Doctoral thesis

Cerebrospinal fluid biomarkers for the study of the pathophysiological pathways in Alzheimer's disease

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Departament de Medicina. Facultat de Medicina
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To the patients, volunteers and their families,
whose generosity has allowed us
to give one more step in the knowledge of
Alzheimer's disease

A los pacientes, voluntarios y sus familias,
cuya generosidad nos ha permitido
dar un paso más en el conocimiento de la
enfermedad de Alzheimer

List of Content

List of articles included in this thesis

The main body of this thesis consists of a compilation of the following articles:

1. **Alcolea D**, Martínez-Lage P, Izagirre A, Clerigué M, Carmona-Iragui M, Alvarez RM, Fortea J, Balasa M, Morenas-Rodríguez E, Lladó A, Grau O, Blennow K, Lleó A, Molinuevo JL. Feasibility of Lumbar Puncture in the Study of Cerebrospinal Fluid Biomarkers for Alzheimer's Disease: A Multicenter Study in Spain. J Alzheimers Dis. 2014;39(4):719-726.
IF 2014: 4.151. Category: Neurosciences Q1

2. Pera M, **Alcolea D**, Sánchez-Valle R, Guardia-Laguarta C, Colom-Cadena M, Badiola N, Suárez-Calvet M, Lladó A, Barrera-Ocampo AA, Sepulveda-Falla D, Blesa R, Molinuevo JL, Clarimón J, Ferrer I, Gelpi E, Lleó A. Distinct patterns of APP processing in the CNS in autosomal-dominant and sporadic Alzheimer disease. Acta Neuropathol. 2013 Feb;125(2):201-213.
IF 2014: 10.762. Category: Clinical Neurology Q1 / Neurosciences Q1 / Pathology Q1

3. **Alcolea D**, Carmona-Iragui M, Suárez-Calvet M, Sánchez-Saudinós MB, Sala I, Antón-Aguirre S, Blesa R, Clarimón J, Fortea J, Lleó A. Relationship between β-Secretase, inflammation and core cerebrospinal fluid biomarkers for Alzheimer's disease. J Alzheimers Dis. 2014;42(1):157-167
IF 2014: 4.151. Category: Neurosciences Q1

4. **Alcolea D**, Vilaplana E, Pegueroles J, Montal V, Sánchez-Juan P, González-Suárez A, Pozueta A, Rodríguez-Rodríguez E, Bartrés-Faz D, Vidal-Piñeiro D, González-Ortiz S, Medrano S, Carmona-Iragui M, Sánchez-Saudinós M, Sala I, Anton-Aguirre S, Sampedro F, Morenas-Rodríguez E, Clarimón J, Blesa R, Lleó A, Fortea J. Relationship between cortical thickness and cerebrospinal fluid YKL-40 in predementia stages of Alzheimer's disease. Neurobiol Aging. 2015 Jun;36(6):2018-2023.
IF 2014: 5.013. Category: Neurosciences Q1 / Gerontology Q1

5. **Alcolea D**, Martínez-Lage P, Sánchez-Juan P, Olazarán J, Antúnez C, Izagirre A, Ecay-Torres M, Estanga A, Clerigué M, Guisasola MC, Sánchez Ruiz D, Marín Muñoz J, Calero M, Blesa R, Clarimón J, Carmona-Iragui M, Morenas-Rodríguez E, Rodríguez-Rodríguez E, Vázquez Higuera JL, Fortea J, Lleó A. Amyloid precursor protein metabolism and inflammation markers in preclinical Alzheimer disease. Neurology. 2015 Aug 18;85(7):626-633.
IF 2014: 8.286. Category: Clinical Neurology Q1

Chapter 1

Introduction, hypotheses, objectives and outline

1. Introduction

1.1. Dementia and Alzheimer's disease

1.1.1. Epidemiology and clinical description

Dementia syndrome is a major cause of dependency among older people, and its prevalence is increasing as population ages.[1,2] The Delphi epidemiologic study estimated in 2005 that at the beginning of the decade 24 million people had dementia worldwide and that this number would double every 20 years reaching more than 80 million affected by 2040.[2] We know today that Alzheimer's disease (AD) is the most common cause of dementia in the western world.[1,3]

AD causes a progressive impairment of cognitive functions, frequently associated with behavioural disturbances that have devastating effects on patients and on their caregivers and families. The typical and most frequent presentation in AD is the amnestic syndrome, in which the patient undergoes a gradual deterioration of episodic memory that affects the ability to acquire and recall recently learnt information.[4] But other clinical presentations are also observed. In the atypical syndromes, the most prominent deficits might be word-finding difficulties (language presentation), spatial cognition alterations (visuospatial presentation) or impaired reasoning, problem solving and behavioural disturbances (frontal variants).[4] As these symptoms progress, they may significantly interfere with the ability to function at work or in usual activities, and it is at these stages when the clinical diagnosis of dementia can be established.

The pathological changes underlying AD are known to start decades before the clinical diagnosis of dementia can be made. In initial stages, the patient experiences a progression from normal cognition or subtle cognitive decline (preclinical stages) to a mild cognitive

impairment (MCI), in which cognitive symptoms are evident but do not have significant functional consequences (prodromal stage). Currently available treatments for AD have been proven to be effective only in dementia stages, and their effect is marginal and only symptomatic,[5–8] i.e. they do not modify the progression of the disease.[9] For these reasons, current treatment strategies in AD are focused on earlier stages, in which they are thought to be more effective to change the course of the disease by targeting fundamental pathophysiological pathways,[10,11] hence the importance of an early and accurate diagnosis.

1.1.2. Pathological overview

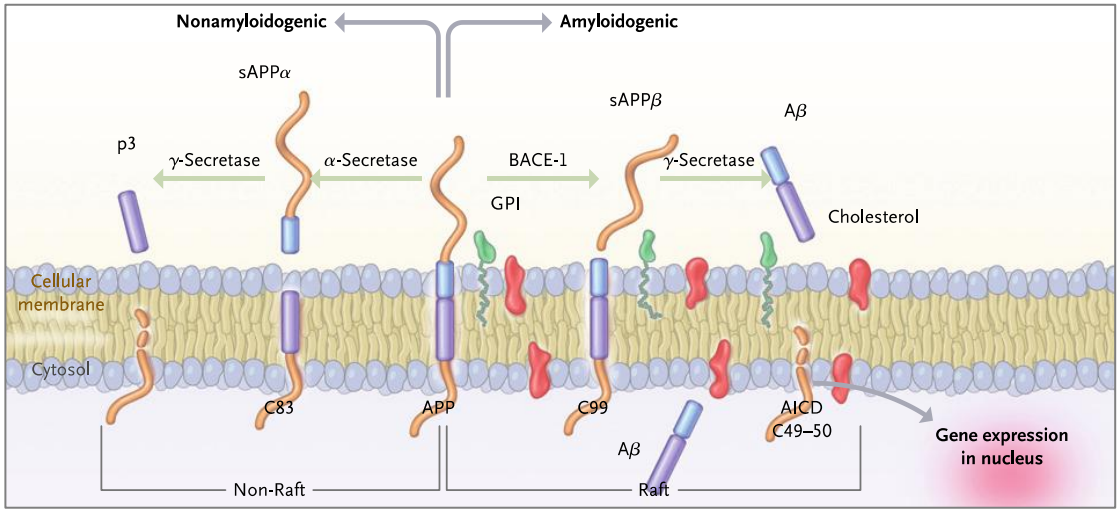
The main neuropathological hallmarks of AD are amyloid plaques and neurofibrillary tangles. Amyloid plaques are formed by accumulation of the aggregated Aβ peptide, which in turn results from the cleavage of the amyloid precursor protein (AβPP) by a β- and a γ-secretase (**Figure 1**).[12] The "amyloid cascade hypothesis" suggests that the chronic imbalance between the Aβ peptide production and clearance in the brain would result in its accumulation in the form of oligomers, fibrils, and amyloid plaques. These aggregates would trigger a cascade of pathogenic events leading to synaptic disfunction and neuronal death.[3,13] This hypothesis has been accepted for decades and supported extensively in the literature. However, a few controversies have also challenged its full validity.[14] Among others, the fact that the amount of amyloid pathology does not correlate well with cognitive decline[15,16], and that amyloid plaques are also found in cognitively normal individuals have suggested that amyloid could be an incidental product of questionable pathogenic consequences.[14]

Neurofibrillary tangles are intracellular inclusions mainly composed by the abnormally hyperphosphorylated form of the microtubule-associated protein Tau.[17] Hyperphosphorylation reduces the affinity of Tau for microtubules, and it might also facilitate its aggregation.[18] Neurofibrillary tangles spread following a specific topographical

distribution that starts in transentorhinal cortex, continues in limbic areas, and in the latest stages, involves neocortical regions.[19–21] Unlike the amyloid plaque burden, the extent of neurofibrillary pathology correlates with the disease severity.[22–24] Neurofibrillary tangles, however, are not specific to AD and can also be observed in different distributions in other neurodegenerative disorders known as tauopathies.[3,18,25]

Figure 1. Normal and pathological proteolytic processing of amyloid precursor protein

Adapted from Querfurth et al.[3] In the amyloidogenic pathway, after the action of the β-site amyloid precursor protein-cleaving enzyme 1 (BACE-1) two products are released: an extracellular soluble fragment (sAPPβ), and a transmembrane C99 peptide, which after the action of a γ-secretase is cleaved into the extracellular Aβ peptide and the amyloid intracellular domain (AICD).



Inflammation is another key process in the pathogenesis of AD.[26–28] Experimental studies support that the activation of the inflammatory cells in the brain, microglia and astrocytes, in AD might be triggered by the abnormal protein aggregates. Microglial cells, the resident phagocytes of the central nervous system, bind to Aβ oligomers and fibrils, which causes the activation of an innate immune response and the release of cytokines and chemokines.[28] Reactive astrocytes are frequently found surrounding amyloid plaques and are believed to exacerbate and perpetuate the inflammatory response and the release of cytotoxic

products.[28,29] The important role of inflammation in the pathogenesis of AD is also supported by the fact that several genes involved in inflammatory pathways have been found to modulate the risk of AD.[28,30–33]

1.1.3. Genetic aspects: Autosomal dominant AD and sporadic AD

The only identified deterministic factors for the development of AD are mutations in the amyloid precursor protein (*APP*) gene or in one of the two presenilin genes (*PSEN1* and *PSEN2*).[34] Mutations in these genes cause a shift in the processing of the AβPP favouring the production of Aβ species that are more prone to aggregate.[35,36] With virtually a complete penetrance, people bearing mutations in *APP*, *PSEN1* or *PSEN2* will develop an AD dementia of an early onset (generally between 30 and 50 years of age), and will transmit the pathogenic mutation to aproximately half of their kindred.[34] However, these autosomal-dominant forms of AD (ADAD) account for a small proportion of all AD cases, estimated between 1 and 5%.[34,37]

For the most common form, known as sporadic AD (SAD), several potential risk genes have been identified. The apolipoprotein E gene (*APOE*) is the most consistent one. Three allele variants exist for this gene: *APOE-ε2*, *APOE-ε3* and *APOE-ε4*. The presence of the ε4 allele increases the lifetime risk of developing AD dementia and lowers the age of onset in a gene-dose dependent manner.[38,39] Subjects with the ε4/ε4 genotype are more than eight times as likely to be affected than subjects with the ε3 alelle.[39] But in the past decade, novel DNA analysis techniques have allowed the identification of other genetic risk factors.[40] The sequencing of entire genomes and exomes has led to the detection of heterozygous rare variants in the triggering receptor expressed on myeloid cells 2 protein gene (*TREM2*).[30] Although rare, these variants have been associated with a moderate-to-high risk of AD (odds ratio, 5.05). On the other hand, the apolipoprotein J (or clusterin) gene (*CLU*), the

phosphatidylinositol-binding clathrin assembly protein gene (*PICALM*) and the complement component (3b/4b) receptor 1 gene (*CR1*) have been associated with sporadic AD in genome-wide association studies.[38,41,42] The risk effect of these genes is low (with odds ratios estimated between 1.2 and 1.5),[37] but their identification have mechanistic implications,[36] because unlike genes that cause ADAD, the protein products of *CLU*, *PICALM* and *CR1* may play a role in the innate immune system response and the Aβ clearance from the brain,[38] and not in its production.

1.2. Biomarkers for Alzheimer's disease

1.2.1. What are biomarkers?

A biomarker is a parameter that can be objectively measured *in vivo* and gives information about a biological or pathogenic process.[11,43] Among other applications, they can help to improve the accuracy of clinical diagnosis, assess the risk of progression, evaluate disease stage, and monitor the effect of a therapeutic agent.[10,11] Biomarkers must reflect features that are specific to the physiopathological processes that take place in the disease and should be validated in pathological cohorts.[44–46] Ideally, they should also be reliable, non-invasive and unexpensive.[46] Different techniques can be used for the obtention of biomarkers. In this section, we describe some of these techniques and the most well-known biomarkers in the field of AD.

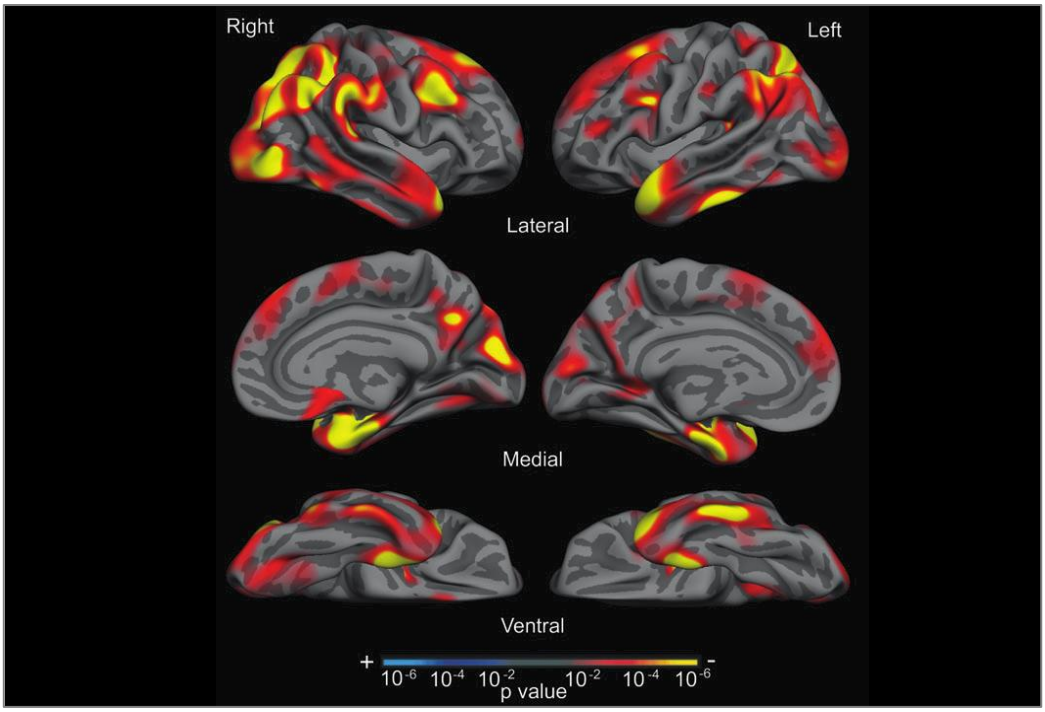
1.2.2. Imaging markers of Alzheimer's disease

The more widely studied imaging markers in AD are structural and functional measures obtained through magnetic resonance imaging (MRI) or positron emission tomography (PET). These techniques can simultaneously give mechanistic and topographical information. Through MRI it is possible to quantify structural parameters, such as cortical volume or

cortical thickness in specific brain areas. The most consistent and well established neuroimaging marker in AD is the atrophy of medial temporal lobe structures,[47–52] which can be visually assessed through semiquantitave rating scales in coronal T1-weighted images.[52,53] Visual rating scales have 80-85% sensitivity and specificity to distinguish patients with AD dementia from cognitively normal controls.[52] Structural MRI mirrors the neuropathological findings in AD, correlates topographically with neuropsychological deficits and has predictive value to detect progression from MCI to AD dementia.[52–54] But in the past few years, more sophisticated and automated analyses of MRI have been developed. These analyses have consistently detected changes in cortical thickness in lateral temporoparietal and midline parietal regions (posterior cingulate/precuneus) of patients with AD.[55,56] This structural pattern is characteristic of the disease and has been termed as the "cortical signature of AD" (**Figure 2**).[56]

Figure 2. Cortical signature of AD

Pattern of cortical thinning measured in structural MRI by Dickerson et al.[56]



Besides structural imaging, MRI scans can also be used to obtain functional information and measure differences in specific neuronal networks. Human networks seem to have a selective vulnerability to neurodegenerative diseases.[57] The disruption of the default mode network, a network that is active during the resting state and inactive while performing a task, has been described as an early finding in AD.[58]

PET imaging uses specific radioactive tracers for the obtention of the images. The scans acquired after the administration of ^{18}F-fluorodeoxyglucose (FDG) give information about neuronal metabolism and synaptic activity. Typically, patients with AD show hypometabolism in temporo-parietal areas.[58] This pattern has proven to be associated with high sensitivity (up to 93%) for the diagnosis of AD in pathological series,[59] and it is also predictive of cognitive decline in cognitively normal elderly.[60] Other more recent PET tracers have given novel imaging potentialities. In the past decade, different amyloid-binding ligands have been used to obtain images of the amyloid burden of the brain *in vivo*,[61,62] and they have shown a high correlation with the density and presence of fibrillar β-amyloid observed in neuropathological studies.[63] More recently, tau imaging ligands have also been developed to detect the aggregation of pathological tau in the brain.[64]

1.2.3. Biochemical markers for Alzheimer's disease

Different biochemical markers can be measured in accessible biological fluids. Peripheral blood, plasma and cerebrospinal fluid (CSF) have been widely studied in the search for biomarkers. Several molecules have been explored as AD biomarkers in these fluids,[10,58] but the Aβ peptides have been the most investigated ones.[65–67] Regarding plasma, most studies have reported no differences in Aβ levels or inconsistent results.[10,58,65,68] The main reasons for the failure of this search might be that the concentration of brain-derived proteins in

peripheral blood is low, and that these potential biomarkers might bind to other, much more abundant proteins.[58,69,70] Another reason could be that Aβ peptides are also produced by peripheral tissues, such as muscle, endothelium or platelets, and therefore, the levels measured in blood or plasma might not reflect the pathological changes in the brain.[10,68,71,72] To overcome this issue, the measurement of protein levels in neutrally derived exosomes (i.e. vesicles released by neurons) in plasma has recently been proposed as a more specific and promising technique that would reflect more accurately the changes that take place in the central nervous system.[73,74]

CSF has been used for over two decades as an accessible biological source to study neurodegenerative diseases. Unlike plasma, CSF is in direct contact with the extracellular space of the brain and might reflect more accurately the pathophysiological processes that take place in AD.[10]

Numerous studies have consistently identified a specific CSF biomarker signature that reflects the neuropathological hallmarks of AD. AD patients typically have decreased levels of CSF $A\beta_{1-42}$ and increased levels of CSF total-tau (t-tau) and phospho-tau (p-tau) compared to cognitively normal controls.[10,11] These three biomarkers, known as core AD biomarkers, have a sensitivity and specificity over 80% in the diagnosis of AD dementia.[10,11] They are also good prognostic markers to predict progression to dementia in patients with MCI.[75,76] Cortical biopsies[77] and *post-mortem* studies[78,79] have shown that levels of CSF $A\beta_{1-42}$ are inversely associated with amyloid plaques and cerebral amyloid angiopathy, while autopsy findings have shown that tau[79] and p-tau[80] levels in CSF correlate with neurofibrillary tangle burden.

Besides core AD biomarkers, other molecules have been investigated in CSF to explore the normal and abnormal pathways that occur in AD. Some of the most promising novel markers are associated with processes like the AβPP processing, neurodegeneration, synaptic loss, oxidative stress or neuroinflammation.[81] Although in many cases the diagnostic performance of these novel markers is not comparable to that of core biomarkers, they can offer new insights about the pathophysiological processes of AD, and in some cases, they are useful to monitor the effect of specific treatments.

BACE (β-site AβPP-cleaving enzyme) is a β-secretase involved in the amyloidogenic proteolytic processing of AβPP.[82] BACE protein levels and β-secretase activity have been found increased in AD *post-mortem* brain tissue,[83] and the brain levels of BACE protein correlate with Aβ deposition.[84] β-secretase activity can also be detected in CSF,[85–91] but studies in patients with AD have yielded variable results. Some studies[87,89] have reported mild increases in the early stages of AD, whereas others[90–92] have found no differences compared to healthy controls. Besides $A\beta_{1-42}$, many other peptides derived from the processing of AβPP have also been investigated in CSF in AD. Some authors[93–95] have reported that sAPPβ levels are slightly elevated in the early stages of AD compared to controls, but others[87,91,92] could not confirm these results.

Neuroinflammation can also be detected in the brain from the early stages of the disease.[26,96] Several inflammatory molecules have been investigated to study this complex process in the CSF in AD.[81] YKL-40, also known as chitinase-3-like-1 protein, is one of these molecules. YKL-40 levels in CSF have been found to be increased in the very early stages of AD,[97–99] although some authors could not replicate this finding.[100]

1.3. Biomarkers for the diagnosis of Alzheimer's disease

1.3.1. Biomarkers in the diagnosis of Alzheimer's disease dementia

Three decades ago, the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) established consensed criteria for the clinical diagnosis of AD.[101] These criteria had a remarkable accuracy compared to neuropathological diagnosis with an average sensitivity of 81% and specificity of 70%.[102] However, in the light of more recent findings, some aspects of these criteria needed to be reviewed. One of these aspects is the incorporation of biomarkers that have proven enough sensitivity and specificity in the diagnosis of AD.

In 2007, the International Working Group (IWG) for New Research Criteria for the diagnosis of AD updated the existing criteria incorporating biomarkers to the clinical diagnosis of AD, and therefore, conceptually merging clinical and pathological diagnosis into a dual clinicobiological entity that can be diagnosed *in vivo*.[103] Following these criteria, a diagnosis of probable AD can be made in the presence of early, gradual and significant episodic memory impairment and the support of one or more biomarkers. Dementia is no longer required for the diagnosis. These criteria were revisited by the same group to update definitions and incorporate new lexicon in 2010[104] and in 2014.[105]

In 2011, in parallel, the National Institute on Aging – Alzheimer's Association (NIA-AA) published another set of operational research criteria that incorporated biomarkers of amyloidosis (amyloid-PET or CSF $A\beta_{1-42}$) and of neurodegeneration (CSF tau, FDG-PET, structural MRI) as tools to evidence or rule out AD.[4] Combining clinical criteria with normal, abnormal or indeterminate biomarkers, different levels of evidence of the AD pathophysiological process can be reached (**Table 1**). The IWG and the NIA-AA criteria are similar in essence, but show small differences in lexicon and framework.[106]

Table 1. NIA-AA criteria for AD dementia incorporating biomarkers

Adapted from McKhann et al.[4]

CORE CLINICAL CRITERIA

ALL-CAUSE DEMENTIA

- Symptoms interfere with ability to function at work or usual activities AND
- Represent a decline AND
- Are not explained by delirium or major psychiatric disorder

Cognitive/behavioural impairment involves a minimum of two of:

- Acquire new information
- Impaired reasoning and handling of complex tasks, poor judgment
- Impaired visuospatial abilities
- Impaired language funtions
- Changes in personality, behavior or comportment

PROBABLE AD DEMENTIA: Meets criteria for dementia AND

- Insidious onset with a clear-cut history of worsening of cognition by report or observation AND
- The initial and most prominent cognitive deficits are evident in one of the following:

- **Amnestic presentation:** impairment in learning and recall and evidence of dysfunction in at least one other cognitive domain
- **Non amnestic presentation:**
  - Language presentation. Other cognitive domains should be affected.
  - Visuospatial presentation. Other cognitive domains should be affected.
  - Executive dysfunction. Other cognitive domains should be affected.

The diagnosis of AD dementia should NOT be applied when there is evidence of:

- Substantial cerebrovascular disease
- Core features of dementia with Lewy bodies
- Prominent features of behavioral variant of frontotemporal dementia
- Prominent features of semantic variant or non-fluent variant of primary progressive aphasia
- Evidence for another concurrent active neurological disease or non-neurological comorbidity or use of medication that could have a substantial effect on cognition

POSSIBLE AD DEMENTIA: Meets all clinical criteria for AD dementia, but:

- **Atypical course:** Sudden onset or insufficient historical detail or objective cognitive documentation of progressive decline
- **Etiologically mixed presentation:**
  - Concomitant cerebrovascular disease
  - Core features of dementia with Lewy bodies
  - Evidence for another concurrent active neurological disease or non-neurological comorbidity or use of medication that could have a substantial effect on cognition

CLINICAL RESEARCH CRITERIA

	Biomarker probability of AD etiology	**Aβ amyloid (amyloid-PET or CSF $A\beta_{1-42}$)**	**Markers of neuronal injury (CSF tau, FDG-PET, sMRI)**
Probable AD dementia clinical criteria	Uninformative	Conflicting Indeterminate Untested	Conflicting Indeterminate Untested
Probable AD dementia pathophysiological process	Intermediate	Unavailable or indeterminate	+
	Intermediate	+	Unavailable or indeterminate
	High	+	+
Possible AD dementia clinical criteria	Uninformative	Conflicting Indeterminate Untested	Conflicting Indeterminate Untested
Possible AD dementia pathophysiological process	High but does not rule out second etiology	+	+
Dementia unlikely due to AD	Lowest	-	-

1.3.2. Biomarkers in the diagnosis of mild cognitive impairment due to AD

The term MCI was proposed to designate an early, but abnormal, state of cognitive impairment but of insufficient severity to constitute dementia.[107,108] Since the term was coined, numerous studies have reported an increased risk of progression to dementia in a subset of patients with MCI. In particular, a specific subtype, the amnestic MCI, was identified as the one with highest risk to progress to dementia.[108]

The incorporation of biomarkers to the clinical diagnosis of MCI helps to determine the likelihood of cognitive and functional progression and establishes support for the underlying etiology of the clinical syndrome.[109] Paralleling the revision of the AD dementia criteria, the MCI concept has also been recently updated (**Table 2**). In the new criteria, the concept of "prodromal AD", proposed by the IWG,[104] and the term "MCI due to AD", suggested by the NIA-AA,[109] aim to represent the earliest symptomatic phase of AD.[110] A recent study estimated that the 3-year progression rate from MCI to AD-type dementia was of 59% in the high AD likelihood group compared to 5% in the low AD likelihood group.[111] Besides the prognostic implications, the identification of subjects at these stages of the disease is also a key point in the design of clinical trials. With a rigorous selection, the intervention with specific treatments at this stage could delay progression of symptoms and potentially prevent the onset of dementia.[110]

Table 2. NIA-AA criteria for mild cognitive impairment incorporating biomarkers

Adapted from Albert et al.[109]

CORE CLINICAL CRITERIA
▪ **Cognitive concern by patient or informant or clinician** ▪ **Objective evidence of impairment in one or more cognitive domain (typically including memory)** ▪ **Preservation of independence in functional abilities** ▪ **Not demented** ▪ **Rule out vascular, traumatic, medical causes of cognitive decline, where possible** ▪ **Provide evidence of longitudinal decline when feasible** ▪ **Report history consistent with AD genetic factors, when relevant**

CLINICAL RESEARCH CRITERIA		
	Aβ amyloid (amyloid-PET or CSF $A\beta_{1-42}$)	**Markers of neuronal injury (CSF tau, FDG-PET, sMRI)**
MCI core clinical criteria	Conflicting Indeterminate Untested	Conflicting Indeterminate Untested
MCI due to AD intermediate likelihood	+ Untested	Untested +
MCI due to AD high likelihood	+	+
MCI unlikely due to AD	-	-

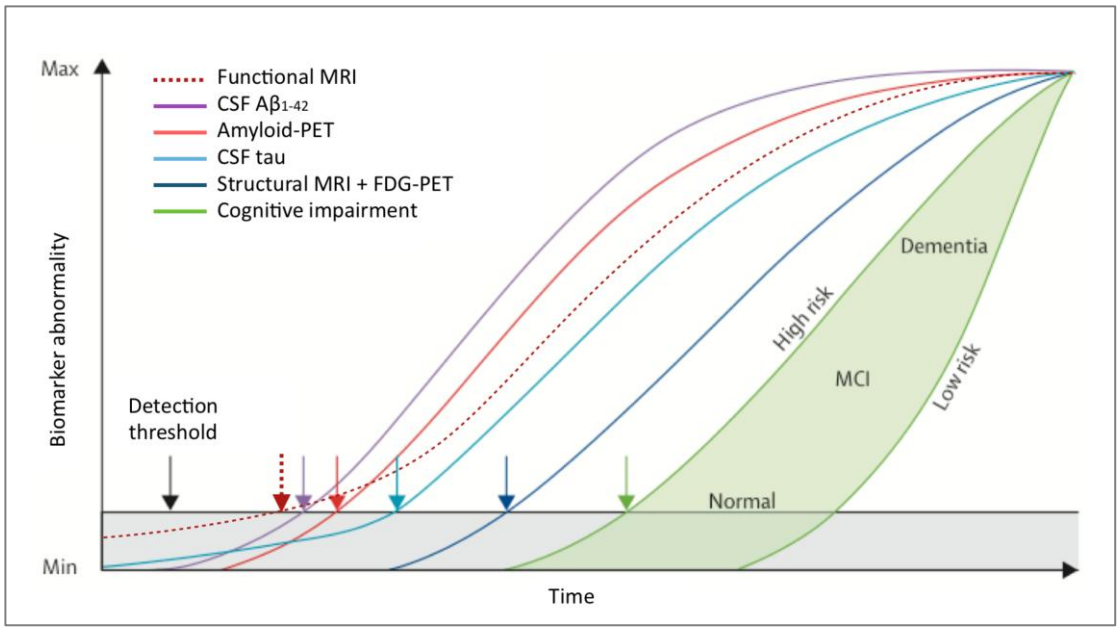
1.3.3. Use of biomarkers to define preclinical stages of Alzheimer's disease

Data from clinicopathological studies indicate that the main neuropathological hallmarks of AD, amyloid plaques and neurofibrillary tangles, can be detected in autopsied older non-demented individuals.[112,113] Together with many recent studies that show changes in biomarkers in cognitively normal subjects before the onset of symptoms, these data support the notion that there is a long preclinical phase in AD. Due to the lack of symptoms at this stage, the study of preclinical AD relies on the use of biochemical or imaging biomarkers to detect changes in the brain. Characterizing this asymptomatic phase is of critical importance in the design of clinical trials,[114] as there is hope that intervention in this preclinical stage will maximize the possibilities of a positive clinical outcome.

Evidence suggest that the most widely validated biomarkers for AD become abnormal in an ordered manner that somehow parallels the pathological changes in the brain (**Figure 3**).[45,115] Some studies have found that biomarkers of synaptic dysfunction, such as functional MRI (fMRI), may demonstrate abnormalities very early, especially in APOE-ε4 carriers,[116] even before the accumulation of amyloid plaques.[117] Aβ accumulation biomarkers (CSF $A\beta_{1-42}$ first, followed by amyloid-PET) become abnormal as early as 10-15 years before the appearance of clinical symptoms.[118–120] Although tau pathology can be found in a proportion of young individuals in subcortical areas,[121] biomarkers of neurodegeneration (structural MRI and CSF tau) are thought to become abnormal later than Aβ biomarkers, and they correlate with clinical performance in MCI and dementia stages.[115,122] In summary, although tau pathology seems to appear earlier than amyloid plaques in the brain in AD, biomarkers of neurodegeneration become abnormal later, likely due to a higher detection threshold.

Figure 3. Hypothetical model integrating pathology and biomarkers in AD

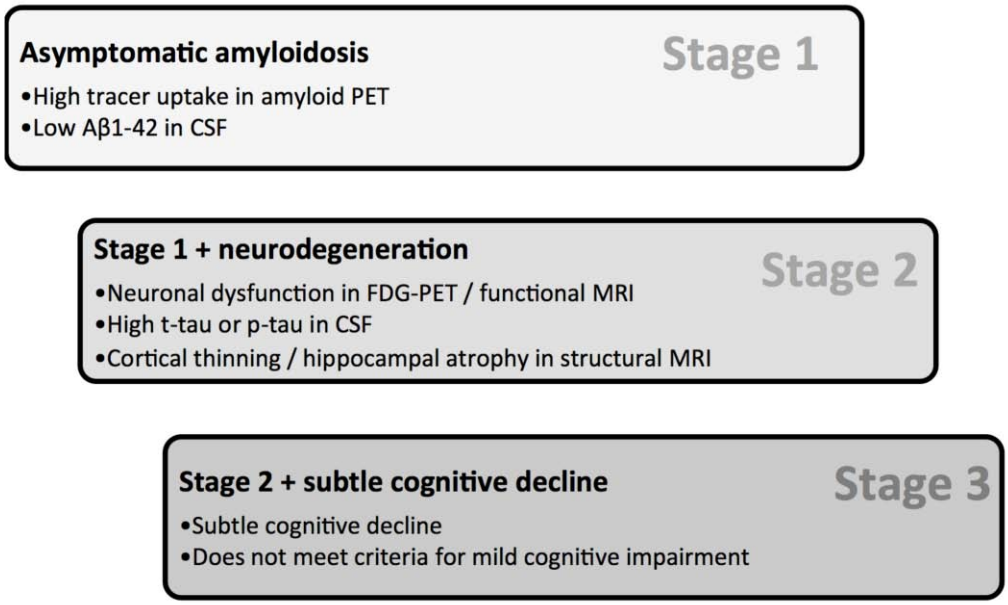
Modified from Jack et al.[115] and Sperling et al.[122]



Based on this scheme, the NIA-AA proposed operational research criteria to define study cohorts at risk of AD dementia.[122] Under these criteria, three stages were defined (**Figure 4**). Stage 1 is defined as asymptomatic cerebral amyloidosis, characterized by low $A\beta_{1-42}$ in CSF or high tracer uptake in amyloid-PET. Stage 2 is defined as stage 1 with additional evidence of synaptic dysfunction detected in functional imaging studies and/or early neurodegeneration detected in CSF (increase of t-tau or p-tau) or in structural imaging studies (hippocampal atrophy or cortical thinning). Stage 3 is defined as a stage 2 plus subtle cognitive decline that does not reach the category of MCI. An additional category, SNAP (suspected non-Alzheimer pathology), has also been recently proposed to label subjects with signs of neurodegeneration in the absence of cerebral amyloidosis.[123]

Figure 4. NIA-AA staging of preclinical AD stages

Adapted from Sperling et al.[122]



1.3.4. Limitations in the use of CSF biomarkers for Alzheimer's disease

Although core CSF biomarkers have a remarkable diagnostic performance, there are some limitations for the generalization of their use in clinical settings. First, CSF can only be obtained through an invasive procedure (i.e. lumbar puncture). Although large studies

assessing the safety of this technique in several contexts have reported no major adverse events,[124] the feasibility of lumbar puncture in neurodegenerative diseases for clinical purposes still remains controversial. Second, due to pre-analytical and analytical factors, there is a wide variability in the absolute measures of CSF biomarkers between laboratories,[125] and therefore the cut-off values used for diagnostic purposes can not be extrapolated from one center to another.[126] To quantify and reduce this variability, the Alzheimer's Association, in collaboration with the University of Gothenburg, launched an international quality control program for CSF biomarkers in 2009.[127] Many other efforts are ongoing to detect and standardize all possible confounding factors and to minimize the variability in the analysis of biomarkers.[128–131]

2. Hypotheses and objectives

2.1. Hypotheses

1. Lumbar puncture is a feasible and safe procedure for the study of CSF biomarkers for AD.
2. The pathophysiological pathways underlying Alzheimer's disease can be studied *in vivo* through biomarkers in CSF.
    a. Biomarkers give information *in vivo* about processes distinctively altered in different neurodegenerative diseases.
    b. Biomarkers that track similar pathophysiological pathways correlate with each other.
3. The pathophysiological pathways underlying Alzheimer's disease can be studied also in preclinical stages.
4. There is a correlation between CSF biomarkers and the structural changes that can be measured in MRI in early stages of AD.

2.2. Objectives

1. To analyze the incidence of complications of lumbar puncture in the study of CSF biomarkers of AD and describe the factors associated with these complications
2. To study the AβPP processing in sporadic and autosomal-dominant AD
3. To study markers of AβPP processing ($A\beta_{42}$, sAβPPβ, β-secretase activity), neuronal damage (total tau, p-tau) and inflammation (YKL-40) in CSF in patients with different symptomatic neurodegenerative conditions
4. To study markers of AβPP processing ($A\beta_{42}$, sAβPPβ, β-secretase activity), neuronal damage (total tau, p-tau) and inflammation (YKL-40) in CSF across preclinical stages of AD
5. To address the relationship between YKL-40, a CSF marker of inflammation, and brain structure and its interactions with core AD biomarkers

3. Outline

This thesis deepens in the knowledge of key aspects of neurodegenerative diseases, and more precisely in AD, both in symptomatic and preclinical stages. This is achieved through the study of CSF biomarkers that reflect *in vivo* the changes that take place in the brain very early in the disease process, and that are the central line of the thesis.

As an introduction, **Chapter 1** sets the framework and general context for this thesis by summarizing the current knowledge on the field of AD and biomarkers. In **chapter 2**, we assessed the feasibility of lumbar puncture, the incidence of complications and their associated factors so as to determine the impact of this procedure in the study of CSF biomarkers of AD. **Chapter 3** analyzes the pathophysiological differences between sporadic

and autosomal dominant AD. CSF biomarkers allowed us to identify *in vivo* some characteristics in the processing of the amyloid precursor protein that complement the information found in neuropathological studies. In **chapter 4**, we study the differences in CSF biomarkers between neurodegenerative diseases that cause dementia in their symptomatic stages. We chose markers related to different pathophysiological processes and studied their relationship. We extended this study to preclinical stages in **chapter 5**. For this aim, we analyzed the same set of biomarkers in a large cohort of cognitively normal participants. **Chapter 6** studies the relationship between one of these biomarkers, YKL-40, and cortical thickness measured by MRI in predementia stages of AD. Lastly, in **chapter 7** we provide a general discussion and formulate the concluding remarks and future perspectives.

In summary, in this thesis we use CSF biomarkers to study AD from a translational perspective in both clinical and preclinical stages and to identify relationships between distinct pathophysiological pathways. This kind of approach is essential to stablish new accurate diagnostic tools, to learn about the processes in the early stages of the disease, and, potentially, to discover new therapeutic targets.

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Chapter 2

Feasibility of lumbar puncture in the study of CSF biomarkers for Alzheimer's disease: a multicentre study in Spain

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Abstract

Background: Lumbar puncture (LP) is increasingly performed in memory units due to the usefulness of cerebrospinal fluid (CSF) biomarkers in the diagnosis of Alzheimer's disease. The feasibility of this procedure in this context, however, is controversial.

Objective: Our aim was to analyze the incidence of complications and their associated factors so as to determine the impact of LP in the study of CSF biomarkers of Alzheimer's disease.

Methods: In the context of a larger international initiative, we prospectively collected data from 689 participants who underwent LP in three memory units in Spain. Data included demographic factors, headache history, subjective attitude towards the procedure, patient positioning, needle characteristics, volume of CSF extracted, attempts needed, and resting time after CSF acquisition. Five to seven days after the procedure we asked participants about complications through a semi-structured telephone interview.

Results: No adverse events were reported in 441 (64.0%) participants. The most frequent complication was headache, reported by 171 (24.8%) subjects. It was severe in only 17 (2.5%). Headache was more frequent in younger participants and when a cutting-edge needle was used. Back pain was present in 111 (16.1%) cases, and it was associated with female gender, cutting-edge needles, increased number of attempts, and longer resting time after LP. No major complications were reported. The use of pen-point needles showed a trend towards a higher frequency of haematic CSF.

Conclusion: LP can be safely performed to study CSF biomarkers. The main complication is headache, associated with younger age and use of cutting-edge needles.

Keywords: Lumbar puncture; post-lumbar puncture headache; biomarkers; cerebrospinal fluid; Alzheimer's disease; dementia.

1. Introduction

Alzheimer's disease (AD) is characterized by deposition of amyloid plaques and neurofibrillary tangles in the brain. Several studies have shown that $A\beta_{42}$, tau and p-tau in cerebrospinal fluid (CSF) can track these neuropathological hallmarks in AD. While cortical biopsies[1] and post-mortem studies[2,3] have shown that levels of CSF $A\beta_{42}$ are inversely associated with amyloid plaques and cerebral amyloid angiopathy, autopsy findings have shown that increased tau[3] and p-tau[4] levels in CSF are associated with a higher neurofibrillary tangle burden. As $A\beta_{42}$, tau and p-tau reflect the underlying pathophysiological process they can help in the diagnosis of AD. For this reason, these biomarkers have now been incorporated into the recently revised diagnostic research criteria for AD,[5–9] and they have also been recommended by the European and Oxford Task Force Groups to evaluate the effects of disease-modifying drugs.[10,11] All this has led to an increased implementation of lumbar puncture (LP) in memory units.

Large studies assessing the safety of LP in several different contexts have reported no major adverse events.[12] The most prevalent complication is post-lumbar puncture headache (PLPH), occurring in up to 40% of cases.[13,14] Other possible complications are back pain, nausea and dizziness.[14,15] Several studies have assessed the technical and constitutional factors associated with LP complications, yielding specific recommendations to minimize their incidence.[13,14] However, some of these studies were performed in the context of LP for anaesthesia,[16–18] and those investigating diagnostic LP are from single centres.[19–26] Our aim was to determine the feasibility of LP to study CSF biomarkers of AD, by analyzing the incidence of complications and their associated factors in a multicentre study in Spain.

2. Methods

We prospectively collected data from 689 subjects who underwent LP for CSF biomarker analysis in specialized memory units at three centres: Hospital Clínic, Barcelona (HC); CITA Alzheimer, San Sebastián (CITA); and Hospital Sant Pau, Barcelona (HSP). The participants were included during the following time periods: January 2009-January 2013 (HC), June 2011-January 2013 (CITA) and March 2011-January 2013 (HSP). The data were collected in the context of a larger international initiative led by the Alzheimer's Association and the University of Gothenburg.[27] The recorded data included demographic factors, previous history of headache, subjective attitude towards the procedure, patient positioning, needle characteristics, amount of CSF extracted, attempts needed for CSF removal, visually haematic CSF staining, and resting time after CSF acquisition.[27]

Neurologists with expertise in the procedure carried out the LP in the setting of research projects on CSF biomarkers that had been approved by the ethics committee at each institution and following the ethical standards recommended by the Helsinki Declaration. All participants received information about the possible complications of LP and signed an informed consent before the procedure. At two of the centres, HC and HSP, LP was performed either in sitting or lying position, using the cutting-edge Quincke needle (20G or 22G) and introducing the bevel parallel to dural fibres. Patient positioning and needle size were decided based on the neurologist's preference according to each patient's characteristics. At the other centre, CITA, the pen-point "atraumatic" needle (Whitacre-22G, without introducer) was used in all but one patient, and all subjects were positioned in left lateral decubitus. CSF was collected by free-flow/dripping at all centres. All participants were given similar recommendations after the procedure. They were advised to rest in the 24 hours after the test and to drink additional fluids.

Between 5 and 7 days after the procedure, we contacted the subjects to ask about complications through a semi-structured telephone interview. We recorded variables concerning headache, local back pain, dizziness, and nausea. The variables recorded for headache were: presence of headache; headache intensity, defined as mild (no treatment or mild analgesics), moderate (patient had to stay in bed for periods of the day), or severe (invalidating or requiring hospitalization); headache duration (<2 days, 2-4 days, >4 days); and headache classification as fulfilling the International Headache Society (IHS) criteria of PLPH[28] or as non-specific.

We analyzed the differences in the frequency of complications using Chi-Square tests and bivariate logistic regression. Variables with p values <0.20 in the bivariate analysis were included in logistic multivariate regression models. We used SPSS Statistics software v.19.0 for statistical analysis.

3. Results

We collected data from 689 participants (HC=275, CITA=240 and HSP=174). **Table 1** summarizes demographic and clinical information. The distribution of age, clinical diagnosis, needle type, patient positioning, and volume of CSF extracted differed between centres.

As displayed in **table 2**, 441 (64.0%) of 689 participants did not have any adverse event after the procedure. We found that 171 of the 689 (24.8%) reported some degree of headache. Of these, 140 (20.3%) fulfilled IHS criteria of PLPH[28] and 31 (4.5%) had non-specific characteristics. Headache was mild in 105 cases (15.2%), moderate in 50 (7.3%), and severe or invalidating in 17 (2.5%). Among all participants, 111 (16.1%) had local back pain and 27 (3.9%) had other mild symptoms such as nausea or dizziness. One subject with a known

previous history of syncope had a convulsive syncope the day after the procedure. No other complications were reported.

Table 1. Demographic and procedure characteristics across the centres

HC: Hospital Clínic, Barcelona. CITA: CITA-Alzheimer, San Sebastián. HSP: Hospital Sant Pau, Barcelona. SCI: Subjective cognitive impairment. MCI: Mild cognitive impairment. AD: Alzheimer's Dementia. Q-20G: Quincke-20Gauge. Q-22G: Quincke-22Gauge. W-22G: Whitacre-22Gauge.

[1]This group consisted of non-AD dementias: Lewy body dementia, frontotemporal dementia, corticobasal syndrome, supranuclear palsy and vascular dementia.

		HC (n=275)	CITA (n=240)	HSP (n=174)	Total (n=689)	P value
Age, mean (SD)		63.48 (9.29)	58.34 (7.51)	66.33 (8.66)	62.41 (9.11)	<0.001
Gender, n (%)	Female	151 (54.9%)	125 (52.1%)	85 (48.9%)	361 (52.4%)	0.45
	Male	124 (45.1%)	115 (47.9%)	89 (51.1%)	328 (47.6%)	
Diagnosis, n (%)	Control	53 (20.0%)	147 (61.5%)	39 (22.4%)	239 (35.3%)	<0.001
	SCI	43 (16.2%)	73 (30.5%)	26 (14.9%)	142 (20.9%)	
	MCI	55 (20.8%)	19 (7.9%)	53 (30.5%)	127 (18.7%)	
	AD	69 (26.0%)	0 (0.0%)	36 (20.7%)	105 (15.5%)	
	Other[1]	45 (17.0%)	0 (0.0%)	20 (11.5%)	65 (9.6%)	
Needle type, n (%)	Q-20G	135 (49.1%)	0 (0.0%)	34 (19.5%)	169 (24.5%)	<0.001
	Q-22G	140 (50.9%)	1 (0.4%)	140 (80.5%)	280 (40.6%)	
	W-22G	0 (0.0%)	239 (99.6%)	0 (0.0%)	240 (34.8%)	
Position, n (%)	Sitting	151 (54.9%)	0 (0.0%)	57 (32.8%)	208 (30.2%)	<0.001
	Lateral decubitus	124 (45.1%)	240 (100.0%)	117 (67.2%)	481 (69.8%)	
Volume (ml), mean (SD)		10.96 (2.15)	9.13 (0.83)	8.88 (1.43)	9.83 (1.88)	<0.001

Table 2. Incidence of complications across the centres

HC: Hospital Clínic, Barcelona. CITA: CITA-Alzheimer, San Sebastián. HSP: Hospital Sant Pau, Barcelona. PLPH: Post-lumbar puncture headache (IHS criteria)[28].

		HC (n=275)	CITA (n=240)	HSP (n=174)	Total (n=689)	P value
Complications	No	155 (56.3%)	186 (77.5%)	100 (57.5%)	441 (64%)	<0.001
	Yes	120 (43.6%)	54 (22.5%)	74 (42.5%)	248 (36%)	
Headache	No Headache	186 (67.6%)	202 (84.2%)	130 (74.7%)	518 (75.2%)	<0.001
	Mild	55 (20.0%)	17 (7.1%)	32 (18.4%)	105 (15.1%)	
	Moderate	21 (7.6%)	19 (7.9%)	10 (5.7%)	50 (7.3%)	
	Severe	13 (4.7%)	2 (0.8%)	2 (1.1%)	17 (2.5%)	
Headache Type	No Headache	186 (67.6%)	202 (84.2%)	130 (74.7%)	518 (75.2%)	<0.001
	Typical PLPH	89 (32.4%)	26 (10.8%)	25 (14.4%)	140 (20.3%)	
	Unspecific	0 (0)	12 (5%)	19 (10.9%)	31 (4.5%)	
Back pain	No	223 (81.1%)	217 (90.4%)	138 (79.3%)	578 (83.9%)	0.003
	Yes	52 (18.9%)	23 (9.6%)	36 (20.7%)	111 (16.1%)	
Dizziness or Nausea	No	267 (97.1%)	229 (95.4%)	166 (95.4%)	662 (96.1%)	0.538
	Yes	8 (2.9%)	11 (4.6%)	8 (4.6%)	27 (3.9%)	

Table 3 shows the association between complications and the variables recorded. Among these variables, we analyzed participants' characteristics and factors related to the procedure. Regarding participants' characteristics, older subjects had a lower incidence of headache (OR 0.95 per year, p<0.001; Figure 2), and women had a higher frequency of back pain (OR 1.95, p=0.003). A previous history of headache was not associated with headache after the procedure (p=0.120). Subjects who reported fear before the procedure were more

likely to present PLPH (OR 2.02, p=0.002) and back pain (OR 1.80, p=0.007). Clinical diagnosis did not affect the occurrence of complications (p=0.98).

Table 3. Bivariate and multivariate analyses for the variables studied

IHS PLPH: Post-lumbar puncture headache according to the IHS criteria.

All variables with p values<0.20 in the bivariate analyses were included in the multivariate logistic regression model.

	Age (years)	Gender Female / Male	Headache History Yes / No	Fear of complications Yes / No	Needle type Whitacre / Quincke	Diameter 20 Gauge / 22 Gauge	Position Sitting / Lying down	More than one attempt Yes / No	Volume (ml)	Rest after procedure ≤1h / >1h
Complications										
Raw OR (IC 95%)	0.98(0.96-0.99)	1.54 (1.13-2.11)	1.35 (0.84-2.14)	1.41 (1.03-1.93)	0.37 (0.26-0.53)	1.76 (1.24-2.51)	1.27 (0.91-1.78)	1.26 (0.89-1.79)	1.02 (0.93-1.10)	0.69 (0.42-1.15)
p value	0.004	0.007	0.211	0.033	<0.001	0.002	0.16	0.193	0.732	0.152
Adjusted OR (IC95%)	**0.95 (0.93-0.97)**	**1.39 (1.00-1.94)**	-	**1.44 (1.02-2.02)**	**0.25 (0.17-0.37)**	**1.13 (1.04-2.21)**	0.78 (0.53-1.19)	1.29 (0.88-1.88)	-	0.64 (0.38-1.30)
p value	<0.001	0.052	-	0.038	<0.001	0.002	0.272	0.192	-	0.102
Headache										
Raw OR (IC 95%)	0.96 (0.94-0.98)	1.18 (0.84-1.68)	1.75 (1.08-2.86)	1.12 (0.79-1.59)	0.43 (0.29-0.65)	1.62 (1.10-2.37)	1.05 (0.72-1.53)	0.79 (0.53-1.19)	1.03 (0.95-1.13)	0.96 (0.56-1.63)
p value	<0.001	0.34	0.023	0.529	<0.001	0.013	0.791	0.255	0.463	0.871
Adjusted OR (IC95%)	**0.93 (0.91-0.96)**	-	1.50 (0.90-2.51)	-	**0.28 (0.18-0.43)**	0.96 (0.62-1.49)	-	-	-	-
p value	<0.001	-	0.120	-	<0.001	0.860	-	-	-	-
IHS PLPH										
Raw OR (IC 95%)	0.96 (0.93-0.98)	1.23 (0.81-1.86)	1.47 (0.81-2.67)	2.05 (1.35-3.11)	0.47 (0.29-0.76)	1.38 (0.86-2.19)	1.24 (0.80-1.92)	0.70 (0.42-1.16)	1.06 (0.95-1.17)	0.54 (0.24-1.20)
p value	<0.001	0.331	0.204	0.001	0.002	0.182	0.343	0.164	0.300	0.130
Adjusted OR (IC95%)	**0.94 (0.92-0.96)**	-	-	**2.02 (1.31-3.12)**	**0.28 (0.17-0.47)**	0.78 (0.45-1.78)	-	0.69 (0.41-1.17)	-	0.47 (0.20-1.08)
p value	<0.001	-	-	0.002	<0.001	0.385	-	0.169	-	0.077
Severe Headache										
Raw OR (IC 95%)	0.94 (0.89-0.99)	1.69 (0.62-4.61)	1.59 (0.45-5.64)	1.02 (0.38-2.72)	0.24 (0.06-1.08)	0.95 (0.30-2.94)	3.42 (1.28-9.11)	0.87 (0.28-2.69)	1.04 (0.84-1.29)	3.17 (1.09-9.25)
p value	0.035	0.306	0.476	0.964	0.063	0.923	0.014	0.805	0.696	0.034
Adjusted OR (IC95%)	**0.92 (0.87-0.97)**	-	-	-	0.30 (0.06-1.60)	-	**4.70 (1.68-13.1)**	-	-	2.77 (0.92-8.36)
p value	0.005	-	-	-	0.160	-	0.003	-	-	0.071
Back pain										
Raw OR (IC 95%)	0.99 (0.97-1.02)	2.13 (1.38-3.27)	1.17 (0.64-2.14)	1.91 (1.27-2.88)	0.41 (0.25-0.68)	1.78 (1.15-2.75)	1.25 (0.81-1.92)	2.04 (1.33-3.12)	1.01 (0.90-1.12)	0.37 (0.16-0.88)
p value	0.614	<0.001	0.604	0.002	<0.001	0.009	0.311	0.001	0.927	0.019
Adjusted OR (IC95%)	-	**1.95 (1.25-3.04)**	-	**1.80 (1.17-2.78)**	**0.38 (0.23-0.64)**	1.10 (0.67-1.81)	-	**1.75 (1.13-2.73)**	-	**0.38 (0.16-0.92)**
p value	-	0.003	-	0.007	<0.001	0.701	-	0.013	-	0.032
Haematic										
Raw OR (IC 95%)	1.02 (0.99-1.04)	1.05 (0.70-1.64)	1.18 (0.61-2.27)	1.07 (0.68-1.68)	3.78 (2.37-6.01)	0.13 (0.05-0.35)	0.51 (0.30-0.90)	2.74 (1.73-4.35)	-	-
p value	0.205	0.838	0.624	0.774	<0.001	<0.001	0.019	<0.001	-	-
Adjusted OR (IC95%)	-	-	-	-	1.93 (0.99-3.73)	**0.10 (0.03-0.32)**	0.48 (0.22-1.05)	**5.33 (3.12-9.10)**	-	-
p value	-	-	-	-	0.052	<0.001	0.065	<0.001	-	-

Among the variables related to the procedure, needle type was highly associated with outcome. The use of pen-point needles was associated with a lower incidence of complications (OR=0.25, p<0.001), and specifically with a lower incidence of PLPH (OR=0.28, p<0.001) and back pain (OR=0.38, p<0.001). Patient positioning during the procedure did not influence the occurrence of general complications (p=0.272) or headache (p=0.791), but the sitting position was associated with a higher incidence of severe headache (OR=4.70, p=0.003). Resting time was not associated with headache (p=0.871), but participants with less than 1 hour of recumbence time had a lower frequency of back pain (OR=0.38, p=0.032). The volume of CSF extracted was not associated with any of the outcome variables.

Despite the lower incidence of clinical complications, the use of pen-point needles showed a trend towards a higher incidence of macroscopic blood staining of CSF than the cutting-edge type (23.0% vs. 7.3%; **Figure 1**). This difference was in the limit of statistical significance after controlling for other variables (OR=1.93, p=0.052, **Table 3**). Haematic CSF was also more frequent when multiple attempts were needed (OR=5.33, p<0.001).

Figure 1. Proportion of general complications, haematic LP, headache and back pain with different needle types

Q-20G: Quincke-20Gauge; Q-22G: Quincke-22Gauge; W-22G: Whitacre-22Gauge

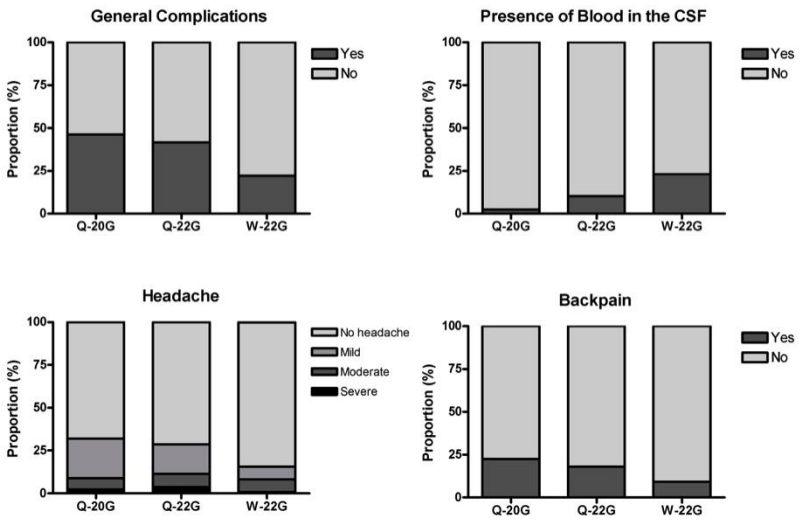
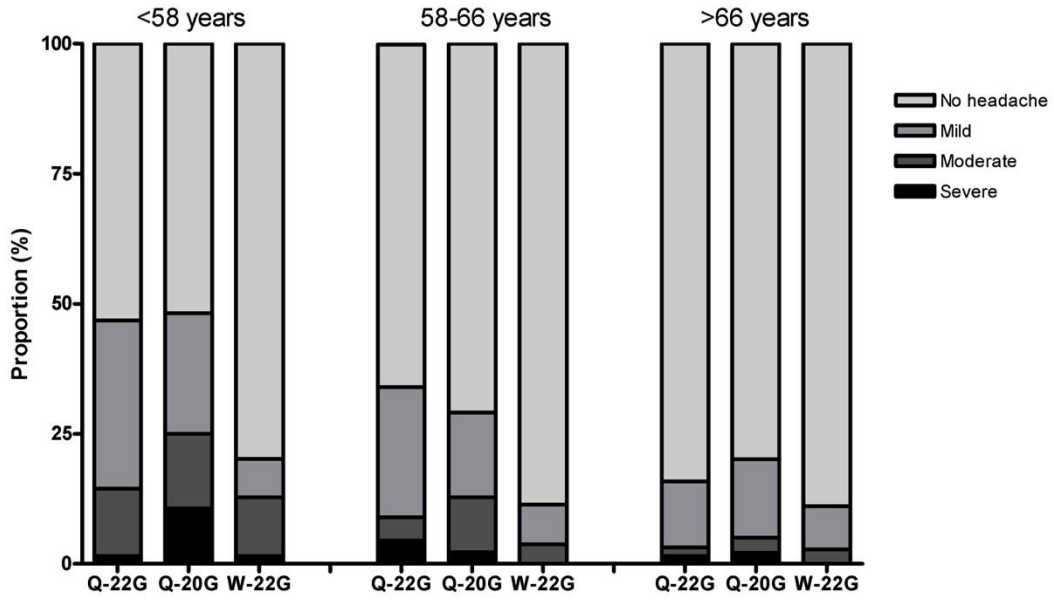


Figure 2. Proportion of headache by age tertiles after using different needle types

Q-20G: Quincke-20Gauge; Q-22G: Quincke-22Gauge; W-22G: Whitacre-22Gauge.



4. Discussion

Our study indicates that LP is a safe technique to evaluate AD biomarkers in CSF. Most participants had no complications. Headache was the most frequent event, occurring in about a quarter of the subjects, but it was mild in most cases and required no treatment or only low doses of commonly-used analgesic drugs. The main factors associated with headache were younger age and the use of cutting-edge needles.

We also confirmed other interesting findings. First, we observed that patient positioning was not associated with the incidence of headache, but the sitting position was associated with headache of severe intensity. Accordingly, we would recommend the lying position as the first option to perform the procedure, especially in young patients, who are at higher risk of

headache. Second, as suggested in previous studies,[22,29] early mobilisation after LP was associated with a lower incidence of back pain and had no influence on the occurrence of headache. And third, we found that the volume of CSF extracted was not associated with the presence of complications.

Consistent with our results, most studies have estimated the frequency of PLPH as between 20 and 40%.[15,20,23,24,30,31] A few have reported significantly lower frequencies,[32] even below 5% in some cases.[19,21,26] This large variability could be explained by methodological differences. The first of these is that the definitions of PLPH differed across studies. Some groups required definite PLPH, fulfilling the IHS criteria,[13] whereas others recorded the appearance of any kind of headache regardless of its characteristics. Second, different methods were used to record complications. Some authors recorded only those complications spontaneously reported by patients,[19] while others, such as ourselves, interviewed the participants systematically after the procedure.[20,23,32] Lastly, there is great variability across studies regarding the age of participants and the technical aspects of the procedure. In this respect, studies in older patients,[19–21] and studies using pen-point needles[23,24,26,30] have consistently found lower frequencies of headache.

As a consequence, pen-point needles have been proposed as the best choice in elective LP,[13,14,24] and more specifically in LP to determine AD biomarkers.[33,34] However, the use of this kind of needle could have other consequences. In our study, pen-point needles showed a trend towards a higher rate of visual haematic staining of CSF. We believe this is an important finding in this context because blood contamination of CSF could potentially alter the biomarker results.[35] To our knowledge, no previous large studies have compared the occurrence of haematic staining of CSF using different needle types. A smaller study assessing this issue (n=61) found no differences in the number of red blood cells in CSF,[36] but

another reported technical difficulties and a higher failure rate when pen-point needles were used, especially in patients with high body mass index.[25] In our series, haematic contamination was only observed at the beginning of the flow. It is unlikely that this interfered with the determination of biomarkers because we systematically discarded the first 1-2 ml of CSF until it was clear, and we centrifuged all samples after collection. Nonetheless, this point should be taken into account when standardizing the needle type for analysis of AD biomarkers.

Finally, besides the technical aspects discussed, psychological factors might also play a role in the appearance of complications. This consideration would be supported by findings from the only double-blind study available to date on the incidence of headache after LP;[37] the author found that frequencies of headache in the diagnostic LP and in the sham LP groups were similar. Furthermore, in our study, participants who reported fearfulness before the procedure had a higher frequency of PLPH. As the IHS criteria for PLPH have not been validated, we cannot be certain that every headache fulfilling these criteria is due to CSF leakage after LP. Thus, tension-type headache or other types of headache related to psychological factors may be misdiagnosed as PLPH.

The main limitation of our study is the lack of randomization in the technical aspects of the procedure. Some of these aspects were intrinsically linked to the centres. The use of pen-point needles was limited to only one centre and conclusions about the type of needle should be taken with caution. Moreover, participants at the three memory units differed in age and clinical diagnosis. Those from CITA were mostly younger healthy volunteers, while those from the other two centres, HC and HSP, had a higher proportion of older patients with cognitive impairment. To overcome the possible biases that these differences between centres could have introduced, we used a multivariate analysis.

In conclusion, in this multicentre study we found that LP can be safely performed to study CSF biomarkers. Headache was the most common adverse effect, and it was associated with younger age and the use of cutting-edge needles. It was rarely severe, and we did not find other major complications. The risk of headache decreased significantly when pen-point needles were used, but the presence of haematic CSF and an increased difficulty in the procedure with the use of this needle are issues that should be addressed in future studies.

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Chapter 3

Distinct patterns of APP processing in the CNS in autosomal-dominant and sporadic Alzheimer disease

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Abstract

Autosomal-dominant Alzheimer disease (ADAD) is a genetic disorder caused by mutations in *Amyloid Precursor Protein* (*APP*) or *Presenilin (PSEN)* genes. Studies from families with ADAD have been critical to support the amyloid cascade hypothesis of Alzheimer disease (AD), the basis for the current development of amyloid-based disease modifying therapies in sporadic AD (SAD). However, whether the pathological changes in APP processing in the CNS in ADAD are similar to those observed in SAD remains unclear. In this study, we measured β-site APP-cleaving enzyme (BACE) protein levels and activity, APP and APP C-terminal fragments in brain samples from subjects with ADAD carrying *APP* or *PSEN1* mutations (n=18), patients with SAD (n=27) and age-matched controls (n=22). We also measured sAPPβ and BACE protein levels, as well as BACE activity, in CSF from individuals carrying *PSEN1* mutations (10 mutation carriers and 7 non-carrier controls), patients with SAD (n=32) and age-matched controls (n=11). We found that in the brain, the pattern in ADAD was characterized by an increase in APP β-C-terminal fragment (β-CTF) levels despite no changes in BACE protein levels or activity. In contrast, the pattern in SAD in the brain was mainly characterized by an increase in BACE levels and activity, with less APP β-CTF accumulation than ADAD. In the CSF, no differences were found between groups in BACE activity or expression or sAPPβ levels. Taken together, these data suggest that the physiopathological events underlying the chronic Aβ production/clearance imbalance in SAD and ADAD are different. These differences should be considered in the design of intervention trials in AD.

Keywords

Amyloid precursor protein, autosomal-dominant Alzheimer disease, β-site APP-cleaving enzyme, presenilin; β-amyloid

1. Introduction

Autosomal-dominant Alzheimer disease (ADAD) is a genetic disorder that accounts for less than 1 % of all AD cases.[1] It is genetically heterogeneous and has been associated with mutations in the amyloid precursor protein (*APP*) gene or in the two presenilin genes (presenilin-1 and -2 or *PSEN1* and *PSEN2*).[1]

Studies in ADAD have been critical to support the amyloid cascade hypothesis, which states that the sequence of pathogenic events leading to AD is primarily initiated by accumulation of β-amyloid (Aβ).[2] The knowledge derived from these studies has been instrumental in guiding the development of the amyloid-based disease-modifying drugs currently being tested in sporadic Alzheimer disease (SAD).

Aβ peptide is the major protein component of amyloid plaques observed in the brain of patients with ADAD and SAD and it is produced via sequential cleavage of APP by two proteases, β- and γ-secretases.[3] The prevailing view about the cause of brain Aβ deposition in ADAD is that *APP* and *PSEN* mutations lead to a chronic increase in the absolute or relative production of the fibrillogenic 42-aminoacid-long form of Aβ (Aβ42) that, over time, leads to formation of brain oligomeric Aβ, deposition of fibrillar Aβ and eventually neurodegeneration.[1] The causes of Aβ accumulation in SAD are far more complex. A predominant view claims that brain Aβ deposition in SAD results from the complex interaction of genetic and environmental factors that end up in a chronic imbalance between Aβ production and clearance. Different mechanisms have been proposed to explain this chronic imbalance in SAD, such as increased,[4–8] altered production[9] or reduced clearance of Aβ.[10] The investigation to elucidate the molecular mechanisms of AD has been complicated by the fact that many studies about the pathogenesis of AD rely on transgenic

mouse models that overexpress ADAD-associated mutations. The results of these investigations are often extrapolated to all forms of AD, irrespective of the underlying causes. Elucidating the differences and commonalities between ADAD and SAD in the human CNS is an important topic as the first intervention trials in preclinical and presymptomatic AD are imminent. Although some previous studies have focussed on the differences in Aβ isoforms between ADAD and SAD in the CNS,[11–14] other aspects of APP processing remain poorly investigated. In this study we focused on BACE protein and activity, and their related cleavage products in a large a collection of well-characterized brain and CSF samples from subjects with ADAD carrying *APP* or *PSEN1* mutations, patients with SAD and age-matched controls.

2. Materials and methods

2.1. Human brain samples

All individuals or relatives had given their written informed consent for research, and the study was approved by the local ethical standards committee on human experimentation. Human brain samples were obtained from the Institut de Neuropatologia, Hospital Universitari de Bellvitge, and the Neurological Tissue Bank of the Biobanc-Hospital Clinic-IDIBAPS. We included samples from 10 patients with ADAD (2 with an APP mutation and 8 with *PSEN1* mutations, mean age 55±8.7 years, **Table 1**),[15–18] 19 patients with SAD (mean age 78±8.0 years, Braak neurofibrillary stage= V-VI, Thal phase of Aβ= 5), and 22 healthy controls (Braak neurofibrillary stage = 0; 12 young controls and 10 elderly controls, mean age 48.7±13.2 and 75.1±6.5 years, respectively). The mean postmortem interval (PMI) was 7.4±4.8 hours. As a confirmation group we included 8 additional cases with the E280A *PSEN1* mutation (mean age 54.5±4.9 years) and 8 age-matched cases with SAD from the University

of Antioquia Brain Bank (**Table 1**).[19,20] For biochemical analyses we used frozen blocks from the frontal association cortex, known to have high density of amyloid plaques.[7,21] For immunohistochemical analyses paraffin-embedded samples from several brain regions were used (see below).

Table 1. Clinical and neuropathological data of ADAD patients from whom brain material was analyzed

NA: not available; NF: neurofibrillary; M: male; F: female.

[a]Source: http://www.molgen.ua.ac.be/ADMutations

Case #	Mutation	Gender	Thal Aβ phase	Braak NF stage	Age at onset	Age at death	APOE genotype	PMI (h)	Effects on Aβ production[a]
1	*APP* I716F	M	5	VI	31	36	33	15	Aβ1-40 ↓ Aβ1-42 ↑ Aβ1-42/Aβ1-40 ↑
2	*APP* A713T	M	5	VI	49	56	33	16	NA
3	*PSEN1* V89L	M	5	VI	48	57	23	9.5	NA
4	*PSEN1* E120G	M	5	VI	34	44	33	5.5	NA
5	*PSEN1* M139T	M	5	V	47	64	33	14.7	Aβ1-42/Aβtot ↑
6	*PSEN1* M139T	M	5	VI	48	57	33	15.2	-
7	*PSEN1* M139T	M	5	VI	45	53	33	5.3	-
8	*PSEN1* P264L	F	5	VI	45	56	44	6	Aβ1-40 = Aβ1-42 ↑ Aβ1-42/Aβ1-40 ↑
9	*PSEN1* P264L	M	5	VI	53	60	34	7.2	-
10	*PSEN1* L286P	F	5	V	35	56	33	5	NA
11	*PSEN1* E280A	F	5	VI	47	54	33	5.5	Aβ1-40 = Aβ1-42 ↑ Aβ1-42/Aβ1-40 ↑
12	*PSEN1* E280A	F	5	VI	42	50	33	7.5	-
13	*PSEN1* E280A	M	5	VI	44	52	33	4.8	-
14	*PSEN1* E280A	M	5	VI	47	56	33	3.3	-
15	*PSEN1* E280A	F	5	VI	49	62	33	4	-
16	*PSEN1* E280A	F	5	VI	37	47	33	2.3	-
17	*PSEN1* E280A	M	5	VI	49	55	44	2.8	-
18	*PSEN1* E280A	F	5	VI	50	60	33	2.8	-

2.2. Human CSF samples

A total of 60 CSF samples were included in this study. CSF samples from *PSEN1* mutation carriers were part of the Genetic Counseling Program (PICOGEN)[22] at the Hospital Clínic, Barcelona. This group included 10 subjects carrying *PSEN1* mutations (5 subjects with ADAD, global deterioration scale 3-5 and 5 presymptomatic mutation carriers), and 7 non-mutation carriers from the same family (**Table 2**). The clinical and CSF data of some of these patients have been previously reported.[23] Adjusted age was defined as the subject's age relative to the median age of onset in the family. We also included 32 CSF samples from patients with dementia due to SAD and 11 age-matched healthy controls (mean age 74.6±5.3 and 67.6±4.0, respectively) obtained at the Hospital Sant Pau, Barcelona.

Table 2. Clinical and demographic data from *PSEN1* mutations carriers from whom CSF was analysed

The age has been omitted in presymptomatic mutation carriers to protect confidentiality. MMSE: Mini-Mental State Examination; NA: not available.

[a]Source: http://www.molgen.ua.ac.be/ADMutations

Group	PSEN1 mutation	Age (years)	MMSE score	CSF Aβ42 levels (pg/ml)	Effects on Aβ production[a]
Healthy controls					
1	-	25.1	29	667	-
2	-	35.4	29	647	-
3	-	34.7	30	691	-
4	-	38.8	29	578	-
5	-	51.7	29	430	-
6	-	43.8	29	734	-
7	-	42.3	28	769	-
Presymptomatic *PSEN1* mutation carriers					
8	M139T	-	30	822	Aβ1-42/Aβtot↑
9	M139T	-	30	753	-
10	M139T	-	28	655	-
11	M139T	-	29	505	-
12	K239N	-	29	1091	NA
Symptomatic *PSEN1* mutation carriers					
13	L235R	46	11	279	NA
14	L282R	46.3	22	199	NA
15	L286P	37.3	28	166	NA
16	L286P	42.6	24	163	NA
17	L286P	44.7	24	165	NA

2.3. BACE-specific enzymatic activity assay

100-200 mg of tissue of the human brain samples was homogenized with the proteoExtractTM Native Membrane Protein Extraction Kit (Calbiochem). BACE1 activity in human brain homogenates was measured as previously described.[4,24] This BACE activity assay was based on an antibody capture assay in which activity was measured via

fluorescent emission after the cleavage of a β-secretase substrate.[4,24] To avoid the detection of other β-secretase activities, BACE was first captured via its C-terminal domain with anti-BACE1 antibody MAB5308 (mouse monoclonal anti-BACE, Chemicon) raised against different epitopes from BACE2. Briefly, 96-well plates were coated overnight with the capture antibody MAB5308 at a dilution of 1:1000 in 100mM carbonate buffer at 4ºC. The plates were washed 3 times with phosphate-buffered saline (PBS, pH 7.0), and then blocked with a blocking reagent (25% BlockAce; Dai-Nippon) for 6 hours. The samples (50 μl of 0.004 wt/vol) were added to the wells containing 50 μl of Superblock® blocking buffer (Pierce) in PBS and incubated for 1 hour at 37ºC. The plates were washed 6 times with PBS, and the enzymatic reaction was carried out by incubation with 10 μM of the fluorogenic β-secretase substrate Arg-Glu(5-[aminoethyl] aminonaphthalene sulfonate [EDANS])-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys (4'-dimethylaminoazo-benzene-4-carboxylate[DABCYL])-Arg (Calbiochem) in acetate buffer at pH 4.1, which is optimal for BACE β-secretase activity.[4,24] Samples were incubated overnight at 37ºC, and the enzymatic reaction was measured using a Victor3 Wallac microplate reader (Perkin-Elmer).

BACE activity in human CSF was measured by incubating 10 μl of sample with 50 μl of BACE substrate (40 μM) overnight at 37ºC in acetate buffer with 100 mM sodium chloride (pH 4.1) containing 0.025% BSA.[25] Fluorescence was measured at different time points with a Victor3 Wallac microplate reader with an excitation wavelength at 355 nm and emission wavelength at 486 nm. The concentration of BACE substrate used was that which best differentiated serial CSF dilutions over different time points. The enzymatic activity was calculated as ΔUF/min from the linear part of the reaction (between 2h and 24h). The activity was completely inhibited by a BACE1 inhibitor verifying the specificity of the assay. The intra-assay and inter-assay coefficients of variation were 1.2% and 5.8%, respectively.

2.4. BACE1 protein, sAPPβ and APP β-CTF assays

BACE1 protein, sAPPα, sAPPβ, and APP β-CTF levels were measured in human brain samples or CSF using commercial kit assays (IBL). These assays are based on a solid-phase sandwitch ELISA using specific anti-BACE or anti-APP antibodies. For sAPPβ levels the cross-reactivity with human sAPPβ-Sw and human sAPPα is 0.25% and 1.41%, respectively, and for APP β-CTF levels the cross-reactivity with human sAPPβ and human sAPPα is ≤0.1%. BACE1 protein and APP β-CTF levels were measured in the membrane brain fraction while sAPPα and sAPPβ levels were measured in CSF.

2.5. Immunohistochemistry procedures

Detailed neuropathological studies were performed on multiple formalin-fixed, paraffin-embedded samples, as previously described.[16] For immunohistochemistry, dewaxed 5-μm thick sections that included hippocampus, parahippocampal and temporo-occipital gyrus were immunostained in an automated stainer (DAKO Autostainer Plus) using the following antibodies: a rabbit anti-APP C-terminal (Sigma-Aldrich) recognizing the C-terminus (amino acids 676-695) of human APP 695, APP751 and APP770 at a dilution of 1:1500; a mouse anti-APP N-terminal (Millipore, clone 22C11) antibody at a dilution of 1:50; and a mouse monoclonal anti-βA4-amyloid (DAKO, clone 6F/3D) antibody at a dilution of 1:400. To further evaluate the neuritic component of amyloid plaques, anti-ubiquitin (DAKO, polyclonal) and anti-hyperphosphorylated tau (Thermo Scientific, mc, clone AT8) antibodies were used at a dilution of 1:400 and 1:200, respectively. APP-immunoreactive structures were assessed semiquantitatively as follows: + mild (1 to 10 conglomerates of dystrophic neurites in one visual field using a 10x objective), ++ moderate (from 10 to 20 neuritic conglomerates), +++ abundant (more than 20 neuritic conglomerates), similarly to the assessment of β-amyloid deposits [1]. For antigen retrieval, sections were immersed for 5

minutes in 98-100% formic acid and heated for 20 minutes in a pressure cooking oven in 0.1M sodium citrate buffer at pH 6.0. The reaction was visualized with 0.05% diaminobenzidine and 0.01% H_2O_2.

2.6. Western blot

Human brain homogenates were electrophoresed in 5% to 16% Tris-Tricine gels, transferred to 0.2 μm nitrocellulose membranes, and detected by immunoblotting with a rabbit anti-APP C-terminal (1:2000; Sigma-Aldrich), a rabbit monoclonal N-terminus anti-BACE (D10E5, 1:1000; Cell Signaling), a mouse monoclonal C-terminus anti-BACE (MAB5308, 1:1000; Chemicon) or mouse monoclonal anti-tubulin (1:20000; Sigma-Aldrich) antibodies. Specificity of the anti-BACE antibodies was verified by Western blot using brain homogenates from P7 BACE1 -/- mice (a kind gift from Bart De Strooper,[26] **Fig. S1**). Incubation with primary antibodies was followed by detection with IR-fluorescent-conjugated antibody (LI-COR Biosciences). All blots were quantified by densitometric analysis and normalized to tubulin (Odyssey software, LI-COR Biosciences).

2.7. *APOE* genotyping

APOE genotype was determined as previously described.[16]

2.8. Statistical analysis

Non-parametric statistical analysis (Kruskal-Wallis) was performed to analyze differences in BACE1, APP β-CTF, sAPPβ protein levels, and BACE activity. Correlation analysis between age and CSF BACE1 activity, APP β-CTF, and sAPPβ protein levels was performed using the

Spearman's Rho test. Statistical significance for all the analyses was set at 5% (α= 0.05). All data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20.0.

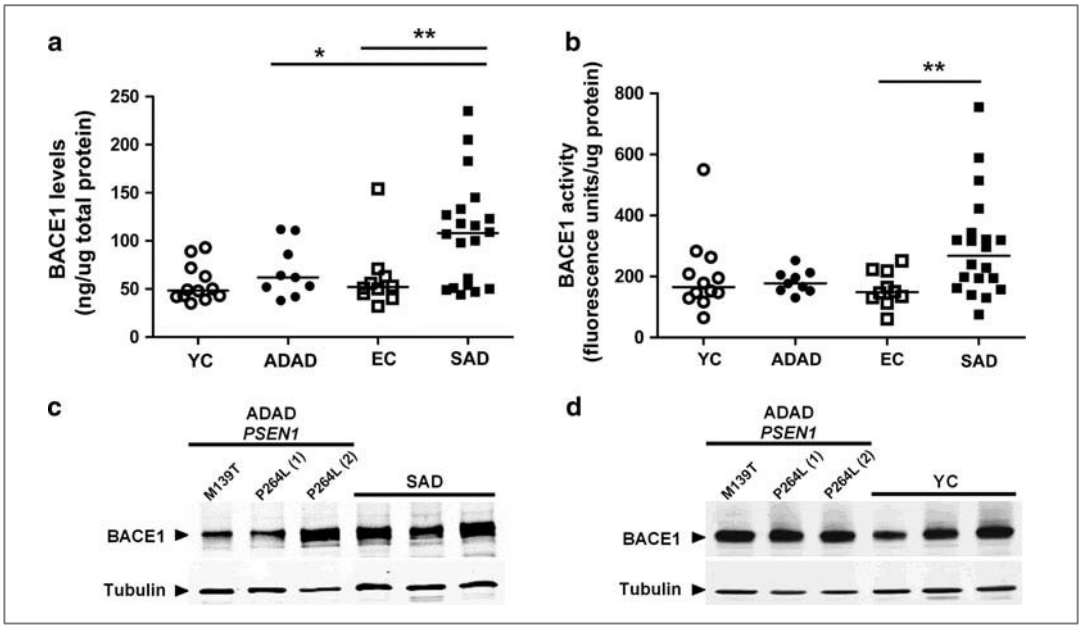
3. Results

3.1. BACE1 protein levels and activity are elevated in SAD but not in ADAD brains

We first examined the initial proteolytic cleavage involved in Aβ generation, performed by BACE1.[3] BACE1 protein levels and activity were measured in homogenates from the frontal cortex from ADAD cases, and compared with those from SAD cases and age-matched controls. Across the entire group (n=51) BACE1 protein levels and activity positively correlated with age (ρ=0.3, p=0.03 and ρ=0.28, p=0.04 respectively). Consistent with other studies,[4] there was no association of BACE1 protein levels or activity with PMI, gender or *APOE* genotype. BACE1 protein levels correlated with BACE1 activity in the entire sample (ρ=0.29, p=0.04). No differences were detected in either brain BACE1 protein levels or activity between ADAD cases and age-matched controls (**Fig. 1a, b**; p=0.91 and p=0.42, respectively). Consistent with previous reports,[4,8,27] we found an increase in BACE1 protein levels (1.91 fold, **Fig. 1a**, p=0.01) and activity (1.76 fold, **Fig. 1b**, p=0.04) in the frontal cortex of SAD cases when compared to age-matched controls. There was a significant increase in BACE1 protein levels (p=0.03) but not in BACE1 activity (p=0.12) in SAD relative to ADAD cases. The levels of BACE1 protein in brain homogenates were also analyzed by Western blot using the specific anti-BACE1 antibody D10E5 (**Fig. S1**). These analyses confirmed the increase in BACE1 protein expression in SAD relative to controls and ADAD, as well as the lack of differences between ADAD and controls (**Fig. 1c, d**).

Figure 1. Brain BACE1 protein levels and activity in ADAD, SAD patients and controls

BACE1 protein levels and activity were measured in brain homogenates from ADAD and SAD patients, and from young (YC) and elderly (EC) controls. There was an increase in BACE1 protein levels (**a**, **p=0.01) and activity (**b**, *p=0.04) in the frontal cortex of SAD cases compared to age-matched elderly controls. No differences were detected between ADAD cases and age-matched controls in either brain BACE1 protein levels or activity (p=0.91 and p=0.42, respectively). There was a significant increase in BACE1 protein levels (**a**, *p=0.03) but not in BACE1 activity (**b**, n.s., p=0.12) in SAD relative to ADAD cases. Western blot analyses using the BACE-specific antibody BC05 confirmed the increased in BACE expression in SAD compared to ADAD (**c**) and the lack of differences between ADAD and controls (**d**). Representative blots are shown.



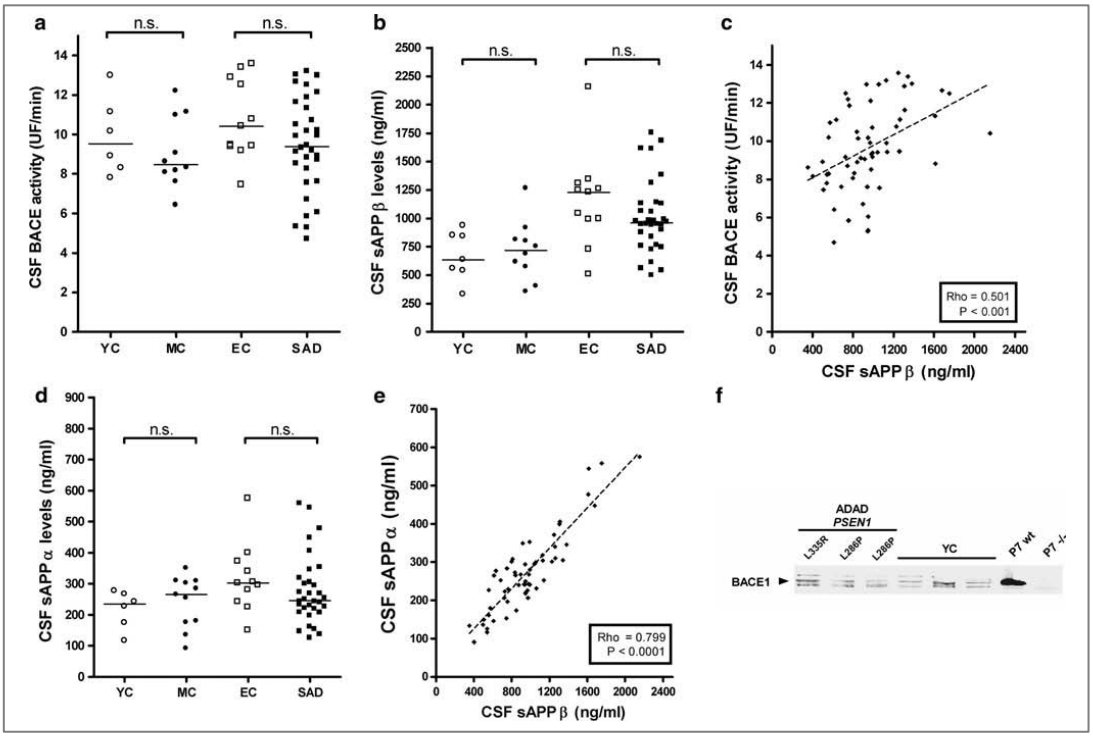
3.2. CSF BACE1 expression and activity are not elevated in *PSEN1* mutation carriers or in SAD dementia cases

We next tested whether an increase in BACE1 expression or activity was present in ADAD in the CSF. We obtained CSF samples from a cohort of subjects recruited from a genetic counseling program for familial dementias.[22,23] We used a fluorogenic CSF BACE1 enzymatic activity assay to measure BACE1 activity in CSF samples from 17 *PSEN1* mutation carriers and non-carriers (**Table 2**), subjects with SAD dementia (n=32) and healthy controls (n=11). CSF

BACE1 activity did not correlate with age, MMSE score or CSF Aβ42 levels in the entire sample (n=60) or with the adjusted age in *PSEN1* mutation carriers (n=10). When analyzed according to clinical or mutation status, no differences in CSF BACE1 activity were detected among *PSEN1* mutation carriers and non-mutation carriers (p=0.85) or between SAD cases and controls (p=0.1, **Fig. 2a**). As an additional measure of APP processing, we analyzed CSF levels of sAPPβ, a soluble fragment generated by BACE cleavage. Levels of sAPPβ correlated positively with age (Rho=0.279; p=0.03) but not with MMSE score or CSF Aβ42 levels in the entire sample. We found no differences in CSF sAPPβ levels between *PSEN1* mutation carriers and non-mutation carriers (p=0.85, **Fig. 2b**) or between SAD dementia cases and age-matched controls (p=0.12, **Fig. 2b**). CSF BACE1 activity and sAPPβ levels showed a positive correlation in the entire subject sample (Rho=0.501, p<0.001, **Fig. 2c**). Almost identical results were found when CSF sAPPα levels were measured (**Fig. 2d**). This is not surprinsing since CSF sAPPβ and sAPPα levels showed a strong positive correlation in the entire subject sample (Rho=0.799, p<0.0001, **Fig. 2e**), as in previously reported studies.[28,29] To examine whether there was any difference in CSF BACE1 protein levels, Western blotting was carried using the specific anti-BACE1 antibody D10E5 and no differences were detected between *PSEN1* mutation carriers and non-mutation carriers (**Fig. 2f**). Overall, no changes in CSF BACE1 activity or expression could be detected in subjects with *PSEN1* mutations or SAD compared to age-matched controls.

Figure 2. CSF BACE1 activity in *PSEN1* mutation carrries, SAD patients and controls

(**a**) CSF BACE1 activity was measured in non-mutation carriers controls (YC), *PSEN1* mutation carriers (MC), elderly controls (EC) and SAD patients. No differences were found between groups in CSF BACE1 activity. (**b**) CSF sAPPβ levels were determined in YC, MC, EC and SAD patients. No differences were found between SAD cases and age-matched controls or between MC and YC. (**c**) CSF BACE1 activity and sAPPβ levels showed a positive correlation in the entire subject sample (ρ = 0.501, p<0.001). (**d**) CSF sAPPα levels were determined in YC, MC, EC and SAD patients. No differences were found between SAD cases and age-matched controls or between MC and YC. (**e**) CSF sAPPβ and sAPPα levels showed a strong positive correlation in the entire subject sample (ρ = 0.799, p<0.0001). (**f**) Western blot analyses of BACE1 protein levels using the specific anti-BACE1 antibody D10E5 showed no differences between *PSEN1* mutation carriers and non-carriers (YC). The specificity of the D10E5 was determined by using brain samples from 7-days old (P7) wt and BACE 1 -/- mice (**Fig. S1**). A representative blot is shown.



3.3. Brain APP β-CTF levels are higher in ADAD than SAD

Using ELISA, we then determined the levels of APP β-CTF, a protein fragment generated from full-length APP in frontal cortex brain homogenates obtained from patients with ADAD, SAD and controls. The APP β-CTF fragment is generated by BACE and processed by γ-secretase to release Aβ peptides.[3] We found that ADAD cases showed a prominent APP β-

CTF accumulation when compared to age matched-controls and to SAD cases (both p<0.001, **Fig. 3a**). SAD cases did not show statistically significant higher APP β-CTF levels than age-matched controls by ELISA (p=0.47, **Fig. 3a**), despite being elevated by Western blot (**Fig. S2**). No differences were found between young and elderly controls. The levels of APP β-CTF were not influenced by age, gender or *APOE* genotype. The results of the ELISA were confirmed by Western blot analysis in our subject sample and in patients with the E280A *PSEN1* mutation (**Fig. 3b, c, d, Fig. S2**). Among ADAD cases, the *APP* A713T and some *PSEN1* mutations (P264L, P286P) displayed higher levels of APP C-terminal fragments than others (M139T, V89L).

Figure 3. Brain APP β-CTF levels are elevated in ADAD

APP β-CTF levels were measured in membrane fractions in brain homogenates from ADAD, SAD and controls (**a**). ADAD cases showed higher APP β-CTF levels than age-matched controls and SAD (**=p<0.01). These differences were confirmed by Western blot in samples from patients with ADAD, SAD and controls. APP CTF accumulation was observed in ADAD cases compared to age matched-controls (**b**) and to SAD cases (**c**, **d**). APP CTF accumulation was also observed by Western blot in SAD cases compared to controls despite the fact that it did not reach statistical significance in the ELISA assay (**e**).

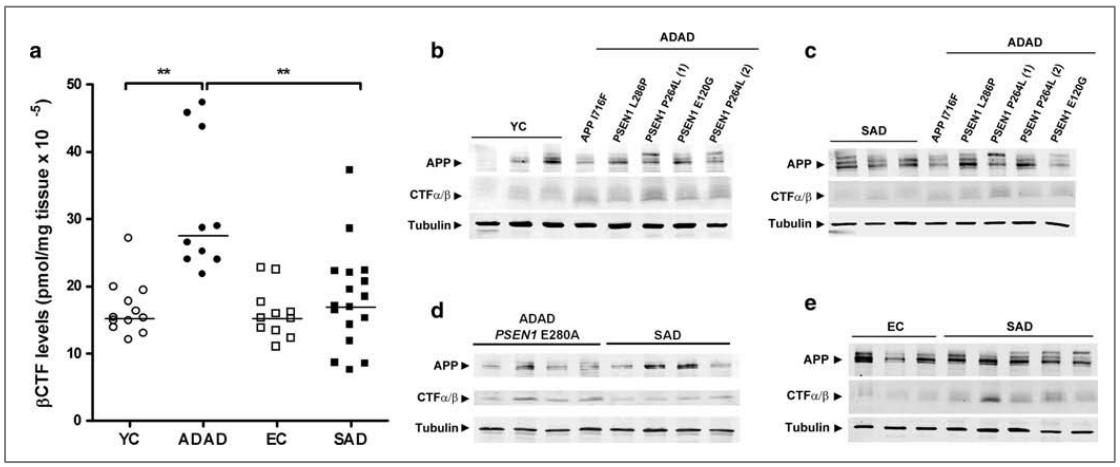


Table 3. Relationship between dystrophic neurites and Aβ deposits in ADAD cases

Case	Mutation	APP-immunoreactive dystrophic neurites	Aβ deposits
1	*APP* I716F	+++	Abundant mature and primitive plaques
2	*APP* A713T	+++	Abundant mature and primitive plaques. Few diffuse plaques
3	*PSEN1* V89L	+++	Abundant mature and primitive plaques
4	*PSEN1* E120G	+++	Abundant mature and primitive plaques
5	*PSEN1* M139T	++	More primitive plaques than mature plaques
6	*PSEN1* M139T	++	Abundant mature and primitive plaques
7	*PSEN1* M139T	+++	Abundant mature and primitive plaques
8	*PSEN1* P264L	+	Predominantly cotton-wool plaques
9	*PSEN1* P264L	++	Abundant cotton-wool plaques mixed with mature and primitive plaques
10	*PSEN1* L286P	+	Predominantly cotton-wool plaques

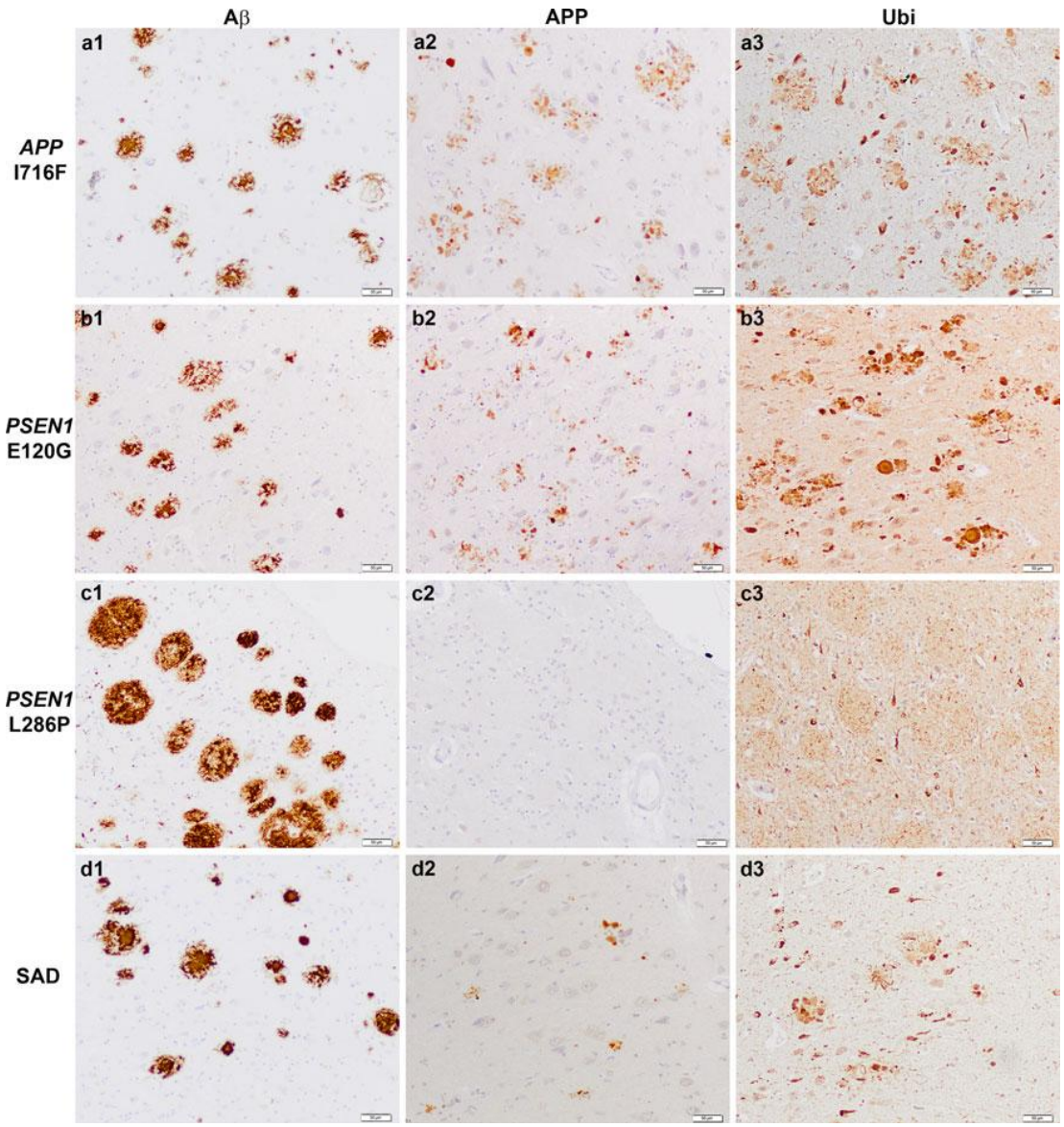
3.4. APP-immunoreactive dystrophic neurites and aggravated neuritic component in ADAD

Since our biochemical data indicated elevated APP β-CTF levels in ADAD compared to SAD and controls, we next evaluated the distribution of APP accumulation. We performed immunohistochemical studies on brain sections from ADAD cases using either an anti-APP C-terminal or an anti-APP N-terminal antibody. As previously described,[30–32] we confirmed that antibodies rose to APP labeled dystrophic neurites of senile plaques in SAD using both, anti C-terminal and anti-N-terminal antibodies. Both APP antibodies also detected APP epitopes in dystrophic neurites of senile plaques in ADAD (**Fig. S3**). Semiquantitative assessment of the neuritic component associated to amyloid plaques in parahippocampal and temporo-occipital cortices revealed that patients with ADAD had a more prominent neuritic component than those with SAD (**Fig. 4, Table 3**) independently of the number of Aβ plaques. This was also observed using anti-ubiquitin (**Fig. 4**) and anti-phosophorylated tau antibodies (**Fig. S3** and data not shown). Cases with predominant cotton-wool plaques

(*PSEN1* P264L, *PSEN1* L286P) showed fewer APP-immunoreactive dystrophic neurites than ADAD cases with neuritic plaques (**Fig. 4**).

Figure 4. APP accumulates in dystrophic neurites in ADAD.

Immunohistochemistry for Aβ, APP and ubiquitin on representative brain sections from ADAD subjects carrying the *APP* I716F (**a1-a3**), the *PSEN1* E120G (**b1-b3**), *PSEN1* L286P (**c1-c3**) mutations and from one patient with SAD (**d1-d3**). Note frequent Aβ deposits, APP- and ubiquitin-positive bulbous dystrophic neurites in subjects with *APP* I716F and *PSEN1* E120G mutations in contrast to the nearly lack of APP-positive neurites in subject with the *PSEN1* L286P mutation where cotton-wool plaques predominate. In the latter case, ubiquitin immunostains delicate intermingled neurites (**c3**). In the SAD case, abundant mature Aβ deposits (**d1**) contrast with the few APP- (**d2**) and prominent ubiquitin-immunoreactive dystrophic neurites. *Bar* 50 µm



4. Discussion

The main finding in the current study is that ADAD and SAD display distinct profiles in BACE protein and activity, and in APP β-CTF levels in the brain. While no apparent increase in brain BACE1 protein levels or activity was observed in ADAD, both were clearly elevated in SAD. Accumulation of APP β-CTF was higher in the brain in ADAD than in SAD and controls. No changes in BACE measures were observed in the CSF between ADAD and SAD.

Research in ADAD has been instrumental as a model to understand the pathogenesis of SAD and to guide the current development of anti-amyloid strategies. The mainstream paradigm claims that ADAD and SAD share similar clinical and pathological phenotypes as well as common mechanisms of disease, irrespective to the initiation factors.[1,33] However, very few studies have investigated the differences in APP processing between ADAD and SAD in the human CNS to support this view. Previous studies have mainly focused on Aβ isoforms, tau or p-tau in the CSF[11,13,14,34] or Aβ isoforms in human brain.[12,14] The findings suggest a specific CSF profile of Aβ isoforms in ADAD, with low levels of Aβ1-37, Aβ1-38, Aβ1-39 and Aβ1-42 compared with SAD.[11,34] Whether other relevant aspects of APP processing differ between ADAD and SAD remains unknown.

BACE1 is a type-I transmembrane protease that is highly expressed in neurons.[35] Previous studies have demonstrated that BACE1 protein levels and activity are elevated approximately two-fold in the brain of SAD patients,[4,6,8,36] suggesting that this feature might initiate or contribute to brain Aβ accumulation.[7] Our data confirmed the increased BACE1 levels and activity in SAD brains, but no increase could be detected in ADAD cases compared to age-matched controls. Although the increase in brain BACE1 protein levels in SAD relative to ADAD cases might reflect a difference in chronological age between groups, the

increment in brain BACE1 activity and protein levels in SAD compared to age-matched controls suggests a disease-specific effect. Our data differ from the only previous study that had examined BACE1 expression or activity in ADAD brains,[37] where the authors reported an increase in BACE1 mRNA levels and activity in 11 ADAD cases carrying 10 different *PSEN1* mutations. The strengths of the current study are the use of age-matched controls without any brain lesion, the selection of a region in which elevated BACE1 activity had been previously detected in SAD,[7] and the investigation of CSF samples obtained from *PSEN1* mutation carriers. Our findings lend support to other studies that reported no change in BACE1 expression or activity in either APP or PS1 mutant-transfected cells or APPxPS1-transgenic mice.[7,24,38]

In contrast with our findings in human brain, we did not find any differences in CSF BACE activity or expression, or in sAPPβ levels between groups. The lack of increase in CSF BACE1 activity or sAPPβ levels in cases with SAD dementia compared to healthy controls is in agreement with most recent studies.[29,39] The present data together with previous work,[25,29,39] suggest that BACE1 activity may become elevated at the stage of mild cognitive impairment, and then decrease over time as disease progresses.[39] Our data obtained in patients with SAD dementia also indicate that CSF BACE1 activity does not parallel brain BACE1 activity, at least in the advanced stage of the disease. While BACE1 activity and protein levels in the brain tend to increase in late stage AD, BACE1 activity in the CSF would stabilize or even decrease, perhaps as a result of a reduction in global neuronal function.[39] More generally, this observation indicates that CSF may not accurately reflect the changes in the local intracellular or extracellular environment.[40,41] Taken together, our results indicate that an increase in brain BACE1 up-regulation is characteristic of SAD, but is not a salient feature in many ADAD-associated mutations. Previous studies[5,6,42] have shown that increased BACE1 expression in SAD is due to post-translational regulation mechanisms and

that BACE1 mRNA levels are unchanged. One possible explanation is that the increase in BACE1 in SAD results from the interaction of Alzheimer pathology with diverse factors associated to ageing, such as oxidative stress, inflammatory changes or microRNA dysregulation, conditions known to increase BACE1 expression and activity in cell culture.[5,43–45]

The lack of increase in BACE1 in ADAD has clinical implications as BACE1 has become an attractive drug target for AD intervention.[35] Although inhibitor development has proved to be highly challenging, some promising BACE1 inhibitors as well as other strategies, such as immunization with anti-BACE1 antibodies, have been developed.[35] The lack of increase in brain or CSF BACE1 expression or activity in ADAD in our study suggests that BACE1 is a less attractive target for families with ADAD than for patients with SAD. Nonetheless, it is still possible that BACE1 inhibition may prove to be effective as a preventive therapy in subjects with *APP* or *PSEN1* mutations. This is a relevant and timely topic since clinical trials in ADAD are imminent.

Another important finding derived from our study is the higher acumulation of APP β-CTFs in the brain of ADAD cases than in SAD patients and controls. The APP β-CTF fragment is generated by BACE and processed by γ-secretase to release Aβ peptides.[3] The main explanation for the APP β-CTF accumulation in SAD has been the overproduction due to increased BACE1 protein levels and activity.[8] However, the lack of elevated BACE1 in ADAD points to other underlying mechanisms. It has been suggested that *PSEN* mutations alter the conformation of the γ-secretase complex.[46,47] This change could be a plausible mechanism by which *PSEN* mutations lead to γ-secretase dysfunction and the formation of longer Aβ peptides in ADAD.[46,48] Since APP β-CTF are processed by γ-secretase, it is possible that elevated APP β-CTF may be the result of a dysfunctional γ-secretase. A recent study has

shown that ADAD-associated mutations do not consistentently affect kinetic activity[48] excluding the possibility that mutations inhibit γ-secretase. However, conformational changes in γ-secretase may subtly slow substrate processivity, which could increase β-CTF in ADAD. Other possible mechanisms underlying APP β-CTF accumulation in ADAD include impaired macroautophagy as it has been shown that APP β-CTF is rapidly cleared by autophagy under physiological conditions.[49] In any case, the accumulation of APP CTFs has been shown to be neurotoxic by itself.[50–53] This phenomenon has also been observed in wild-type mice or transgenic APP mouse models after treatment with classical γ-secretase inhibitors[54,55] or after inactivation of *PSEN1*.[56] In both these situations, APP CTFs accumulate at the presynaptic terminals, likely impairing synaptic plasticity and long-term memory.[55,56] Interestingly, APP CTF accumulation has been postulated, together with inhibition of Notch processing,[48] as a possible mechanism underlying cognitive side effects in patients with AD treated with the γ-secretase inhibitor Semagacestat.[3,54] Although the precise cause of APP CTF accumulation in ADAD deserves further investigation, it is likely that this feature acts as an active component of the disease that may contribute to the metabolic and cytoskeletal derangement and neurodegeneration.

Our findings also demonstrate that the neuritic component is more prominent in ADAD cases than in SAD. It has previously been shown that full-length APP accumulates in dystrophic neurites in SAD[30–32,57,58] and that this accumulation is an early event that occurs prior to tau accumulation.[30] Our results extend these findings to ADAD and show a more severe neuritic component in ADAD than in SAD. The wide variety of neuronal proteins found in AD in dystrophic neurites has been increasingly recognized as a failure of the autophagic-lysosomal pathway.[59] In addition, it has been shown that PS1 is essential for lysosomal proteolysis and autophagy and that PS1-null or PS-ADAD fibroblasts display marked autophagy impairment.[59,60] This defect could account for our observation of

numerous and enlarged dystrophic neurites in ADAD as compared to SAD. Finally, the contribution of APP-immunoreactive dystrophic neurites to parenchymal amyloid deposition seems unlikely because at least one half of diffuse plaques, which may represent the earliest stage of the amyloid plaque, do not contain APP-immunoreactive neuritic profiles[30,31] and we did not observe APP epitopes in dystrophic neurites in ADAD cases with cotton-wool plaques.

The main limitation in the present study is that as only the frontal cortex region was analyzed for BACE1 activity and protein levels, we cannot exclude the possibility that other brain areas might have shown different results. Besides, our study only included cases carrying either two *APP* mutations or nine *PSEN* mutations, and whether our findings are generalizable to all ADAD cases requires further investigation. Finally, it is worth mentioning that the two APP mutations investigated herein are close to the γ-secretase cleavage site, and they were predicted to affect γ-secretase processing on a similar way to *PSEN* mutations. It is possible that other APP mutations located outside of the γ-secretase cleavage site may have shown different effects.

In summary, the data presented herein reinforce the different physiopathological mechanisms underlying the Aβ production/clearance imbalance in SAD and ADAD. These differences in APP processing may contribute to explain the lack of alignment between studies in humans and in AD animal models. A deeper understanding of the common and divergent fundamental pathogenic mechanisms in ADAD and SAD is needed to fine tune and accelerate drug development in AD.

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The authors declare that they have no conflict of interest.

6. Supplementary material

Figure S1. Specificity of the anti-BACE antibodies used in this study

The specificity of two anti-BACE antibodies (D10E5, N-terminus, Cell Signaling; MAB5308, C-terminus, Chemicon) was tested using brain homogenates from a SAD case, 7-days old (P7) wild type and BACE1 -/- mice (a kind gift from Bart De Strooper). Western blot analysis showed absence of BACE1 immunoreactivity (arrowhead) in samples from P7 BACE1 -/- mice.

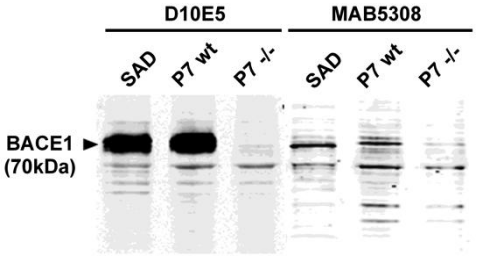


Figure S2. Western blot analyses of APP CTFs in human brain samples

APP-FL and APP CTFs were detected by using a rabbit APP C-terminal antibody. Cell lysates from CHO cells overexpressing APP treated with the γ-secretase inhibitor DAPT were used as a control (**a**). Densitometric analysis of the ratio APP-CTFα-β/APP-FL from YC and ADAD (**b**), SAD and ADAD (**c**), and EC and SAD (**d**). Values represent the mean of al least three indendent experiments. Values are expressed as a % of controls (**b, d**) or SAD (**c**).

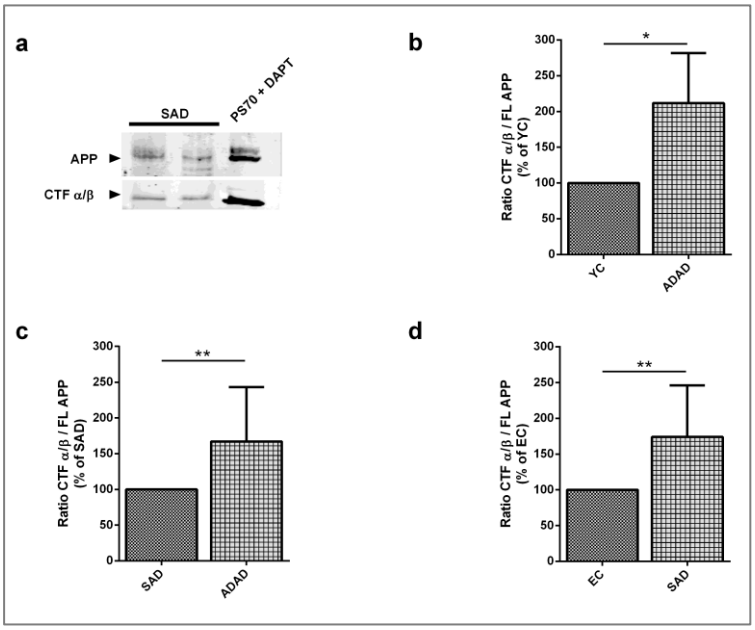
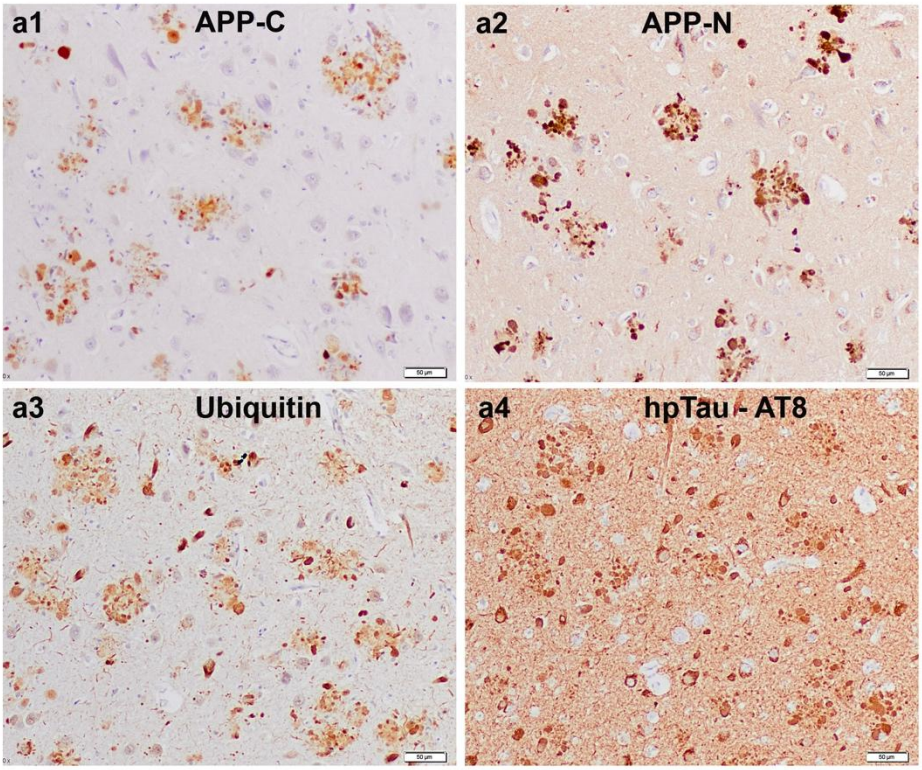


Figure S3. Anti-APP antibodies label dystrophic neurites of senile plaques in ADAD

Both anti-C-terminal (**a1**) and anti-N-terminal (**a2**) APP antibodies detect dystrophic neurites in a patient with the *APP* I716F mutation. In addition to APP, tau (**a3**) and ubiquitin (**a4**) antibodies label the neuritic component of amyloid plaques. *Bar* 50 µm



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Chapter 4

Relationship between β-secretase, inflammation and core CSF biomarkers for Alzheimer's disease

J Alzheimers Dis. 2014;42(1):157-167

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Abstract

Background: Biomarkers in the cerebrospinal fluid (CSF) can track specific pathophysiological pathways underlying Alzheimer's disease. The connection between these biomarkers remains unclear.

Objective: To study six CSF biomarkers in a clinical cohort of patients with different neurodegenerative conditions.

Methods: We measured markers of Amyloid-β Precursor Protein (AβPP) processing ($Aβ_{42}$, sAβPPβ, β-secretase activity), neuronal damage (total tau, p-tau) and inflammation (YKL-40) in CSF from 194 participants with the following diagnoses: subjective cognitive impairment or non-amnestic mild cognitive impairment (na-SCI, n=44), amnestic mild cognitive impairment (aMCI, n=45), dementia of the Alzheimer type (DAT, n=59), frontotemporal dementia (FTD, n=22) and 24 cognitively normal controls. We compared biomarkers between clinical groups and CSF-profile groups, and we analyzed the correlation between biomarkers.

Results: CSF levels of sAβPPβ were decreased in FTD patients compared to the other groups. YKL-40 was elevated in DAT and FTD, and also in aMCI patients with evidence of the Alzheimer's pathophysiological process. CSF $Aβ_{42}$ correlated positively with β-secretase activity (R_S=0.262) and sAβPPβ (R_S=0.341). CSF YKL-40 correlated positively with total tau (R_S=0.467) and p-tau (R_S=0.429). CSF p-tau and sAβPPβ contributed significantly to distinguish DAT from FTD.

Conclusions: CSF biomarkers of AβPP processing correlate with each other and are decreased in FTD. The inflammatory marker YKL-40 is increased in different neurodegenerative diseases, even in early stages, and it correlates with biomarkers of neurodegeneration. This suggests that inflammation is a common feature in AD and FTD. A combination of CSF biomarkers tracking distinct pathophysiological processes may be useful to classify subjects with neurodegenerative conditions.

Keywords: Biological markers; cerebrospinal fluid; amyloid β-protein precursor; inflammation; YKL-40; β-secretase; Alzheimer disease; frontotemporal dementia

1. Introduction

Cerebrospinal fluid (CSF) biomarkers are progressively being incorporated into clinical care and clinical trial settings in Alzheimer's disease (AD) and other neurodegenerative diseases. In AD, many studies have consistently found a biological signature in CSF characterized by low levels of Aβ42, and high levels of total tau and phosphorylated tau (p-tau).[1] This signature has proven to be highly sensitive and specific to distinguish patients with dementia of the Alzheimer type (DAT) from healthy controls,[1,2] and also to predict progression to dementia in patients with mild cognitive impairment (MCI),[3–6] and long-term outcome in subjects with preclinical AD.[7] As a result, CSF Aβ42, total tau and p-tau were introduced in the 2011 revised research criteria of AD to detect the AD pathophysiological process in subjects with dementia or MCI.[8,9] Under these criteria, patients with a CSF signature of AD are placed in the category of "mild cognitive impairment due to AD" and "dementia with evidence of the AD pathophysiological process". CSF biomarkers have also been incorporated in clinical trials in AD to ensure target engagement, and to improve the selection of subjects with underlying AD pathology. CSF biomarkers can also be used to detect other pathologies that frequently coexist with AD pathology.[10]

Besides core CSF biomarkers of AD ($A\beta_{42}$, total tau and p-tau), other markers have been investigated as tools to explore the normal and abnormal pathways that occur during the disease.[11] One of these markers is BACE (β-site AβPP-cleaving enzyme), a β-secretase involved in the amyloidogenic proteolytic processing of AβPP. Its cleavage generates the β-C-terminal fragment (that is subsequently processed by γ-secretase to generate $A\beta_{42}$) and the soluble fragment sAβPPβ.[12,13] It has been found that β-secretase activity and BACE protein levels are increased in AD post-mortem brain tissue,[14,15] and the brain levels of BACE

protein correlate with Aβ deposition.[16] β-secretase activity can also be detected in CSF,[15,17–23] but studies in patients with AD have yielded variable results. Some studies[19,21] have reported mild increases in β-secretase activity in the CSF of patients in the early stages of AD, whereas others have not found differences compared to healthy controls.[15,22–24] Similarly, the CSF levels of sAβPPβ have also been investigated as an indicator of AβPP processing in research studies. Some authors have reported that sAβPPβ levels are slightly elevated in the early stages of AD compared to controls,[25–27] but others could not confirm these results.[15,19,23,24] Although their diagnostic value is yet to be determined, sAβPPβ levels are consistently being used as a measure of target engagement in clinical trials with β-secretase inhibitors.

Another important pathogenic process that can be detected in the brain in the early stages of the disease is neuroinflammation.[28,29] Inflammation can be studied indirectly through the analysis of CSF, and several molecules have been proposed for this purpose.[11] One of these molecules is YKL-40, also known as chitinase-3-like-1 protein. This inflammatory molecule, produced by astrocytes, has been investigated as a marker of glial activation in plasma and in CSF in conditions such as cancer or multiple sclerosis.[30,31] YKL-40 levels in CSF have been found to be increased in the very early stages of AD,[32–34] although other authors could not replicate these results.[35]

A large body of evidence from studies in human brain and animal models of AD has shown that AβPP processing, inflammation and neuronal damage are highly interrelated processes. First, BACE is known to be a stress-response protein, up-regulated in the brain under inflammatory conditions.[36] Second, some AβPP metabolites derived from β-secretase cleavage have been shown to be neurotoxic and cause neuronal damage.[15,37–39] Finally, experimental studies have found that inflammatory mediators can accelerate the aggregation of $A\beta_{42}$ and tau, and also contribute to the perpetuation of neuronal

damage.[28,40] However, the precise link between these pathways *in vivo* has not been fully elucidated, and the usefulness of a combination of these markers in the diagnosis of neurodegenerative diseases needs to be clarified. In this study, we aimed to analyze the relationship between CSF markers of AβPP processing, inflammation and the core biomarkers of AD in a clinical cohort of patients with different neurodegenerative conditions.

2. Methods

2.1. Study participants and clinical classification

We prospectively included 194 subjects from the Memory Unit at Hospital Sant Pau evaluated between January 2009 and October 2012 (**Table 1**). Participants had the following clinical diagnoses: subjective cognitive impairment (SCI, n=31), non-amnestic mild cognitive impairment (naMCI, n=13), amnestic mild cognitive impairment (aMCI, n=45), dementia of the Alzheimer's type (DAT, n=59) and frontotemporal dementia (FTD, n=22), comprising behavioral variant FTD (n=12), progressive non-fluent aphasia (n=7) and semantic dementia (n=3). We included a group of cognitively normal controls (NC, n=24) recruited among patients' caregivers. All participants were evaluated by neurologists with expertise in neurodegenerative diseases, and all underwent formal cognitive evaluation using a previously published neuropsychological battery.[41] Subjects diagnosed with SCI had memory complaints but their neuropsychological evaluation was in normal range for age and education. Subjects diagnosed with MCI met Petersen's criteria[42] and were classified as aMCI (if memory domain was impaired) or naMCI (if memory was normal but other domains were impaired). Patients with a diagnosis of DAT met the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association.[43] Patients diagnosed with behavioral variant FTD fulfilled the

new revised criteria,[44] and those with progressive non-fluent aphasia or semantic dementia fulfilled the primary progressive aphasia international consensus criteria.[45] Finally, subjects were classified as NC when they did not have cognitive complaints and the results of the neuropsychological evaluation were in normal range for age and education. In order to minimize the number of diagnostic categories with small number of subjects, the groups of SCI and naMCI were pooled together in a single group (na-SCI) for the analysis, as there were no differences in any of the variables studied (**Supplementary Table 1**). All participants gave their written consent, and the study was approved by the local ethics committee following the ethical standards recommended by the Helsinki Declaration.

2.2. CSF analyses

CSF was obtained through lumbar puncture as described,[46] and samples were collected following international consensus recommendations.[47] Briefly, CSF was collected in polypropylene tubes and immediately centrifuged (1900-2000g x 10min) to avoid any possible haematic contamination. All samples were processed in the first 2 hours after extraction and stored in 1.5 ml polypropylene tubes at -80ºC until analysis. We used commercially available ELISA kits to determine levels of $A\beta_{42}$ (Innotest™ β-Amyloid$_{1-42}$, Innogenetics), total tau (Innotest™ hTAU Ag, Innogenetics), p-tau$_{181}$ (Innotest™ Phospho-Tau$_{181P}$, Innogenetics), sAβPPβ (Human sAPPβ-w highly sensitive, IBL) and YKL-40 (MicroVue™, Quidel) following the manufacturers' recommendations. We also measured CSF β-secretase activity as previously described.[15] Briefly, we incubated the CSF sample with a fluorogenic β-secretase substrate (β-Secretase Substrate IV, Fluorogenic, Calbiochem®) and measured the fluorescence at different time points. The highest intra- and inter-assay coefficients of variation for all the experiments were 5.4% and 15% (**Supplementary Table**

2). Our laboratory has experience in CSF biomarker determination and participates in the Alzheimer's Association external quality control program for CSF biomarkers.[48]

2.3. CSF classification

In addition to the clinical diagnosis, patients were classified according to the evidence of AD pathophysiological process based on the core CSF AD biomarkers. For this purpose we used the ratio between total tau and $A\beta_{42}$ (tau/$A\beta_{42}$ ratio) with a cut-off point of 0.52. The diagnostic accuracy of this ratio was assessed using a receiver operating characteristic curve (ROC) in a subset of participants of this study: 45 patients with DAT and 20 age-matched controls. The area-under-the-curve for the tau/$A\beta_{42}$ ratio in this subset of patients was 97.3%. A cut-off point of 0.52 had a sensitivity of 91.1% and a specificity of 95.0% to classify patients with DAT and normal controls. Subjects in the present study were classified as having evidence (CSF-AD; tau/$A\beta_{42}$>0.52) or no evidence (CSF-no AD; tau/$A\beta_{42}$≤0.52) of an AD pathophysiological process.

2.4. Genetic analysis

APOE was genotyped according to previously described methods.[49]

2.5. Statistical analysis

As some of the biomarkers did not follow a normal distribution we used non-parametric tests for the analyses. We compared groups through Kruskal-Wallis, followed by the Mann-Whitney U test, and determined age-adjusted Spearman rank correlations (R_S) between biomarkers. One subject with na-SCI with unusually high β-secretase activity (>4-fold standard deviations above the mean value) was considered an outlier and excluded from the analysis. We used the Mann-Whitney U test to compare the levels of biomarkers between

APOEε4 carriers and non-carriers, and also between participants with CSF-AD and CSF-no AD within each clinical category. Additionally, we performed a multivariate analysis through a logistic regression within the patients with dementia to study the effect of individual biomarkers in the classification of DAT against FTD. We transformed variables when necessary to obtain a normal distribution. Age, sex and the biomarkers studied were introduced as co-variables in a forward stepwise logistic regression model. As some of the variables were highly correlated, we assessed multicollinearity and excluded those variables with a variance inflation factor above 5. Finally, ROC curves were used to compare the diagnostic power of the final multivariate combination and the individual biomarkers. Statistical significance for all the analyses was set at 5% (α=0.05) and results were corrected for multiple comparisons (Bonferroni) when necessary. All the statistical analyses were performed using the IBM SPSS Statistics software v.19.0 for Windows.

3. Results

3.1. CSF biomarkers across diagnostic groups

Table 1 shows the demographic, clinical, and CSF biomarker data for all diagnostic groups. **Figure 1** compares the levels of the CSF biomarkers across the groups. We first investigated β-secretase activity and sAβPPβ levels, two CSF markers of AβPP processing. There were no differences in β-secretase activity and sAβPPβ between na-SCI, aMCI, DAT and NC. There was a mild decrease in β-secretase activity in FTD patients compared to the groups of na-SCI (p=0.004), and in the limit of statistical significance compared to aMCI (p=0.009) and NC (p=0.011). Levels of sAβPPβ were significantly lower in the group of FTD compared to NC (p<0.001), na-SCI (p<0.001), aMCI (p<0.001) and DAT (p<0.001). Next, we measured the CSF levels of YKL-40, a marker of neuroinflammation. The levels of YKL-40 in the different diagnostic groups showed a bimodal distribution (**Figure 1F**). NC and na-SCI had lower YKL-

40 levels (median 201ng/ml and 196ng/ml, respectively) than aMCI, DAT and FTD (median 251ng/ml, 260ng/ml and 239ng/ml, respectively).

Table 1. Demographic factors and biomarker results for the different diagnostic groups

Unless otherwise specified, values are expressed as median (interquartilic range).

NC: cognitively normal controls; na-SCI: non-amnestic and subjective cognitive impairment; aMCI: amnestic mild cognitive impairment; DAT: dementia of the Alzheimer type; FTD: frontotemporal dementia; MMSE: mini-mental status examination; IDDD: interview for the deterioration of daily living in dementia

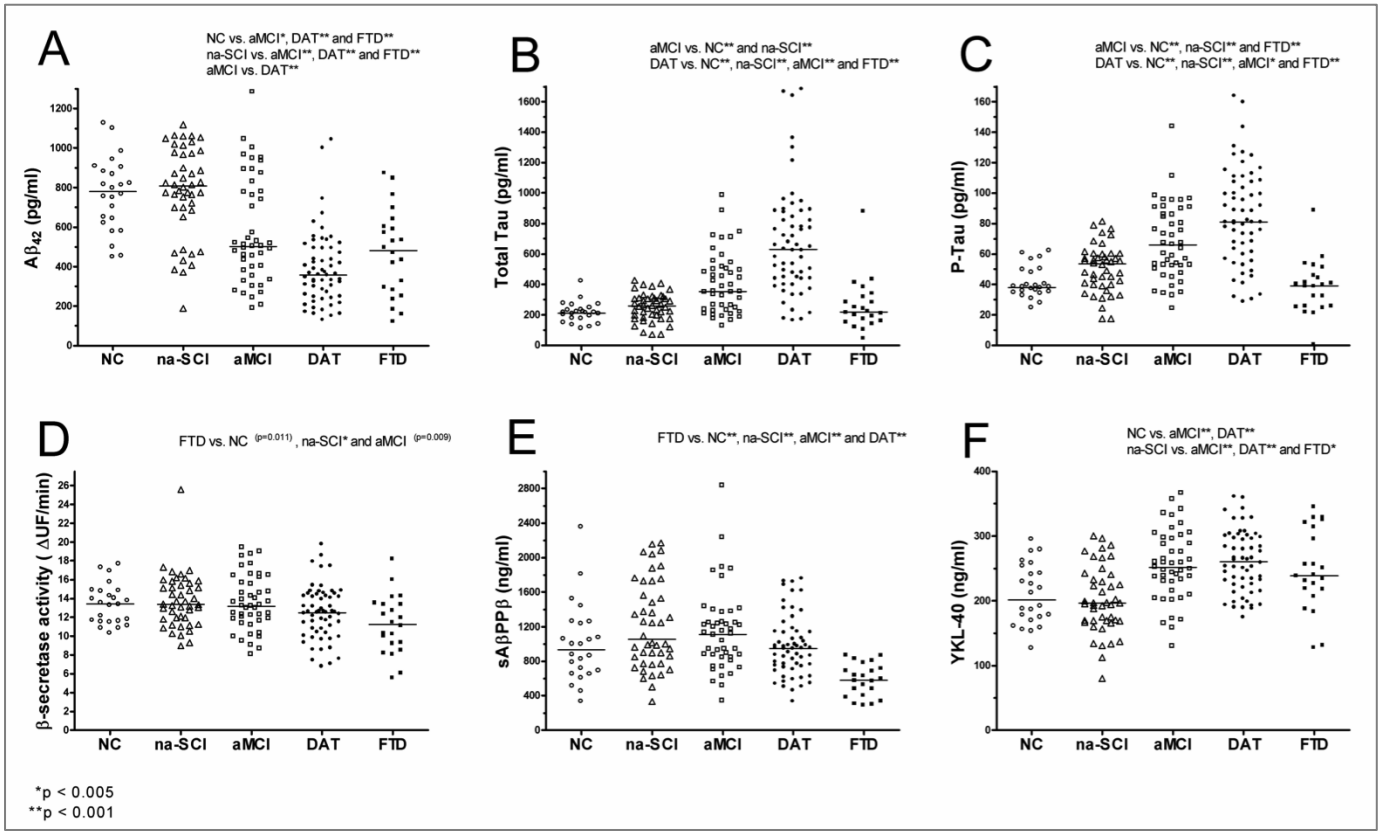
[a]Based on Kruskal-Wallis. [b]Based on Chi-square test

	NC	na-SCI	aMCI	DAT	FTD	P value
Number of subjects	24	44	45	59	22	-
Age, y	64 (57-69)	63 (57-67)	70 (66-76)	71 (64-77)	63 (59-71)	<0.001[a]
Sex , %women	54.2%	63.6%	48.9%	64.4%	31.8%	0.064[b]
APOE, %APOEε4 carriers	21.7%	20.5%	44.2%	50.9%	31.8%	0.009[b]
MMSE	30 (29-30)	29 (28-29)	28 (26-29)	22 (20-24)	25 (21-27)	<0.001[a]
IDDD	33 (33-33)	35 (33-37)	36 (34-38)	44 (38-52)	56 (43-69)	<0.001[a]
$A\beta_{42}$, pg/ml	780 (629-891)	808 (692-975)	502 (380-778)	357 (247-491)	482 (281-600)	<0.001[a]
Total tau, pg/ml	211 (174-237)	260 (191-311)	352 (232-501)	631 (435-871)	217 (156-315)	<0.001[a]
Phospho tau_{181}, pg/ml	38 (35-49)	54 (40-60)	66 (52-86)	81 (61-107)	39 (26-46)	<0.001[a]
β-secretase activity, ΔUF/min	13 (12-15)	13 (12-15)	13 (12-16)	12 (10-14)	11 (9-13)	0.009[a]
sAβPPβ, ng/ml	930 (665-1241)	1053 (811-1646)	1112 (838-1248)	947 (738-1138)	579 (399-710)	<0.001[a]
YKL-40, ng/ml	201 (171-247)	196 (169-237)	251 (229-295)	260 (224-297)	239 (217-313)	<0.001[a]
CSF-AD profile (tau/$A\beta_{42}$>0.52), %	4.2%	11.4%	62.2%	91.5%	36.4%	<0.001[b]

Figure 1. Biomarker results across the different clinical groups

Solid lines correspond to median values. Only statistically significant differences are displayed. Statistical significance after Bonferroni correction was set at p<0.005.

NC: cognitively normal controls; na-SCI: non-amnestic and subjective cognitive impairment; aMCI: amnestic mild cognitive impairment; DAT: dementia of the Alzheimer type; FTD: frontotemporal dementia

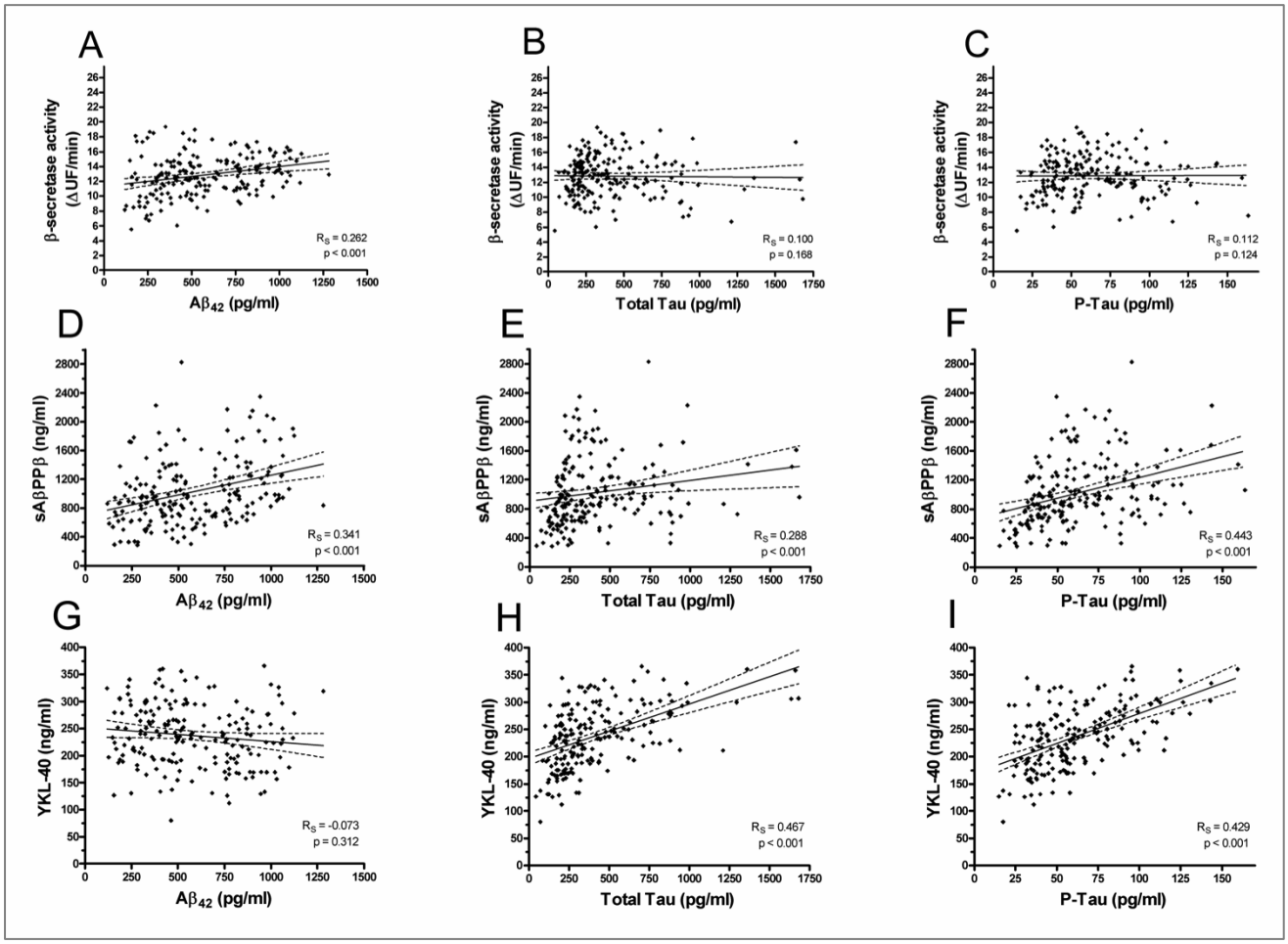


3.2. Correlations between CSF biomarkers

We investigated the relationship between CSF biomarkers by generating partial correlations. The levels of $A\beta_{42}$, total tau, p-tau and YKL-40 correlated significantly with age, so all calculations were adjusted by age. As displayed in **Figure 2**, in the entire sample the levels of $A\beta_{42}$ correlated positively with β-secretase activity (R_S=0.262; p<0.001) and with sAβPPβ (R_S=0.341; p<0.001), whereas YKL-40 correlated positively with total tau (R_S=0.467; p<0.001) and with p-tau (R_S=0.429; p<0.001). There was no correlation between β-secretase activity and YKL-40 (p=0.556; **Supplementary Table 3**).

Figure 2. Age-adjusted partial correlation between different biomarkers

The levels of Aβ42 correlated positively with β-secretase activity and with sAβPPβ, whereas YKL-40 correlated positively with total tau and with p-tau. Solid lines indicate the linear regression and dotted lines indicate 95% CI. RS: Spearman rho coefficient



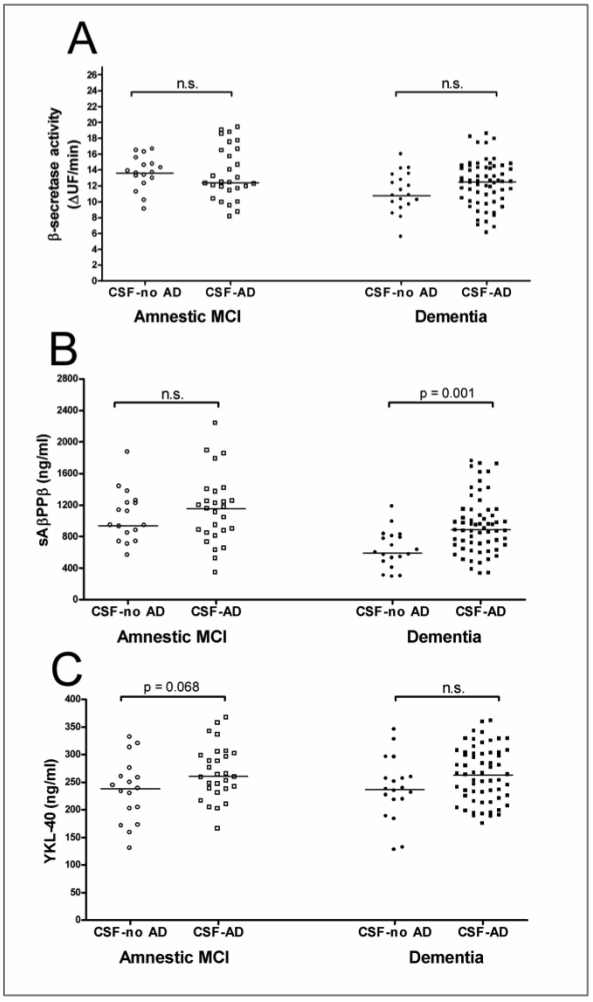
3.3. Relationship of AβPP processing and YKL-40 with the CSF AD profile and with APOE genotype

To understand the relationship between the different pathophysiological processes investigated herein, we classified subjects according to their CSF profile based on the ratio tau/$A\beta_{42}$ with a cut-off point of 0.52. **Figure 3** shows the comparison between subjects with CSF evidence (CSF-AD; tau/$A\beta_{42}$>0.52) and subjects with no CSF evidence of AD pathophysiological process (CSF-no AD; tau/$A\beta_{42}$≤0.52) in the groups of aMCI and dementia. In the group of aMCI, there was a trend towards higher levels of YKL-40 in subjects with CSF-

AD compared to subjects with CSF-no AD (p=0.068). There were no differences in β-secretase activity or in sAβPPβ levels within this group. In the pooled group of subjects with dementia, there were no differences in the levels of YKL-40 or in β-secretase activity between subjects with CSF-AD and subjects with CSF-no AD, but the latter group had lower levels sAβPPβ (p=0.001). We also compared CSF biomarkers according to the APOEε4 status within each clinical category. There were no differences in sAβPPβ, β-secretase activity or YKL-40 levels regarding APOEε4 status within any clinical group (**Supplementary Table 4**).

Figure 3. β-secretase activity, sAβPPβ and YKL-40 in amnestic MCI and dementia patients by CSF profile

CSF-AD: CSF evidence of Alzheimer's disease pathophysiological process (tau/Aβ42> 0.52); CSF-no AD: no CSF evidence of Alzheimer's disease pathophysiological process (tau/Aβ42≤0.52); n.s.: not significant



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3.4. Classification of dementias using biomarkers

We assessed the usefulness of combining the six biomarkers to distinguish DAT from other dementias. For this multivariate approach, total tau and sAβPPβ were logarithmic transformed, and p-tau was square root transformed to obtain normality. After testing for collinearity, total tau was not included in the analysis as it was highly correlated to p-tau (variance inflation factor > 5). The remaining biomarkers, age and sex were included as co-variables in the initial forward stepwise logistic regression model. In our study, the biomarkers that significantly contributed to the classification of subjects with dementia were p-tau and sAβPPβ (**Figure 4A**). **Figure 4B** shows the ROC curves comparing the multivariate combination with the individual core AD biomarkers. As shown, the area under the curve of the multivariate model was higher than any of the individual standard biomarkers and than the tau/$A\beta_{42}$ ratio.

Figure 4. Classification of patients with dementia (DAT vs. FTD) using six CSF biomarkers

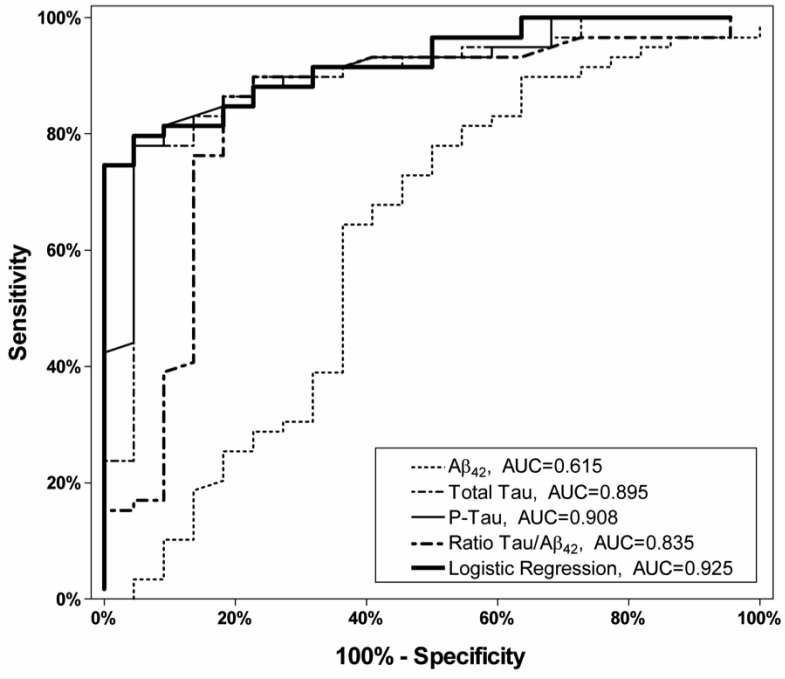
A. Biomarkers significantly contributing to the classification in the forward stepwise logistic regression model

Initially, Aβ42, p-tau, β-secretase activity, sAβPPβ, YKL-40, age and sex were included as co-variables in the forward stepwise logistic regression model. Total tau was not included in the analysis because of high collinearity with p-tau (variance inflation factor >5). In our study, the biomarkers that significantly contributed to the classification of subjects with dementia were p-tau and sAβPPβ. **B. Receiver operating characteristic (ROC) curves comparing the multivariate combination (Logistic Regression), the individual AD biomarkers, and the tau/Aβ42 ratio.** The area under the curve of the multivariate model was higher than any of the individual core biomarkers and than the tau/Aβ42 ratio. AUC: area under the curve

A

	B	S.E.	P value	Exp(B) – IC95%
Age, years	-	-	0.419	-
Sex (male=0; female=1)	-	-	0.188	-
$A\beta_{42}$, ng/ml	-	-	0.348	-
P-Tau$^{1/2}$, pg/ml	-0.988	0.286	0.001	0.372 (0.212 - 0.653)
Log(sAβPPβ), ng/ml	-5.585	2.556	0.029	0.004 (0 - 0.563)
β-secretase activity, ΔUF/min	-	-	0.900	-
YKL-40, ng/ml	-	-	0.127	-
Constant	22.196	7.377	0.003	4.36E9
Goodness-of-fit test (Hosmer-Lemeshow)			p=0.253	
Nagelkerke R^2			0.621	

B



4. Discussion

The main finding in our study is that markers of AβPP processing and markers of neuroinflammation in the CSF show distinct profiles in different types of cognitive disorders. On the one hand, sAβPPβ levels and β-secretase activity were decreased in patients with FTD but not in the group of DAT. YKL-40, on the other hand, was increased in patients with FTD and also in DAT, even in the early stages. Moreover, the combination of p-tau, sAβPPβ and YKL-40 had a higher diagnostic power to distinguish patients with DAT from FTD.

β-secretase activity and levels of sAβPPβ in the CSF have been previously studied as markers of AβPP processing.[15,17–27,50] Several studies have described an elevation in β-secretase activity in DAT and MCI patients compared to controls.[17–19,51,52] However, these findings have not been consistent across studies.[15,22–24,53,54] In our study, there were no differences in β-secretase activity between DAT, aMCI and controls, but we found a mild decrease in FTD patients compared to na-SCI and in the limit of statistical significance compared to NC and aMCI. Regarding sAβPPβ, we found lower levels of sAβPPβ in the group of FTD compared to the other groups. Furthermore, in the pooled group of dementias (comprising DAT and FTD), patients without CSF evidence of an AD pathophysiological process (CSF-no AD) had lower sAβPPβ levels than those with CSF evidence of an underlying AD pathophysiology (CSF-AD). These results are in agreement with other studies that have used a similar approach.[25,26,50] As AβPP and BACE are proteins highly expressed in neurons, the decrease in sAPPβ levels in FTD could merely reflect a pronounced neuronal loss typically observed in this disorder. However, expression of BACE in the brain has been found elevated in FTD[55] to a similar degree as in AD, and why reduced sAβPPβ levels in CSF are observed only in FTD remains to be determined. In any case, these differences highlight the complex dynamic nature of CSF biomarkers in neurodegenerative diseases.

YKL-40 is an inflammatory marker that has been reported to be increased in the CSF in DAT, FTD[32] and in other neurological diseases with inflammation in the CNS, such as multiple sclerosis.[31] Other authors did not find differences between DAT and controls.[35] These discrepancies across studies have been attributed to pre-analytical or analytical confounding factors and differences in study populations. We found increased levels of YKL-40 in the CSF of patients with DAT and FTD, but also in those with aMCI, compared to normal controls. This finding suggests that neuroinflammation is a common pathophysiological process present in distinct neurodegenerative conditions. Moreover, in the group of aMCI there was a trend towards an increase in YKL-40 in patients with CSF evidence of an AD pathophysiological process. This finding is in agreement with other studies,[33] and indicates that neuroinflammation can be detected in the early stages of AD. Therefore, CSF YKL-40 could be useful to distinguish those subjects with MCI caused by an underlying neurodegenerative disease from other etiologies, in particular from MCI due to non-neurodegenerative conditions, such as pharmacological, medical or psychiatric causes.

Other interesting findings in our study are derived from the analysis of correlations between different CSF biomarkers. First, sAβPPβ and β-secretase activity showed a mild positive correlation with each other and with $A\beta_{42}$, supporting the fact that these biomarkers might be tracking the AβPP pathophysiological process. Second, in agreement with other studies,[32,33] we found a moderately positive correlation between YKL-40, total tau and p-tau, but not with markers of AβPP processing. This fact suggests that inflammation and neurodegeneration are closely related processes in neurodegenerative diseases.

Neuropathological studies have emphasized the many pathological processes that usually coexist in the elderly[56] and in neurodegenerative disorders.[57] From a diagnostic point of view, the combination of different CSF biomarkers able to reflect each contributing

mechanism is likely to be the most appropriate approach in neurodegenerative dementias. In this study we found that p-tau and sAβPPβ were the biomarkers that best contributed to distinguish DAT from FTD, and the combination had a higher diagnostic accuracy than any of the individual core biomarkers or the tau/$A\beta_{42}$ ratio. The inclusion of one marker of neuronal damage and one of AβPP processing in this model highlights the importance of measuring the different coexisting processes when classifying neurodegenerative dementias.

The strengths of this study are the inclusion of subjects with a thorough clinical and neuropsychological evaluation, covering two different neurodegenerative dementias, and the use of six CSF biomarkers of different pathophysiological processes. The main limitations are the lack of neuropathological confirmation and the small sample size in some groups. Due to the limited size of the FTD group, we could not assess the usefulness of these six biomarkers to distinguish between different clinical phenotypes within this heterogeneous group.

In conclusion, we found that markers of AβPP processing, markers of neuroinflammation and core biomarkers of AD in CSF are closely interconnected in neurodegenerative diseases. Our results suggest that markers of AβPP processing are decreased in CSF in FTD, while this decrease is not detectable in AD. We also found evidence suggesting that inflammation is a common feature in FTD and AD, even in early stages, and that it is tightly correlated with neuronal damage. Finally, a combination of markers of different neuropathological processes may help in the diagnostic classification of neurodegenerative dementias.

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Disclosure of Conflict of Interest: The authors declare no conflict of interest.

Additional contributions: We are grateful to the participants of this study and their families, and we thank Laia Muñoz and Carolyn Newey for technical and editorial assistance.

6. Supplementary Material

Supplementary Table 1. Comparison between the groups with subjective cognitive impairment (SCI) and with non-amnestic mild cognitive impairment (naMCI)

There were no differences in any of the variables studied. Therefore, these two groups were analyzed together as a single category (na-SCI) to minimize the number of diagnostic groups. Unless otherwise specified, values are expressed as median (interquartilic range). MMSE, mini-mental status examination; IDDD, interview for the deterioration of daily living in dementia

[a] Based on the Mann-Whitney U test. [b] Based on Chi-square test

	SCI	naMCI	p value
Number of subjects	31	13	-
Age, y	63 (57-68)	59 (57-66)	0.653[a]
Sex ,%women	58.06%	76.92%	0.241[b]
APOE, %APOEε4 carriers	22.58%	15.38%	0.594[b]
MMSE	29 (28-29)	28 (27-29)	0.386[a]
IDDD	35 (33-38)	33 (33-35)	0.110[a]
$A\beta_{42}$, pg/ml	784 (685-986)	815 (595-927)	0.887[a]
Total tau, pg/ml	263 (200-320)	245 (147-292)	0.335[a]
Phospho tau_{181}, pg/ml	53 (41-60)	54 (37-61)	0.959[a]
β-secretase activity, ΔUF/min	13.2 (11.8-15.1)	14.8 (11.6-16.4)	0.174[a]
sAβPPβ, ng/ml	1094 (774-1757)	1012 (800-1646)	0.827[a]
YKL-40, ng/ml	197 (169-232)	192 (165-260)	0.928[a]

Supplementary Table 2. Intra- and inter-assay coefficients of variation (CV) for the different biomarkers' assays

	CV intra-assay	CV inter-assay	Num. of assays
$A\beta_{42}$	4.5%	15.0%	9
Total tau	5.4%	7.4%	11
Phospho tau_{181}	1.8%	8.1%	10
sAβPPβ	2.2%	11.2%	7
β-secretase activity	4.6%	14.1%	6
YKL-40	2.9%	2.9%	6

Supplementary Table 3. Age-adjusted partial correlation between different biomarkers in the sample

		$A\beta_{42}$	Total tau	Phospho tau_{181}	β-secretase activity	sAβPPβ	YKL-40
$A\beta_{42}$	**R_S**	-	-0.359	-0.212	0.262	0.341	-0.073
	P value	-	<0.001	0.003	<0.001	<0.001	0.312
Total tau	**R_S**	-0.359	-	0.886	0.100	0.288	0.467
	P value	<0.001	-	<0.001	0.168	<0.001	<0.001
Phospho tau_{181}	**R_S**	-0.212	0.886	-	0.112	0.443	0.429
	P value	0.003	<0.001	-	0.124	<0.001	<0.001
β-secretase activity	**R_S**	0.262	0.100	0.112	-	0.399	-0.043
	P value	<0.001	0.168	0.124	-	<0.001	0.556
sAβPPβ	**R_S**	0.341	0.288	0.443	0.399	-	0.181
	P value	<0.001	<0.001	<0.001	<0.001	-	0.012
YKL-40	**R_S**	-0.073	0.467	0.429	-0.043	0.181	-
	P value	0.312	<0.001	<0.001	0.556	0.012	-

Supplementary Table 4. Biomarker values within clinical groups according to *APOE* genotype status

NC: cognitively normal controls; na-SCI: non-amnestic and subjective cognitive impairment; aMCI: amnestic mild cognitive impairment; DAT: dementia of the Alzheimer type; FTD: frontotemporal dementia. Values are expressed as median (interquartilic range). P values based on the Mann-Whitney U test

		$A\beta_{42}$ (pg/ml)	Total tau (pg/ml)	Phospho tau_{181} (pg/ml)	β-secretase activity (ΔUF/min)	sAβPPβ (ng/ml)	YKL-40 (ng/ml)
NC	**APOEε4-**	803 (641-880)	205 (147-231)	38 (35-48)	13.4 (11.5-14.8)	1017 (784-1254)	215 (177-257)
	APOEε4+	702 (577-906)	212 (211-274)	40 (38-50)	12.1 (10.8-13.7)	683 (507-712)	186 (169-242)
	p value	0.745	0.290	0.446	0.325	0.199	0.638
na-SCI	**APOEε4-**	814 (700-986)	233 (175-306)	49 (36-58)	13.6 (11.3-15.4)	999 (774-1481)	196 (169-230)
	APOEε4+	776 (485-887)	323 (274-380)	61 (48-74)	13.1 (12.1-15.1)	1563 (860-1765)	197 (169-268)
	p value	0.439	0.018	0.025	0.988	0.226	0.529
aMCI	**APOEε4-**	766 (510-913)	327 (204-443)	59 (46-86)	13.2 (11.7-14.4)	1155 (854-1389)	254 (235-287)
	APOEε4+	431 (276-501)	456 (261-541)	72 (52-91)	12.4 (10.3-15.6)	935 (720-1216)	247 (208-301)
	p value	<0.001	0.040	0.299	0.751	0.107	0.922
AD	**APOEε4-**	380 (257-530)	578 (396-880)	88 (60-108)	12.5 (10.3-14.1)	868 (620-1131)	258 (233-295)
	APOEε4+	350 (238-435)	637 (474-818)	81 (67-107)	12.4 (11.1-14.5)	958 (763-1138)	262 (214-297)
	p value	0.324	0.727	0.988	0.727	0.379	0.750
FTD	**APOEε4-**	527 (281-640)	213 (148-338)	32 (25-41)	10.2 (8.5-13.3)	624 (476-778)	250 (217-324)
	APOEε4+	468 (233-569)	240 (156-281)	44 (40-54)	12.7 (9.9-14.1)	377 (301-635)	233 (206-313)
	p value	0.535	1.000	0.142	0.447	0.106	0.535

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Chapter 5

Amyloid precursor protein metabolism and inflammation markers in preclinical Alzheimer disease

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Abstract

Objective: To investigate CSF markers involved in amyloid precursor protein processing, neuronal damage and neuroinflammation in the preclinical stages of Alzheimer's disease (AD) and subjects with suspected non-Alzheimer pathology (SNAP).

Methods: We collected CSF from 266 cognitively normal volunteers participating in a cross-sectional multicenter study (the SIGNAL study) to investigate markers involved in amyloid precursor protein processing (Aβ42, sAPPβ, β-secretase activity), neuronal damage (t-tau, p-tau) and neuroinflammation (YKL-40). We analyzed the relationship between biomarkers, clinical variables and the APOE genotype, and we compared biomarker levels across the preclinical stages of the NIA-AA classification: stage 0, 1, 2, 3, and SNAP.

Results: The median age in the whole cohort was 58.8 years (range 39.8 – 81.6). Subjects in stages 2-3 and SNAP had higher levels of YKL-40 than those in stages 0 and 1. Subjects with SNAP had higher levels of sAPPβ than subjects in stage 0 and 1. No differences were found between stages 0, 1, 2-3 in sAPPβ or β-secretase activity in CSF. Age correlated with t-tau, p-tau and YKL-40. It also correlated with Aβ42, but only in APOEε4 carriers. Aβ42 correlated positively with t-tau, sAPPβ and YKL-40 in subjects with normal Aβ42.

Conclusions: Our findings suggest that inflammation in the CNS increases in normal aging and is intimately related to markers of neurodegeneration in the preclinical stages of AD and SNAP. sAPPβ and β-secretase activity are not useful diagnostic or staging markers in preclinical AD.

Search terms: [26] Alzheimer's disease; [319] Cerebrospinal fluid; [38] Assessment of cognitive disorders/dementia; [36] Cognitive aging; Biomarkers.

1. Introduction

Patients with Alzheimer's disease (AD) have lower levels of CSF Aβ42 and higher levels of CSF total-tau (t-tau) and phospho-tau (p-tau) than cognitively normal controls.[1] However, other biomarkers have been investigated to track concomitant pathologies and secondary pathophysiological processes in AD.[2,3] It is not known whether the markers of amyloid precursor protein (APP) processing, sAPPβ levels and β-secretase activity are altered in the preclinical stages of AD, and only a few studies[4–6] have investigated the role of the inflammatory marker YKL-40 (also known as chitinase 3-like 1) in preclinical AD. In this large multicentre study, we measured these markers to evaluate the changes that occur in these relevant pathophysiological pathways during the preclinical stages of AD and in individuals with SNAP (suspected non-Alzheimer pathology), a category recently proposed to label subjects with signs of neurodegeneration in the absence of cerebral amyloidosis.[7]

2. Methods

2.1. Study participants and clinical classification

We included 266 cognitively normal subjects who were included in the SIGNAL study (www.signalstudy.es) and evaluated between April 2011 and November 2013 at one of five centers in Spain: CITA Alzheimer, San Sebastián (CITA); Hospital Sant Pau, Barcelona (HSP); Hospital Marqués de Valdecilla, Santander (HMV); Hospital Gregorio Marañón, Madrid (HGM), and Hospital Virgen de la Arrixaca, Murcia (HVA). The participants were volunteers who were enrolled after hearing about the study through the media or from relatives who were attended at one of the study centers.

All participants had a Minimental State Examination (MMSE) score ≥ 24 and normal memory performance, assessed by the Free and Cued Selective Reminding Test (FCSRT, total immediate score ≥ 36 and free immediate recall subscore ≥ 19).[8] For further classification,

an episodic memory composite score was calculated as the sum of the transformed Z-scores of FCSRT total immediate and free immediate recall subscores. Significant impairment in other cognitive domains was excluded through a formal cognitive evaluation as previously described.[9]

We excluded volunteers who had evidence of focal brain lesions or a past medical history of stroke or any other neurologic or psychiatric condition, and also those who were taking steroid, immunosuppressant, anticholinergic, antiepileptic, neuroleptic or anticoagulant drugs were excluded from the study.

Of the 372 participants evaluated in the SIGNAL study, 266 met the inclusion/exclusion criteria and were included in the analysis.

2.2. CSF classification

All participants underwent a lumbar puncture to obtain CSF samples. We used CSF Aβ42, t-tau and p-tau levels to classify preclinical stages of AD according to the NIA-AA criteria.[10] Participants were classified as: stage 0 (Aβ42≥550pg/ml, t-tau≤350pg/ml and p-tau≤61pg/ml), stage 1 (Aβ42<550pg/ml, t-tau≤350pg/ml and p-tau ≤61pg/ml), stage 2 (Aβ42<550pg/ml and either t-tau>350pg/ml or p-tau>61pg/ml), or stage 3 (stage 2 plus subtle cognitive decline, defined as an episodic memory composite score in the lowest 10th percentile).[11] For the analysis, stages 2 and 3 were combined due to the low number of subjects in each group. Subjects with Aβ42≥550pg/ml and either t-tau>350pg/ml or p-tau>61pg/ml were classified as SNAP.

2.3. Standard protocol approvals, registrations, and patient consent

All participants gave their written consent, and the study was approved by the local ethics committee at each center.

2.4. CSF analyses

CSF was obtained by lumbar puncture and collected following international consensus recommendations.[12,13] Briefly, CSF was collected in polypropylene tubes and immediately centrifuged (1900-2000g x 10min) to avoid any haematic contamination. All samples were stored in polypropylene tubes at -80ºC and shipped on dry ice to HSP for analysis. We used commercially available ELISA kits to determine levels of Aβ42 (Innotest™ β-Amyloid$_{1-42}$, Fujirebio-Europe, Gent, Belgium), t-tau (Innotest™ hTAU Ag, Fujirebio-Europe, Gent, Belgium), p-tau (Innotest™ Phospho-Tau$_{181P}$, Fujirebio-Europe, Gent, Belgium), sAPPβ (Human sAPPβ-w highly sensitive, IBL, Gunma, Japan) and YKL-40 (MicroVue™, Quidel, San Diego, CA, USA) following the manufacturers' recommendations. We also measured CSF β-secretase activity as previously described.[6,14] Briefly, we incubated the CSF sample with a fluorogenic β-secretase substrate (β-Secretase Substrate IV, Fluorogenic, Calbiochem® - Merck, Darmstadt, Germany) and measured fluorescence at different time points. Our laboratory has experience in CSF biomarker determination and participates in the Alzheimer's Association external quality control program for CSF biomarkers.[15] The intra- and inter-assay coefficients of variation for all biomarkers were lower than 10% and 20%, respectively. The performance of the assays is described in more detail in **Supplementary Table 1**.

2.5. CSF cut-off points

We applied a cut-off point of 550pg/ml for Aβ42, 350pg/ml for t-tau and 61pg/ml for p-tau. The diagnostic accuracy of these cut-off points had been previously assessed in a cohort of 45 patients who were clinically diagnosed with the Alzheimer type dementia (diagnosis was made prior to the CSF biomarkers' analysis) and 20 age-matched controls (age range: 50-79

years) from HSP. Their sensitivity and specificity were 88.9%/85.0% for Aβ42, 84.4%/95.0% for t-tau and 75.6%/95.0% for p-tau.

2.6. Genetic analysis

APOE was genotyped according to previously described methods.[16,17]

2.7. Statistical analysis

We assessed normality of the variables through D'Agostino's K^2 test. As some variables did not follow a normal distribution, we used non-parametric tests for bivariate analysis (Kruskal-Wallis, followed by Mann-Whitney U test). If required, variables were log-transformed to achieve a normal distribution for the multivariate analyses. Statistical significance for all tests was set at 5% (α=0.05). We used Bonferroni's correction for multiple comparisons when necessary. All group comparisons were adjusted for age and center as possible confounder factors. All the statistical analyses were performed using the R statistical software (http://www.R-project.org).

3. Results

3.1. CSF biomarkers are influenced by age and APOE genotype in cognitively normal subjects

Table 1 summarizes the demographics, clinical characteristics and CSF biomarkers of all the participants in the study. The median age in the whole cohort was 58.8 years (range 39.8 – 81.6). As shown in **Figure 1**, we found a correlation between age and t-tau, p-tau, and YKL-40. We also found a correlation between age and Aβ42 in APOEε4 carriers but not in APOEε4 non-carriers. There was no association between any of the biomarkers and gender.

We also analyzed the relationship between CSF biomarkers and cognitive scores in cognitively normal subjects. There was no significant correlation between MMSE or the FCSRT scores and any of the biomarkers (data not shown).

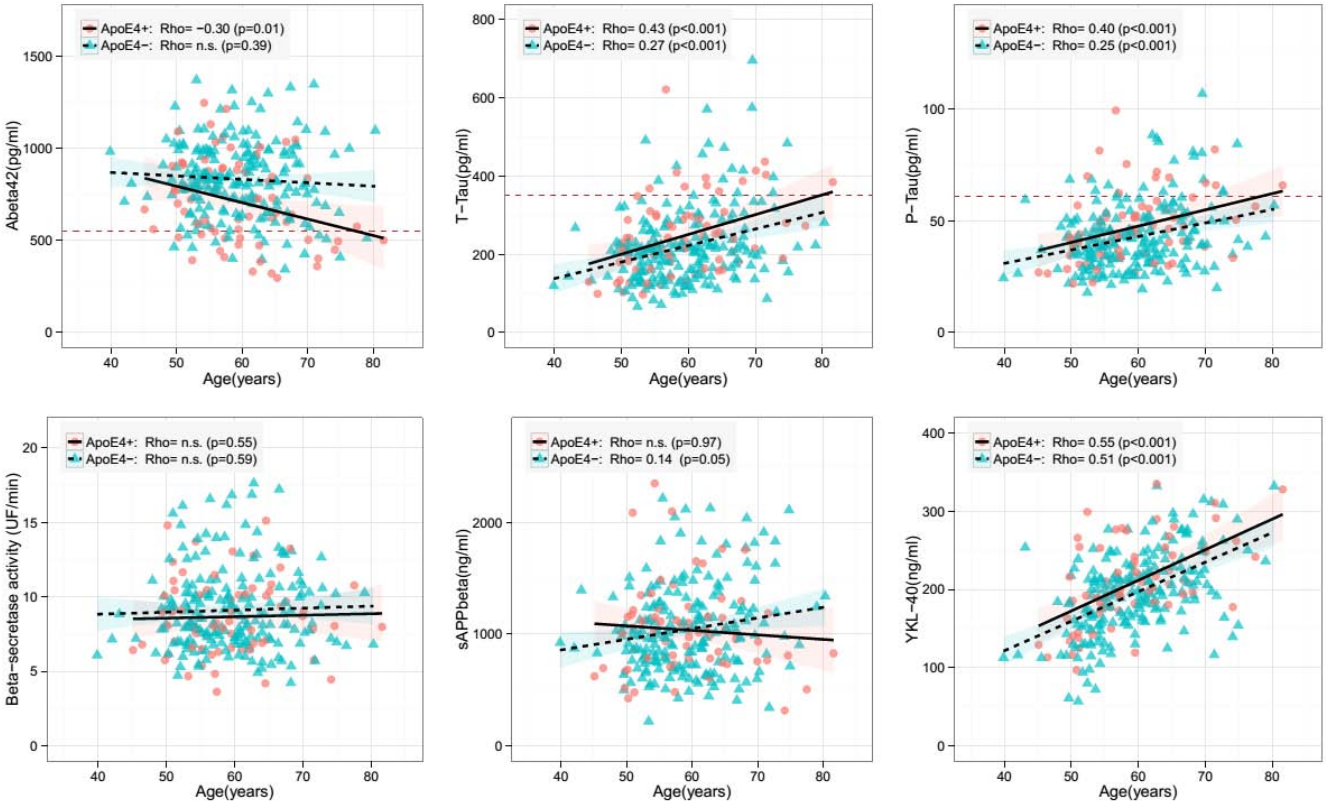
Table 1. Demographic and biomarker characteristics of the participants

Unless otherwise specified, data are shown as median (median average deviation). SNAP: suspected non-Alzheimer pathology. MMSE: mini-mental state examination. CSF: cerebrospinal fluid. UF: Fluorescence Units.

	All participants	Stage 0	Stage 1	Stages 2-3	SNAP
n	266	203	26	10	27
Age, years	58.77 (7.77)	57.29 (6.49)	60.63 (7.91)	66.25 (6.66)	63.73 (8.15)
Gender, (% female)	59.0%	59.1%	69.2%	40.0%	55.6%
APOEε4, %	25.28%	20.30%	46.15%	80.00%	22.22%
MMSE	29 (1.48)	29 (1.48)	29 (1.48)	29 (1.48)	29 (1.48)
CSF Aβ42, pg/ml	810.75 (236.85)	832.50 (160.12)	491.25 (48.93)	415.25 (43.00)	1026.50 (299.49)
CSF t-tau, pg/ml	212.75 (89.70)	204.50 (61.53)	142.50 (63.75)	409.25 (45.22)	388.00 (57.08)
CSF p-tau, pg/ml	41 (14.83)	39.50 (9.64)	31.50 (14.08)	71.00 (7.41)	66.50 (8.90)
CSF β-Secretase activity, UF/ml	8.29 (2.44)	8.22 (2.20)	7.47 (3.62)	8.72 (2.33)	9.12 (1.92)
CSF sAPPβ, ng/ml	990.71 (362.86)	980.09 (330.27)	704.01 (355.22)	881.10 (304.48)	1481.28 (521.23)
CSF YKL-40, ng/ml	196.77 (48.68)	192.24 (44.36)	177.42 (32.94)	283.40 (40.74)	240.12 (53.94)

Figure 1. Relationship of CSF biomarkers with age and APOE genotype

Age correlated with t-tau, p-tau and YKL-40, regardless of gender or APOE status. Age correlated with Aβ42 in APOEε4 carriers only. Dashed red lines indicate the cut-off values used in this study (Aβ42: 550pg/ml; t-tau: 350pg/ml; p-tau: 61pg/ml).



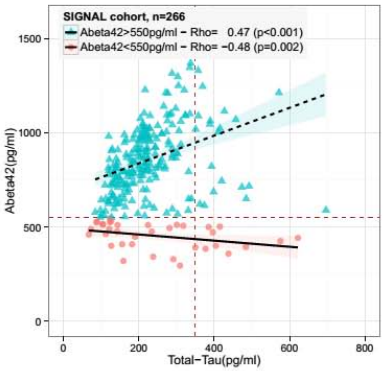
3.2. Correlation between CSF biomarkers depends on the Aβ42 status

We took advantage of the large sample size of this cohort of cognitively normal subjects to analyze the relationship between core CSF biomarkers. As shown in **Figure 2A**, we found that the correlation between Aβ42 and tau differed depending on the Aβ42 status. In subjects with Aβ42 below 550pg/ml, higher levels of t-tau were associated with lower Aβ42 levels. However, in subjects with Aβ42 levels above 550pg/ml, higher t-tau was associated with higher Aβ42. These results remained significant after excluding subjects in the SNAP category.

Figure 2. Correlation between CSF biomarkers

Figure **2A** shows the correlation between Aβ42 and t-tau (n=266). Figure **2B** shows the correlation of Aβ42 and t-tau with the other biomarkers. All correlations were analyzed independently in two groups according to Aβ42 levels. Dashed red lines indicate the cut-off values used in this study (Aβ42: 550pg/ml; t-tau: 350pg/ml).

A



B

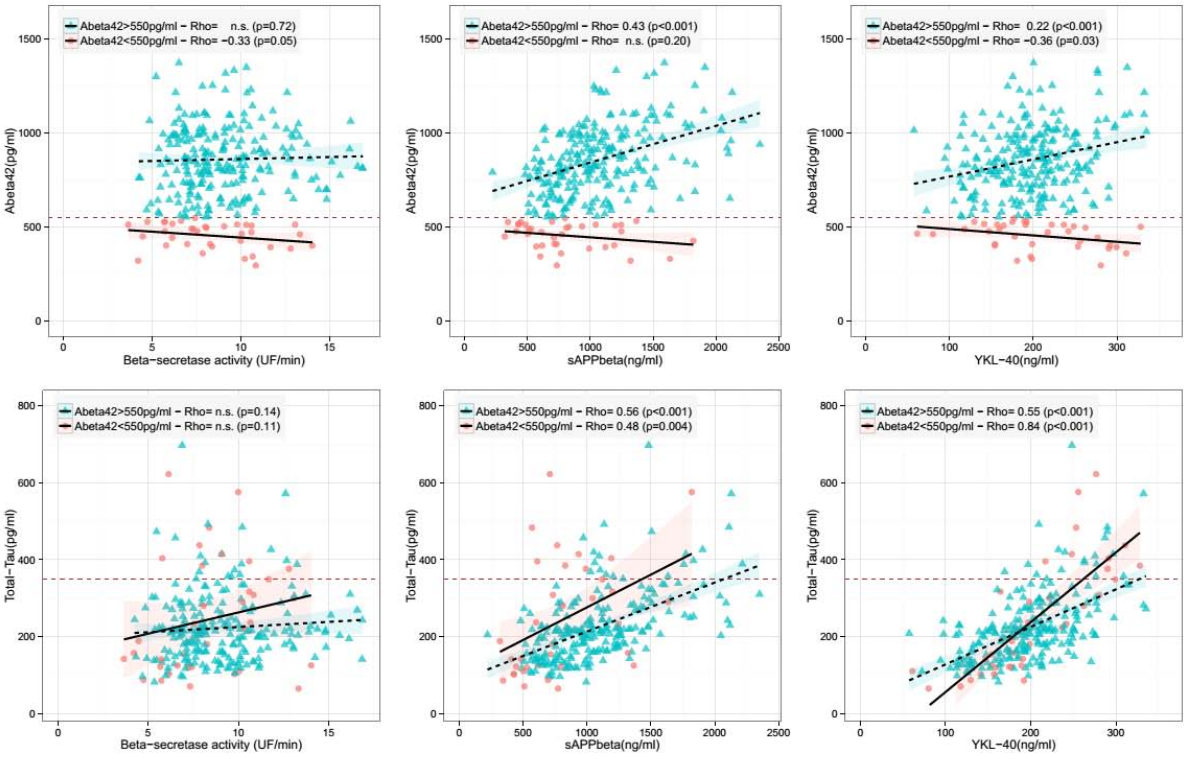


Figure 2B shows the correlation of Aβ42 and of t-tau with the other CSF biomarkers studied.

There were no significant correlations between β-secretase activity and Aβ42 or t-tau. In

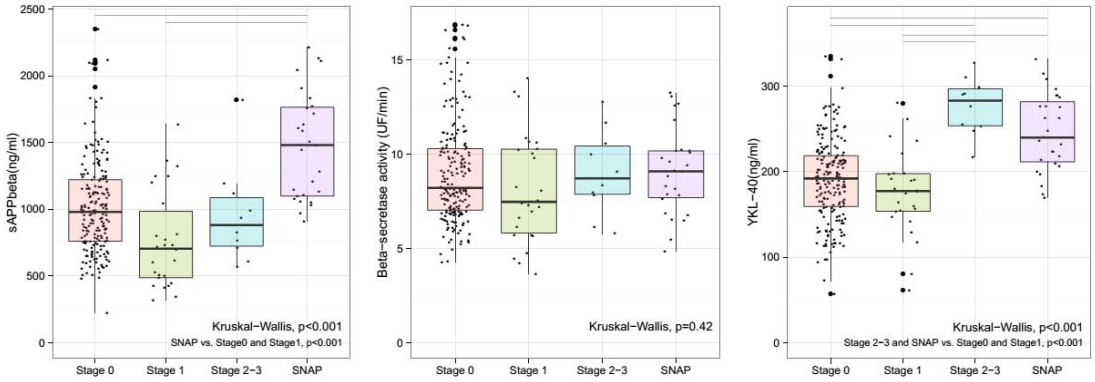
subjects with Aβ42 levels above the cut-off, sAPPβ showed a significant correlation with both Aβ42 and t-tau. The directionality of the correlation between YKL-40 and Aβ42 differed between subjects that had Aβ42 levels above and below the cut-off point. YKL-40 correlated with t-tau regardless of the Aβ42 status. The correlation of p-tau with the other CSF biomarkers was similar to the correlations found with t-tau (data not shown).

We repeated all the analysis applying a 5% confidence interval[18] in the cut-off point for Aβ42 (522.5 - 577.5pg/ml) and obtained similar results (data not shown).

3.3. Preclinical stages of AD and SNAP show different profiles in CSF β-secretase activity, sAPPβ and YKL-40

To investigate the differences in these additional CSF biomarkers in preclinical AD, we classified the participants into the NIA-AA stages. As shown in **Figure 3**, there were no differences in CSF sAPPβ between stage 0, 1 and 2-3. The levels of sAPPβ in the SNAP group were significantly higher than in stage 0 or stage 1. We found no differences in β-secretase activity between groups. Regarding YKL-40, participants in the groups of stage 2-3 and SNAP had higher values than subjects in stage 0 or stage 1. As there were significant differences between groups regarding age and center, all results were adjusted for these two variables as possible confounder factors.

Figure 3. Levels of sAPPβ, β-secretase activity, and YKL-40 across preclinical stages of AD



4. Discussion

The study of this middle-aged cohort has three key findings. First, participants in preclinical stages of AD and subjects with SNAP showed different profiles in CSF YKL-40 and sAPPβ levels, whereas CSF β-secretase activity showed no differences. Second, the correlations between biomarkers differed depending on the Aβ42 status. Third, our findings confirmed the observation that CSF biomarkers in cognitively normal subjects are influenced by age and APOE genotype.[19–22]

The levels of CSF β-secretase activity and sAPPβ have been previously studied as markers of APP processing in clinical cohorts of patients with MCI and dementia.[6,14,23–30] Some authors found that CSF β-secretase activity and/or sAPPβ were mildly increased in MCI and early AD,[25,26,31–33] although subsequent studies by our group and others found no differences between MCI, dementia of the Alzheimer type and cognitively normal controls.[6,14,27,28,30] In the present study we measured for the first time β-secretase activity and sAPPβ levels in CSF across the preclinical stages of AD based on the NIA-AA classification and in subjects with SNAP. We found no differences between stages 0, 1 and 2-3, but sAPPβ levels in the group of SNAP were higher than in stages 0 and 1. We found no differences in β-secretase activity

between groups. Our findings suggest that CSF β-secretase activity and sAPPβ are not useful biomarkers for the diagnosis or staging in preclinical AD. However, CSF sAPPβ levels have proven to be a good marker to ensure target engagement in clinical trials with BACE1 inhibitors in AD.[34]

YKL-40 has been studied as a CSF marker of neuroinflammation in the AD continuum and in other degenerative dementias.[4–6,35,36] These studies have consistently found an increase in CSF YKL-40 levels in degenerative dementias and a correlation between CSF YKL-40 levels and markers of neurodegeneration, such as tau and p-tau, even in preclinical stages of AD.[4] In the present study, we further extend these findings by showing that CSF YKL-40 levels are higher in subjects with preclinical AD stages 2-3 and SNAP than in preclinical AD stages 0 and 1. The similar levels observed in preclinical AD and SNAP also suggest that neuroinflammation can emerge through a non-amyloid related pathway, and that it is also detectable in CSF in preclinical stages in non-amyloid neurodegenerative disorders. As previously shown in other studies,[4–6,36] we found a correlation between CSF YKL-40 and t-tau in the entire cohort. This correlation was also significant when the Aβ42 positive and Aβ42 negative groups were analyzed independently, indicating that the correlation is not driven by subjects with the AD pathological process. Moreover, CSF YKL-40 levels correlated with age in our cohort of cognitively normal subjects, regardless of the APOEε4 status. This finding suggests that low-grade inflammatory processes are present in the aging brain even in the absence of AD. Taken together, these findings reinforce the idea that CSF YKL-40 levels increase with aging, preclinical AD and SNAP and correlate closely with markers of neurodegeneration.

Making use of the large sample size of this cohort of cognitively normal subjects, we investigated the correlation between core AD biomarkers. As expected, we found a

correlation between Aβ42 and t-tau. However, the directionality of this correlation differed between subjects with Aβ42 above or below the cut-off point. To our knowledge, this finding has not been reported previously, and it could explain some inconsistencies found across studies in the relationship of other biomarkers with Aβ42 and t-tau. The mechanisms underlying this correlation in the absence of the pathological process of AD require further investigation. One possible explanation is that, Aβ42 and t-tau levels in CSF in subjects without the AD pathological process reflect general neuronal/synaptic integrity and function. Because APP and tau are highly expressed proteins in neurons, their levels in CSF could reflect the overall synaptic function. Another possibility is that Aβ42 and t-tau levels in CSF correlate because they are subject to common mechanisms of brain clearance. It has recently been described that interstitial solutes, including Aβ, are cleared through a paravascular pathway.[37] This clearance system becomes progressively impaired with normal aging in mouse models.[38] Aβ42 and t-tau levels could therefore correlate in normal aging because they reflect the age-related changes in the clearance system through this common paravascular pathway.

One of the main strengths of our study is the large sample size. This allowed us to perform correlation analysis and to detect differences in CSF biomarkers between preclinical stages. Moreover, all subjects underwent an extensive neuropsychological evaluation to ensure that their cognition was preserved. The study also has some limitations. Being a multicenter study, some of the findings may have been influenced by center-driven characteristics. To minimize the impact of inter-center variability, however, we applied a common protocol for CSF collection, the analysis of all CSF biomarkers was centralized in one laboratory, and the results were adjusted by age and center as possible confounder factors. Another possible limitation is the low prevalence of preclinical AD (roughly 15%) and SNAP (10%) in this cohort. This likely occurred because the participants in our study were younger than those

in other studies. And lastly, although the main objective of our study was to analyze the relationship between biomarkers in the CSF, it would have been interesting to have neuropathological information or additional surrogate biomarkers (i.e. amyloid or tau imaging) of these subjects for a more accurate classification.

In this cohort of cognitively normal subjects, we found that levels of CSF YKL-40 were increased in preclinical stages 2-3 and in subjects with SNAP, and that YKL-40 levels correlated with t-tau levels in preclinical AD and normal aging. These findings suggest that inflammation is intimately related to markers of neurodegeneration in normal aging and in the very early stages of the AD pathological process and SNAP. Moreover, Aβ42 and t-tau levels in CSF correlated positively in normal aging, suggesting that they are influenced by common production and clearance mechanisms. This relationship should be taken into account in studies with cognitively normal subjects.

5. Acknowledgements

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6. Supplementary material

Supplementary Table 1. CSF assay details and procedures

CV: coefficient of variation

	Number of assays	Number of batches	Intra-assay CV (%)	Inter-assay CV (%)
CSF Aß42	19	8	2.73%	13.99%
CSF t-tau	21	9	2.45%	8.2%
CSF p-tau	20	8	2.34%	9.78%
CSF ß-secretase activity	14	1	8.67%	19.15%
CSF sAPPß	15	3	5.00%	8.19%
CSF YKL-40	13	2	3.62%	3.83%

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Chapter 6

Relationship between cortical thickness and cerebrospinal fluid YKL-40 in predementia stages of Alzheimer's disease

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Abstract

Cerebrospinal fluid (CSF) YKL-40 has been described as a marker of glial inflammation. We aimed to study the relationship between YKL-40 and brain structure and its interactions with core Alzheimer's disease (AD) biomarkers. We measured cortical thickness (CTh) and CSF biomarkers (Aβ42, t-tau, p-tau, YKL-40) of 80 cognitively normal controls (CN) and 27 patients with amnestic mild cognitive impairment. Subjects were classified as Aβ42+ (<550pg/ml) or Aβ42- (>550pg/ml). CTh difference maps were derived from interaction and correlation analyses in the whole sample and within clinical groups. There was a strong correlation between YKL-40 and markers of neurodegeneration (t-tau and p-tau). In the whole sample, we found a negative correlation between YKL-40 and CTh in AD vulnerable areas in Aβ42+ subjects, but not in Aß42- participants. Our results suggest that YKL-40 could track the inflammatory processes associated to tau-related neurodegeneration in the presence of the AD pathophysiological process.

Keywords: Cortical thickness; YKL-40; neuroinflammation; preclinical Alzheimer's disease; CSF biomarkers; structural MRI.

1. Introduction

Alzheimer's disease (AD) is a complex disease where multiple pathophysiological processes coexist.[1] Different cerebrospinal fluid (CSF) and neuroimaging biomarkers allow us to track these distinct processes.[2,3] In CSF, the levels of ß-Amyloid 1-42 (Aβ42) reflect the amyloid deposition in the brain in subjects with AD pathophysiological process,[4–6] whereas the levels of total tau (t-tau) and phospho-tau (p-tau) correlate with neurodegeneration, neuronal loss and cortical neurofibrillary burden.[6,7] Neuronal loss can also be studied measuring cortical thickness with magnetic resonance imaging (MRI).[8,9] Together, these biomarkers have been used to detect biological evidence of the AD pathophysiological process. Therefore, the use of biomarkers allows to stage the preclinical phase of AD,[10] to identify patients with mild cognitive impairment due to AD,[11] and to increase the level of certainty in the diagnosis of dementia due to AD.[12]

Inflammation is another common factor in the pathogenesis of AD[13] and can also be studied through biomarkers. Several molecules have been investigated in plasma and in CSF as markers of inflammation in AD.[2] One of them, YKL-40 (also known as chitinase 3-like 1 protein) has been described as a marker of glial inflammation.[14,15] In previous studies, we and others have found that levels of CSF YKL-40 are higher in AD and in other neurodegenerative conditions than in cognitively normal controls.[3,14–17] Some of these studies also found changes in pre-dementia stages, where YKL-40 has been reported to be higher in subjects with CSF evidence of the AD pathophysiological process.[3,17] Additionally, previous studies have reported a strong correlation between CSF YKL-40 and markers of neuronal degeneration (t-tau and p-tau).[3,14,16,17]

Multimodal studies are essential to disentangle the complex pathophysiological processes that occur in the early stages of AD. In this respect, different studies have explored the

relationship between biomarkers in CSF and structural markers in magnetic resonance imaging (MRI).[18–21] Specifically, a pattern of atrophy in AD vulnerable areas has been described associated with CSF markers of neurodegeneration (t-tau and p-tau). We described a CSF Aß42-p-tau interaction affecting brain structure in preclinical AD,[18] emphasizing the need to consider interactions in order to capture pathogenic synergies.

To our knowledge, the relationship between CSF YKL-40 and brain structure has not been previously assessed. In this respect, we hypothesize that YKL-40 in CSF, due to its strong correlation with markers of neurodegeneration, is also associated with structural markers in MRI such as cortical thickness (CTh). In particular, our aims were to analyze the relationship between CSF YKL-40 (and markers of neurodegeneration) and CTh in a large cohort of non-demented subjects, and to investigate how CSF Aß42 can affect this relationship.

2. Material and Methods

2.1. Study participants and clinical classification

We included 80 cognitively normal (CN) subjects and 27 patients with amnestic mild cognitive impairment (aMCI) evaluated at Hospital de Sant Pau, Barcelona (HSP, n=82) and Hospital Marqués de Valdecilla, Santander (HMV, n=25). All subjects underwent a lumbar puncture and 3 Tesla MRI. CN subjects had a neuropsychological evaluation[22] in normal range for age and education, and subjects with aMCI met Petersen's criteria[23]. All participants gave their written consent, and the study was approved by the local ethics committee at each center.

2.2. CSF analyses

CSF was obtained through lumbar puncture and collected following international consensus recommendations as described.[24,25] Briefly, CSF was collected in polypropylene tubes and immediately centrifuged (1900-2000g x 10min) to avoid haematic contamination. All samples were stored in polypropylene tubes at -80ºC and shipped in dry ice to HSP, where they were analyzed. We used commercially available ELISA kits to determine levels of Aβ42 (Innotest^{TM} β-Amyloid$_{1-42}$, Fujirebio-Innogenetics), t-tau (Innotest^{TM} hTAU Ag, Fujirebio-Innogenetics), p-tau (Innotest^{TM} Fujirebio-Phospho-Tau$_{181P}$, Innogenetics) and YKL-40 (MicroVue^{TM}, Quidel) following the manufacturers' recommendations. Our laboratory has experience in CSF biomarker determination and participates in the Alzheimer's Association external quality control program for CSF biomarkers.[3,26]

2.3. CSF classification

According to the CSF analysis, participants were classified as Aβ42+ (CSF Aβ42<550pg/ml) or Aβ42- (CSF Aβ42>550pg/ml). The internal diagnostic accuracy of this cut-off point had been previously assessed in a cohort of 45 patients with dementia of the Alzheimer type and 20 age-matched controls and had a sensitivity of 91.1% and a specificity of 75.0%.[3]

2.4. MRI acquisition

HSP procedure: 3T MRI scanner (PHILIPS 3.T X SERIES ACHIEVA). A high-resolution three-dimensional structural dataset was acquired with the following parameters: T1-weighted magnetization-prepared rapid gradient-echo, repetition time 8.1 msec, echo time 3.7 msec, 160 slices, matrix size 240x234; slice thickness 1 mm, voxel size 0.94x0.94x1 mm.

HMV procedure: 3T MRI scanner (PHILIPS 3.T X SERIES ACHIEVA). A high-resolution three-dimensional structural dataset was acquired with the following parameters: T1-weighted

magnetization-prepared rapid gradient-echo, repetition time 8.2 msec, echo time 3.8 msec, 160 slices, matrix size 240x234; slice thickness 1 mm, voxel size 0.94x0.94x1 mm.

2.5. Cortical thickness analysis

Cortical reconstruction of the structural images was performed at HSP with the FreeSurfer software package, version 5.1 (http://surfer.nmr.mgh.harvard.edu). The procedures have been fully described elsewhere.[9] Estimated surfaces were inspected to detect errors in the automatic segmentation procedure. One hundred and twenty-four participants with a valid 3T T1 MRI were initially included in the study. Seventeen were excluded because of segmentation errors, and 107 were included in the cortical thickness analysis.

2.6. Statistical analysis

Group analyses were made using R statistical software.[27] Correlations between biomarkers were assessed using Pearson's correlation test. Cortical thickness analyses were performed using linear modelling of the thickness maps as implemented in FreeSurfer. We included as covariates all clinical and demographic variables that were significantly different between groups. Additionally, to avoid covariates, we re-run all the analyses in a subset of 27 CN subjects matched in age, gender, education and center to the group of 27 aMCI.

A Gaussian kernel of 10mm full-width at half maximum was applied. We performed correlation and interaction analyses to study the relationship between cortical thickness and CSF biomarkers. For the resulting maps, an initial vertex-wise threshold was set to $p=0.05$ to find clusters. Then we tested Monte Carlo simulation with 10,000 repeats in Qdec (family-wise error [FWE], $p<0.05$). Only regions that survived FWE correction are presented in the figures.

3. Results

3.1. Clinical characteristics and CSF biomarkers for AD

Table 1 displays the demographics, clinical characteristics and CSF results of the participants. Patients in the aMCI group were older than CN participants (t=-5.38; p<0.001). There were no differences in gender (Chi-square=0.025; p=0.87), education (t=1.46; p=0.15) or in MMSE score (t=1.53; p=0.13) between clinical groups. Compared to CN, the group of aMCI had lower levels of Aβ42 (t=1.98; p=0.05), and higher levels of t-tau (t=-2.20; p=0.03) and p-tau (t=-2.52; p=0.02) in CSF.

Table 1. Demographics and biomarker characteristics of the participants

Unless otherwise specified, values are presented as mean (standard deviation). CN: Cognitively normal subjects; aMCI: amnestic mild cognitive impairment; Aβ42-: CSF Aβ42>550pg/ml; Aβ42+: CSF Aβ42<550pg/ml; MMSE: mini-mental state examination; CSF: cerebrospinal fluid. *p=0.05 compared to CN. **p <0.05 compared to CN. [a]p<0.05 compared to CN Aβ42-. [b]p=0.05 compared to aMCI Aβ42-. [c]p<0.05 compared to aMCI Aβ42-

	All	**CN**	CN Aβ42-	CN Aβ42+	**aMCI**	aMCI Aβ42-	aMCI Aβ42+
n	**107**	**80**	71	9	**27**	15	12
Age, years	**62.27 (9.10)**	**59.99 (8.57)**	59.6 (8.66)	63.12 (7.53)	**69.02** ** **(7.16)**	69.09 (7.97)	68.93 (6.35)
Gender, Female (%)	**74 (69.2%)**	**55 (68.8%)**	49 (69.0%)	6 (66.7%)	**19 (70.4%)**	10 (66.7%)	9 (75.0%)
Education, years	**12.93 (5.06)**	**13.39 (4.87)**	13.54 (4.76)	12.22 (5.89)	**11.59 (5.43)**	12.53 (5.11)	10.42 (5.82)
APOEε4+, n (%)	**37 (34.6%)**	**25 (31.3%)**	19 (26.8%)	6[a] (66.7%)	**12 (44.4%)**	4 (26.7%)	8[b] (66.7%)
MMSE	**28.43 (3.88)**	**28.65 (4.38)**	28.68 (4.64)	28.44 (1.24)	**27.78 (1.53)**	27.73 (1.39)	27.83 (1.75)
CSF Aβ42, pg/ml	**730.57 (204.11)**	**755.94 (182.60)**	793.73 (155.18)	457.78[a] (76.44)	**655.39* (246.37)**	839.53 (157.81)	425.21[c] (87.94)
CSF total-Tau, pg/ml	**251.1 (176.85)**	**219.79 (148.64)**	214.01 (100.49)	265.33 (356.65)	**343.89** ** **(220.04)**	256.3 (112.61)	453.38[c] (273.43)
CSF P-Tau, pg/ml	**48.02 (26.09)**	**43.55 (23.30)**	42.88 (15.11)	48.83 (57.70)	**61.26** ** **(29.69)**	47.5 (15.50)	78.46[b] (34.59)
CSF YKL-40, ng/ml	**210.39 (50.27)**	**200.37 (47.34)**	202.69 (44.94)	182.12 (63.54)	**240.07** ** **(47.64)**	230.32 (44.16)	252.27 (50.90)

YKL-40 was higher in aMCI than in CN subjects (t=-3.75; p<0.001). Within each clinical group, we compared YKL-40 levels between Aβ42+ and Aβ42- subjects and found no differences in CN (t=0.94, p=0.37) or in aMCI (t=-1.18, p=0.25).

We analyzed the relationship between YKL-40 and other CSF biomarkers. In the whole sample, there was no correlation between YKL-40 and Aβ42 (p=0.99). However, YKL-40 showed a strong correlation with t-tau (r=0.55; p<0.001) and p-tau in CSF (r=0.66; p<0.001). These correlations were also significant when we analyzed independently the group of aMCI (t-tau: r=0.70, p<0.001; p-tau: r=0.79, p<0.001), and of CN (t-tau: r=0.56, p<0.001; p-tau: r=0.59, p<0.001). Similar correlations were found when the groups were categorized in Aβ42+ and Aβ42- subjects (data not shown).

3.2. CSF neurodegenerative biomarkers and YKL-40 are associated with cortical atrophy

We then analyzed the relationship between CTh and CSF biomarkers in the whole cohort. As shown in **Figure 1**, YKL-40, t-tau and p-tau had a negative correlation with CTh (FWE corrected p<0.05), especially in middle and inferior temporal areas, whereas CSF Aβ42 did not correlate with brain structure (not shown). As displayed, the patterns of atrophy for YKL-40, t-tau and p-tau were similar. There was a significant association between tau biomarkers and CTh in the precuneus. The same pattern was observed in this area between YKL-40 and CTh, but this cluster did not survive FWE. The correlation between YKL-40 and CTh disappeared completely when results were adjusted by CSF p-tau and vice versa.

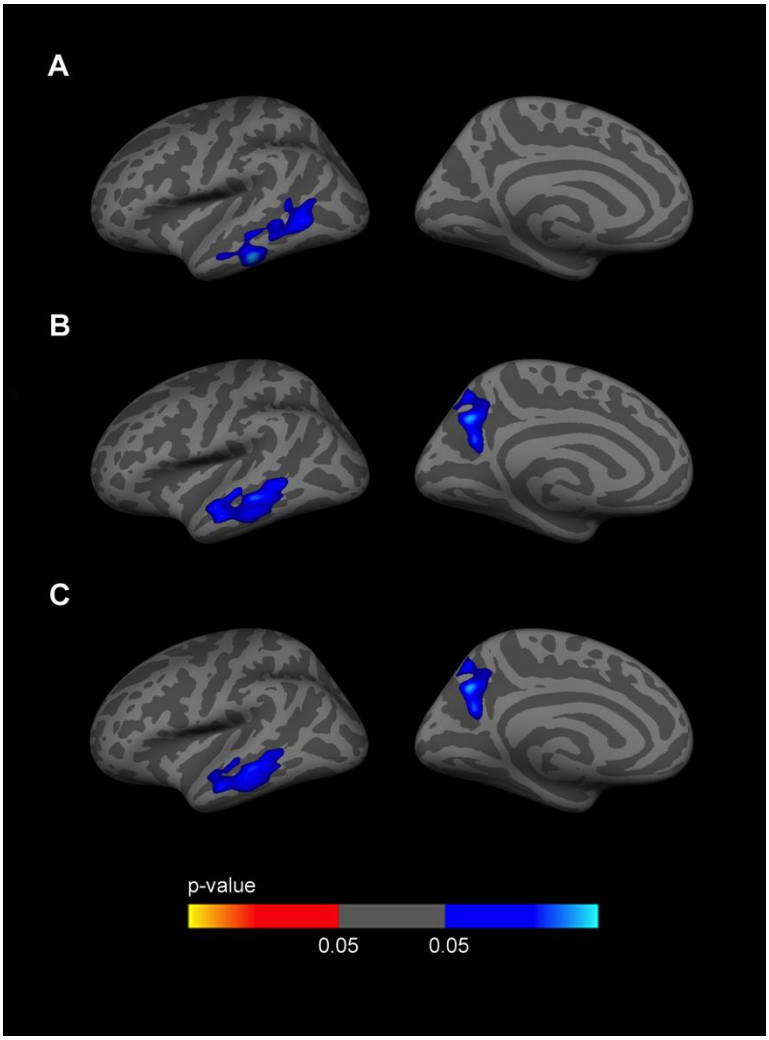
To ensure that our results were not dependent on sample sizes and to avoid including covariates, we re-run all the analyses in a subset of 27 CN subjects matched in age, gender,

education and center to the group of 27 aMCI. The results were not significantly different from the analyses in the whole sample (not shown).

Figure 1. Correlation between cortical thickness (CTh) and cerebrospinal fluid (CSF) biomarkers in the whole cohort (n=107)

There is a significant negative correlation between CTh and CSF YKL-40 in middle and inferior temporal areas (Figure 1A). The pattern is similar to the one found with CSF total tau (Figure 1B) and CSF phospho-tau (Figure 1C). No significant correlations were found between CTh and CSF ß-Amyloid 1-42 (not shown).

All analyses were adjusted by age and center. Only regions that survived FWE correction are presented.



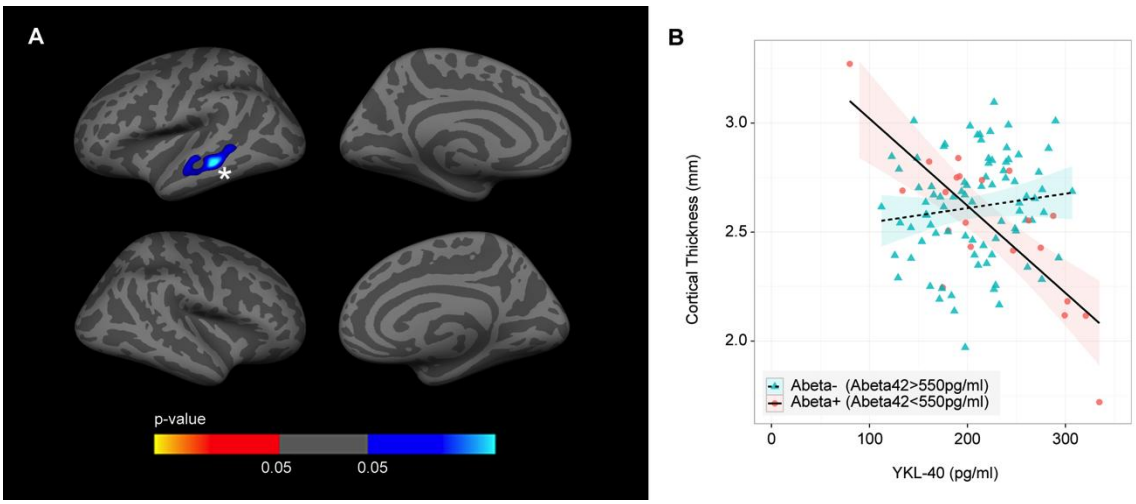
3.3. Interaction of CSF Aß42 with markers of neurodegeneration and YKL-40

We conducted an interaction analysis to assess whether the relationship of CTh with YKL-40 and p-tau was affected by the amyloid status (Aβ42+ vs. Aβ42-) in the whole cohort. As displayed in **Figure 2**, the interaction analyses showed a negative correlation of cortical thickness with YKL-40 in the Aβ42+ subjects, but not in the Aβ42- group. A similar tendency was obtained for p-tau (**Supplementary Figure 1**).

The results in the subset of 54 matched subjects were not significantly different from the analyses in the whole sample (not shown).

Figure 2. Interaction analyses of ß-Amyloid 1-42 (Aβ42) on the correlation between cortical thickness (CTh) and cerebrospinal fluid (CSF) YKL-40 (n=107)

Areas in which the correlation between CSF YKL-40 and CTh is modified by CSF Aβ42 status (Figure **2A**) are shown. Only regions that survived FWE correction are presented. The scatterplot (Figure **2B**) shows the relationship between CSF YKL-40 and CTh by CSF Aβ42 status at the maximum significant vertex in the laterotemporal cluster (asterisk).



3.4. Exploratory analysis in preclinical AD

As an exploratory analysis, we analyzed the correlation between YKL-40 and CTh specifically in the group of CN subjects according to their Aβ42 status. As shown in Supplementary Figure 2, the correlation between YKL-40 and CTh observed in the whole cohort was also significant when the group of CN subjects with low Aβ42 (Aβ42+, n=9) were analyzed independently. No correlations were found in the Aβ42- group.

4. Discussion

This is the first study analyzing the structural correlates of YKL-40 in the pre-dementia stages of AD. Our main findings are that, similarly to t-tau and p-tau, CSF YKL-40 is associated with cortical thinning in middle and inferior temporal areas, areas vulnerable to the AD pathophysiological process and described in AD cortical signatures,[8] and that this association is present in subjects with low CSF Aβ42 only. These findings suggest that CSF YKL-40 track the inflammatory processes associated to tau-related neurodegeneration.

We first investigated the relationship between YKL-40 and core CSF biomarkers of AD in our cohort. We found a strong positive correlation between YKL-40 and CSF markers of neurodegeneration (t-tau and p-tau), but no correlation between YKL-40 and Aβ42 in CSF. These findings are in agreement with previous studies,[3,14,15,17] and suggest that YKL-40 is a marker of neuroinflammation closely related to the process of neurodegeneration, and not to amyloid deposition.

In the analysis of the structural correlates of CSF YKL-40, we found that this biomarker is strongly associated with cortical thinning. The YKL-40-related pattern of atrophy in AD vulnerable areas had an extensive overlap with the one found for t-tau and p-tau, also described in previous studies.[18,20,21] Importantly, the correlation between YKL-40 and cortical

thickness disappeared completely after adjusting by CSF p-tau (and vice versa), indicating that both biomarkers track structural changes in similar areas. These findings are also in agreement with the strong correlation of YKL-40 with t-tau and p-tau found in the CSF analyses, and reinforce the idea that YKL-40 reflects an inflammatory process associated with tau-associated neurodegeneration.

The interaction analyses by Aβ42 status revealed that the amyloid status, assessed by CSF Aβ42 levels, modulates the effect of YKL-40 on brain structure. That is, the correlation of CSF YKL-40 with CTh was significant in Aβ42+ subjects only, whereas the effect of YKL-40 over brain structure in Aβ42- subjects was non-significant. We had previously described a similar interaction effect between p-tau and Aβ42 on brain structure in a different cohort of cognitively normal subjects,[18] and we found that p-tau was associated with cortical thinning in the presence of low CSF Aβ42 only. Other authors have described similar interaction effects between CSF Aβ42, CSF p-tau and brain structure.[20] In the present study, analyzing p-tau and CTh in a different cohort, we observe the same tendency (**Supplementary Figure 1**). Regarding YKL-40, there are no previous structural studies assessing this specific issue. However, our findings are in agreement with other authors that have used a similar approach and recently described that different CSF biomarkers show correlations with brain structure in the presence of low CSF Aβ42 only.[28]

Altogether, our results support the idea that tau-related neurodegeneration and YKL-40-related neuroinflammation affect brain structure mainly in the presence of brain amyloidosis. This effect is also in line with in vitro and animal studies showing that accumulation of Aβ42 induces neuroinflammation[13,29] and enhances tau pathology.[30,31]

The main strength of our study is the large size of our cohort with homogeneous acquisition and analyses procedures. This study was performed in the context of a larger multicenter study (SIGNAL study – www.signalstudy.es) that aimed to harmonize the methodology employed in the application of AD biomarkers (CSF and MRI) across centers in Spain. Specifically, in our study, both MRI scanners are from the same manufacturer and model, as were the acquisition parameters. Reconstruction and analyses of the images were performed in one center only, all the CSF samples were analyzed by the same person, and CSF preanalytical procedures were harmonized across centers.

This study has also some limitations. Firstly, although many efforts were put in avoiding inter-center variability, we did not perform a full harmonization protocol for the acquisition of MRI (i.e. MRI phantom analysis). Secondly, the number of CN subjects with low CSF Aβ42 (and therefore in the preclinical stages of AD) is small. Therefore, the analyses in this subgroup of participants (described in the results section 3.4) should be considered as exploratory and the results interpreted with caution. However, the fact that the correlations are significant, even within this small group of subjects, points out the biological consistency of our results. Lastly, we assessed one specific CSF biomarker of inflammation (YKL-40), but other markers could be linked to other stages of the disease. Further studies analyzing other radiological or biochemical markers of inflammation could be of help to stage the chronology of this complex pathophysiological process, and to determine its relationship with amyloid deposition and neurodegeneration.

In conclusion, CSF YKL-40 could track an inflammatory process associated to tau-related neurodegeneration. These two processes are strongly associated with cortical thinning in AD vulnerable areas in preclinical and prodromal AD.

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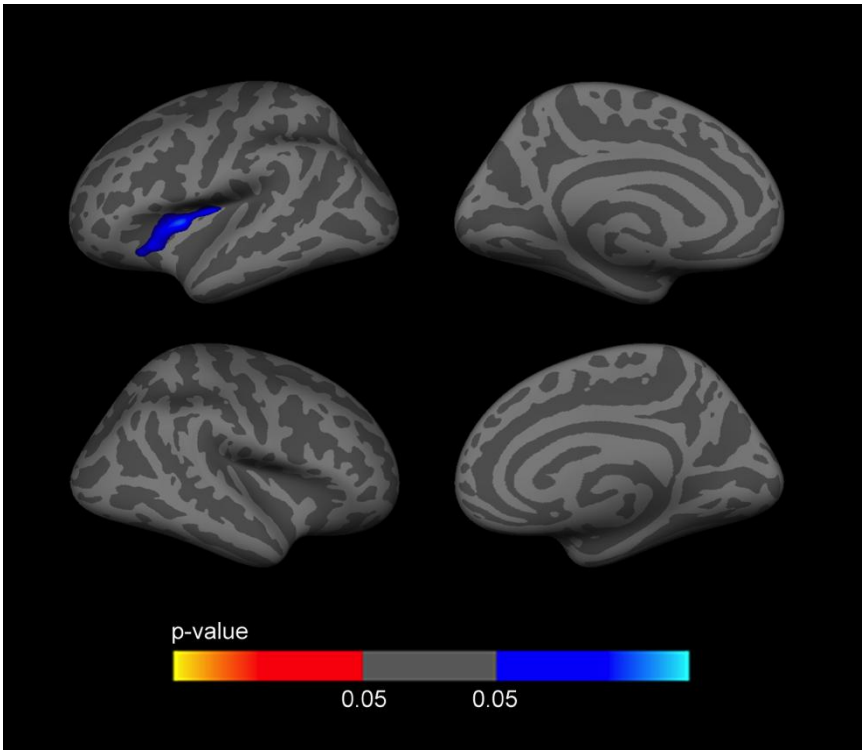
The authors declare that they have no financial conflicts of interest in connection with this article.

6. Supplementary material

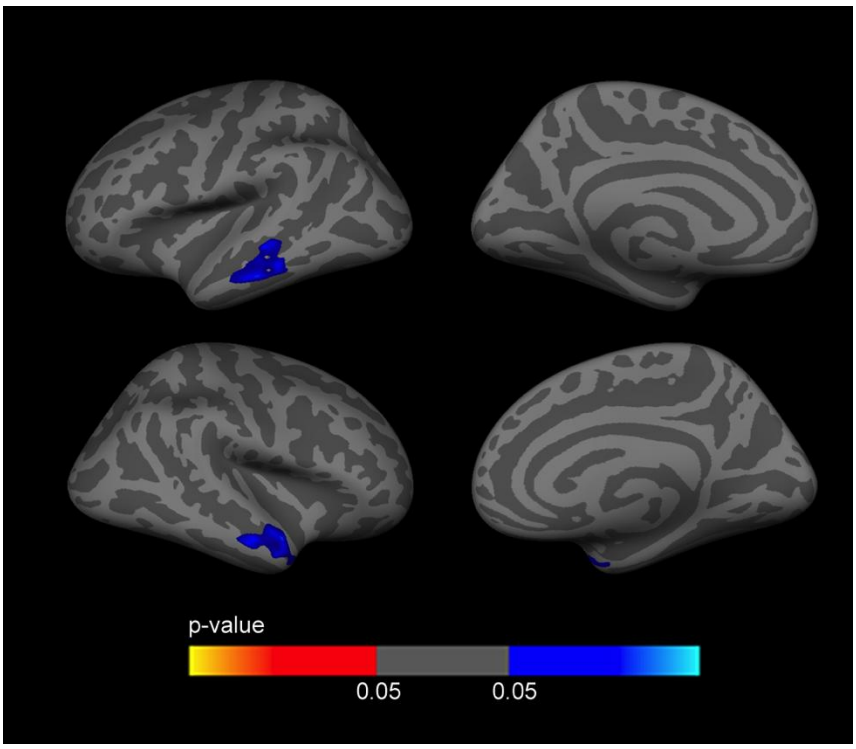
Supplementary Figure 1. Interaction analyses of ß-Amyloid 1-42 (Aβ42) on the correlation between cortical thickness (CTh) and cerebrospinal fluid (CSF) phospho-tau (p-tau)

Areas in which the correlation between CTh and CSF p-tau is modified by Aß42 status are shown. Similarly to YKL-40, there was a correlation between CSF p-tau and CTh in Aβ42+ subjects but not in Aβ42- subjects. Only regions that survived FWE correction are presented.



Supplementary Figure 2. Correlation between cortical thickness (CTh) and cerebrospinal fluid (CSF) YKL-40 in ß-Amyloid 1-42 positive (Aβ42+) cognitively normal subjects (CN) (n=9).

There was a negative correlation between CSF YKL-40 and CTh in temporal areas in the group of CN subjects with low Aβ42 (Aβ42+). No correlations were found in the Aβ42- group (not shown). Only regions that survived FWE correction are presented.



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Chapter 7

General discussion, concluding remarks and future directions

1. General discussion

For many decades, the advances in Alzheimer's disease (AD) research have depended on neuropathological descriptions. The need of *post-mortem* neuropathological examination has made these advances difficult and slow over more than a century, and we have learnt from neuropathological studies that many pathophysiological processes usually coexist in neurodegenerative disorders.[1] The use of biomarkers in the past 10-15 years represents a radical change in the approach of the disease allowing an earlier and more accurate diagnosis and, as a consequence, a more efficient development of clinical trials.[2,3] Additionally, biomarkers are also a valuable tool to assess different pathophysiological pathways *in vivo* and to characterize the preclinical stages of the disease.[4] In this thesis, we take advantage of several cerebrospinal fluid (CSF) biomarkers to study AD from a translational perspective in both clinical and preclinical stages.

To achieve this aim, we first performed a study to assess the feasibility of lumbar puncture (LP), an inevitable procedure for the collection of CSF. This technique has been used for decades for other diagnostic or therapeutic purposes, and large studies have previously assessed its safety in different contexts, such as epidural or intradural anesthesia.[5–10] Due to its invasive nature, several concerns have been raised about the use of this procedure in older population for the diagnosis of AD. This has been one of the reasons, among other issues (such as standardization of the assays), that have hampered the use of LP, and therefore, CSF biomarkers, in clinical settings. In our multicentre study, described in **chapter 2**, we found no major complications derived from this procedure. Consistently with most studies, headache was the most frequent event, occurring in about a quarter of the participants. In the majority of cases, headache was mild and required no treatment or only low doses of commonly used analgesic drugs. The main factors associated with a higher incidence of headache were a younger age and the use of cutting-edge needles. We also

found that patient positioning (sitting or lying) and the volume of CSF extracted were not associated with the incidence of headache. All these results led us to conclude that LP is a safe technique for the study of CSF biomarkers for neurodegenerative diseases.

After our article was published, two other large studies assessing this issue have been performed. One of them (Duits et al, *Alzheimer's and Dementia, in press*), including nearly four thousand participants, confirmed that the use of cutting-edge needles and a younger age were associated with higher incidence of headache. In the second one (n=338),[11] the needle type was not assessed, as the study protocols called for atraumatic needles only. However, the use of larger needles (22G compared to 24G) was associated with a higher proportion of patients that needed a blood patch and with a trend towards higher incidence of headache 24 hours after the procedure. Consistently with our study, there was no association between post-LP headache and the patient position at the moment of LP, and in this study the effect of volume of CSF extracted was only significant for volumes above 30ml. Taking all these results together, ours and other studies have contributed to the elaboration of consensus guidelines to perform LP for CSF sampling in neurological patients (Engelborghs et al., *submitted*).

The study described in **chapter 3** illustrates how the analysis of CSF biomarkers offers a way to study mechanistic pathways *in vivo*. In this study, we merged pathological information and CSF biomarkers to analyze differences in the pathophysiology of sporadic (SAD) and autosomal-dominant AD (ADAD). Under the hypothesis that the mechanisms that lead to Aβ accumulation might be different in these two forms of the disease, we first studied the AβPP processing by measuring BACE protein levels, β-secretase activity and AβPP β-CTF levels in the brain of patients with confirmed neuropathological SAD or ADAD. We found that BACE protein levels and β-secretase activity were elevated in SAD but not in ADAD brains, and that

brain AβPP β-CTF levels were higher in ADAD than in SAD. The AβPP β-CTF fragment is generated after the action of BACE, and it is further processed by γ-secretase to release Aβ peptides.[12] As the levels and activity of BACE were not found increased in ADAD, the accumulation of β-CTFs in the brain of these patients might respond to a decreased processing of these fragments by γ-secretase or to a reduced clearance, perhaps due to impaired macroautophagic mechanisms.

The analysis of CSF biomarkers in **chapter 3** complemented the results observed in the neuropathological part of the study. We measured sAβPPβ levels and β-secretase activity in the CSF of patients with SAD and of *PSEN1* mutation carriers and found no significant differences compared to age-matched controls. These findings indicate that, in these dementia stages, CSF β-secretase activity does not parallel brain β-secretase activity, as the latter was found increased in the brain of patients with SAD. One possible hypothesis for this discrepancy is that β-secretase activity in the CSF would stabilize (or even decrease) as the disease progresses, perhaps as a result of a reduction in global neuronal function. In fact, other CSF studies have suggested that β-secretase activity might become elevated at earlier stages (i.e. mild cognitive impairment), and then decrease as disease progresses.[13–15]

We next studied CSF biomarkers of AβPP processing ($Aβ_{1-42}$, sAβPPβ, β-secretase activity) together with markers of neurodegeneration (t-tau, p-tau) and neuroinflammation (YKL-40) in different cognitive disorders including early stages, and found that these markers showed distinct profiles. As described in **chapter 4**, there were no differences in β-secretase activity and sAβPPβ between amnestic mild cognitive impairment (MCI), dementia of the Alzheimer type (DAT) and cognitively normal controls. However, sAβPPβ levels and β-secretase activity were decreased in patients with frontotemporal dementia (FTD) compared to DAT and controls. These findings are consistent with other studies.[16–18] As AβPP and BACE are proteins highly expressed in neurons, the decrease in sAβPPβ levels in FTD could merely

reflect a marked neuronal loss observed in this disorder. The fact that this decrease is not observed in AD, in which neuronal loss is also a typical neuropathological characteristic, needs further study. The differences in the AβPP processing between these two diseases could be a plausible explanation. The increase in brain BACE1 protein levels or activity observed in the brain of patients with SAD (and described in **chapter 3**), could lead to an overproduction of sAβPPβ that would compensate the decrease associated to neuronal loss.

Another interesting result of this study was the increase in CSF YKL-40 levels both in FTD and DAT, even in stages prior to AD dementia (MCI). This result is in agreement with previous studies,[19,20] and supports the idea that neuroinflammation is a common pathway in these two neurodegenerative diseases. On the other hand, the analyses of correlations between biomarkers showed that YKL-40 correlated with t-tau and p-tau, suggesting a close relationship between neuroinflammation and neurodegeneration.

We next decided to extend the study of this set of biomarkers to preclinical stages. To achieve this, as described in **chapter 5**, we analyzed the same set of biomarkers in 266 CSF samples of cognitively normal participants from five centres in Spain who were classified according to the preclinical stages of the NIA-AA: stage 0, 1, 2, 3, and suspected non-Alzheimer pathology (SNAP).[4,21] We found differences in CSF YKL-40 and sAPPβ levels, but not in β-secretase activity accross stages. In line with the results described in **chapter 4**, the study of the preclinical stages suggests that CSF YKL-40 correlates closely with markers of neurodegeneration from the very early stages of the disease, even before symptoms appear. According to our results, the levels of CSF sAPPβ and β-secretase activity might not be useful biomarkers for the diagnosis or staging of preclinical AD.

Other interesting findings were derived from the analysis of correlations between biomarkers in this cohort. We observed that the correlations between biomarkers differed depending on the $A\beta_{1-42}$ status. Taking into consideration the typical CSF signature of AD, characterized by low $A\beta_{1-42}$ and high tau compared to controls, these two biomarkers would be expected to correlate inversely. However, the expected negative correlation was only found in the group with $A\beta_{1-42}$ below the cut-off. Conversely, in the group that had $A\beta_{1-42}$ above the cut-off, CSF $A\beta_{1-42}$ and t-tau had a positive correlation. This difference in the directionality of correlations could explain some inconsistencies found across biomarker studies, and suggests that the relationship between biomarkers depends on whether the pathophysiological process of AD has started or not.

Furthermore, taking advantage of this large cohort of cognitively normal participants, we could also assess the influence of age, sex and *APOE* genotype on CSF biomarkers. Consistently with other studies, we found that age correlated with CSF levels of t-tau, p-tau and YKL-40. This could also explain part of the correlation found between each other, but the fact that their correlation was still significant after age-adjustment, suggests other mechanistic associations. Age also correlated with $A\beta_{1-42}$, but only in *APOE-ε4* carriers. Another recently published multicentre study gathers information about over 1000 cognitively normal subjects covering a wide age range over the life span.[22] In this study, *APOE-ε4* carriers showed stronger age-related changes in CSF $A\beta_{1-42}$, t-tau and p-tau than *APOE-ε4* non-carriers. Consistently with our findings, the effect was greatest on CSF $A\beta_{1-42}$, starting during the fifth decade of life. However, the differences in CSF t-tau and p-tau due to *APOE* genotype were not significant until the seventh decade of life.

In the previous studies of this thesis, comprising both clinical (**chapter 4**) and preclinical (**chapter 5**) stages of AD, we consistently found a strong correlation between markers of

neurodegeneration (t-tau, p-tau) and the marker of neuroinflammation YKL-40. In **chapter 6**, we hypothesized that YKL-40 would also be associated with structural markers in MRI such as cortical thickness. We confirmed this hypothesis by finding that higher levels of CSF YKL-40 were associated with cortical thinning in middle and inferior temporal areas, regions vulnerable to the AD pathophysiological process. Moreover, the amyloid status seemed to modulate the effect of YKL-40 on brain structure, similarly to what we found in the study of CSF biomarkers described in **chapter 5**. Altogether, these findings suggest that CSF YKL-40 might track the inflammatory processes associated to tau-related neurodegeneration in the context of the pathophysiological process of AD.

2. Concluding remarks

In conclusion, biomarkers are powerful tools that allow an earlier and a more accurate diagnosis of AD, which is the first step in the development of clinical trials and finding an effective treatment. In this thesis, we have described the relationship between different pathophysiological pathways in AD by studying biomarkers in clinical and in preclinical stages, and by analyzing the structural correlates of some of these biomarkers. A better knowledge of these and other pathways, and the relationship between them *in vivo*, can eventually lead to the identification of novel therapeutic targets and/or guide the application of the ones under investigation.

The main conclusions of this thesis are the following:

1. **Lumbar puncture is a safe technique to evaluate AD biomarkers in CSF.**
    a. Headache was the most frequent event, occurring in about a quarter of the subjects, but it was mild in most cases.

b. The main factors associated with headache were younger age and the use of cutting-edge needles.

2. **Autosomal-dominant AD (ADAD) and sporadic AD (SAD) display distinct profiles in the processing of amyloid-β precursor protein.**
    a. No apparent increase in brain BACE protein levels or activity was observed in ADAD, while both were clearly elevated in SAD.
    b. Accumulation of AβPP β-CTF was higher in the brain in ADAD than in SAD and controls.
    c. No changes in BACE measures were observed in the CSF between ADAD and SAD.

3. **Markers of AβPP processing and markers of neuroinflammation in the CSF show distinct profiles in different symptomatic cognitive disorders.**
    a. CSF sAβPPβ levels and β-secretase activity were decreased in patients with FTD but not in the group of DAT.
        i. CSF sAβPPβ and β-secretase activity showed a mild positive correlation with each other and with $A\beta_{1\text{-}42,}$ suggesting that these biomarkers might be tracking the AβPP pathophysiological process.
    b. YKL-40 was increased in patients with FTD and also in DAT, even in the early symptomatic stages.
        i. We found a moderate correlation between YKL-40, total tau and p-tau, indicating that inflammation and neurodegeneration are closely related processes in neurodegenerative diseases.

4. **Markers of AβPP processing and markers of neuroinflammation in the CSF show distinct profiles across presymptomatic stages of AD.**
    a. Whereas sAPPβ was increased in the SNAP group, we found no differences in β-secretase activity between groups.
    b. Paralelling markers of neurodegeneration, CSF YKL-40 was found elevated in SNAP and in Stages 2-3.
    c. CSF biomarkers in cognitively normal subjects are influenced by age and APOE genotype.
    d. The correlations between biomarkers differed depending on the $A\beta_{1-42}$ status.

5. **Similarly to t-tau and p-tau, CSF YKL-40 is associated with cortical thinning in middle and inferior temporal areas, areas vulnerable to the AD pathophysiological process and described in AD cortical signatures.**
    a. This suggests that CSF YKL-40 tracks the inflammatory processes associated to tau-related neurodegeneration.
    b. This association is present in subjects with low CSF $A\beta_{1-42}$ only.

3. Future directions

This thesis has contributed important results to the study of CSF biomarkers in AD, and it has also opened new questions that need to be addressed. In the study described in **chapter 4**, we found a decrease in sAβPPβ in the group of FTD compared to DAT and to cognitively normal controls. This finding should be replicated in a larger cohort and including the entire phenotypical spectrum of FTD. To assess these issues, we are currently analyzing a much larger collection of CSF samples from FTD patients recruited in different centers in Spain. As

mentioned in the general discussion, one possible explanation is that the decrease in CSF sAβPPβ in FTD is due to a massive neuronal loss. To test this hypothesis, we are also working on the structural correlates of this biomarker in MRI, and have obtained striking preliminary results.

Another key aspect of this thesis has been the study of neuroinflammation through the analysis of YKL-40 in CSF. This biomarker has been described associated to inflammation in conditions such as multiple sclerosis, cancer or neurodegenerative diseases. We also found an increase in prodromal AD (**chapter 4**) and preclinical stages (**chapter 5**). Although attributed to glial activation, the expression of this protein in the brain in neurodegenerative diseases needs further characterization. We are currently working on the neuropathological description of YKL-40 immunoreactivity in the brain of cognitively normal controls, patients with AD and subjects with other neurodegenerative conditions. We are also investigating the relationship between CSF YKL-40 and brain structure in MRI that we analyzed in **chapter 6**. Going one step further, we are analyzing these markers in an enlarged cohort of preclinical AD subjects.

We are also interested in the evaluation of longitudinal changes in CSF biomarkers across neurodegenerative diseases, and more importantly along preclinical stages of AD. For this aim, we need to obtain CSF from the same participants at different time points. To this date, more than 50 participants have already contributed with more than one CSF extraction in our centre. Moreover, a multicentre approach that will substantially enlarge the sample size is ongoing.

One of the key points in the study of biomarkers is that they are obtained in living subjects. Thus, at the moment of their analysis, the only diagnosis available is usually based on clinical

criteria. For this reason, a clinical follow-up (and eventually a *post-mortem* neuropathological examination) are crucial to confirm the diagnosis of the participants in biomarker studies and to examine the underlying correlations with biological processes. In our centre, we started the CSF biomarker collection in 2009. To this date more than 680 participants have collaborated in our program with at least one CSF extraction, and all participants are encouraged to take a clinical follow-up and brain donation. This will help to clarify their role in clinical practice and in the design of clinical trials, as it is clear that the development of biomarkers is a critical step to allow early diagnosis and eventually to find a cure for AD.

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Chapter 8

List of abbreviations

AD	Alzheimer's disease
ADAD	Autosomal-dominant Alzheimer's disease
AICD	Amyloid intracellular domain
APOE	Apolipoprotein E
Aβ	Amyloid-β
$Aβ_{1-40}$ or $Aβ_{40}$	Amyloid-β 1-40
$Aβ_{1-42}$ or $Aβ_{42}$	Amyloid-β 1-42
Aβ42-	Subjects with CSF Aβ42 above the cutoff
Aβ42+	Subjects with CSF Aβ42 below the cutoff
AβPP or APP	Amyloid-β precursor protein
BACE	β-site amyloid precursor protein-cleaving enzyme
CITA	Centro de Investigación y Terapias Avanzadas para la enfermedad de Alzheimer, San Sebastián
CLU	Clusterin
CN	Cognitively normal controls
CNS	Central Nervous System
CR1	Complement component (3b/4b) receptor 1
CSF	Cerebrospinal fluid
CSF-AD	Subjects who have evidence of the Alzheimer's disease pathophysiological process in their cerebrospinal fluid
CSF-no AD	Subjects who do not have evidence of the Alzheimer's disease pathophysiological process in their cerebrospinal fluid
CTh	Cortical Thickness
CV	Coefficient of variation
DAT	Dementia of the Alzheimer type
ELISA	Enzyme-Linked ImmunoSorbent Assay
FCSRT	Free and Cued Selective Reminding Test
FDG	^{18}F-fluorodeoxyglucose
fMRI	Functional magnetic resonance imaging
FTD	Frontotemporal dementia
FWE	Family-wise error
HC	Hospital Clínic, Barcelona
HGM	Hospital Gregorio Marañón, Madrid
HMV	Hospital Marqués de Valdecilla, Santander
HSP	Hospital Sant Pau, Barcelona
HVA	Hospital Virgen de la Arrixaca, Murcia
IDDD	Interview for the deterioration of daily living in dementia
IHS	International Headache Society
IWG	International Working Group for new research criteria for the diagnosis of Alzheimer's disease
LP	Lumbar puncture
MCI	Mild cognitive impairment
MMSE	Mini-Mental State Examination
MRI	Magnetic resonance imaging
naMCI	Non-amnestic mild cognitive impairment

NC	Cognitively normal controls
NIA-AA	National Institute on Aging – Alzheimer's Association
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's disease and Related Disorders Association
OR	Odds ratio
p-tau	Phospho-tau
PET	Positron emission tomography
PICALM	Phosphatidylinositol-binding clathrin assembly protein
PICOGEN	Information and Genetic Counseling Program
PLPH	Post-lumbar puncture headache
PMI	Postmortem interval
PSEN	Presenilin
ROC	Receiver Operating Characteristic
SAD	Sporadic Alzheimer's disease
sAβPPα or sAPPα	Soluble N-terminal fragment of AβPP after cleavage by α-secretase
sAβPPβ or sAPPβ	Soluble N-terminal fragment of AβPP after cleavage by β-secretase
SCI	Subjective Cognitive Impairment
sMRI	Structural magnetic resonance imaging
SNAP	Suspected non-Alzheimer pathology
t-tau	Total tau
TREM2	Triggering receptor expressed on myeloid cells 2
UF	Fluorescence Units
YKL-40	Chitinase-3-like-1 protein
β-CTF	β-C-terminal fragment

Chapter 9

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Chapter 10

Certificate of direction

Doctoral thesis

Cerebrospinal fluid biomarkers for the study of the pathophysiological pathways in Alzheimer's disease

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