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NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
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‘Amyloid beta 1-43 som potensiell biomarkør for påvisning av tidlig Alzheimers sykdom’

Alzheimers sykdom (AD) er en stor utfordring for helsevesenet og for pasientene som rammes, og forekomsten av AD er ventet å øke de kommende årene grunnet en økende andel eldre i befolkningen. Det er viktig å kunne identifisere hvilke pasienter med redusert korttidshukommelse som har AD som årsak til glemsomheten, slik at man kan forutsi hvem som vil ha nytte av medikamentell behandling når denne blir tilgjengelig. Nye diagnosekriterier for AD er utviklet og muliggjør påvisning av sykdommen før pasienten har utviklet demens og er hjelpetrengende i hverdagen, ved hjelp av kognitive tester og analyse av biomarkører i spinalvæske eller ved bruk av billeddiagnostikk. Det er i dag vanlig å analysere amyloid beta 1-42 (Aβ42) og tau-proteiner i spinalvæske. Studier viser at peptidet amyloid beta 1-43 (Aβ43) har stor tendens til å aggregere og avleires i amyloide plakk, og derfor kan ha betydning i tidlig fase av AD.

I de tre studiene ble det analysert spinalvæske fra Nevrologisk forskningsbiobank og Trønderbrainstudien ved St. Olavs Hospital og NTNU, samt fra Akershus Universitetssykehus der det også ble gjort billeddiagnostikk. Deltakere i studiene ble delt inn i grupper bestående av kognitivt friske, individer med subjektivt eller objektivt redusert korttidshukommelse, og pasienter med AD.

I den første artikkelen fant vi at Aβ43 var signifikant bedre enn Aβ42 til å skille mellom pasienter med redusert korttidshukommelse som utviklet AD innen kort tid og de som ikke gjorde det. Nivåene av Aβ43, men ikke Aβ42, sank hos pasientene med AD i løpet av de to årene de ble fulgt. I artikkel to, der nivået av Aβ43 ble sammenlignet mellom yngre (≤ 62 år) og eldre (≥ 68 år) deltakere, var Aβ43 lavere for yngre enn eldre pasienter med AD, en forskjell som ikke ble funnet for Aβ42. Personer med subjektivt eller objektivt redusert korttidshukommelse ble inkludert i artikkel tre. Her samvarierte både Aβ43 og Aβ42 med mengden amyloide plakk i hjernen, men resultatene tilsa ikke at Aβ43 var bedre enn den mer etablerte markøren Aβ42 for å skille de to gruppene.

Våre resultat tyder ikke på at Aβ43 er bedre enn Aβ42 for å separere friske fra pasienter med tidlig AD. Det er imidlertid ønskelig med større studier for å undersøke om Aβ43 bør analyseres i tillegg til Aβ42 for å identifisere individer med størst risiko for å utvikle demens grunnet AD innen kort tid. Fremtidige studier bør inkludere kognitivt friske individer, samt personer med subjektivt og objektivt redusert korttidshukommelse, som har patologiske biomarkørnivå.

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Biveiledere: Sigrid Botne Sando og Geir Bråthen

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Trondheim March 2017

Camilla Lauridsen

List of papers

Paper I

Camilla Lauridsen, Sigrid Botne Sando, Adiba Shabnam, Ina Møller, Guro Berge, Gøril Rolfseng Grøntvedt, Inger Johanne Bakken, Øyvind Salvesen, Geir Bråthen, Linda Rosemary White

Cerebrospinal fluid levels of amyloid beta 1-43 in patients with amnestic mild cognitive impairment or early Alzheimer's disease: a 2-year follow-up study

Published in Frontiers in Aging Neuroscience 2016 Mar 1;8:30. doi: 10.3389/fnagi.2016.00030. eCollection 2016.

Paper II

Camilla Lauridsen, Sigrid Botne Sando, Ina Møller, Guro Berge, Precious Kwadzo Pomary, Gøril Rolfseng Grøntvedt, Øyvind Salvesen, Geir Bråthen, Linda Rosemary White

Cerebrospinal fluid Aβ43 is reduced in early-onset compared to late-onset Alzheimer's disease, but has similar diagnostic accuracy to Aβ42

Manuscript submitted (Since published in Frontiers in Aging Neuroscience 2017 Jun 28;9:210. doi: 10.3389/fnagi.2017.00210. eCollection 2017).

Paper III

Ina S. Almdahl, Camilla Lauridsen, Per Selnes, Lisa F. Kalheim, Cristopher Coello, Beata Gajdzik, Ina Møller, Marianne Wettergreen, Ramune Grambaite, Atle Bjørnerud, Geir Bråthen, Sigrid B. Sando, Linda R. White, Tormod Fladby

Cerebrospinal Fluid Levels of Amyloid Beta 1-43 Mirror 1-42 in Relation to Imaging Biomarkers of Alzheimer's Disease

Published in Frontiers in Aging Neuroscience 2017 Feb 7;9:9. doi: 10.3389/fnagi.2017.00009. eCollection 2017

List of abbreviations

Aβ	amyloid beta
Aβ40	amyloid beta 1-40
Aβ42	amyloid beta 1-42
Aβ43	amyloid beta 1-43
AD	Alzheimer's disease
aMCI	amnestic mild cognitive impairment
APOE	apolipoprotein E gene
APP	amyloid precursor protein
CSF	cerebrospinal fluid
CV	coefficient of variation
DTI	diffusion tensor imaging
ELISA	enzyme-linked immunosorbent assay
EOAD	early-onset Alzheimer's disease
FDG	fluorodeoxyglucose
FLUT	flutemetamol
GFAP	glial fibrillary acidic protein
IWG	International Working Group
LOAD	late-onset Alzheimer's disease
MCI	mild cognitive impairment
MMSE	mini mental state examination
MRI	magnetic resonance imaging
NF-L	neurofilament light
NIA-AA	National Institute on Aging - Alzheimer's Association
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
PET	positron emission tomography
p-tau	phosphorylated tau protein
SCD	subjective cognitive decline
t-tau	total tau protein

1. General introduction

1.1 Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia in senescence (about 70 % of cases (1)), and is characterized by a slow but progressive and devastating loss of cognitive functions. 'Dementia' is a collective term used to describe diseases that affect the cognitive functions of the brain, to a degree that the patients can no longer be independent. The cognitive modalities affected can be memory, judgment, planning, orientation, information processing, attention, execution and language. According to the NINCDS-ADRDA criteria for AD diagnosis from 1984, two or more areas of cognition must be affected, and there must be progressive worsening of cognitive function (2). There is an ongoing search for effective treatment strategies, and early diagnosis through analysis of biomarkers with high diagnostic accuracy will be crucial when effective treatment becomes available.

Although the symptoms were described by the ancient Greeks (3), the disease bears the name of Alois Alzheimer, who in 1906 described progressive dementia symptoms in his patient, Auguste D. (4, 5). He was also the first to describe the histopathological findings in her brain when it was examined post mortem; both the amyloid plaques consisting mainly of amyloid beta (Aβ) peptides (6), and neurofibrillary tangles consisting largely of hyperphosphorylated tau (7). To this day, these observations are required for a 'definite' AD diagnosis (2).

The majority of patients develop AD in old age. In a study from central Norway, where the samples in papers I and II were collected, an average age at onset of AD dementia around 74 years was found (8). An arbitrary cut-off age of 65 years is often used to separate early-onset AD (EOAD) from the more common late-onset AD (LOAD), and the late-onset variant is 10-20 times more prevalent than the early-onset variant (9). Although there are studies that have shown the pathological burden of amyloid plaques and neurofibrillary tangles to be greater in EOAD than in patients with LOAD (10, 11), others have indicated the amount of amyloid deposition to be similar, though with variation in anatomical distribution (12, 13). On the other hand, similar amyloid burden and distribution between EOAD and LOAD has also been found (14).

1.2 AD prevalence

AD prevalence increases almost exponentially with age (15), with the incidence doubling every 5-6 years from the age of 65 (16). AD is becoming more prevalent as an increasing proportion of the world's population survives to old age. Worldwide, an estimated 35.6 million people suffered from dementia in 2010, and dementia is predicted to affect 65.7 million people worldwide by 2030, with the number doubling every 20 years, and the largest increase is likely to be in low- and middle-income countries (15). The increased prevalence of AD, in addition to immense human suffering for both patients and their relatives and caregivers, will put increased financial burden on health care systems (17).

1.3 AD risk factors

Aging is thus the greatest risk factor for AD (16). Other modifiable AD risk factors include depression, midlife hypertension, physical inactivity, diabetes, midlife obesity, hyperlipidemia and smoking (18). Fewer years of education has been shown to increase AD risk (19), whereas complex cognitive activities seem to reduce dementia incidence (20). To date, AD risk reduction is mostly based on lifestyle changes and controlling or treating other medical conditions that may trigger AD (21), such as midlife hypertension, hypercholesterolemia (22) and diabetes (23).

The *APOE* ε4 allele is the most important genetic risk factor for AD (24). The *APOE* allele exists in three common isoforms; ε2, ε3 and ε4, of which the ε3 variant is the most frequent (25). Carrying the *APOE* ε4 allele increases the risk of AD, and risk is increased about 10- to 15-fold if an individual is homozygous (26). Additionally, an earlier age at onset can be expected (8). The *APOE* ε4 isoform is thought to increase cerebral deposition of Aβ (27), perhaps by clearing Aβ less effectively from the brain than the other isoforms (28).

1.4 AD diagnosis

Ante mortem diagnosis of the disease is currently based on clinical examination and assessment, with supporting evidence obtained from analysis of biomarkers (see section 1.7) that reflect the pathology considered to be at the core of the disease process. The NINCDS-ADRDA diagnostic criteria from 1984 require symptoms of dementia to be present (i.e. daily function affected so that independence can no longer be preserved) for AD to be diagnosed (2). This means that an AD diagnosis can be given only in a late stage of the disease. However, in recent years there has been increasing awareness that the AD process begins many (perhaps as many as 20) years before symptoms become manifest. Being able to diagnose AD at the earliest possible disease stage will be of crucial importance when effective treatment becomes available, to stop or delay neurodegeneration.

Suggested new diagnostic criteria for AD have been published from both American (NIA-AA) (29) and European working groups (IWG-2) (30). These criteria are largely similar, and permit the diagnosis of AD before symptoms of dementia have developed.

1.5 Mild cognitive impairment and subjective cognitive decline

Reduced cognitive function in advance of fulminant dementia is sometimes, but not always, a transitional stage between normal aging and dementia, and is known as mild cognitive impairment (MCI) (31), see Figure 1. The independence of daily living is kept during MCI, but the patient experiences reduced function in a cognitive domain such as memory, language or attention, and the execution of complex tasks can be impaired (32, 33). Patients diagnosed with amnestic MCI (aMCI) have increased risk of converting to AD, and an annual progression rate of 18.2% has been found (34). However, MCI patients may progress instead to another dementia (especially if diagnosed with non-amnestic MCI), have a stable MCI, or revert to normal cognitive function (34, 35), as illustrated in Figure 1.

However, since MCI is defined according to criteria based on symptoms, it follows that there must have been a degenerative process in advance of MCI. This preclinical stage of AD has been termed both subjective cognitive decline (SCD) and subjective cognitive impairment

(SCI). SCD will be used in this work. SCD is defined as a subjectively-experienced cognitive decline while performance on cognitive tests is still within the 'normal' range (36), see Figure 2. Although unspecific, several studies have shown that SCD is associated with abnormal AD biomarkers and an increased risk for cognitive decline (36), though the annual conversion rate to MCI has been found to be only 6.7 % (37). SCD is thereby a very heterogeneous entity, and not all believe it should be viewed as a proxy to preclinical AD (38).

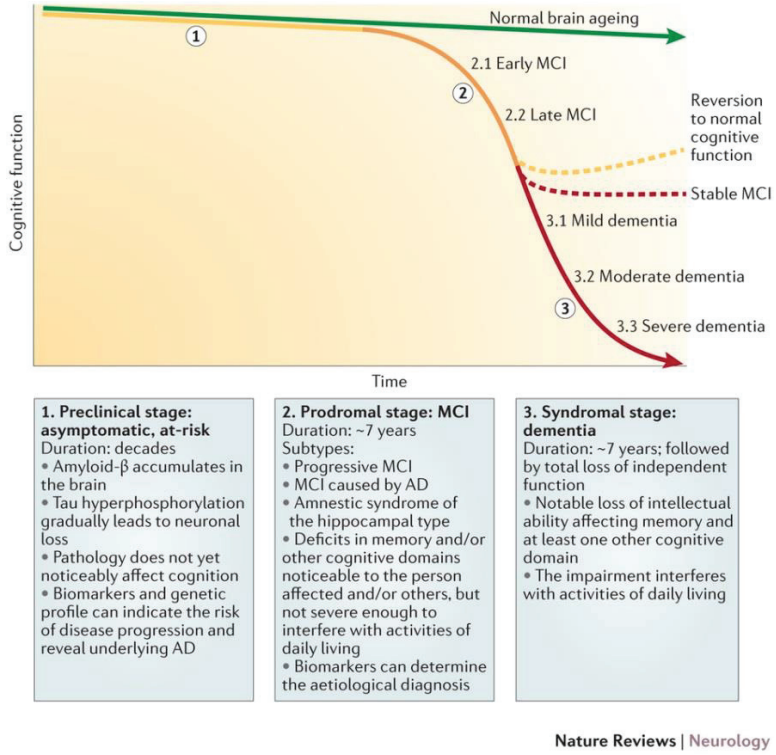

Figure 1. *Various stages of MCI and AD dementia. Figure from (39).*

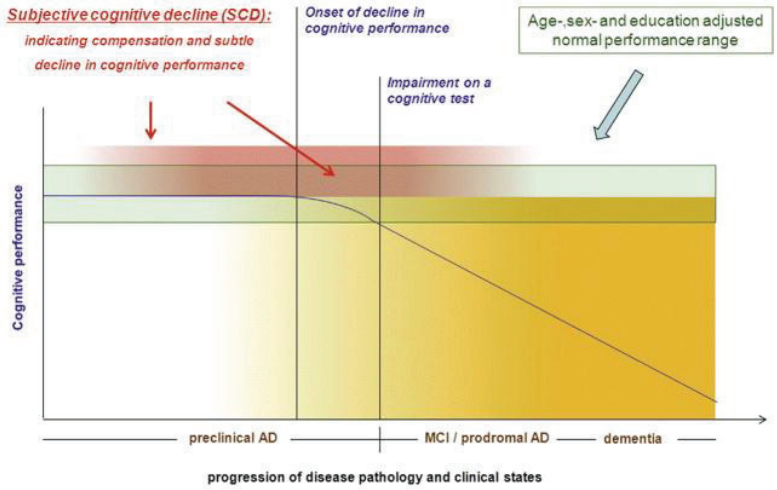

Figure 2. *SCD is considered to be a stage with subtle cognitive decline without clear symptoms, before MCI can be diagnosed. Figure from (36).*

The proposed new diagnostic criteria assume that AD can be diagnosed before dementia has developed, and that diagnosis will be based on a combination of cognitive impairment and one or more abnormal biomarkers for AD (29, 30). In the IWG-2 criteria (30), cerebrospinal fluid (CSF) biomarkers and amyloid positron emission tomography (PET) are central due to their diagnostic performance for early diagnosis. The rationale for the new diagnostic criteria is that the pathological process starts long (decades) before dementia symptoms appear (40), and therefore a positive 'AD signature' biomarker pattern combined with impaired episodic memory will be sufficient to diagnose a patient with prodromal AD (MCI due to AD).

The IWG criteria were designed for research use, whereas the NIA-AA criteria were designed for both clinical and research use (41). Biomarker performance needs to be further validated before biomarkers can be utilized in a clinical routine setting, and both pre-analytical and analytical factors need to be identified and standardized before cut-off-values can be established.

1.6 Pathophysiological mechanisms of AD

Amyloid beta (Aβ) peptides and tau proteins are integral to AD pathology, and amyloid plaques and neurofibrillary tangles can be found before there are any apparent clinical symptoms (42).

1.6.1 Aβ peptides

Amyloid plaques are mostly composed of Aβ peptides (6), which are produced from sequential enzymic cleavage of the transmembrane amyloid precursor protein (APP) (43), illustrated in Figure 3. Soluble Aβ peptides are produced during normal cellular metabolism (44), and in addition to being a source of Aβ peptides, APP is thought to function as a cell surface receptor (reviewed by (45)).

APP can be processed via two pathways: non-amyloidogenic and amyloidogenic, as shown in Figure 3. In the non-amyloidogenic pathway, proteases with α-secretase activity are involved in the formation of a soluble form of APP (sAPP-α) (46) when cleavage takes place close to the cell membrane. A second cleavage by the γ-secretase complex within the cell membrane then takes place (47), and this non-amyloidogenic pathway precludes the formation of aggregation-prone Aβ peptides in the brain.

In the amyloidogenic pathway, the extracellular cleavage of APP is made by the enzyme β-secretase (48) and liberates soluble APP-β. The β C-terminal fragment (β-CTF) remains within the cell membrane, and the γ-secretase complex produces Aβ peptides that vary in amino acid length from β-CTF, depending on the exact point of cleavage (47). Amyloid beta 1-40 (Aβ40) is the most abundant amyloid beta protein in CSF (49), about 10x the concentration of the amyloid beta 1-42 (Aβ42) variant, but the two extra hydrophobic amino acids compared to Aβ40 make Aβ42 more prone to aggregation (50).

Aβ peptides are released as monomers, which may aggregate into dimers, trimers, oligomers and then insoluble Aβ fibrils (51), see Figure 4.

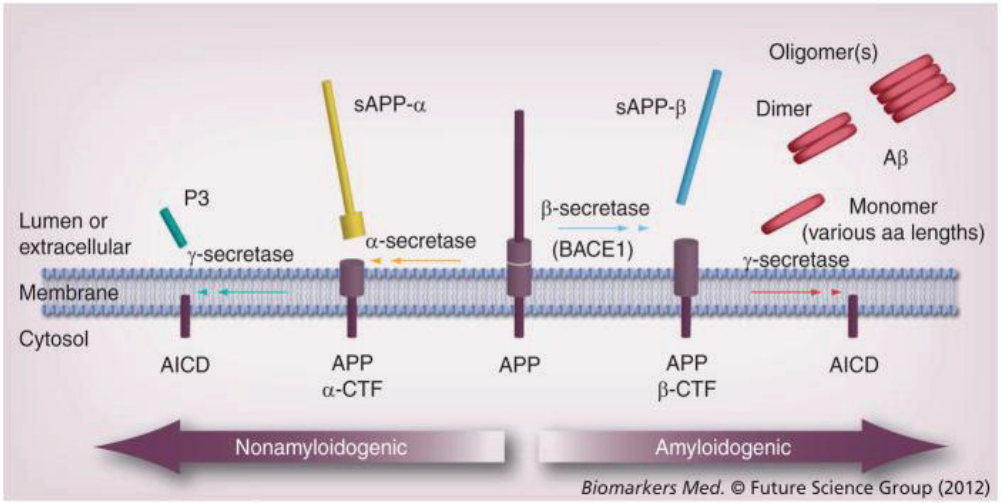

Figure 3. *In the non-amyloidogenic processing pathway (left part of figure), the amyloid precursor protein (APP) is cleaved by α-secretase to release sAPP-α from the membrane. A subsequent cleavage by γ-secretase releases P3. In the amyloidogenic pathway (right part of figure), APP is cleaved by a β-secretase to release sAPP-β. The remaining membrane-bound C-terminal fragment (CTF) is thereafter cleaved by γ-secretase to release monomeric Aβ peptides that may aggregate to form amyloid plaques. Figure from (52).*

Compared to Aβ42, amyloid beta 1-43 (Aβ43) has an additional threonine at the C-terminal, and is thought to be even more aggregation-prone than Aβ42 (50, 53, 54). Aβ42 and Aβ43 have been suggested to be produced by different routes of enzymic cleavage, where three amino acids are cleaved off the peptides Aβ48 and Aβ49 in two steps to produce Aβ42 and Aβ43, respectively (55).

1.6.2 Aβ oligomers

Aβ oligomers consist of 2-12 peptides (Figure 4) and have been proposed to contribute to the pathology of AD (56), despite low levels in CSF. Oligomers have for instance been demonstrated to impair synaptic plasticity (57, 58), increase oxidative stress (59), impair axonal transport in neuronal cell cultures (60), and stimulate tau hyperphosphorylation (61).

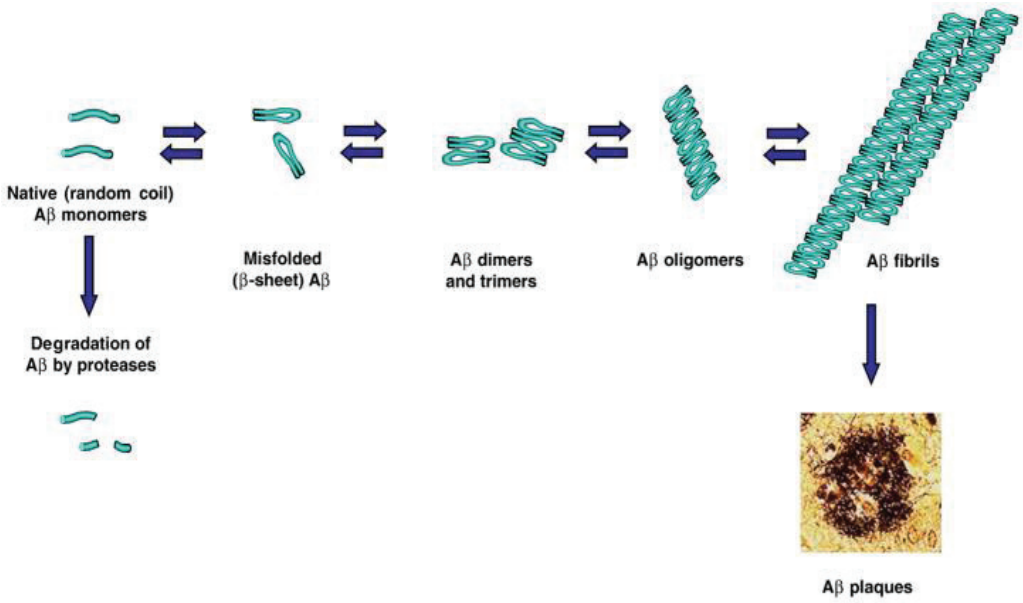

Figure 4. *Misfolding and aggregation of Aβ monomers into polymeric fibrils, a process most likely for aggregation-prone Aβ peptides. Soluble native protein undergoes a conformational change into β-sheets that easily oligomerize and form insoluble Aβ fibrils. Figure from (51).*

1.6.3 The amyloid cascade hypothesis

'The amyloid cascade hypothesis' has been the most widely acclaimed hypothesis for AD, shown in Figure 5. Increased deposition of mainly Aβ42 is hypothesized to be a pathological triggering event, thought to be a result of increased Aβ production caused by a mutation in the *PSEN1/2* or *APP* genes or Down syndrome / trisomy 21, or reduced Aβ clearance, which is thought to be important for sporadic AD (56). Activation of glial cells and neuroinflammation, hyperphosphorylation of tau with neurofibrillary tangle formation, and synaptic dysfunction are thought to lie 'downstream' of increased amyloid accumulation in the brain, eventually leading to neurodegeneration and dementia (56).

Some potential limitations to the amyloid cascade hypothesis have been put forward, especially regarding sporadic AD (62). One aspect is that Aβ burden has been demonstrated to correlate poorly with disease progression and severity (63). Furthermore, it is unclear whether Aβ deposition is a cause or a consequence of AD neurodegeneration, and it has been suggested that the formation of amyloid plaques is a protective mechanism (64).

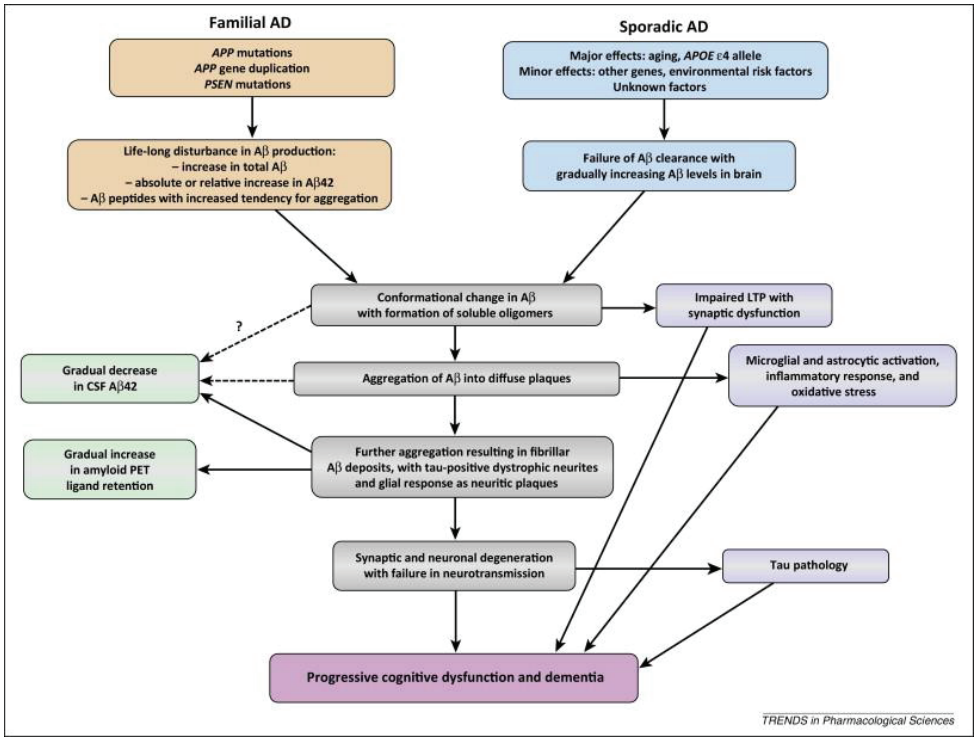

Figure 5. *The amyloid cascade hypothesis. In familial AD (upper left), there is a life-long overproduction of Aß peptides. In the more common sporadic AD (upper right), reduced amyloid clearance is thought to underlie increased Aß levels in the brain. Several pathological reactions, including the formation of tau pathology (neurofibrillary tangles), are thought to lie 'downstream' to Aß alterations in this hypothetical model. Figure from (65). LTP: long-term potentiation*

1.6.4 Tau proteins

In addition to amyloid plaques, neurofibrillary tangles are the other pathological core feature of AD, consisting of hyperphosphorylated tau protein (7, 66). Tau is a protein found in neurons where it stabilizes microtubules that are part of the transport system and cytoskeleton. The tau gene gives rise to six different isoforms in the brain of adult humans as a result of alternative mRNA splicing, and posttranslational modifications of tau proteins include phosphorylation and glycosylation (67). In AD brain, tau proteins are hyperphosphorylated at several sites (68), including threonines 181 and 231, which are often utilized for analytical purposes. Tau hyperphosphorylation makes microtubules more unstable (69) and the degree of tau phosphorylation is regulated by the relative activity of

specific kinases and phosphatases, as reviewed (70). Several experiments in both cell cultures and animal models of AD have shown that increased activity of glycogen synthase kinase 3β (GSK3β) results in hyperphosphorylation of tau and increased Aβ production. Hence, this enzyme is hypothesized to have a role in AD pathogenesis (71).

Histopathologically, tau pathology has been shown to spread in a stereotypical manner, starting in the transentorhinal region and then spreading to the hippocampus, amygdala, and neocortex (72), and forming the basis of the histopathological stages for brain AD pathology.

1.7 Biomarkers

When objectively measured, a biomarker can give an indication about normal or disease processes or be used to monitor the effect of medical treatment (73). The 1998 Consensus report stated that an ideal biomarker for AD should have at least 80 % sensitivity (i.e. the ability to identify patients with AD pathology) and 80 % specificity (i.e. the ability to differentiate those with AD pathology from healthy controls or patients with other forms of dementia) (74). The biomarker should also be reproducible, reflect the underlying pathology of the dementia, have a clearly defined cut-off and be non-invasive. To validate a potential biomarker, the results must be reproduced by other researchers and published (73). A diagnostic biomarker will, by identifying a certain molecule or structure, indicate the presence of a certain condition or disease, whereas a prognostic biomarker is used to predict the outcome or progression of a certain condition or disease (75).

1.8 Potential biomarkers for AD

In the following sections, a brief introduction is given for potential and more well-established AD biomarkers that were analyzed in this thesis, before CSF biomarkers for AD are discussed in more depth.

Early diagnosis of AD pathology through biomarkers of high sensitivity and specificity is already important, and will be crucial when effective treatment exists. The highest diagnostic accuracy is most likely achieved through a combination of different biomarker modalities, and the most widely used biomarkers for AD are currently biochemical (Aβ42 and tau levels in CSF), and topographical (structural magnetic resonance imaging (MRI), amyloid positron emission tomography (PET), and ^{18}F-fluorodeoxyglucose (FDG)-PET).

1.8.1 Neuroimaging biomarkers for AD

Imaging biomarkers for AD have similar sensitivity and specificity to CSF biomarkers (76), but may have more limited availability, PET methods in particular being expensive. In contrast to CSF biomarkers, imaging methods provide topographical information.

Cerebral magnetic resonance imaging

MRI methods are widely used as AD biomarkers today, and studies have suggested that diagnostic performance is similar to that of CSF biomarkers (76). Reduced volume of structures such as the hippocampus is thought to reflect loss of neurons (77). The volume of the entorhinal cortex has been demonstrated to be reduced already in individuals with subjective memory impairment compared to controls (78). Both reduced entorhinal and hippocampal volume by MRI have been demonstrated to predict conversion from MCI to AD, in combination with other measures such as age and cognitive tests (79).

When measured by MRI, whole brain atrophy rates tend to correlate well with the decline in cognitive test performance (80, 81). In one study, neurodegeneration both preceded and paralleled measurable cognitive decline, and clinical symptoms seemed to be linked to the degree of neurodegeneration rather than the amount of amyloid deposition (80).

Positron emission tomography

The presence of amyloid brain deposits in cognitively normal individuals, as visualized by PET imaging, has been associated with a significantly increased future risk of AD (82). The agreement between CSF Aβ42 levels and amyloid PET uptake has repeatedly been found to be very high, as reviewed (65). An inverse linear correlation between CSF Aβ42 levels and uptake of ^{11}C-PiB (83, 84) or ^{18}F-flutemetamol (85) has been found in the brain, and uptake of amyloid tracer correlated positively with post mortem amyloid plaque burden (86). However, CSF Aβ42 levels have been found to be abnormal early in AD, also before amyloid PET is positive (87). Compared to CSF Aβ42, amyloid PET has the advantage of indicating the anatomical location of amyloid deposition.

FDG-PET is an imaging method visualizing uptake of the glucose analog FDG into neurons, as an indirect measure of neuronal metabolism. Hypometabolism seen by FDG-PET has been found to be closely related to reduced function of synapses and neurons, as reviewed by e.g. (88). A bilateral temporo-parietal reduction in metabolism has been found for AD patients (89), as well as bilateral hypometabolism in the posterior cingulate, precuneus, temporo-parietal and frontal cortices in patients with aMCI and AD, compared to controls (90). Sensitivity and specificity were found to be high for separating patients with early AD from control individuals (91).

Diffusion tensor imaging

Diffusion tensor imaging (DTI) is an MRI method that is a promising biomarker for AD (92), as well as being a predictor of dementia in patients with SCD or MCI (93). The principle is based on the random diffusion of water molecules being hindered by physical barriers such as cell membranes or axons. Neurodegeneration has been found to cause microstructural white matter changes resulting in altered DTI measures in individuals with SCD and MCI (94), and in MCI and AD (95). It has been demonstrated that white matter deterioration is most pronounced in fiber tracts connected to areas with AD pathology, such as in the cingulum-angular bundle that connects with the hippocampus (95).

1.8.2 Biomarkers in CSF

Core biomarkers

CSF is in direct contact with the extracellular space and closely mirrors the biochemical processes in the brain, which makes analysis of proteins in CSF useful when searching for biomarkers for AD. The three core CSF AD biomarkers (i.e. Aβ42, total tau (t-tau) and phosphorylated tau (p-tau)) have been validated against brain pathology post mortem, yielding high diagnostic accuracy (96). Characteristic findings in CSF of AD patients are reduced Aβ42 levels and increased levels of t-tau and p-tau when compared to non-demented controls (97).

CSF Aβ42 is approximately 50% reduced in AD patients compared to controls (97, 98), with a corresponding sensitivity of 80% and a specificity of 82% (76). When the first symptoms of dementia appear there is already a substantial Aβ load in brain (99), and CSF Aβ42 levels are reduced and stable five to ten years before conversion to AD dementia (100), a reduction hypothesized to reflect sequestration of Aβ42 into amyloid plaques (83, 84, 101).

CSF t-tau has been found to be increased approximately 250 % in AD patients compared to controls (97, 98), with a sensitivity of 82% and a specificity of 90% (76). It is hypothesized that CSF t-tau is a marker of damage to cortical axons and neuronal cell death in general (102), as increased levels are also found as a result of stroke (103), brain trauma (104) and Creutzfeldt-Jakob disease (105).

CSF p-tau is in many studies found to be increased about 200% in AD patients (97), with a sensitivity of 80% and specificity of 83% (76). Increased CSF p-tau is thought to reflect the phosphorylation state of tau protein and the presence of neurofibrillary tangles (96, 106). CSF p-tau levels can be used to differentiate AD from non-AD dementias (107, 108).

Amyloid beta 1-43

In addition to CSF core biomarkers, levels of amyloid beta 1-43 (Aβ43) were analyzed in all three papers in this thesis. Aβ43 has been found to be the first amyloid peptide to deposit in a mouse model (109) and is theorized to provide a 'seed' for subsequent Aβ42 deposition (53). Despite its low level in brain tissue, Aβ43 may therefore play a role in early AD pathogenesis (110). CSF Aβ43 has been found to be decreased in MCI and AD patients

compared to healthy controls, as well as positively correlated to CSF Aβ42 (111), and to have similar diagnostic accuracy compared to Aβ42 for the separation of controls from AD patients (112).

1.8.3 Temporal alterations of core biomarkers

Longitudinal changes

Various models of the temporal relationship for abnormality of biomarkers have been suggested (113), exemplified in Figure 6. AD is currently considered a disease where the pathophysiological changes start long (decades) before clinical symptoms appear, both in sporadic AD (40) and in autosomal dominant AD (114), based on the analysis of CSF and imaging biomarkers from clinically healthy individuals, and post mortem histopathological examination. CSF Aβ42 levels are reduced early in the disease process and remain stable thereafter, whereas tau levels seem to increase gradually, closer to the conversion to AD dementia (100). When cognitive impairment (most often impaired episodic memory) is noticeable, there is extensive neuronal loss in vulnerable cortical areas (115). Studies have shown that CSF tau levels increase slightly with disease progression and can reflect disease stage (116, 117). This conclusion is in accordance with the results from another study, where the number of neurons and neurofibrillary tangles was found to correlate with patient cognitive status as opposed to the much weaker association between plaque burden and cognitive status (118). However, CSF biomarkers are primarily diagnostic, and other methods are considered more suitable for reflection of disease stage (119).

Predictive abilities of core CSF biomarkers

Patients with aMCI displaying a positive AD CSF biomarker profile have the highest risk of progressing to AD, and progression also occurs earlier (100, 119-122). P-tau 181 seems to predict cognitive decline in MCI patients better than t-tau (123), and high t-tau levels indicate a more rapid cognitive decline in patients already diagnosed with AD (124). A combination of CSF Aβ42 and p-tau has been shown to be the strongest predictor of future AD in healthy elderly controls (125). MCI patients with 'normal' biomarker levels do not seem to have an increased risk of developing AD (119), further indicating that biomarker analyses will be helpful in identifying which MCI patients run the greatest risk of suffering from AD in the future.

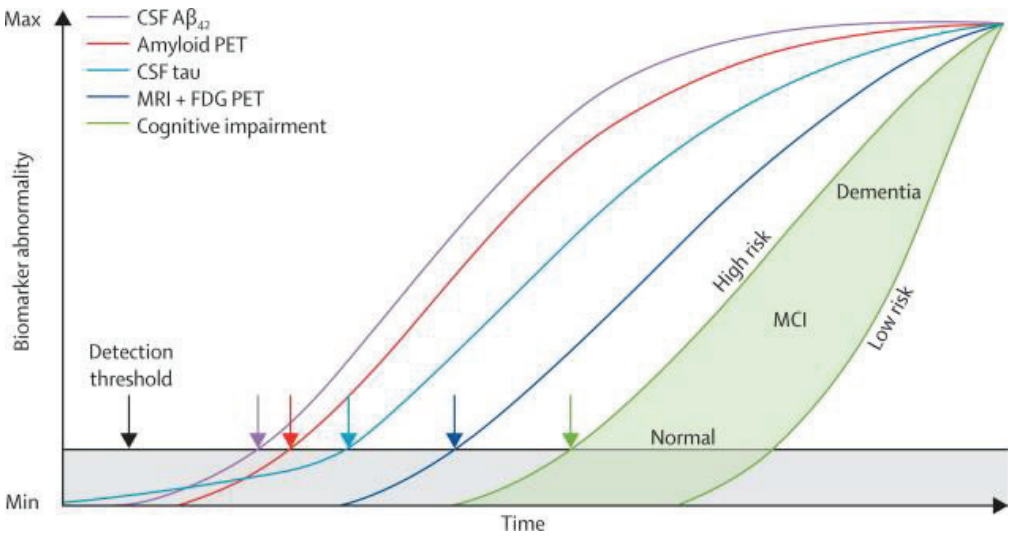

Figure 6. *AD biomarkers have been found to be abnormal before cognitive symptoms are measurable. These changes appear in a temporally ordered manner. The y-axis indicates the degree of abnormality. Time (x-axis) will vary from person to person. Those with high risk (genetic risk alleles, low cognitive reserve, comorbidity, unhealthy lifestyle) can be placed to the left in the green field. Those with reduced risk can be placed to the right in the green field. In this version of the model, tau is hypothesized to become abnormal first, although beneath the detection level. From (113).*

1.9 CSF analytes and age

AD is often separated according to age, whereby onset prior to age 65 years is considered to be early-onset AD (EOAD), while onset from an age of 65 years (which is much more common) is termed late-onset AD (LOAD). There is disagreement between studies from the two AD groups regarding the pathological burden of plaques and tangles (10-14), so it is uncertain whether putative differences would be reflected in CSF levels of analytes. No statistically significant differences were found between levels of the core biomarkers Aβ42, t-tau and p-tau in patients with EOAD compared to LOAD in two studies (126, 127). Although several investigations have shown no significant correlations between core biomarkers and age in AD patients (121, 126, 128), one study found CSF Aβ43 to be correlated positively with age (112).

Older controls have been found to have decreased CSF Aβ42 levels compared to younger controls (126), and CSF Aβ42 levels in controls were negatively correlated with age in another study (128). Conversely, CSF t-tau and p-tau correlated positively with age in healthy elderly individuals (129-132). In one study, it was concluded that increased age in cognitively-intact individuals was associated with more 'AD-like' CSF biomarker levels. Certainly the difference in biomarker levels between younger controls and younger patients with AD is more marked than the difference found between older patients and controls (133). Supporting these results, histopathological brain examination has revealed a converging amount of AD pathology between controls and demented patients in very advanced age (134).

Several other proteins have also been investigated as putative biomarkers for AD, both alone and in combination with the core biomarkers. Neurofilament light (NF-L) is expressed in large myelinated axons (135), and has been found to be increased in CSF from patients with AD compared to controls (97, 136). Another intermediate filament, glial fibrillary acidic protein (GFAP), is often used as a glial marker, and CSF levels of both these proteins have been shown to correlate with age in healthy individuals (137). CSF GFAP has been found to increase in AD in one study (138), but similar levels between controls and AD patients have also been found (139).

YKL-40 (also known as chitinase 3-like protein 1) is a protease secreted mainly from astrocytes and has been suggested to have several physiological functions, such as modulating the immune response, cell proliferation and tissue remodelling, as reviewed (140). It is also considered to be a marker for gliosis and neuroinflammation (141), and was therefore another of the analytes measured in paper II. CSF YKL-40 has been shown to increase throughout middle age in controls, suggesting that some degree of neuroinflammation occurs in normal aging (142). Although one study found a correlation between YKL-40 and age in controls (131), another did not (143). However, several papers have shown YKL-40 to be increased in AD patients compared to non-demented controls (139, 141, 143).

The function of progranulin in brain remains poorly understood, but based on results largely from animal models, it is expected to have anti-inflammatory and neuroprotective abilities (144). Again, although levels of progranulin in CSF have been found to increase significantly

in CSF with age (145), another study indicated no significant alteration in patients with AD compared to controls, and no correlation with age (146).

2. Aims of the thesis

- To assess usefulness for CSF Aβ43 as a biomarker for early AD or for the progression from aMCI to AD, in comparison with the diagnostic performance of well-established CSF Aβ42. To investigate longitudinal levels of CSF Aβ43 and CSF Aβ42 levels and ratios with these amyloid peptides and t-tau.

- To compare CSF levels of Aβ43 and Aβ42 in addition to several other analytes (t-tau, p-tau, NF-L, YKL-40, GFAP and progranulin) between younger and older groups of controls and patients, and assessment of the analytes' ability to separate controls from AD patients in both the younger and older groups.

- To assess whether CSF Aβ43 levels reflect amyloid deposition as visualized by ^{18}F-FLUT PET, and if CSF Aβ43 levels correlate with MRI or ^{18}F-FDG PET measures in individuals with SCD or MCI, compared to the established marker CSF Aβ42.

3. Materials and methods

3.1 Study participants

Papers I and II

In these papers, patients were recruited through the Department of Neurology, St. Olav's Hospital (University Hospital of Trondheim) after being referred to the clinic by general practitioners, and were diagnosed by a neurologist. Patients with early AD were diagnosed according to the NINCDS-ADRDA criteria (2), and patients with aMCI according to the International Working Group on Mild Cognitive Impairment criteria (33).

Samples of CSF were either taken from the SAMBA study (papers I and II) or also the Neurological Research Biobank at the University Hospital of Trondheim (paper II). SAMBA is a substudy of the larger Trønderbrain project, in which genetic and metabolic aspects of dementia are studied. The SAMBA study was started in 2009, and inclusion continued until 2014 with a total of 62 elderly controls, and 108 patients diagnosed either with aMCI or early AD at inclusion. Patients were followed over a two-year period, during which time some patients diagnosed with aMCI progressed to AD. In paper I, this subgroup was designated "pMCI". The remaining patients with aMCI did not progress to AD in the same period and were designated "sMCI". In paper II, all patients included had AD. Samples from the Research Biobank had been taken routinely at clinical work-up, but were surplus and patients had given written informed consent to their use in research. The biobank has been licensed by the Norwegian Directorate for Health Affairs.

In paper I, elderly volunteers were recruited as controls from societies for retired people in central Norway, or were caregivers not genetically related to the patient. These control individuals were also examined by a neurologist and were healthy for their age without signs of a neurological disorder. In paper II some of the control samples were obtained from the Neurological Research Biobank. These latter individuals had been referred to the clinic for suspected neurological conditions, but none was subsequently found. For all controls, CSF cell count, glucose and protein were within standard physiological limits. For paper II, both controls and AD patients were divided into younger (≤ 62 years) and older (≥ 68 years) groups to emphasize potential age-group differences in analyte levels.

The neurological examination performed on SAMBA study participants included a comprehensive assessment of cognitive function, which included the Mini Mental State Examination (MMSE) (147), as well as cerebral MRI (section 3.3). Diagnostic assessment and biomarker analysis were independent of each other.

Paper III

In paper III, study participants were diagnosed with either SCD or MCI, and all came from two different cohorts at Akershus University Hospital. Individuals in Cohort 1 were predominantly diagnosed with SCD and underwent amyloid PET in addition to MRI imaging, whereas those in Cohort 2 were predominantly diagnosed with MCI and underwent MRI imaging, including diffusion tensor imaging (DTI).

In Cohort 1, forty individuals were referred by their general practitioner to the hospital's memory clinic or recruited through newspaper advertisements. All subjects were assessed with ^{18}F-FLUT PET either at the time of inclusion or at a second assessment two years after first inclusion in the project. Clinical assessment, lumbar puncture, and MRI were done within 3.5 months of ^{18}F-FLUT PET. Thirty-one of the 40 subjects also underwent ^{18}F-FDG PET. MCI was defined based on the core criteria in the recommendation from the National Institute on Aging-Alzheimer's Association (NIA-AA) (29). Documented impairment greater than expected for the person's age, gender, and educational level in one or more cognitive domains was found for these patients on several cognitive tests, as reported in paper III. SCD was defined according to the recommendations by the Subjective Cognitive Decline Initiative Working Group (36).

In Cohort 2, 50 individuals were referred to the memory clinic of Akershus University Hospital by their general practitioner. All subjects underwent lumbar puncture and MRI at inclusion. The subjects were assessed with clinical interview, routine physical examination, blood screening, and a battery of cognitive tests. Subjects in Cohort 2 were defined as having MCI if objective cognitive impairment was evident on at least one of the cognitive screening tests specified in paper III. Subjects without objective cognitive impairment on the same cognitive tests were classified as having SCD.

3.2 Body fluid analyses

Analysis of Aβ43 in CSF

The common denominator for all three articles in this thesis was the analysis of CSF Aβ43, performed at the Neurobiological Laboratory, Department of Neuromedicine and Movement Science, Norwegian University of Science and Technology, and the Department of Neurology, Trondheim University Hospital. For paper III, all biochemical and medical imaging analyses except the analysis of CSF Aβ43 were performed elsewhere, as reported.

For details of CSF sampling and storage, see the respective papers. All CSF samples were thawed in ice-water prior to analysis, and analyzed in duplicate. Commercially-available ELISA monoplex kits for Aβ43 (IBL) were run according to the manufacturers' instructions. Cross-reactivity for Aβ42 in the Aβ43 ELISA was given as <1%. Although this would contribute slightly to measurements for Aβ43, it would apply for both control and patient groups. Aβ43 was reported to have 50x less affinity than Aβ42 for the antibodies in the Aβ42 kit. The measurement range for the Aβ43 assay was reported to be 2.34-150 pg/ml, and all analyzed samples fell within this range. For the Aβ43 assay in paper I, an internal control was run on 5 of 6 plates, yielding an inter-assay CV of 14.4%, and an intra-assay CV of 5.1%.

Analysis of other CSF analytes

For papers I and II, CSF samples were analysed using ELISA monoplex kits according to the manufacturers' instructions (Aβ43 (IBL, cat. no. 27710), Aβ42 (Innogenetics, cat. no. 80324), t-tau (Innogenetics, cat. no. 80323), p-tau (Innogenetics, cat. no. 80317), NF-L (UmanDiagnostics, cat. no. 10-7001), YKL-40 (Bio-Techne, cat. no. DC3L10, CSF diluted 1:400), GFAP (BioVendor, cat. no. RD192072200R) and progranulin (Adipogen Life Sciences, cat. no. AG-45A-0018YEK-KI01, CSF diluted 1:15)).

APOE genotyping

In papers I and II, DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, cat.no. 51106), together with the spin protocol provided. Random samples of isolated DNA were checked for purity using NanoDrop technology. *APOE* genotyping was performed using the Fast Start DNA Master HybProbe Kit (Roche) in combination with the LightMix ApoE C112R R158C kit (TiB MolBiol) according to the manufacturer's instructions, followed by *APOE* genotyping with LightCycler technology (Roche).

3.3 Medical imaging

For papers I and II, cerebral MRI at 3T according to the Alzheimer's Disease Neuroimaging Initiative (ADNI) protocol (http://www.adni-info.org/Home.html) was carried out. For paper III, MRI and PET imaging methods were performed at Akershus University Hospital and Aleris in Oslo. See paper III for further details.

3.4 Statistical analyses

See the respective papers for further details.

3.5 Ethics

All research conformed to the Helsinki Declaration, and projects were approved by the Regional Committees for Research Ethics (approval 2010/226 REK Midt, 2013/467 REK Midt, 2013/150 REK Sør-Øst, 2009/2550 REK Sør-Øst).

4. Review of results

Paper I

Cerebrospinal fluid levels of amyloid beta 1-43 in patients with amnestic mild cognitive impairment or early Alzheimer's disease: a 2-year follow-up study

Camilla Lauridsen, Sigrid Botne Sando, Adiba Shabnam, Ina Møller, Guro Berge, Gøril Rolfseng Grøntvedt, Inger Johanne Bakken, Øyvind Salvesen, Geir Bråthen, Linda Rosemary White

Introduction: Biomarkers that will reliably predict the onset of Alzheimer's disease (AD) are urgently needed. Although cerebrospinal fluid (CSF) amyloid beta 1-42 (Aβ42), total tau and phosphorylated tau can be used to complement the clinical diagnosis of AD, amnestic mild cognitive impairment (aMCI), the prodromal phase of AD, is heterogeneous. Biomarkers should be able to determine which patients with aMCI are at greatest risk of AD. Histological studies and animal models indicate that amyloid beta 1-43 (Aβ43) aggregates early, and may play a role in the pathological process of AD. We have examined levels of CSF Aβ43 in a two-year longitudinal study of aMCI and early AD.

Materials and methods: CSF was collected at baseline, and after one and two years from patients with AD (n=19), and patients with aMCI (n=42). Of these, 21 progressed to AD during the two years of study, whereas 21 did not. Controls (n=32) were lumbar punctured at baseline only. CSF analyses of Aβ43, Aβ42 and total tau were carried out with ELISA.

Results: At baseline, CSF Aβ43, CSF Aβ42 and ratios with total tau could be used to separate controls from all three patient groups. CSF Aβ43, but not Aβ42, could separate patients with aMCI who progressed to AD during the two years of follow-up, from those that did not. The CSF total tau/Aβ43 ratio had a slightly but significantly larger area under the receiver operating characteristic curve when compared to the CSF total tau/Aβ42 ratio. CSF Aβ43 levels, but not Aβ42 levels, decreased from baseline to two years in the AD group.

Discussion and conclusion: CSF Aβ43 was demonstrated to be significantly reduced in patients already by the time that aMCI or AD was diagnosed, compared to controls, and this change must have occurred during the preclinical period. Since our results suggested that CSF Aβ43 distinguishes between subgroups of patients with aMCI better than CSF Aβ42, it

may prove to be a useful additional biomarker for identifying aMCI patients at greatest risk of AD.

Paper II

Cerebrospinal fluid Aβ43 is reduced in early-onset compared to late-onset Alzheimer's disease, but has similar diagnostic accuracy to Aβ42

Camilla Lauridsen, Sigrid Botne Sando, Ina Møller, Guro Berge, Precious Kwadzo Pomary, Gøril Rolfseng Grøntvedt, Øyvind Salvesen, Geir Bråthen, Linda Rosemary White

Introduction: Amyloid beta 1-43 (Aβ43) may be a useful additional biomarker for diagnosing Alzheimer's disease (AD). We have investigated cerebrospinal fluid (CSF) levels of Aβ43 in patients with early-onset AD in contrast to levels in late-onset AD. For comparison, in addition to the 'core' biomarkers, several other analytes were also determined (YKL-40, neurofilament light, glial fibrillary acidic protein, progranulin).

Materials and Methods: CSF samples were obtained from patients with early-onset AD (age≤62, n=66), late-onset AD (age≥68, n=25), and groups of cognitively intact individuals (age≤62, n=41, age≥68, n=39). Core CSF AD biomarkers (amyloid beta 1-42 (Aβ42), total tau, phosphorylated tau) were analyzed, as well as levels of Aβ43 and other analytes, using commercially-available enzyme-linked immunosorbent assays.

Results: CSF Aβ43 was significantly reduced in early-onset AD compared to late-onset AD, whereas the levels of Aβ42 in the two AD groups were not significantly different. Aβ43 and all core biomarkers were significantly altered in patients with AD compared to corresponding controls. Relationships between the Aβ peptides and tau proteins, YKL-40, neurofilament light, glial fibrillary acidic protein and progranulin were also investigated without finding marked associations. Aβ43 did not improve diagnostic accuracy in either AD group compared to Aβ42.

Discussion: CSF Aβ43, but not Aβ42 levels, seem to vary significantly with age in patients with AD. If CSF levels of Aβ peptides reflect amyloid deposition in brain, the possibility arises that there is a difference between Aβ43 and Aβ42 deposition in younger compared to older brain. However, the level of Aβ43 in CSF shows no improvement over Aβ42 regarding diagnostic accuracy.

Paper III

Cerebrospinal Fluid Levels of Amyloid Beta 1-43 Mirror 1-42 in Relation to Imaging Biomarkers of Alzheimer's Disease

Ina S. Almdahl, Camilla Lauridsen, Per Selnes, Lisa F. Kalheim, Cristopher Coello, Beata Gajdzik, Ina Møller, Marianne Wettergreen, Ramune Grambaite, Atle Bjørnerud, Geir Bråthen, Sigrid B. Sando, Linda R. White, Tormod Fladby

Introduction: Amyloid beta 1-43 (Aβ43), with its additional C-terminal threonine residue, is hypothesized to play a role in early Alzheimer's disease pathology possibly different from that of amyloid beta 1-42 (Aβ42). Cerebrospinal fluid (CSF) Aβ43 has been suggested as a potential novel biomarker for predicting conversion from mild cognitive impairment (MCI) to dementia in Alzheimer's disease. However, the relationship between CSF Aβ43 and established imaging biomarkers of Alzheimer's disease has never been assessed.

Materials and Methods: In this observational study, CSF Aβ43 was measured with ELISA in 89 subjects; 34 with subjective cognitive decline (SCD), 51 with MCI, and four with resolution of previous cognitive complaints. All subjects underwent structural MRI; 40 subjects on a 3T and 50 on a 1.5T scanner. Forty subjects, including 24 with SCD and 12 with MCI, underwent ^{18}F-Flutemetamol PET. Seventy-eight subjects were assessed with ^{18}F-fluorodeoxyglucose PET (21 SCD/7 MCI and 11 SCD/39 MCI on two different scanners). Ten subjects with SCD and 39 with MCI also underwent diffusion tensor imaging.

Results: Cerebrospinal fluid Aβ43 was both alone and together with p-tau a significant predictor of the distinction between SCD and MCI. There was a marked difference in CSF Aβ43 between subjects with ^{18}F-Flutemetamol PET scans visually interpreted as negative (37 pg/ml, n = 27) and positive (15 pg/ml, n = 9), $p < 0.001$. Both CSF Aβ43 and Aβ42 were negatively correlated with standardized uptake value ratios for all analyzed regions; CSF Aβ43 average *rho* -0.73, Aβ42 -0.74. Both CSF Aβ peptides correlated significantly with hippocampal volume, inferior parietal and frontal cortical thickness and axial diffusivity in the corticospinal tract. There was a trend toward CSF Aβ42 being better correlated with cortical glucose metabolism. None of the studied correlations between CSF Aβ43/42 and imaging biomarkers were significantly different for the two Aβ peptides when controlling for multiple testing.

Conclusion: Cerebrospinal fluid Aβ43 appears to be strongly correlated with cerebral amyloid deposits in the same way as Aβ42, even in non-demented patients with only subjective cognitive complaints. Regarding imaging biomarkers, there is no evidence from the present study that CSF Aβ43 performs better than the classical CSF biomarker Aβ42 for distinguishing SCD and MCI.

5. Discussion

The search for precise diagnostic biomarkers for the earliest, including the pre-symptomatic, phase of AD is ongoing. It is crucial that when effective medical intervention becomes available, it should be given to those individuals on a path towards AD as early as possible before neurodegeneration has become widespread. If we can identify the earliest changes with the help of biomarkers, medication could be given well before the dementia phase. The core biomarkers in CSF have already been shown to be good predictors of which patients with MCI will develop AD (100, 119-122, 125). Research on changes in biomarker levels in patients with MCI or early AD may also increase our understanding of the pathophysiological mechanisms resulting in AD. This is an underlying intention of this work.

Since Aβ43 is thought to be more aggregation-prone than Aβ42 (50, 53, 54), as well as to be the first Aβ peptide to deposit in a mouse model (109), perhaps thereby providing a 'seed' for subsequent Aβ42 deposition (53), it seemed reasonable to further investigate CSF levels of Aβ43 as well as Aβ42 in the present work. In this thesis, the potential for CSF Aβ43 as a biomarker for early AD was explored from different angles: for separation of patients with aMCI that progressed to AD within two years from those who did not, for levels measured in early- compared to late-onset AD, and for the association with brain imaging biomarkers. All results were compared to CSF Aβ42. Other possible biomarkers were also measured for comparison.

The suggested new diagnostic criteria for AD postulate that it can be diagnosed early with support from biomarkers when the patient experiences an early and significant memory impairment, such that aMCI and at least one abnormal AD biomarker is sufficient for AD to be diagnosed (29, 30). In the IWG-2 criteria, the core AD biomarkers in CSF and amyloid PET are regarded as most relevant for an early diagnosis of AD (30), in line with the temporal changes in biomarkers for AD postulated in the model from Jack and colleagues (113). The studies in the present work were therefore conducted on CSF samples from individuals with no cognitive decline, SCD, MCI or early AD.

Despite the modest study size in paper I, CSF Aβ43 with or without t-tau in the equation separated individuals with non-progressing aMCI from those with aMCI that progressed to AD during the two-year study period. This separation was not found for CSF Aβ42 alone.

Patients in the aMCI group that did not progress to AD during the two-year study period may have had heterogeneous underlying causes for the aMCI diagnosis; some are known to have progressed to AD after the study ended, and some will likely never convert to any neurodegenerative disease (34, 35). However, two years is a relatively short follow-up time when it comes to a slowly progressing disease like AD, and a longer follow-up period could have provided additional data regarding conversion to AD.

Even though both Aβ42 and Aβ43 may be important in the early stages of disease and involved in plaque formation (50, 148), it is possible that these two markers reflect slightly different aspects of early AD pathology, and that CSF amyloid marker levels change at different time-points during the disease course. Whereas CSF Aβ42 levels are reduced and stable five to ten years before AD dementia (100), CSF Aβ43 continued to fall during later aMCI (difference between "sMCI" and "pMCI" in paper I). On the other hand, the CSF t-tau/Aβ42-ratio had higher sensitivity than the t-tau/Aβ43-ratio for separating the two aMCI groups. When effective treatment becomes available, a test with high sensitivity (i.e. the ability to identify patients with AD pathology) will be important for starting medical intervention as soon as possible, so the establishment of reliable biomarkers for routine diagnostic support is important.

In paper II, CSF levels of a number of markers in younger compared to older individuals were studied. Several analytes, including Aβ43 and the 'core' biomarkers, were analyzed and compared in younger (≤ 62 years) and older (≥ 68 years) groups of patients with AD, and with healthy elderly controls of corresponding age. No difference in diagnostic accuracy between Aβ43 and Aβ42 for the separation of controls and AD patients was found in either age group. However, decreased levels of CSF Aβ43 and YKL-40 in patients with early-onset compared to late-onset AD were found. For YKL-40, this increase is most likely a result of an increased concentration in CSF during ageing, since we found a similar increase for older compared to younger controls. For Aβ43, the reason for the decrease in patients with early-onset AD is less obvious as levels were not significantly different between the two control groups. It may be speculated whether this result, apart from the obvious age difference,

could be caused by regional differences in amyloid deposition in brain in early- compared to late-onset AD, as found previously in amyloid imaging studies (12, 13). That such anatomical differences should affect amyloid peptide concentrations in CSF seems unlikely. If there nevertheless was a difference, it seemed to affect only CSF Aβ43, as a similar result was not observed for CSF Aβ42. If the theory regarding lower levels of amyloid peptides in CSF being the result of increased deposition in brain is correct (84), this would suggest that more Aβ43 is deposited in the younger AD brain than in older patients. Subsequently, since the information was not given in paper III, a personal communication from Ina Selseth Almdahl confirmed that no significant correlation between age and amyloid deposition had been found in Cohort 1 in that paper ($r=0.27$, $p=0.09$, $n=39$, not published). However, the individuals in paper III had SCD or MCI rather than AD dementia so a direct comparison is not possible.

Paper I also demonstrated that Aβ43 seems to be more dynamic than Aβ42 during the late stages of aMCI/early stage of AD, as Aβ43 levels are decreased longitudinally in patients with AD, as well as being reduced in patients with aMCI that are close to converting to AD dementia, compared to those in the "sMCI" group, as mentioned above.

In papers I and II, where cognitively normal individuals were used as controls, there is always the risk that some of them will have pathological biomarker levels (as was in fact the case) and be at risk of eventually developing AD given enough time. This is a risk in most AD-biomarker studies and can potentially reduce diagnostic accuracy. In general, there will always be some uncertainty present when it comes to diagnosing dementia in its earliest phases. For the participants in papers I and II, the MCI and AD diagnoses were made by a neurologist experienced in dementia, supporting diagnostic consistency both within and between groups.

In paper III, CSF Aβ43 levels were found to be inversely associated with cortical amyloid burden in a similar way to Aβ42. This suggested the possibility for increased deposition of amyloid in brain parenchyma when CSF levels of Aβ peptides are reduced, as found earlier for Aβ42 (84). Nothing in the results supported the hypothesis that the amyloidogenic impact in participants with SCD or MCI was significantly different when comparing CSF levels of the two peptides. Moreover, Aβ43 did not seem to have any diagnostic advantage compared to Aβ42 in separating the two groups.

Participant groups in paper III are likely to be heterogeneous as individuals were in the earliest stages of subjective or objective cognitive impairment. The annual progression rate from aMCI to AD has been reported to be 18.2% (34), whereas the annual progression from SCD to MCI has been found to be only 6.7% (37). Such figures suggest that many of those included will not develop dementia. Although several studies have shown that persons with SCD are at a slightly increased risk for progression to AD compared to non-complainers (36), others have suggested that the underlying causes of SCD are too heterogeneous for the condition to be viewed as preclinical AD (38). Nevertheless, it is probably during the very early stages of cognitive impairment, and in cognitively intact individuals with a pathological biomarker profile, that future medical intervention should start in order to have the best chance of stopping or slowing neurodegeneration. Due to the lack of a control group in paper III, no assessment could be made for the ability of the biomarkers to separate controls from participants with SCD or MCI.

The lack of procedural standardization for CSF analyses using ELISA methods in the laboratory has thus far prohibited the establishment of uniform cut-off values for potential CSF biomarkers (149). ELISA analyses of CSF biomarkers in the present three studies were performed with what is largely a manual procedure, and results may to some degree depend on the laboratory techniques and training of the laboratory personnel, with room for intra- and inter-laboratory variations. Within our own studies there was usually little intra- and inter-assay variation. It was clearly an advantage that ELISAs were carried out at a single laboratory, even though several personnel participated. However, such potential variations can also occur in imaging studies where results are obtained through software analysis. Sometimes this is performed automatically by the software, but some procedures need to be performed by the operator, introducing the possibility of differences in style. One general limitation to CSF biomarkers compared to imaging methods is the lack of anatomical precision. Nevertheless, CSF samples are relatively easy to obtain, and analyzing amyloid peptide levels in CSF is much cheaper than amyloid PET imaging. It has been shown that CSF Aβ42 levels start to drop before increased amyloid deposition can be seen with amyloid PET imaging (87), as illustrated in Figure 6 in the Introduction, and the analysis of CSF Aβ42 can therefore be considered useful for the early diagnosis of AD.

Overall, it seems unlikely from our results that Aβ43 is better than Aβ42 for the separation of controls and patients with AD (including those that convert from aMCI to AD). While the question remains as to whether Aβ43 is better than Aβ42 for identifying potential convertors from aMCI to AD, the analysis of CSF Aβ43 in addition to Aβ42 should be more comprehensively studied in such patients. Similarly, larger cohorts of cognitively normal individuals and those with SCD with pathological biomarkers should be investigated. The reduced level of CSF Aβ43 in patients with early-onset compared to late-onset AD that was not found for CSF Aβ42 also needs confirmation. If confirmed, an investigation of the clinical or diagnostic implications will be important.

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Paper I

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Cerebrospinal Fluid Levels of Amyloid Beta 1–43 in Patients with Amnestic Mild Cognitive Impairment or Early Alzheimer's Disease: A 2-Year Follow-Up Study

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Introduction: Biomarkers that will reliably predict the onset of Alzheimer's disease (AD) are urgently needed. Although cerebrospinal fluid (CSF) amyloid beta 1–42 (Aβ42), total tau, and phosphorylated tau can be used to complement the clinical diagnosis of AD, amnestic mild cognitive impairment (aMCI), the prodromal phase of AD, is heterogeneous. Biomarkers should be able to determine which patients with aMCI are at greatest risk of AD. Histological studies and animal models indicate that amyloid beta 1–43 (Aβ43) aggregates early, and may play a role in the pathological process of AD. We have examined levels of CSF Aβ43 in a 2-year longitudinal study of aMCI and early AD.

Materials and Methods: Cerebrospinal fluid was collected at baseline, and after one and 2 years from patients with AD ($n = 19$), and patients with aMCI ($n = 42$). Of these, 21 progressed to AD during the 2 years of study, whereas 21 did not. Controls ($n = 32$) were lumbar punctured at baseline only. CSF analyses of Aβ43, Aβ42, and total tau were carried out with ELISA.

Results: At baseline, CSF Aβ43, CSF Aβ42 and ratios with total tau could be used to separate controls from all three patient groups. CSF Aβ43, but not Aβ42, could separate patients with aMCI who progressed to AD during the 2 years of follow-up, from those that did not. The CSF total tau/Aβ43 ratio had a slightly but significantly larger area under the receiver operating characteristic curve when compared to the CSF total tau/Aβ42 ratio. CSF Aβ43 levels, but not Aβ42 levels, decreased from baseline to 2 years in the AD group.

Discussion and Conclusion: CSF Aβ43 was demonstrated to be significantly reduced in patients already by the time that aMCI or AD was diagnosed, compared to controls, and this change must have occurred during the preclinical period. Since our results suggested that CSF Aβ43 distinguishes between subgroups of patients with aMCI better than CSF Aβ42, it may prove to be a useful additional biomarker for identifying aMCI patients at greatest risk of AD.

Keywords: Alzheimer's disease, amnestic mild cognitive impairment, biomarkers, amyloid beta 1–43, amyloid beta 1–42, cerebrospinal fluid, diagnostic accuracy

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INTRODUCTION

According to the proposed new diagnostic criteria for Alzheimer's disease (AD), early AD can be diagnosed if the prodromal phase (amnestic mild cognitive impairment, aMCI) is present, and at least one biomarker is positive, though these biomarkers have yet to be validated (Albert et al., 2011; McKhann et al., 2011; Dubois et al., 2014). Biomarkers with high sensitivity and specificity that reflect the underlying pathology of AD will therefore be important in identifying patients with aMCI who are most likely to progress to AD. Such identification will be crucial when efficient treatment becomes available.

Levels of amyloid beta 1–42 (Aβ42) in cerebrospinal fluid (CSF) correlate inversely with brain parenchymal Aβ42 load, as visualized by positron emission tomography (PET) (Fagan et al., 2006; Tolboom et al., 2009). Additionally, CSF Aβ42 levels have repeatedly been found to be reduced in patients with MCI and AD, compared to healthy controls (Sunderland et al., 2003; Hansson et al., 2006). CSF Aβ42 levels are usually reduced years before clinical symptoms of dementia appear (Buchhave et al., 2012), and CSF Aβ42 is therefore considered a candidate biomarker for early AD, alone or in combination with other CSF biomarkers such as total tau (t-tau) representing axonal degeneration, or phosphorylated tau representing tau hyperphosphorylation (Hansson et al., 2006; Tapiola et al., 2009). The combination of CSF Aβ42 and t-tau has been found to predict incipient AD with a sensitivity $\geq$88% and specificity $\geq$82% (Hansson et al., 2006; Hertze et al., 2010), while CSF Aβ42 alone predicted AD in patients with MCI with a sensitivity of 79% and specificity of 65% (Mattsson et al., 2009).

Compared to Aβ42, amyloid beta 1–43 (Aβ43) has an additional threonine at the C-terminal, and is thought to be more aggregation-prone than Aβ42 (Jarrett et al., 1993; Saito et al., 2011; Conicella and Fawzi, 2014). Aβ43 was found to be the first amyloid beta peptide to deposit in mutant amyloid precursor protein transgenic mice (Zou et al., 2013), and Aβ43 was theorized to provide a 'seed' for subsequent Aβ42 deposition (Conicella and Fawzi, 2014), suggesting that both peptides are likely to be involved in early plaque formation (Jarrett et al., 1993; Parvathy et al., 2001). Aβ43 may therefore play a role in AD pathogenesis despite its low level in brain tissue (Sandebring et al., 2013), as it has been shown to have equal or even greater neurotoxicity than Aβ42 in PS1-R278I knock-in mice (Saito et al., 2011). In a study from human brain tissue, Aβ43 was frequently found in plaques, and no amyloid peptides longer than Aβ43 were found (Welander et al., 2009).

To date there are few studies assessing CSF Aβ43 for its potential as a biomarker in early AD. It has been demonstrated that CSF Aβ43 was decreased in MCI and AD patients, as well as being positively correlated to CSF Aβ42 (Kakuda et al., 2012), despite Aβ43 and Aβ42 being produced by different enzymic routes (Qi-Takahara et al., 2005). CSF Aβ43 was found to be slightly inferior to Aβ42 for separating controls from AD patients (Bruggink et al., 2013).

In the present study, we examined CSF Aβ43 levels at baseline, and longitudinal levels over a 2-year period, in samples from patients with aMCI that progressed to AD during this period, as opposed to those that did not. Levels in patients with AD from baseline were also studied, and results for healthy control individuals were available at baseline. Comparisons were made with CSF levels of Aβ42, including correlations, and ratios with CSF t-tau.

MATERIALS AND METHODS

Subjects

Patients were recruited through the Department of Neurology, St. Olav's Hospital (University Hospital of Trondheim) and assigned to this study from 2009 onward. Patients were referred to the clinic by general practitioners, and were diagnosed by a neurologist (SBS). Patients with early AD ($n = 19$) were diagnosed according to the NINCDS–ADRDA criteria (McKhann et al., 1984), and patients with aMCI ($n = 42$) according to the International Working Group on Mild Cognitive Impairment Criteria (Winblad et al., 2004). Patients were followed over a 2-year period. During this time, 21 patients diagnosed with aMCI progressed to AD. This subgroup is subsequently designated "pMCI" in the results, for convenience. The remaining 21 patients with aMCI did not progress to AD, and are similarly designated "sMCI". All patients were ethnic Norwegians, with sufficient sight and hearing to complete the cognitive tests. Exclusion criteria were a present psychiatric or malignant disease, use of anti-coagulating medication, or high alcohol consumption.

Thirty-two elderly volunteers were recruited as controls from societies for retired people in central Norway, or were caregivers not genetically related to the patient. These control individuals were also examined by SBS and were healthy for their age without signs of a neurological disorder. Four had first-degree relatives with dementia. Controls were examined at baseline and after 2 years, but CSF samples were obtained only at baseline.

The neurological examination performed on both patients and controls included the Mini Mental State Examination (MMSE) (Folstein et al., 1975), as well as cerebral MRI at 3T, at baseline, and after 2 years. Apolipoprotein E (*APOE*) genotyping was performed on blood samples from all study participants as described elsewhere (Berge et al., 2014). Diagnostic assessment and biomarker analysis were independent of each other. The demographic data are shown in **Table 1**.

Sampling of CSF

Cerebrospinal fluid was usually collected early in the morning with patients lying on their side, and the puncture was at the L4/L5 or L5/S1 level. The first 2.5 mL CSF was used for routine clinical investigation. Aliquots of CSF (1 mL) were then collected directly into 2 mL polypropylene cryovials (Corning) immersed in ice-water. Samples were not centrifuged unless contaminated by blood (four samples), and were frozen within 30 min of lumbar puncture, and stored at −80°C until analysis.

ELISA Assays

Cerebrospinal fluid was analyzed using ELISA monoplex kits [Aβ43 (IBL, cat. no. 27710), Aβ42 (Innogenetics, cat. no. 80324),

TABLE 1 | Demographic data.

			aMCI at baseline		
		Controls	sMCI	pMCI	AD at baseline
Individuals included (n)		32	21	21	19
Gender (% females)		75.0	52.4	57.1	47.4
Age at inclusion (years)		67.3 ± 3.7	65.5 ± 6.4	63.8 ± 4.3	65.6 ± 6.1
Age at onset (years)		N/A	63.0 ± 7.1	61.1 ± 4.2	62.4 ± 6.2
Duration of symptoms (years)		N/A	2.5 ± 1.5	2.8 ± 1.0	3.2 ± 1.9
% *APOE* ε4 carriers		31.0	47.6	76.2[#]	84.2[##]
Education (years)		13.7 ± 3.2	12.9 ± 3.6	13.2 ± 3.7	12.1 ± 3.8
MMSE score	at baseline	29.5 ± 0.7*	28.0 ± 1.4*	26.6 ± 1.9*	22.9 ± 2.9*
	after 1 year	N/A	28.1 ± 1.3	24.5 ± 2.5**	19.4 ± 4.2**, $n = 18$
	after 2 years	N/A	28.1 ± 1.3	22.5 ± 2.8**, $n = 20$	16.9 ± 5.3**, $n = 14$

Results for continuous variables are given as the mean ± SD. [#]Significantly different to the control group ($p = 0.002$). [##]Significantly different to the control group ($p < 0.001$) and the sMCI group ($p = 0.015$). Significant difference between all groups at baseline (all pairwise comparisons: $p \leq 0.010$).** Significantly reduced levels compared to baseline ($p \leq 0.001$). AD: Alzheimer's disease, aMCI: amnestic mild cognitive impairment, sMCI: patients with mild cognitive impairment that did not progress to AD over 2 years, pMCI: patients with mild cognitive impairment that progressed to AD over 2 years, APOE ε4, apolipoprotein E gene ε4 allele; MMSE, Mini Mental State Examination; N/A, not applicable.*

and total tau protein (Innogenetics, cat. no. 80323)]. Cross-reactivity for Aβ42 in the Aβ43 ELISA was given as <1%. Although this would contribute slightly to measurements for Aβ43, it would be a constant for both control and patient groups. Aβ43 was reported to have 50x less affinity than Aβ42 for the antibodies in the Aβ42 kit.

Samples were thawed in ice-water prior to analysis, and kits were run according to the manufacturers' instructions. No samples underwent more than one freeze-thaw cycle, and all were analyzed undiluted, in duplicate. Both control and patient samples were included on each plate, and samples from patients in the three groups were evenly distributed across the plates. For the Aβ43 assay, an internal control was run on 5 of 6 plates, yielding an inter-assay CV of 14.4%, and an intra-assay CV of 5.1%.

Statistical Analysis

Statistical analyses were carried out using SPSS version 22 (IBM), Stata version 13.1., and R version 2.13.1. For all analyses, *p*-values <0.05 were considered statistically significant.

Demographic data and MMSE scores at baseline were assessed using Pearson's chi-square test for dichotomous variables, or one-way ANOVA for continuous variables, followed by the least-significant difference (LSD) *post hoc* test for pairwise comparisons if significant *p*-values were obtained. Longitudinal MMSE-scores were analyzed in a mixed linear model. Biomarker concentrations at baseline, after 1 year and after 2 years, were log-transformed to the natural logarithm (ln) to approximate to a normal distribution before comparison of groups by one-way ANOVA, followed by the LSD *post hoc* test for pairwise group comparisons. Pearson's correlation coefficient (*r*) for correlations between biomarkers was also calculated from log-transformed values.

Receiver operating characteristic (ROC) curves were made to assess the diagnostic accuracy for individual biomarkers, or combinations of them, and the area under each ROC curve (AUC) was calculated.

Youden's index [(sensitivity + specificity)-1] was determined to find exploratory cut-offs (not shown) where the sum of sensitivity and specificity was maximized, for biomarkers or ratios of biomarkers. Pairwise comparisons of AUC between Aβ43 and Aβ42, or ratios including these two biomarkers and total tau protein, were made for pairs of diagnostic groups (DeLong method). Longitudinal data have been analyzed using a mixed linear model. To account for repeated measurements, de-identified patient ID was included as a random effect. The combination of group and time was included as a fixed effect. All biomarker concentrations were log-transformed to the natural logarithm (ln) to approximate to a normal distribution before analysis in the mixed linear model.

Possible confounding factors included age, gender, and *APOE* genotype. Age and gender were found not to be confounding factors in the present study, and correction for *APOE* genotype would have resulted in many small groups, so was not done. There are results indicating that CSF Aβ42 levels reflect amyloid deposition in the brain independent of *APOE* ε4 status (Lautner et al., 2014), suggesting no need for different cut-offs for CSF Aβ42 based on *APOE* ε4 status (Molinuevo et al., 2014).

Ethics

The study was conducted according to the Helsinki Declaration. Written, informed consent was obtained from all patients or suitable proxies, and from all control individuals. The biobank is licensed by the Norwegian Directorate for Health Affairs, and the research project was approved by the Regional Committee for Medical Research Ethics, as well as by the Norwegian National Committees for Medical Research Ethics (approval 2013/150).

RESULTS

Demographic Data

Demographic data are summarized in **Table 1**. There were no significant differences in gender distribution between the four

participant groups (overall $p = 0.183$), despite a preponderance of females in the control group. Neither the age at inclusion nor the duration of education was significantly different between the four groups. Moreover, no significant difference in the age at onset of symptoms, or duration of symptoms was found between the patient groups.

There was a higher prevalence of the *APOE* ε4 allele in the pMCI subgroup, and amongst patients with AD, compared to the control group (both $p \leq 0.002$). There was also a higher prevalence of the *APOE* ε4 allele in the AD group compared to the sMCI subgroup ($p = 0.015$). The distribution of *APOE* ε4 alleles was not significantly different between controls and the sMCI subgroup, between patients with AD and the pMCI subgroup, or between sMCI and pMCI subgroups.

At baseline, MMSE-scores varied between all four participant groups (all pairwise comparisons: $p \leq 0.010$). There were decreases in MMSE-scores from baseline to 2 years in the pMCI and AD groups (both $p < 0.001$), but not in the sMCI group.

Biomarker Data at Baseline and Diagnostic Accuracy

Means and standard deviations of CSF Aβ43, Aβ42, t-tau/Aβ43, and t-tau/Aβ42 in all groups are shown in **Table 2**. Means and error bars in all groups at baseline are shown graphically in Supplementary Figures S1A–S1D.

Overall, all four analytes or ratios varied between groups at baseline (all $p < 0.001$). The CSF Aβ43 level in the control group was higher than in any patient group (all $p < 0.001$). The control group was also significantly different from all patient groups for the other three analytes or ratios (all $p \leq 0.007$). There were differences between AD and the sMCI subgroup for Aβ43 and the t-tau/Aβ43 and t-tau/Aβ42 ratios (all $p \leq 0.009$), but for Aβ42 there was only a weak trend ($p = 0.086$). Aβ43 and the t-tau/Aβ43 and t-tau/Aβ42 ratios varied between the sMCI and pMCI subgroups (all $p \leq 0.008$), but no difference for Aβ42 levels was found. No significant differences were found between patients with AD and the pMCI subgroup for any analytes or ratios.

Cerebrospinal fluid Aβ43 and Aβ42 were correlated in all four participant groups at baseline (all $p \leq 0.004$, $r = 0.621$–0.853), as were t-tau/Aβ43 and t-tau/Aβ42 (all $p < 0.001$, $r = 0.903$–0.959). After 1 year, Aβ43 and Aβ42 were still correlated in the sMCI and pMCI groups (both $p \leq 0.001$, $r = 0.736$–0.912), but not the AD group. After 2 years, Aβ43 and Aβ42 were correlated in all three patient groups (all $p \leq 0.019$, $r = 0.690$–0.868). T-tau/Aβ43 and t-tau/Aβ42 were correlated in all three patient groups both after 1 year and after 2 years (all $p < 0.001$, $r = 0.847$–0.969).

Receiver operating characteristic plots (not shown) were created for baseline levels of biomarkers or ratios thereof, and statistics were derived for pairwise comparisons of groups (**Table 3**). Both CSF Aβ43 and Aβ42 showed similar specificity in distinguishing control individuals from patients with AD, but the sensitivity was slightly higher for Aβ43. CSF Aβ43 and Aβ42 were also excellent at separating controls from patients in the pMCI subgroup, with AUCs over 0.90. Sensitivity and specificity were all over 90%, except for slightly lower sensitivity of Aβ42. For separating controls from patients in the sMCI subgroup, all AUC values were over 0.7, and specificity was high (87–100%), whereas sensitivity was much lower (53–74%). On the other hand, the sMCI subgroup was well separated from AD patients by the t-tau/Aβ43-ratio, with a slightly higher sensitivity than the corresponding t-tau/Aβ42-ratio. Aβ43 alone also separated these two groups (AUC 0.73, $p = 0.024$), while Aβ42 alone was not significant. The pMCI and AD patient groups were not separated significantly by any of the biomarkers or ratios thereof. However, the sMCI and pMCI subgroups were separated by ratios t-tau/Aβ43 and t-tau/Aβ42 (AUC 0.81 and 0.72, respectively, p-values 0.001 and 0.018, respectively). T-tau/Aβ43 had higher specificity (90%), whereas t-tau/Aβ42 had higher sensitivity (85%). When comparing AUC between sMCI and pMCI subgroups, the t-tau/Aβ43-ratio gave a larger AUC than the t-tau/Aβ42-ratio ($p = 0.040$), but for all other comparisons of Aβ43 and Aβ42, or comparisons of ratios t-tau/Aβ43 and t-tau/Aβ42 between two and two diagnostic groups, there were no significant differences in AUC.

Longitudinal Biomarker Levels

Longitudinal biomarker data are given in **Table 2**. Means and error bars for CSF Aβ43, Aβ42, t-tau/Aβ43, and t-tau/Aβ42 in all groups at baseline, after 1 year and after 2 years are shown in Supplementary Figures S1A–S1D.

After 1 year, group levels varied for CSF Aβ43 and the t-tau/Aβ43 and t-tau/Aβ42 ratios (all $p \leq 0.003$), but not for CSF Aβ42. For CSF Aβ43, the t-tau/Aβ43 and t-tau/Aβ42 ratios, there were differences between AD and the sMCI subgroup (all $p \leq 0.003$), and the sMCI and pMCI subgroups (all $p \leq 0.004$).

After 2 years, there were variations in group levels for CSF Aβ43 and the t-tau/Aβ43 and t-tau/Aβ42 ratios (all $p \leq 0.031$), but not for CSF Aβ42. For CSF Aβ43, the t-tau/Aβ43, and t-tau/Aβ42 ratios, there were differences between AD and the sMCI subgroup (all $p \leq 0.036$), and the sMCI and pMCI subgroups (all $p \leq 0.016$). No significant differences were found between patients with AD and the pMCI subgroup for any analytes or ratios at either one or 2 years.

Figures 1A–D show estimated longitudinal biomarker levels for the mixed linear model, for each participant group. The model has not been corrected for age at inclusion or gender, as these factors were not found to significantly affect the model.

CSF Aβ43 showed a decrease from baseline to 1 year after inclusion in both pMCI ($p = 0.023$) and AD groups ($p = 0.005$), but no significant changes were observed from year one to year two after inclusion in these two groups. In the AD group, CSF Aβ43 levels were decreased from baseline to 2 years after inclusion ($p = 0.003$), and levels were stable over 2 years in the sMCI group. For CSF Aβ42, levels were stable over 2 years in all three patient groups.

For the t-tau/Aβ43-ratio, levels were stable over 2 years in the AD group. In the sMCI and pMCI groups, the ratio increased from baseline to 2 years (both $p = 0.002$). For sMCI, the increase was significant from year one to year two ($p = 0.004$), but not from baseline to 1 year after inclusion. For pMCI, the increase was significant from baseline to year one ($p = 0.010$), but not from year one to year two.

TABLE 2 | Cerebrospinal fluid (CSF) biomarker data.

				aMCI at baseline					
		Controls	***n***	**sMCI**	***n***	**pMCI**	***n***	**AD**	***n***
Aβ43 (pg/ml)	at baseline	44.6 ± 13.1#	32	32.0 ± 23.6	20	20.0 ± 12.4**	21	18.8 ± 7.5**	18
	after 1 year	N/A		31.9 ± 22.1	20	17.8 ± 8.1**	20	17.0 ± 6.6**	19
	after 2 years	N/A		31.4 ± 22.0	20	18.7 ± 9.4*	20	16.7 ± 7.1****E**	15
Aβ42 (pg/ml)	at baseline	1065.5 ± 273.1#	25	663.3 ± 348.6	20	528.5 ± 178.3	20	475.7 ± 169.8	15
	after 1 year	N/A		622.4 ± 355.3	18	512.3 ± 175.3	19	443.2 ± 155.2	14
	after 2 years	N/A		631.4 ± 373.5	18	529.7 ± 199.3	19	476.7 ± 167.1	14
t-tau/Aβ43	at baseline	7.5 ± 7.6#	24	17.3 ± 16.0	19	47.6 ± 40.0***	20	45.1 ± 25.3***	14
	after 1 year	N/A		17.9 ± 16.9	17	60.1 ± 53.3***	18	49.5 ± 23.9***	14
	after 2 years	N/A		21.4 ± 22.4 **A**	18	70.9 ± 89.3****B**	19	47.8 ± 37.9*	11
t-tau/Aβ42	at baseline	0.35 ± 0.47#	23	0.85 ± 1.00	20	1.70 ± 1.76**	20	1.88 ± 1.65***	15
	after 1 year	N/A		0.91 ± 1.00	18	2.04 ± 2.22**	19	1.96 ± 1.62**	14
	after 2 years	N/A		1.12 ± 1.47 **C**	18	2.61 ± 4.36* **D**	19	1.55 ± 0.72*	14

Results are given as the mean ± SD. Significant group differences (analyzed with ANOVA, followed by LSD post hoc test, of log-transformed biomarker data): **#** *Control group levels at baseline were significantly different from all three patient groups (all $p \leq 0.007$). *Significantly different from the sMCI group ($p < 0.05$). **Significantly different from the sMCI group ($p < 0.01$). ***Significantly different from the sMCI group ($p \leq 0.001$). A graphic representation of the data is given in Supplementary Figures S1A–S1D. Longitudinal analyses (mixed linear model) of log-transformed biomarker data: increased from baseline to 2 years:* ***A*** *($p = 0.002$),* ***B*** *($p = 0.002$),* ***C*** *($p < 0.001$),* ***D*** *($p = 0.003$). Decreased from baseline to 2 years:* ***E*** *($p = 0.003$). AD, Alzheimer's disease; aMCI, amnestic mild cognitive impairment; sMCI, patients with mild cognitive impairment that did not progress to AD over 2 years; pMCI, patients with mild cognitive impairment that progressed to AD over 2 years; Aβ43, amyloid beta 1–43; Aβ42, amyloid beta 1–42; t-tau, total tau; N/A, not applicable.*

TABLE 3 | Diagnostic accuracy of CSF biomarkers at baseline.

	Controls vs. AD	**Controls vs. pMCI**	**Controls vs. sMCI**	**sMCI vs. AD**	**pMCI vs. AD**	**sMCI vs. pMCI**
Aβ43	AUC: 0.97 Sens: 93 Spec: 96	AUC: 0.93 Sens: 90 Spec: 100	AUC: 0.75 Sens: 53 Spec: 100	AUC: 0.73 Sens: 86 Spec: 68	AUC: n.s.	AUC: 0.71 Sens: 80 Spec: 68
Aβ42	AUC: 0.96 Sens: 86 Spec: 96	AUC: 0.94 Sens: 85 Spec: 96	AUC: 0.81 Sens: 63 Spec: 92	AUC: n.s.	AUC: n.s.	AUC: n.s.
t-tau/Aβ43	AUC: 0.94 Sens: 93 Spec: 91	AUC: 0.91 Sens: 90 Spec: 96	AUC: 0.72 Sens: 58 Spec: 91	AUC: 0.83 Sens: 79 Spec: 90	AUC: n.s.	AUC: 0.81* Sens: 75 Spec: 90
t-tau/Aβ42	AUC: 0.95 Sens: 86 Spec: 96	AUC: 0.91 Sens: 90 Spec: 96	AUC: 0.78 Sens: 74 Spec: 87	AUC: 0.78 Sens: 64 Spec: 90	AUC: n.s.	AUC: 0.72* Sens: 85 Spec: 63

**For comparison of sMCI vs. pMCI subgroups, the AUC of the t-tau/Aβ43-ratio is larger than the AUC of the t-tau/Aβ42-ratio ($p = 0.040$, DeLong method). AD, Alzheimer's disease; pMCI, patients with mild cognitive impairment that progressed to AD over 2 years; sMCI, patients with mild cognitive impairment that did not progress to AD over 2 years; Aβ43, amyloid beta 1–43; Aβ42, amyloid beta 1–42; t-tau, total tau; AUC, area under the ROC curve; Sens, sensitivity (%); Spec, specificity (%); n.s., not significant.*

For the t-tau/Aβ42-ratio, levels were stable over 2 years in the AD group. In the sMCI and pMCI subgroups, the ratio increased from baseline to 2 years thereafter (both $p \leq 0.003$). For sMCI, there was an increase from year one to year two ($p = 0.015$), but not from baseline to 1 year after inclusion. For pMCI, there was an increase from baseline to year one ($p = 0.018$), but not from year one to year two.

DISCUSSION

Patients with aMCI do not necessarily progress to AD. However, being able to distinguish individuals that are at greatest risk of progressing to dementia before brain atrophy becomes so pronounced that symptoms significantly impair cognitive function is an important goal for biomarker research. In the present study, half the patients initially diagnosed with aMCI progressed to AD during the 2 years of study. This pMCI subgroup was therefore comparatively homogeneous, given that patients were all in a prodromal phase, close to the development of Alzheimer dementia, which probably explains why the pMCI and AD groups could not be separated by any biomarker or combination of biomarkers at baseline. The ability to separate out such patients within the total aMCI group will be urgent once more effective treatment becomes available to prevent, or at least slow, disease progression.

At baseline, as well as after one and 2 years, CSF Aβ43 with or without t-tau in the equation separated patients with aMCI not progressing to AD within the 2 years of study from patients that did progress to AD, as well as from those diagnosed with

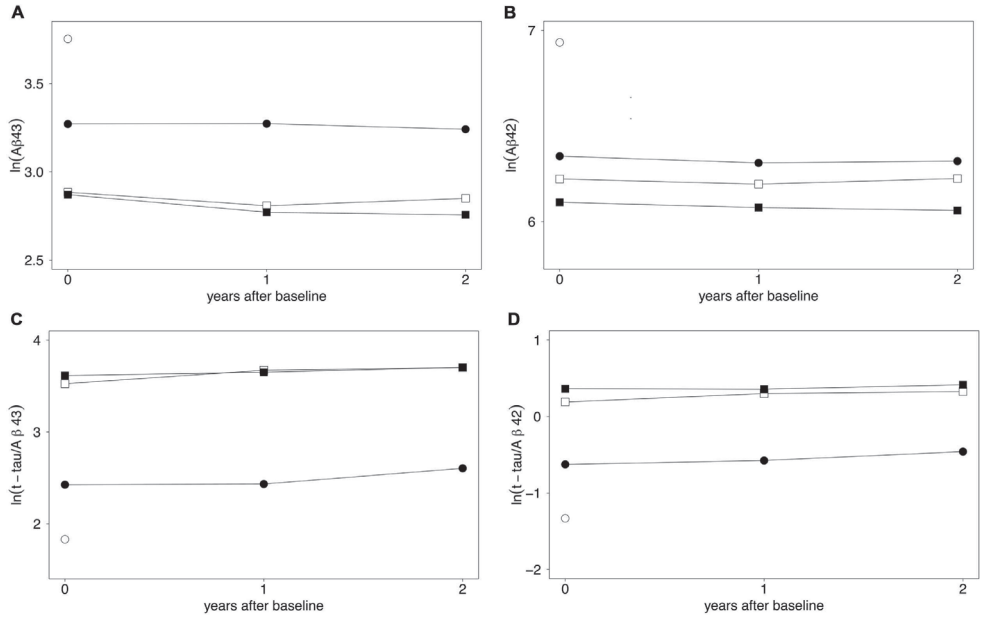

FIGURE 1 | (A–D) Longitudinal biomarker levels expressed by a mixed linear model. Estimates of mean log-transformed biomarker levels of **(A)** CSF Aβ43, **(B)** CSF Aβ42, **(C)** t-tau/Aβ43, and **(D)** t-tau/Aβ42 over 2 years are shown. ○ control group (baseline only), ● sMCI, □ pMCI, ■ AD. For clarity, the spread of results is not shown. Means and standard deviations are given in **Table 2**. All analytes were already significantly changed compared to the control level at baseline. Decreased Aβ43 levels over 2 years were found in the AD group ($p = 0.003$). Increased levels over 2 years were found for t-tau/Aβ43 and t-tau/Aβ42 ratios in the sMCI group ($p \leq 0.002$) and in the pMCI group ($p \leq 0.003$). Differences between groups at the three time-points are shown in **Table 2** and Supplementary Figures S1A–S1D. Aβ43, amyloid beta 1–43; Aβ42, amyloid beta 1–42; t-tau, total tau; sMCI, patients with mild cognitive impairment that did not progress to Alzheimer's disease during the 2 years; pMCI, patients with mild cognitive impairment that progressed to AD during the 2 years of study; AD, Alzheimer's disease.

AD at baseline. No such significant separation was found for CSF Aβ42 alone. Therefore, and because t-tau/Aβ43 also seemed to be at least as good as t-tau/Aβ42 for diagnostic accuracy at baseline in this study, CSF Aβ43 could be a useful biomarker for identifying patients with aMCI at greatest risk of AD. However, even though t-tau/Aβ43 in the present study had a slightly and significantly larger area under the ROC curve for distinguishing between the two aMCI subgroups at baseline, t-tau/Aβ42 was better for identifying patients in an early stage of AD (higher sensitivity).

To the best of our knowledge, this is the first longitudinal study of CSF Aβ43 in connection with aMCI and AD. A significant reduction in the CSF Aβ43 level over the 2 years following baseline was observed in the AD group, whereas no significant longitudinal changes in CSF Aβ43 levels were observed in the sMCI and pMCI groups. The longitudinal changes during the 2 years of study were substantially less in all patient groups compared to the concentration difference between the control group and patient groups at baseline. This is an indication that the reduction of CSF Aβ43 is most pronounced during the preclinical phase of disease, which may take place slowly over many years. It is certainly clear that changes in CSF Aβ43 concentration over the 2 years of our study are slow (according to our data no more than 0.5–1% annually). Since the standard deviation was relatively small in the AD group, the reduction was significant. However, this was not the case in the pMCI or sMCI subgroups where standard deviations were larger, and indeed there was hardly any change over 2 years in the sMCI subgroup.

The sMCI subgroup was probably heterogeneous, and although it contained individuals who have converted to AD since the end of the study (data not shown), it probably also contains individuals with control levels that will never convert to any neurodegenerative disease (Nettiksimmons et al., 2014; Tifratene et al., 2015). Overall, the annual small change in such a group is likely to be difficult to detect statistically.

Despite the heterogeneity of the sMCI subgroup, baseline mean levels of CSF Aβ43 (but not CSF Aβ42) distinguished significantly between the two aMCI subgroups, even though the groups were small. Contrary to CSF Aβ43, CSF Aβ42 levels were stable from baseline to 2 years in all three patient groups, in agreement with an earlier longitudinal study showing that CSF Aβ42 levels were stable over 4 years in MCI patients (Mattsson et al., 2012), and in agreement with results showing fully reduced CSF Aβ42 levels several years before dementia symptoms appear (Buchhave et al., 2012).

CSF Aβ43 and Aβ42 were highly correlated in all four participant groups at baseline. Aβ43 and Aβ42 are suggested to be produced from different routes of enzymic cleavage, where three amino acids are cleaved off the peptides Aβ48 and Aβ49 in two steps to produce Aβ42 and Aβ43, respectively (Qi-Takahara et al., 2005). The close correlation of Aβ42 and Aβ43 in the present study supports a connection between the two synthetic pathways, as shown earlier (Kakuda et al., 2012).

This is a small study with groups of limited size. In an earlier study of similar size that compared AD patients with controls, CSF Aβ43 was found to have similar specificity (97%) but much lower sensitivity (52%) and lower AUC (0.77) than in the current study (Bruggink et al., 2013). Such differences between studies arise easily when comparing small groups, and the present data should therefore be confirmed in a larger material. However, one of the main strengths of the present study is that all patients were diagnosed by a single neurologist, ensuring consistency of diagnoses between and within groups. Additionally, diagnoses were confirmed by a second neurologist.

CONCLUSION

Our findings suggest that CSF Aβ43, either alone or in a ratio with CSF t-tau, may be a useful additional discriminator for patients with aMCI that will progress to AD within a short period of time.

AUTHOR CONTRIBUTIONS

CL planned and performed the laboratory work, analyzed data, and wrote the manuscript. SS performed the clinical examination of all study participants together with GG. AS, IM, and GBe planned and performed the laboratory work. IB and ØS advised on statistical analysis. SS, GBr, and LW contributed to the conceptual design of the study, and supervised the project. All authors contributed to critical revision and finalization of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2016.00030

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Cerebrospinal fluid levels of amyloid beta 1-43 in patients with amnestic mild cognitive impairment or early Alzheimer's disease: a 2-year follow-up study

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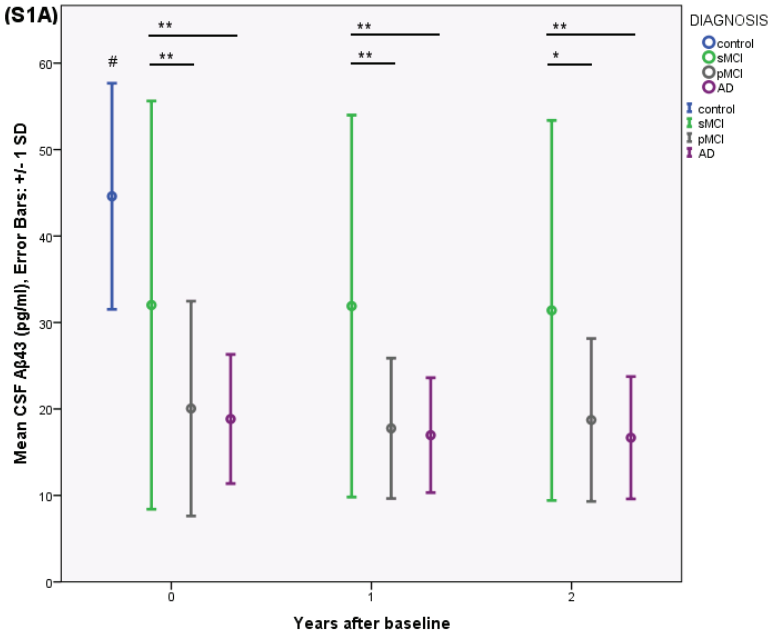
Supplementary Figure S1A-D. Biomarker concentrations in cerebrospinal fluid at baseline, after one year and after two years. Means and error bars representing +/- 1 standard deviation are given for all four participant groups. **(S1A)**: Aβ43, **(S1B)**: Aβ42, **(S1C)**: t-tau/Aβ43, **(S1D)**: t-tau/Aβ42. Values for mean ± standard deviation are given in Table 2. Statistical analyses of group differences were performed on log-transformed values (ANOVA followed by LSD post hoc test). #Control group levels at baseline were significantly different from all three patient groups (all p≤0.007). *p<0.05, **p<0.01, ***p≤0.001. Aβ43: amyloid beta 1-43, t-tau: total tau, sMCI: patients with mild cognitive impairment that did not progress to Alzheimer's disease over two years, pMCI: patients with mild cognitive impairment that progressed to AD over two years, AD: Alzheimer's disease, SD: standard deviation.



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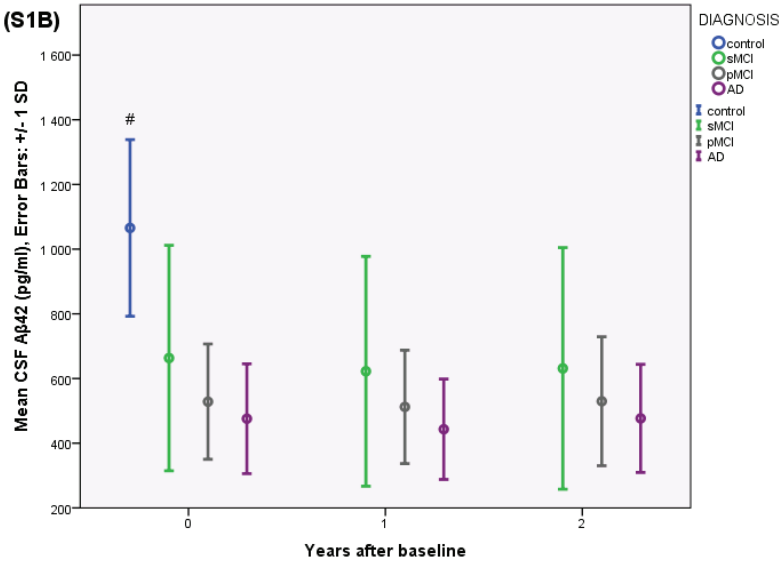
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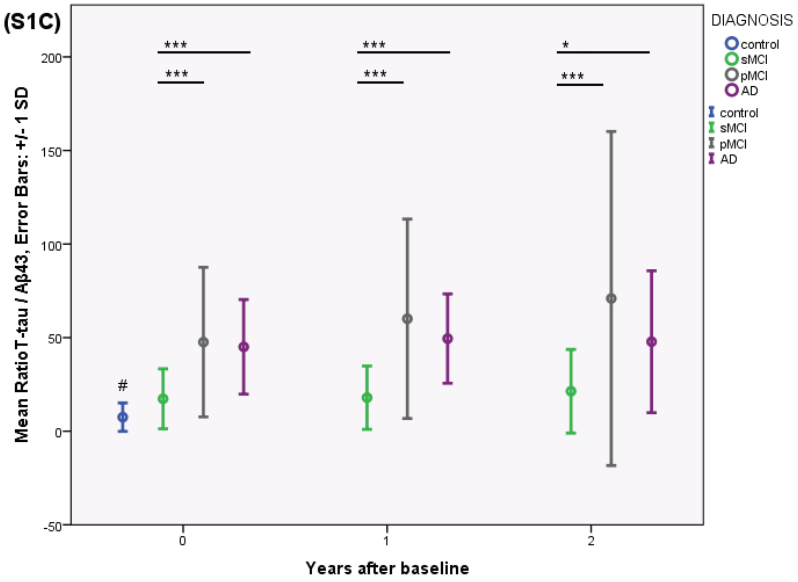
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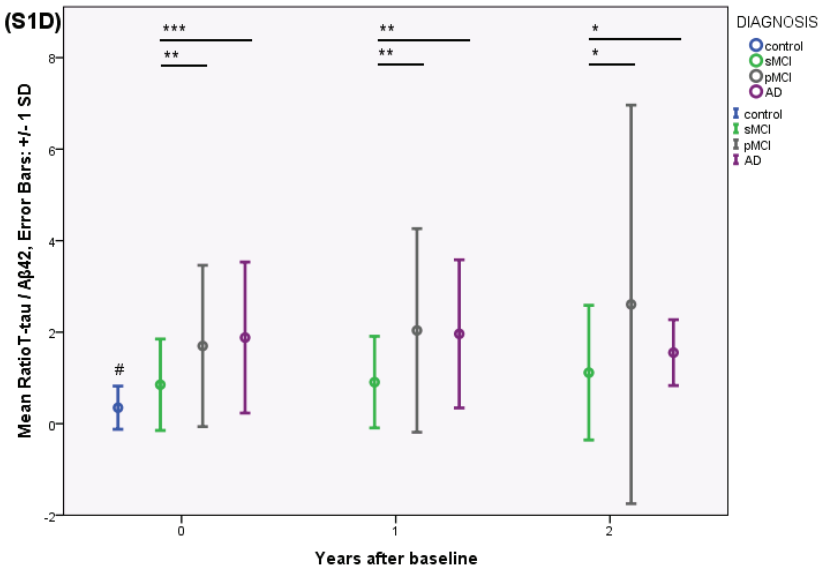
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DIAGNOSIS
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Paper II

Cerebrospinal fluid Aβ43 is reduced in early-onset compared to late-onset Alzheimer's disease, but has similar diagnostic accuracy to Aβ42

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Abstract

Background: Amyloid beta 1-43 (Aβ43) may be a useful additional biomarker for diagnosing Alzheimer's disease (AD). We have investigated cerebrospinal fluid (CSF) levels of Aβ43 in patients with early-onset AD in contrast to levels in late-onset AD. For comparison, in addition to the 'core' biomarkers, several other analytes were also determined (YKL-40, neurofilament light, glial fibrillary acidic protein, progranulin).

Material and Methods: CSF samples were obtained from patients with early-onset AD (age ≤ 62, n=66), late-onset AD (age ≥ 68, n=25), and groups of cognitively intact individuals (age ≤ 62, n=41, age ≥ 68, n=39). Core CSF AD biomarkers (amyloid beta 1-42 (Aβ42), total tau, phosphorylated tau) were analyzed, as well as levels of Aβ43 and other analytes, using commercially-available enzyme-linked immunosorbent assays.

Results: CSF Aβ43 was significantly reduced in early-onset AD compared to late-onset AD, whereas the levels of Aβ42 in the two AD groups were not significantly different. Aβ43 and all core biomarkers were significantly altered in patients with AD compared to corresponding controls. Relationships between the Aβ peptides and tau proteins, YKL-40, neurofilament light, glial fibrillary acidic protein and progranulin were also investigated without finding marked associations. Aβ43 did not improve diagnostic accuracy in either AD group compared to Aβ42.

Discussion: CSF Aβ43, but not Aβ42 levels, seem to vary significantly with age in patients with AD. If CSF levels of Aβ peptides reflect amyloid deposition in brain, the possibility arises that there is a difference between Aβ43 and Aβ42 deposition in younger compared to older brain. However, the level of Aβ43 in CSF shows no improvement over Aβ42 regarding diagnostic accuracy.

Keywords

early-onset Alzheimer's disease, biomarkers, tau, YKL-40, neurofilament light, glial fibrillary acidic protein, progranulin

Introduction

Alzheimer's disease (AD) is often separated according to age, whereby onset prior to age 65 years is considered to be early-onset AD, while onset from an age of 65 years (which is much more common) is termed late-onset AD. Imaging studies exploring the possibility of differences between the early and late forms of AD have been conflicting. Although the pathological burden of amyloid plaques and neurofibrillary tangles has been shown to be greater in early-onset AD than in patients with late-onset AD (Ho et al., 2002; Marshall et al., 2007), others have indicated the burden to be similar between the two groups, though with variation in anatomical distribution of amyloid (Ossenkoppele et al., 2012; Cho et al., 2013). Similar amyloid burden and distribution between early- and late-onset AD has also been found (Rabinovici et al., 2010). Whether such putative differences in the distribution of pathology affect levels of the 'core' CSF biomarkers for AD (Blennow and Zetterberg, 2009) amyloid beta 1-42 (Aβ42) and total tau (t-tau) and phosphorylated tau (p-tau) protein is not known. Similar CSF levels of these biomarkers have been found in early- and late-onset AD (Bouwman et al., 2009; Chiaravalloti et al., 2016). Additionally, several studies have shown no correlation between the core biomarkers and age in AD patients (Bouwman et al., 2009; Mattsson et al., 2009; Popp et al., 2010).

However, healthy individuals display increased AD pathology with increasing age (Savva et al., 2009). Older control individuals have been found to have decreased CSF Aβ42 levels compared to younger controls (Bouwman et al., 2009), and the level was negatively correlated with age (Popp et al., 2010). Conversely, CSF t-tau and p-tau correlate positively with age in healthy elderly individuals (Blomberg et al., 2001; Glodzik-Sobanska et al., 2009; Jaworski et al., 2009; Alcolea et al., 2015).

CSF amyloid beta 1-43 (Aβ43) deposits in amyloid plaques in AD brain tissue (Welander et al., 2009; Sandebring et al., 2013), correlates positively with age in patients with AD (Bruggink et al., 2013), and correlates closely with CSF Aβ42 both in patients and control individuals (Bruggink et al., 2013; Lauridsen et al., 2016). As far as we know, no study as yet has compared CSF levels of Aβ43 in early-onset AD with late-onset AD.

We have therefore investigated Aβ43 and Aβ42 in CSF from well-characterized cohorts of patients with early- and late-onset AD. The cut-off between these subtypes of AD has been

accepted as 65 years of age, but in the present study we excluded patients with age at onset in the five-year period 63-67 years to highlight potential age differences. Thus only patients with early-onset AD ≤62 years of age, or patients with late-onset AD who were aged ≥68 years were included.

In addition to the Aβ species in CSF from these two subgroups of patients with AD and corresponding control groups, levels of t-tau and p-tau were also determined. For further comparison, levels of several other analytes that have been investigated with respect to AD, and to age, were also assessed. These included two other cytoskeletal intermediate filaments; neurofilament light (NF-L) (Petzold et al., 2007; Vagberg et al., 2015; Olsson et al., 2016) and glial fibrillary acidic protein (GFAP) (Vagberg et al., 2015; Wennstrom et al., 2015), found respectively in neurons and glia, YKL-40 (also known as chitinase 3-like protein 1), a protease secreted mainly by astrocytes and considered a marker for gliosis and neuroinflammation (Craig-Schapiro et al., 2010), and progranulin, a growth factor believed to have anti-inflammatory and neuroprotective abilities (Jing et al., 2016).

Methods

Subjects

Study patients were ethnic Norwegians referred to the Department of Neurology, St. Olav's Hospital (Trondheim University Hospital) by general practitioners, and diagnosed by a neurologist. Some patients were initially diagnosed with amnestic mild cognitive impairment (aMCI, n=14) according to the International Working Group on Mild Cognitive Impairment criteria (Winblad et al., 2004), but all later developed AD within the next two years. Patients with AD were diagnosed according to the NINCDS-ADRDA criteria (McKhann et al., 1984), final total n=91, whereof 66 were aged ≤62 years at onset (early-onset AD) and 25 were aged ≥68 years at onset of symptoms (late-onset AD).

As controls, CSF samples were obtained either from non-demented elderly volunteers (n=35) recruited from societies for retired people or caregivers not genetically related to the patient, or from samples stored in the Neurological Research Biobank at the hospital (n=45). These latter individuals had been referred to the clinic for suspected neurological conditions, but none was subsequently found. Of the total 80 control individuals, 41 were aged ≤ 62 years, and 39 were aged ≥ 68 years. For the control groups, CSF cell count, glucose and protein were within standard physiological limits.

The neurological examination performed on most study participants included the Mini Mental State Examination (MMSE) (Folstein et al., 1975). MMSE was performed on all patients, but for many control individuals there had been no reason to carry out an MMSE during the clinical work-up, and where MMSE was available, the minimum score was 28. For the same reason, *APOE* genotype was not available for most younger controls. The demographic data are shown in Table 1.

Sampling of CSF

CSF was collected with patients lying on their side, and lumbar puncture carried out at the level L4/L5 or L5/S1. The first 2.5 mL CSF was used for routine clinical investigation. Aliquots of CSF were collected directly into polypropylene cryovials (Corning) immersed in ice-water. No samples used in this study were contaminated by blood, and so were not centrifuged. All samples were frozen within 30 minutes of lumbar puncture and stored at -80^{o}C until analysis. Ten samples were thawed and then frozen again before core biomarkers were

analyzed. One freeze-thaw cycle has previously been shown to not significantly affect core biomarker results (Le Bastard et al., 2015).

ELISA assays

CSF samples were analysed using ELISA monoplex kits according to the manufacturers' instructions (Aβ43 (IBL), Aβ42 (Innogenetics), t-tau (Innogenetics), p-tau (Innogenetics), NF-L (UmanDiagnostics), YKL-40 (Bio-Techne, CSF diluted 1:400), GFAP (BioVendor) and progranulin (Adipogen Life Sciences, CSF diluted 1:15)). Samples were thawed in ice-water prior to analysis, and all samples were analyzed in duplicate. Cross-reactivity for Aβ42 in the Aβ43 ELISA was given as <1%. Although this would contribute slightly to measurements for Aβ43, it would be a constant for both control and patient groups. Aβ43 was reported to have 50x less affinity than Aβ42 for the antibodies in the Aβ42 kit.

Statistical analysis

Statistical analyses were carried out using SPSS version 24 (IBM) and Stata version 13.1. Due to multiple testing, p-values <0.01 were considered statistically significant. Distribution of gender between groups and the distribution of the *APOE* ε4 allele between groups were assessed with Pearson's χ^2 (chi-square) test. Differences in age at inclusion between patients with early- or late-onset of AD and respective control groups, as well as for MMSE scores and duration of disease, were assessed with the independent samples Mann-Whitney U test for pairwise comparisons. CSF analyte levels were log-transformed to the natural logarithm (ln) to approximate a normal distribution before further analysis. Analyte levels were compared for younger and older participants within control and AD patient groups using t-tests for independent samples. It was necessary to adjust for age when comparing analyte levels between controls and patients. Hence, analyte levels for the group of younger controls were compared with those of early-onset AD patients, and analyte levels for older controls were compared with those of late-onset AD patients in a univariate general linear model. In this model, log-transformed analyte levels were used as the dependent variable, participant status as a fixed factor, and age at inclusion as a covariate. Correlations between analytes, or between analytes and age at inclusion, were calculated with Pearson's r. Associations are only tentative as some type 2 errors can occur even employing a significance level of p<0.01 as in the present study. Patterns as a whole have been considered more informative than

individual correlations. To investigate potential differences in diagnostic accuracy, receiver operating characteristic (ROC) curves were made for Aβ43 and Aβ42, and the area under each ROC curve (AUC) was calculated. Youden's index was found to determine where the sum of sensitivity and specificity was maximized. AUC was compared between Aβ43 and Aβ42 for controls and AD patients in corresponding groups (DeLong method (DeLong et al., 1988)). Ratios between analytes were calculated but did not provide additional useful information, and are not considered further. Ratio data are given in Supplementary Table 1.

Ethics Statement

The study was conducted according to the Helsinki Declaration. Written, informed consent was obtained from all patients or suitable proxies, and from all control individuals. The Neurological Research Biobank has been licensed by the Norwegian Directorate for Health Affairs, and the research was approved by the Regional Committee for Medical Research Ethics (approval 2010/226 REK Midt, 2013/467 REK Midt, 2013/150 REK Sør-Øst).

Results

When comparing participant groups, there were no significant differences in the distribution of gender. The median age at inclusion in both younger and older control groups was significantly lower than for corresponding patient age groups. There was no significant difference in the duration of disease between the two patient groups. No significant differences were found in MMSE scores between individuals in the respective control or patient groups. Both patient groups had significantly lower median MMSE scores than their respective control group. There was increased frequency of the *APOE* ε4 allele in combined patient compared to combined control groups (p=0.001) (Table 1).

CSF levels of the various analytes are shown in Tables 1 and 2A, and scatter plots for amyloid peptides are shown in Figure 1 and in Supplementary Figure S1A-F for the other analytes. Additionally, correlations between CSF levels of Aβ peptides and other analytes, and between Aβ peptide levels and age were calculated. CSF Aβ43 was significantly decreased in patients with early-onset AD compared to late-onset AD, but no significant difference was found between the two patient groups for Aβ42. There were highly significant reductions in the levels of both Aβ43 and Aβ42 in CSF of patients with AD compared to controls. No significant differences in levels of Aβ43 or Aβ42 were found between the two control groups. Both CSF Aβ43 and Aβ42 were excellent at separating corresponding controls from patients in the AD groups, with AUCs of 0.93 or better and no significant difference in AUCs between Aβ43 and Aβ42 (Table 2B). Aβ43 and Aβ42 correlated significantly with each other in all four participant groups ($r=0.58$-0.85, $p\leq0.006$).

A significant positive association between Aβ42 and age at inclusion was found in younger controls ($r=0.55$, $p=0.001$) and in early-onset AD ($r=0.38$, $p=0.002$). For older controls a trend was found for a negative correlation ($r=-0.42$, $p=0.012$), but this was lost in late-onset AD. For Aβ43, a positive correlation with age at inclusion was found in the early-onset AD group ($r=0.43$, $p=0.002$), but the association was not significant in the other three participant groups.

Results for t-tau and p-tau were similar in nature, and both correlated with each other in all groups ($r=0.76$-0.93, all $p<0.001$). Their levels were significantly increased in patients compared to the corresponding control group, but there was no difference between patients

with early- or late-onset AD. However, the older control group had significantly higher levels of the tau species compared to younger controls (Table 1). Associations between tau proteins and Aβ peptides were found only in younger controls ($r=0.43$, $p=0.016$ to $r=0.52$, $p=0.003$), not older controls or either AD group.

YKL-40 was not significantly increased in patients compared to the respective control group. However, a significant increase was found between early- and late-onset AD, as well as between younger and older controls. There was a pattern for a relationship between the Aβ peptides and YKL-40 in younger controls and early-onset AD, but correlation coefficients were low (all $r=0.33$-0.44, $p<0.05$ except for Aβ43 and YKL-40 in early-onset AD, $p=0.003$).

A highly significant increase in the level of NF-L was found in early-onset AD compared to younger controls, but this difference was lost between late-onset AD and older controls. There was no difference between the two groups of patients, but older controls had significantly higher levels of NF-L compared to younger controls. Levels of GFAP in patients with early-onset AD were significantly higher than in younger controls. Older controls also had significantly increased levels compared to the younger controls, but no significant differences between the patient groups were found. No significant group differences in progranulin levels were found in this material.

Discussion

The most interesting result in this study is that the AD-related reduction in CSF levels of Aβ43 was more marked in early-onset compared to late-onset AD, and therefore seems to be age-related. This difference was not found for Aβ42. As expected, there was a clear and highly significant reduction in the concentration of both Aβ43 and Aβ42 in the CSF of the patient groups compared to corresponding controls. However, the data do not suggest that Aβ43 has better diagnostic accuracy for AD than Aβ42.

The increased deposition of parenchymal Aβ species in the AD brain has been suggested as the reason for the reduced amounts of Aβ peptides measured in CSF (usually Aβ42), based on the idea that less may be available for passage over the brain-CSF barrier (Fagan et al., 2006). Recent results from imaging studies showed that although CSF Aβ43 is strongly associated with cerebral amyloid deposits, even at early stages of clinical cognitive impairment (subjective cognitive decline (SCD) and MCI), there were no relative differences in deposition between Aβ42 and Aβ43, and thus that Aβ43 provided no diagnostic improvement over the established marker Aβ42 (Almdahl et al., 2017). Also in the present study comparing early- and late-onset AD versus comparable control groups, no improvement to diagnostic accuracy was found for Aβ43 compared to Aβ42.

It is not immediately obvious why Aβ43 would be reduced more in early-onset than late-onset AD, other than that there is an age difference between the patient groups. However, two studies comparing amyloid imaging in early- and late-onset AD report regional (though not identical) differences in amyloid deposition (Ossenkoppele et al., 2012; Cho et al., 2013). It is therefore possible there are age-related differences in the deposition of Aβ43 and Aβ42. This again might produce differences in CSF concentrations of the peptides in early-onset compared to late-onset AD as found in the present study, though with the information available this remains speculative. We did not find a similar reduction for CSF Aβ42 in early-onset AD, and this result is very similar to previously published data (Gronning et al., 2012).

Several studies have compared age and Aβ42 levels in CSF in healthy individuals with little or no correlation found (Hansson et al., 2006; Bouwman et al., 2009; Mattsson et al., 2009; Popp et al., 2010). Our data indicated a significant positive correlation between Aβ42 and age in younger controls, but the trend was a negative correlation in older controls (which

overall would give no significant correlation, as is usually found). Indeed, Shoji et al. have shown that Aβ42 levels in CSF follow a very shallow U-shape over life, falling between childhood and around 30 years, flattening out and increasing in older age (Shoji et al., 2001). It is therefore perhaps not surprising if studies carried out over a fairly large age span find no overall correlation between Aβ42 and age. Several studies have shown no such correlation in patients with AD (Bouwman et al., 2009; Mattsson et al., 2009; Popp et al., 2010). Our present data found no correlation between CSF Aβ42 and age in late-onset AD, but a weak correlation in connection with early-onset AD. Regarding Aβ43 and age, little has been published but a weak positive correlation has been found in late-onset AD, though not in controls (Bruggink et al., 2013). We found no significant correlation between Aβ43 and age in controls or late-onset AD, though a weak significant correlation in early-onset AD.

Generally speaking, our results for the core biomarkers in controls and in AD, as well as the association with age, agree broadly with previous studies (Blomberg et al., 2001; Bouwman et al., 2009; Glodzik-Sobanska et al., 2009; Popp et al., 2010; Alcolea et al., 2015; Chiaravalloti et al., 2016; Olsson et al., 2016). Age is also important for other substances analyzed in the present study. In recent years several reports have shown that YKL-40 increases throughout middle-age in cognitively healthy individuals, suggesting that a certain level of neuroinflammation is physiological in normal aging (Alcolea et al., 2015; Sutphen et al., 2015), as well as being an aspect of AD (Wennstrom et al., 2015). The present study agrees with the finding of increased YKL-40 levels with increased age. NF-L is well known to be increased in the CSF of patients with AD (Petzold et al., 2007; Olsson et al., 2016), and the present results are in accordance with this but only in connection with early-onset AD. No significant difference was found for CSF NF-L between late-onset AD and older controls. However, since NF-L increases during normal aging (Vagberg et al., 2015), this was probably an effect of ageing. Similarly, we found an increase in CSF GFAP in patients with early-onset AD compared to younger controls, but again perhaps due to the increase in CSF levels of GFAP with age (Vagberg et al., 2015), this difference was lost between late-onset AD and older controls. The results for the older groups agree with one study (Wennstrom et al., 2015), but not with another study that found increased GFAP levels in AD patients compared to controls (Jesse et al., 2009). No changes in the concentration of progranulin in CSF from

patients with AD were found compared to controls, as previously demonstrated (Morenas-Rodriguez et al., 2016).

Taken together, few differences were detected between early- and late-onset AD when analyzing CSF for potential markers of disease, even though the two groups had been clearly defined with respect to a difference in age. When comparing patients and controls, more differences were associated with early-onset rather than late-onset AD, perhaps because both patients and controls tend to suffer more co-morbidities with increasing age which can cloud differences between patients with AD and controls. The main strength of the present study was to employ clinically well-defined patient and control cohorts which were large enough to distinguish differences and similarities in Aβ43 and Aβ42. In light of an earlier report (Lauridsen et al., 2016), future studies should probably concentrate on examining CSF Aβ43 and Aβ42 in relation to early stages of the AD process, including amnestic MCI, subjective cognitive decline, and cognitively intact individuals who have a pathological pattern of core biomarkers in CSF, or increased amyloid deposition in brain.

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Conflicting interests

The authors report no conflicting interests.

Author contributions

CL planned and performed the laboratory work together with IM, PKP, and GBe. SBS was responsible for clinical aspects together with GRG and GBr. CL analyzed the data and ØS advised on statistical analysis. LRW was responsible for study design and data collation

together with CL and SBS. LRW, SBS and GBr supervised the project. CL and LRW wrote the manuscript. All authors contributed to critical revision and finalization of the manuscript.

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Table 1. Demographic and CSF biochemical data. Demographic data are given as the median (range) for continuous variables, CSF biochemical data are given as the mean ± SD, with the number of analyses. Statistical analysis was performed with pairwise group comparisons of log-transformed analyte levels between controls and AD patients aged ≤62 years (age-adjusted), and between controls and AD patients aged ≥68 years (age-adjusted), as well as between younger and older groups of controls, and younger and older groups of patients with AD. [A]significantly different to younger controls, [B]significantly different to older controls, [C]significantly different to late-onset AD patients. *p<0.01, **p<0.001. [#]Increased frequency of the *APOE* ε4 allele in patient compared to control groups (p=0.001). Abbreviations: AD = Alzheimer's disease, N/A = not applicable, MMSE = Mini Mental State Examination, *APOE*= apolipoprotein E, y = years, t-tau = total tau, p-tau = phosphorylated tau, NF-L = neurofilament light, GFAP = glial fibrillary acidic protein.

	Controls age ≤62	Early-onset AD age ≤62	Controls age ≥68	Late-onset AD age ≥68
Total n	41	66	39	25
Gender (female/male)	21/20	37/29	23/16	15/10
Age at inclusion (y)	57 (47-62)	61 (51-67) [A**]	71 (68-84)	76 (71-84) [B*]
Age at onset (y)	N/A	58 (47-62)	N/A	73 (68-82)
Duration (y)	N/A	3 (1-11)	N/A	2 (1-5)
MMSE score	29 (28-30) 12	24 (10-30) [A**] 63	29 (28-30) 33	23 (12-29) [B**] 25
APOE genotype (% with an ε4 allele, total n genotyped)	37.5 8	74.2 [#] 62	45.2 31	72.2 [#] 18
t-tau (pg/ml)	246.5 ± 99.5 [B*], 32	767.8 ± 485.5 [A**], 64	348.0 ± 166.8, 37	646.9 ± 418.6 [B*], 25
p-tau (pg/ml)	42.6 ± 18.1 [B**], 32	98.1 ± 39.5 [A**], 64	60.8 ± 20.8, 37	98.8 ± 51.4 [B*], 25
YKL-40 (ng/ml)	139.1 ± 55.6 [B**], 38	206.0 ± 97.4 [C*], 45	237.3 ± 73.2, 34	287.3 ± 109.8, 23
NF-L (pg/ml)	567.2 ± 190.0 [B**],41	1497.8 ± 814.5 [A**], 51	1381.4 ± 1419.3, 39	1882.0 ± 2122.2, 24
GFAP (pg/ml)	1227.5 ± 475.3 [B*], 13	1889.8 ± 1072.1[A*], 14	1786.8 ± 608.3, 25	2210.8 ± 903.1, 21
Progranulin (pg/ml)	4844.2 ± 1349.8, 37	4855.3 ± 1395.5, 38	5358.8 ± 977.1, 8	5403.6 ± 1064.9, 21

Table 2A. Levels of amyloids in cerebrospinal fluid. Data are given as the mean ± SD, with the number of analyses. Abbreviations: AD = Alzheimer's disease, Aβ = amyloid beta. Statistical analysis was performed with pairwise group comparisons of log-transformed analyte levels between controls and AD patients aged ≤62 years (age-adjusted), and between controls and AD patients aged ≥68 years (age-adjusted), as well as between younger and older groups of controls, and younger and older groups of patients with AD. [A] significantly different to corresponding control group, [B] significantly different to patients with late-onset AD **p<0.001.

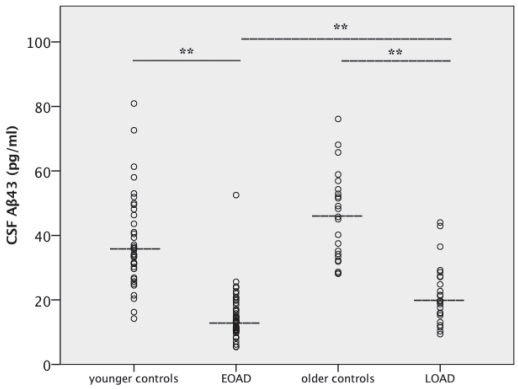
	Controls age ≤62	Early-onset AD age ≤62	Controls age ≥68	Late-onset AD age ≥68
Aβ43 (pg/ml)	38.0 ± 14.6 37	14.8 ± 7.3 [A**, B**] 50	45.8 ± 13.7 23	21.8 ± 9.4 [A**] 24
Aβ42 (pg/ml)	844.9 ± 220.9 31	474.9 ± 142.0 [A**] 64	967.5 ± 247.2 36	539.6 ± 159.9 [A**] 25

Table 2B. Diagnostic accuracy of β-amyloids for the separation of controls and patients. Abbreviations: Aβ = amyloid beta, AUC: area under the receiver operating characteristic curve.

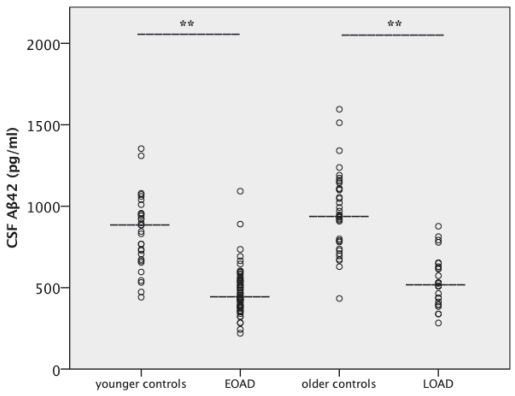
	Age ≤62 years	Age ≥68 years
CSF Aβ43	AUC: 0.96 sensitivity: 96% specificity: 89%	AUC: 0.94 sensitivity: 79% specificity: 100%
CSF Aβ42	AUC: 0.93 sensitivity: 92% specificity: 84%	AUC: 0.93 sensitivity: 84% specificity: 94%

Figure 1A-B. Amyloid levels in cerebrospinal fluid. Scatter plots for all four participant groups with median lines added for each group. Values for mean ± 1 SD are given in Table 2A. Statistical analysis was performed with pairwise group comparisons of log-transformed analyte levels between controls and AD patients aged ≤62 years (age-adjusted), and between controls and AD patients aged ≥68 years (age-adjusted), as well as between younger and older groups of controls, and younger and older groups of patients with AD. **(1A)** Aβ43, **(1B)** Aβ42. **Significantly different at the p<0.001 level. Abbreviations: AD = Alzheimer's disease, Aβ = amyloid beta, EOAD: early-onset AD, LOAD: late-onset AD.

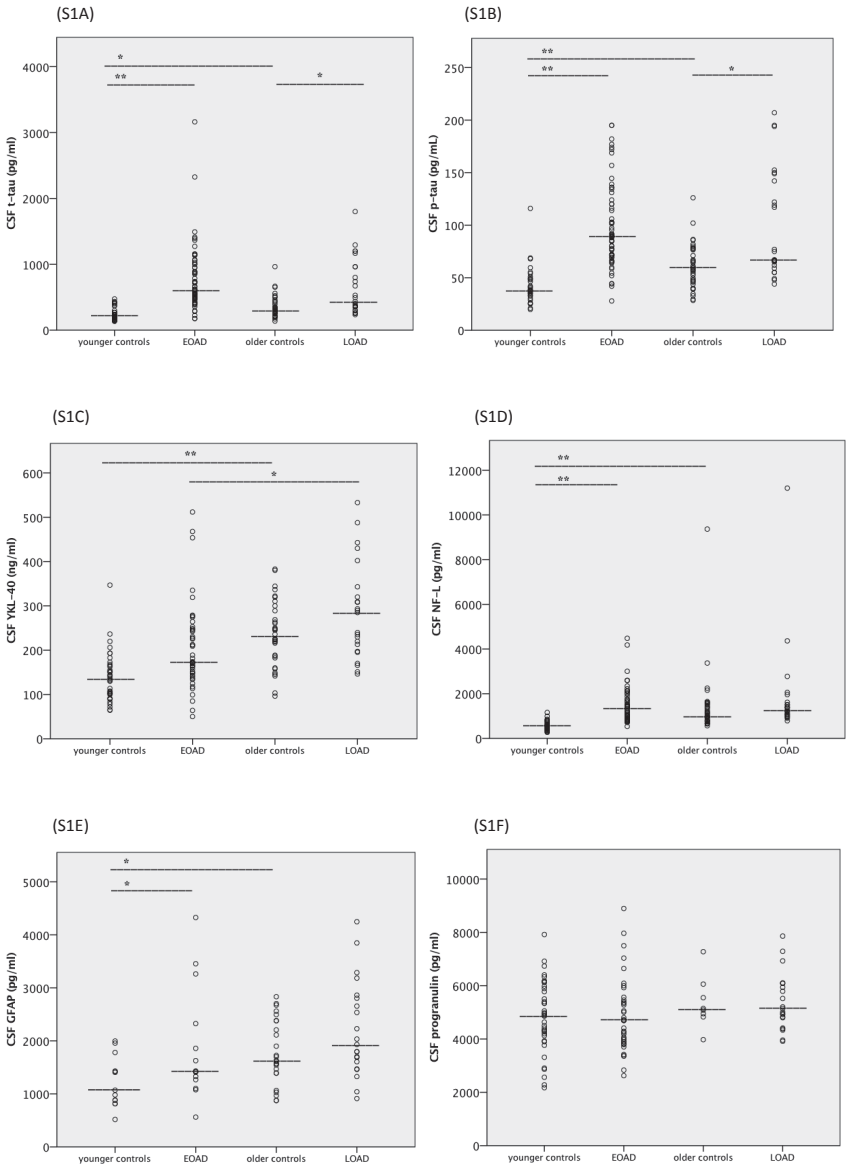
(1A)



(1B)



Supplementary Figure S1A-F. Analyte levels in cerebrospinal fluid. Scatter plots for all four participant groups with median lines added for each group. Values for mean ± 1 SD are given in Table 1. Statistical analysis was performed with pairwise group comparisons of log-transformed analyte levels between controls and AD patients aged ≤62 years (age-adjusted), and between controls and AD patients aged ≥68 years (age-adjusted), as well as between younger and older groups of controls, and younger and older groups of patients with AD. (S1A) t-tau, (S1B) p-tau, (S1C) YKL-40, (S1D) NF-L, (S1E) GFAP, (S1F) progranulin. *Significantly different at the p<0.01 level. **Significantly different at the p<0.001 level. Abbreviations: AD = Alzheimer's disease, t-tau = total tau, p-tau = phosphorylated tau, NF-L = neurofilament light, GFAP = glial fibrillary acidic protein, EOAD: early-onset AD, LOAD: late-onset AD.



Supplementary Table 1. Ratios of the analytes in cerebrospinal fluid. Data are given as the mean ± SD, with the number of analyses. Statistical analysis was performed with pairwise group comparisons of log-transformed analyte levels between controls and AD patients aged ≤62 years (age-adjusted), and between controls and AD patients aged ≥68 years (age-adjusted), as well as between younger and older groups of controls, and younger and older groups of patients with AD. [A] significantly different to corresponding control group, p≤0.001, [B] significantly different to patients with late-onset AD, p≤0.001, [C] significantly different to older control group, p<0.001. Abbreviations: AD = Alzheimer's disease, Aβ = amyloid beta, t-tau = total tau protein, p-tau = phosphorylated tau protein, NF-L = neurofilament light, GFAP = glial fibrillary acidic protein.

	Controls ≤62	Early-onset AD ≤62	Controls ≥68	Late-onset AD ≥68
Aβ42 / Aβ43	24.0 ± 6.7 27	35.6 ± 12.6 [A, B] 48	22.7 ± 5.5 21	26.6 ± 6.0 24
t-tau / Aβ43	7.2 ± 2.8 28	57.6 ± 31.0 [A, B] 48	6.9 ± 3.6 22	34.3 ± 33.5 [A] 24
t-tau / Aβ42	0.3 ± 0.1 31	1.7 ± 1.3 [A] 64	0.4 ± 0.4 36	1.3 ± 1.1 [A] 25
YKL-40 / Aβ43	4028 ± 2198 37	15863 ± 8478 [A] 43	4936 ± 1921 21	15957 ± 9588 [A] 23
YKL-40 / Aβ42	164.6 ± 62.3 [C] 28	460.1 ± 246.7 [A] 43	272.7 ± 149.7 31	595.2 ± 296.3 [A] 23
Progranulin / Aβ43	142.1 ± 60.9 37	410.6 ± 203.9 [A] 38	122.9 ± 39.9 8	310.4 ± 136.2 [A] 21
Progranulin / Aβ42	6.2 ± 2.3 27	11.0 ± 3.0 [A] 36	6.4 ± 1.9 8	11.4 ± 3.6 [A] 21
NF-L / GFAP	0.6 ± 0.4 13	0.9 ± 0.6 14	0.9 ± 0.7 25	0.9 ± 0.6 21

Paper III

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ORIGINAL RESEARCH
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Cerebrospinal Fluid Levels of Amyloid Beta 1-43 Mirror 1-42 in Relation to Imaging Biomarkers of Alzheimer's Disease

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Introduction: Amyloid beta 1-43 (Aβ43), with its additional C-terminal threonine residue, is hypothesized to play a role in early Alzheimer's disease pathology possibly different from that of amyloid beta 1-42 (Aβ42). Cerebrospinal fluid (CSF) Aβ43 has been suggested as a potential novel biomarker for predicting conversion from mild cognitive impairment (MCI) to dementia in Alzheimer's disease. However, the relationship between CSF Aβ43 and established imaging biomarkers of Alzheimer's disease has never been assessed.

Materials and Methods: In this observational study, CSF Aβ43 was measured with ELISA in 89 subjects; 34 with subjective cognitive decline (SCD), 51 with MCI, and four with resolution of previous cognitive complaints. All subjects underwent structural MRI; 40 subjects on a 3T and 50 on a 1.5T scanner. Forty subjects, including 24 with SCD and 12 with MCI, underwent ^{18}F-Flutemetamol PET. Seventy-eight subjects were assessed with ^{18}F-fluorodeoxyglucose PET (21 SCD/7 MCI and 11 SCD/39 MCI on two different scanners). Ten subjects with SCD and 39 with MCI also underwent diffusion tensor imaging.

Results: Cerebrospinal fluid Aβ43 was both alone and together with p-tau a significant predictor of the distinction between SCD and MCI. There was a marked difference in CSF Aβ43 between subjects with ^{18}F-Flutemetamol PET scans visually interpreted as negative (37 pg/ml, $n = 27$) and positive (15 pg/ml, $n = 9$), $p < 0.001$. Both CSF Aβ43 and Aβ42 were negatively correlated with standardized uptake value ratios for all analyzed regions; CSF Aβ43 average *rho* −0.73, Aβ42 −0.74. Both CSF Aβ peptides correlated significantly with hippocampal volume, inferior parietal and frontal cortical thickness and axial diffusivity in the corticospinal tract. There was a trend toward CSF Aβ42 being better correlated with cortical glucose metabolism. None of the studied correlations between CSF Aβ43/42 and imaging biomarkers were significantly different for the two Aβ peptides when controlling for multiple testing.

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Conclusion: Cerebrospinal fluid Aβ43 appears to be strongly correlated with cerebral amyloid deposits in the same way as Aβ42, even in non-demented patients with only subjective cognitive complaints. Regarding imaging biomarkers, there is no evidence from the present study that CSF Aβ43 performs better than the classical CSF biomarker Aβ42 for distinguishing SCD and MCI.

Keywords: Alzheimer's disease, amyloid beta 1-43, cerebrospinal fluid, positron emission tomography, magnetic resonance imaging, mild cognitive impairment

INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia. Treatment of this devastating disease will depend on biomarkers that can reliably identify individuals who will develop dementia due to AD in the future. A previous study following patients with mild cognitive impairment (MCI) for 2 years, found that the baseline cerebrospinal fluid (CSF) levels of amyloid-beta 1-43 (Aβ43) could distinguish patients that converted to AD dementia from those that did not, suggesting that CSF Aβ43 could be a useful addition to the more well-studied CSF biomarkers amyloid beta 1-42 (Aβ42), total tau (t-tau), and tau phosphorylated on position 181 (p-tau) (Kandimalla et al., 2011, 2013; Lauridsen et al., 2016). Aβ43 differs from Aβ42 by one C-terminal threonine residue, and is the product of an alternative γ-secretase cleavage pathway from the amyloid precursor protein (APP) (Takami et al., 2009). Findings from studies of neuropathology, genetics and animal models have resulted in the hypothesis that Aβ43 could play a role in AD pathogenesis out of proportion to its low levels in the brain. With its additional C-terminal beta-branched amino acid, Aβ43 could theoretically be expected to be more prone to aggregation than Aβ42. Experiments *in vitro* have yielded conflicting results: some report that Aβ43 indeed aggregates faster than Aβ42 and with a higher potential for seeding aggregation of other Aβ species (Saito et al., 2011; Conicella and Fawzi, 2014), others that Aβ43 aggregates slower with later amyloid nucleation and that it is inefficient in cross-seeding Aβ42 (Chemuru et al., 2016). Whether these experiments reflect the true aggregational process in the human brain is uncertain (Vandersteen et al., 2012). Cerebral deposition of Aβ43 is frequently present both in sporadic and familial AD (Welander et al., 2009; Keller et al., 2010; Sandebring et al., 2013) as a component of both neuritic and diffuse extracellular plaques (Iizuka et al., 1995; Parvathy et al., 2001; Miravalle et al., 2005). Some *PSEN1* mutations associated with familial AD are known to cause an overproduction of Aβ43 (Nakaya et al., 2005; Shimojo et al., 2008). In a transgenic mouse model based on such a *PSEN1* mutation, Aβ43 appeared to have greater neurotoxicity than Aβ42 with short-term memory impairment occurring with rising levels of Aβ43 even before plaque formation (Saito et al., 2011). Aβ43 has also been shown to deposit ahead of Aβ42 in the brain of mutant APP transgenic mice (Zou et al., 2013).

Information is sparse regarding CSF Aβ43 as a potential clinical biomarker, especially in early stages of cognitive impairment. Previously, it has been shown that CSF Aβ43 levels are decreased in MCI and AD dementia as compared to controls, with a strong correlation between CSF levels of Aβ43 and Aβ42 (Kakuda et al., 2012; Lauridsen et al., 2016). At the late stage of dementia, CSF Aβ43 and Aβ42 appear to have equal diagnostic accuracy for discriminating AD dementia from non-demented controls (Bruggink et al., 2013). Clinically more important, however, is the ability of biomarkers to single out non-demented patients that are on a trajectory toward AD dementia. A meta-analysis combining the classical CSF biomarkers; Aβ42 with t-tau and/or p-tau, yielded a mean sensitivity of 84% and a mean specificity of 63% for the distinction between stable and progressive MCI (Ferreira et al., 2014). Enhancement of this diagnostic performance would obviously be an advantage. Lauridsen et al. (2016) found that when used in a ratio with t-tau, substituting Aβ42 with Aβ43 gave a slight, but significant improvement of the diagnostic accuracy for this distinction, a finding that warrants further exploration. The use of CSF biomarkers in clinical routine is impeded by the invasiveness of lumbar puncture and by the high between-center variability particularly in the measurement of Aβ42. Imaging biomarkers are often more readily available, provide complimentary information as well as improve the predictive accuracy for dementia conversion when combined with CSF biomarkers (Vemuri et al., 2009). To our knowledge, CSF Aβ43 has not been described in relation to imaging biomarkers.

Positron emission tomography (PET) imaging allows visualization of cerebral Aβ aggregates *in vivo*. Uptake of amyloid-binding PET tracers, like ^{18}F-Flutemetamol (^{18}F-FLUT), correlates inversely with CSF Aβ42 levels (Fagan et al., 2006; Li et al., 2015), and positively with Aβ plaque burden observed post-mortem (Ikonomovic et al., 2008). It remains to be determined whether the relationship with amyloid PET is the same for CSF Aβ43. In addition to amyloid pathology, development of AD is characterized by neurodegeneration. Neurodegenerative changes in AD identifiable by magnetic resonance imaging (MRI) include gray matter atrophy of the hippocampus and vulnerable cortical regions (Whitwell et al., 2008; Sabuncu et al., 2011), and microstructural white matter changes resulting in increased mean, radial and axial diffusivity and reduced fractional anisotropy on diffusion tensor imaging (DTI) (Selnes et al., 2013; Amlien and Fjell, 2014; Lee et al., 2015). Several studies have reported correlations between CSF Aβ42 and structural MRI, while others have found no association, with methodological differences suggested as a possible reason for the discrepancy (Vemuri and Jack, 2010; Li et al., 2014). Neurodegeneration is also related to changes in cerebral metabolism as assessed by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) PET imaging (Fouquet et al., 2009). In AD dementia

cortical ^{18}F-FDG uptake has been reported to correlate with CSF Aβ42 levels (Vukovich et al., 2009; Yakushev et al., 2012) and ^{18}F-FDG PET imaging appears to have high prognostic value in MCI (Shaffer et al., 2013; Perani et al., 2016).

The objectives of this study were to explore firstly whether CSF Aβ43 reflects cerebral amyloid deposits as visualized by ^{18}F-FLUT PET, and secondly whether CSF Aβ43 correlates with MRI and ^{18}F-FDG PET imaging findings of neurodegeneration in non-demented patients with cognitive complaints.

MATERIALS AND METHODS

Subject Recruitment

Cohort 1 – Amyloid PET cohort:

Forty subjects were included in the Dementia Disease Initiation (DDI) project at Akershus University Hospital between March 2013 and March 2016. They were referred by their general practitioner to the hospital's memory clinic or recruited through newspaper advertisements. Inclusion criteria were complaints of decline in cognitive capacity compared with a previously normal state, age 40–79 and Scandinavian first language. Exclusion criteria were established dementia, neurodevelopmental disorders, known brain injury including recognized previous stroke, as well as any serious somatic or psychiatric disorder or drug use that could significantly influence cognitive capacity. All subjects were assessed with ^{18}F-FLUT PET either at the time of inclusion ($n = 22$) or at a second assessment 2 years after first inclusion in the project ($n = 18$). Clinical assessment, lumbar puncture, and MRI were done within 3.5 months of ^{18}F-FLUT PET. Thirty-one of the 40 subjects also underwent ^{18}F-FDG PET. Patients were interviewed and examined by a physician trained in diagnosing cognitive disorders. A clinical report form was used to collect information about current cognitive symptoms both from the participant and a knowledgeable informant. Standardized cognitive testing, physical examination, and blood screening were completed. MCI ($n = 12$) was defined based on the core criteria in the recommendation from the National Institute on Aging-Alzheimer's Association (NIA/AA; Albert et al., 2011). Documented impairment greater than expected for the person's age, gender, and educational level in one or more cognitive domains was operationalized by a score 1.5 standard deviations or more below the normative mean on at least one of the following tests; the delayed recall task of the CERAD Word List Test (Fillenbaum et al., 2008), Trail Making Test B (TMTB) (Reitan and Wolfson, 1985), Controlled Oral Word Association Test (COWAT) (Benton and Hamsher, 1989) and the silhouettes task from the Visual Object and Space Perception (VOSP) Battery (Warrington and James, 1991) or a score below 28 on MMSE (Folstein et al., 1975). All subjects maintained independent functioning in social, and if appropriate, occupational settings, and had a global Clinical Dementia Rating score of $\leq$0.5 (Morris, 1997). Subjective cognitive decline (SCD) ($n = 24$) was defined according to the recommendations by the Subjective Cognitive Decline Initiative Working Group (Jessen et al., 2014), with normal performance on standardized cognitive tests operationalized by a score above 1.5 standard deviations below the normative mean on the above mentioned tests. Four subjects had been classified as having SCD at inclusion, but did not have cognitive complaints 2 years later when they underwent ^{18}F-FLUT PET. They had normal performance on cognitive tests and were classified as cognitively normal with resolution of previous cognitive complaints (CN).

Cohort 2 – MRI and DTI cohort:

Fifty subjects were included in the MCI project at Akershus University Hospital between January 2007 and February 2013 after having been referred to the hospital's memory clinic by their general practitioner. Inclusion criteria were cognitive complaints for at least 6 months and age 40–79. Exclusion criteria included established dementia, major psychiatric disorder, drug abuse, significant solvent exposure, and anoxic brain damage. All subjects underwent lumbar puncture and MRI at inclusion. The subjects were assessed with clinical interview, routine physical examination, blood screening, and a battery of cognitive tests. One subject was found to have been included in Cohort 1 and was therefore excluded from Cohort 2 when data from both cohorts were analyzed together (total number of unique subjects in the study $n = 89$). Subjects in Cohort 2 were defined as having MCI ($n = 39$) if objective cognitive impairment was evident on at least one of the following screening tests; MMSE score below 28, score equivalent to mild impairment on one or more of the items of the Cognistat (Kiernan et al., 1987) or score >1 on I-Flex (Royall et al., 1992). Subjects without objective cognitive impairment on the same screening battery were classified as having SCD ($n = 11$).

Ethics Statement

The study was conducted in accordance with the Helsinki Declaration. All participants gave written informed consent. The Regional Committee for Medical and Health Research Ethics, South East Norway, approved the study (approval 2009/2550 and 2013/150).

CSF Collection and Storage

Lumbar puncture was performed generally between 8 a.m. and noon, at the L3/L4, L4/L5, or L5/S1 interspace and without any serious adverse events. The first 4 ml CSF was used for routine clinical investigations. The next 1.5 and 4.5 ml CSF were collected in two polypropylene tubes and centrifuged at 2000 × *g* for 10 min within 4 h of collection. The 1.5 ml CSF was stored at −80°C prior to analysis of the traditional CSF biomarkers Aβ42, t-tau and p-tau. In Cohort 1, the 4.5 ml CSF was aliquoted into 450 μl polypropylene tubes before storage at −80°C, while in Cohort 2 the 4.5 ml CSF was stored at −80°C, before later being thawed and aliquoted, with further storage at −80°C prior to determination of Aβ43. Consequently, the samples underwent one freeze-thaw cycle before determination of Aβ43 in Cohort 1 and two in Cohort 2, with the exception of two samples in Cohort 2 where due to lack of CSF in the biobank, remaining CSF after analysis of the traditional CSF biomarkers was used also for analysis of Aβ43, resulting in three freeze-thaw cycles.

ELISA Assays and *APOE* Genotyping

Cerebrospinal fluid levels of Aβ42, t-tau, and p-tau were quantified with commercially available ELISAs; Innotest® β-amyloid 1–42 (Vanderstichele et al., 2000), Innotest® hTau Ag (Blennow et al., 1995), and Innotest® phosphoTau (181P) (Vanmechelen et al., 2000) (Fujirebio Europe, Gent, Belgium), and carried out in accordance with the manufacturers' instructions at the national reference laboratory for these tests at the Department of Interdisciplinary Laboratory Medicine and Medical Biochemistry, Akershus University Hospital. The laboratory lists the following cut-off values for abnormality (modified from Sjögren et al., 2001); t-tau > 300 pg/ml for age < 50 years, >450 pg/ml for age 50–69 years, and >500 pg/ml for age ≥ 70 years, p-tau ≥ 80 pg/ml and Aβ42 < 550 pg/ml.

Aβ43 in CSF was analyzed at the laboratory of the Department of Neuroscience, Norwegian University of Science and Technology, Trondheim, Norway, with an ELISA monoplex kit; Aβ1-43, RE59711 (IBL, Hamburg, Germany) run according to the instructions given by the manufacturer. The antibodies included in the kit were anti-human Aβ (38–43) rabbit IgG affinity purity and anti-human Aβ (82E1) mouse IgG MoAb Fab' affinity purity. According to the manufacturers of the kits the cross-reactivity for Aβ42 in the Aβ43 ELISA is <1% and the antibodies in the Innotest® β-amyloid 1–42 have 50x less affinity for Aβ43 compared to Aβ42. Samples of CSF were analyzed undiluted and in duplicate. The measurement range for the kit was reported to be 2.34–150 pg/ml. All samples analyzed in the study (7.51–66.49 pg/ml) fell within this range. Intra- and inter-assay variations have been reported previously (Lauridsen et al., 2016). As this study continued from the previously published material, these values were not calculated again.

Apolipoprotein E (*APOE*) genotyping was performed on EDTA blood samples from all subjects at the Gene Technology Division, Department of Interdisciplinary Laboratory Medicine and Medical Biochemistry, Akershus University Hospital according to the laboratory's routine protocol using real-time PCR combined with a TaqMan assay (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

MRI Imaging Acquisition and Processing

In Cohort 1, MRI scans were acquired on a Philips Achieva 3 Tesla system. A single 3D turbo field echo sequence was acquired for morphometric analysis with the following sequence parameters: TR/TE/TI/FA = 4.5 ms/2.2 ms/853 ms/8°, matrix = 256 × 213, 170 slices, thickness = 1.2 mm, in-plane resolution of 1 mm × 1.2 mm. In Cohort 2, MRI was performed on a Siemens Espree 1.5 T scanner. One 3D magnetization-prepared rapid gradient echo T1-weighted sequence was obtained with the following specifications: TR/TE/TI/FA = 2400 ms/3.65 ms/1000 ms/8°, matrix = 240 × 192, 160 slices, thickness = 1.2 mm, in-plane resolution of 1 mm × 1.2 mm. The pulse sequence used for DTI was: b = 750, 12 directions repeated five times, five b0-values per slice, TR = 6100 ms, TE = 117 ms, number of slices = 30, slice thickness = 3 mm (gap = 1.9 mm), in-plane resolution = 1.2 × 1.2 mm^2, bandwidth = 840 Hz/pixel. Cortical reconstruction and volumetric segmentation was performed with the FreeSurfer image analysis suite version 5.3.0[1]. This includes segmentation of the subcortical white matter and deep gray matter volumetric structures (Fischl et al., 2002) and parcellation of the cortical surface (Fischl et al., 2004) according to a previously published scheme labeling cortical sulci and gyri (Desikan et al., 2006), and thickness values are calculated over the cortical mantle. The thickness value of the entorhinal cortex (ERC) was calculated using a method based on ultra-high resolution *ex vivo* applied to *in vivo* MRI, as implemented in FreeSurfer (Fischl et al., 2009). In addition to hippocampal volume and cortical thickness of the ERC, the thickness of the following cortical regions of interest (ROIs) known to be atrophic relatively early in the development of AD were selected for analysis; the temporopolar, middle temporal, posterior cingulate, inferior parietal, and inferior frontal cortex. For analyses of the total hippocampal volume, the sum of the right and left hippocampal volumes as permillage (‰) of the estimated total intracranial volume was used. For each cortical ROI, the average of the measurements from the right and left hemisphere was used. Image processing for DTI has been described previously (Kalheim et al., 2016). DTI data were missing for one subject (n = 49). The fractional anisotropy, mean, radial, and axial diffusivities were assessed in the following four tracts, selected based on previous reports of DTI changes in AD and MCI (Amlien and Fjell, 2014; Lee et al., 2015) and calculated as an average of the metrics from the right and left hemispheres; the cingulum bundles (average of the cingulum-cingulate gyrus bundle and the cingulum-angular bundle), the corpus callosum-forceps bundles (average of the corpus callosum bundles to forceps major and minor, respectively), the uncinate fasciculus and the corticospinal tract.

PET Imaging Acquisition, Processing, and Interpretation

In Cohort 1 ^{18}F-FLUT and ^{18}F-FDG PET/CT imaging were performed on the same GE Discovery 690 PET/CT scanner on two separate days. Subjects received a bolus injection of 185 MBq (5 mCi) tracer and after resting were positioned head-first supine in the scanner. A low-dose CT scan was acquired first for attenuation correction. Subjects fasted at least 6 h in advance and blood glucose was measured routinely before ^{18}F-FDG injection (all subjects had blood glucose below 8.0 mmol/l). PET scanning in 3D-mode commenced 45 min after injection of ^{18}F-FDG and 90 min after ^{18}F-FLUT. PET data were acquired for 10 min for ^{18}F-FDG and for 20 min (four frames of 5 min) for ^{18}F-FLUT. Acquired data were corrected for random events, dead time, attenuation, scatter, and decay. PET volumes were reconstructed with an iterative algorithm (VUE Point FX SharpIR with six iterations, 24 subsets for ^{18}F-FDG, four iterations, 16 subsets for ^{18}F-FLUT) and smoothed with a post-reconstruction 3D Gaussian filter of 3 mm full-width at half maximum. Image format for ^{18}F-FDG was 256 × 256 (pixel size 1 mm × 1 mm), for ^{18}F-FLUT 192 × 192 (pixel size 1.3 mm × 1.3 mm), with slice

[1]http://surfer.nmr.mgh.harvard.edu/

thickness 3.75 mm. In Cohort 2 ^{18}F-FDG PET/CT-scans were acquired as previously described (Coello et al., 2013).

Visual interpretation of the ^{18}F-FLUT images was done by trained readers and the scans were classified as positive or negative in line with the manufacturer's guidelines. For the automated quantitative assessment, motion correction of the dynamic ^{18}F-FLUT PET was performed using frame by frame rigid registration, then the frames were summed to a single time-frame image and registered to the anatomical MRI volume using a six-parameter rigid registration as implemented in the Spatial Parametrical Mapping (SPM 12, Wellcome Trust Centre for Neuroimaging, UCL, UK) toolbox. Due to missing dynamic images one subject had to be excluded from the automated quantitative analyses ($n = 39$). Five cortical ROIs known to harbor substantial amyloid plaques in AD were selected for analysis of ^{18}F-FLUT uptake: the precuneus and posterior cingulate combined, anterior cingulate, prefrontal, inferior parietal and lateral temporal cortex. The average ^{18}F-FLUT uptake in each of these ROIs was calculated incorporating values from both hemispheres. The average uptake in the cerebellar cortex, which is usually devoid of amyloid pathology in early AD, was used as the reference region after eroding voxels at the segmentation boundaries to avoid influence due to inaccurate segmentation or co-registration. Regional standardized uptake value ratios (SUVRs) for ^{18}F-FLUT were created by dividing the average uptake in each ROI by the average uptake in the cerebellar cortex.

The same ROIs that were used for the structural MRI analyses were selected for study of ^{18}F-FDG activity. Uptake in the cerebellar white matter was used as the reference region after first eroding the cerebellar white matter mask. SUVRs were calculated by dividing the average uptake of ^{18}F-FDG per voxel in each ROI to the average uptake in the cerebellar white matter.

Statistical Analysis

The statistical analyses were performed using IBM SPSS version 23 (Chicago, IL, USA) unless otherwise stated. All tests were two-sided and *p*-values below 0.05 were considered significant. Distribution of the variables and whether normal distribution could be assumed were assessed by histograms and a Shapiro–Wilk test. Levene's statistics were calculated to assess the homogeneity of variance in each variable for parametric tests. For comparisons of CSF biomarker levels, demographical data and neuropsychological test results between SCD and MCI, a χ^2 test was used for categorical variables, an independent sample *t*-test for continuous variables with normal distribution, and a Mann–Whitney *U* test for continuous variables with non-normal distribution. Binary logistic regression models were created with the SCD/MCI distinction as the dependent variable and with either CSF tau, p-tau or one of the imaging biomarkers as a covariate. In significant models, CSF Aβ43 and Aβ42 were then in turn added as a second covariate. Bivariate correlation and partial correlation controlling for age were assessed between CSF biomarkers, MRI, ^{18}F-FLUT and ^{18}F-FDG variables. Spearman's rank coefficients (*rho*) of the correlations between an imaging variable and Aβ43, and the imaging variable and Aβ42, were compared with an asymptotic *z* test using software available from http://quantpsy.org (Lee and Preacher, 2013). To detect potential interrelating effects of aging, all analyses were done both unadjusted and with age as a covariate. Controlling for gender and educational length was not done, as these factors were not found to have significant impact in linear regression models of imaging measures as a function of Aβ. *APOE* genotype was related to CSF Aβ43 levels, but was not found to be a significant factor in regression models that already included either Aβ42 or Aβ43, and was therefore not included as a covariate. Differences in baseline CSF Aβ43 and Aβ42 levels between groups based on the result of the ^{18}F-FLUT PET were assessed using an independent samples *t*-test. Receiver operating characteristic (ROC) curves for the prediction of a positive ^{18}F-FLUT scan were plotted for both Aβ peptides, and area under the curve (AUC) was calculated. Cut-off values for Aβ43 and Aβ42 yielding the best combination of sensitivity and specificity, were determined by maximal Youden's index. Differences in AUC were assessed using MedCalc statistical software (MedCalc software, Mariakerke, Belgium).

RESULTS

Demographics, Cognition, and CSF Biomarkers: Comparison between SCD and MCI

Demographical characteristics, cognitive scores and CSF biomarker levels in the SCD and MCI groups are reported in **Table 1**. The frequency of the *APOE*ε4 genotype was similar in both SCD and MCI. The pattern of relative levels of the two CSF Aβ peptides was similar with respect to *APOE* genotype, with significantly lower mean peptide levels in the group with *APOE*ε4/ε4 (**Figure 1**). No significant difference for the correlations between *APOE* allele and Aβ43 and Aβ42 levels was found. There was a strong positive correlation between the CSF measurements of Aβ43 and Aβ42 (all subjects *rho* 0.88, $p < 0.001$) with no significant difference between the groups (SCD *rho* 0.81 and MCI *rho* 0.86). In the MCI group both Aβ43 and Aβ42 correlated inversely with t-tau and p-tau without any significant difference between the two amyloid peptides (MCI $n = 51$, Aβ43:t-tau *rho* −0.45, $p = 0.001$, Aβ42:t-tau *rho* −0.35, $p = 0.01$, Aβ43:p-tau *rho* −0.38, $p = 0.007$, Aβ42:p-tau *rho* −0.40, $p = 0.003$). In the SCD group, however, there were no significant correlations between Aβ43/Aβ42 and t-tau and p-tau. For all subjects ($n = 89$) the overall correlation coefficients for Aβ43:t-tau was *rho* −0.28, Aβ42:t-tau *rho* −0.25, Aβ43:p-tau *rho* −0.29, and for Aβ42:p-tau *rho* −0.37, without any significant differences between Aβ43 and Aβ42. Adjustment for age did not significantly change the correlations between the CSF biomarkers. In binary logistic regression models for the distinction between SCD and MCI, both Aβ43, Aβ42, and p-tau were statistically significant predictors when entered into the model as the only CSF

TABLE 1 | Demographics, cognitive scores, and cerebrospinal fluid (CSF) biomarkers in SCD and MCI.

	SCD		MCI		*p*
n	34		51		
Gender m/f, *n*	15/19		22/29		–
Age	64.5	[9]	65	[10]	–
Years of education	14	[4]	14	[5]	–
*APOE*ε4 (%)	47		45		–
MMSE *total score*	29	[1]	28	[2]	<0.001
RAVLT delayed recall *t-score*	57	[20]	47	[18]	<0.001
TMT B *t-score*	49	[9]	44	[11]	0.005
COWAT *t-score*	52	[13]	49	[16]	–
CSF Aβ43 *pg/ml*	37	[22]	24	[19]	0.004
CSF Aβ42 *pg/ml* (% below cut-off)	981	[488] (12)	679	[388] (26)	0.009 (0.17)
CSF t-tau *pg/ml* (% above cut-off)	312	[155] (6)	335	[267] (31)	0.13 (0.006)
CSF p-tau *pg/ml* (% above cut-off)	57	[25] (6)	69	[34] (35)	0.001 (0.003)

Data are presented as the median [interquartile range], unless otherwise stated. The cut-off values for CSF Aβ42, t-tau, and p-tau are those of the national reference laboratory: Aβ42 < 550 pg/mL, p-tau ≥ 80 pg/mL, and t-tau > 300 pg/mL for age < 50 years, >450 pg/mL for age 50–69 years, and >500 pg/mL for age ≥ 70 years. APOEε4 is presented as the percentage of subjects with at least one ε4-allele. The scores on TMTB, COWAT, and RAVLT were missing for one subject in Cohort 2. "–" p-value > 0.15. RAVLT, Rey Auditory Verbal Learning Test (Schmidt, 1996); TMT B, Trail Making Test B; COWAT, Controlled Oral Word Association Test; MCI, Mild cognitive impairment; SCD, subjective cognitive decline; APOE, Apolipoprotein E genotype.

biomarker. In a multivariate model with p-tau, inclusion of Aβ43 added significantly to the prediction, while Aβ42 did not (**Table 2**).

Amyloid PET

Based on visual interpretation nine of the 40 ^{18}F-FLUT PET scans were deemed to be positive (five SCD, four MCI), two borderline positive (one CN and one MCI), two borderline negative (one SCD and one MCI), and 27 negative (18 SCD, six MCI, three CN). The mean CSF Aβ43 in these four groups were 15, 22, 32, and 37 pg/ml, respectively, and the mean CSF Aβ42 547, 657, 1074, and 1042 pg/ml. The mean difference [95% CI] in CSF concentration between subjects with positive and negative scans was 22 pg/ml [13,31] for Aβ43 and 495 pg/ml [380,611] for Aβ42, $p < 0.001$ for both. ROC curves for prediction of a positive scan gave AUC 0.97 for both Aβ43 and Aβ42. The best cut-off value was ≤24 pg/ml for Aβ43 with sensitivity 100% and specificity 93% for a positive scan, and ≤679 pg/ml for Aβ42 with sensitivity 100% and specificity 89%. In the group with negative scans, 16/27 (59%) had *APOE* genotype *APOE*ε3/ε3 and 9/27 (33%) *APOE*ε3/ε4, the same in the group with positive scans was 2/9 (22%) and 5/9 (56%), but the differences were not statistically significant. ^{18}F-FLUT SUVR based on the automated quantitative assessment of the scans was not a significant predictor of the SCD/MCI distinction in binary logistic regression models with and without age as a covariate. Mean overall ^{18}F-FLUT SUVR for the five ROIs was 1.29, 95% CI [1.17–1.40] in the SCD group and 1.40, 95% CI [1.23–1.58] in the MCI group ($p = 0.26$). There were highly significant inverse correlations between CSF Aβ43 and ^{18}F-FLUT SUVR in all the examined ROIs and the correlations became stronger with adjustment for the effect of age (**Table 3**). The same was true for Aβ42, and there were no significant differences between the correlation coefficients for the two Aβ peptides. The correlations with overall ^{18}F-FLUT SUVR remained strong also when analyzing only subjects diagnosed with SCD ($n = 23$; *rho* −0.64, $p = 0.001$ for both Aβ peptides, adjusted for age *rho* −0.69, $p < 0.001$ for Aβ43, and *rho* −0.67, $p = 0.001$ for Aβ42). When excluding subjects with visually interpreted definitely positive scans from the analysis ($n = 31$), there were still significant correlations with overall SUVR; for Aβ43 unadjusted *rho* −0.37, $p = 0.04$, adjusted for age *rho* −0.52, $p = 0.004$, for Aβ42 unadjusted *rho* −0.39, $p = 0.03$, adjusted for age *rho* -0.51, $p = 0.004$. CSF t-tau and p-tau were not significantly correlated with overall ^{18}F-FLUT SUVR (t-tau *rho* 0.29, $p = 0.07$, p-tau *rho* 0.24, $p = 0.15$).

Hippocampal Volume and Cortical Thickness

Magnetic resonance imaging data from the two cohorts were analyzed separately due to the use of different scanners for the imaging acquisition. There were no significant differences in mean hippocampal volume or thickness in the six cortical ROIs between SCD and MCI in either of the cohorts (Supplementary Table) and none of the ROIs were significant predictors of the SCD/MCI distinction in binary logistic regression models even after adjustment for age. In Cohort 1 there were no significant correlations between Aβ43/42 CSF levels and the structural MRI measurements. In Cohort 2 there were unadjusted moderate positive correlations for both CSF Aβ43 and Aβ42 with total hippocampal volume, thickness of the middle temporal, inferior parietal and inferior frontal cortices with statistical significance after correction for multiple testing in seven ROIs. CSF Aβ43 correlated significantly also with thickness of the ERC and CSF Aβ42 with posterior cingulate cortical thickness. After adjustment for effects of age, however, only the correlations with hippocampal volume, inferior parietal and inferior frontal cortical thickness were nominally significant (**Table 4**). In the SCD group CSF Aβ43 correlated significantly with hippocampal volume and CSF Aβ42 with thickness of the

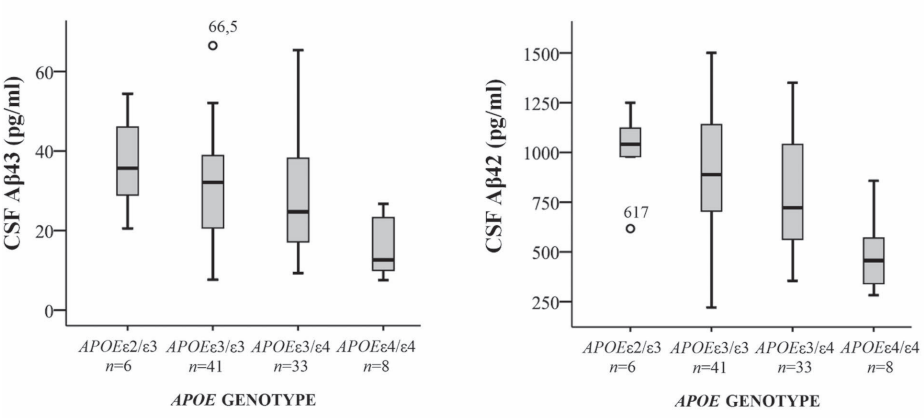

FIGURE 1 | Box-plots of cerebrospinal fluid (CSF) Aβ43 and Aβ42 according to *APOE* genotype. All subjects from both cohorts have been included, except for one subject with genotype *APOE*ε2/ε4. CSF Aβ43 for this subject was 17 pg/ml and CSF Aβ42 598 pg/ml. The whiskers represent the range, except for the two outliers. There were significant differences in mean CSF Aβ43 and Aβ42 levels between the *APOE*ε4/ε4-group and the three other groups (all $p<0.01$). *APOE*, Apolipoprotein E genotype.

TABLE 2 | Specification of logistic regression models for the SCD/MCI distinction with one or two CSF biomarkers as covariates together with age.

Model	χ^2	Nagelkerke R^2	Sensitivity	Specificity		OR	*p*
Aβ43	10.2, $p = 0.006$	0.15	88%	50%		0.95	0.004
Aβ42	8.6, $p = 0.01$	0.13	88%	44%		0.998	0.008
p-tau	10.8, $p = 0.005$	0.16	80%	50%		1.03	0.008
t-tau	3.4, $p = 0.18$						
p-tau + Aβ43	15.9, $p = 0.001$	0.23	84%	56%	p-tau	1.03	0.03
					Aβ43	0.96	0.03
p-tau + Aβ42	13.8, $p = 0.003$	0.20	88%	53%	p-tau	1.02	0.04
					Aβ42	0.998	0.09

OR, Odds ratio; MCI coded 1, SCD 0. n = 85.

TABLE 3 | Correlation analyses between ^{18}F-FLUT SUVRs and Aβ43 or Aβ42 in CSF, unadjusted and adjusted for age.

	Unadjusted		Adjusted for age	
	Aβ43	**Aβ42**	**Aβ43**	**Aβ42**
Prefrontal SUVR	−0.60**	−0.64**	−0.71**	−0.73**
Precuneus – Posterior cingulate SUVR	−0.67**	−0.70**	−0.74**	−0.75**
Anterior cingulate SUVR	−0.61**	−0.67**	−0.71**	−0.75**
Inferior parietal SUVR	−0.65**	−0.70**	−0.72**	−0.76**
Lateral temporal SUVR	−0.54*	−0.59**	−0.64**	−0.67**
Average of the five SUVRs	−0.63**	−0.67**	−0.73**	−0.74**

*Data presented are Spearman's rank coefficients for correlations between ^{18}F-FLUT SUVRs (standardized uptake value ratios) and CSF levels of Aβ43 and Aβ42, respectively. Dynamic ^{18}F-FLUT data were missing for one subject, n = 39. *Correlations with p-values < 0.001. **Correlations with p-values < 0.0001. There were no statistically significant differences in correlation coefficients between CSF Aβ43 and Aβ42.*

posterior cingulate cortex. In the MCI group there were no significant correlations after correction for age. None of the correlations were significantly different between CSF Aβ43 and Aβ42, there was only a trend toward the correlation coefficient for hippocampal volume being stronger with CSF Aβ43 than Aβ42 ($p = 0.05$ unadjusted for age, $p = 0.07$ adjusted for age) in the SCD group. Both CSF Aβ43 and Aβ42 were significant predictors of hippocampal volume in linear regression both with and without age in the model. T-tau was a significant predictor when modeled alone and with age, but not when either of the CSF Aβ peptides were entered into the same model.

TABLE 4 | Correlations between CSF Aβ43, CSF Aβ42, hippocampal volume, and cortical thickness in SCD and MCI subjects together or separately in Cohort 2.

		All		SCD $n = 11$		MCI $n = 39$	
		Aβ43	**Aβ42**	**Aβ43**	**Aβ42**	**Aβ43**	**Aβ42**
Hippocampus volume, ‰		0.52**	0.52**	0.66*	0.26	0.51**	0.51**
	Age-adjusted	0.33*	0.30*	0.64*	0.29	0.28	0.25
Entorhinal cortex thickness		0.39**	0.29*	0.29	−0.05	0.35*	0.23
	Age-adjusted	0.25	0.10	0.20	−0.09	0.23	0.07
Posterior cingulate cortex thickness		0.37*	0.39**	0.47	0.66*	0.23	0.28
	Age-adjusted	0.22	0.23	0.43	0.72*	0.02	0.04
Temporopolar cortex thickness		0.20	0.27	0.29	0.17	0.13	0.24
	Age-adjusted	0.05	0.12	0.24	0.17	−0.07	0.04
Middle temporal cortex thickness		0.46**	0.51**	0.52	0.34	0.43**	0.51**
	Age-adjusted	0.27	0.32*	0.49	0.39	0.18	0.26
Inferior parietal cortex thickness		0.50**	0.52**	0.55	0.45	0.47**	0.51**
	Age-adjusted	0.30*	0.29*	0.54	0.56	0.22	0.24
Inferior frontal cortex thickness		0.41**	0.41**	0.43	0.34	0.39*	0.40*
	Age-adjusted	0.32*	0.31*	0.42	0.34	0.25	0.24

*Data presented are Spearman's rank coefficients for bivariate correlation and partial correlation correcting for age. n = 50. *Nominal significance ($p < 0.05$), **Significant correlations with correction for multiple testing in seven regions of interest ($p < 0.05/7$). None of the correlation coefficients were significantly different between CSF Aβ43 and Aβ42.*

Diffusor Tensor Imaging

Diffusor tensor imaging was only available for Cohort 2. There were no statistically significant differences in the DTI metrics of the selected tracts between the SCD and MCI groups and in logistic regression models none of the DTI metrics were significant covariates for the SCD/MCI distinction even after adjustment for age. Both CSF Aβ43 and Aβ42 were inversely correlated with axial diffusivity in the corticospinal tract and this was the only correlation that maintained significance after correction for multiple testing. Both Aβ peptides showed a nominal significant negative correlation with mean diffusivity in the cingulum bundles and corticospinal tract. There was also a nominal significant negative correlation for CSF Aβ42 and radial diffusivity, but a positive correlation with fractional anisotropy in the cingulum bundles. Comparing the two Aβ peptides, the positive correlation with fractional

TABLE 5 | Correlations between CSF Aβ43, CSF Aβ42, and diffusion tensor imaging metrics in the selected tracts.

	Unadjusted			Adjusted for age		
	Aβ43	**Aβ42**	***p***	**Aβ43**	**Aβ42**	***p***
FA Cingulum	0.35*	0.53**	0.007*	0.18	0.40*	0.01*
FA Corticospinal	0.11	0.17	–	−0.10	−0.06	–
FA Callosum-Forceps	0.21	0.30*	–	−0.02	0.06	–
FA Uncinate fasciculus	−0.02	0.01	–	−0.19	−0.18	–
DR Cingulum	−0.43**	−0.55**	–	−0.25	−0.39*	–
DR Corticospinal	−0.29*	−0.32*	–	−0.11	−0.12	–
DR Callosum-Forceps	−0.29*	−0.33*	–	−0.07	−0.10	–
DR Uncinate fasciculus	−0.04	−0.03	–	0.18	0.23	–
DA Cingulum	−0.40*	−0.32*	–	−0.27	0.16	–
DA Corticospinal	−0.36*	−0.34*	–	−0.43**	−0.42**	–
DA Callosum-Forceps	−0.36*	−0.34*	–	−0.24	−0.21	–
DA Uncinate fasciculus	−0.28	−0.23	–	−0.09	0.004	–
MD Cingulum	−0.45**	−0.52**	–	−0.30*	−0.37*	–
MD Corticospinal	−0.44**	−0.41**	–	−0.34*	−0.30*	–
MD Callosum-Forceps	−0.31*	−0.34*	–	−0.11	−0.12	–
MD Uncinate fasciculus	−0.13	−0.10	–	0.10	0.17	–

*Data presented are Spearman's rank coefficients for bivariate correlation and partial correlation correcting for age, and significant p-values for the test of difference between the correlation coefficients for CSF Aβ43 and Aβ42. Diffusion tensor imaging measurements were missing for one subject, n = 49. FA, fractional anisotropy; DR, radial diffusivity; DA; axial diffusivity, MD; mean diffusivity. *Nominal significance ($p < 0.05$), **Significance with Bonferroni correction for multiple testing ($p < 0.05/16$).*

anisotropy in the cingulum bundles was slightly stronger for CSF Aβ42 compared to CSF Aβ43, though it did not reach significance after strict Bonferroni correction for multiple testing (**Table 5**).

Cortical Glucose Metabolism

As the ^{18}F-FDG PET scans were obtained on different scanners in the two cohorts, the data were analyzed in each cohort separately. There were no significant differences in overall ^{18}F-FDG SUVR between the SCD and MCI groups and adjustment for age did not change this. There were no significant correlations between ^{18}F-FDG SUVRs and either CSF Aβ43 or CSF Aβ42 in Cohort 1 ($n = 28$). In the larger Cohort 2 ($n = 50$) both CSF Aβ43 and Aβ42 appeared to be correlated with glucose metabolism in the hippocampus and several of the cortical ROIs, but this changed after correction for the effect of age when only the correlation between CSF Aβ42 and ^{18}F-FDG uptake in the entorhinal cortex was significant after correction for multiple testing. Looking at the SCD and MCI subjects separately, the correlation between CSF Aβ43 and ^{18}F-FDG uptake in the posterior cingulate cortex was nominally significant after correction for age, while the only significant correlation after correction for multiple testing was that between CSF Aβ42 and ^{18}F-FDG uptake in the posterior cingulate cortex in the MCI group (**Table 6**). By direct comparison none of the differences in correlation coefficients between CSF Aβ42 and Aβ43 were statistically significant. Unadjusted there was an inverse relation between p-tau and overall ^{18}F-FDG uptake (average of the seven ROIs), but after correction for age there were no significant correlations with either p-tau or t-tau.

DISCUSSION

The interest in CSF Aβ43 as a biomarker first arose from experimental data suggesting that this peptide could be more prone to aggregation than Aβ42, and thus potentially have importance for amyloidogenesis in AD (Saito et al., 2011; Zou et al., 2013; Conicella and Fawzi, 2014; Burnouf et al., 2015). Our results show that CSF Aβ43 levels are inversely correlated with cortical amyloid deposits, even at the stage of SCD and before extensive amyloid pathology is evident. However, results revealed nothing to support the hypothesis that the amyloidogenic impact of Aβ43 is different to that of Aβ42. The strength of the correlation between CSF Aβ42 and amyloid load was comparable in the present study to that reported in other studies (Jagust et al., 2009; Tolboom et al., 2009; Landau et al., 2013; Palmquist et al., 2014). Investigating the potential role of CSF Aβ43 in very early AD pathology is difficult. It has been suggested that CSF Aβ42 levels start to drop prior to the increase in amyloid tracer uptake (Fagan et al., 2006; Mattsson et al., 2015), but contradictory results have also been presented (Landau et al., 2013). ^{18}F-FLUT only binds Aβ when it has formed extensive β-sheet formations in insoluble fibrils, and does not bind to the soluble Aβ oligomers that are suggested more likely to be the main neurotoxic culprit in AD (Selkoe and Hardy, 2016). Whether Aβ43 in CSF may have an impact on the quantity and toxicity of oligomers cannot be answered by the current imaging techniques. Recently, the first successful use of a monoclonal antibody-based PET ligand, capable of binding soluble Aβ protofibrils, was demonstrated in two AD mouse models (Sehlin et al., 2016). Future use of similar radioligands in humans could possibly elucidate the impact on oligomers.

TABLE 6 | Correlations between CSF Aβ43, CSF Aβ42, and ^{18}F-FDG SUVRs in Cohort 2.

		All		SCD $n = 11$		MCI $n = 39$	
		Aβ43	Aβ42	Aβ43	Aβ42	Aβ43	Aβ42
Hippocampus		0.32*	0.42**	0.37	0.53	0.35*	0.43**
	Age-adjusted	0.12	0.22	0.39	0.54	0.01	0.08
Entorhinal		0.35*	0.47**	0.26	0.60	0.34*	0.43**
	Age-adjusted	0.25	0.38**	0.29	0.60	0.14	0.24
Posterior cingulate		0.40**	0.53**	0.08	0.30	0.61**	0.69**
	Age-adjusted	0.16	0.31*	0.05	0.30	0.37*	0.46**
Temporopolar		0.28	0.36*	0.22	0.48	0.22	0.31
	Age-adjusted	0.14	0.23	0.26	0.49	−0.03	0.05
Middle temporal		0.37*	0.44**	0.13	0.31	0.40*	0.46**
	Age-adjusted	0.16	0.24	0.16	0.31	0.10	0.14
Inferior parietal		0.42**	0.50**	0.27	0.42	0.51**	0.56**
	Age-adjusted	0.19	0.27	0.30	0.42	0.22	0.24
Inferior frontal		0.25	0.37*	0.06	−0.01	0.38*	0.50**
	Age-adjusted	−0.05	0.09	0.06	−0.01	−0.02	0.13
Average of all six cortical ROIs		0.41**	0.51**	0.07	0.35	0.48**	0.55**
	Age-adjusted	0.21	0.31*	0.09	0.35	0.19	0.26

*Data presented are Spearman's rank correlation coefficients for bivariate correlations and partial correlations controlling for age. *Nominal significance ($p < 0.05$), **Significance with Bonferroni correction for multiple testing ($p < 0.05/7$). None of the differences in correlations coefficients between CSF Aβ43 and Aβ42 were statistically significant.*

Measurements of Aβ43 in CSF have not previously been described in relation to cerebral imaging findings, while CSF Aβ42 has been extensively studied. CSF Aβ42 has been shown previously to correlate with hippocampal volume in several cross-sectional studies (Apostolova et al., 2010; Wang et al., 2015). Some longitudinal studies have reported no association between CSF Aβ42 and hippocampal volume at baseline, but an association with subsequent hippocampal atrophy (Schuff et al., 2009; Tosun et al., 2010; Stricker et al., 2012; Mattsson et al., 2014). Other studies have shown no association either at baseline (de Souza et al., 2012) or longitudinally (Henneman et al., 2009; Tarawneh et al., 2015). One explanation for the inconsistency is that the rates of alteration of analytes in CSF and imaging biomarkers are neither parallel nor linear. As a result the correlations between biomarkers will change over time with disease progression (Insel et al., 2016). Divergences in how the various disease stages are defined will further contribute to this variability. We found that the correlation with hippocampal volume tended to be stronger in the SCD group in Cohort 2, especially for CSF Aβ43. Previous studies have shown that brain amyloid load is related to hippocampal volume in cognitively healthy elderly (Dickerson et al., 2009) and in SCD, but not in MCI and AD (Bourgeat et al., 2010; Chételat et al., 2010a). Similarly, Fagan et al. (2009) found that CSF Aβ42 correlated with whole-brain volume in elderly subjects without cognitive impairment, but not in MCI and AD, suggesting that the association between atrophy and amyloid could be present only early in the disease process. Many studies have described a strong correlation between CSF tau and hippocampal atrophy in MCI and AD (Henneman et al., 2009; Apostolova et al., 2010; de Souza et al., 2012; Tarawneh et al., 2015). In the current study, we found that hippocampal volume was better predicted by either CSF Aβ43 or Aβ42 than by t-tau or p-tau, which is in line with former studies in SCD and healthy elderly individuals. That the result differs from past reports in patients with MCI may possibly be attributed to the younger age of our MCI subjects compared to many of the previously published MCI cohorts.

White matter changes are also known to be related to CSF biomarkers (Amlien and Fjell, 2014). CSF Aβ42 has been shown to be positively associated with fractional anisotropy and inversely with mean diffusivity (Gold et al., 2014; Li et al., 2014) as found also in the current study. Both DTI and ^{18}F-FLUT PET have been reported to be superior to the core CSF biomarkers in predicting the conversion from MCI to dementia (Selnes et al., 2013; Shaffer et al., 2013; Perani et al., 2016). Therefore, it was particularly interesting to compare these biomarkers with CSF Aβ43 that in a previous study was suggested to have the same quality. Surprisingly, fractional anisotropy in the cingulum fibers appeared to be better correlated with CSF Aβ42 than Aβ43 and the trend was the same for cortical glucose metabolism.

The current study has several limitations. The use of different MRI and PET-scanners in the two cohorts made direct comparisons between cohorts challenging. Cortical thickness was measured to be higher in Cohort 2 than in Cohort 1 for several of the ROIs even though p-tau levels were on average higher in Cohort 2. Correcting for age resulted in only a slight reduction in the between cohort differences in these ROIs. It is known that differences in scanner field strength and possibly also scanner settings like pulse sequence, can impact on regional cortical thickness measurements (Han et al., 2006; Govindarajan et al., 2014; McCarthy et al., 2015), which may have contributed to the described differences between the cohorts. The cortical thickness measures were obtained by automated segmentation using the freely available and widely used software FreeSurfer. FreeSurfer version, operating system and workstation used in the processing can also impact the cortical thickness measurements (Gronenschild et al., 2012), but were identical for the two cohorts in this study. Some have suggested that there could be a transitional phase in the development of AD with increased thickness of certain cortical areas (Chételat et al., 2010b; Fortea et al., 2011; Molinuevo et al., 2012), but this has mainly been described in pre-clinical stages. Because we suspected a significant scanner effect, the imaging data were analyzed in each cohort separately, with a lower number of subjects in each analysis as a consequence. The cohorts came from somewhat different populations; all subjects in Cohort 2 were consecutively recruited from a memory clinic, while Cohort 1 also included subjects recruited by advertisements. Greater variability due to partly community based recruitment and fewer subjects with abnormal CSF biomarkers could be the reason why no correlation with neurodegenerative imaging biomarkers was found in Cohort 1. The study is also limited by the fact that we only included subjects that had already developed cognitive symptoms (which are only subjective in the case of SCD). We could therefore not assess CSF Aβ43 in pre-clinical stages of AD such as in cognitively normal subjects with positive ^{18}F-FLUT PET, nor could we evaluate the impact of biomarkers on the distinction between controls and SCD. This ought to be assessed in future studies.

CONCLUSION

In this first description of CSF Aβ43 in relation to imaging biomarkers, we found that CSF levels of Aβ43 are inversely correlated with fibrillary Aβ accumulation in the brain and more weakly positively correlated with biomarkers of neurodegeneration including hippocampal volume. However, none of the studied correlations between CSF Aβ and imaging measurements were significantly different between the two Aβ peptides when controlling for multiple testing. We conclude that in respect to imaging, CSF Aβ43 does not appear to contribute any added value over the well-established CSF biomarker Aβ42 in distinguishing individuals with SCD from those with MCI.

AUTHOR CONTRIBUTIONS

IA planned the study, recruited and clinically examined study participants, performed the statistical analyses and wrote the manuscript. CL planned the study and performed ELISA of CSF Aβ43 together with IM. PS and LK processed the MRI and ^{18}F-FDG PET images and performed clinical examinations of participants. CC processed the ^{18}F-FLUT PET images. BG visually interpreted ^{18}F-FDG and ^{18}F-FLUT PET scans. MW

carried out laboratory work and administered the CSF biobank. RG did neuropsychological assessments of participants. AB supervised imaging acquisition. LW, SS, GB, and TF supervised the project. All authors critically revised and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2017.00009/full#supplementary-material

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SUPPLEMENTARY TABLE: Hippocampal volume and cortical thickness values in the SCD and MCI groups in the two cohorts.

	SCD			MCI			SCD vs MCI	
	Cohort 1	Cohort 2	Cohort 1 vs 2 (*p*)	Cohort 1	Cohort 2	*Cohort 1 vs 2 (p)*	*Cohort 1 (p)*	*Cohort 2 (p)*
n	24	10		12	39			
Age	65.5 (9)	63 (15)	-	63.5 (10)	65 (11)	-	-	-
CSF Aβ43	32 (22)	41 (23)	-	24 (21)	24 (18)	-	-	0.01
CSF Aβ42	973 (560)	1002 (482)	-	772 (510)	659 (350)	-	-	-
CSF t-tau	320 (167)	295 (147)	-	329 (207)	335 (293)	-	-	-
CSF p-tau	53 (25)	61 (15)	0.02	53 (23)	74 (31)	0.02	-	-
Hippocampal volume, ‰	5.36 (1.04)	4.85 (0.72)	-	5.38 (1.30)	4.85 (1.16)	-	-	-
Entorhinal cortex, *mm*	3.56 (0.34)	3.46 (0.42)	-	3.59 (0.24)	3.30 (0.46)	0.02	-	-
Posterior cingulate cortex, *mm*	2.17 (0.21)	2.39 (0.30)	0.002	2.17 (0.22)	2.31 (0.26)	0.02	-	-
Temporopolar cortex, *mm*	3.40 (0.42)	3.70 (0.29)	-	3.37 (0.49)	3.65 (0.39)	0.02	-	-
Middle temporal cortex, *mm*	2.50 (0.25)	2.77 (0.31)	<0.001	2.53 (0.36)	2.76 (0.31)	0.003	-	-
Inferior parietal cortex, *mm*	2.08 (0.17)	2.33 (0.19)	<0.001	2.14 (0.18)	2.21 (0.30)	0.03	-	-
Inferior frontal cortex, *mm*	2.19 (0.17)	2.49 (0.29)	<0.001	2.17 (0.18)	2.44 (0.20)	<0.001	-	-

Data presented are median (interquartile range) and significant *p*-values for the comparison between cohorts for the SCD and MCI groups separately and for the comparison between SCD and MCI for each cohort separately. Hippocampal volume is the combined volume of the right and left hippocampus as parts per thousand (‰) of the estimated total intracranial volume. "-" No statistical significance ($p>0.05$).