alltid nytt starttall som angitt ved retesting. Ved 6. adm. bruk oppgavesett 1 osv. Sett ring rundt dagens starttall, skriv ned tallsvar. Unngå følgefeil: Gi poeng når svar er minus 7 fra forrige tall, uavhengig av om forrige tallsvar var rett eller galt.

12. **Nå vil jeg at du trekker 7 fra .....** [*Gir ikke PAS tallsvar, si:* **Hva er ..... minus 7?**] [*Rett etter tallsvar, si*]: **Og så fortsetter du å trekke 7 fra tallet du kom frem til, helt til jeg sier stopp.** [*Instruksjon gis kun én gang. Ikke informer underveis om subtraksjonstall eller hvor langt PAS har kommet*]. Ved færre enn 5 tallsvar, gå til distraksjonsbetingelsen.

Oppgavesett:	1	2	3	4	5		
Starttall: **Nå vil jeg at du trekker 7 fra ...**	**80**	**50**	**90**	**40**	**60**		
Og så fortsetter du å trekke 7 fra tallet du kom frem til, helt til jeg sier stopp →	73	43	83	33	53	_____	0☐ 1☐
	66	36	76	26	46	_____	0☐ 1☐
Ved behov si: **Og så videre**	59	29	69	19	39	_____	0☐ 1☐
Ved behov si: **Og så videre**	52	22	62	12	32	_____	0☐ 1☐
Ved behov si: **Og så videre**	45	15	55	5	25	_____	0☐ 1☐

Etter 5 subtraksjoner, si: **Fint, det holder** [Gå til distraksjonsbetingelsen].

Obligatorisk distraksjonsbetingelse – OBS, er ikke poenggivende!
Bruk alltid distraksjonsbetingelsen for å sikre tilstrekkelig tidsopphold med distraksjon. Dette for å fremme reell kartlegging av langtidshukommelse fremfor arbeidshukommelse på oppgave 13. Be PAS telle baklengs fra 100 ca. 30 sekunder med følgende instruksjon: **Nå vil jeg at du teller baklengs fra 100 på denne måten: 99, 98, 97..., helt til jeg sier stopp. Vær så god!** [*Etter ca. 30 sek. si:*] **Fint, det holder.**

UTSATT GJENKALLING

13. **Hvilke 3 ord var det jeg ba deg om å huske?** [*Ikke gi ledetråder/stikkordshjelp, sett ring rundt dagens ordsett*]

Oppgavesett:	1	2	3	4	5		
	HUS	STOL	SAFT	KATT	FLY	_____	0☐ 1☐
	KANIN	BANAN	LAMPE	AVIS	EPLE	_____	0☐ 1☐
	TOG	NÅL	BÅT	LØK	SKO	_____	0☐ 1☐

Nevnes mer enn 3 ord, må PAS velge hvilke 3 ord som skal være svaret, rekkefølge er uten betydning. Gi *kun* poeng for dagens ordsett og eksakt gjengivelse, dvs. bolighus, hytte, kaninen, kaniner, hare, togbane, lokomotiv e.l. gir ikke poeng.

BENEVNING

14. **Hva heter dette?** [*Vis stimuliarket riktig vei og pek på blyanten*] _____ 0☐ 1☐

15. **Hva heter dette?** [*Vis stimuliarket riktig vei og pek på armbåndsuret*] _____ 0☐ 1☐

Alternative poenggivende svar: Penn, gråblyant, fargeblyant, ur, klokke, klokkerem e.l. Bruk kun stimuliarket i farger med blyant og armbåndsur, ikke andre objekter, gjelder også retesting. Eneste unntak er testing av sterkt synshemmete eller blinde, hvor stimuliobjektene blyant og armbåndsur kan presenteres taktilt med konkreter.

TL

PAS

TL

PAS

FRASEREPETISJON

16. **Gjenta *ordrett* det jeg sier. Er du klar?** [*Si tydelig*]: **«ALDRI ANNET ENN OM OG MEN».**

Gi poeng når hele frasen gjengis korrekt med alle 6 ord i riktig rekkefølge. Dialektvarianter godtas.

TL kan si frasen 3 ganger, men gi *kun* poeng etter 1. presentasjon. Antall presentasjoner: _____ stk.

ALDRI ANNET ENN OM OG MEN _____ 0☐ 1☐

3-LEDDET KOMMANDO

Legg et ubrukt A4-ark på bordet midt foran PAS, kortsiden mot PAS. For å unngå at PAS starter før hele instruksjonen er gitt, legg egen hånd på arket til all instruksjon er gitt. Gi poeng for hver korrekt utførte delhandling.

17. **Hør godt etter, for jeg skal be deg gjøre 3 ting i en bestemt rekkefølge. Er du klar?**
Ta arket med én hånd [*pause*]**, brett arket på midten kun én gang, med en eller begge hender** [*pause*]**, og gi arket til meg** [*pause*]**. Vær så god!** [*Instruksjon gis kun én gang, enkeltledd kan ikke repeteres*]

TAR ARKET MED KUN *EN* HÅND _____ 0☐ 1☐

BRETTER ARKET PÅ MIDTEN KUN *EN* GANG _____ 0☐ 1☐

GIR ARKET TIL TL (gi også poeng om arket legges på bordet tydelig foran TL) _____ 0☐ 1☐

LESNING

18. **Nå vil jeg at du gjør det som står på arket** [*Vis PAS teksten*]. PAS må lukke øynene for poeng. Lukker ikke PAS øynene, kan instruksjon gjentas 2 ganger til. Hver presentasjon gir mulighet for poeng. Antall presentasjoner: _____ stk.

LUKK ØYNENE DINE _____ 0☐ 1☐

SETNINGSGENERERING

Legg nedre del av neste side MMSE-NR skjema med kortsiden foran PAS, og gi vedkommende en blyant.

19. **Skriv en meningsfull setning her.** [*Pek på øvre del av neste side*] _____ 0☐ 1☐

Skrives imperativsetning med kun ett ord, f.eks. «Spis», si: **Skriv en lengre setning.** Skrives intet eller tidligere gitt setning/frase, f.eks.«Lukk øynene dine» eller «En meningsfull setning», si: **Skriv en setning du lager selv.** Skriver ikke PAS noe nå heller, si: **Skriv om været.**

Setningen må gi mening, men trenger ikke ha objekt og tidvis heller ikke subjekt eller verb, se manualeksempler. Ignorer stave- og grammatikalske feil. Gi poeng ved rett utførelse etter supplerende instruksjon og for spørresetning, om kriterier ellers er innfridd.

FIGURKOPIERING

Legg figurarket som vist med figurspissene mot PAS over øvre del av neste side (over setningen PAS skrev), viskelær ved siden av (skal ikke brukes som linjal). PAS får ikke rotere eller flytte på figurarket som TL må sørge for at blir liggende til PAS er ferdig.

20. **Kopier figuren så nøyaktig du kan her.** [*Pek på nedre del av neste side*] _____ 0☐ 1☐

Du kan bruke viskelær. Ta deg god tid. Si fra når du er ferdig.

Er PAS misfornøyd med utførelse, oppfordre til å korrigere/tegne figuren på nytt, maks. 3 poenggivende forsøk. Gi poeng når to 5-kantede figurer former en 4-sidet figur der 5-kantene overlapper: 5-4-5. Rotert utførelse, størrelsesforskjell mellom 5-kantene eller hvor de overlapper er ikke avgjørende for skåring om kriterier ellers er innfridd, se skåringseksempler i manual. Er TL i tvil om utførelse er korrekt, be PAS tegne figuren på nytt.

TL

PAS