# Clinical study of novel fluid biomarkers in Alzheimer's disease and other neurodegenerative disorders

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# Clinical study of novel fluid biomarkers in Alzheimer's disease and other neurodegenerative disorders

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## ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia worldwide. Diagnosis during life can be made using imaging and fluid biomarkers reflecting the hallmark lesions *i.e.,* amyloid and tau pathologies, and neurodegeneration. 'Core AD' cerebrospinal fluid (CSF) biomarkers, including measurements of amyloid beta 1–42 (Aβ42) and of phosphorylated tau (p-tau) and total tau (t-tau), are now being used in the routine clinical work up of cognitive impairment. However, those biomarkers present with limited availability, invasiveness and high financial costs. Moreover, they do not reflect the other processes underlying AD, such as synaptic demise, axonal damage or neuroinflammation. Recently developed novel blood-based biomarkers appear to be very promising for AD diagnosis. Additionally, new markers tracking non-amyloid and tau mechanisms are now available, showing potential for AD and non-AD dementia exploration.

The aim of this thesis was to investigate novel CSF and blood candidate biomarkers in patients from clinical settings, including amyloid and tau specific and – unspecific markers, for positive and differential diagnosis of AD. We studied a memory clinic cohort issued from the Center of Cognitive Neurology, Paris University, France, including cognitively impaired patients and neurological controls who had undergone CSF and plasma sampling for exploration of a cognitive complaint.

Firstly, we explored plasma neuregulin 1, a presynaptic protein member of the epidermal growth factor family. Plasma neuregulin 1 levels were increased in AD patients compared with control subjects. Its levels were associated with CSF synaptic markers and cognitive status, showing its potential to easily monitor synaptic impairment. Secondly, we performed a paired CSF and plasma comparison of two neurofilament markers, neurofilament's light chain (NfL) and phosphorylated heavy chains (pNfH), both axonal damage markers. Plasma NfL, the most studied plasma marker in neurodegenerative disorders, performed as well as its CSF counterparts and was associated with cognition and cerebral atrophy. Thirdly, we explored plasma glial acidic fibrillary protein (GFAP), a marker of astrocytic activation. Plasma GFAP levels had high potential to identify amyloid positive status at an early stage in AD in two selected cohorts but also in our memory clinic patients. Fourthly, we explored Galectin-3, a marker of microglial activation in AD brain and CSF. An increased expression of Gal-3 was associated with amyloid plaques in neuropathological examination. CSF Galectin-3 levels were increased in AD and clustered with other neuroinflammation CSF biomarkers. Finally, we compared a series of plasma biomarkers for AD diagnosis: we showed that plasma p-tau measurements including p-tau181 (Thr181 phosphorylation) and p-tau231 (Thr231 phosphorylation), and plasma GFAP displayed a higher specificity to AD, compared with plasma amyloid Aβ42/Aβ40 ratio, total tau and NfL. Combinations of plasma biomarkers improved positive and differential diagnosis of AD. Blood based markers were also associated with cognition and medio-temporal atrophy and cerebral white matter lesions.

In conclusion, the work included in this thesis demonstrates that novel CSF and blood-based biomarkers show potential to contribute to AD diagnosis in clinical settings, and to monitor amyloid and tau lesions, synaptic impairment, axonal damage and neuroinflammation processes. However, their exact interpretation and the conditions of their use in clinical routine still warrant further study.

**Keywords:** dementia; Alzheimer's disease; biomarkers; cerebrospinal fluid; plasma biomarkers; memory clinic

**Étude clinique de nouveaux biomarqueurs liquidiens dans la maladie d'Alzheimer et autres maladies neurodégénératives.**

## RÉSUMÉ

La maladie d'Alzheimer (MA) est la plus fréquente des pathologies neurodégénératives. Des biomarqueurs liquidiens et imageriques sont disponibles, reflétant les lésions caractéristiques de la maladie, à savoir l'accumulation anormale des protéines amyloïdes et tau, et la neurodégénérescence. Les biomarqueurs du liquide cérébrospinal (LCS), mesurant les niveaux du peptide béta-amyloïde 1-42 (Aβ42) et de la protéine tau (forme phosphorylée phospho-tau, et forme totale), sont utilisés en pratique clinique pour l'exploration des troubles cognitifs. Cependant, ils restent peu accessibles, invasifs, coûteux et ne reflètent pas les autres processus sous-jacents de la maladie, tels que la perte synaptique, les dommages axonaux et la neuro-inflammation. De nouveaux biomarqueurs prometteurs ont récemment été identifiés, incluant des biomarqueurs sanguins, moins invasifs, et des marqueurs des mécanismes non amyloïdes et tau de la MA.

L'objectif de cette thèse était d'étudier de nouveaux biomarqueurs candidats de la MA, incluant des marqueurs du LCS et du plasma, spécifiques ou non des lésions amyloïdes et tau, dans une cohorte clinique de centre mémoire. À cette fin, des patients atteints de troubles cognitifs ainsi que des sujets témoins ayant eu un prélèvement de LCS et de plasma pour l'exploration d'une plainte cognitive, ont été inclus au Centre de Neurologie Cognitive, Hôpital Lariboisière Fernand Widal, Paris, France.

Dans une première étude, nous nous sommes intéressés à une protéine présynaptique, la neuréguline 1, et avons montré que son taux plasmatique était plus élevé chez les patients avec une MA que ceux retrouvés chez les témoins. Il était également associé aux marqueurs synaptiques du LCS et à l'état cognitif, démontrant son potentiel pour le suivi de l'atteinte synaptique. Nous avons ensuite comparé les taux cérébrospinaux et plasmatiques de deux marqueurs de dommages axonaux : les taux de neurofilaments à chaîne légère (NfL) et à chaîne lourde phosphorylée (pNfH). Nous avons mis en évidence que le dosage des NfL dans le plasma, marqueur le plus étudié dans les troubles neurocognitifs, était aussi performant que leur mesure dans le LCS et qu'il était corrélé aux troubles cognitifs et à l'atrophie cérébrale. Troisièmement, nous avons étudié deux marqueurs de neuro-inflammation. Nous avons démontré que les concentrations plasmatiques de la protéine acide fibrillaire gliale (GFAP), un marqueur d'activation astrocytaire, identifiaient les individus amyloïde-positifs dès les stades précoces de la maladie, dans deux cohortes de recherche, mais également chez nos patients de centre mémoire. Ensuite, nous avons exploré Galectine-3 (Gal-3), une protéine microgliale. En analyse neuropathologique, une expression accrue de Gal-3 était observée autour des plaques amyloïdes dans le tissu cérébral. Nous avons démontré que Gal-3 était mesurable dans le LCS et que ses niveaux étaient augmentés dans la MA et corrélaient à d'autres marqueurs neuro-inflammatoires du LCS, ce qui en fait un potentiel marqueur microglial. Finalement, nous avons comparé une série de biomarqueurs plasmatiques pour le diagnostic positif et différentiel de la MA : les mesures plasmatiques de protéine phospho-tau et de GFAP discriminaient correctement les patients avec une MA de ceux atteints d'autres pathologies neurodégénératives, comparés aux niveaux plasmatiques d'Aβ42, de tau totale et des NfL. De plus, la combinaison de ces biomarqueurs plasmatiques améliorait leur performance diagnostique dans la MA.

En conclusion, les travaux réalisés dans le cadre de cette thèse démontrent que les nouveaux biomarqueurs cérébrospinaux et plasmatiques ont le potentiel de contribuer au diagnostic de la MA. Bien que leur interprétation et les conditions de leur utilisation en pratique clinique doivent encore être précisées, ils permettent de suivre les lésions amyloïdes et tau de manière

moins invasive, mais également la perte synaptique, les dommages axonaux et la neuro-inflammation.

**Mots-clés:** maladies neurodegénératives; maladie d'Alzheimer; biomarqueurs; liquide cérébrospinal; biomarqueurs plasmatique; centre mémoire.

## LIST OF PAPERS

This thesis is based on the following studies, referred to in the manuscript by their Roman number.

**I. Plasma neuregulin 1 as a synaptic biomarker in Alzheimer's disease: a discovery cohort study.**

Vrillon A, Mouton-Liger F, Martinet M, Cognat E, Hourregue C, Dumurgier J, Bouaziz-Amar E, Brinkmalm A, Blennow K, Zetterberg H, Hugon J, Paquet C.

Alzheimers Res Ther. 2022 May 23;14(1):71. doi:10.1186/s13195-022-01014-7. PMID: 35606871.

**II. Comparison of CSF and plasma NfL and pNfH for Alzheimer's disease diagnosis. A memory clinic study.**

Vrillon A, Ashton NJ, Karikari TK, Hourregue C, Cognat E, Dumurgier J, Zetterberg H, Paquet C, Blennow K.

*Manuscript*

**III. Differences Between Plasma and Cerebrospinal Fluid Glial Fibrillary Acidic Protein Levels Across the Alzheimer Disease Continuum.**

Benedet AL*, Milà-Alomà M*, Vrillon A*, Ashton NJ, Pascoal TA, Lussier F, Karikari TK, Hourregue C, Cognat E, Dumurgier J, Stevenson J, Rahmouni N, Pallen V, Poltronetti NM, Salvadó G, Shekari M, Operto G, Gispert JD, Minguillon C, Fauria K, Kollmorgen G, Suridjan I, Zimmer ER, Zetterberg H, Molinuevo JL, Paquet C, Rosa-Neto P, Blennow K, Suárez-Calvet M. **co-first author.*

JAMA Neurol. 2021 Dec 1;78(12):1471-1483. doi: 10.1001/jamaneurol.2021.3671. PMID: 34661615.

**IV. Galectin-3 is elevated in CSF and is associated with Aβ deposits and tau aggregates in brain tissue in Alzheimer's disease**

Boza-Serrano A*, Vrillon A*, Minta K, Paulus A, Camprubí-Ferrer L, Garcia, M, Andreasson U, Antonell A, Wennström M, Gouras G, Dumurgier J, Cognat E, Molina-Porcel L, Balasa M, Vitorica J, Sánchez-Valle R, Paquet C, Venero JL Blennow K, Deierborg T. **co-first author.*

Acta Neuropathol. 2022 Jul 27. doi: 10.1007/s00401-022-02469-6. Epub ahead of print. PMID: 35895141.

**V. Plasma biomarkers for diagnosis of Alzheimer's disease in clinical settings: a memory clinic cohort study.**

Vrillon A, Ashton NJ, Karikari TK, Lantero-Rodriguez J, Götze K, Hourrègue C, Dumurgier J, Cognat E, Zetterberg H, Paquet C, Blennow K.

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*“From now on, we live in a world where man has walked on the Moon. It's not a miracle; we just decided to go.”*
*Jim Lovell*

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*Enfin, ce travail est dédié aux patients, à leurs familles et à tous ceux qui les accompagnent.*

# Table of Contents

**Summary of figures**

**Abbreviations**

| | |
|---|---|
| AD | Alzheimer’s disease |
| ALS | Amyotrophic lateral sclerosis |
| APOE | Apolipoprotein E |
| APP | Amyloid precursor protein |
| AUC | Area under the curve |
| Aβ | Amyloid beta |
| BACE 1 | Beta-site APP cleaving enzyme 1 |
| BBB | Brain blood barrier |
| bvFTD | Behavioral variant frontotemporal dementia |
| CAA | Cerebral amyloid angiopathy |
| CBD | Cortico basal degeneration |
| CERAD | Consortium to establish a registry for Alzheimer’s disease |
| CI | Cognitively impaired |
| CJD | Creutzfeldt-Jakob disease |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| CU | Cognitively unimpaired |
| C9ORF72 | Chromosome 9 open reading frame 72 |
| DAM | Disease-associated microglia |
| DLB | Dementia with Lewy bodies |
| EEG | Eletroencephalography |
| Elisa | Enzyme-linked immunosorbent assay |
| EOAD | Early onset Alzheimer's disease |
| FDG | [$^{18}$F]fluorodeoxyglucose |
| FTD | Frontotemporal dementia |
| FTLD | Frontotemporal lobar degeneration |
| FUS | Fused in sarcoma |
| Gal-3 | Galectin-3 |
| GAP 43 | Growth-associated protein 43 |
| GBA | Glucosylceramidase Beta |
| GFAP | Glial fibrillary acidic protein |
| GWAS | Genome wide association study |
| HRP | Horseradish peroxidase |
| IL6 | Interleukin 6 |
| IL8 | Interleukin 8 |
| LOAD | Late onset Alzheimer's disease |
| MAPT | Microtubule-associated protein tau |
| MCI | Mild cognitive impairment |
| MMSE | Mini mental state examination |
| MRI | Magnetic resonance imaging |
| MS | Mass spectrometry |
| MSA | Multiple system atrophy |
| NDDs | Neurodegenerative diseases |
| NfL | Neurofilament light |
| NFTs | Neurofibrillary tangles |
| NIA-AA | National Institute on Aging and Alzheimer’s Association |
| NRG1 | Neuregulin1 |

| | |
|---|---|
| PD | Parkinson's disease |
| PET | Positron emission tomography |
| PCA | Posterior cortical atrophy |
| pNfH | phosphorylated neurofilament heavy |
| p-tau | phosphorylated tau |
| PPA | Primary progressive aphasia |
| PrP | Prion protein |
| PSEN | Presenilin |
| PSP | Progressive supranuclear palsy |
| ROC | Receiver operating characteristic |
| Simoa | Single molecule assay |
| SNAP-25 | Synaptosomal associated protein 25 |
| SNCA | Synuclein alpha |
| SV2A | Synaptic vesicle glycoprotein 2A |
| svPPA | Semantic variant PPA |
| TBI | Traumatic brain injury |
| TNFα | Tumor necrosis factor alpha |
| TDP-43 | Transactivation response DNA binding protein 43 |
| TREM2 | Triggering receptor expressed on myeloid cells 2 |
| Tukey's HSD test | Tukey's honest significant difference test |
| t-tau | Total tau |
| VaD | Vascular dementia |
| WML | White matter lesions |

# 1. INTRODUCTION

## 1.1 General considerations on neurodegenerative diseases

### 1.1.1 Common pathophysiological mechanisms and characteristics of neurodegenerative diseases

Neurocognitive disorders are an acquired, progressive and significant impairment of cognitive functions, affecting memory, language, visual functions (1). Dementia relates to a cognitive impairment that severely impacts on the patient's daily life, whereas mild cognitive impairment (MCI) doesn't impact patient autonomy (1). A wide range of causes of dementia exists or coexists and establishing a reliable diagnosis is crucial to determine the prognosis and the proposed treatment. The major cause of neurocognitive disorders is neurodegenerative diseases (NDDs) that are characterized by abnormal accumulation of abnormal proteins defined by the term 'proteinopathy' leading to neuronal death, in a process defined as neurodegeneration (2). Neurodegeneration can initiate in different brain regions, with selective loss of neurons in motor, sensory or cognitive systems, and affects different cell types depending on the pathology. The large group of neurodegenerative diseases display common pathophysiological processes and clinical evolution as well as contrasting specificities and heterogeneity mainly linked to the type of the proteinopathy and the pattern of neurodegeneration. Alternatively, neurocognitive impairment can also be due to other causes different from neurodegenerative disorder, such as vascular, metabolic or toxic causes. These alternative causes are important to identify as they may benefit from specific treatment.

The most frequent NDDs are Alzheimer's disease (AD), dementia with Lewy bodies (DLB), and fronto-lobar temporal dementia (FTLD). Although these diseases are characterized by clinical symptoms, anatomic vulnerability and specific proteinopathy, they share many fundamental processes. Indeed, neurodegenerative disorders associate with i/loss of proteostasis leading to abnormal aggregation and deposits of misfolded proteins, ii/progressive synaptic, axonal and neuronal dysfunction and loss, and iii/a long clinically silent phase when protein abnormalities that define neurodegenerative diseases are already present before the onset of clinical features.

At a molecular level, NDDs are characterized by aggregation and deposits of misfolded proteins principally in the CNS but also in peripheral organs. Therefore, they can be referred to and classified as proteinopathies (3) (**Fig. 1**). The most common proteinopathies include amyloidosis, tauopathies, α-synucleinopathies, prion protein, transactivation response DNA binding protein 43 (TDP-43) and fused in sarcoma (FUS). Other proteins are mostly found within hereditary disorders, comprising proteins encoded by genes linked to neurological trinucleotide repeat disorders (e.g., huntingtin, ataxins, atrophin-1), neuroserpinopathy or ferritin-related NDDs.
Many pathophysiological processes are linked to the protein accumulation including neuroinflammatory processes, deficiency of the ubiquitin – proteosome – autophagy system, oxidative stress with free radicals formation, mitochondrial dysfunction, impaired bioenergetics, dysfunction of neurotrophins, disruptions of neuronal Golgi apparatus and of axonal transport, leading to a progressive neuronal dysfunction and death. Will then ensue a disruption of larger brain networks and ultimately of the cognitive function they support.

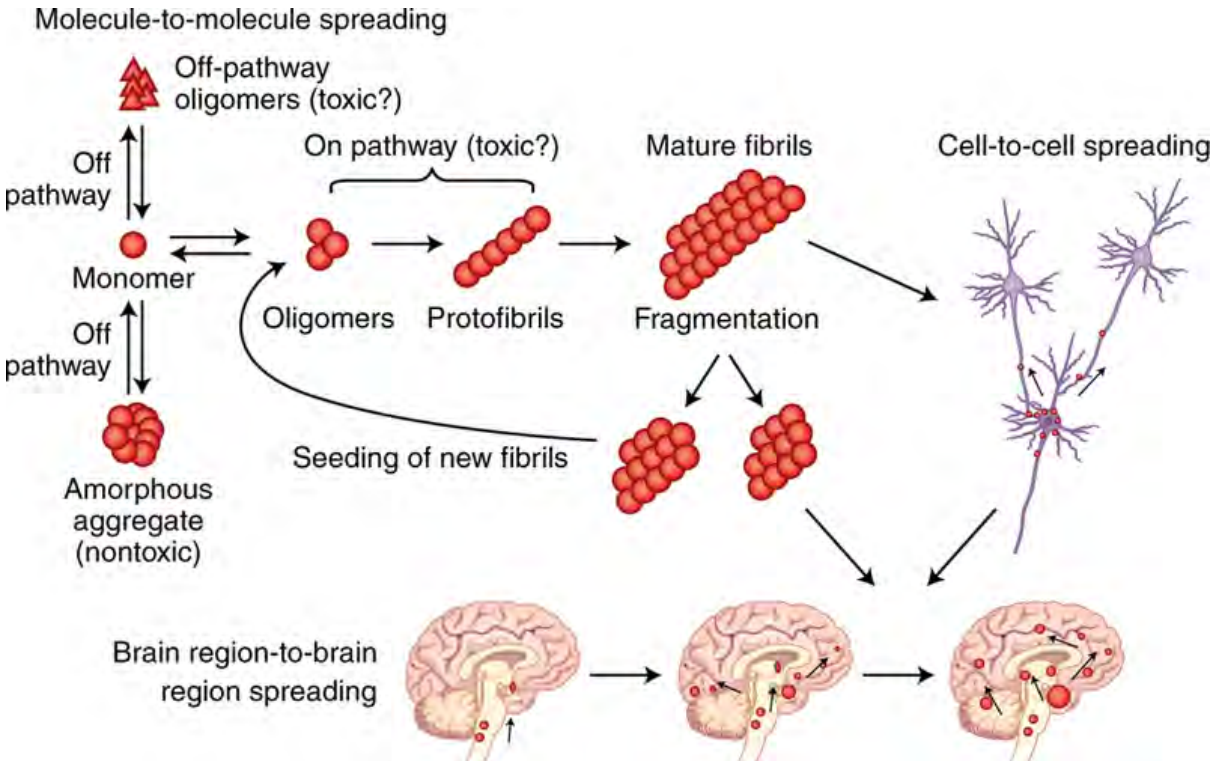



**Figure 1, Protein aggregation and spreading in neurodegenerative diseases**
*Reproduced from Soto et al., Nature Neuroscience 2018* (4) *Proteins initially in monomeric form can misfold then aggregate. Spreading of protein misfolding occurs along the disease progression at different levels, including molecule-to-molecule, cell-to-cell and brain region-to-brain region processes.*

Definitive diagnosis of NDDs is based on the neuropathological examination of the brain, where the identification of the type and the neuroanatomical distribution of the proteins aggregates is performed (5). Neuropathological studies have demonstrated that most of the patients present with copathologies (*i.e.,* more than one pathogenic process occurring in their brain at the time of neuropathological exams) (6). This heterogeneity is translated into clinical presentation, as only few patients present with pure syndromes, while most of them have mixed clinical features.
The process of clinical and pathological phenotyping has substantially improved classifications of NDDs after decades of clinicopathological correlation. Based on the clinical features (affected cognitive domains, associated motor deficits, evolution of the deficits), classifications have initially been proposed with clear separate typical diseases: typically AD presenting as amnestic syndrome of the hippocampal type, FTD as behavioral impairment, *etc.* Progress in clinicopathological correlations has now established that different pathologies can cause similar clinical syndromes and conversely, very different phenotypes can be associated to the same underlying pathology (**Fig. 2**). Moreover, NDDs can be induced by more than one abnormal proteinopathy. As an example, mounting evidence from neuropathological studies shows TDP-43 and α – synuclein co-pathologies in the brains of individuals meeting pathological diagnostic criteria for AD (7).

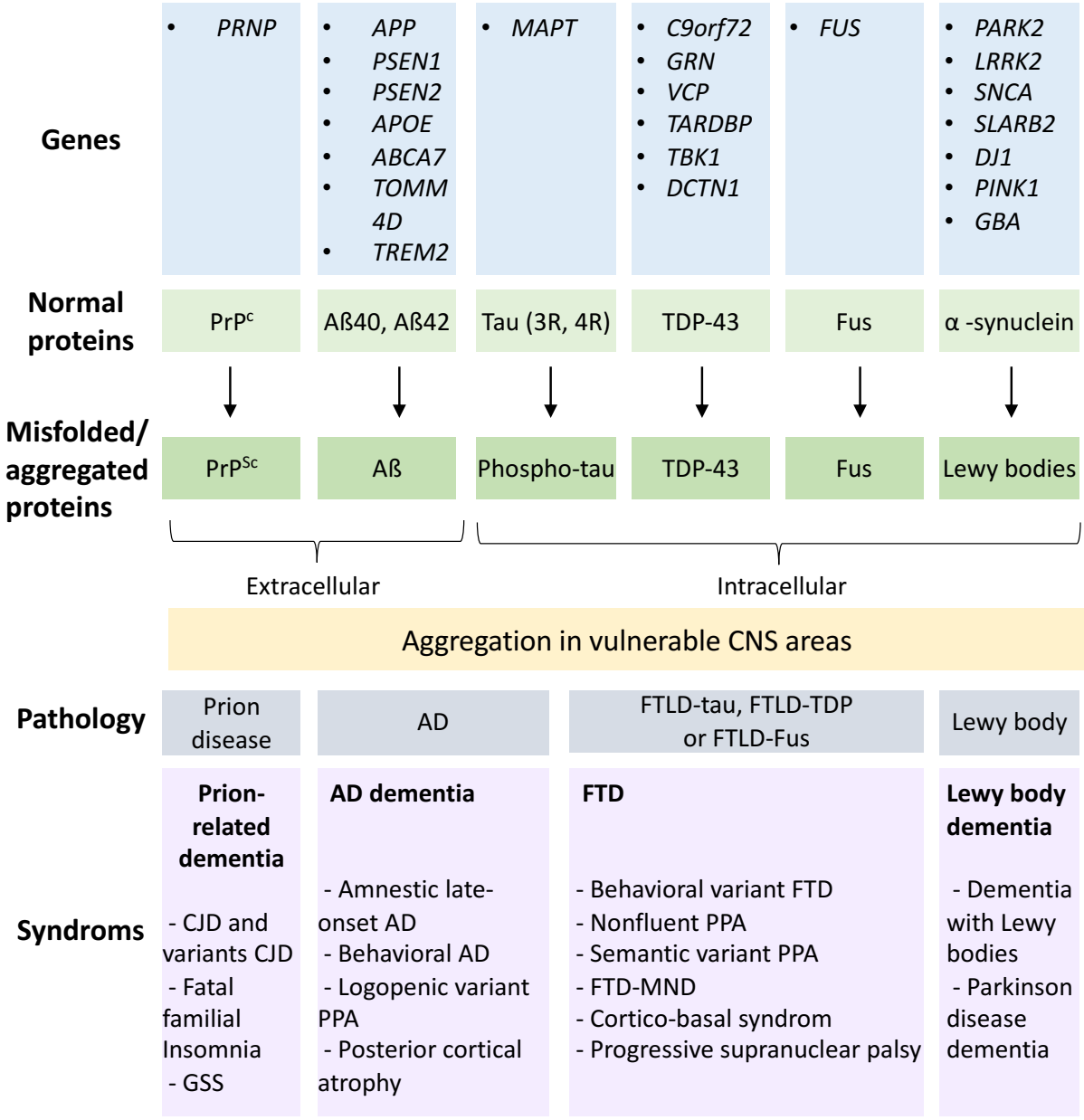



**Figure 2, Clinico-pathological spectrum of neurodegenerative proteinopathies**
*Adapted from Elahi et al., Nature Reviews 2017* (8). *Four major categories of pathological diseases can be defined: prion disease, Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD) and Lewy body diseases (LBD). Each pathology manifests as a variety of clinical syndromes, sometimes featuring overlapping symptoms. Aβ, amyloid-β; CJD, Creutzfeldt – Jakob disease; FTD – MND, FTD with motor neuron disease; GSS, Gerstmann Straussler-Scheinker; PPA, primary progressive aphasia; PrP, prion protein.*

Taken together, all these aspects challenge diagnosis during life and robust markers for improving diagnosis are still sought after for most NDDs. Thus, the use of biomarkers has become crucial for classification of neurodegenerative syndromes into distinct molecular and structural subtypes. Indeed, rigorous syndromic classification using imaging, fluid and genetic markers can improve the prediction of the underlying pathology.

### 1.1.2 Fluid biomarkers for neurodegenerative diseases

Neurodegeneration represents the underlying process for most evolutive cognitive disorders. Due to the difficult access to the brain, its complexity and the long time period of disease progression, measurable markers are needed to identify, monitor and predict NDDs. A biomarker can be defined as 'any substance, structure, or process that can be measured in the body or in its products and influence or predict the incidence or outcome of disease' (9). Biomarkers can serve different purposes including evaluating disease risk, guiding clinical diagnosis, evaluating prognosis and monitoring therapeutic response.

Biomarkers data can be acquired through imaging, fluid collection or electrophysiology. Different bodily fluids can be analyzed for detecting or quantifying biomarkers, including blood, cerebrospinal fluid (CSF), saliva or urine. Saliva and urine are excellent sources of biomarkers because of their abundance in non-invasive sampling. However, urine has proved to be inadequate as it is anatomically distant from the CNS and difficultly conveys cerebral changes. For salivary biomarkers, the use of different sampling methods makes it difficult to obtain reproducible results in research studies. Because of its close relation with the brain, CSF can reflect biochemical changes occurring in the brain. Hence, CSF has been the most studied fluid for biomarker discovery for NDDs. The CSF compartment surrounds the brain, occupying the subarachnoid spaces and the ventricular system, and filling the central canal of the spinal cord. CSF is mainly secreted by specialized cells of the choroid plexus, whose functions are to filter plasma, retain high molecular weight components in the plasma and secret liquid, salts, and lower molecular weight components of the plasma into the CSF (10). CSF participates in brain homeostasis as it provides nutrients as well as waste removal of metabolic products out of the brain. Through the blood – CSF barrier, which restricts the exchange of molecules and proteins, the CSF is relatively isolated from the peripheral vascular system (11). Thus, it represents a useful reflect of CNS. A sample of CSF can be taken by performing a lumbar puncture to measure markers of interest.
Aside from CSF, research effort is currently focusing on the development of more accessible biomarkers. Principally, plasma has been recently studied to identify new CNS biomarkers. Blood-based biomarkers would be of great utility because of their accessibility, although the blood's relative distance from the brain and its contact with the periphery represent potential limitations for CNS-specific biomarkers.

## 1.2 Alzheimer's disease

Alzheimer's disease (AD) represents the most common cause of dementia, estimated to account for more than 60% of all dementia cases worldwide. AD currently affects roughly 55 million people worldwide (12). In France, around one million patients was estimated to be living with AD in 2020 (Inserm Data). Due to increasing age and size of the population, the number of affected individuals is likely to further increase.

### 1.2.1 Clinical features

At dementia stage, AD is characterized by the impairment of cognitive functions and macroscopically by a massive brain atrophy, related to neuronal degeneration. This stage derives from pathological changes in the brain starting to appear more than 20 years before the symptoms become overt. Thus, AD should be described as a *continuum*. This *continuum* starts

with a preclinical phase, when the affected individual remains asymptomatic but starts to exhibit abnormal biomarkers and measurable brain changes. This initial phase is followed by a mild cognitive impairment (MCI) phase, when the first cognitive symptoms start to appear, although not severe enough to impact on the patient's daily activity performance (13). The disease then progresses to the dementia stage, with impaired communication, disorientation, changes in behavior and poor judgment, ultimately leading to the inability to perform everyday activities (1).
The clinical phenotypes commonly associated with AD are the amnestic syndrome classically defined as of 'the hippocampal type', the posterior cortical atrophy variant (PCA) and the logopenic variant primary progressive aphasia (PPA):
—amnestic syndrome is characterized by impairment of episodic memory, where the affected person will forget recent events. This form of memory can be evaluated in recognition paradigms using the remember/know procedure, first developed by Tulving *et al,.* (14). Impairment in spatiotemporal orientation appears along disease evolution, as well as impairment of executive functions (decision-making deficits, loss of cognitive flexibility, working memory deficits).
—PCA is a clinical-radiologic syndrome in which patients present with visuoperceptual symptoms, such as diminished ability to interpret, locate, or reach for objects under visual guidance as well as deficits in numeracy, literacy, and praxis (15). These symptoms are related to occipital, parietal, and occipitotemporal cortices atrophy. Although episodic memory and insight are initially relatively preserved, the progression of PCA ultimately leads to a more diffuse pattern of cognitive dysfunction.
—PPA is a clinical syndrome characterized by progressive language impairment remaining isolated for years prior to the development of impairment in other domains. It is associated with heterogeneous underlying neuropathology (16). The logopenic form has been shown in clinicopathologic studies to be most often linked to AD pathology. In logopenic PPA, word retrieval difficulty, sentence repetition deficit and phonological impairment are prominent.
Rarer presentations of AD can include the AD behavioral variant or dysexecutive variant, corticobasal syndrome and the other variants of PPA (17).
Evolution of AD can eventually lead to a clinical picture of agnosia, aphasia and apraxia. Along disease progression, AD is also characterized by the occurrence of neuropsychiatric symptoms that can present as anxio-depressive symptoms, sleep disorders, delusions, paranoia.
When the disease occurs before 65 years of age, it is called early-onset AD (EOAD), while late-onset AD (LOAD) occurs in subjects over 65 years of age. EOAD is estimated to represent 5 to 10% of AD cases (12). EOAD patients are more likely to experience an aggressive clinical course and to display an atypical clinical presentation (18).

### 1.2.2 Molecular pathological findings

The neuropathology of AD manifests in several features (19). At the macroscopic level, AD is traditionally considered a gray matter disease due to the prevalent neuronal pathological hallmarks in the gray matter. However, white matter degeneration and demyelination are also important pathological features of AD (20). The degeneration occurring in the AD brain can be explicitly observed as atrophy involving shrinkage of cerebral cortex and hippocampus along with the enlargement of the ventricles.
At the microscopic level, the neuropathological hallmarks of AD are multiple and include the classically described amyloid plaques, and neurofibrillary tangles as well as cerebral amyloid angiopathy, neuronal and synaptic loss, white matter degeneration, demyelination, oligodendrocyte degeneration, granular lipid inclusions and glial responses (astrogliosis and microgliosis). Amyloid plaques are the first feature of Alzheimer's disease (**Fig. 3, A**). They

are composed of aggregated amyloids, insoluble fibrous proteins that present with β-sheet-rich secondary structures, which can be detected by specific dyes, such as Congo red (21). The most common amyloidosis is a proteolytic product of the amyloid precursor protein (APP), referred to as Aβ. In AD, immunohistochemical staining reveals extracellular deposition of amyloid plaques (consisting of several truncations, 38 – to 43 amino acids, of Aβ peptide) which can be in different morphologies (diffuse and dense-core) (22). The Aβ peptide 1–42 (Aβ42) is the main plaque component, as prone to form toxic oligomers, whereas the most abundant Aβ specie is Aβ 1–40 (Aβ40), both in normal and AD brains. The earliest amyloid plaques are non-compacted, diffuse, amyloid deposits (23). Amyloid plaques have heterogeneous cellular components, including neuronal processes, the 'dystrophic neurites'. The five amyloid 'phases' as reported by Thal *et al*., describe the topography of Aβ deposits during disease progression, with phases 1 and 2 having plaques restricted to the neocortex and hippocampus, spreading to the striatum in phase 3, and to the brainstem and cerebellum in phases 4 and 5, respectively (24).

Neurofibrillary tangles (NFTs) are the second main lesion defining AD pathology (**Fig. 3, B**) (25). NFTs are composed predominantly of abnormally hyperphosphorylated tau protein, a microtubule-associated protein primarily located in the neuronal axons (26,27). Tau aggregates are present not only in the neuronal perikarya as NFTs but also in dystrophic neuronal processes, in neuritic plaques. As NFTs expend, the neurons displaying the inclusion die leaving an extracellular 'ghost' NFT. The Braak classification defines NFTs progression stages: stage I begins in the transentorhinal cortices, spreading to the hippocampus and limbic cortices (stages II – III) and multimodal association cortices (stages IV – V), the primary cortices being the last affected (stage VI) (28). Alternative patterns of the tau pathology have been described in a significant subset of patients, with 'limbic predominant' pathology or 'hippocampal-sparing' pathology types (29).

Cerebral amyloid angiopathy (CAA), which is characterized by the accumulation of amyloid fibrils in the walls of small to medium-sized leptomeningeal and intracortical arterial blood vessels, is also frequently observed in the AD brain (30).

A significant advance in the neuropathological diagnosis of AD, and thereby for enabling both cross-sectional and longitudinal studies, has been the development of imaging technic to detect the localization and the density of neuritic plaques and of NFTs (31). Both can be assessed using a semiquantitative method proposed by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (32).

Neuropathological exam also demonstrates the presence of neuroinflammation along AD lesions. Amyloid plaques are surrounded by proofing active glial cells, microglia and astrocytes, with altered morphologies. Microglia is frequently observed in association with dense plaques (33). Similarly, reactive astrocytes occupy peri-plaque positions (34).

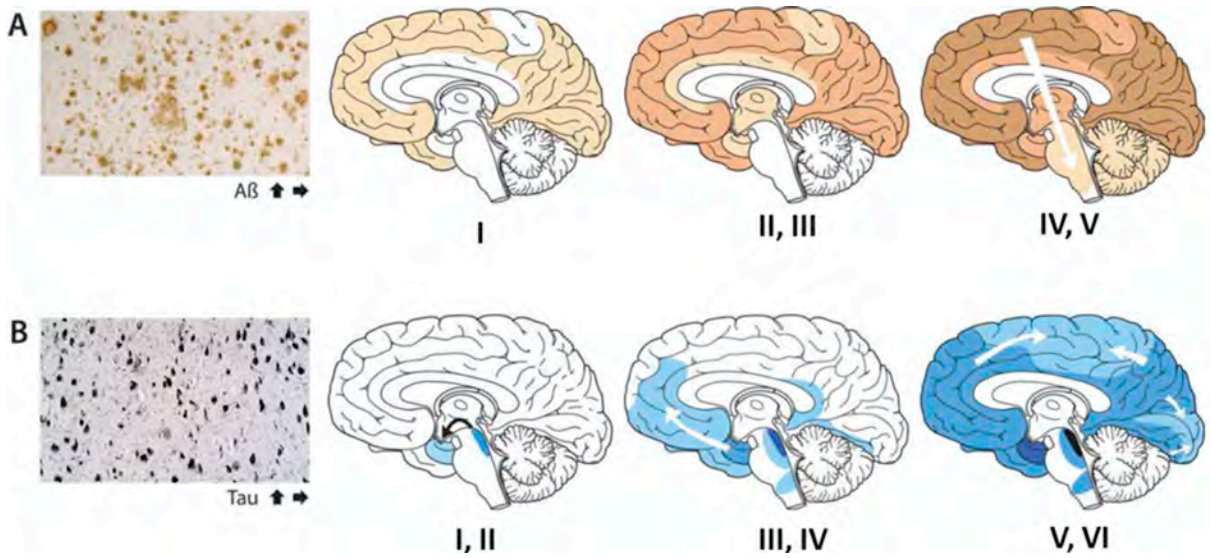



**Figure 3, Pathological hallmarks of Alzheimer's disease and lesions distribution**
*From Jouanne et al., Eur J Med Chem 2017* (35). ***A)*** *Amyloid-β (Aβ) plaques spread in the AD brain according to a pattern described by Thal (stages I to V)* (24)*;* ***B)*** *Neurofibrillary tangles (NFTs) pathology progresses in AD according to Braak stages (stages I toVI)* (28).

1.2.3 Physiopathological hypothesis

*Amyloid cascad hypothesis*

Following the identification of Aβ protein as a core element of AD pathology and of the genetic variants linked to autosomal dominant forms of the disease (all in genes involved in Aβ metabolism), the amyloid cascade hypothesis was introduced (36). This hypothesis introduced the idea that an imbalance in the production or clearance of Aβ would be the primary trigger in AD leading to subsequent amyloid plaques, tau tangles, oxidative stress, and neuroinflammation, resulting in the neuronal loss underlying the symptoms. The APP normally cleaved by alpha-secretase (non-amyloidogenic pathway) would be aberrantly processed by beta- and gamma-secretase (amyloidogenic pathway), resulting in accumulation of Aβ peptides (**Fig. 4**).
Evidence in favor of this hypothesis can be found in familial form of AD relating to mutations in the APP, or in the presenilin-1 (PSEN1) and presenilin-2 genes (PSEN2), the key catalytic subunits of gamma–secretase. Down syndrome caused by the existence of three copies of the APP gene on chromosome 21 being the leading cause of genetic AD also supports this hypothesis (37). AD mice models carrying familial human *App* and *Psen* mutations present with a clinical phenotype mirroring certain aspects of AD.

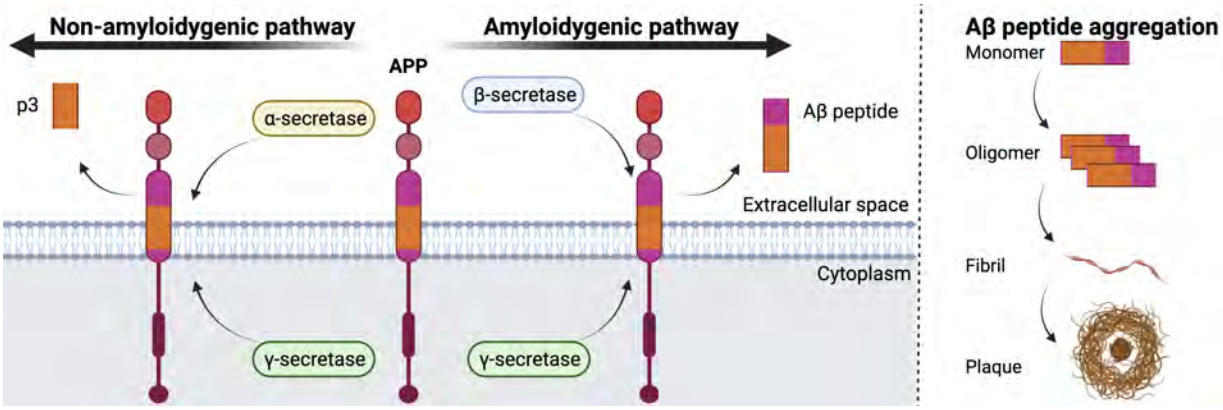



**Figure 4, Amyloid processing and aggregation processes**
*Figure courtesy of Dr. J. Nilsson, created with Biorender.com. The amyloid precursor protein (APP) is a single transmembrane protein. In the non-amyloidogenic pathway (left), APP is cleaved by A Disintegrin and metalloprotease (ADAM) family proteases to yield an extracellularly released soluble APP fragment (P3 peptidic fragment). In the amyloidogenic pathway (right), APP is first cleaved by β-secretase (β-APP-cleaving enzyme-1 or BACE1). The obtained fragment is subsequently cleaved by the γ-secretase complex (comprising Presenilin 1 or 2). This proteolytic processing releases Aβ peptides into the extracellular space. Aβ (far right) oligomerization occurs via distinct intermediates, including Aβ monomers, oligomers, protofibrils, fibrils and plaques.*

However, there are several limits to this hypothesis. At autopsy, about 20–40% of cognitively intact elderly subjects meet some neuropathological criteria for AD, and CSF biomarker or PET imaging studies can demonstrate AD profile in cognitively healthy individuals (38). There is also a limited association between amyloid plaque and NFT burden and cognitive status. Lastly, drug trials targeting amyloid lesions, *i.e.,* Aβ aggregates and associated proteins, have had very limited efficiency (39).
In contrast to the lack of correspondence between amyloid plaque burden and dementia, there is growing evidence in favor of alternative hypotheses with other proteins or processes, such as tau, neuroinflammation or oxidative stress, potentially being also central mechanisms of AD pathogenesis.

*Tau hypothesis*

Some elements indicate that the main factor underlying the development and progression of AD could be the tau protein. Tau is one of the microtubule-associated proteins that regulate the stability of tubulin assembly (**Fig. 5**). Tau is encoded by the *MAPT* gene and presents as six isoforms (with length ranging from 352 to 441 amino acids) in the adult human brain (27). The tau protein sequence includes an amino-terminal region, a mid-region, a microtubule-binding region, and a carboxy terminus. The microtubule-binding region consists of three or four pseudo-repeat domains (R1–R4). The mid-region domain displays several threonine and serine residues, that are targets for phosphorylation by specific kinases.

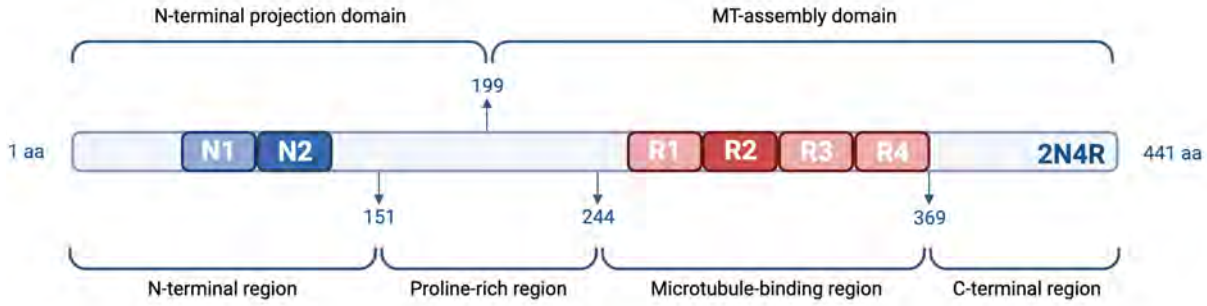



**Figure 5, Schematic representation of the tau protein**
*Figure courtesy of Dr. J. Lantero-Rodriguez, created with Biorender.com. This figure displays the largest tau isoform (441 amino acids). The N-terminal projection domain, including an acidic and a proline-rich region, interacts with cytoskeletal elements to regulate microtubules disposition in axons. The C-terminal part, including the microtubule-binding domain, regulates microtubules polymerization.*

Although the phosphorylation of tau has a physiological function in assuring microtubule assembly, increased phosphorylation above a certain threshold has pathological consequences (40–42). In AD brains, abnormal hyperphosphorylation causes the detachment of tau proteins leading to microtubule disassembly (**Fig. 6**). The tau accumulation in an hyperphosphorylated state induces aggregation in the NFTs, insoluble pathological inclusions. Aberrant interaction of stabilized tau with filamentous actin induces mis-stabilization of actin, synaptic and mitochondrial dysfunction, subsequently leading to neurodegeneration (43,44). Analyses of a large number of human brains across the lifespan could show that the tau pathology begins about a decade before the formation of Aβ plaques (45). Recent studies using positron emission tomography (PET) have shown that the spatial patterns of tau tracer binding are closely linked to the patterns of neurodegeneration and the clinical progression in AD patients (46).

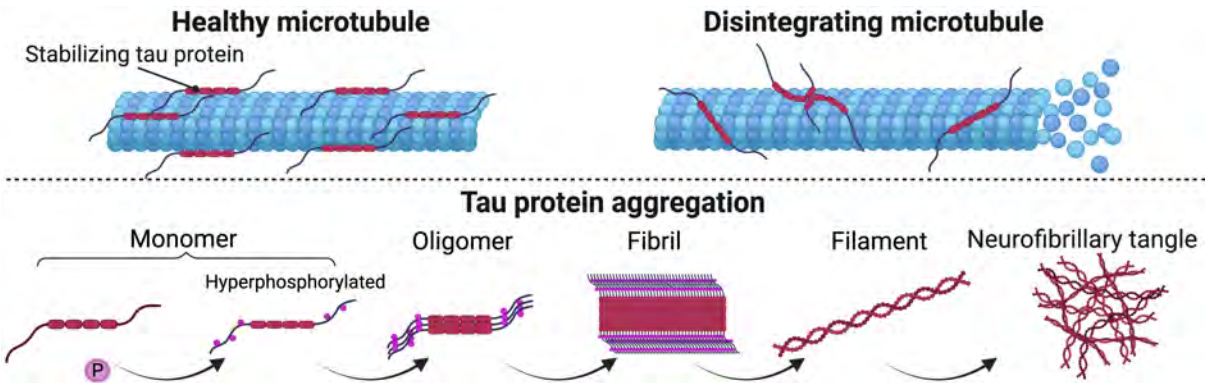



**Figure 6, Tau aggregation and microtubule dysfunction**
*Figure courtesy of Dr. J. Nilsson, created with Biorender.com. Tau abnormal hyperphosphorylation causes the detachment of the protein leading to microtubule disassembly. Hyperphosphorylation of tau and microtubule detachments are followed by conformational changes with abnormal levels of unbound hyperphosphorylated misfolded tau. These free pathological monomers will then bind into oligomers. Oligomers will constitute seeding core to constitute large filaments and subsequently, tangles. Both soluble oligomeric tau and NFTs have been found to be neurotoxic through a number of pathways.*

*Neuroinflammation hypothesis*

It is also well established that there is a neuroinflammatory component to AD, but the inflammation has most often been assumed to be a consequence of, or response to, the pathophysiological changes. More recently, innate immune system related events have been demonstrated to be a key regulator of the disease pathogenesis, possibly both in detrimental and protective manners (47). Reactive astrogliosis and microgliosis are prominent pathological features of AD. Whole genome sequencing and genome-wide association studies (GWAS) analyses revealed that a number of immune-related, apoptotic and proinflammatory genes including triggering receptor expressed on myeloid cells 2 (*TREM2*), apolipoprotein E (*APOE*), *CD33* or interleukin-1β are risk factors for AD (48). AD research has also particularly highlighted the importance of the innate immune-associated events that are primarily driven by the resident microglia in the CNS (49).
Evidence has also been put forth that supports a role of infectious agent in AD pathogenesis. Reactivated Herpes simplex 1 (HSV-1) in the brain could be a triggering factor in AD pathogenesis (50,51). HSV-1 encephalitis primarily affects the entorhinal cortex and the hippocampus, similarly to NFTs. Furthermore, HSV-1 kinase has been implicated in tau hyperphosphorylation, and neuropathological studies have shown a strong correlation between the presence of HSV-1 DNA in human brains and risk of AD. Other pathogens, including other human herpes viruses, spirochetes or hepatitis, have also been suspected of being implicated in AD pathogenesis (52).

Other alternative hypotheses to explain the neuropathology of AD include oxidative stress (53). Increased DNA and protein oxidation have been observed in AD brain. Moreover, Aβ peptides have been shown to be capable of generating free radicals. Oxidative damage occurs early in the disease process, preceding a high plaque load and has been liked to abnormal phosphorylation of tau and, mitochondrial dysfunction.

### 1.2.4 Genetic forms and genetic risk factors

*Autosomal dominant forms of AD*

In rare cases, AD is fully inherited, related to an autosomal dominant mode of inheritance. Three genes: *APP*, *PSEN1* and *PSEN2*, coding respectively for amyloid precursor protein (APP), presenilin-1 (PSEN1) and presenilin-2 (PSEN2), have been found to be responsible for those familial forms of AD (54). Familial AD is overall characterized by high penetrance, early onset and fastest progression of the disease (55).
Pathogenic mutations in APP account for <1% of EOAD patients. Missense mutations and whole gene duplications have been identified (https://www.alzforum.org/mutations) mostly, located near the β- or γ-secretase cleavage sites or in the Aβ sequence in the APP protein. These mutations appear to result in overproduction of either total Aβ or in a shift in the Aβ40/Aβ42 ratio toward the Aβ42 peptide. PSEN1 and PSEN2 are both essential proteins of the γ-secretase complex, which catalyzes the cleavage of membrane proteins including the APP protein. Mutant γ-secretase increases Aβ1–42 levels, while decreasing Aβ1–40 levels, leading to an increased Aβ42/Aβ40 ratio. Mutations in *PSEN1* are the most common cause of familial EOAD explaining around 6% of EOAD patients, and are characterized by the earliest onset ages. In comparison, PSEN2 mutations are rare, explaining <1% of EOAD patients, and may show incomplete penetrance.

*Sporadic AD and genetic susceptibility*

The vast majority of cases of AD are late-onset 'sporadic' forms, with no obvious familial aggregation. However, AD appears to display high heritability, between 60% and 80% (56). Numerous susceptibility genes (or loci) and genetic variants associated with a higher risk of developing AD have been identified.

Among those, polymorphism in the *APOE* gene is a major genetic risk factor in LOAD (57). In the CNS, APOE is a glycoprotein abundantly expressed by astrocytes, microglia and vascular cells (58). It is implicated in the distribution of cholesterol and other lipids to neurons and in between cells. Three single nucleotide polymorphisms lead to a different combination of the amino acid cysteine (Cys) and arginine (Arg) at positions 112 and 118, thus resulting in three isoforms of the protein: APOE2 (Cys112, Cys158), APOE3 (Cys112, Arg158) and APOE4 (Arg112, Arg158). Thus, the association of the different isoforms of the protein are combined in six possible genotypes: ε 2/ε2, ε3/ε3, ε4/ε4, ε2/ε3, ε3/ε4 and ε4/ε2. The ε3 allele is the most frequently reported allele, while the ε 2 is the least common. The ε4 allele has been demonstrated to be a strong risk factor for AD in an allele number-dependent manner. It is estimated that carrying one *APOE* ε 4 allele increases the risk up to 3–4 fold and that carrying two ε4 alleles that risk up to 9–15 fold (59,60). Moreover, ε 4 carriership is associated with a younger age of onset of disease, carrying one ε4 allele bringing the onset of AD forward by 2–5 years and carrying two ε 4 alleles by 5–10 years (61). It also appears to interact with sex to modulate AD risk (60).

At a neuropathological level, the ε4 allele is associated with increased tau pathology, greater synaptic pathology and increased risk of CAA (62–64).

There is strong evidence from clinical and basic research that a major pathway by which APOE4 increases the risk of AD is by driving earlier and more abundant amyloid pathology in the brains of ε 4 allele carriers. The amino acid variations in the *APOE* isoforms substantially modify the protein structure and modulate its binding properties to both lipids and receptors. This has implications for Aβ clearance, with APOE4 being less efficient than APOE2 or APOE3. This decrease in efficiency of Aβ clearance in the brain has been reported throughout the different clearance pathways: through the brain blood barrier (BBB), cellular uptake, enzymatic degradation, CSF absorption, *etc.,* to result in the higher Aβ burden in *APOE* ε 4 carriers.

Moreover, emerging evidence suggests that *APOE* genotype also affects multiple pathways modulating AD pathogenesis: tau pathology, microglial response to amyloid pathology, synaptic integrity, lipid transport, glucose metabolism and cerebrovascular function.

Conversely, the ε 2 allele confers a decreased risk relative to the common ε 3 allele (65). Evidence from both human and animal studies suggests that it exerts its effect through both Aβ-dependent and independent mechanisms (66).

Genetic variants of *TREM2*, a cell surface receptor expressed exclusively by microglia in the brain, have also been linked to AD. Association between *TREM2* and AD was initially established through the identification of a rare heterozygous missense mutation in *TREM2*, p.R47H, that increased AD risk by about a 2 to 4.5-fold (67).

More recently, high-throughput genomic approaches have enabled the identification of other genetic factors. Up to today, more than 40 loci of interest have been associated with the risk of developing AD (48,68). Many of these genes have a role in brain development, cytoskeletal organization, and immune functions. Combinations of variants in these genes have been proposed as markers for genetic risk of developing overt AD establishing 'polygenic risk scores' (69,70). However, these signals account for less than 50% of the genetic variance of AD, leaving most of the genetic risk mischaracterized (71).

### 1.2.5 Current clinical diagnosis criteria

The 2014 IWG-2 criteria guidelines for diagnosis of typical AD recommend that the diagnosis should be based on the clinical-biological phenotype (72). A diagnosis of AD requires both a clinical phenotype of AD and biomarker evidence of amyloid positivity. A typical AD phenotype is defined as an amnestic syndrome, with gradual decline in memory function and evidence of impaired episodic memory using an established test. Amyloid positivity can be defined using CSF (measurements of Aβ42 or Aβ42/Aβ40 ratio) or amyloid PET. Atypical clinical features or history or other major causes of memory impairment should be excluded (non-AD dementia, vascular disease, major depressive episode). The uncommon phenotypes (posterior, logopenic or behavioral variants of AD) require *in vivo* evidence of both amyloid and tau pathologies (in CSF or PET) to retain an AD diagnosis. According to the clinical presentation, the physician could evaluate that AD is associated to another pathology: to diagnose a mixed AD, evidence of both tau and amyloid pathology is required, with evidence of the associated disease, cerebrovascular disease or dementia with Lewy bodies.
In the 2018, NIA – AA diagnostic framework for research, AD diagnosis was centered exclusively around a biological definition of disease according to Amyloid Tau Neurodegeneration (ATN) scheme (73). In the absence of cognitive symptoms, the presence of abnormal amyloid β and tau biomarkers can be defined as preclinical AD. However, there is actually no clinical recommendation to investigate AD biomarkers at preclinical stage, given the current inability to predict reliably clinical evolution of cognitively unimpaired individuals with positive biomarker status and the absence of available preventive treatment.

## 1.3 Non-AD dementia

### 1.3.1 Frontotemporal dementia

Frontotemporal dementia (FTD) is a term that describes a group of clinical syndromes that can present as executive functions impairment, changes in behavior and/or language modification (74). FTD is an early-onset dementia (<65 years in 75% of the cases). FTD results from the degeneration of frontal and temporal lobes, described as frontotemporal lobar degeneration (FTLD) when pathologically confirmed (75).

Clinically, FTD patients can present with two main syndromes: behavioral variant FTD (bvFTD) and PPA. The PPA variants most frequently associated with FTLD are semantic variant (svPPA) or progressive nonfluent aphasia (PNFA). The third variant of PPA, the logopenic variant is in most cases associated with AD pathology and only in a minority of cases with FTLD. bvFTD is characterized by changes in personality and behavior (apathy, disinhibition, personality changes) and executive deficits. Semantic dementia is associated with alteration of semantic knowledge and naming. Patients with PNFA produce dysfluent and distorted speech (speech apraxia), without grammar structure (agrammatism). FTD can also largely overlap with motor neuron diseases such as amyotrophic lateral sclerosis (FTD-MND/ALS) and parkinsonian disorders like progressive supranuclear palsy (PSP) and corticobasal syndrome (CBS).
FTLD can also be classified based on the nature of the protein aggregates observed at histopathology (76). Most frequently (around 40% of all FTLD cases), tau inclusions are found in neurons or both neurons and glial cells (FTLD-tau). TAR DNA-binding protein 43 (TDP-

43) accumulations are found in around 50% of cases. Fused-in-sarcoma (FUS) associated FTD are less commonly found. Only few cases have no discernible pathological inclusions.
FTD is a highly heritable disorder, though with varying heritability in the different clinical syndromes and subtypes (77). Mutations on three main genes, namely microtubule-associated protein tau (MAPT), progranulin (GRN) and chromosome 9 open reading frame 72 (C9orf72) account for around 60% of familial cases. C9orf72 is the most common genetic cause of frontotemporal dementia and amyotrophic lateral sclerosis (78).

To date, no specific fluid biomarkers are available for the diagnosis of FTD. No current biomarker is validated to detect tau pathology in primary tauopathies or TDP-43 pathology. The CSF AD core biomarkers do not significantly show changes across tauopathies. Thus, they are used as support criteria, for differential diagnosis with AD.
CSF neurofilament light (NfL), a marker of neuronal damage now also quantified in blood, has been shown to be significantly increased in DFT and in PSP (79,80). Imaging modalities such as magnetic resonance imaging (MRI) and PET are used to evaluate patterns of atrophy and pathological changes. FDG-PET, assessing hypometabolism, and volumetric MRI, measuring the gray matter atrophy, constitutes a useful tool for diagnosis of FTD.

### 1.3.2 Dementia with Lewy bodies

Dementia with Lewy bodies (DLB) is the second most common form of age-associated dementia, accounting for more than 20% of all dementia cases. With Parkinson's disease (PD), multiple system atrophy (MSA) and progressive autonomic failure (PAF), DLB can be grouped in the neurodegenerative class of α-synucleinopathies, defined by the presence of aggregates of α-synuclein as the main pathological feature.
Diagnostic of probable DLB at dementia stage or MCI stage is based on the association of cognitive impairment, cognitive fluctuations, visual hallucinations to parkinsonian symptoms, dysautonomia and sleep disorders (81). Rapid eye movement (REM) sleep behavior disorder and severe neuroleptic sensitivity are also key features of the disease.
DLB is mainly a sporadic disease with unknown etiology, although a higher occurrence in families with a history of dementia, including DLB, has been reported (82). To date, no high penetrance pathogenic mutations have been identified. However, genetic risk variants have been associated with DLB, including variants in the alpha-synuclein (*SNCA*), *APOE* and in the *APP* genes. The association of DLB with glucocerebrosidase (*GBA*) has also been identified, with DLB patients being 8 times more likely to be carriers of *GBA* mutations than controls (83). Recent GWAS studies indicated that LBD shares risk profiles and pathways with AD and PD (84).
DLB is characterized by the intraneuronal accumulation of aggregated protein α-synuclein, which forms the so-called Lewy-bodies (LB), and Lewy neurites (LN) inside neuronal processes. α-synuclein is a presynaptic protein primarily involved in regulating the fusion and clustering of vesicles to the presynaptic plasma membrane, an essential step for neurotransmitter release, as well as for synaptic vesicle recycles. Moreover, approximately 50% of the patients also show high levels of AD neuropathological changes. A staging system has been proposed by Braak *et al.*, describing a sequential spread, starting in the medulla oblongata and progressing in the brainstem, then to the limbic system and subsequently to the neocortex (85).
Diagnosis is supported by a series of major and indicative biomarkers, although there are no specific diagnostic biomarkers yet (81). These biomarkers are mainly represented by imaging modalities (MRI, PET, electroencephalography – EEG – ). The combination of FDG-PET and MRI shows to be useful in discriminating DLB from other dementia (86). CSF AD core

biomarkers can be used to detect an amyloid copathology. α-synuclein quantified in CSF using seeding assays show promising performance for positive diagnosis of synucleinopathy, but no assay has been validated for clinical use yet (87).

### 1.3.3 Vascular Dementia

Dementia caused by impaired cerebral blood flow or other cerebrovascular disease is referred to as vascular dementia (VaD) (88). As VaD may affect different brain regions, cognitive symptoms presented are of a wide range. However, VaD often presents with impairment in executive functions (89). VaD can occur as a consequence of severe brain injury from a stroke, ischemic or hemorrhagic (90). In addition, small cerebral vessel disease may also result in VaD (91). Stroke and small cerebral vessel disease impair blood delivery to brain tissue, as well as the other cerebral support functions *i.e.,* integration with neurons, microglia, and astrocytes to maintain the integrity of the blood-brain barrier and clear waste products (92). White matter lesions (WML) can be the result of many processes and diseases, e.g., cardiovascular factors (*i.e.,* hypertension, diabetes mellitus), vasculitis, genetic causes… The common elements throughout are the demyelination and axon loss in subcortical structures, accompanied by narrowing of the lumen of the small penetrating arterioles in the white matter. The different pathological hallmarks in WML associate periventricular-WML (*i.e.,* reduced axons and myelin of the white matter near the ventricle wall) and the deep-WML (*i.e.,* the central part of white matter, more widespread in the brain) (93). The association with impairment in cognitive and functional capacity is probably due to disruption in cortical-subcortical connections, especially in the thalamus-cortex connection.

VaD frequently associates with other causes of dementia. Mixed dementia is the term used to refer to dementia caused by the combination of AD's pathology and of cerebrovascular lesions (94). Some studies estimate mixed dementia to account for approximately 20 to 40% of all dementia cases (95). In neuropathological studies, around 20% of confirmed AD cases are associated with significant cerebrovascular lesions (96,97). In regards to clinical symptoms, the association of vascular lesions is established to constitute a factor of progression of cognitive decline in AD (98).

As cognitive disorders of vascular etiology constitute a heterogeneous group with diverse clinical manifestations, the *continuum* of vascular cognitive impairment is recognized by the categories of mild cognitive vascular disorder, and VaD in the criteria proposed by the VASCOG statement (99). Clinical and neuroimaging criteria have also been proposed for establishing vascular etiology.

Although many blood biomarkers for acute ischemic stroke have been reported, there are actually no reliable blood biomarkers for VaD. Several families of candidate biomarkers have been studied relying on underlying physiopathological processes, including biomarkers of inflammatory response, of CNS tissue injuries and of coagulation and thrombosis as well as circulatory miRNAs (100).

## 1.4 Validated fluid biomarkers for AD diagnosis

Fluid biomarkers have been validated for the diagnosis of AD, along with imaging biomarkers, and reflect the underlying progression of the disease (**Fig. 7**). In clinical practice, they are now used for positive diagnosis and differential diagnosis with other NDDs. They include CSF Aβ42 and total and phosphorylated tau protein levels in CSF (101). The combined use of these biomarkers, with high sensitivity and specificity, contribute to the early identification of AD,

particularly in individuals with MCI, with higher diagnostic certainty. Fully automated methods have also been validated for use in clinical practice with established cut points for AD diagnosis (102,103).

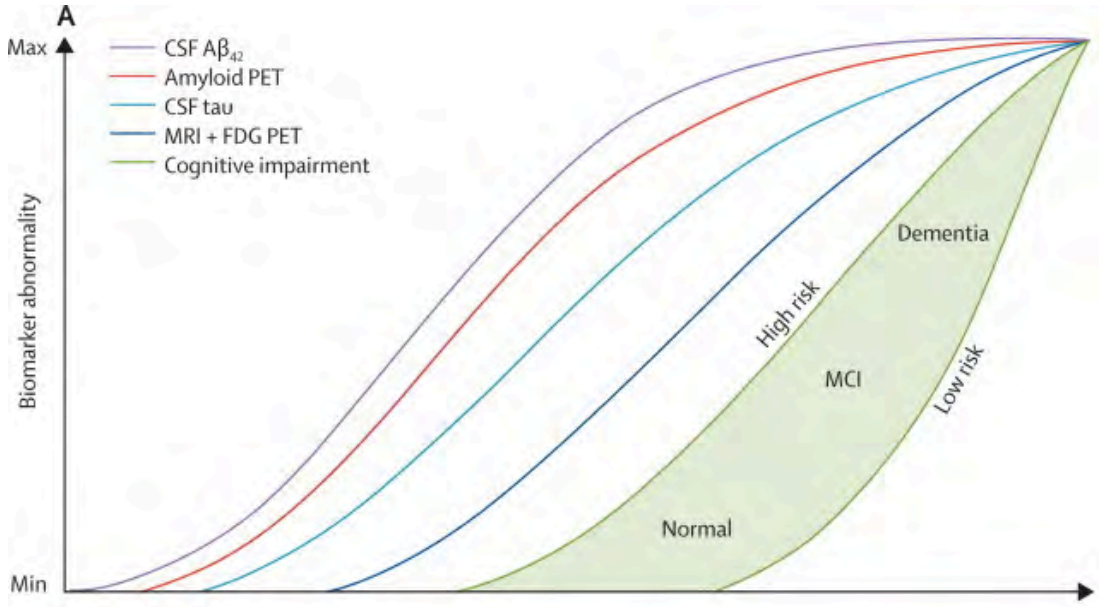



**Figure 7, Biomarkers model of the AD pathological cascade**
*Jack et al., Lancet Neurology 2013* (104). *Jack et al., proposed a hypothetical model of the major biomarkers of AD. CSF Aβ42 becomes positive before amyloid PET, which is followed by CSF tau markers. FDG PET and MRI are depicted coincidentally as the last biomarkers to become abnormal, but they are also those that track most closely with progressive cognitive impairment.*

### 1.4.1 CSF Aβ42

The 42 amino acid form of amyloid-β (Aβ42) is the major constituent of senile plaques, used as a marker for amyloid metabolism and measurable in CSF. It has been shown to be consistently decreased in AD, from approximately 50% in large meta-analyses (105). Low Aβ42 which associate to Aβ fibrillar deposits is considered the earliest biomarker of AD (104). Great evidence exists that lower CSF Aβ42 levels reflect amyloid deposition, such as neuritic plaques and CAA. *Post-mortem* CSF Aβ42 levels correlate with the plaques density in the hippocampus and the neocortex measured at autopsy (106). CSF Aβ42 levels display a sensitivity greater than 90% across studies for detecting cortical Aβ deposition in the whole AD *continuum*, including preclinical stages (107,108).
The lower concentrations are commonly hypothesized to be related to the sequestration of Aβ42 in plaques, with only the soluble fraction of the protein detectable in CSF (109). Other possible hypotheses include a decrease in the rate of Aβ42 production, increased clearance of Aβ42, or oligomerization of Aβ monomers. CSF Aβ42 is also significantly associated with CAA lesions (110).
CSF Aβ42 levels show a consistent association with amyloid load measured using amyloid PET, with a concordance around 90% in large studies (111). CSF Aβ42 appears to be an earlier biomarker for amyloid deposition than PET, as suggested by studies following up trajectories of both biomarkers in cognitively unimpaired subjects (112). Individuals with low CSF Aβ42

but normal PET amyloid tend to show increased rate of amyloid accumulation at follow-up PET, signaling earlier change in CSF than in PET.
In unimpaired individuals and in individuals with subjective cognitive impairment (SCI), low CSF amyloid markers predict future cognitive decline (113,114). In asymptomatic carriers of familial AD mutations, low CSF Aβ42 levels predict the apparition of overt symptoms (115). Other proteins issued from APP cleavage, such as Aβ38, Aβ40, sAPPα and sAPPβ have been measured in the CSF of AD patients but show inconsistent modifications in AD (116,117). However, combined used of CSF Aβ42 and CSF Aβ40 in a ratio improved the diagnosis accuracy (118). Indeed, CSF Aβ42/Aβ40 shows better concordance to amyloid PET imaging. The most commonly accepted hypothesis is that CSF Aβ40 can serve as a proxy to normalize for Aβ production. Reduction in Aβ42 can be identified more accurately in high Aβ production whereas low Aβ42 will not be misinterpreted as amyloidosis in low producers.

### 1.4.2 CSF phosphorylated-tau

The identification of tau as the main component of NFTs prompted the study of tau proteins as a biomarker candidate for AD. Tau functions are regulated by numerous post-translational modifications, including phosphorylation (119). The measurement of phosphorylated species of tau has been achieved in the CSF. Profiling of tau in the CSF using mass spectrometry has revealed that the soluble p-tau pool released into CSF is mostly composed of mid-region and N-terminal fragments (120,121). Multiple species phosphorylated at different epitopes can be quantified, both under physiological and pathological conditions (**Fig. 8**). Interestingly, evidence exists that the pathological hyperphosphorylation in AD differs among the maturing stages of neurofibrillary changes (122). Some phosphorylation sites have been reported to occur in cluster and some seem to be mutually exclusive in AD brain tissue, supporting a temporally ordered addition of phosphate to tau (123). Additionally, the relative abundance of phosphorylated fragments has been shown to vary according to phosphorylation sites (124,125).

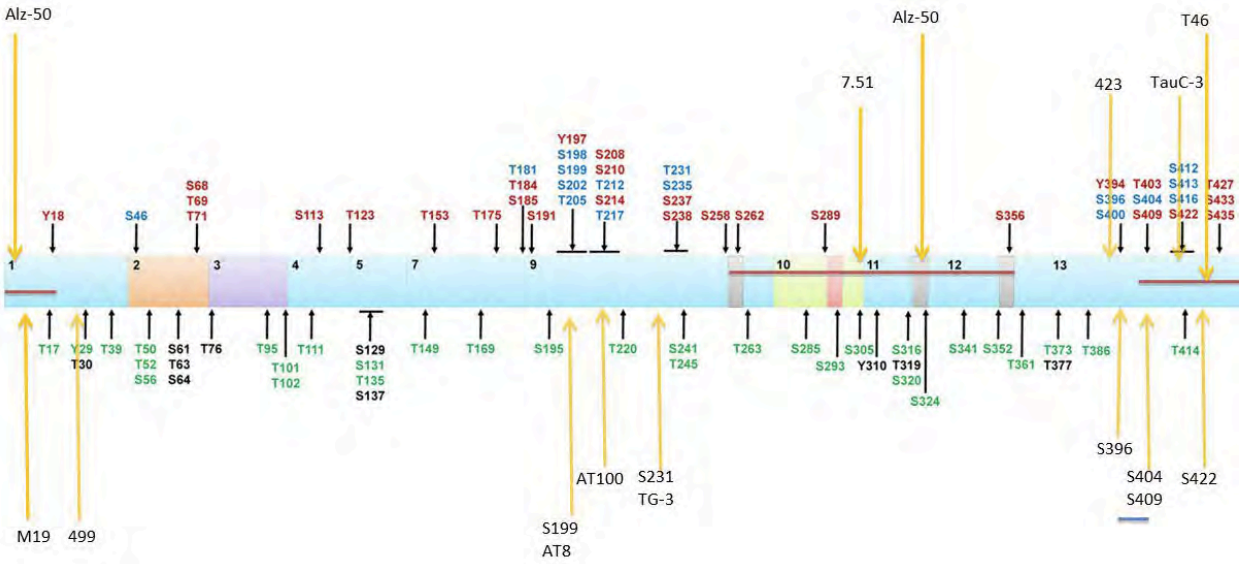



**Figure 8, Sites of physiological and pathological tau phosphorylation and epitope of antibodies allowing for measurement of p-tau biomarkers**
*From Luna-Munoz et al., 2013* (126). *Tau is phosphorylated at multiple sites, including specific AD-related phosphorylation (red), specific physiological phosphorylation (green) and those*

*found both in normal and AD brain (blue). Some putative phosphorylation sites have also been reported but with no in vivo or in vitro confirmation (black). Antibodies have been developed targeting specific phosphorylation sites (yellow arrows) and allow for the measurement of p-tau species in blood and CSF.*

Among the multiple phosphorylation sites, the most commonly used assays for phosphorylated tau (p-tau) detect fragments phosphorylated at threonine 181 (p-tau181).
Measurements of CSF p-tau181 have shown a marked increase in AD. Mean change in AD is around 250% of age-matched controls (105). CSF p-tau levels display significant correlation with tau pathology, including neocortical tangle counts or neuritic plaque counts and PET measures (127,128). However, recent studies found more consistent association of CSF p-tau with Aβ accumulation (129). Indeed, changes in CSF p-tau observed in AD appears to occur earlier than tau aggregation measured at neuropathological examination or using PET imaging (130). CSF p-tau measurements predict cognitive decline at the very prodromal phase of the disease (131). CSF p-tau shows high specificity for AD, not being increased or only very marginally in other tauopathies or other neurodegenerative disorders. This evidence supports that CSF p-tau levels reflect the phosphorylation levels of tau in AD brain and not general neurodegeneration or neuronal injury. CSF tau phosphorylated at threonine 231 (p-tau231) and serine 217 (p-tau 217) have also been intensively studied and show similar performance in AD diagnosis at the symptomatic phase (132). At very early stages, CSF p-tau231 was shown to correlate better with early Aβ accumulations when compared with CSF ptau181 and p-tau217, most likely reflecting the sequential phosphorylation process in the brain (133).

### 1.4.3 CSF total tau

Tau has been extensively investigated in the CSF and other fluids as the main component of NFTs. The first immunoassays measuring tau in CSF used an enzyme-linked immunosorbent assay (Elisa) and could detect all CNS tau isoforms regardless of the phosphorylation state, thus was designed as total tau, t-tau (134).
CSF t-tau is established as a biomarker of neurodegeneration in AD. It has been consistently found increased in AD (105). The mean increase observed is around 250% compared to age-matched controls. CSF t-tau levels have been shown to predict a more rapid disease progression (135). CSF t-tau levels are related to temporal, parietal and frontal hypometabolism in AD measured using FDG-PET (136).
However, CSF t-tau changes are not AD specific. Rather, t-tau reflects unspecific processes of axonal damages and neuronal degeneration.
CSF t-tau levels are also modified in acute brain injury, including stroke or traumatic brain injury (TBI). The highest levels are found in CJD where the neurodegeneration is the most intense (increase around 10-20 fold higher than in AD) (137). In acute axonal injuries, CSF t-tau display very dynamic changes, characterized by a rapid increase after injury followed by a stabilization period, before returning to normal levels (138,139).
However, most non-AD dementia display normal or only slightly increased CSF tau levels, including DLB, VAD or Parkinson's disease (140). It has been hypothesized that CSF t-tau increase in AD is caused, at least partly, by a change in tau metabolism in response to Aβ (120).

The combination of these biomarkers, commonly designated as ‘CSF core AD biomarkers’ has been demonstrated to provide the highest values of sensitivity and specificity for AD diagnosis. The specificity and sensitivity to differentiate AD dementia from control subjects is around 90% (141). Several studies have demonstrated that the use of CSF biomarkers increases diagnostic performance, compared with purely clinical evaluation (142,143). However,

regarding differential diagnosis, the combination of the AD biomarkers yielded high sensitivity (around 87%) but lower specificity (around 80%) in distinguishing AD from other dementias, including FTD, DLB, VaD, CJD and PD (141). At MCI stage, AD core CSF biomarkers combination demonstrates good diagnostic value to identify AD compared with controls (143–145). Very high (up to 95%) diagnostic sensitivity for the combination of low Aβ42 and high CSF t-tau/p-tau in predicting AD-MCI stage was demonstrated (146). However, to differentiate AD MCI from MCI of other causes, specificity appeared significantly lower (median specificity of 63% in a meta-analysis including 11 studies) (141).

### 1.4.4 Core AD CSF biomarkers limitations

In June 2021, the IWG proposed than an AD diagnosis should be restricted to people presenting with specific AD phenotypes and displaying amyloid and tau positive biomarkers (147). Cognitively unimpaired individuals displaying positive AD biomarkers should be considered only at-risk for progression to AD. Indeed, several limitations in the use of CSF biomarkers for diagnosis of AD exist.

Amyloid and tau CSF biomarkers do not associate perfectly with cognitive decline. All stages of AD brain lesions (including Aβ and tau lesions) are found in around one third of individuals aged at least 70 years in systematic post-mortem examination, regardless of cognitive status (148). Thus, the prevalence of these lesions exceeds the prevalence of cognitive impairment. In medio-temporal regions, neurofibrillary tangles are highly prevalent in almost all cognitively unimpaired people aged 70 years or older (38,149).

Large post-mortem cohorts have reported similar burden of AD brain lesions in unimpaired and impaired individuals (150,151). Similar findings were made using PET imaging. Large studies observed a large overlap in amyloid and tau PET burden between cognitively healthy and cognitively impaired individuals (152,153). Both amyloid and diffuse (*i.e.,* outside the medial temporal lobe) tau pathologies were found in around 25% of cognitively unimpaired older individuals in a large cohort of more than 500 individuals (154). Their prognostic value remains limited in asymptomatic subjects, as there is a high variability of decline rates among individuals with positive AD biomarkers.

In symptomatic AD patients, only CSF t-tau has shown consistent association to cognitive decline. Thus, they have low potential as prognostic biomarkers.

A positive pattern of AD biomarkers can be observed in other brain diseases in which AD pathology is present as a copathology (155). Significant amyloid pathology in association to α-synuclein is observed in up to around 80% of DLB cases, 50% of PSP and 20% of tau-related DLFT (156). At MCI stage, around 50% of DLB patients display an A+ CSF profile and up to 70% at dementia stage (157). In C9Orf72 related FTD cases series, up to 25% of patients displayed an A+ profile (158). Conversely, in cases when AD is believed to be the primary diagnosis, it can be associated with other pathologies. Pure AD pathology was seldom observed in a large neuropathological series of demented subjects (7). This is especially important in older individuals with the increasing prevalence of copathology (6). Both the early and late clinical features of AD can be affected by copathologies (159).

There are still interrogations on the use of the core CSF biomarker for early diagnosis of AD. One goal of early diagnosis is the identification of SCI or MCI individuals who will convert to AD dementia or other forms of dementia over time, to introduce prevention measures or early treatment. An increasing number of patients seen at memory clinics seek diagnosis at MCI stage. CSF core biomarkers present consistently a better sensitivity than specificity *i.e.,* the absence of evidence of amyloid pathology was likely to exclude a later diagnosis of AD. However, their specificity remains moderate (141). Thus, in clinical practice, there is still uncertainty of the value of the biomarkers in the management of patients with SCI or MCI in

routine. A negative CSF profile, in people with MCI, is likely to reflect the absence of AD pathology as the etiology underlying their clinical symptoms. However, a positive result cannot formally allow concluding that the presence of AD is the cause of the symptoms. Indeed, MCI covers many pathological disorders and can be related to multiple causes including psychiatric diseases, infectious or inflammatory disorders, drug abuse, sleep apnea…
Finally, CSF analysis requires a lumbar puncture (LP), which is rather safe and well tolerated. However, it is time consuming, costly and can sometimes result in side effects, including postpuncture headache. Their use for the follow-up is thus limited.

## 1.5 Novel biomarkers: plasma markers and non-amyloid and tau markers

AD biomarker field has known great progress these last decades, mostly in two directions (**Fig. 9**). Firstly, a lot of focus has been put on the development of non-invasive blood-based biomarkers. Secondly, there has been significant advancement in the identification of biomarkers reflecting the different molecular pathophysiological mechanisms at play in the brain during AD.

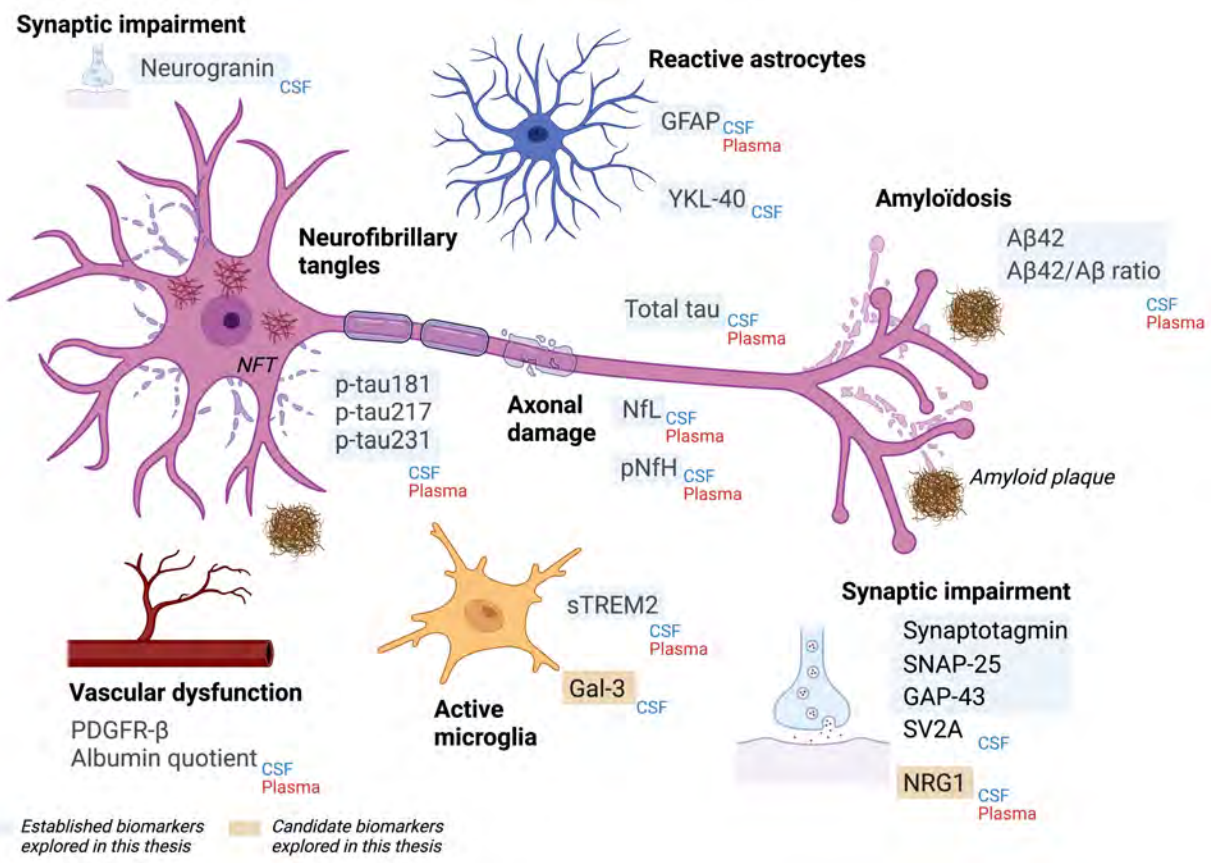



**Figure 9, CSF and plasma biomarkers of the different physiopathological processes of AD**

*Figure created with Biorender.com. This figure summarizes the biomarkers explored in this thesis that reflect different pathologies in AD, including amyloid deposition, tau pathology, axonal damage, neuroinflammation and synaptic impairment.*

### 1.5.1 Plasma markers of amyloid and tau pathology

Advances in CSF biomarkers and PET imaging analyses have the potential to improve the accuracy of positive and differential diagnostic of AD. However, these methods have limitations regarding their use as clinical diagnostic tools, including high cost, limited availability and invasiveness. Blood-based biomarkers would ideally circumvent these limitations. Blood testing is already established and well accepted in clinical routine, requiring no further introduction and training for health-care professionals, and can be easily performed in a variety of settings, including primary care and community medicine centers. Blood testing also allows for repeated monitoring of markers of interest.
The measurement of cerebral proteins of interest in the brain has revealed challenging due to multiple factors. The variability in the brain blood barrier passage, the rich plasma matrix, the low concentration of circulating CNS antigens in the periphery and the alteration of markers levels due to circulating proteases have hindered the development of plasma markers. However, recent advances in ultrasensitive technologies have made possible the measurement of CNS markers in blood.

#### *1.5.1.1 Plasma amyloid biomarkers*

Several platforms are now available to detect and quantify Aβ present in peripheral blood. Immunoprecipitation mass spectrometry (IP-MS) methods have been developed to extract Aβ from plasma, which is then quantified using MS-based quantification (160,161). Novel immunoassays have also become available, which would be easier to implement in clinical settings (162). Overall, a reduction in the Aβ42/Aβ40 ratio is consistently measured in plasma in AD (163–165). Reductions in plasma Aβ42/Aβ40 levels using IP-MS are observed in subjects with positive amyloid PET – compared with amyloid PET-negative subjects. Plasma Aβ42/40 was also shown to predict Aβ-PET status in cognitively normal individuals, especially when combined with age and APOE ε4 status (166). Immunochemical tests, however, demonstrated weaker performance for identifying individuals with abnormal intracerebral Aβ status measured through CSF Aβ42/40 levels and Aβ-PET at prodromal stages (163).
Moreover, the size of the difference observed between groups remains moderate (8–15% *vs* 40–60% in CSF) with a significant overlap between groups. A significant percentage of amyloid-positive subjects' plasma values lie close to the cut-off (167). As Aβ is ubiquitously expressed in extra-cerebral tissues, peripheral Aβ also contributes to variability in measured levels in plasma.

#### *1.5.1.2 Plasma tau biomarkers*

Levels of phosphorylated tau can now be measured in plasma at multiple sites, including at amino acids 181 (p-tau181), 217 (p-tau217) and 231 (p-tau231), using multiple platforms. Plasma p-tau levels constitute a small fraction, but proportional, to the CSF levels in the same subjects, suggesting that brain-derived p-tau is released into blood through the CSF. Phosphorylated tau fragments measured in the blood appear to be constituted mostly by N-terminal fragments up to the start of the microtubule-binding region (120).
Plasma p-tau181 has shown high performance in differentiating AD dementia from other dementia in neuropathologically confirmed cases (168,169). It is an early biomarker as it could accurately identify AD pathology in subjects whose blood was sampled eight years prior to death and autopsy (170). Other p-tau species, p-tau217 and p-tau231, demonstrated similar accuracy for differential diagnosis of AD (171,172). P-tau measured in plasma is associated to both Aβ plaques and tau tangles, measured at post-mortem examination or using PET imaging

(171,173,174). Similarly to CSF tau, plasma p-tau appears to be specifically increased in AD and not in other tauopathies including FTD, PSP or CBD. Plasma p-tau could differentiate individuals converting from MCI to dementia du to AD compared to those remaining stable or converting to non-AD dementia (168). Importantly, plasma performance of p-tau181 was nearly similar to that of CSF p-181 to identify risk of progression to AD dementia.
Plasma p-tau shows very early increase on the AD *continuum*, already occurring at preclinical stage in cognitively unimpaired individuals (175). Elevated baseline plasma P-tau181 levels (adjusted for age, sex, and education levels) were associated with an increased risk of progression to AD dementia in cognitively normal individuals, during a follow-up of 5 years (168). Furthermore, plasma p-tau could be combined with clinical measures (age, cognitive testing) and APOE status to predict conversion to AD dementia in individuals with SCI or MCI with a high accuracy (area under the curve [AUC] of 0.91 in receiver operating characteristics [ROC] curve analysis) (176). It was also reported to increase around the estimated age of symptom onset (estimated through kindred age at onset) in AD familial forms (177).

Plasma levels of t-tau protein have also been successfully quantified using novel ultra-sensitive assays, and increased levels have been reported in AD patients (178–180). In a meta-analysis, plasma t-tau levels showed a strong association with AD (105). However, the correlations with CSF appeared weak (178,181). Moreover, there is a large overlap between levels found in control subjects, AD and non-AD dementia, with limited effect sizes. Hypotheses about this variability include interference caused by peripheral expression of tau or rapid metabolism and fragmentation of tau in plasma, resulting in fragments that could not be measured.
In the ADNI cohort, higher plasma t-tau levels predicted a higher rate of cognitive decline (178). It was also associated with brain atrophy measured with MRI, and with hypometabolism on FDG-PET. New analytical techniques may be needed as using a sensitive immunoassay with electrochemiluminescence detection, one study showed a strong association of plasma t-tau with tau PET, as well as high concordance with CSF (182). In addition, assays targeting other epitopes on the protein have also reported interesting diagnostic performance (179). Moreover, higher plasma t-tau levels have been found associated with cognitive decline, independently of Aβ levels, suggesting a potential use as a non disease-specific marker for screening or follow-up (180). Plasma t-tau measurements also show consistent modifications in acute neuronal injury, including stroke, cardiac arrest or traumatic brain injury (183).

Rapid and significant progress has been made in the development of blood-based biomarkers for AD due to technical advances. From a clinical practice perspective, plasma amyloid and tau biomarkers could be incorporated into primary care to determine which patients with cognitive decline should be referred to secondary settings for advanced care and additional investigations with CSF or imaging markers. In clinical trials, plasma biomarkers would serve screening for inclusion as well as drug target-engagement and outcome markers.

### 1.5.2 New biomarkers of non-amyloid and tau processes

Biomarkers based on the core pathological proteins associated with AD are established as useful diagnostic biomarkers. However, biomarkers for staging clinical severity, monitoring the progression of the disease and treatment response in clinical trials are still lacking. Moreover, markers of other proteinopathies and of other pathophysiological processes taking place in NDDs are still lacking. The measurement of many proteins involved in the pathophysiological processes of AD has now been achieved and several display potential as new biomarkers, both in CSF and plasma.

#### 1.5.2.1 Axonal and neuronal damage biomarkers

Biomarkers of neurodegeneration reflect nonspecific and event-ending pathologies that are commonly observed in many neurodegenerative disorders. Thus, they cannot be used for differentiating in between neurodegenerative disorders. However, they have the potential to contribute to diagnosis and follow-up as well as to treatment monitoring.

The cellular cytoskeleton is a major component of cellular architecture and its disorganization in neurons has been identified to be a key element in the pathophysiology of NDDs (184,185). The cytosqueleton is constituted through the polymerization of elementary protein units, into macromolecular structures. These structures can be classified through their diameter measured in electron microscopy: actin microfilaments (8nm), microtubules (24 nm) and intermediate filament also called neurofilament in neurons (Nf) (10 nm) (186). Nfs consist of three neurofilament proteins of different molecular weights (light chain, NfL), medium chain (NfM) and heavy chain (NfH). The assembly of the filaments is notably regulated through phosphorylation (187). The cytoskeleton elements have been found to constitute a source of fluid biomarkers for NDDs (**Fig. 10**).

NfL chains are the most abundant component of large myelinated axons, which is released into CSF and systemic circulation when neurodegeneration or neuronal injury occur (188). High NfL concentrations have consistently been found in CSF in a variety of neuroinflammatory and neurodegenerative diseases including AD (189). High-level are also observed after acute brain injury (stroke, traumatic brain injury or cardiac arrest) (190). NfL levels can also be measured in plasma and correlate very robustly with those in CSF, suggesting that plasma levels are an accurate reflect of brain state (191). NfL levels are consistently increased in AD compared with healthy controls, both at MCI and dementia stages (192,193). Individuals with SCI already were reported to display higher levels than controls and elevated NfL levels predicted a faster rate of cognitive and functional decline (194).

Similarly to CSF, however, plasma NfL levels increase are not specific for AD and are observed in other neurodegenerative disorders, in particular in FTD, atypical parkinsonian disorders and ALS (195–197). Higher levels of plasma NfL predict faster disease progression and faster progression of brain atrophy in most neurodegenerative disorders. Very interestingly, plasma NfL has shown potential in differentiating NNDs from their non-neurodegenerative mimics (198).

Plasma NfL is also starting to be used as a biomarker of disease activity and therapeutic response in several neurological diseases, including multiple sclerosis, spinal muscular atrophy (199,200). As the first disease modifying therapies are being validated for AD, plasma NfL should be a candidate for monitoring therapeutical response.

For plasma NfL to be used in a clinical setting, reference values to indicate neurodegeneration need to be established. Several studies have already proposed cut-offs for routine use, that should prompt the translation of plasma NfL to clinics (201,202).

Phosphorylated neurofilament heavy chain (pNfH), another component of neurofilaments, has been less studied as a biomarker in AD. pNfH dimerizes with NfL to form heteropolymer in the neuronal cytoskeleton. Similarly to NfL, when neurons are damaged, pNfH are released into the CSF and then in the blood. pNfH levels in CSF and plasma have been extensively examined in ALS, where they are dramatically increased, similarly to NfL levels (203). Moreover, in ALS, plasma pNfH levels discriminate ALS patients from mimics, and they display a prognostic value as higher levels predict faster decline and shorter survival time (204). In AD, CSF and plasma were found to be increased in AD compared to controls (205,206). Data are still lacking regarding the utility of pNfH in plasma or in CSF for positive diagnosis and differential of AD.

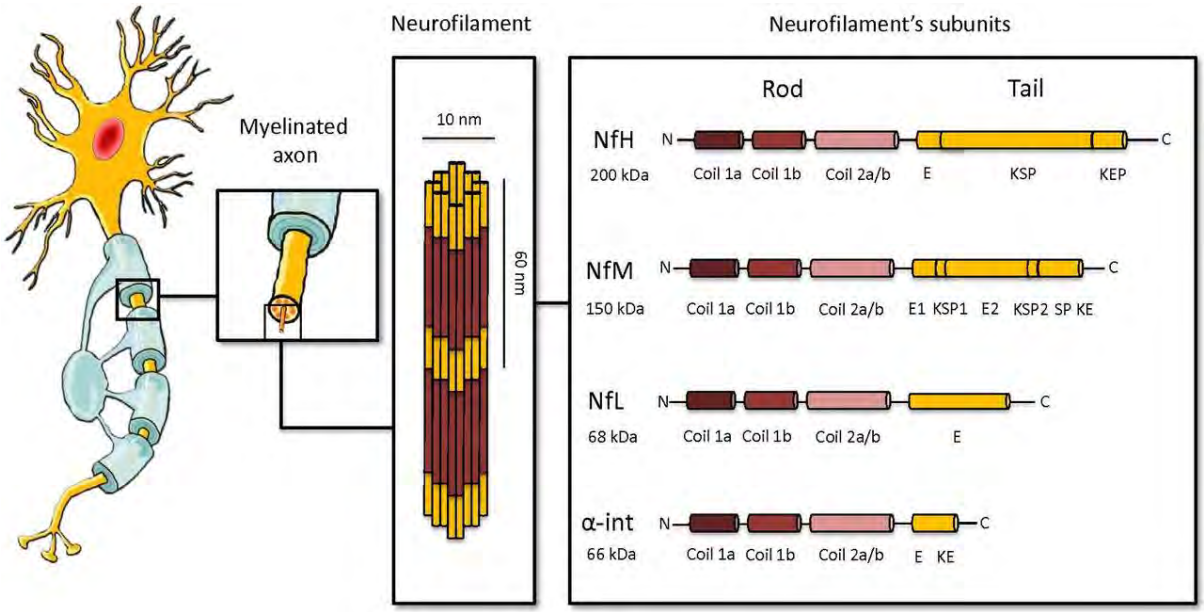



**Figure 10**, **Neurofilaments in central nervous system**
*From Heckler et al., J Neurophysiol. 2022* (207). *Large calibre myelinated axons abundantly express neurofilaments (Nfs). Nfs are made of subunits including neurofilament light chain (NfL), neurofilament middle chain (NfM), neurofilament heavy chain (NfH) and α-internexin (α-int). Upon axonal injury, different neurofilament elements are released in CSF and plasma and can be measured using immuno assays.*

1.5.2.2 Synaptic Biomarkers

It is established that synaptic degeneration is an early event in AD, and loss of synapses precedes cognitive impairment. Synapse loss also correlates better with cognitive decline than NFTs and Aβ plaques (208–210). Recent advances in mass spectrometry and immunoassays have allowed the accurate quantification of synaptic proteins in CSF (**Fig. 11**). There are currently 3 main presynaptic biomarkers, growth-associated protein 43 (GAP-43), synaptosomal-associated protein 25 (SNAP-25) and synaptotagmin, and one postsynaptic marker, neurogranin that have been studied in AD (211) Other synaptic markers of interest include synaptic vesicle glycoprotein 2A (SV2A), neuronal pentraxins or β-synucleins.
Neurogranin has been the most investigated. This intracellular protein is concentrated in the dendritic and postsynaptic compartment of synaptic spines in neurons (212). Neurogranin consistently shows increased levels in the CSF in AD patients and predicts a more rapid progression to major cognitive impairment (213,214).
GAP-43 is a presynaptic protein involved in memory and information storage (215). GAP-43 levels in the CSF were reported to be significantly increased in AD patients compared to controls and in other neurodegenerative disorders.
Synaptotagmin is a calcium sensor vesicle protein, implicated in neurotransmitter release. CSF synaptotagmin levels have been found to increase in AD from MCI stage and to be correlated with cognitive decline (216). SNAP-25 is a presynaptic protein from the SNARE family with a key role in vesicular exocytosis. It is known to be decreased in the AD brain tissue. A number of studies have found that CSF SNAP-25 levels are significantly higher in AD (217). The SV2a protein, expressed in synaptic vesicles, was the only synaptic marker reported decreased in the CSF in AD in a preliminary study (218). In PET, reductions of SV2A binding in medial temporal and neocortical brain regions demonstrated synaptic loss in early AD compared to controls (219).

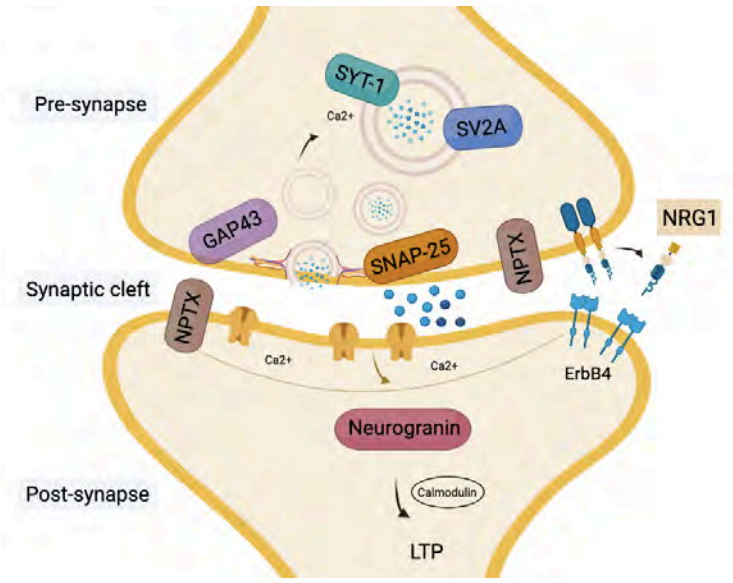



**Figure 11, Schematic overview of synaptic biomarkers with altered levels in AD**
*Adapted from Camporesi et al.,* (211). *Figure created with Biorender.com.This figure is a schematic representation of the most studied synaptic biomarkers in AD. Most of the candidate biomarkers are presynaptic proteins, with the exception of neurogranin and neuronal pentraxins which has also been described in the presynaptic compartment. Many proteins are involved in synaptic vesicle assembly and neurotransmitters release, like synaptotagmin-1 (SYT1 1), synaptophysin, SNAP-25, and SV2A. GAP-43 shows high density in the presynaptic terminal, modulating synaptic vesicle trafficking. Neurogranin is highly expressed in dendritic spine promoting synaptic plasticity through long-term potentiation. In this thesis, we explore in the plasma NRG1, an epidermic growth factor protein which is released in the synaptic cleft and interacts with ErbB4 receptors.*

A mass spectrometry panel measuring simultaneously 17 synaptic proteins (including neurogranin) found significant differences between AD subjects and controls (220). Current CSF synaptic biomarkers have been mostly studied in AD but there is evidence that some of them are also altered in other NNDs (221,222). However, bigger changes in AD suggest a higher level of synaptic and neuronal damage in AD, possibly Aβ-mediated.
CSF synaptic markers show modifications already very early on, from preclinical stage (223). CSF neurogranin, GAP-43, synaptotagmin and SNAP-25 levels were reported increased even in individuals in the earliest stages of Aβ deposition measured on PET imaging. In addition, higher CSF neurogranin and GAP-43 levels were significantly associated with lower cortical thickness in AD-related brain regions in cognitively unimpaired individuals. Regarding relationship to cognition, CSF levels of synaptic markers displayed a significant association with cognition across several consistent studies (216).
However, there is actually no available blood-based synaptic marker. Accessible markers for synaptic dysfunction could allow detecting the neurodegeneration onset and measure its intensity and progression. They would also constitute ideal therapeutical response biomarkers as they closely relate to cognition. Being able to easily measure changes in synaptic proteins might bring new insights into pathological mechanisms occurring during neurodegeneration, as different synaptic markers could reflect different processes at play at the synapse.

In this work, we explore plasma measurements of neuregulin-1 (NRG1), a protein of the neuregulin (NRG) family expressed at the synapse. NRGs belong to the epidermal growth

factors (EGF) family, participating in the regulation of normal cells and tumor cell growth and survival through the ErbB family receptor tyrosine kinases (224).
NRG1 exists in six isoforms that all contain an EGF-like domain essential for regulating biological activities (**Fig. 12**). NRG1 binds to the dimerized ErbB receptors to signal from the extracellular to the intracellular space. NRG1 is preferentially expressed in the cerebral cortex and in the limbic region including the hippocampus (225). NRG is a substrate of BACE 1, the sole β-secretase that generates Aβ peptides by cleavage of APP. NRG1 is indispensable for neural and cardiac developpement (226). During adult life, NRG1 contributes to synapse formation, neurite growth and plasticity. It was also shown to promote excitatory synapse development and function of GABAergic neurons (227).

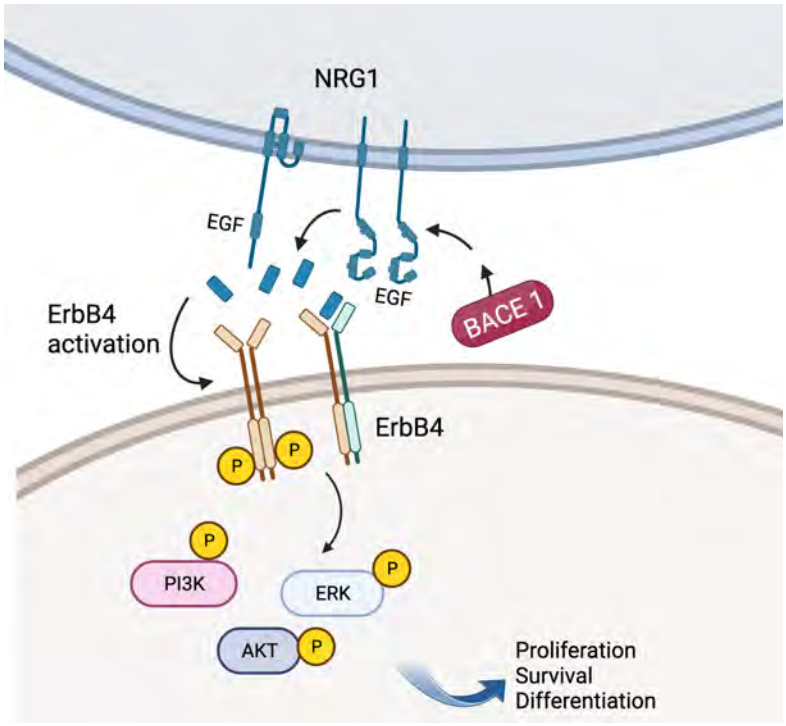



**Figure 12, BACE1-dependant NRG1 and ErbB4 signaling**
*Figure created with BioRender.com. Neuregulin 1 (NRG1) can be released from the membrane by cleavage by β-site amyloid precursor protein cleaving enzyme 1 (BACE 1). NRG1 ligand interaction with ErbB4 increases their affinity and induces homodimerization and heterodimerization of ErbB4, thus activating the tyrosine kinase domain and allowing the phosphorylated activation of ErbB receptors in the cytoplasmic region. These processes regulate multiple intercellular signal transduction and participate in a wide range of biological processes in the CNS, including proliferation, survival, differentiation and synaptic plasticity. To note, only two of the six isoforms of NRG1 are pictured for easier visualization.*

In pathological conditions, NRG1 was first reported to be associated with the cognitive deficits observed in schizophrenia in genetic studies (228). In AD, animal and human studies support alterations in NRG1 signaling. In AD human brain and in AD mice models, NRG1 was shown to be upregulated in reactive astrocytes and microglia surrounding neuritic plaques (229). NRG1-ligands ErbB4 and phospho-ErbB4 immunoreactivity was increased in neurons of the hippocampal AD brains as compared to controls (230). In 2020, our team demonstrated that CSF NRG1 levels were higher in AD subjects with confirmed AD pathology (231). Conversely, non-AD MCI and non-AD dementia patients presented with similar CSF NRG1

levels as controls. CSF NRG1 levels positively correlated with CSF Aβ42 levels. Moreover, it correlated with cognitive testing and the CSF synaptic biomarkers GAP 43 and neurogranin. Interestingly, an inverse correlation between the cognitive decline over time and CSF NRG1 baseline levels was found in AD patients, which could indicate a protective effect of NRG1 regarding cognitive decline.
Interestingly, NRG1 can be measured in blood: one study reported increased levels of plasma NRG1 in subjects with dementia, including AD cases, however, without biomarker evidence of AD pathology (232).

The available evidence on CSF synaptic biomarkers shows potential for the possible use of these proteins as indicators of synaptic alteration in AD. They could contribute to improve the diagnosis of AD at an early stage as well as to monitor clinical progression. Their investigation in blood should be performed to assess their potential as accessible biomarkers.

#### 1.5.2.3 Neuroinflammation markers

Neuroinflammation is a prevalent feature in AD (233). Markers of activation of CNS innate immune cells and of the release of neuroinflammatory factors have been extensively studied across NDDs. CSF markers of astrocytosis and microglial activation are now available and show alterations in AD. However, development of plasma markers of brain immune system activation has proven challenging as there is a high extra-cerebral expression of inflammatory markers, making the blood tests less reflective of cerebral changes. Glial fibrillary acidic protein (GFAP), YKL-40 and sTREM2 have been the most studied. New markers are currently being investigated, such as galectin-3 (Gal-3).

*GFAP*

GFAP is the main intermediate filament protein expressed in astrocytes (234). GFAP is a vital component of the astroglial cytoskeleton and exerts several other functions in the cell, including regulation of neuronal proliferation, isolation of damaged tissue as well as blood flow regulation following cerebral lesions, functions known as astrogliosis. Astrogliosis is observed around Aβ plaques and GFAP expression in brain is correlated with Aβ plaques density (235). GFAP levels measured in blood and CSF appear increased in AD, reflecting the astrocytic activation in response to the Aβ pathology (236,237). Interestingly, plasma GFAP was reported to display a better performance in identifying Aβ pathology than CSF GFAP (238). However, astriogliosis is most likely not a linear process during AD as PET imaging studies reported that, as the Aβ load increases along AD progression, astrogliosis decreases (239,240). CSF and plasma GFAP kinetics along AD *continuum* are not well characterized to this regard.
To be noted, high GFAP levels are also observed after traumatic brain injury and cerebrovascular injury, constituting potential confounders (241,242). Regarding other neurodegenerative disorders, although GFAP is not entirely AD-specific, the magnitude of change in FTD or alpha-synucleinopathies was reported as relatively small compared with that occuring in AD (243,244).

*YKL-40*

YKL-40 (also referred to as Chitinase 3-like 1) is a glycoprotein produced by inflammatory cells (245). In the brain, during neuroinflammatory processes, its expression has been found to be abundant in reactive astrocytes and, to a lesser extent, in microglial cells. In, CSF YKL-40

is moderately elevated in AD, already at the prodromal stage (246,247). Plasma levels of YKL-40 have already been reported to correlate with their CSF measurements. Moreover, plasma YKL-40 has been reported to be positively associated with memory performance and negatively associated with brain Aβ deposition, in favor of a potential protective effect (247). Levels of YKL-40 might also be altered in other neurodegenerative conditions, as already reported in frontotemporal dementia or Creutzfeldt-Jakob disease (248).

*Soluble TREM2*

TREM2 is expressed in microglial cells, and is known to regulate modulating phagocytosis, cytokine production, cell proliferation, and cell survival. Genetic TREM2 variants are associated with increased risk of AD (249). Soluble TREM2 (the secreted ectodomain of TREM2, sTREM2) can be measured in CSF. Although the findings are still conflicting, several studies reported that AD patients display higher levels compared with controls (250). Moreover, sTREM2 levels in CSF correlated with tau pathology and neurodegeneration measured in CSF with p-tau and tau dosages. At prodromal stage, sTREM2 levels associated with a slower cognitive decline in a disease-stage dependent manner (251). Studies measuring plasma sTREM2 levels in AD have reported variable results, with normal or increased levels compared to controls (252,253). A hypothesis is that TREM2-related inflammatory activity alterations could be specific to given AD stages and differ along AD *continuum*.

*Galectin-3*

Galectins are β-glycan-binding ligand molecules expressed in the brain and recognized as potential modulators of the brain microglia, immunosurveillance, and neuroinflammation (254). Galectin-3 (Gal-3) is a chimera-type protein of the galectin family, encoded by the *LGALS3* gene, expressed in the CNS in neurons and astroglial cells. Gal-3 plays a crucial role in the neuroinflammatory response by mediating activated microglia (**Fig. 13**). Gal-3 participates in the regulation of microglial state (through M1or M2 polarization) to ensure tissue homeostasis (255). Gal-3 can activate microglia *via* interferon γ and further induces the production of proinflammatory cytokines *via* the JAK-STAT pathway (256). Gal-3 also binds to microglial Toll-like receptor 4 as well as to TREM2 (257). Gal-3 appears to exert both proinflammatory or anti-inflammatory effects. In animal models of encephalitis, gal-3 is engaged in macrophages polarization favoring disease exacerbating inflammatory response (258). It was also shown to be induced in microglial phenotypes that are implicated in cell debris and damaged axons clearance as well as axon regeneration (259).

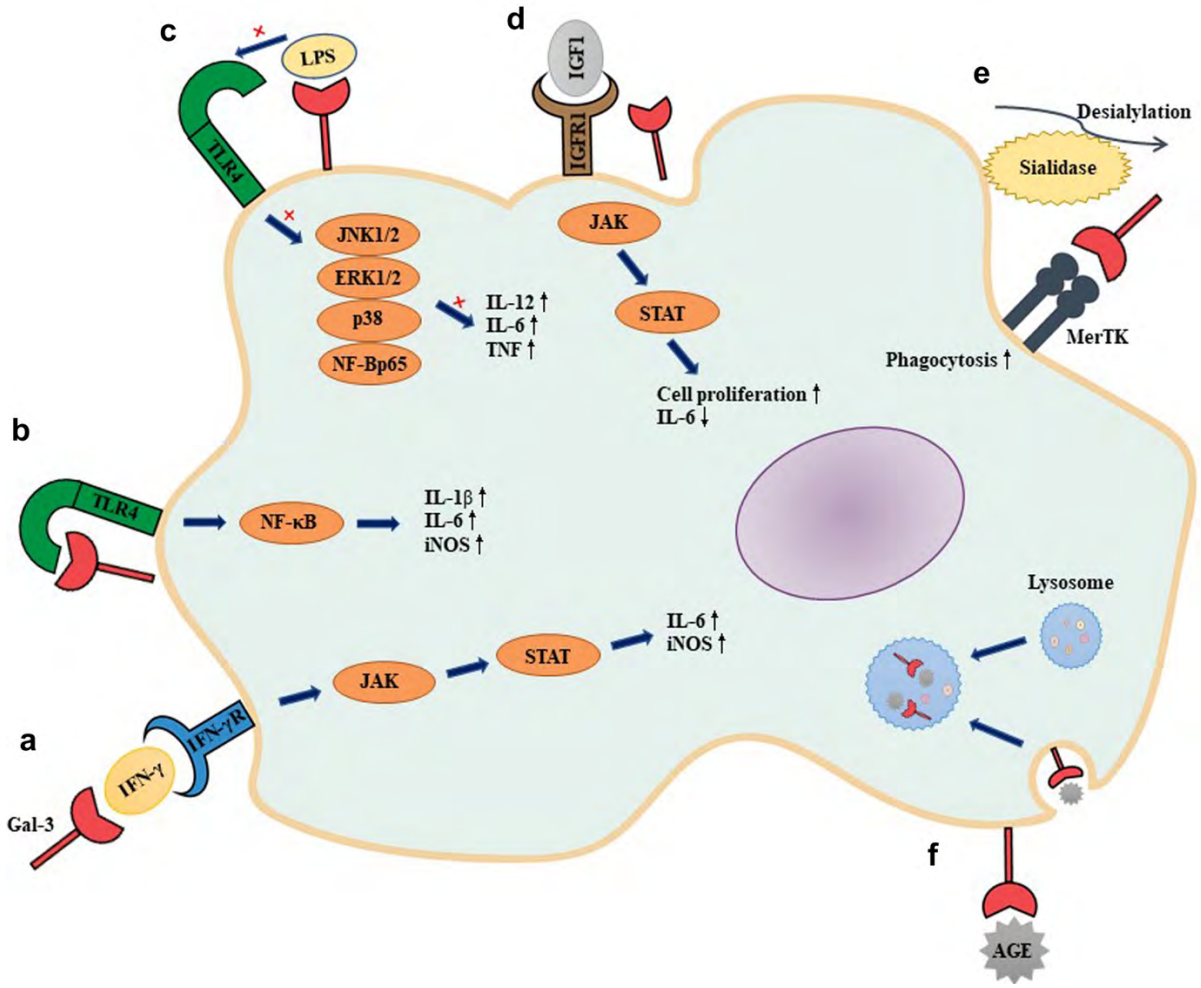



**Figure 13, Mechanisms of galectin-3 microglial activation**
*Figure from Tan et al., Cell & Bioscience 2021* (260). *The molecular mechanisms of microglial modulation by galectin-3 (Gal-3) are multiple.* ***a)*** *Gal-3 induces the production of proinflammatory cytokines through IFN γ and the JAK-STAT pathway.* ***b)*** *Gal-3 can bind to toll-like receptor 4 (TLR4) inducing a proinflammatory response.* ***c)*** *Gal-3 can inhibit downstream proinflammatory cytokine production by binding to lipopolysaccharide (LPS).* ***d)*** *Gal-3 binds IGFR1 receptor activating the JAK-STAT pathway.* ***e)*** *Gal-3 promotes phagocytosis through the receptor Mer tyrosine kinase (MerTK).* ***f)*** *Gal-3 binds with advanced glycosylation end products (AGEs) to degrade their toxicity.*

Experimental and clinical studies have demonstrated the implication of Gal-3 in AD (261). Variants in the *LGALS3* gene might be associated with the decline of cognitive function in older adults. In 5xFAD (familial AD) mice, Gal-3 expression was found to be upregulated (262). In AD brain, Gal-3 immunoreactivity was increased compared with controls and colocalized with Aβ plaque-associated microglia (262). Moreover, Gal-3 staining showed colocalization and direct interaction with sTREM2 at the microglia.
Previous experimental studies have suggested that Gal-3 enhances inflammation in the pathogenesis of AD. Gal-3 expression was found upregulated after Aβ injection in rats hippocampus (263). Gal-3 induced microglial activation through the production and release of proinflammatory factors, such as IL6, IL8 and TNFα in mice AD models (262).
Gal-3 can be detected in CSF and plasma. A pilot study suggested increased levels in AD patients compared to controls in both CSF and plasma (264).

All in all, evidence of neuroinflammation in AD is strongly supported by altered levels of inflammatory markers, pathological evidence and AD genetic risk factors implicating immune functions. CSF and plasma markers of astrocytosis and microglial activation appears very promising in this context. However, the exact nature of the inflammatory response within the CNS in AD remains unclear. Most likely, the different phenomena of neuroinflammation take place differentially along AD initiation and progression, with variable individual susceptibilities. The different processes reflected by the alterations of neuroinflammation fluid biomarkers are not well characterized. Thus, their use and interpretation for diagnosis, prognosis or stratification of AD patients are not framed and there is no clinically validated neuroinflammatory biomarker currently.

### 1.5.3 Clinical validation of new biomarkers

Novel biomarkers described above, measured in plasma or in CSF, show potential to complement actual validated CSF biomarkers for AD. However, translation to clinical use still requires validation regarding population of use, as well as standardization of pre-analytical and analytical conditions.

In plasma, a major issue is the frequent existence of associated comorbidities in older adults, including cardiovascular, respiratory, hepatic, renal and rheumatic diseases, which might modify the protein levels. Current cohorts with both CSF and blood samples available are largely based on research centers. Most frequently, individuals with comorbidities (HIV, cancer, inflammatory disorders) are excluded from research cohorts. A recent study found higher plasma p-tau181 and p-tau217 levels in individuals with chronic kidney disease, hypertension, stroke, and myocardial infarction (265). Plasma p-tau levels were also found to be associated with body mass index, years of education, diabetes, atrial fibrillation, and cancer, before adjustment for age and sex. Plasma biomarkers will need to be evaluated in less selected cohorts, including memory centers, general neurological department and primary care.
Cohorts which are used for biomarkers evaluation and validation should also reflect the settings for which the biomarkers are intended, *i.e.,* memory clinics, neurological departments or primary care. Specialized memory clinic settings, even if they enroll more diverse patients than in pure research cohorts, are characterized by referral bias with higher prevalence of NDDs. Recent epidemiological data evaluates the prevalence of AD in older adults, with or without cognitive impairment at around 10% (12). Thus, new cohorts might be needed for the purposes of blood-based biomarkers assay development.
Moreover, some of the improvements in diagnostic performance recorded for plasma tests during recent years may also be due to improved diagnostic accuracy regarding the study participants, using PET or CSF. This has made it less likely that the control group contains individuals with preclinical amyloid pathology. Studies in memory clinics or population-based cohorts without prior stratification by CSF or PET biomarkers are still lacking to ascertain the diagnostic potential of plasma markers.
Lastly, the measurements of these markers of interest rely on research-grade assays developed in independent laboratories using specific methods. Reliable assays with robust replication in different settings, including comparative studies in real-life cohorts, standardization efforts, and development of reference materials and methods will be needed before introduction in clinical settings.

# 2. AIMS

## 2.1 General aim

Our general aim was to investigate novel CSF and blood candidate biomarkers in patients from clinical settings, including amyloid and tau – specific and – unspecific markers, for positive and differential diagnosis of AD. Furthermore, we explored their relation to cognition and morphological imaging.

## 2.2 Specific aim of each article

**Paper I** characterizes NRG1 in plasma and evaluates if plasma NRG1 could be used as a novel synaptic biomarker for AD.

**Paper II** investigates NfL and pNfH in CSF and plasma for clinical use for AD differential diagnosis.

**Paper III** investigates plasma GFAP as a biomarker for AD diagnosis along the whole AD *continuum*, from preclinical stage to dementia.

**Paper IV** characterizes Gal-3 both in brain and CSF in the AD and explores if CSF Gal-3 could be a relevant CSF marker for neuroinflammation in AD.

**Paper V** compares the diagnosis performance of multiple plasma biomarkers in clinical settings for positive and differential diagnosis of AD.

# 3. MATERIAL & METHODS

## 3.1 Material

### 3.1.1 Cohorts studied in this work

All our studies analyzed CSF and plasma samples from a memory clinic cohort issued from the Centre of Cognitive Neurology, Lariboisière Fernand-Widal Hospital, Paris, France. We retrospectively included patients who had undergone CSF and plasma biobanking as well as a comprehensive neurological examination, between 2012 and 2019, in the context of the exploration of a cognitive complaint.

*Cohort characterization*

Patients were referred in routine care at our memory clinic by general practitioners or by neurologists, psychiatrists or any other medical specialist for exploration of a cognitive complaint or of a cognitive decline. At the clinic, patients underwent a thorough clinical examination involving personal, medical and family histories collection, neurological examination, neuropsychological assessment, CSF biomarkers analysis, morphological brain MRI and plasma and CSF biobanking (266).
Mini-Mental State Examination (MMSE) was used as a general measure of cognition in all studies (267). MRI was obtained during the diagnosis workup, within <1 year of CSF and plasma collection. Medio-temporal atrophy was visually rated using the Scheltens visual scale independently by two physicians (268). Fazekas score for white matter lesions was rated by a single operator (269). All patients were genotyped for APOE using standard polymerase chain reaction (270).
CSF core biomarkers (Aβ42/Aß40 ratio, p-tau181 and t-tau) were used for the diagnosis. They were measured with the available method in clinical routine at the time of the analysis (Innotest® or Cobas, Roche Diagnostics International Ltd®).

*Diagnosis criteria*

The diagnosis for each patient was made during multidisciplinary consensus meetings (associating neurologists, neuropsychologists, gerontologists and biochemists) considering neuropsychological assessment, morphological and functional imaging and CSF results. Diagnosis criteria used were the 2014 IWG-2 criteria for AD dementia and the NIA-AA MCI criteria of 2011 for MCI due to AD (AD-MCI) (72,271). Patients with DLB were diagnosed according to recent clinical criteria by McKeith *et al* (81). FTD patients included subjects with behavioral variant FTD and semantic dementia (16,272). They displayed a normal CSF Aβ ratio. VaD patients were diagnosed according VASCOG criteria and presented with a normal CSF Aβ ratio (99). Non-AD MCI group included amyloid-negative patients with psychiatric disorders, sleep apnea or systemic disease (273). The individuals with no evidence of underlying neurocognitive disease were enrolled as neurological controls when they fulfilled the following conditions: (i) the neuropsychological assessment found preserved global cognition (*i.e.,* normative or subnormative scores for age, sex and level of education), (ii) brain MRI did not find any sign of atrophy (iii) they displayed a normal CSF biomarker profile and (iv) no cognitive decline was observed during the follow-up.
In **Paper IV**, the CSF study was a pilot study focusing solely on AD and not on other dementia. Were only included AD subjects at both MCI and dementia stages and neurological controls.

*Other cohorts included*

In **Paper III**, two other cohorts were analyzed: the Trial Cohort from McGill University, Canada, (https://triad.tnl-mcgill.com) and the ALFA+ cohort from the Barcelona Beta research center, Spain (https://www.barcelonabeta.org/en/alzheimer-research/studies/alfa%2B) (274,275). The TRIAD cohort follows individuals with cognitive impairment at all stages, from SCI to dementia, as well as cognitively unimpaired individuals, highly phenotyped with CSF and blood markers, MRI imaging and amyloid and tau PET. The ALFA + cohort is a, prospective and observational cohort for the early identification of biomarkers of AD in 500 cognitively healthy people. Detailed cohort's description is reported in **Paper III**.
In **Paper IV,** the brain study included control and AD brain samples, issued from the Neurological tissue Bank, Biobanc-Hospital Clinic IDIBAPS, Barcelona, Spain and from the Netherlands Brain Bank. Their detailed characteristics are described within the paper.

3.1.2 Samples used in this work

CSF and plasma were collected in a less than 4-hour interval, in the morning and under fasting conditions. All samples were processed in a 4-hour delay after collection. CSF was collected according standardized validated protocol. Briefly, CSF was collected *via* lumbar puncture through L3-L4 or L4-5 spaces and collected in polypropylene tubes. The first millimeters were used for routine analysis including cytology, and the measure of core AD biomarkers. The 7 and 8 the millimeters were collected for biobanking. Insoluble materials were subsequently removed by centrifugation at 2000g at 4 degrees for 10 minutes. The supernatant was transferred in polyprolene aliquots and stored at -80 °c pending analysis.
Plasma was obtained through whole blood collected by venipuncture in tubes coated with anticoagulant ethylenediaminetetraacetic (EDTA). Whole blood was centrifuged at 3500g at 4°c for 20 minutes. The obtained plasma transferred in polypropylen aliquot and stored at -80 °c.

In **Paper IV**, immunochemistry was performed on frozen hippocampal and cerebral cortical tissue. Sections of formalin-fixed and glucose-immersed hippocampal tissue were also analyzed.

3.1.3 Ethical Approval

All patients or their legal relatives gave written informed consent to their participation in the study. Collection and analysis of samples were approved by the local ethic committee of Bichat University, Paris, France (CEERB GHU Nord n°10-037). The TRIAD cohort, McGill University was approved by the Douglas Institute Research Ethics Board and written consent was obtained from all participants (Protocols: IUSMD 16-60 and 16-61). The ALFA+ study (ALFA-FPM-0311) was approved by the Independent Ethics Committee 'Parc de Salut Mar', Barcelona. For brain study, written informed consent for the use of tissue and clinical data for research purposes was obtained from all patients or their next of kin following the International Declaration of Helsinki and Europe's Code of Conduct for Brain Banking. The medical ethics committee of VU medical center in Amsterdam and the IRB of Clinic Hospital in Barcelona gave approval.

## 3.2 Methods

### 3.2.1 Enzyme linked immunosorbent assay

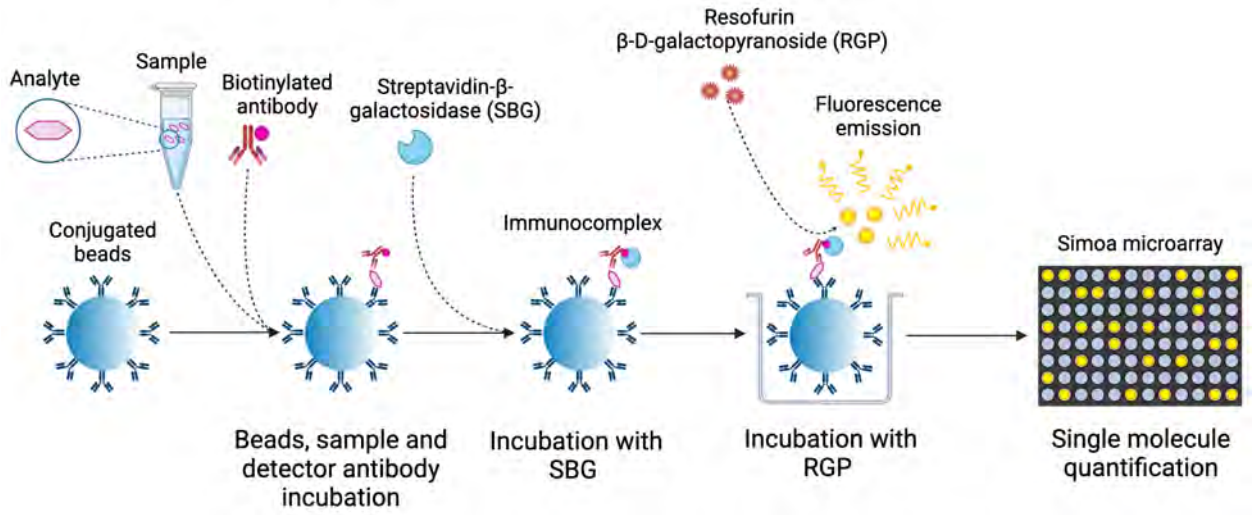
In all papers, sandwich enzyme linked immunosorbent assay (Elisa) was used to measure our different analytes of interest.
Elisa is an immunoassay that relies on the use of antibodies specific to the same target. The most commonly used Elisa is the sandwich Elisa that uses two antibodies targeting the same antigen, allowing for high specificity. A 'capture antibody' is coated on a 96-wells microtiter plate. The remaining binding sites are blocked using a blocking solution with high protein concentration such as bovine serum albumin. The samples are added to the plate and the target antigen is bound by the capture antibody. A second antibody targeting the same antigen is used, the so-called detection antibody. The detection and capture antibody typically target different epitope of the antigen. The plate is washed between each step to remove any excess of proteins or antibodies nonspecifically bound. Different strategies can be used to detect the antigen-antibody complex. The detection antibody can be conjugated to biotin which will react to a streptavidin conjugated horse radish peroxydase (HRP) is then added. A chromogenic substrate 3,3′,5,5′ tétraméthylbenzidine (TMB) will then be added. The reaction will induce a color change proportional to the amount of the HRP bound, hence to the target analyte concentration. The color change following the HRP enzymatic reaction can be detected and quantified using an absorbance spectrophotometer.

In **Paper I**, a commercial kit provided by R& D system (Human NRG1-beta 1/HRG1-beta 1 DuoSet, #DY377-05) was used to measure plasma NRG1 levels.
In **Paper II**, CSF NfL levels were measured using a in-house Elisa developed at the University of Gothenburg, Sweden (276).
In **Papers II and IV**, plasma and CSF YKL-40 levels were measured using a commercial kit provided by R& D system (Human Chitinase 3-like 1 Quantikine Elisa Kit #DC3L10)
In **Paper IV**, Elisa plates from Abcam (ab269555) were used to measure the levels of Gal-3 in tissue homogenates. CSF Gal-3 levels were measured using a kit from thermofischer Scientific (Galectin 3 Human Elisa Kit #BMS279-4).

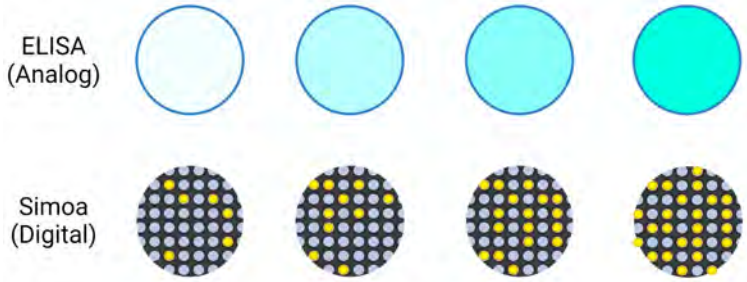
### 3.2.2 Single molecule array

Single molecule array (Simoa) is a technology developed and validated by Quanterix in 2016 (**Fig. 14**) (277). The Simoa technique is an immunoassay with a functional principle common to the Elisa. However, the Simoa technique has a highly increased sensibility, in the range of PicoG/L. It is a semi-automated method, improving precision and consistency and it also requires less volume as common immunoassays.
In brief, a capture antibody is conjugated to paramagnetic beads. It is incubated with the samples. The number of the beads exceeds the analyte concentration. The beds containing analytes follow the poisson distribution: at low concentration of the analyte, only one molecule of analyte will bind to each bead. In the next step, the beads are incubated with a biotinylated detection antibody and then with B-D-galactopyranoside streptavidin (SBG). Only the beads that have successfully formed an immunocomplex are able to bind the enzyme. Then, beads are transferred to a microarray, where each bead fits in a microwell. An enzymatic substrate, resofuriin B-D galactopyranoside (RGP) is added. Fluorescence will only be generated in wells containing the enzyme, indicating the presence of an immunocomplex. The concentration of the analyte is computed from the number of fluorescent wells in relation to the total number of

wells containing beads. This result is computed in digital mode, in comparison with the Elisa technique that provides analog measurements.




Single Molecule Array (Simoa)




Quantification method: comparison to Elisa

**Figure 14, Steps of the Simoa technique and technique of quantification**
*Figure courtesy of Dr. Juan Lantero-Rodriguez, created with Biorender.com. Illustration of the main steps of Single Molecule Array (Simoa) immunoassays and a comparison of the quantification methods of Simoa and Elisa immunoassays.*

In **Papers II, III, IV** and **V**, commercial kits from Quanterix® were used for measurements of plasma NfL and GFAP (2-plex B, No. 103520), pNfH (CSF and plasma, No. 102669), CSF GFAP (No. 102336), Aβ 42, Aβ 40 and total tau (plasma, Neurology 3-plex A No. 101995), on a Simoa HD-1 analyzer. In the **Paper V**, in-house assays developed on the Simoa platform at the University of Gothenburg, Sweden, were used to measure p-tau231 and plasma p-tau181, both in CSF and plasma, on a Simoa HD-1 analyzer (171,173).
Tau and phospho-tau immunoassays explored in this thesis are depicted **Fig. 15**.

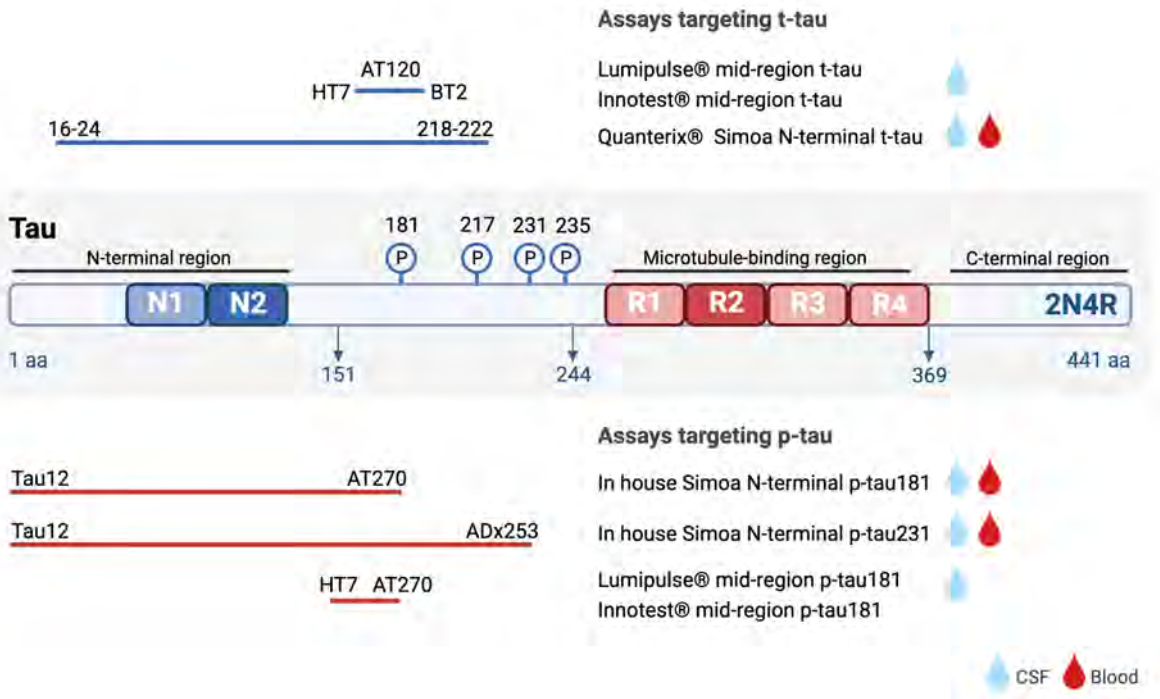



**Figure 15, Total tau and phospho-tau immunoassays**
*Adapted from Dr. J. Lantero-Rodriguez, created in Biorender.com. This figure displays the different CSF and blood immunoassays targeting total tau (t-tau) and phosphorylated forms of tau (p-tau) explored in this thesis. Lumipulse® and Innotest® are measuring CSF t-tau mid-region fragments, whereas the blood and CSF t-tau assay from Quanterix targets N-terminal t-tau. In-house p-tau181 and p-tau 231 Simoa assays, developped by the University of Göteborg, target N-terminal p-tau fragments whereas Lumipulse® and Innotest® commercial assays target mid-region p-tau fragments.*

### 3.2.3 Lumipulse

In **Papers II, III, IV and IV**, CSF core biomarkers (Aβ 42/Aß40 ratio, p-tau181 and t-tau) were measured using the clinically validated Lumipulse® G1200 (Fujirebio) (278). Patients were classified according to the ATN scheme, in normal biomarker profile (A-T-N-), AD continuum (A+T-N – , A+T+N- and A+T+N+) and suspected non-amyloid pathology (SNAP; including A- participants with T and/or N positivity), according clinically validated cut-offs: Aß42/Aß40 <0.61 pg/mL; p-tau181<61 pg/mL; t-tau <450 pg/mL.

### 3.2.4 Mesoscale discovery assay

Mesoscale discovery assays (MSD) are immunoassays using the principle of electrochemiluminescence**.** The majority of MSD immunoassays are designed as sandwich assays. Microplates with high binding carbon electrodes in the bottom are coated with a capture antibody targeting the analyte of interest. The samples are then added to the plate as well as SULFO-TAG – conjugated detection antibody. Electricity is applied to the plate electrodes by an MSD instrument leading to light emission by the SULFO-TAG labels. Light intensity is then measured to quantify analytes in the sample.

In **Paper IV**, MSD was used to measure the levels of Aβ42, p-tau, and t-tau in the RIPA fraction of the human brain. The plates were developed and read using a QuickPlex Q120

reader (Meso Scale Diagnostics). CSF sTREM-2 levels were measured using a in-house MSD kit developed at the university of Gothenburg as previously described by Alosco *et al.,* (279).

### 3.2.5 Western blotting

Western blotting was performed to measure our proteins of interest. Western blot allows the separation and detection of proteins from a complex matrix. Western blot starts with gel electrophoresis to separate the proteins of the sample. After electrophoresis, proteins were transferred from the gel to a membrane. After a phase of blocking, the membrane was then incubated with a primary and a secondary antibody. The primary antibody specifically recognizes proteins of interest. The secondary antibody binds to the primary antibody and is labeled with a molecule for the generation of a detection signal. A substrate is then added leading to a chemiluminescent reaction with the release of light that can be quantified with an adapted reader.

In **Paper IV**, Gal-3, Aβ42, p-tau and t-tau were measured using WB. Electrophoresis was performed using polyacrylamide gel and SDS-PAGE solution. Transfer was done using nitrocellulose membranes (Bio-Rad). Reading was performed using SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoScientific, Spain) and imaged using a ImageQuant LAS-4000 biomolecular imager (GE Healthcare)

### 3.2.6 Immunohistochemistry

In **Paper IV**, immunohistochemistry was performed at the Neuroinflammation Lab, University of Lund, Sweden, on human AD brain as well as 5-FAD mice models to study our protein of interest gal-3.
Immunochemistry was performed on sections of formalin-fixed and glucose-immersed tissue. Hippocampal sections 40 µm-thick were washed (3 × 15 min) in 0.1 M KPBS and then incubated in 0.1 M KPBS, Tween 20 0.25% and normal donkey serum 5% for one hour at room temperature. For immunofluorescence labeling (Iba1, Gal-3, Aβ, or p-tau), sections were first incubated with the primary antibodies followed by the corresponding Alexa 488/555/647 secondary antibodies (1:1000 dilution, AlexaFluor, Life Technologies). After mounting and drying on slides, the sections were incubated in 0.6 g Sudan Black (Sigma) dissolved in 70% ethanol for 5 min. Subsequently, the sections were washed in PBS and mounted with the mounting medium. When imaging, the camera settings were adjusted at the start of the experiment and maintained for uniformity. A Nikon Eclipse Ti confocal microscope (Nikon, Japan) and NIS elements software (Nikon, Japan) were used to take pictures at a 20x magnification. Primary antibodies included anti-Iba1 (Wako, 019-19741), Gal-3 (R&D, AF1197), Aβ (6E10, Covance), p-tau (Thermofisher, MN120). Respective secondary antibodies were Alexa 488/555/647 (AlexaFluor Technologies). NIS Element Analysis software (Nikon, Japan) was used to evaluate plaque size and shape.

### 3.2.7 Statistical Analysis

Statistical analysis was performed using: Graphpad Prism for Mac Software (GraphPad software La Jolla CA USA), SPSS (IBM SPSS statistics, Armonk NY IBM Corp), Scistat (MedCalc Software Ltd, Ostend,Belgium) and R programing language (R core team – 2013 – , R: a language and environment for statistical computing. R Fondation for statistical programing, Vienna, Austria, http://www.R-program.org/). Graphs were created using

Graphpad Prism and R. A two-sided p-value <0.05 was overall considered statistically significant.

*Analysis of biomarkers concentrations*

Patient data was analyzed in 5 groups: NC, AD-MCI, non-AD MCI, AD dementia and non-AD dementia. Patients were also dichotomized according to Aβ42/Aβ 40 in Aβ-positive and Aβ-negative groups. Group differences for biomarkers results were assessed with one-way ANCOVA adjusted for age and sex, with post-hoc Tukey's HSD test. In **Paper IV**, biomarkers levels were compared between NC and AD.
Correlations between biomarkers were analyzed using Spearman's rank correlation or through adjusted analysis using linear regression.

*ROC analysis*

Diagnostic accuracy of studied biomarkers was assessed with receiver operating characteristic curves (ROC) to determine areas under the curve (AUC). In all papers, ROC analysis was used in order to determine cut-offs for CSF biomarkers for differentiating between controls and the different diagnosis groups. In **Paper IV**, Youden index was used to identify the cut-off maximizing specificity and sensitivity. In **Paper V**, binary logistic regression was performed with diagnosis as the dependent variable, including blood biomarkers as explicative variables. To find the most adequate combination of markers, linear regressions with stepwise backward elimination were performed. All models were adjusted for age and sex. Probabilities from the models were saved as variables. ROC analyses were then used to evaluate the performance of each single blood biomarker and of combinations of markers.

*Association of biomarkers with cognition*

Association of our markers of interest with MMSE score were studied using linear regression adjusted on age, sex and level of education.

*Mediation analysis*

In **Paper III,** a mediation analysis was performed to study the association of CSF GFAP with with CSF Aβ ratio and p-tau, using the R package "mediation". Plasma and CSF GFAP were, successively, the outcome variable and Aβ and tau biomarkers (CSF or PET) the predictor variables, adjusting for age, sex and diagnosis. Standardized indirect effects were computed for each 1000 bootstrapped samples. 95% confidence intervals were computed by calculating the indirect effects at 2.5th and 97.5th percentiles.

*Principal component analysis*

In **Paper IV**, principal component analysis was performed to explore the patterns between the different CSF biomarkers. After exclusion of outliers and evaluation of the suitability of the data set (Kaiser-Meyer-Olkin and Bartlett's tests), we performed principal component analysis in the whole cohort and in A+T+ group. Components were retained if their eigenvalues was superior to one. Variables with a loading factor superior to |0.4| were considered representative of the component. Each component was interpreted post-analysis according to the physiopathology of the biomarkers included in the component. Component values were compared in between groups using linear regression adjusting for age and sex.

### 3.2.8 Thesis author's contribution to this work

All biomarkers measurements in Paris Cohort samples, the processing of the data and the statistical analysis for Paris Cohort were carried out by the author of this thesis.
Plasma GFAP and other markers measurements in Alfa+ and TRIAD cohorts in **Paper III** were realized by the Neurochemi Lab, Gothenburg, Sweden.
Mediation analysis in **Paper III** was carried out by Dr. Andrea L. Benedet, Neurochemi Lab, Gothenburg, Sweden.
The animal work in **Paper IV** was carried out by Dr. Antonio Bozza-Serrano, Lund University, Sweden.

# 4. RESULTS

## 4.1 Paper I

### 4.1.1 Rationale

Synaptic dysfunction and loss have been shown to be the best correlate of cognitive decline, among AD features. Measurements of synaptic damage or loss are therefore expected to closely correlate to cognitive status. Thus, the identification and validation of synaptic biomarkers for follow-up, for establishing prognosis and as potential endpoint for trials in the longer term is highly sought. No blood-based synaptic biomarker is presently available, despite the fact that, as intended as a follow-up and therapeutic response biomarker, ideal synaptic biomarkers should be easily accessible and non-invasive to be repeatedly measured.
Among synaptic proteins, evidence from human and animal studies suggest that neuregulin 1 (NRG1) signaling may be altered in AD and may affect cognitive functions, as presented in the introduction section. NRG1 levels have been investigated in the brain and in the CSF suggesting alterations in AD. Our team has previously explored NRG1 levels in patients and controls who had undergone core AD CSF biomarkers analysis (231). In this work, NRG1 i) was increased in AD from MCI stages, ii) was specifically increased in AD compared with non-AD dementia, iii) was associated with cognitive status and iv) correlated with established synaptic biomarkers. NRG1 has been measured in plasma in studies related to schizophrenia and levels were altered in individuals at increased risk of schizophrenia (280,281).
Thus, in **Paper I**, we decided to investigate NRG1 plasma levels in AD in a pilot study.

### 4.1.2 Study

The study cohort comprised neurological controls (NC, n=20), AD at the stages of MCI (AD-MCI, n=27) and dementia (AD dementia, n=35) as well as non-AD MCI (n=19) and non-AD dementia (n=26) patients from our memory clinic. Plasma NRG1 levels were measured using the Elisa technique.

Firstly, we compared plasma NRG1 levels across groups (**Fig. 16, a-b**). Plasma NRG1 levels were higher in AD, at MCI and dementia stages, compared to NC ($P<0.005$). Non-AD dementia also demonstrated higher levels than NC ($P=0.014$). Dichotomizing the cohort in Aβ-positive (Aβ+) and Aβ-negative (Aβ-) individuals based on the CSF Aβ42/Aβ40 ratio, we found that Aβ+ displayed higher levels than Aβ- individuals ($P=0.023$).
Then, we investigated the performance of plasma NRG1 when discriminating inbetween groups (**Fig. 16, c**). Plasma NRG1 displayed interesting AUCs to distinguish respectively between AD-MCI and controls (AUC=88,3%) and between AD-dementia and controls (AUC=87.6%). To identify AD at MCI stage, the diagnosis performance remained interesting (AD-MCI *vs* non-AD MCI, AUC=86.4%). However, at dementia stage, diagnosis potential of plasma NRG1 for AD was poor (AUC=69.3%).
CSF NRG1 showed a moderate association with plasma NRG1 in the whole cohort ($\beta=0.372$, $P=0.056$), after adjustment for age and sex (**Fig. 17, a**). This correlation was sustained in the Aβ+ group ($\beta=0.292$, $P=0.034$). Plasma NRG1 levels showed a weak correlation with CSF Aβ42/Aβ40 ratio ($\beta=-0.197$, $P=0.043$, **Fig. 17, b**). Its association with CSF p-tau and CSF p-tau were stronger, especially for t-tau (p-tau: $\beta=0.361$, $P<0.001$; t-tau: $\beta=0.423$, $P<0.001$, **Fig. 17, c-d**). These correlations were also observed focusing on the Aβ+ subgroup.
We studied the association of plasma NRG1 with three established biomarkers for synaptic impairment in AD: GAP-43, neurogranin and SNAP-25(**Fig. 17, e-g**). After adjustment for age and sex, plasma NRG1 levels were associated with synaptic markers levels ($\beta=0.278$-$0.355$) in

the whole cohort and in the Aβ+ group (β=0.322-0.434). Cognitive status was evaluated using MMSE score at blood sampling time point. Plasma NRG1 levels showed a weak inverse association to MMSE score in the whole cohort (β=-0.188, P=0.038). Interestingly, this association appeared stronger in the Aβ+ subgroup (β=-0.255, P=0.037) (**Fig. 17, h**).

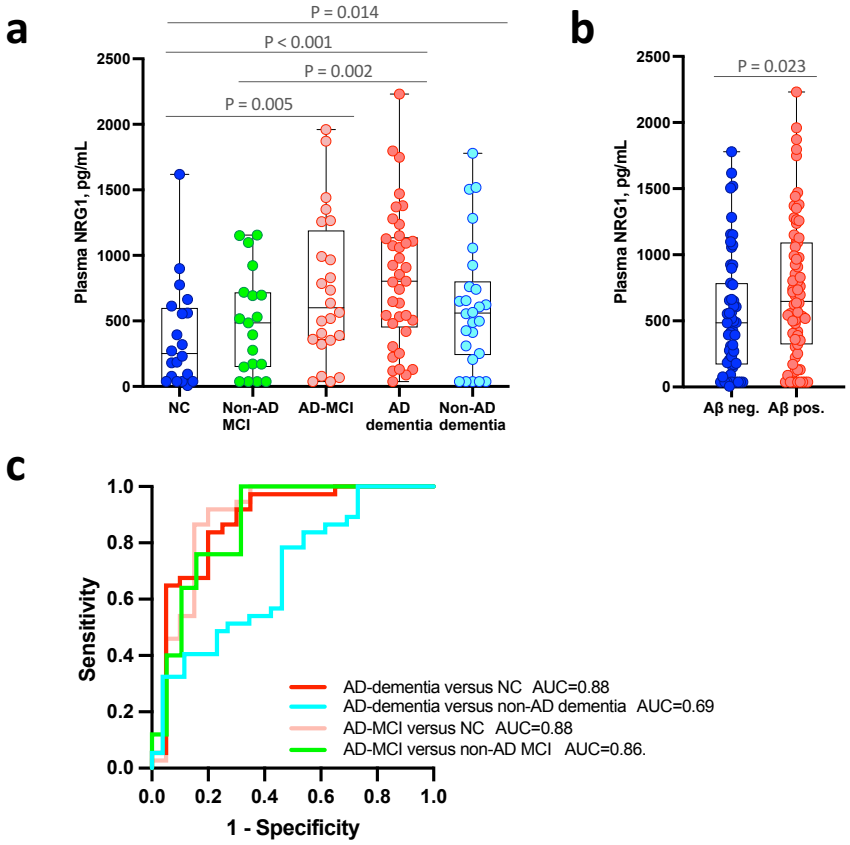



**Figure 16, Plasma NRG1 levels across groups and diagnosis performance**
*Plasma NRG1 levels were compared between groups using one-way ANCOVAs adjusted on age and sex.* ***a)*** *Plasma NRG1 was increased in AD, at both MCI and dementia stages, as well as in non AD dementia compared to neurological controls (NC).* ***b)*** *Aβ+ patients displayed higher levels than Aβ- patients.* ***c)*** *Plasma NRG1 displayed moderate AUC in ROC analysis to differentiate groups, but for distinguishing between AD dementia and NC group.*

Earlier studies on plasma NRG1 had shown increased levels in clinically diagnosed AD subjects. In this pilot study, we report that plasma NRG1 levels were modified in subjects with evidence of underlying AD pathology. Additionally, plasma NRG1 was associated with core AD CSF biomarkers and synaptic markers as well as with cognitive status measured with MMSE. Plasma NRG1 levels were also altered in non-AD dementia, indicating that it could reflect synaptic degeneration beyond AD.

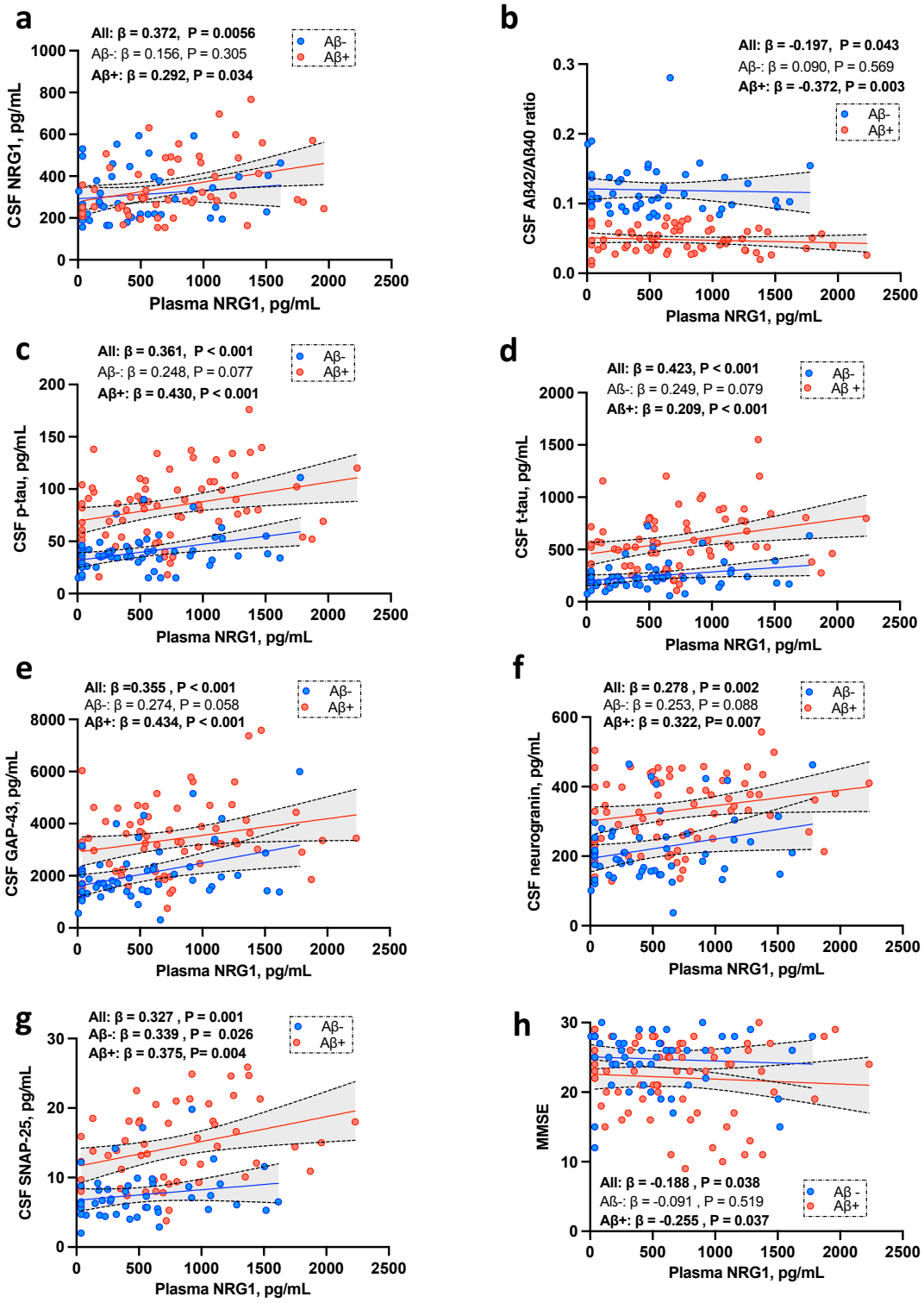



**Figure 17, Association of plasma NRG1 with CSF markers and with cognition**

*Association of plasma NRG1 with CSF markers levels and MMSE was studied using linear regression adjusted on age and sex.* ***a)*** *Plasma NRG1 was associated moderately with its CSF counterpart, in the whole cohort and in Aβ+ patients. Plasma NRG1 was associated with CSF AD core biomarkers:* ***b)*** *Aβ42/Aβ40 ratio;* ***c)*** *p-tau;* ***d)*** *t-tau in the whole cohort. The different associations were observed in the Aβ+ subgroup. Plasma NRG1 was associated with CSF synaptic biomarkers:* ***e)*** *GAP-43;* ***f)*** *neurogranin and* ***g)*** *SNAP-25;* ***h)*** *Plasma NRG1 displayed an inverse association with MMSE score in the whole cohort and in the Aβ+ subgroup.*

## 4.2 Paper II

### 4.2.1 Rationale

Neurofilament light chain (NfL) levels constitute a well-characterized marker of neuronal injury and neurodegeneration, both in CSF and plasma, in a wide range of neurological disorders. NfL have the potential to contribute to dementia diagnosis challenges faced in clinical practice, differentiating for example, idiopathic PD from atypical parkinsonism, and FTD from clinical phenocopies (201,282). Plasma NfL levels have been demonstrated to be a robust marker, showing little sensitivity to variations in pre-analytical and analytical conditions (283,284). In consequence, there has been an increasing interest in introducing plasma NfL measurements into clinical routine. Establishment of cut-offs for clinical diagnosis and reference values by age classes are already being investigated (201,202).
Phosphorylated neurofilament heavy chain (pNfH) levels have been less extensively studied in NDDs apart from ALS, where they are thought to reflect specific physiopathological processes of the disease. Thus, their potential for use in clinical settings is not well established and they have not been directly compared with NfL in this regard.
In **Paper II**, we aimed at comparing the performance of NfL and pNfH for AD diagnosis in paired blood and CSF samples.

### 4.2.2 Study

Our biomarkers of interest were measured in a sample of our memory clinic cohort, including neurological controls (NC, n=22), non-AD MCI (n=38), AD-MCI (n=36), AD dementia (n=64) and non-AD dementia patients (n=28, including FTD [n=12], DLB [n=13] and VaD, [n=3]). CSF and plasma pNfH and plasma NfL levels were measured using the same Simoa platform; CSF NfL levels were measured using Elisa.
All markers correlated with age (*rho*=0.259-0.451). Plasma and CSF NfL measurements displayed a strong correlation with each other (*rho*=0.77) whereas it was moderate between CSF and plasma pNfH (*rho*=0.52). pNfH and NfL were correlated in CSF as well as in plasma (CSF: *rho*=0.71; plasma *rho*=0.51).
Levels of biomarkers across clinical groups are displayed **Fig. 18**. CSF pNfH and CSF NfL were higher in all MCI and dementia groups compared with NC (overall, $P<0.05$). AD dementia and non-AD dementia patients displayed the highest increase compared with NC. Plasma pNfH levels showed higher levels in all diagnosis groups compared with NC (overall $P<0.05$), except for non-AD MCI. Plasma NfL levels were higher in all groups including non-AD MCI compared with NC. The fold changes for both plasma markers in the AD and non-AD dementia groups were in the same range as their CSF counterparts.
Then, we studied the performance of our markers to differentiate between groups using ROC analysis (**Fig. 19, a**). CSF and plasma pNfH and NfL showed good accuracy to discriminate AD dementia and AD-MCI from NC with overall AUCs>0.80. There was no difference between CSF NfL and pNfH performance. Plasma pNfH displayed a lower AUC to differentiate AD-dementia from NC compared to its CSF counterpart (comparison of AUcs using Delong test: P=0.038); CSF and plasma NfL performed the same. Both pNfH and NfL displayed only a weak accuracy in differentiating non-AD MCI from NC or from AD-MCI (AUCs=0.61-0.77).
Regarding non-AD dementia, diagnosis accuracy of markers was overall moderate looking at the non-AD dementia group at a whole (AUC=0.58-0.81, **Fig. 19,b**). However, CSF pNfH and NfL discriminated FTD from NC with high accuracy (respectively AUC=0.96-0.98). Plasma

NfL had the same performance as CSF (AUC=0.90) but plasma pNfH showed a lower AUC than CSF pNfH (AUC=0.81; Delong *vs* CSF pNfH, P=0.0139). Both CSF markers also had high AUCs for discriminating DLB and FTD (CSF pNfH, AUC=0.83; CSF NfL, AUC=0.93) whereas plasma displayed lower performance (AUC=0.58-0.79). For distinguishing AD dementia from FTD or DLB, our markers displayed moderate performance (AUCs=0.66-0.83).

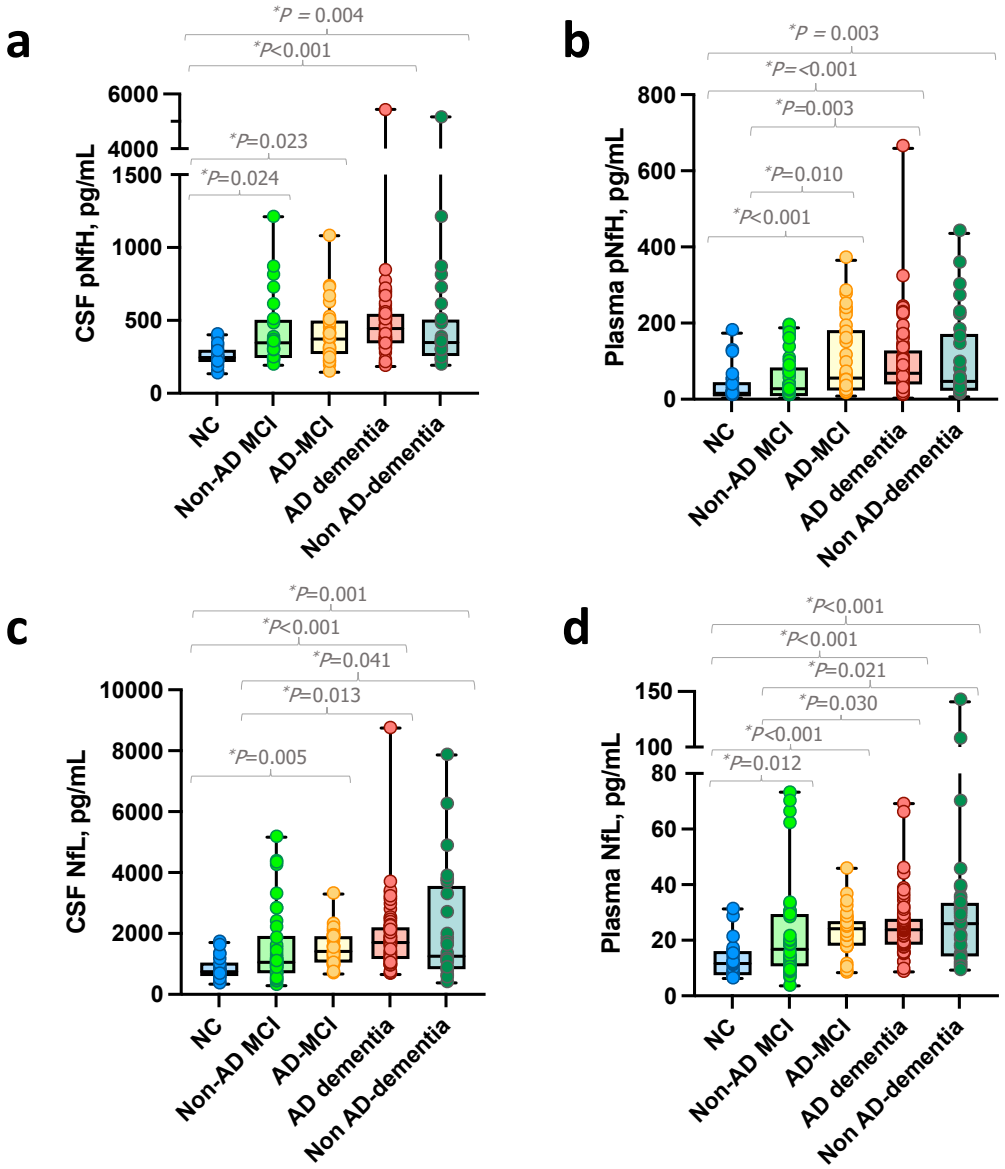



**Figure 18, Plasma and CSF levels of NfL and pNfH across diagnosis groups**
***a)*** *CSF pNfH levels;* ***b)*** *Plasma pNfH levels;* ***c)*** *CSF NfL levels;* ***d)*** *Plasma NfL levels across groups. Biomarkers levels were compared across groups using one-way ANCOVA followed by post-hoc Tukey's HSD test, adjusting for age and sex and for multiple comparaisons. CSF pNfH and NfL levels were higher in all groups compared to NC. In plasma, NfL showed similar differences as CSF, whereas plasma pNfH did not differ between NC and non-AD MCI.*

Regarding cognition, CSF NfL, plasma NfL and plasma pNfH showed a significant negative association with MMSE score in linear regression adjusted for age and sex, whereas CSF pNfH did not (**Fig. 20, a&d**). Looking at subgroups, only CSF NfL remained associated with MMSE in the non-AD-MCI group (β=0.721, P=0.004).

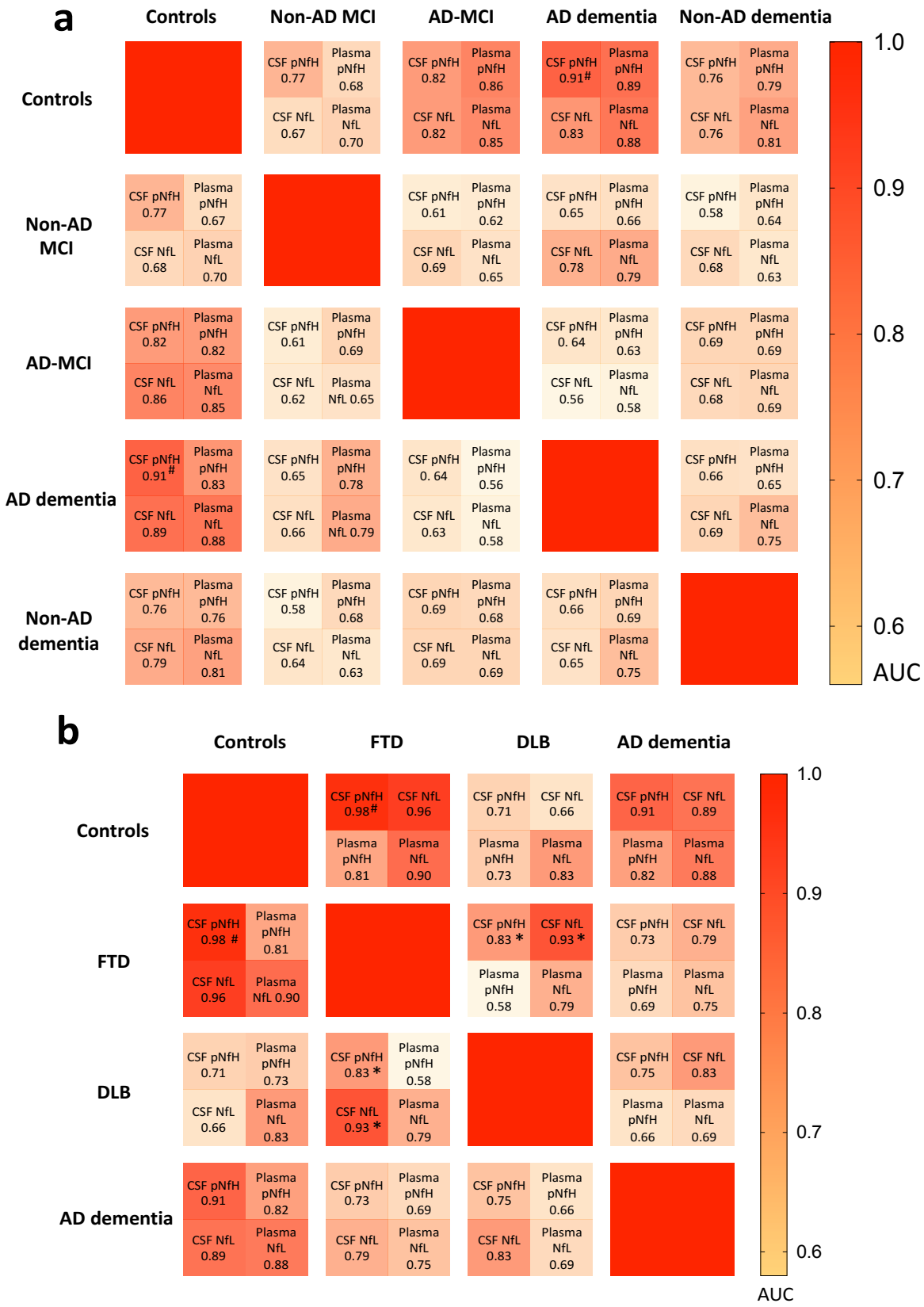



**Figure 19, Performance of Nfs markers in distinguishing between groups**

*Area under the ROC curves (AUC) matrix depicting AUCs of each marker for differentiating inbetween groups a) including neurological controls (NC), non-AD MCI, AD-MCI, AD dementia and non-AD dementia ;* ***b)*** *including NC, DLB, FTD and AD dementia. # and * indicate significantly different AUCs between plasma and CSF for one given marker.*

We then investigated the association of Nf markers with medio-temporal atrophy, rated visually on morphological MRI using Scheltens score and with white matter lesions evaluated with Fazekas scale (whole cohort, **Fig. 20, b-c, e- f;** subgroups analysis not pictured). All explored Nf markers were correlated with Scheltens score in the whole cohort. The association of CSF and plasma NfL with Scheltens score was respectively higher compared with CSF and plasma pNfH. CSF and plasma NfL remained both associated with Scheltens in the AD patients (CSF: β=0.265, P=0.012; plasma: β=0.246, P=0.022). Conversely, pNfH levels were not associated with Scheltens score in any subgroup. Regarding white matter lesions, only NfL measured in CSF and plasma correlated with Fazekas score. In the AD group, a weak correlation was observed between plasma NfL and Fazekas score (β=0.244, P=0.048). No association was found between pNfH and Fazekas score in the whole or in subgroups.

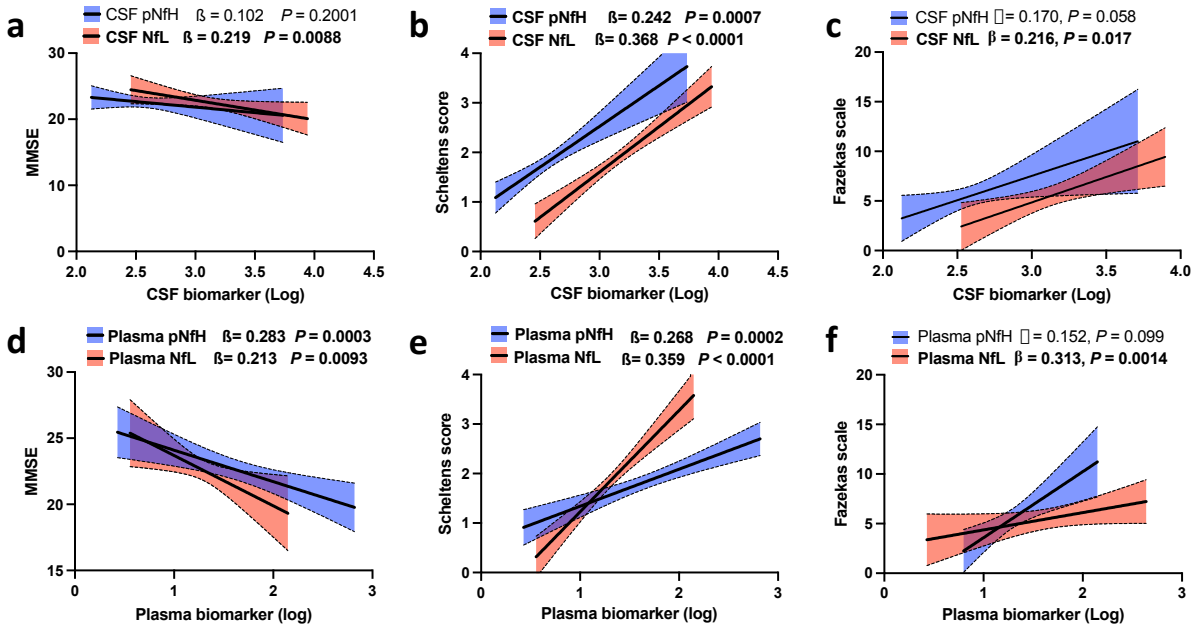



**Figure 20, Association of Nfs markers with cognition and imaging in the whole cohort**
*The association of our markers with MMSE, Scheltens and Fazekas scores was studied using linear regression, adjusted on age and sex.* ***a,d)*** *CSF NfL, plasma NfL and CSF pNfH were associated significantly with MMSE in the whole cohort.* ***b,e)*** *All four markers were associated with Scheltens score in the whole cohort.* ***c,f)*** *Only plasma and CSF NfL were associated to Fazekas score in the whole cohort.*

The present study explored the differential performances of NfL and pNfH in a cohort issued from clinical practice. Using ultrasensitive assays, we explored our biomarkers of interest in paired CSF and plasma samples from well-phenotyped patients representative of a memory clinic setting. Both pNfH and NfL levels were strongly associated in CSF and plasma. In CSF, our markers presented similar performance for distinguishing inbetween clinical groups. Plasma NfL performed similarly to CSF NfL to distinguish AD from non-AD dementia and displayed slightly higher AUCs than plasma pNfH. NfL levels also displayed a stronger association to cognitive score and to medio-temporal lobe atrophy. In conclusion, pNfH and NfL measured in CSF or plasma constitute potential markers of neurodegeneration for AD. Plasma NfL displayed equal performance as its CSF measure. Plasma pNfH did not outperform or present added value compared with plasma NfL.

## 4.3 Paper III

### 4.3.1 Rationale

Astrocytes demonstrate dynamic changes in response to AD pathology and enter a reactive state, the so-called reactive astrogliosis (285). This astrocytic activation is characterized by molecular, functional and morphological changes, defining the different astrocytes activation phenotypes (286). Changes in expression of astrocytic proteins, including GFAP, YKL-40 or S100B can be measured in brain tissue or in fluids of AD patients. Neuropathological investigations have shown the presence of reactive astrocytes overexpressing GFAP in close contact to Aβ plaques (287). Levels of GFAP in CSF are known to be consistently increased in AD. Additionally, CSF GFAP levels were found to inversely correlate with cognition evaluated with MMSE. However, plasma GFAP measurements have shown more variability. A significant heterogeneity can be found across studies in the size of the increase observed in AD. The specificity to AD compared to other neurodegenerative disorders has also been variable, especially since modifications in plasma GFAP levels are observed in acute neuronal injury. Moreover, if studies have investigated AD at various stages, at very early and advanced stages, no unique study has studied this biomarker along the whole spectrum. Finally, the differential association of plasma GFAP to amyloid and to tau pathology remains unclear.
In the **Paper III**, our goal was to characterize plasma GFAP as a biomarker of astrocytic activation in AD along the whole AD *continuum*, from preclinical to dementia and to study how it compares to CSF GFAP. We also further investigate its link with amyloid and tau pathology. Our clinical-based sample was leveraged to confirm findings in a less selected population.

### 4.3.2 Study

The **Paper III** reports the evaluation of plasma GFAP as a biomarker for AD diagnosis in a multicenter study encompassing the full AD *spectrum*. We measured CSF and plasma GFAP in a cross-sectional study on three cohorts: the TRIAD Cohort including 300 selected individuals comprised of cognitively unimpaired (CU) adults, and MCI and dementia patients, who had undergone tau and amyloid PET (MGill University, Canada); the ALFA+ cohort with 384 participants CU individuals at elevated risk for AD, explored with amyloid PET (Barcelona Beta Center, Barcelona, Spain). The last cohort was our memory clinic based cohort, Paris Cohort, from which 166 patients were analyzed, including cognitively impaired patients and neurological controls. Plasma and CSF GFAP levels were measured using the same Simoa platform at Gothenburg University, Sweden.
Plasma GFAP levels increased with age in the three cohorts and were higher in females after adjustment for age and diagnosis. No association was observed with ApoE4 after adjustment for Aβ status. There was a positive correlation, ranging from moderate to strong, between plasma and CSF GFAP levels in the 3 cohorts (*rho*=0.37–0.66, P<0.05).
Levels of plasma and CSF GFAP were higher across the AD *continuum* compared to controls and non-AD dementia (**Fig. 21).** Plasma GFAP levels increased in a stepwise manner along the AD *continuum*: in TRIAD and Paris cohorts, plasma GFAP increased continuously in AD-MCI (79–128% increase compared with controls) and AD dementia (107–133% increase compared with controls).
Plasma GFAP levels appear increased very early on in AD as levels were already higher at preclinical stage, before symptom onset: Aβ+ CU displayed higher levels than Aβ- CU in both TRIAD and ALFA + cohorts (32–54% increase compared to Aβ- CU).

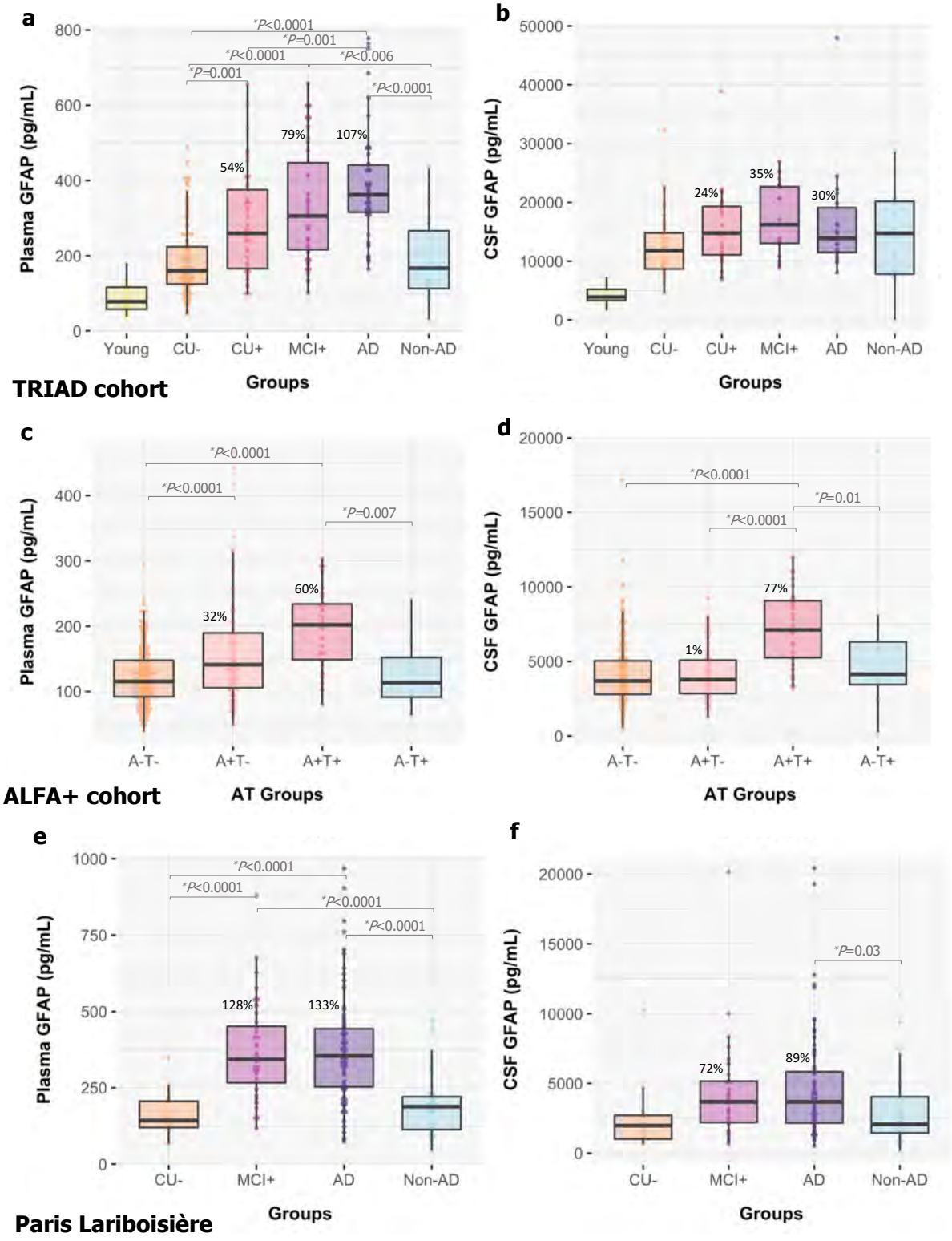



**Figure 21, Plasma and CSF GFAP levels group comparisons**

***a)*** *Plasma GFAP levels and* ***b)*** *CSF GFAP levels in TRIAD.* ***c)*** *Plasma GFAP levels and* ***d)*** *CSF GFAP levels in ALFA+.* ***e)*** *Plasma GFAP levels and* ***f)*** *CSF GFAP levels in Paris cohort. Group comparisons were computed with a one-way ANCOVA adjusted for age and sex. The Tukey's HSD test was used for post hoc pairwise comparisons.Fold changes are depicted for the AD continuum groups and were established against Aβ- CU individuals in TRIAD and Paris cohorts, and againts Aβ- and tau- CU in ALFA+ cohort as the reference groups.*

We observed higher increase sizes in plasma than in CSF: respective increases at AD-MCI and at AD dementia stages *vs* controls were of 79–128% and 107–133% in plasma compared to 35–72 % and 30–89 % in CSF. Plasma GFAP levels modifications appeared AD-specific in our study: patients with FTD and DLB in TRIAD and Paris cohorts displayed similar levels with controls (**Fig. 21**).

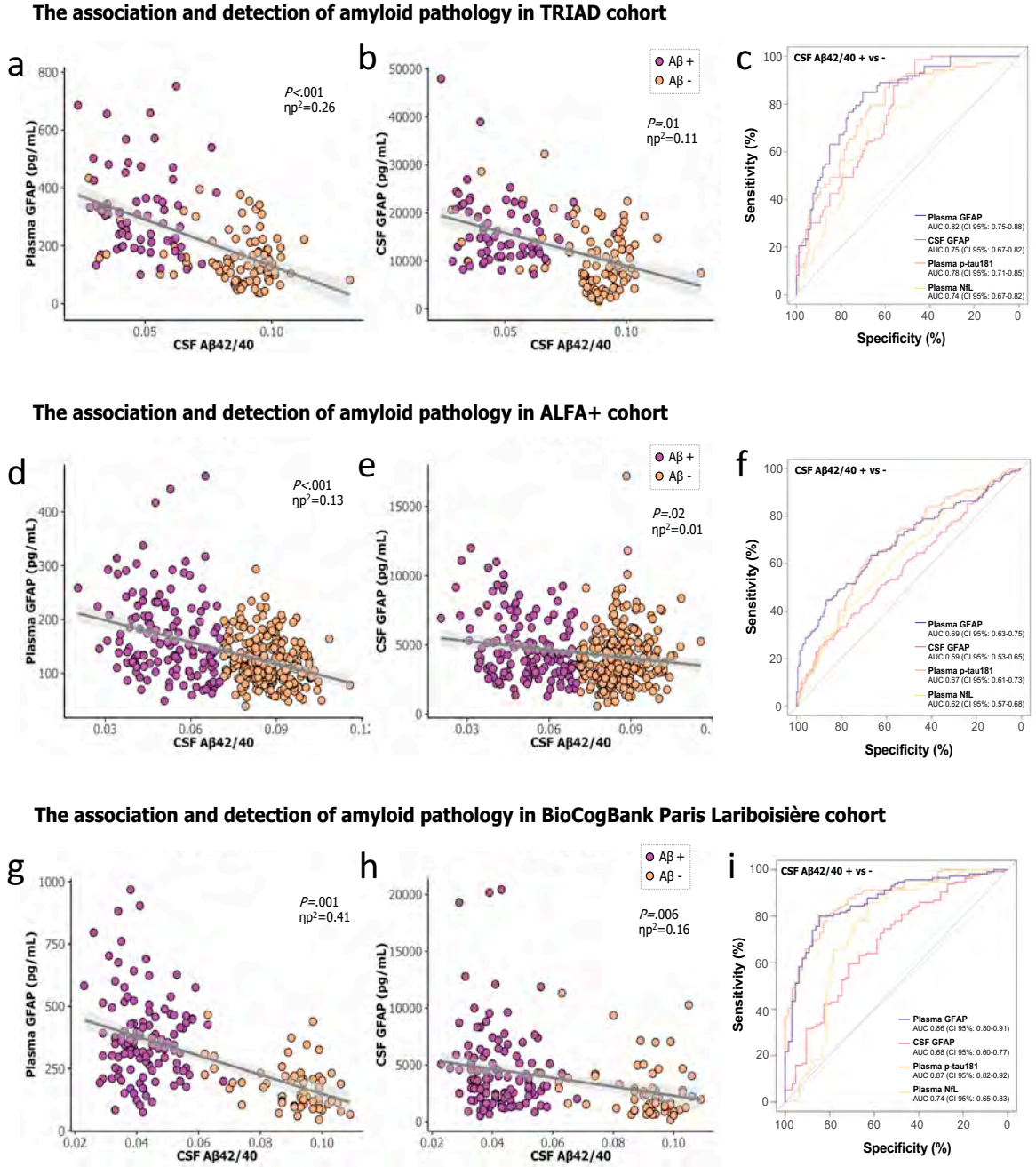



**Figure 22, Association of plasma and CSF GFAP protein levels with Aβ pathology and discriminative accuracies**

*Association of CSF Aβ42/40 ratio with* ***a)*** *Plasma GFAP levels and* ***b)*** *CSF GFAP levels in TRIAD;* ***d)*** *Plasma GFAP levels and* ***e)*** *CSF GFAP levels in ALFA+;* ***g)*** *Plasma GFAP levels and* ***h)*** *CSF GFAP levels in Paris cohort, computed with linear models adjusted by age, sex, and clinical diagnosis (the latter only for TRIAD and Paris cohorts). Performances of biomarkers to identify Aβ status in* ***c)*** *TRIAD cohort;* ***f)*** *ALFA+ cohort;* ***i)*** *Paris cohort was evaluated using ROC analysis and computing of AUCs.*

Plasma GFAP levels were associated both with amyloid and tau biomarkers (**Fig. 22**). Plasma GFAP correlated with CSF Aβ ratio in the 3 cohorts, within both Aβ+ and Aβ- subgroups. The strength of the association was similar in TRIAD and our memory clinic cohort (TRIAD, *ηp2*=0.26; Paris Cohort, ηp2=0.41). Plasma GFAP levels were also associated with amyloid load measured with PET in TRIAD and ALFA +. Interestingly, the association of plasma GFAP with Aβ markers was stronger than that of CSF.

We then investigated how plasma and GFAP levels identify Aβ status using ROC analysis (**Fig. 22)**. Plasma GFAP discriminated Aβ+ from Aβ- individuals (defined by PET or CSF) with high AUCs around between 0.82–0.86, even at preclinical stage. Performance in our clinical samples was similar to the performance in the TRIAD cohort (Paris Cohort, AUC=0.86 *vs* the TRIAD cohort, AUC=0.82). In comparison to plasma, CSF displayed poorer performance, especially at preclinical stage (AUC=0.59–0.75). In the three cohorts, AUC for plasma GFAP to identify Aβ status was similar to the one of plasma p-tau and better than plasma NfL. Interestingly, the combination of plasma GFAP with plasma p-tau181 improved accuracy to discriminate Aβ status (Paris cohort: combination of plasma GFAP and p-tau181, AUC=0.93 *vs* plasma GFAP sole, AUC=0.86, P<0.05).

Regarding tau markers, plasma GFAP significantly associated with p-tau in CSF and plasma in the three cohorts and with tau PET load in the TRIAD cohort.

Next, we performed a mediation analysis to investigate the association of plasma GFAP with amyloid and tau and to assess if these were mediated by Aβ status (**Fig. 23**). In the 3 cohorts, the association of plasma GFAP with tau (measured by CSF or PET) was mediated by Aβ, for around 60% of the association. In our Paris sample, the association of CSF p-tau with plasma GFAP levels was mediated by CSF Aβ ratio, with accounting indirectly for 63% of the plasma GFAP-CSF p-tau association.

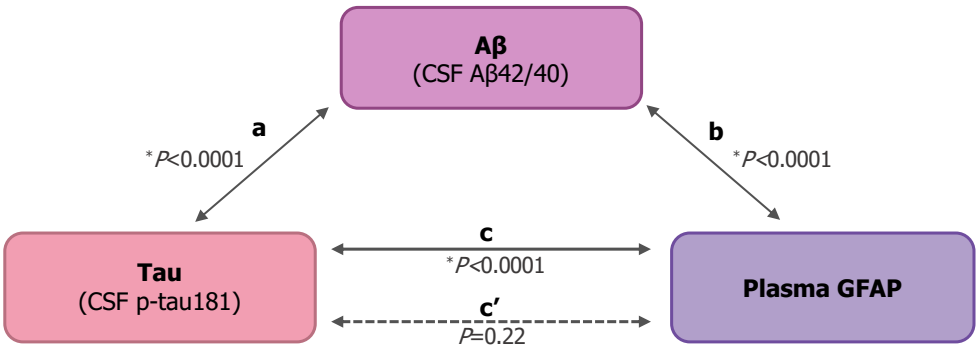



**Figure 23, Mediation analysis of Aβ and tau association with plasma GFAP**
*In mediation analysis, CSF p-tau181 and plasma GFAP levels association lost significance (**c'**, P=0.22) after adjustement for CSF Aβ ratio effect (**a-b**, indirect effect of CSF Aβ ratio evaluated at 63 % of the association, P<0.0001).*

Overall, this study brings evidence that plasma GFAP is a specific biomarker for AD pathology. Plasma GFAP levels increased in a stepwise manner throughout the AD *continuum*. They showed a strong correlation with Aβ measured in CSF or with PET. The stronger correlation with Aβ than with tau, and the results of the mediation analysis further strengthened the idea of an Aβ-induced astrocytic activation. Importantly, plasma GFAP levels showed similar performance in our clinical-based cohort samples issued from routine care. We also showed that plasma GFAP could increase diagnosis performance when combined with other plasma biomarkers such as plasma p-tau. Plasma GFAP could successfully contribute to a non-invasive AD diagnosis in a blood biomarker panel or to screening in primary care.

## 4.4 Paper IV

### 4.4.1 Rationale

Evidence from transcriptomic data and from genetic studies has highlighted the role of the innate immune system in AD (288,289). At a pathological level, reactive microglial cells surrounding amyloid plaques are a hallmark lesion of AD pathology. Indeed, microglia is known to be activated in AD. It has been demonstrated to display a specific AD phenotype, called disease-associated microglia, or DAM (290). There is evidence that Gal-3, a beta-galactosidase expressed in the microglia, participates to that DAM phenotype (291). A recent study by Boza-Serrano *et al*., demonstrated several facts pointing to the key role of Gal-3 in microglial activation in AD: i/Gal-3 was upregulated in the AD cortex, ii/in immunostaining, Gal-3 clustered around Aβ plaques and iii/in AD mouse models, the lack of Gal-3 lessened Aβ burden and improved cognition (262). Interestingly, CSF Gal-3 can be measured in plasma and CSF, and it has been explored as a biomarker in various diseases, including CNS disorders (292). However, to our knowledge, it had not been evaluated before in AD with confirmed underlying evidence of amyloid pathology.
The aim of this clinico-pathological study was double: first, to further explore Gal-3 relation to amyloid pathology in human AD brain; secondarily, to explore if CSF Gal3 levels could constitute an AD biomarker.

### 4.4.2 Study

The first part of this study analyzed humain brain tissue in 10 controls and 20 AD patients (11 early onset AD cases and 9 genetic AD cases). Using Elisa then Western blot, Gal-3 levels were found to be elevated in AD samples compared to controls. The increase was higher in hippocampus than in cortex. Levels did not differ between genetic and early onset AD.
Immunohistochemistry identified a population of microglial cells clustered around amyloid plaques with high Gal-3 expression. It could be distinguished Gal-3 positive and Gal-3 negatives plaques, with different morphology (**Fig. 24**). Moreover, we also found Gal-3 positive microglial cells in contact with p-tau agregates in senile plaques.This results confirmed the upregulation of Gal-3 in cortex and hippocampus in AD and its association to amyloid and senile plaques.

To complement these findings, we explored Gal-3 CSF levels and other CSF biomarkers in AD patients. 155 participants were recruited from our memory clinic, including n=36 controls (with normal CSF profile) and n=119 AD patients (with AD CSF profile). CSF Gal-3 levels were measured using an Elisa commercial kit.
CSF Gal-3 levels were higher in AD compared to controls (P=0.030), conversely to CSF sTREM2 levels which did not differ between AD patients and controls (**Fig. 25, a-b**). Gal-3 displayed a moderate AUC (0.79) to discriminate AD cases from controls, similar to CST sTREM 2 (AUC=0.78). We then studied its association to other CSF neuroinflammation biomarkers. CSF Gal-3 was moderately correlated with sTREM2, GFAP and YKL-40 levels in CSF (*rho*=0.326–0.378). These correlations were also observed in the AD subgroup.
CSF Gal-3 correlated with core AD CSF markers in the whole cohort, with no significant difference in the association with Aβ ratio and p-tau (*rho*=|-0.248–0.362) and similar results were found focusing on the AD group. Interestingly, CSF Gal-3 was also correlated to CSF synaptic markers neurogranin and GAP-43 in the whole cohort and in subgroups.

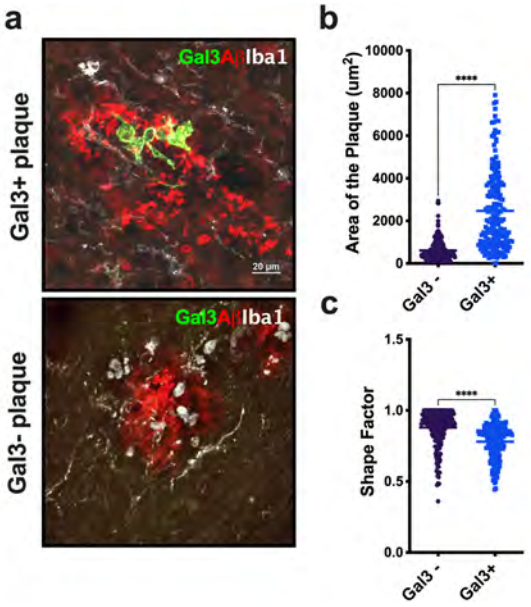



**Figure 24, Identification of Gal-3 positive and Gal-3 negative plaques**

***a)*** *Gal-3 positive microglial cells were associated with larger and irregular amyloid plaques.* ***b)*** *Gal-3 positive plaques were larger than Gal-3 negative plaques.* ***c)*** *Gal-3 positive plaques were more irregularely shaped. Non-parametric t-tests were performed. ****P<0.0001. (n=3 (healthy controls), n=8 (AD). Gal-3-negative plaques, n=212; Gal-3-positive plaques, n=197)*

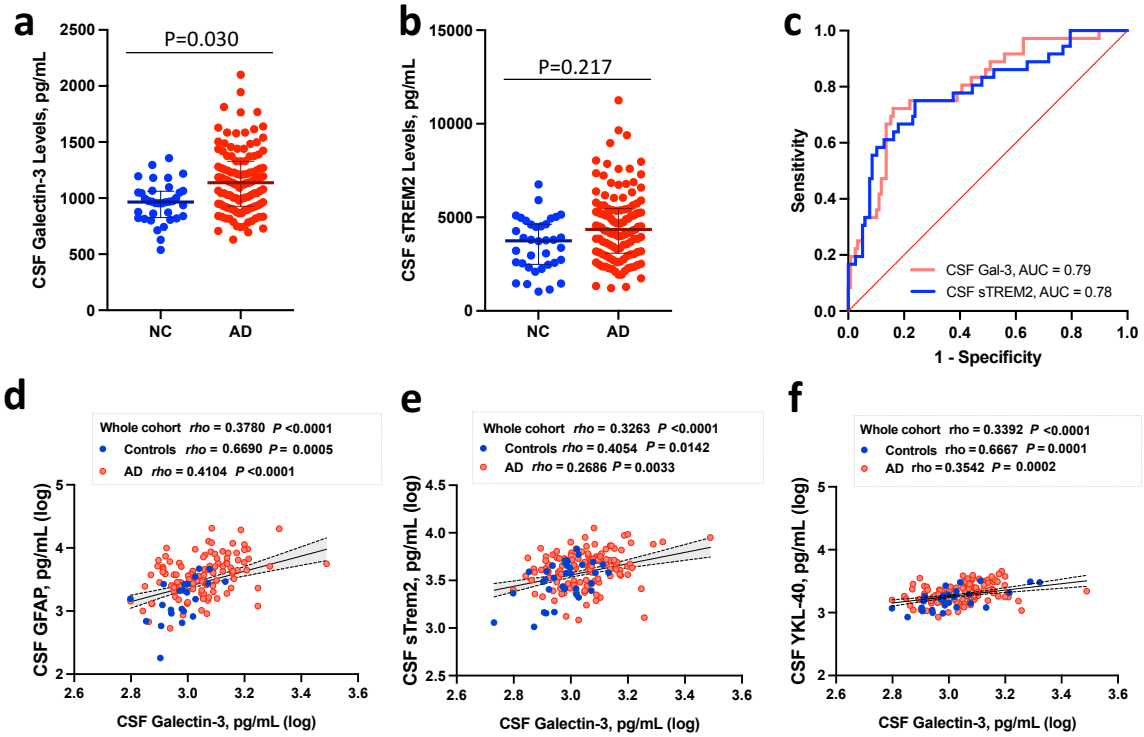



**Figure 25, CSF Gal-3 levels, diagnostic performance and association to neuroinflammation CSF markers**

***a)*** *CSF Gal-3 levels and* ***b)*** *sTREM2 levels were compared between NC and AD patients using one way ANCOVA adjusted on age and sex.* ***c)*** *CSF Gal-3 and sTREM2 levels accuracy to discriminate AD from NC using ROC analysis. CSF Gal-3 Spearman rho correlation with CSF neuroinflammation markers:* ***d)*** *CSF GFAP,* ***e)*** *CSF sTREM2 and* ***f)*** *CSF YKL-40.*

Regarding cognition, an inverse U-shaped relation was observed between MMSE and Gal-3 CSF levels in AD patients, with higher levels being found in subjects with intermediate MMSE. Lower Gal-3 levels were observed in patient with high and low MMSE respectively.
Lastly, we performed principal component analysis to explore the relations between these biomarkers (**Fig. 26**). We could identify two principal components explaining 71% of the variance in the data. A 'core AD component' clustering AD core CSF biomarkers and synaptic markers explained 56% of the variance whereas CSF Gal-3 clustered with GFAP, sTREM2 and YKL-40 in a 'neuroinflammation component', accounting for 14% of the variance. Core AD component differentiated AD from controls whereas the neuroinflammation component did not significantly differ between the two groups. However, there was a stepwise increase in the neuroinflammatory component along the *AD continuum* (component 2 was higher in A+T+N+ CSF profile group compared to A+T-N- CSF profile group).

**a**

| | Component 1 | Component 2 |
|---|---|---|
| **Variance explained** | 56 % | 14 % |
| **Eigen value** | 5.0 | 1.3 |
| **Loading values** | | |
| Gal-3 | | 0.769 |
| sTREM2 | | 0.767 |
| YKL-40 | | 0.566 |
| GFAP | | 0.754 |
| Aß40/42 ratio | 0.842 | |
| p-tau | 0.898 | |
| t-tau | 0.869 | |
| GAP-43 | 0.720 | |
| neurogranin | 0.818 | |

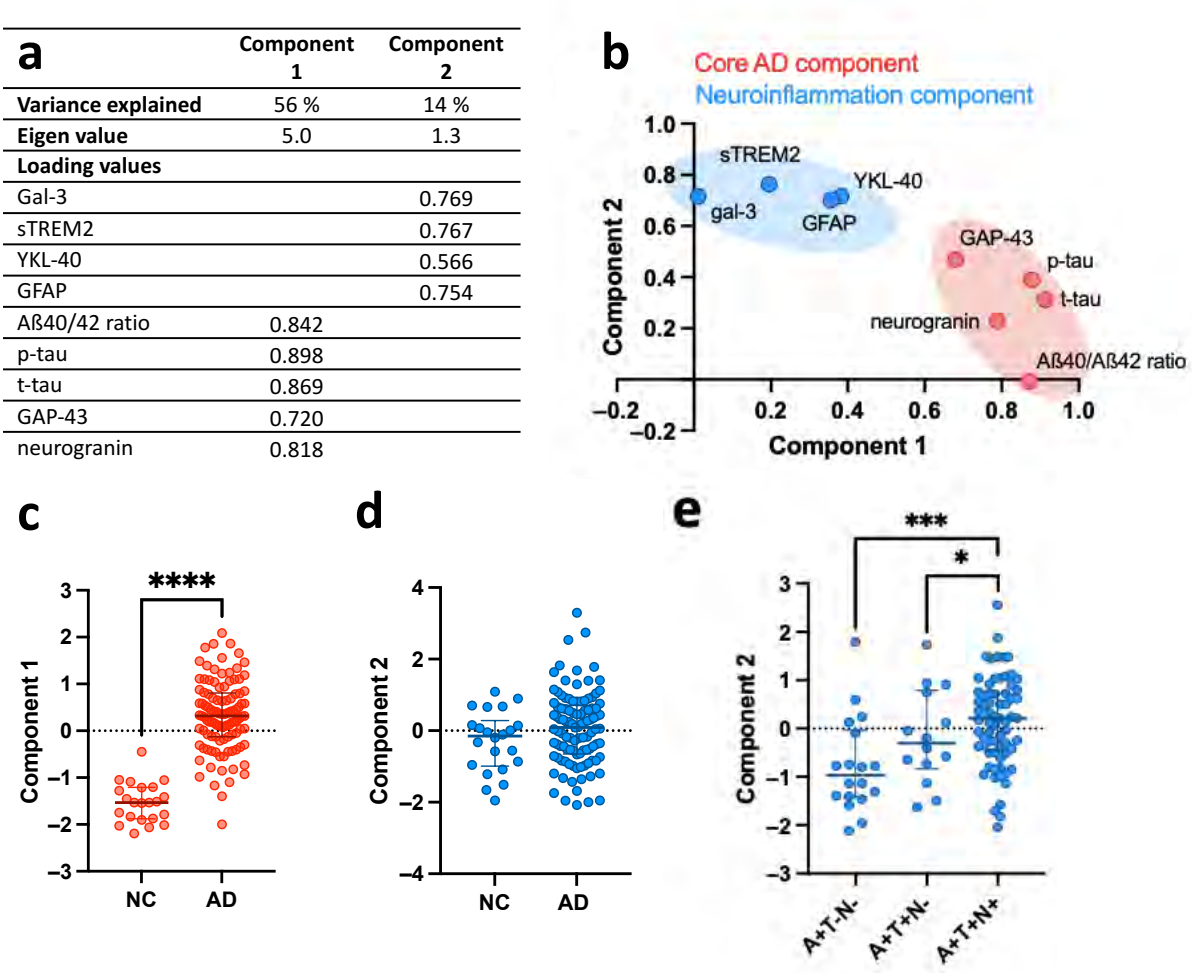



**Figure 26, Clustering of AD and neuroinflammation CSF biomarkers in principal component analysis**

***a-b)*** *We performed a principal component analysis in the whole cohort, after exclusion of outliers. CSF Gal-3 clustered in a "neuroinflammation component" with CSF GFAP, sTREM2 and YKL-40. Core AD CSF biomarkers and synaptic markers correlated in an "core AD component".* ***c)*** *Component 1 "core AD component" levels in neurological controls (NC) and AD patients.* ***d)*** *Component 2 "neuroinflammation component" levels in NC and AD patients. Components were compared between AD and NC using one way ANCOVA adjusted on age and sex.* ***e)*** *Component 2 was similarly compared across the ATN groups in the AD continuum.*

In conclusion, our results support the implication of Gal-3 in AD physiopathological process, promoting a microglial activation which is satellite of the amyloid deposits. The other important finding of this study is that CSF Gal-3 displays potential as a biomarker to monitor microglial activation in AD. Indeed, CSF Gal-3 levels were higher in AD patients and were associated with AD core CSF biomarkers. In principal component analysis, CSF Gal-3 clustered in a neuroinflammatory component with other CSF neuroinflammation biomarkers. CSF Gal-3 did not appear, however, to be useful for positive diagnosis of AD.

## 4.5 Paper V

### 4.5.1 Rationale

Blood-based biomarkers are now available in AD and are suggested as an alternative to CSF biomarkers for investigation of amyloid, tau and neurodegeneration status. Plasma Aβ42/40 ratio has been demonstrated to be altered in preclinical AD and to identify amyloid status in cognitively unimpaired individuals (129). Plasma measurements of p-tau181, p-tau217 or p-tau231 demonstrated overall high diagnostic accuracy in differentiating AD from other NDDs in research studies (171–173). In several studies, the performance of plasma p-tau biomarkers was comparable or only minimally inferior to established CSF p-tau measurements. Plasma GFAP, although a marker of astrocytic activation, is highly associated to amyloid status across the whole AD spectrum, as reported in the **Paper III** (243)**.** Plasma NfL (**Paper II**) and to a more limited extent, plasma t-tau, accurately detect neurodegeneration (201). However, all these promising findings still need additional confirmation in the different populations where they could be implemented, *i.e*, in primary care, in specialized memory clinics or for use in clinical trials. In memory clinic practice, it is not yet sure if these biomarkers should be used as stand-alone tests, in a panel combining several biomarkers, or as an aid for the decision to perform CSF analysis, or rather for screening for referral to the clinic.
The aim of our study was to perform a head-to-head comparison of plasma biomarkers for AD in a cohort issued from a memory clinic, outside of a standardized research setting. We studied their accuracy to distinguish AD from non-AD related cognitive impairment, defined biologically by CSF biomarkers. Additionally, we studied their association to cognition and imaging.

### 4.5.2 Study

We included samples and data from a total of 203 patients of our memory clinic including neurological controls (NC, n=22), AD-MCI (n=42) and non-AD MCI (n=37), AD-dementia (n=71) and non-AD dementia (n=31 in total, including DLB, n=14; FTD, n=12; VaD, n=4; CJD, n=1). We measured a panel of biomarkers, in plasma and in CSF: Aβ42/Aβ40 ratio, p-tau181, p-tau231, t-tau, NfL and GFAP. All plasma biomarkers were measured on the Simoa platform. The core AD biomarkers (CSF Aβ 42/40, p-tau181 and t-tau) measured using the clinically validated Lumipulse® platform were considered as the gold-standard.
The age of the patients affected the levels of all plasma markers (*rho*= 0.229–0.430, P<0.005) except for plasma t-tau (*rho*=-0.055, P=0.434). Plasma GFAP levels were higher in women than in men in unadjusted analysis (P<0.001).
In the whole cohort, the Spearman *rho* correlation coefficients between the corresponding plasma and CSF biomarkers levels were: 0.362 for Aβ42/Aβ40 ratio, 0.733 for p-tau181, 0.585 for p-tau231, 0.155 for t-tau, 0.666 for NfL and 0.422 for GFAP (**Fig. 27**). No association between CSF and plasma levels remained for Aβ42/Aβ40, t-tau and GFAP looking at AD subgroups. Significant correlations remained between CSF and plasma levels in both AD-MCI and AD-dementia groups for p-tau181, p-tau231 and NfL.

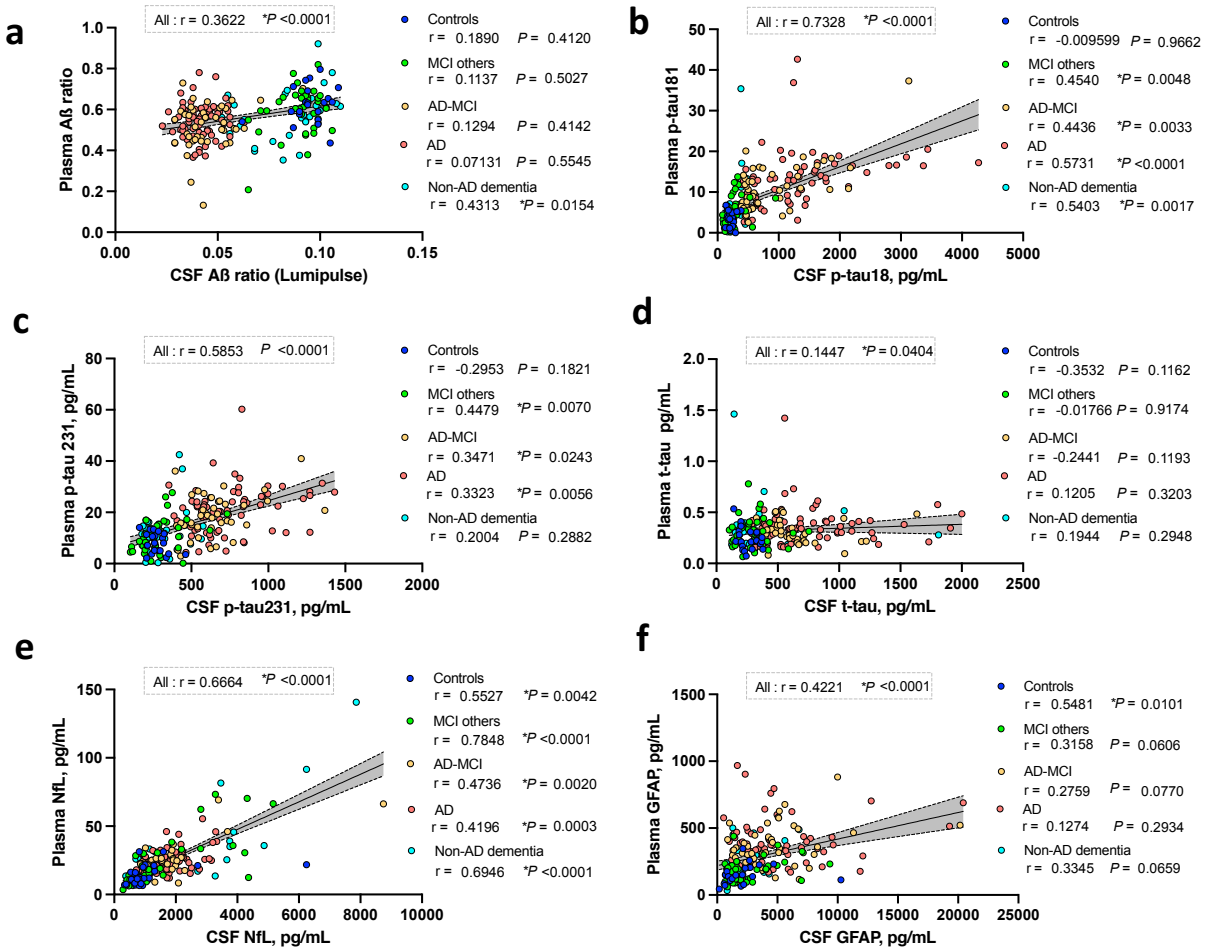



**Figure 27, Biomarker's correlation between plasma and CSF levels**
*Correlation between CSF and plasma levels were computed for each biomarkers, in the whole cohort and in subgroups, using Spearman rho correlations, including including* ***a)*** *Aß42/Aß40 ratio;* ***b)*** *p-tau181;* ***c)*** *p-tau231;* ***d)*** *t-tau;* ***e)*** *NfL and* ***f)*** *GFAP. All plasma markers were associated with their CSF counterpart. The strongest associations were found for p-tau181, NfL, p-tau231 and GFAP.*

Plasma Aβ42/Aβ40 ratio was lower in AD-MCI and AD-dementia compared to NC (P<0.05, **Fig. 28**). It was lower in AD-MCI *vs* non-AD MCI (P=0.001). However it did not differ between AD-dementia and non-AD dementia. Plasma p-tau181 and plasma p-tau231 displayed similar patterns: levels were significantly higher in AD-MCI and in AD-dementia compared to all non-AD groups (p-tau181: P<0.001; p-tau231, P<0.002). Levels did not significantly differ between NC, non-AD-MCI and non-AD dementia. Plasma t-tau levels were higher at dementia stages, in both AD and non-AD dementia, compared to NC (P<0.020). Plasma NfL levels were higher in AD-MCI, AD-dementia and non-AD dementia compared to NC (overall P<0.001). Both AD-MCI and AD-dementia groups displayed higher levels compared to non-AD MCI (P<0.015). Plasma GFAP levels were higher in AD-MCI and AD-dementia compared to all non-AD groups (overall P<0.001). Levels did not significantly differ between non-AD groups.

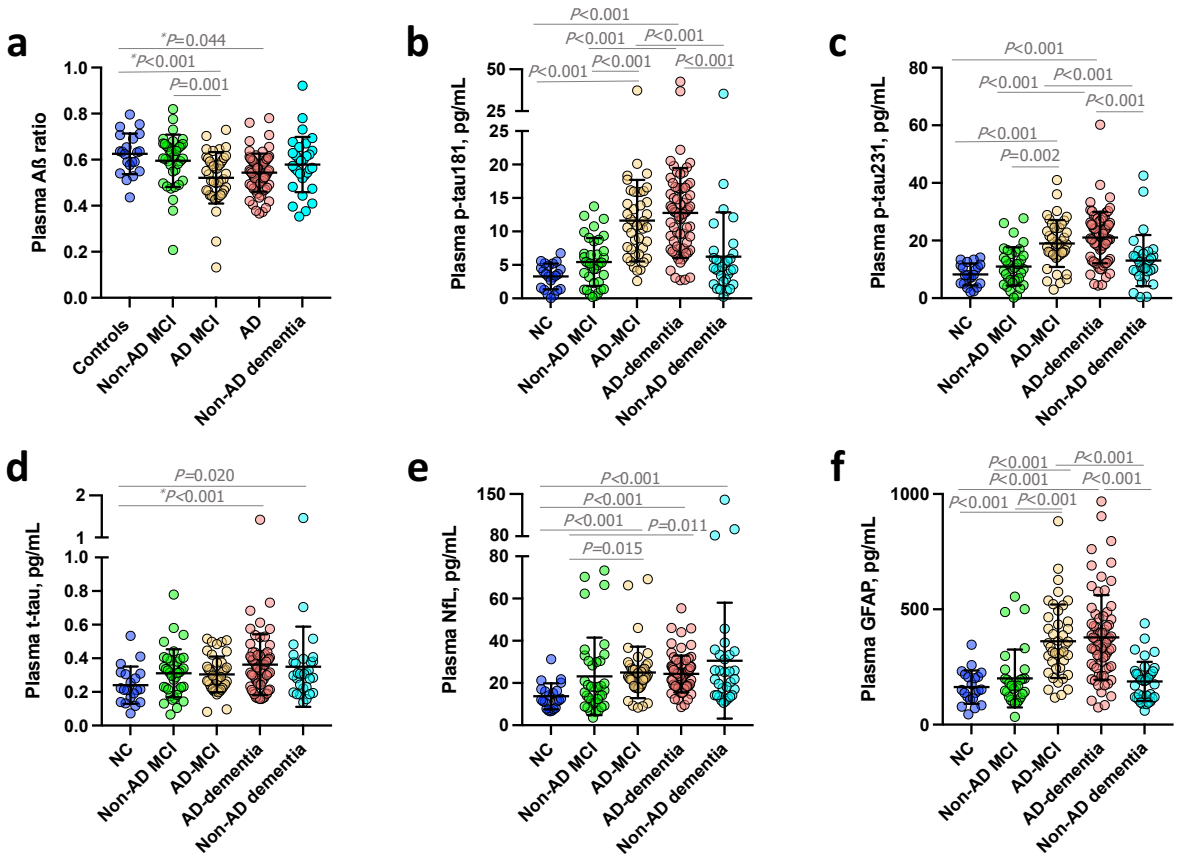



**Figure 28, Levels of plasma biomarkers across groups**

*Levels of plasma biomarkers were compared across groups using a one-way ANCOVA with post-hoc Tukey's test, adjusting on age and sex, including* ***a)*** *plasma Aβ42/Aβ40 ratio;* ***b)*** *plasma p-tau181;* ***c)*** *plasma p-tau231;* ***d)*** *plasma t-tau;* ***e)*** *plasma NfL;* ***f)*** *plasma GFAP.*

We first studied our plasma biomarkers performance as stand-alone tests to differentiate inbetween groups (**Fig. 29**).

To distinguish between AD and controls, the highest AUCs were obtained for plasma p-tau181 (0.95), plasma p-tau231 (0.92) and GFAP (0.92). More moderate AUCs were yielded by plasma Aβ42/Aβ40 (0.81), t-tau (0.84) and NfL (0.87). To differentiate AD-dementia from non-AD dementia, plasma p-tau181 (0.85), p-tau231 (0.82) and GFAP (0.81) still performed the best compared to plasma Aβ42/Aβ40 ratio, t-tau and NfL (0.69–0.72).The same plasma markers performed the best to differentiate AD-MCI from controls, as in dementia stage: p-tau 181 (0.92), p-tau231 (0.89) and GFAP (0.91). Plasma markers displayed overall lower AUCs to distinguish AD-MCI from non-AD-MCI: p-tau181 (0.84), p-tau231 (0.81) and GFAP (0.85) and Aβ42/Aβ40 ratio, t-tau and NfL (0.66–0.74).

The combination of plasma biomarkers increased their diagnosis value (**Fig. 30**). We performed stepwise backward regressions to identify the best and most parsimonious combination. As plasma p-tau181 and p-tau231 showed high correlation to one another (Spearman *rho*=0.72), only one plasma p-tau measure was included in the models. The p-tau markers revealed interchangeable, yielding similar AUCs.

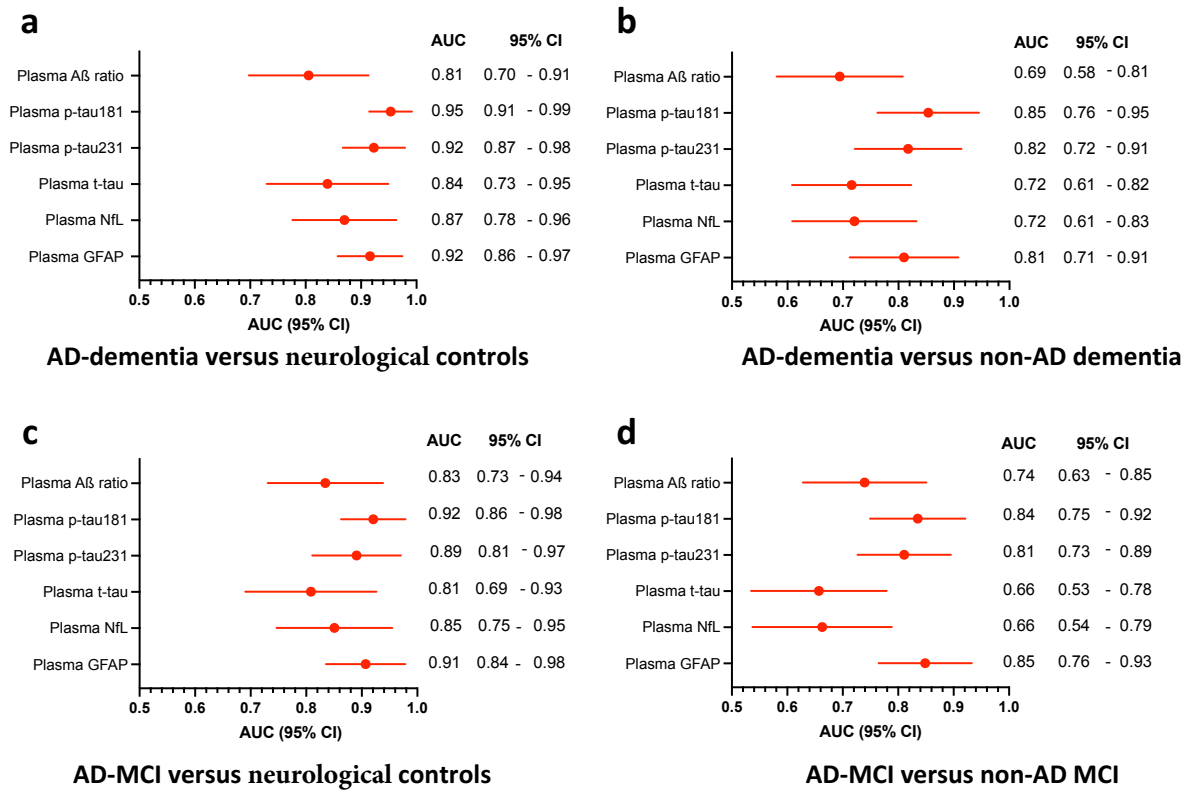



**Figure 29, Plasma biomarkers accuracy in distinguishing diagnosis groups**
*Forest plot-of markers area under the curve (AUC) in distinguishing* ***a)*** *AD-dementia vs controls;* ***b)*** *AD-dementia vs non-AD dementia;* ***c)*** *AD-MCI vs controls and* ***d)*** *AD-MCI vs non AD-MCI. Accuracy of each biomarkers was studied using ROC curve analysis.*

To distinguish AD from NC, the association of p-tau181 and NfL yielded a high performance (0.97). To distinguish AD-dementia from non-AD dementia, the association of p-tau181 and NfL (0.87) did not differ statistically from the association of p-tau181, NfL and GFAP (0.91). To differentiate AD-MCI from NC, the association of p-tau181 and NfL showed a high performance (0.93). To discriminate AD-MCI from non AD-MCI, it was the combination of p-tau181, GFAP and NfL that showed the best potential (0.91). All these combinations increased the diagnosis performance compared to individual markers.
Lastly, we investigated the association of our biomarkers with cognition and medio-temporal lobe atrophy. In plasma, the following plasma markers showed an association with MMSE: GFAP (β=-0.249), t-tau (β=-0.248), p-tau231 (β=-0.163) and p-tau181 (β=-0.138). Regarding medio-temporal atrophy, several plasma markers were associated with Scheltens score: NfL (β=0.321), GFAP (β=0.284), p-tau181 (β=0.155) and p-tau231 (β=0.150).

In this study, we investigated a panel of plasma and CSF biomarkers of AD pathophysiology and their relationship with cognition in our memory-clinic based cohort. We observed that p-tau231, p-tau181 and GFAP measured in plasma displayed high specificity to AD in our cohort of unselected patients. Combination of biomarkers associating mainly p-tau231, p-tau18, GFAP and NfL improved AD diagnosis. Mostly, the association of a plasma p-tau measurement (p-tau231 or p-tau181) with plasma NfL could discriminate the best inbetween AD and non-AD related MCI and dementia. Moreover, the same markers associated to cognition and imaging measured with tools available in clinical routine.

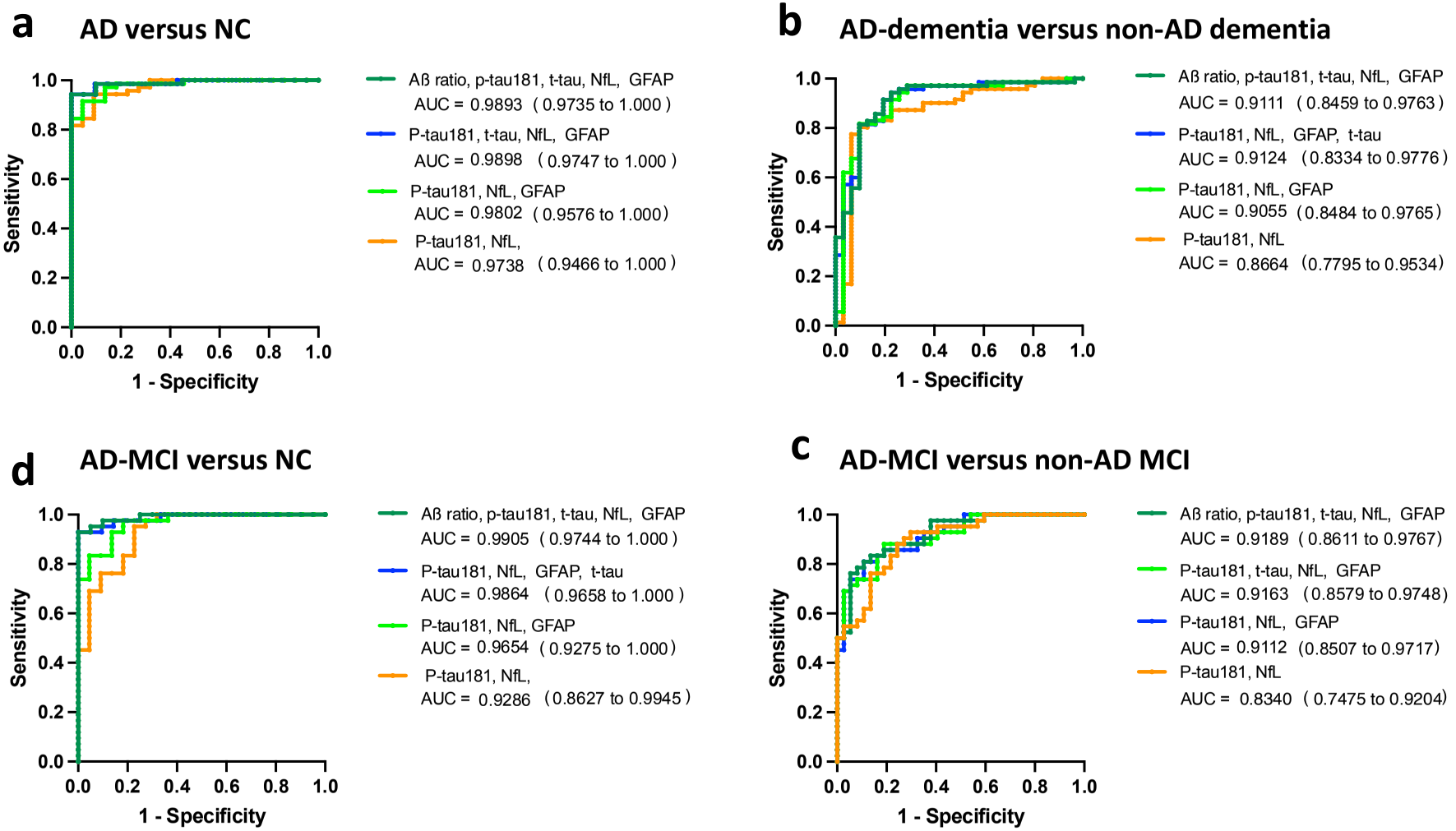



**Figure 30, Plasma biomarkers combinations accuracy in distinguishing diagnosis groups**

*Plasma biomarkers were combined using backward stepwise linear regression to select the best combination to distinguish in between* ***a)*** *AD-dementia and controls;* ***b)*** *AD-dementia and non-AD dementia;* ***c)*** *AD-MCI and NC;* ***d)*** *AD-MCI and non-AD MCI. The use of plasma p-tau231 instead of p-tau-181 in the differents models showed similar results.*

# 5. GENERAL DISCUSSION

Neuropathological studies, and, more recently, biological and imaging studies, have demonstrated that in absence of the use of specific biomarkers, the diagnosis error rate in AD dementia diagnosis was about 30% (170,293,294). In anti-amyloid trials, it constituted a major issue with the inclusion of 30% of non-AD patients without the therapeutic target confounding the results (295). Accurate and timely diagnosis is a crucial stake both in clinical practice for the information and the management of the patient and in research. Since the use of amyloid positive, AD diagnosis was clearly improved in clinical practice and its use as criteria for inclusion in anti-amyloid trials, the difference between treated and placebo groups have evolved positively even if it is not yet significant enough. In parallel, in research, other fluid biomarkers have significantly contributed to improving our understanding of the pathophysiology of AD and other dementias and are opening wide possibilities for the validation of new biomarkers reflecting various aspects of NDDs.

However, significant gaps remain to be filled. In the clinical use, the set-up of accessible (with large availability and technically simple) and reliable validated biomarkers is a priority. Apart from diagnosis, these are needed to perform accurate differential diagnosis, to predict the evolution of the patient and to biologically evaluate the therapeutic impact of modifying therapies. In clinical research, biomarkers to objectify the presence of AD pathological lesions and to monitor the engagement of the target are indispensable as well as to evaluate other processes (synaptic and axonal degeneration, inflammation…). Furthermore, new biomarkers have to be validated in daily clinical practice. New markers are often discovered and validated in a very selected homogeneous population with clear diagnoses and strict selection criteria, to demonstrate their potential as a biomarker. However, when the test is more widely applied, its diagnostic performance is susceptible to reveal itself lower, and sources of variability arising in 'real-life' need to be identified. Validation of novel markers for dementia will require these several points to be further examined to allow for their clinical use and implementation in the various clinical settings.

This thesis takes place in this 'iterative process of fluid biomarker development and validation' for dementia (296). The studies presented in this manuscript explored blood and CSF biomarkers of both amyloid and tau processes and of other processes in a real-world memory clinic cohort. **Papers I** and **IV** explored in blood and CSF, two novel candidate biomarkers, respectively for synaptic impairment and neuroinflammation. **Papers II** and **III** explored plasma biomarkers of neurodegeneration and of astrocytosis for use in clinical settings. Lastly, **Paper V** compared a panel of plasma biomarkers for AD positive and differential diagnosis.

## 5.1 Moving forward to blood

The pressing need of easily usable biomarkers of NDDs led us to move from CSF to blood biomarkers offering better accessibility. However, to what extent blood biomarkers constitute an accurate proxy for CSF biomarkers is not entirely established.
In our work, we explored different plasma biomarkers reflecting various pathophysiological processes to determine if they were a good reflect of CSF results. In summary, plasma Aβ measurements, t-tau and GFAP displayed a weak association with their CSF levels while plasma p-tau measurements (p-tau181, p-tau231), plasma NfL and pNfH had a stronger one.

Blood is a highly complex biofluid, containing a wide range of different molecules, including proteins, peptides, nucleic acids, lipids and metabolites. Each of the different cellular compartments of blood (erythrocyte, lymphocytes, platelets) is a potential source of markers

of interest and can introduce variability into measurements. Conversely, CSF is a relatively acellular and contained environment. Moreover, the BBB passage may also induce variability, all the more as there is evidence of BBB impairment in AD (297,298).
Our results show that our biomarkers are variably affected upon their metabolism in blood. Our data on plasma Aβ adds evidence to the already existing literature on the moderate association of Aβ plasma levels to their CSF counterpart (163). Blood Aβ levels most likely reflect the Aβ metabolism of both the CNS and in the periphery. It is established that Aβ clearance exists in the periphery, mostly through the liver, kidneys and gastrointestinal tract (299). It has also been shown that vascular cells produce and release Aβ into the blood (300).
The type of the assay might also affect the concordance between plasma and CSF levels, as plasma Aβ measurements with mass spectrometry display higher association to CSF compared to Simoa or other immunoassays, most likely through a lower sensitivity to matrix effect (163,164).
Similarly, plasma t-tau levels showed weak or no association with their CSF levels, in line with the previous literature. This is suspected to be related to a differential expression of tau fragments in each compartment. Firstly, tau fragments are known to be sensitive to peripheral degradation (301). Secondly, tau also originates from the periphery (heart, testis) and CNS-derived tau is estimated to represent 20% of plasma tau (302,303).
GFAP, which is a brain and BBB specific marker, displayed low correlation between plasma and CSF levels, both in **Paper III** and **Paper IV**, but surprisingly plasma GFAP seemed to have more discriminative power in NDDs than that of CSF GFAP. This was previously reported both in dementia and in other neurological disorders, such as MS (238). Astrocytes, that release GFAP, are a component of the BBB, which is altered in AD and thus may modify its release in plasma (304). The possibility of a direct lymphatic release through astrocytes end-feet has also been discussed (305). Finally, a recent study has reported a higher sensitivity of CSF GFAP to pre-analytical conditions (especially the number of freeze/thaw cycles), than that of plasma GFAP (306). A 'hook effect' through protein aggregates has also been discussed as an explanation for the stability of GFAP in plasma (307).
Conversely, in **Paper IV**, plasma p-tau measurements showed high association to their CSF levels. This was already reported in large studies (171,173). Conventional immuno-assays initially lacked the sensitivity to detect the very small amounts of p-tau available in blood. Using mass spectrometry, a study by Barthélémy *et al*., have investigated CSF and plasma tau isoforms' profile relationships (303). Truncated tau fragments, but no full-length tau, were both detected in CSF and plasma, matching other prior studies (120). CSF and plasma p-tau217 and p-tau181 levels were found to correlate whereas it was not the case for t-tau. The new immunoassays available target tau fragments with phosphorylation at specific epitopes, to insure a good specificity to CNS of p-tau markers (308). Nevertheless, as discussed below, plasma p-tau levels have been reported to be impacted by hepatic and renal functions, similarly to t-tau, indicating that it can be sensitive to renal clearance and circulating albumin levels (309).
Similarly, plasma NfL and pNfH levels showed a significant correlation with their CSF levels in line with existing studies, despite being several fold lower than their CSF levels (310,311). NfL levels have been shown to increase in the plasma through a passive transport over the BBB (312). To be noted, plasma pNfH levels displayed a weakest association to their CSF levels than NfL in **Paper II**. Hypotheses include differential BBB passage and alterations of pNfH in blood levels, promoted by phosphorylation (313).

All in all, our results show that plasma biomarkers differentially reflect CNS changes and that they can be sensitive to non-neurological peripheral changes and blood matrix effect. This should be kept in mind when framing each biomarker purpose for clinical use.

## 5.2 Exploring non-amyloid and non-tau processes

So far, in clinical practice, biomarkers for prognosis and follow-up in AD on one hand, and for diagnosis and prognosis for non-AD NDDs, on the other hand, remain an unmet medical need. In this thesis, one of our aim was to explore biomarkers of non-amyloid and tau processes that would inform both AD and non-AD dementia mechanisms and that could contribute to establishing diagnosis and prognosis.
As stated in the introduction and as demonstrated in our results, several processes involved in AD are accessible using biological biomarkers such as synaptic and/or axonal damages and neuroinflammation.

Regarding synaptic process, it is a very complex and precise machinery in which many molecules (pre-synaptic and/or post-synaptic) could become potential biological biomarkers. Currently, in research, numerous potential biological biomarkers have been studied mainly in the CSF, in a double attempt to find biomarkers of the underlying pathology and to explore pathological mechanisms (211). It has been established that CSF synaptic biomarker levels are correlated with brain synaptic damage. However, while some are used in clinical trials (such as neurogranin), none of them are validated for daily clinical practice.
Several key questions remain to be addressed regarding their validation. Technically, as for other biomarkers, a blood biomarker would be crucial and the validation of sensitive, specific, reproducible and easy-to-use assays will be necessary for introduction in clinical settings. Additionally, we will have to define 1/what would be the objective of those biomarkers in clinical practice, 2/what is the exact nature of the unique or multiple changes that the different markers reflect in AD and other NDDs, and which marker and assay would be the most informative.
In this context, we focused on NRG1, which had a close link with BACE1 and was correlated with cognition in AD measured in CSF (231). Our following step was to explore plasma NRG1 levels as reported in **Paper I**. Plasma NRG1 levels measured in plasma were modified in subjects with underlying AD evidence, with a similar pattern of changes in CSF and plasma.
The correlation between CSF and blood levels was significant, albeit moderate, which could be related to variability in BBB passage or to confounding NRG1 issued from the periphery, notably from the peripheral nervous system (314). Still the significant association of plasma NRG1 with other synaptic markers suggests that it offers a correct reflection of the CSF state. Plasma NRG1 appears particularly interesting as other very studied synaptic biomarkers such as neurogranin or GAP-43, that seem to be reliable in CSF, are not fitting as plasma biomarkers, due to a peripheral contribution (315). More precisely in the synaptic machinery, a meta-analysis of 57 synaptic markers revealed that presynaptic makers were affected more than postsynaptic markers (209). Thus, as a pre-synaptic biomarker, plasma NRG1 may display wider amplitude changes that could be captured in blood.
In our cohort, plasma NRG1 did not display specificity to AD compared to non-AD groups. Plasma NRG1 had a low performance in identifying AD, at both MCI and dementia stage, similarly to CSF NRG1 (231). The specificity of synaptic biomarkers to AD is still debated and could vary between markers. In Tible *et al.*, we found that a panel of CSF synaptic biomarkers was significantly higher in AD than in other dementia (216). The pre-synaptic marker GAP-43 was found to show an AD-specificity in a cohort of 662 individuals (316). Similarly, neurogranin was also reported to be unaltered in non-AD dementia in large cohorts (317). However, after adjusting for bias in patient selection, in a cohort enrolling non AD-dementia including DLB, FTD, VaD and CJD, a recent study found altered levels in these groups. Higher neurogranin levels were associated with high t-tau, in AD and CJD (318). This could indicate that, at least for NRG1 or neurogranin, some synaptic biomarkers would not be

suitable as a differential diagnosis biomarker for AD, but would rather constitute a general marker of synaptic loss.
Altogether, our results support further explorations of plasma NRG1 as a potential complement to other CSF and imaging markers for identification of synaptic pathology, as it is one of the only plasma biomarker so far. It could also be used for prognostic, staging and as a biomarker of therapeutic response in disease-modifying therapies.

Regarding axonal damage, a great evidence is now available that neurofilament markers (and more specifically NfL) constitute non-specific CSF and plasma biomarkers of neuronal injury. Neurofilament markers have been extensively studied in neurocognitive disorders, neurodegenerative or not and their pattern of changes is well established, significantly more than for synaptic biomarkers (201). However, if NfL and pNfH offer both a reflection of axonal damage. A direct comparison of their diagnostic performance, relationship with cognition and imaging is still lacking for several NDDs including AD.
Thus, in **Paper II**, we compared NfL and pNfH measured in CSF and plasma. Our results add to the existing literature showing that plasma NfL levels were elevated in MCI and dementia, compared to control subjects (201,319). Conversely, evidence about pNfH levels in NDDs is scarce. pNfH has been more studied in ALS where it has been directly implicated in the pathogenesis of motor neuron impairment (320). Thus, it has appeared that it has a specific potential as a diagnostic and prognostic biomarker in motoneuron disease, especially in detecting ALS mimics (311,321). Regarding AD, modified levels of pNfH have already been reported at MCI and dementia stages (322). In accordance with prior reports in smaller cohorts, both CSF and plasma pNfH were increased in AD, already at MCI stage in our cohort.
Both markers, in plasma and CSF, associated moderately with cognition. Moreover, plasma NfL showed a higher association with Scheltens score, both in the whole cohort and in AD subgroups, while pNfH did not. Plasma NfL levels were already reported to associate with gray/white matter volume in voxel-wise analyses in AD in cross-sectional and longitudinal studies (323). Our results suggest that this association can be observed with simpler clinical imaging tools.
Regarding differential diagnosis of AD, both NfL and pNfH showed weak discriminatory power between AD- and non-AD dementia. Indeed, neurofilaments are established as general markers of neurodegeneration and thus present limited value from a differential diagnostic perspective (201). Whereas pNfH seem to present an added value in ALS as it is more directly related to underlying physiopathological processes, it is likely to constitute a general marker of neurodegeneration in AD.
Overall, both NfL and pNfH proved to be sensitive and reliable biomarkers of neuronal damage. However, CSF and plasma pNfH did not outperform or add significant value to plasma NfL for identification of AD.

Multiple fluid biomarkers of neuroinflammation, including microglial markers, astrocytic markers and general markers (cytokines), have been measured and are modified in AD (324–326). However, their link with the disease trajectory or their value for diagnosis and monitoring in AD are overall not well characterized yet.
The astrocytic biomarker GFAP is considered to be released in plasma and in CSF by the cerebral astrocytes upon their activation. In **Papers III and IV**, we reported that plasma GFAP levels displayed high performance to identify AD, very close to that of p-tau. Plasma GFAP levels were also closely correlated to Aβ pathology, measured with CSF and PET. Several prior studies and a recent meta-analysis supported those results (238,325,327). Some authors have even suggested plasma GFAP to be used as a marker of amyloid process (A) in the 'AT (N)' scheme (238). Compared to amyloid-β plaques, the potential associations between reactive

astrocytes and neurofibrillary tangles have been much less studied (328). In our study, plasma GFAP levels association with tau pathology was mediated by Aβ, which had already been described (238). This suggests that the astrocytosis reflected by plasma GFAP levels is specifically associated with amyloid-β pathology. Thus, plasma GFAP could be used to detect Aβ-positivity. Given that plasma Aβ levels are not an efficient biomarker, plasma GFAP could be proposed to detect Aβ-positivity in clinical practice.

Another characteristic of neuroinflammation in AD is the presence of a microglial activation. Activated microglia can be found in AD brains, around Aβ deposits, maintaining an inflammatory state by secreting pro-inflammatory markers and cytokines (329). AD genetic risk factors have pointed to a protective function of microglia, lowering the incidence of AD. Conversely, there is also evidence that microglia contributes to neuronal damage in AD. It is also unclear if microglial activation occurs as a consequence of Aβ deposits or if it could serve as a triggering factor for depositions (330,331). Biochemical markers of microglial activation that reflect the inflammatory state of the CNS, could be useful for the understanding of the complex role of microglia along the different stages of AD.

In **Paper IV**, Gal-3, a beta-galactosidase binding protein involved in microglial activation was associated to Aβ plaques in AD brain and its CSF levels were increased in AD. Our data showed an upregulation of Gal-3 in cortical and hippocampal tissue from sporadic and genetic AD. We observed that Gal-3-positive microglia contained a notable number of Aβ inclusions, highlighting their phagocytic capacity associated with the pathology (332).

We reported higher CSF Gal-3 levels in AD patients compared to control subjects. CSF Gal-3 levels correlated with tau and p-tau181 levels, two indicators of pathology progression in AD. Indeed, microglial activation progresses along with tau deposition across the different Braak stages, indicating the synergy of both processes (333). Moreover, the combination of neuroinflammatory microglial activity and tau deposition measured by PET has been reported to predict cognitive decline in AD (334). CSF Gal-3 levels significantly correlated with CSF markers of synaptic dysfunction, GAP-43, and neurogranin. Microglia is implicated in maintaining functional synaptic connections and plasticity under physiological conditions. In AD, microglia has been proved to participate to synapse loss through phagocytosis or release of synaptotoxic factors (335,336). The correlations with the neuroinflammatory markers, CSF sTREM, GFAP and YKL-40, were weaker than with neuronal or synaptic markers, suggesting that Gal-3 monitors complementary inflammatory processes differently than those monitored by GFAP, sTREM-2 or YKL-40.

In principal component analysis, CSF Gal-3 clustered with the other neuroinflammatory factors. Interestingly, we observed a U-shape relation between the core AD component (clustering the core AD biomarkers and the synaptic markers) and the neuroinflammatory component, which might indicate two different stages of inflammatory response throughout the pathology. A similar pattern has been reported for the microglial marker sTREM2 (337). In autosomal dominant AD, sTREM2 biomarker change has been demonstrated to appear within the amyloid cascade immediately after the first pathological changes in Aβ and to be initially protective and to slow cognitive decline (338). Thus, our data support that Gal-3 is a mediator of the microglial pro-inflammatory phenotype in AD and that its measure in CSF could be used to monitor microglial activation. However, its kinetic during AD progression still need to be characterized to establish if it has a prognostic value.

Overall, neuroinflammation biomarkers are being actively developed and may contribute to informing neuroinflammatory processes in AD, including astrocytosis and microglial activation. However promising, the potential place and utility of these biomarkers in clinical routine must be precisely defined. How they could contribute to diagnosis, follow-up and prognosis and if they would be helpful to monitor the effectiveness of disease-modifying

therapies is likely to vary between biomarkers. These questions should be studied in large cohorts including several inflammatory biomarkers.

## 5.3 Performance of plasma biomarkers for AD diagnosis in clinical settings

As presented, multiple plasma biomarkers have emerged over the last decade from deeply phenotyped research cohorts. From those studies, results are promising. However, generating real-world evidence is essential to shape their real utility in the future. Studies from the 'real world' would have to answer an important question: which biomarker could inform the best on the etiology of a cognitive impairment when a patient is seeking care for a cognitive concern?

In **Paper V**, plasma p-tau assays could best identify AD, both at MCI and AD stage, as stand-alone markers. They displayed high AUCs to discriminate AD *vs* non-AD cause of cognitive impairment, which is the question to which physicians are confronted with in clinical practice. Increased levels of plasma p-tau in AD have been demonstrated in numerous studies, across multiple analytical platforms (171–173,308). Blood p-tau measurements also appear specific to AD: p-tau181, p-tau217 and p-tau231 have been shown to accurately discriminate between AD and non-AD cases, not only when diagnosed clinically but also when neuropathologically confirmed (171,172). In a neuropathological study on 312 individuals, plasma p-tau181 and plasma p-tau231 showed the strongest overall sensitivity and specificity for AD neuropathological changes compared with plasma Aβ42/Aβ40, t-tau and NfL (339). A potential limitation of plasma p-tau could be that the elevation may occur later in response to amyloid accumulation than that of the Aβ42/Aβ40 ratio. However, p-tau appears to rise in response to amyloid pathology, more than tau pathology and significant modifications are still seen prior to symptom onset (175). Indeed, in our cohort, plasma p-tau identified AD with the same performance at MCI and at dementia stage. Additionally, p-tau181 and p-tau231 did not significantly differ in diagnosis performance and displayed similar results in biomarkers combination. This is supported by the existing literature, showing that p-tau181, p-tau217 and p-tau231 could be used interchangeably in symptomatic AD (176). In comparative studies, p-tau231 seems to change the earliest, followed by p-tau217 and then p-tau181. However, these changes occur already at preclinical stage (175).
The second-best performing biomarker studied in **Paper V** was plasma GFAP, coherently with our finding in **Paper III**. It displayed better performance than its CSF levels, potentially through direct plasma release by astrocytes of the BBB. In a recent study, GFAP had the highest AUC in differentiating between Aβ+ and Aβ- cognitively impaired older adults, compared to other plasma biomarkers (340).
Plasma Aβ42/Aβ40 measures showed significant diagnostic performance but were outperformed by p-tau measurements as already reported in the literature. We observed a small fold change between Aβ-positive and Aβ-negative patients in our clinical cohort, resulting in large overlap between the groups. This also seems to align with the AD pathophysiology, with Aβ proteins starting to change and plateauing early, making them less informative at later stages of overt cognitive change, while p-tau continues to increase through the AD continuum to the dementia stage (130). Moreover, the nature of assay platforms required for optimal results (mass spectrometry particularly) is likely to limit its use as such in clinical settings (163,164).
Plasma NfL, as a general marker of neurodegeneration, differentiated well AD-MCI and AD-dementia from control subjects. However, its value in discriminating AD from non-AD both at MCI and dementia stage was weak, in **Papers II** and **V.**

Plasma tau levels displayed a large overlap between groups and this reflects in their low diagnostic performance observed in ROC analysis. In previous studies, blood total tau has been shown to contribute to diagnosis only in disorders with high increases in CNS tau with significant release into plasma, such as traumatic brain injury, stroke, or CJD (341,342). There is also evidence that it could have prognostic use (343).

We explored whether a combination of blood biomarkers would be useful in AD diagnosis for clinical use. In **Paper V,** the association of biomarkers increased diagnosis accuracy. To differentiate AD-dementia from non-AD dementia, a promising AUC was found of 0.86 for the combination of p-tau181 and NfL. At MCI stage, the association of three biomarkers reached an AUC of 0.90 to identify AD (p-tau181, NfL and GFAP). There is prior evidence in the literature that combination of plasma biomarkers, generally reflecting different pathophysiological processes of AD, could accurately predict cognitive decline and conversion to dementia (165,176,344).
Coherently, p-tau markers were included in the best performing combinations. This result further adds to the growing literature of plasma p-tau measurements as crucial markers to track AD at symptomatic phase. In a clinical study, it was found that of all plasma biomarker combinations (including p-tau217, plasma Aβ ratio, NfL, GFAP), p-tau217 alone was not inferior to any combination for discriminating progression to AD dementia (345).
GFAP contributed to distinguishing AD MCI from non-AD MCI. There is now large evidence suggesting a close relationship between Aβ and GFAP, including the results of our **Paper III**, rather than between GFAP and p-tau (238,346). So, as plasma GFAP shows stronger association to CSF Aβ than plasma Aβ, it might contribute to the combination by giving independent information on Aβ status.
However, in our cohort, adding plasma Aβ ratio and plasma tau did not result in improved identification of AD. In prior studies, plasma Aβ ratio was shown to improve prediction of conversion in cognitively unimpaired older adults, combined with plasma p-tau217 and NfL (165). Similarly, plasma Aβ ratio combined with p-tau217 could detect early amyloidosis, when adding NfL did not improve diagnostic performance (344). We can make the hypothesis that plasma Aβ ratio plateaus early in disease evolution and so its diagnostic value decreases at overt symptom phases. Conversely, NfL levels which are only slightly increased at very prodromal stages have higher values at MCI and dementia stages. Regarding plasma t-tau, it was shown in a prior study not to add diagnostic value to plasma NfL to discriminate between AD and FTD in a large clinical cohort, most likely providing some degree of overlapping information with each other (347).

Nevertheless, our plasma biomarkers performance remained slightly lower than their demonstrated performance in research cohorts. Plasma p-tau used a standalone marker yielded AUC between 0.90 and 0.96 in differentiating AD-dementia *vs* other dementia in selected research cohorts (172). Similarly, the performance of plasma Aβ and NfL were lower than previously reported (163).
Several hypotheses can be made on these findings. Most research has been conducted in relatively healthy individuals, apart from their neurocognitive disorder. Cohorts such as ADNI include subjects with concurrent neuropsychiatric disorder or somatic disease, such as inflammatory disease, heart failure or HIV infection (*https://clinicaltrials.gov/ct2/show/NCT00106899*). Conversely, evidence exists that there is a high prevalence of comorbid medical conditions and of somatic complaints amongst people with dementia (348,349). Pre-existent neuropsychiatric conditions such as depression, bipolar disorder, neurovascular events or alcohol misuse are highly frequent comorbidities encountered in clinical practice (350). It has been shown that those conditions can affect

biomarkers measurements. Heart failure and kidney or liver failures have been shown to alter plasma p-tau levels through modification of protein metabolism in plasma (309). NfL is associated with kidney function in cognitively unimpaired subjects (351). Evidence exists that synaptic biomarkers are modified in psychiatric disorders such as depressive or bipolar disorders (352,353). A capital point will be, for each marker, to understand if comorbidities confound the interpretation of the biomarker levels (hypothesis that could be made for kidney failure and p-tau levels) or if comorbidities affect the underlying process measured by the biomarker. Additionally, variability in pre-analytical conditions in clinical routine could affect the biomarker performances, in comparison to highly controlled research settings.

Exploration of plasma biomarkers association with cognitive status can inform on their potential value in monitoring cognitive decline and therapeutical response in clinical trial.
In the **Paper V**, high plasma GFAP, p-tau and t-tau concentrations were independently associated with worse cognitive performance at a cross-sectional level. In prior studies, GFAP was found to be correlated with executive functions, and specifically with processing speed among subjects with a CSF AD profile (354). In cohort of 300 cognitively unimpaired subjects, higher baseline serum GFAP levels were associated with a steeper rate of decline in the domains of memory, attention, and executive functioning (355).
Plasma p-tau measurements also showed an association with cognition, adding to the existing evidence of their clinical relevance. Plasma p-tau concentrations increase with clinical disease severity in large cohort studies (356,357). Studies have demonstrated that longitudinal measurements of plasma p-tau have shown associations with cognition. Individuals with high baseline concentrations of plasma p-tau have higher odds of cognitive deterioration (168).
The association of neurodegeneration markers with cognition is inconsistently supported by the literature (358–360). Several studies compared plasma t-tau levels to plasma NfL as two biomarkers of neurodegeneration regarding cognition, and plasma NfL was overall found to display stronger association with general cognition and cognitive subdomains (361). Conversely, in our study, plasma NfL did not associate with cognition in the whole cohort. We cannot exclude that it could be related to the use of MMSE score, a routine screening test widely used in clinical settings. Precise neuropsychological assessment data will contribute to a clear characterization of biomarkers association with cognition.

Morphological MRI is integrated in the IWG diagnosis criteria of 2014 for assessment of atrophy of critical brain regions as a supportive criterion for AD diagnosis (72). While research studies have shown an association between plasma biomarkers and the neurodegeneration processes associated with AD, it remains unclear how plasma biomarkers relate to imaging findings in clinical practice. Thus, we explored how some of our plasma biomarkers associate with the two imaging scores most frequently used in clinical routine for brain atrophy and white matter lesions, respectively Scheltens score and Fazekas score.
In the **Paper II**, plasma NfL levels showed a significant association to both medio-temporal atrophy and white matter lesions scores. Our work in **Paper V** reproduced this finding with a significant association of plasma NfL with Scheltens score. There is significant evidence in the literature that NfL levels, in CSF and plasma, associate with global brain atrophy (192,362). Higher NfL levels are associated to the progression of brain atrophy in AD but also in other dementia such as FTD or VaD (195,363). In the presence of Aβ pathology, it was found in voxel-analysis that NfL levels are specifically associated with reduced gray matter density in AD vulnerable regions (364). NfL levels also associate with white matter alterations, as demonstrated in CSF (365). In plasma, NfL levels have been shown to associate with white matter volume loss in voxel-wise analysis in both cognitively unimpaired and cognitively impaired subjects (323). In AD, white matter injury and atrophy, in the temporal, parietal,

frontal lobes and in the corpus callosum, is a common feature (366,367). Our findings support further the use of plasma NfL to track ongoing neurodegeneration in clinical routine, in both AD and non-AD related cognitive impairment. Indeed, NfL appears to be a significant indicator of brain changes measured with MRI which is an established tool in routine care in neurocognitive disorders.

Plasma p-tau measurements showed significant association with Scheltens score. Plasma p-tau231 and p-tau181 levels have previously been reported to associate with hippocampal atrophy (171,368,369). In cross-sectional analysis, plasma p-tau181 correlates with gray matter loss in the regions commonly affected in AD such as the medial temporal lobe, the precuneus, and the anterior cingulate. Additionally, it was shown that plasma p-tau181 predicts neurodegeneration in these specific regions in AD (370). This finding was only observed in Aβ-patients, conversely to plasma NfL which was associated with neurodegeneration independently of Aβ status in the same study. This finding on the association of p-tau plasma measurements with neurodegeneration adds further to its potential as a marker for AD diagnosis and follow-up.

The available evidence on plasma GFAP levels relation with brain atrophy is scarce. In the **Paper V**, plasma GFAP levels correlated positively with medio-temporal atrophy. In a recent study, plasma GFAP associated with higher gray matter volumes at the earliest stages of the AD continuum, which reverted later during the course of the disease (371). As demonstrated in the **Paper III**, plasma GFAP is a marker of amyloidosis and it appears coherent that it would associate more with early morphological imaging changes.

Overall, these findings indicate that, despite not providing structural information, plasma p-tau, NfL and GFAP levels associate, at least to some extent, with neurodegeneration. Therefore, they can be useful and cost-effective biomarkers to predict AD-related neurodegeneration.

## 5.4 Limitations of fluid biomarkers

Despite their high potential for research and clinical use, there are still limitations of fluid biomarkers that should be noted.

First, most biomarker studies are based on clinically diagnosed cases, as there are no fluid biomarkers for non-AD dementia, which likely induces a significant percentage of misdiagnosis. Several biomarkers have been neuropathologically validated in research cohorts, such as p-tau measurements, amyloid ratio and NfL (170,171,339,372). However, data are still scarce for some biomarkers such as GFAP and other neuroinflammation biomarkers as well as for synaptic biomarkers. Non-AD dementia cases with neuropathological validation are especially lacking to assert the potential for differential diagnosis of the different markers.

A general limitation of fluid biomarkers is their inability to address brain region-specific changes, which may limit their use for staging of disease severity and their prognostic utility. PET imaging, including Aβ and tau, and morphological imaging provide a more direct assessment of disease topography and stage. Plasma p-tau levels have been shown to increase along the progression of the disease (173). NfL levels also increase with the disease progression, with the limitation that they are not disease specific (192). However, this is not established for other plasma biomarkers such as Aβ42/Aβ40 ratio, that could mimic CSF amyloid ratio that display more of a bimodal distribution (Aβ-positive *vs* Aβ-negative). In our **Paper V**, we did not find any significant difference in marker levels between AD-MCI and AD-dementia for any of our plasma biomarkers after adjustment on age and sex. Thus, it does

not appear guaranteed that those markers could track disease progression in unselected patients in clinical routine.
An important caveat is that the broad spectrum of dementia shares pathophysiological alterations and consequently combined pathologies. Thus, this overlap in pathologies between different dementia essentially precludes the possibility of finding a biomarker with perfect specificity for NDDs diagnosis. Thus, we can make the hypothesis that the combination of plasma biomarkers with imaging or electrophysiology biomarkers will be needed to increase the diagnostic accuracy as compared with the sole use of fluid biomarkers.

## 5.5 Strengths and weaknesses of this work

The use of data derived from a real-world tertiary department constitutes a significant strength of this work. The included patients presented with diverse clinical presentations and diagnostic uncertainty. All patients who underwent CSF and plasma sampling during their care and were willing to participate were included. There was no additional exclusion criteria.
The cohort was well phenotyped with cognitive and imaging data using tools accessible in clinical practice.
Paired plasma and CSF samples allowed for direct comparisons of each fluid biomarker performance, to evaluate blood based biomarkers against their CSF counterpart. Similarly, we could perform direct comparisons between all the markers in the **Paper V**.
Most measurements were performed using robust and ultrasensive assays including Simoa and mass spectrometry. Plasma NfL, pNfH, p-tau and GFAP were measured on Simoa which is by far the most common platform used for these markers. Using the same platform limited the bias of technical variability in the comparisons.
Lastly, the inclusion of our cohort in a multicenter study in the **Paper III**, with research cohorts, Mc Gill TRIAD and ALPHA preclinical AD cohorts, enabled the evaluation of the impact of the setting (research or clinical practice) in plasma GFAP performance for AD diagnosis.

Several limitations should be noted about our work. There were a limited number of control subjects that we could include from our memory clinic. Indeed, our department receives subjects with a cognitive complaint, apart from rare observational studies enrolling cognitively unimpaired subjects. We set very strict criteria to define controls (normative cognitive assessment, normal CSF profile and imaging, no decline at a follow-up) for subjects that had a SCI. However, we cannot exclude that these individuals could be at the early preclinical stage of AD or non-AD dementia. The non-AD dementia group was relatively small, as non-AD dementia diagnoses are significantly uncommon compared to AD. We could still investigate the performance of biomarkers for AD differential diagnosis in grouping cases in a 'non-AD dementia' group.
We did not have neuropathological or genetic confirmation of diagnosis in our cohort. All included AD cases were sporadic. We also relied on clinical diagnosis for DLB and FTD. Nevertheless, all cases of non-AD dementia fulfilled the most recent diagnosis criteria for the respective diseases.
Non-AD MCI group was quite diverse as it included very various etiologies of cognitive impairment. It precluded us to draw a conclusion on the performance of our biomarkers in identifying the underlying etiology. However, we could evaluate their values to rule out AD pathology in this group.
Even if we had cognitive and imaging data, we did not study our biomarkers of interest in regards to the potential comorbidities of the included patients, such as cardiovascular comorbidities or kidney function, that are now known to possibly impact biomarkers levels.

Finally, we did not have longitudinal data to evaluate the predictive values of our biomarkers and their value in a follow-up. Further investigations in longitudinal samples will be needed to explore the association of plasma biomarkers with disease progression in clinical cohorts.

## 5.6 Conclusion and perspectives

In conclusion, the different studies included in this thesis participate in a first step, from research to clinical practice, to bring the required real-world evidence needed to introduce new biomarkers for AD in clinical practice.
Our goal was to contribute to the further understanding of the potential of different fluid markers, focusing on blood-based biomarkers, for dementia diagnosis in memory clinic settings. The novel biomarkers investigated displayed overall high performance to identify AD, very close to that of validated AD CSF biomarkers. We confirm that, in clinical practice, they have the potential to improve detection and diagnosis in AD by increasing convenience and acceptability, as well as reducing costs. Given the actual level of evidence, we can make the hypothesis that some of these blood-based biomarkers should enter clinical practice in the coming decade. Additionally, novel biomarkers reflecting non–amyloid and tau processes are promising for both AD and non AD dementia for research and for clinical purpose.
In the future, several future challenges remain to be met to introduce new markers in the physician toolbox. First, assay-related factors, such as clear characterization of specificity and selectivity for each marker and platform, have to be considered. Secondarily, preanalytical factors (*i.e.*, patients-related factors, blood collection, sample handling, and storage), and analytical factors (*i.e.*, internal and external quality control, constitution of reference materials and gold standards against which to validate the assays) should also be harmonized. As with any biomarker, interpretation of blood-based biomarkers should be made in the correct clinical context. While research criteria tend to address AD as a pure biomarker construct, in clinical practice, biomarkers are used as part of a diagnostic process to aid diagnosis in patients with cognitive symptoms. Clinical guidelines will be needed to inform clinicians. Additional studies to confirm a possible extension of their use to less specialized settings, such as primary care, will also be needed.

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# 8. APPENDIX

## 8.1 Appendix 1 - Résumé substantiel en français

*Introduction*

Auparavant définie selon trois phases cliniques successives (phase préclinique, troubles cognitifs légers [TCL] et troubles cognitifs majeurs [TCM]), la maladie d'Alzheimer (MA) est désormais considérée comme un *continuum* qui peut être caractérisé par des biomarqueurs biologiques et/ou d'imagerie, mettant en évidence *in vivo* les processus physiopathologiques de la maladie, *i.e.,* la formation de plaques amyloïdes, de plaques neurofibrillaires avec des agrégats de protéine tau hyperphosphorylée et la neurodégénérescence. Ces marqueurs incluent l'analyse du liquide cérébrospinal (LCS) mesurant le ratio amyloïde Aβ42/Aβ40, les taux de protéine tau phosphorylée (p-tau) et totale (t-tau) et l'imagerie en tomographie à émission de positons (TEP) amyloïde et tau. Les biomarqueurs du LCS sont utilisés en pratique clinique depuis maintenant près de 10 ans en France, pour confirmer le diagnostic de MA et écarter les diagnostics différentiels.
Néanmoins, l'usage de ces biomarqueurs est limité par leur caractère coûteux, invasif et peu accessible. La plus faible concentration périphérique des protéines issues du système nerveux central (SNC) a longtemps limité leur dosage en périphérie mais le développement ces dernières années de techniques plus sensibles a permis de dépasser cette barrière. Une grande avancée récente dans le domaine des biomarqueurs de la MA est donc le développement de dosages plasmatiques, dont certains démontrent une performance diagnostique proche de celle du LCS. Dans des cohortes de recherche, les taux plasmatiques du ratio amyloïde Aβ42/Aβ40, de p-tau et de t-tau, ainsi que ceux des neurofilaments à chaine légère (NfL), permettent d'identifier les patients avec un profil MA dans le LCS et possèdent de bonnes performances diagnostiques. De plus, de nouveaux biomarqueurs évaluant les processus autres qu'amyloïde et tau, comme la perte synaptique, les lésions axonales et neuronales, et la neuroinflammation, sont en cours d'exploration. Néanmoins, ces nouveaux biomarqueurs n'ont été que très peu explorés dans des cohortes en conditions de vie réelle, issues de centre mémoire ou de médecine générale.

*Objectifs de ce travail*

L'objectif de ce travail de thèse a été d'étudier de nouveaux biomarqueurs candidats dans le LCS et le plasma de patients provenant d'une cohorte clinique de centre mémoire, comprenant des marqueurs spécifiques et non spécifiques des processus amyloïde et tau, pour le diagnostic positif et différentiel de la MA. En outre, nous avons visé à explorer leur relation avec la cognition et l'imagerie morphologique.
L'**étude I** a exploré un nouveau biomarqueur plasmatique candidat d'atteinte synaptique. L'**étude II** a comparé deux biomarqueurs plasmatiques de dégât axonal et neuronal. Les **études III** et **IV** se sont intéressées à deux marqueurs de neuroinflammation, explorant respectivement l'activation astrocytaire et la réaction microgliale dans la MA. Enfin, l'**étude V** a comparé un panel de biomarqueurs plasmatiques pour le diagnostic positif et différentiel de la MA en pratique clinique courante.

*Matériel et méthodes*

Tous les travaux décrits dans ce manuscrit ont analysé des échantillons de LCS et de plasma provenant d'une cohorte de centre mémoire, issue du Centre de Neurologie Cognitive, Hôpital Universitaire Lariboisière Fernand-Widal, Paris, France. Nous avons inclus rétrospectivement

les patients ayant bénéficié d'un prélèvement de LCS et de plasma entre 2012 et 2019, dans le cadre de l'exploration d'une plainte cognitive. La cohorte a inclus des patients avec une MA aux stades de TCL et TCM, ainsi que des patients avec d'autres maladies neurodégénératives (MND) telles que la maladie à corps de Lewy (MCL) ou la démence frontotemporale (DFT) et des patients avec un TCL non lié à une MA. Des sujets contrôles sans argument pour une pathologie neurocognitive ont été également inclus. Le diagnostic reçu reposait sur l'examen neurologique détaillé, l'évaluation neuropsychologique, l'IRM cérébrale et sur le dosage des biomarqueurs LCS de la MA.

*Etude I*

L'**étude I** a exploré la mesure plasmatique de la protéine synaptique neuréguline 1 (NRG1). Le dysfonctionnement et la perte synaptique sont une caractéristique centrale et précoce de la MA, étroitement associée aux symptômes cognitifs. La protéine NRG1 est un facteur de croissance et de différenciation impliqué dans le développement et le maintien de la transmission synaptique. Nous avions préalablement montré que l'augmentation de la concentration de NRG1 dans le LCS dans la MA est associée à l'atteinte cognitive et aux biomarqueurs MA du LCS. Des biomarqueurs plasmatiques reflétant l'atteinte synaptique seraient d'un grand intérêt clinique.
Notre objectif a donc été de mesurer la concentration plasmatique de NRG1 chez des patients atteints de MA et d'étudier son association avec les marqueurs validés du LCS ainsi qu'avec des biomarqueurs synaptiques du LCS. Cette étude rétrospective a porté sur 127 participants, dont des patients atteints de MA au stade de TCL (n = 27) et de TCM (n = 35), de MND non-MA (n = 26, Aβ-négatif), de TCL non-MA (n = 19) et des sujets contrôles (n = 20). Les concentrations plasmatiques et cérébrospinales de NRG1, ainsi que les biomarqueurs MA du LCS (ratio Aβ42/Aβ40, phospho-tau et tau total), ont été mesurés par ELISA. Les marqueurs synaptiques du LCS ont été mesurés par ELISA pour GAP-43 et neurogranine, et par immunoprécipitation combinée à la spectrométrie de masse pour SNAP-25.
La concentration plasmatique de NRG1 était plus élevée chez les patients atteints de MA aux stades de TCL et de TCM que chez les contrôles (respectivement, $P = 0,005$ et $P < 0,001$). Nous avons ensuite étudié la performance du taux plasmatique de NRG1 pour différencier les groupes en analyse ROC, *via* le calcul de l'aire sous la courbe (AUC). La concentration plasmatique de NRG1 permettait de différencier les sujets contrôles des patients avec une MA aux stades de TCL et TCM avec des AUC respectives de 0.88 et de 0.87. Le taux plasmatique de NRG1 était corrélé au taux de NRG1 dans le LCS ($\beta = 0,372$, $P = 0,0056$, ajusté sur l'âge et le sexe). Le taux plasmatique de NRG1 était associé aux biomarqueurs MA du LCS dans l'ensemble de la cohorte et chez les patients Aβ-positifs ($\beta = -0,197 – 0,423$). Le taux plasmatique de NRG1 était également corrélé aux niveaux de 3 marqueurs synaptiques dans le LCS : GAP-43, neurogranine et SNAP-25 ($\beta = 0,278 – 0,355$). La concentration plasmatique de NRG1 était inversement corrélée au MMSE dans l'ensemble de la cohorte et chez les patients Aβ-positifs (tous, $\beta = -0,188$, $P = 0,038$ ; Aβ+ : $\beta = -0,255$, $P = 0,038$).
Au final, le taux plasmatique de NRG1 apparaît augmenté chez les patients atteints de MA et corrélé avec les biomarqueurs MA et synaptiques du LCS, et à l'état cognitif. Ainsi, NRG1 mesurée dans le plasma constitue un biomarqueur non invasif prometteur pour suivre l'atteinte synaptique dans la MA.

*Etude II*

Les taux de neurofilaments à chaîne légère (NfL) plasmatiques constituent un biomarqueur prometteur des lésions axonales et neuronales dans les atteintes du SNC. Les NfL plasmatiques

présentent un fort potentiel pour contribuer au diagnostic des maladies neurodégénératives, avec des performances similaires à celles des taux du LCS. Les taux de neurofilaments à chaîne lourde, et spécifiquement la forme phosphorylée (pNfH), ont été principalement étudiés dans la sclérose latérale amyotrophique (SLA) dans laquelle ils semblent un biomarqueur prometteur. Néanmoins, leur intérêt dans la MA reste incertain.
Notre objectif a donc été de comparer la performance des taux plasmatiques de NfL et pNfH dans le diagnostic positif et différentiel de la MA en pratique clinique courante.
Dans une étude transversale, rétrospective et monocentrique, nous avons mesuré les niveaux de NfL et de pNfH dans le LCS et le plasma dans notre cohorte de centre mémoire (n = 188), comprenant des sujets contrôles (n = 22), des patients atteints de la MA au stade de TCL (n = 36) et de TCM (n = 64), des patients avec un TCL non lié à une MA (n = 38) et des patients avec une MND autre (n = 28). Les taux de NfL et de pNfH plasmatiques et les taux de pNfH dans le LCS ont été mesurés en technique Simoa et les taux de NfL dans le LCS en méthode ELISA.
Les taux dans le LCS et dans le plasma de NfL et de pNfH étaient corrélés à l'âge (Spearman *rho* = 0,259 – 0,451, P < 0,003). La corrélation entre les concentrations dans le LCS et dans le plasma était plus forte pour les NfL que pour les pNfH (respectivement, *rho* = 0,77 et *rho* = 0,52). Les taux de NfL dans le LCS et du plasma, et les taux de pNfH dans le LCS, mais pas les taux de pNfH dans le plasma, étaient corrélés aux niveaux de p-tau dans le LCS chez les patients avec une MA. Tous les marqueurs étaient augmentés chez les patients atteints de MA, de MND autre ainsi que chez les patients avec un TCL non liés à la MA. Les taux de NfL et de pNfH du LCS, et les NfL plasmatiques montraient une bonne performance pour différencier la MA aux stades de TCL et de TCM par rapport aux sujets témoins (0,82 – 0,91). Les taux de pNfH plasmatique démontraient des AUCs globalement plus faibles pour la discrimination entre les groupes par rapport aux taux de pNfH du LCS. Au stade de TCL, les 4 marqueurs avaient une performance modérée pour séparer les patients atteints de MA des autres patients (0,61 – 0,77). Similairement, au stade de TCM, les marqueurs avaient une performance modérée pour séparer les patients avec une MA de ceux avec une autre MND (0,58 – 0,81).
Nos marqueurs montraient une association modérée avec l'état cognitif dans l'ensemble de la cohorte. En imagerie, les taux plasmatiques de NfL étaient significativement associés avec le degré d'atrophie médio-temporale dans l'ensemble de la cohorte et spécifiquement chez les patients avec une MA.
En conclusion, les taux de NfL et de pNfH dans le LCS ainsi que les taux de NfL plasmatiques semblent aussi performants dans le diagnostic positif et différentiel de la MA dans notre cohorte de pratique clinique courante. En revanche, contrairement à leur usage dans la SLA, les taux de pNfH plasmatiques n'ont pas démontré de valeur ajoutée par rapport aux taux de NfL plasmatiques.

*Etude III*

La protéine acide fibrillaire gliale (GFAP) est un marqueur d'activation astrocytaire qui est augmenté dans le LCS et le plasma des personnes atteintes de MA. Cependant, il n'est pas clair s'il existe des différences dans le niveau de GFAP plasmatique aux différents stades de la MA et si la performance diagnostique du taux plasmatique est similaire à celle de la GFAP mesurée dans le LCS.
L'objectif de l'**étude III** a été d'étudier le taux plasmatique de GFAP tout au long du *continuum* de la MA, de la phase préclinique à celle des TCM, en comparaison au taux de GFAP dans le LCS. Cette étude observationnelle et transversale a recueilli des données dans 3 centres. La cohorte Translational Biomarkers in Aging and Dementia (TRIAD) (Université Mc Gill, Montréal, Canada) comprenait des personnes atteintes de MA à tous les stades. Les résultats

ont été confirmés dans l'étude Alzheimer and Familles ALFA+ (Barcelona Beta Research Center, Barcelone, Espagne), qui incluait des personnes atteintes de la MA au stade préclinique, et dans notre cohorte de centre mémoire (Centre de Neurologie Cognitive, Paris, France) qui incluait des personnes atteintes de MA aux stades de TCL et de TCM.
Les concentrations de GFAP dans le plasma et le LCS ont été mesurées en technique Simoa. Les autres marqueurs mesurés étaient le ratio plasmatique amyloïde Aβ42/40, p-tau181, NfL, la protéine 1 de type chitinase-3 (YKL40) et le récepteur soluble sTREM2 dans le LCS, ainsi que les niveaux plasmatiques de p-tau181 et de NfL. Des données de TEP amyloïde étaient disponibles dans les cohortes TRIAD et ALFA+, et des données de TEP tau étaient disponibles pour la cohorte TRIAD. Au total, 300 participants de la cohorte TRIAD, 384 participants de la cohorte ALFA+ et 187 patients du Centre de Neurologie Cognitive, Paris, Lariboisière, ont été inclus.
Le taux plasmatique de GFAP était significativement plus élevé chez les personnes avec une MA au stade préclinique par rapport aux sujets contrôles (individus Aβ-négatifs sans troubles cognitifs). Le taux plasmatique de GFAP était également plus élevé chez les individus aux stades symptomatiques de la MA, aux stades de TCL et de TCM, par rapport aux sujets contrôles, et également par rapport aux sujets avec un TCL ou un TCM non liés à une MA. L'amplitude de l'augmentation du taux plasmatique de GFAP était systématiquement plus élevée que celle du taux de GFAP mesuré dans le LCS. Le taux plasmatique de GFAP distinguait mieux les individus Aβ-positifs des individus Aβ-négatifs que GFAP mesurée dans le LCS (AUC pour la GFAP plasmatique : 0,69 – 0,86 ; AUC pour GFAP mesurée dans le LCS, 0,59 – 0,76). De plus, le niveau de GFAP plasmatique était associé à la pathologie tau uniquement chez les individus présentant une pathologie Aβ concomitante en analyse de médiation.
Notre étude suggère donc que le taux plasmatique de GFAP constitue un biomarqueur sensible et spécifique pour détecter et suivre l'activation astrocytaire et les lésions amyloïdes, même chez les individus aux premiers stades de la MA.

*Etude IV*

Galectine-3 (Gal-3) est une protéine de liaison de la bêta-galactosidase impliquée dans l'activation microgliale dans le SNC. Il est démontré que Gal-3 participe à l'activation microgliale délétère observée dans la MA. Gal-3 est principalement exprimée par les cellules microgliales et a été mise en évidence autour des plaques d'Aβ dans le cerveau humain. La délétion de Gal-3 entraîne une réduction de la pathologie amyloïde dans des modèles murins de MA. Dans l'**étude IV**, nous avons visé i/ à explorer l'implication de Gal-3 dans la physiopathologie de la MA et ii/ à évaluer Gal-3 mesurée dans le LCS comme marqueur de la MA.
Pour mieux comprendre l'importance de l'inflammation associée à Gal-3 dans la MA, nous avons cherché à étudier la réponse inflammatoire de Gal-3 dans des tissus cérébraux humains. Tout d'abord, nous avons mesuré les niveaux de Gal-3 dans le cortex et l'hippocampe de patients atteints de MA à un stade précoce, incluant des cas génétiques et sporadiques. Nous avons observé que les niveaux de Gal-3 étaient significativement plus élevés dans le cortex et l'hippocampe des sujets atteints de MA. L'immuno-histochimie a révélé que les cellules microgliales exprimant Gal-3 étaient associées à des plaques amyloïdes de plus grande taille et de forme plus irrégulière et à des neurones contenant des inclusions neuro-fibrillaires.
Nous avons ensuite analysé les niveaux de Gal-3 dans le LCS de patients atteints de MA (n = 119) par rapport à des individus témoins (n = 36). Le niveau de Gal-3 dans le LCS était élevé chez les patients atteints de MA par rapport aux témoins et plus fortement corrélé avec la protéine tau (p-tau181 et t-tau) et les marqueurs synaptiques (GAP-43 et neurogranine) ainsi

que, plus discrètement, au ratio amyloïde Aβ42/Aβ40. Enfin, une analyse en composantes principales des biomarqueurs de la MA a révélé que Gal-3 dans le LCS était associée aux autres marqueurs neuroinflammatoires du LCS, notamment sTREM2, GFAP et YKL40. Cette composante neuroinflammatoire était plus élevée chez les patients avec un profil Amyloïde (A)+, Tau (T) + et Neurodégénerescence (N) + (A+T+N+) que dans le groupe A+T-N-.
En conclusion, Gal-3 semble se distinguer comme un facteur important dans l'activation microgliale pathologique observée dans la MA. De plus, Gal-3 est mesurable dans le LCS, ce qui en fait un potentiel biomarqueur d'activation microgliale, pour le monitoring de la neuroinflammation et à plus long terme, pour l'évaluation de la réponse thérapeutique dans des essais anti-inflammatoires.

*Etude V*

Des biomarqueurs sanguins sont désormais disponibles dans la MA et sont proposés comme alternative aux biomarqueurs du LCS. Plusieurs marqueurs apparaissent très prometteurs. Le dosage du ratio amyloïde plasmatique Aβ42/40 est altéré dans la MA dès le stade préclinique. Les différentes mesures de p-tau (p-tau181, p-tau217 ou p-tau231) ont démontré une précision diagnostique globalement élevée pour différencier la MA des autres MND dans des cohortes de recherche. Les mesures de GFAP, bien qu'un marqueur d'activation astrocytaire, sont fortement associées au statut amyloïde dans la MA, comme le montre l'**étude III**. Les taux de NfL plasmatiques (**étude II**) permettent de suivre la neurodégénérescence. Cependant, tous ces résultats doivent encore être confirmés dans les différentes populations où ils pourraient être utilisés, c'est-à-dire en médecine générale, dans les services spécialisés ou dans le cadre des essais thérapeutiques. Il n'est pas encore établi si ces biomarqueurs doivent être utilisés comme des tests individuels, dans un panel combinant plusieurs biomarqueurs, ou comme une aide à la décision pour effectuer une analyse du LCS, ou encore pour le dépistage. L'objectif de l'**étude V** était d'effectuer une comparaison directe de biomarqueurs plasmatiques de la MA dans une cohorte issue d'un centre mémoire, en dehors d'un cadre de recherche standardisé. Nous avons étudié leur capacité à distinguer la MA des troubles cognitifs non liés à la MA, définis biologiquement par les biomarqueurs MA du LCS.
Nous avons inclus 203 patients de notre cohorte de centre mémoire, incluant des sujets contrôles (n = 22), des patients avec un TCL lié à une MA (n = 42) et non-MA (n = 37), un TCM, lié à une MA (n = 71) et et d'autres MND (n=31). Nous avons mesuré une série de biomarqueurs, dans le plasma et dans le LCS : le ratio amyloïde Aβ42/Aβ40, p-tau181, p-tau231, t-tau, NfL et GFAP. Tous les biomarqueurs plasmatiques étaient mesurés en méthode Simoa. Le profil MA était établi grâce à la mesure des biomarqueurs MA du LCS (Aβ42/Aβ40, p-tau181 et t-tau) en méthode Lumipulse®.
Les niveaux de biomarqueurs plasmatiques étaient associés à l'âge (Spearman *rho* = 0,229 – 0,430, $P < 0,005$) sauf pour t-tau. Dans l'ensemble de la cohorte, les taux plasmatiques étaient significativement corrélés à leurs taux dans le LCS (Spearman *rho* = 0,36 – 0,73). Néanmoins, cette association était plus forte pour les mesures de p-tau et de NfL.
Le ratio Aβ42/Aβ40 plasmatique était diminué chez les patients avec une MA ou avec une autre MND par rapport aux contrôles ($P < 0,05$). Il était également diminué chez les patients avec un TCL lié à une MA par rapport à ceux avec un TCL non lié à une MA ($P = 0,001$). Les taux plasmatiques de p-tau181 et de p-tau231 étaient significativement plus élevés chez les patients MA, aux stades de TCL et TCM, par rapport à tous les autres groupes ($P < 0,002$). Les taux plasmatiques de t-tau étaient plus élevés aux stades de TCM, lié à une MA ou non, par rapport aux contrôles ($P < 0,020$). Les taux plasmatiques de NfL étaient plus élevés dans les cas de TCL-MA, et chez les patients avec un TCM, MA ou non-MA, par rapport aux contrôles

(P < 0,001). Les taux plasmatiques de GFAP étaient plus élevés dans les groupes MA que dans tous les groupes non MA (P < 0,001).
Nous avons ensuite étudié la performance de nos biomarqueurs plasmatiques pour différencier les groupes à l'aide d'analyses ROC établissant l'AUC de chaque biomarqueur ou combinaison de biomarqueurs.
Pour distinguer les patients avec une MA au stade de TCM des contrôles, les AUC les plus élevées ont été obtenues pour plasma p-tau181, plasma p-tau231 et GFAP (0,91 – 0,95). Des AUC plus modérées ont été obtenues pour le plasma Aβ42/Aβ40, t-tau et NfL (0,80 – 0,87). Au stade de TCM, pour différencier MA et non MA, les dosages de p-tau181, p-tau231 et GFAP démontraient la meilleure performance (0,80 – 0,85) par rapport à ceux du ratio Aβ42/Aβ40, de t-tau et aux NfL (0,69 – 0,72). Pour discriminer les patients avec un TCL lié à une MA des contrôles, p-tau 181, p-tau231 et GFAP étaient les meilleurs marqueurs (0,89 – 0,92). Les biomarqueurs distinguaient moins exactement les TCL liés à une MA des TCL non-MA (0,73 – 0,85).
La combinaison des biomarqueurs plasmatiques augmentait leur valeur diagnostique. L'association de p-tau181 et des NfL distinguait les sujets avec une MA, au stade de TCM ou de TCL, des sujets contrôles, avec une performance élevée (0,93 – 0,97). De même, l'association de p-tau181 et des NfL distinguait les TCM MA des TCM non-MA (0,86). Au stade de TCL, pour identifier la MA, c'est l'association de p-tau181 et de GFAP qui montrait le meilleur potentiel (0,90). L'utilisation de p-tau231 à la place de p-tau181 démontrait des résultats similaires. Les combinaisons de biomarqueurs étaient statistiquement supérieures aux marqueurs utilisés individuellement.
Enfin, nous avons étudié l'association de nos biomarqueurs avec la cognition et l'atrophie cérébrale médio-temporale. Mesurés dans le plasma, les taux de GFAP, t-tau, p-tau231 et p-tau181 étaient associés avec le score MMSE. Concernant l'atrophie médio-temporale, les concentrations plasmatiques de NfL, GFAP, p-tau181 et p-tau231 étaient associées au score de Scheltens.

*Conclusion*

Les nouveaux biomarqueurs étudiés dans ce travail de thèse ont montré un potentiel élevé pour identifier la MA chez des patients non sélectionnés issus de la pratique courante, venant renforcer la littérature déjà existante. Les marqueurs sanguins ont le potentiel d'améliorer la détection et le diagnostic de la MA en augmentant l'accès et l'acceptabilité des tests, ainsi qu'en réduisant leurs coûts. Compte tenu du niveau de preuve actuel, nous pouvons émettre l'hypothèse que certains de ces biomarqueurs sanguins devraient entrer dans la pratique clinique au cours des prochaines années. En outre, de nouveaux biomarqueurs reflétant les processus non amyloïdes et tau sont prometteurs pour les maladies neurodégénératives, de type MA et non MA, pour la recherche mais également à des fins clinique.

## 8.2 Appendix 2 - Diagnostic criteria

### 8.2.1 Diagnostic criteria for Alzheimer's disease: the IWG-2 criteria

**Panel 1: IWG-2 criteria for typical AD (A plus B at any stage)**
**Dubois et al, Lancet Neurol. 2014**

**A Specific clinical phenotype**

•Presence of an early and signifi cant episodic memory impairment (isolated or associated with other cognitive or behavioural changes that are suggestive of a mild cognitive impairment or of a dementia syndrome) that includes the following features:
•Gradual and progressive change in memory function reported by patient or informant over more than 6 months
•Objective evidence of an amnestic syndrome of the hippocampal type,* based on significantly impaired performance on an episodic memory test with established specificity for AD, such as cued recall with control of encoding test

**B In-vivo evidence of Alzheimer's pathology (one of the following)**

•Decreased Aβ1–42 together with increased T-tau or P-tau in CSF
•Increased tracer retention on amyloid PET
•AD autosomal dominant mutation present (in PSEN1, PSEN2, or APP)

**Exclusion criteria† for typical AD**

*History*
•Sudden onset
•Early occurrence of the following symptoms: gait disturbances, seizures, major and prevalent behavioural changes

*Clinical features*
•Focal neurological features
•Early extrapyramidal signs
•Early hallucinations
•Cognitive fluctuations

*Other medical conditions severe enough to account for memory and related symptoms*
•Non-AD dementia
•Major depression
•Cerebrovascular disease
•Toxic, inflammatory, and metabolic disorders, all of which may require specific investigations
•MRI FLAIR or T2 signal changes in the medial temporal lobe that are consistent with infectious or vascular insults

AD=Alzheimer's disease. *Hippocampal amnestic syndrome might be difficult to identify in the moderately severe to severe dementia stages of the disease, in which *in vivo* evidence of Alzheimer's pathology might be sufficient in the presence of a well characterised dementia

syndrome. †Additional investigations, such as blood tests and brain MRI, are needed to exclude other causes of cognitive disorders or dementia, or concomitant pathologies (vascular lesions).

**Panel 2: IWG-2 criteria for atypical AD (A plus B at any stage)**
**Dubois et al, Lancet Neurol. 2014**

**A Specific clinical phenotype (one of the following)**

•Posterior variant of AD (including):
- An occipitotemporal variant defined by the presence of an early, predominant, and progressive impairment of visuoperceptive functions or of visual identification of objects, symbols, words, or face
- A biparietal variant defined by the presence of early, predominant, and progressive difficulty with visuospatial function, features of Gerstmann syndrome, of Balint syndrome, limb apraxia, or neglect

•Logopenic variant of AD defined by the presence of an early, predominant, and progressive impairment of single word retrieval and in repetition of sentences, in the context of spared semantic, syntactic, and motor speech abilities
•Frontal variant of AD defined by the presence of early, predominant, and progressive behavioural changes including association of primary apathy or behavioural disinhibition, or predominant executive dysfunction on cognitive testing
•Down's syndrome variant of AD defined by the occurrence of a dementia characterised by early behavioural changes and executive dysfunction in people with Down's syndrome

**B In-vivo evidence of Alzheimer's pathology (one of the following)**

•Decreased Aβ1–42 together with increased T-tau or P-tau in CSF
•Increased tracer retention on amyloid PET
•Alzheimer's disease autosomal dominant mutation present (in PSEN1, PSEN2, or APP)

**Exclusion criteria* for atypical AD**

*History*
•Sudden onset
•Early and prevalent episodic memory disorders

*Other medical conditions severe enough to account for related symptoms*
•Major depression
•Cerebrovascular disease
•Toxic, inflammatory, or metabolic disorders

AD=Alzheimer's disease. *Additional investigations, such as blood tests and brain MRI, are needed to exclude other causes of cognitive disorders or dementia, or concomitant pathologies (vascular lesions).

**Panel 3: IWG-2 criteria for mixed AD (A plus B)**
**Dubois et al, Lancet Neurol. 2014**

**A Clinical and biomarker evidence of AD (both are required)**

•Amnestic syndrome of the hippocampal type or one of the clinical phenotypes of atypical AD
•Decreased Aβ1–42 together with increased T-tau or P-tau in CSF, or increased tracer retention on amyloid PET

**B Clinical and biomarker evidence of mixed pathology**

*For cerebrovascular disease (both are required)*
•Documented history of stroke, or focal neurological features, or both
•MRI evidence of one or more of the following: corresponding vascular lesions, small vessel disease, strategic lacunar infarcts, or cerebral haemorrhages

*For Lewy body disease (both are required)*
•One of the following: extrapyramidal signs, early hallucinations, or cognitive fluctuations
•Abnormal dopamine transporter PET scan

AD=Alzheimer's disease.

8.2.2 Diagnosis of mild cognitive impairment due to Alzheimer's disease: National Institute on Aging-Alzheimer's Association workgroups

**Summary of clinical and cognitive evaluation for MCI due to AD**
**Albert et al., Alzheimers Dement. 2011**

**Establish clinical and cognitive criteria**

- Cognitive concern reflecting a change in cognition reported by patient or informant or clinician (*i.e.,* historical or observed evidence of decline
- over time)
- Objective evidence of Impairment in one or more cognitive domains, typically including memory (*i.e.,* formal or bedside testing to establish
- level of cognitive function in multiple domains)
- Preservation of independence in functional abilities
- Not demented

**Examine etiology of MCI consistent with AD pathophysiological process**

- Rule out vascular, traumatic, medical causes of cognitive decline, where possible
- Provide evidence of longitudinal decline in cognition, when feasible
- Report history consistent with AD genetic factors, where relevant

Abbreviations: AD, Alzheimer's disease; MCI, mild cognitive impairment.

**MCI criteria incorporating biomarkers**

| Diagnostic category | Biomarker probability of AD | Aβ etiology (PET or CSF) | Neuronal injury (tau, FDG, sMRI) |
|---|---|---|---|
| MCI-core clinical criteria | Uninformative | indeterminant | indeterminant |
| MCI due to AD—intermediate likelihood | Intermediate | Positive | Untested |
| MCI due to AD—high likelihood | Highest | Positive | Positive |
| MCI—unlikely due to AD | Lowest | Negative | Negative |

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid beta peptide; PET, positron emission tomography; CSF, cerebrospinal fluid; FDG, fluorodeoxyglucose; sMRI, structural magnetic resonance imaging.

8.2.3 Diagnosis of dementia with Lewy bodies: Fourth consensus report of the DLB Consortium

**Revised criteria for the clinical diagnosis of probable and possible dementia with Lewy bodies (DLB), McKeith et al., Neurology 2017**

Essential for a diagnosis of DLB is dementia, defined as a progressive cognitive decline of sufficient magnitude to interfere with normal social or occupational functions, or with usual daily activities. Prominent or persistent memory impairment may not necessarily occur in the early stages but is usually evident with progression. Deficits on tests of attention, executive function, and visuoperceptual ability may be especially prominent and occur early.

**Core clinical features (*The first 3 typically occur early and may persist throughout the course*.)**

Fluctuating cognition with pronounced variations in attention and alertness. Recurrent visual hallucinations that are typically well formed and detailed. REM sleep behavior disorder, *which may precede cognitive decline*.
One or more spontaneous cardinal features of parkinsonism: these are bradykinesia (defined as slowness of movement and decrement in amplitude or speed), rest tremor, or rigidity.

**Supportive clinical features**

Severe sensitivity to antipsychotic agents; postural instability; repeated falls; syncope or other transient episodes of unresponsiveness; severe autonomic dysfunction, e.g., constipation, orthostatic hypotension, urinary incontinence; hypersomnia; hyposmia; hallucinations in other modalities; systematized delusions; apathy, anxiety, and depression.

**Indicative biomarkers**

Reduced dopamine transporter uptake in basal ganglia demonstrated by SPECT or PET. Abnormal (low uptake) $^{123}$iodine-MIBG myocardial scintigraphy. Polysomnographic confirmation of REM sleep without atonia.

**Supportive biomarkers**

Relative preservation of medial temporal lobe structures on CT/MRI scan. Generalized low uptake on SPECT/PET perfusion/metabolism scan with reduced occipital activity 6 the cingulate island sign on FDG-PET imaging. Prominent posterior slow-wave activity on EEG with periodic fluctuations in the pre-alpha/ theta range.

**Probable DLB can be diagnosed if:**

a. Two or more core clinical features of DLB are present, with or without the presence of indicative biomarkers, or

b. Only one core clinical feature is present, but with one or more indicative biomarkers.

Probable DLB should not be diagnosed on the basis of biomarkers alone.

**Possible DLB can be diagnosed if:**

a. Only one core clinical feature of DLB is present, with no indicative biomarker evidence, or

b. One or more indicative biomarkers is present but there are no core clinical features.

**DLB is less likely:**

a. In the presence of any other physical illness or brain disorder including cerebrovascular disease, sufficient to account in part or in total for the clinical picture, although these do not exclude a DLB diagnosis and may serve to indicate mixed or multiple pathologies contributing to the clinical presentation, or

b. If parkinsonian features are the only core clinical feature and appear for the first time at a stage of severe dementia.

DLB should be diagnosed when dementia occurs before or concurrently with parkinsonism. The term Parkinson disease dementia (PDD) should be used to describe dementia that occurs in the context of well-established Parkinson disease. In a practice setting the term that is most appropriate to the clinical situation should be used and generic terms such as Lewy body disease are often helpful. In research studies in which distinction needs to be made between DLB and PDD, the existing 1-year rule between the onset of dementia and parkinsonism continues to be recommended.

8.2.4 Revised diagnostic criteria for the behavioural variant of frontotemporal dementia

**International consensus criteria for behavioural variant FTD (FTDC)**
**Rascovsky et al., Brain 2011**

**I. Neurodegenerative disease**

The following symptom must be present to meet criteria for bvFTD
A. Shows progressive deterioration of behaviour and/or cognition by observation or history (as provided by a knowledgeable informant).

**II. Possible bvFTD**

Three of the following behavioural/cognitive symptoms (A–F) must be present to meet criteria. Ascertainment requires that symptoms be persistent or recurrent, rather than single or rare events.
A. Early* behavioural disinhibition [one of the following symptoms (A.1–A.3) must be present]:
- A.1. Socially inappropriate behaviour
- A.2. Loss of manners or decorum
- A.3. Impulsive, rash or careless actions

B. Early apathy or inertia [one of the following symptoms (B.1–B.2) must be present]:
- B.1. Apathy
- B.2. Inertia

C. Early loss of sympathy or empathy [one of the following symptoms (C.1–C.2) must be present]:
- C.1. Diminished response to other people's needs and feelings
- C.2. Diminished social interest, interrelatedness or personal warmth

D. Early perseverative, stereotyped or compulsive/ritualistic behaviour [one of the following symptoms (D.1–D.3) must be present]:
- D.1. Simple repetitive movements
- D.2. Complex, compulsive or ritualistic behaviours
- D.3. Stereotypy of speech

E. Hyperorality and dietary changes [one of the following symptoms (E.1–E.3) must be present]:
- E.1. Altered food preferences
- E.2. Binge eating, increased consumption of alcohol or cigarettes
- E.3. Oral exploration or consumption of inedible objects

F. Neuropsychological profile: executive/generation deficits with relative sparing of memory and visuospatial functions [all of the following symptoms (F.1–F.3) must be present]:
- F.1. Deficits in executive tasks
- F.2. Relative sparing of episodic memory
- F.3. Relative sparing of visuospatial skills

**III. Probable bvFTD**

All of the following symptoms (A–C) must be present to meet criteria.

A. Meets criteria for possible bvFTD

B. Exhibits significant functional decline (by caregiver report or as evidenced by Clinical Dementia Rating Scale or Functional Activities
Questionnaire scores)
C. Imaging results consistent with bvFTD [one of the following (C.1–C.2) must be present]:
C.1. Frontal and/or anterior temporal atrophy on MRI or CT
C.2. Frontal and/or anterior temporal hypoperfusion or hypometabolism on PET or SPECT

**IV. Behavioural variant FTD with definite FTLD Pathology**

Criterion A and either criterion B or C must be present to meet criteria.
A. Meets criteria for possible or probable bvFTD
B. Histopathological evidence of FTLD on biopsy or at post-mortem
C. Presence of a known pathogenic mutation

**V. Exclusionary criteria for bvFTD**

Criteria A and B must be answered negatively for any bvFTD diagnosis. Criterion C can be positive for possible bvFTD but must be negative for
probable bvFTD.
A. Pattern of deficits is better accounted for by other non-degenerative nervous system or medical disorders
B. Behavioural disturbance is better accounted for by a psychiatric diagnosis
C. Biomarkers strongly indicative of Alzheimer's disease or other neurodegenerative process

8.2.5 Diagnostic criteria for the semantic variant PPA

**Diagnostic criteria for the semantic variant PPA**
**Gorno-Tempini et al., Neurology 2011**

**I. Clinical diagnosis of semantic variant PPA**

Both of the following core features must be present:

1. Impaired confrontation naming
2. Impaired single-word comprehension

At least 3 of the following other diagnostic features must be present:

1. Impaired object knowledge, particularly for low frequency or low-familiarity items
2. Surface dyslexia or dysgraphia
3. Spared repetition
4. Spared speech production (grammar and motor speech)

**II. Imaging-supported semantic variant PPA diagnosis**

Both of the following criteria must be present:

1. Clinical diagnosis of semantic variant PPA
2. Imaging must show one or more of the following results:

a. Predominant anterior temporal lobe atrophy
b. Predominant anterior temporal hypoperfusion or hypometabolism on SPECT or PET

**III. Semantic variant PPA with definite pathology**

Clinical diagnosis (criterion 1 below) and either criterion 2 or 3 must be present:

1. Clinical diagnosis of semantic variant PPA
2. Histopathologic evidence of a specific neurodegenerative pathology (e.g., FTLD tau, FTLD TDP, AD, other)
3. Presence of a known pathogenic mutation

Abbreviations: AD Alzheimer disease; FTLD frontotemporal lobar degeneration; PPA, primary progressive aphasia.

8.2.6 Diagnostic criteria for vascular cognitive disorders: a VASCOG statement

**Proposed criteria for Mild Cognitive Disorder and Dementia (or Major Cognitive Disorder)**
**Sachdev et al., Alzheimer Dis Assoc Disord. 2014**

**Mild cognitive disorder**

A. Acquired decline from a documented or inferred previous level of performance in one or more cognitive domains (listed in table 1)
as evidenced by the following:

a. Concerns of a patient, knowledgeable informant or a clinician of mild levels of decline from a previous level of cognitive functioning. Typically, the reports will involve greater difficulty in performing the tasks, or the use of compensatory strategies; and

b. Evidence of modest deficits on objective cognitive assessment based on a validated measure of neurocognitive function, (either formal neuropsychological testing or an equivalent clinical evaluation) in one or more cognitive domains listed in table 1. The test performance is typically in the range between 1 and 2 standard deviations below appropriate norms (or between the 3rd and 16th percentiles) when a formal neuropsychological assessment is available, or an equivalent level as judged by the clinician.

B. The cognitive deficits are not sufficient to interfere with independence (*i.e.,* instrumental activities of daily living are preserved), but
greater effort, compensatory strategies, or accommodation may be required to maintain independence.

**Dementia* or Major Cognitive Disorder:**

A. Evidence of substantial cognitive decline from a documented or inferred previous level of performance in one or more of the
domains outlined above. Evidence for decline is based on:

a. Concerns of the patient, a knowledgeable informant, or the clinician, of significant decline in specific abilities; and

b. Clear and significant deficits in objective assessment based on a validated objective measure of neurocognitive function (either formal neuropsychological testing or equivalent clinical evaluation) in one or more cognitive domains. These typically fall two or more standard deviations below the mean (or below the 3rd percentile) of people of similar age, sex, education, and sociocultural background, when a formal neuropsychological assessment is available, or an equivalent level as judged by the clinician.

B. The cognitive deficits are sufficient to interfere with independence (e.g., at a minimum requiring assistance with instrumental activities of daily living, *i.e.,* more complex tasks such as managing finances or medications).

*Note that the DSM-IV6 and ICD-107 concept of dementia requires deficits in at least two domains, one of which being memory.

## 8.3 Appendix 3 - "Biomarqueurs de la maladie d'Alzheimer : des avancées très rapides"

Agathe Vrillon, Claire Paquet. Biomarqueurs de la maladie d'Alzheimer : des avancées très rapides,La Presse Médicale Formation,Volume 3, Issue 1, Part 1,2022,Pages 49-56, ISSN 2666-4798, https://doi.org/10.1016/j.lpmfor.2022.01.013.

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# Biomarqueurs de la maladie d'Alzheimer : des avancées très rapides

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## Résumé

Alors que les premiers traitements, modifiant la charge lésionnelle dans la maladie d'Alzheimer (MA), sont en cours de développement avec des résultats cliniques de plus en plus prometteurs, le développement et la validation de biomarqueurs cliniquement pertinents pour le diagnostic de la MA ont fait l'objet de récentes recherche. Une meilleure compréhension des mécanismes de la maladie et les améliorations technologiques ont conduit à la découverte de nouveaux biomarqueurs, reflétant les principaux processus de la MA, tels que l'agrégation de protéines anormales, l'altération synaptique, la neurodégénérescence et la neuroinflammation. Les marqueurs du liquide céphalorachidien (LCR) et la tomographie par émission de positons ou l'IRM, qui permettent de détecter in vivo les lésions caractéristiques de la MA, ont considérablement amélioré le diagnostic. Néanmoins, leur coût, leur disponibilité limitée et leur caractère invasif ont restreint leur utilisation dans la pratique clinique. La mesure dans le plasma des peptides amyloïdes-β et de la protéine tau-phosphorylée était auparavant limitée par le manque de sensibilité des techniques disponibles. Des méthodes innovantes, dotées d'une sensibilité et d'une spécificité supérieures, ont récemment rendu possible leur mesure ; les taux d'Aβ et de p-tau plasmatiques sont altérés dans la MA et s'associent au déclin cognitif. Au-delà des pathologies amyloïdes et tau, de nouveaux biomarqueurs, reflétant des aspects supplémentaires de la physiopathologie de la MA, ont également été développés dans le LCR, le plasma et via l'imagerie. Les neurofilaments mesurés dans le LCR et le plasma sont maintenant bien confirmés comme marqueur robuste de neurodégénérescence. Parmi les autres candidats, les protéines synaptiques dans le LCR apparaissent spécifiques de la MA et prédisent le déclin cognitif. Par ailleurs, des études récentes dans les liquides biologiques et en imagerie ont permis d'identifier des marqueurs prometteurs pour évaluer la neuroinflammation dans MA. Au total, l'utilisation combinée de nouveaux biomarqueurs pourrait permettre de mieux caractériser les changements pathologiques associés à la MA. Bien que ces techniques doivent encore être validées, les nouveaux marqueurs, en particulier plasmatiques, apparaissent prometteurs pour le diagnostic de la MA en pratique clinique, ainsi que pour l'évaluation des nouvelles thérapies.


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## Summary

### Biomarkers of Alzheimer's disease: Recent advances

*As the development of disease-modifying treatments for Alzheimer's disease (AD) has known significant improvement, development and validation of clinically relevant biomarkers for AD diagnosis have been the focus of recent research efforts. Increased comprehension of disease mechanisms and technological improvements have led to the discovery of innovative biomarkers reflecting the multiple processes underlying AD such as protein aggregation, synaptic impairment, neurodegeneration, and neuroinflammation. Cerebrospinal fluid (CSF) and positron emission tomography or MRI-based neuroimaging markers allowing for in vivo detection of AD pathology have greatly improved the diagnosis accuracy. Nevertheless, their cost, limited availability and invasiveness have limited their use in the clinical setting. Measuring changes in plasma in markers of the hallmarks of AD, including amyloid-β peptides and phosphorylated tau, had been challenged by the lack of sensibility of the available techniques. However, innovative assays with superior sensitivity and specificity have recently made their measurement possible, and Aβ and p-tau show consistent modification in blood in AD and associate with cognitive decline. Beyond the amyloid and tau pathologies, novel biomarkers reflecting additional aspects of AD pathophysiology have also been developed in CSF, plasma and imaging. Neurofilaments levels in CSF and plasma are now well established as robust markers of neurodegeneration. Other candidates include synaptic proteins levels in CSF that seem specific for AD and predict future cognitive deterioration. Further, recent studies in PET and in fluid compartments have identified promising candidates for monitoring neuroinflammation across AD* continuum. *All in all, a combination of new biomarkers could enhance their utility for characterizing AD-associated pathological changes. While these technologies still require validation, new markers, especially in plasma, appear promising for the diagnosis and prognosis in clinical practice and for outcome assessments in clinical trials for AD.*

En 2018, le *National Institute on Aging and Alzheimer's Association* (NIA-AA) a mis à jour les critères diagnostiques de la maladie d'Alzheimer (MA) en recherche, passant d'une définition clinique à une définition biologique [1]. Auparavant définie selon trois phases cliniques successives (phase préclinique, troubles cognitifs mineurs [MCI] et troubles cognitifs majeurs), la MA est désormais considérée comme un continuum qui peut être identifié par des biomarqueurs biologiques et/ou imageriques, mettant en évidence in vivo les processus physiopathologiques de la MA, i.e. agrégâts extraneuronaux de peptide Abéta (ou plaques amyloïdes), les dégénérescences neurofibrillaires composées d'agrégats de protéine tau hyperphosphorylée intraneuronaux. Ces marqueurs, associant l'analyse du liquide cérébro-spinal (LCS) et l'imagerie en Tomographie à émission de positons (TEP), sont utilisés en pratique clinique pour poser un diagnostic positif et écarter les diagnostics différentiels, dès le stade précoce de MCI, depuis maintenant près de 10 ans.

En plus de leur fonction diagnostique majeure [2], les biomarqueurs de la MA sont des outils essentiels dans le développement thérapeutique pour évaluer les effets pharmacodynamiques des molécules étudiées, démontrer l'atteinte de la cible, aider à la sélection des participants pour les essais thérapeutiques, et en évaluer l'efficacité. Dans un contexte où la première thérapie anti-amyloïde, l'aducanumab, a été approuvée par la *Food and Drug Administration* aux États-Unis en juin 2021, le développement et la validation clinique de biomarqueurs sont plus que jamais primordiaux. Toutefois, l'usage des biomarqueurs actuels est limité par leur caractère coûteux, invasif et peu accessible. La faible concentration périphérique des protéines, issues du système nerveux central (SNC), a longtemps limité la découverte de biomarqueurs périphériques, mais le développement récent de dosage innovants ultrasensibles lève progressivement cette barrière. La grande révolution, dans le domaine des biomarqueurs dans la MA, cette dernière décennie, est donc le développement de biomarqueurs sanguins, dont certains démontrent une performance diagnostique proche de celle du LCS [3]. De nouveaux biomarqueurs, évaluant les processus autres que les dépôts protéiques, comme l'atteinte neuronale et synaptique ou la neuroinflammation, sont également en cours d'exploration.

Cette revue vise à faire un état des lieux des principaux biomarqueurs, actuels et potentiels, dans la MA, spécifiques ou non, biologiques, imageriques et électrophysiologiques.

## Biomarqueurs

### Biomarqueurs du liquide cérébrospinal

Le LCS est le meilleur reflet des processus métaboliques cérébraux en raison de son contact direct avec le cerveau ; par conséquent, il est devenu un fluide utile pour le diagnostic de la MA et les seuls biomarqueurs cliniquement validés sont mesurés dans le LCS.

#### *Aß40, Aß42, tau, phospho tau*

La « signature biologique ou biochimique » de la MA dans le LCS consiste en une diminution du peptide amyloïde béta 1-42 (Aβ42) et une augmentation de la protéine tau dans sa forme phosphorylée (p-tau) et sa forme totale (t-tau). Ces dosages, développés il y a plus de 20 ans, sont maintenant très bien caractérisés et validés comme une aide au diagnostic clinique dans la MA [1,4,5].

Il est établi que le peptide Aβ42 mesuré dans le LCS présente une corrélation inverse avec la charge cérébrale en plaques amyloïdes. Le rapport Aβ42/Aβ40 utilisé pour normaliser le taux d'Aβ42 dans le LCS en fonction de la charge amyloïde totale, représentée de manière fiable par les taux d'Aβ40 (peptide amyloïde le plus abondant dans le LCS) a démontré une sensibilité et une spécificité supérieures à celles du peptide Aβ42 seul. La protéine p-tau (forme p-tau181, phosphorylée à la sérine 181) est considérée comme un reflet de la présence d'une pathologie cérébrale tau, reflétant les lésions neurofibrillaires, tandis que la protéine t-tau est un marqueur de lésion neuronale et de neuro-dégénérescence. Récemment, des mesures de la p-tau à d'autres épitopes ont été réussies, notamment p-tau217 et p-tau231. Ces dosages ont mis en évidence que p-tau217 et p-tau231 augmentent dans le LCS avant p-tau181 durant la phase préclinique, ouvrant la voie à une mise en évidence encore plus précoce du processus tau [6,7]. La concentration de t-tau dans le LCS est élevée dans d'autres pathologies impliquant des dommages cérébraux importants, comme la maladie de Creutzfeldt-Jacob, les accidents vasculaires cérébraux, l'arrêt cardiaque ou les lésions cérébrales traumatiques, ce qui en fait un marqueur moins spécifique que Aß42 et p-tau. Pour interpréter le taux de t-tau dans le LCS, il est donc très important d'être à distance d'au moins 3 mois d'un dommage cérébral aigu (AVC, épilepsie, traumatisme…).

Lorsque ces trois marqueurs (Aβ42, t-tau et p-tau) sont mesurés ensemble, ils présentent une meilleure spécificité et sensibilité qu'utilisés seuls pour préciser le risque d'évoluer vers une phase symptomatique de la MA [8]. Le système ATN (Amyloid/Tau/Neurodegeneration), proposé en 2018, permet d'évaluer ce risque en combinant les biomarqueurs du LCS, les imageries en TEP amyloïde et tau et l'imagerie morphologique (IRM) [1]. Ces biomarqueurs sont indiqués en fonction du contexte [9] et ont démontré leur impact en pratique clinique, non seulement dans la MA, mais aussi pour tous les patients qui ont besoin d'un diagnostic différentiel [10].

Les avancées récentes importantes pour les biomarqueurs de la MA dans le LCS comprennent la validation de méthodes de dosage entièrement automatisées (Lumipulse®, Roche Cobas®), et la mise en place de protocoles standardisés, qui favorisent la fiabilité et la disponibilité des dosages.

#### *Marqueurs de dégâts axonaux et neuronaux*

Les chaînes légères des neurofilaments (NfL) sont une protéine intraneuronale et l'un des composants principaux du cytosquelette axonal ; sa présence dans le LCR indique donc une atteinte ou une dégénérescence neuronale [11]. Dans la MA, les concentrations de NfL dans le LCS augmentent dès les premiers stades de la maladie et s'accroissent au fil du temps avec le déclin cognitif et la progression de l'atrophie et les modifications de la substance blanche.

Les études sur les formes génétiques autosomiques dominante de MA montrent que les taux de NfL dans le LCS augmentent déjà environ 15 ans avant l'apparition des premiers symptômes. Ce marqueur n'est pas spécifique de la MA ; son augmentation est plus importante dans les maladies neurodégénératives avec une neurodégénérescence intense comme la maladie de Creutzfeld Jakob ou la dégénérescence frontotemporale. Dans plusieurs pathologies neurologiques, notamment la sclérose en plaques et l'amyotrophie spinale, des traitements efficaces modificateurs de la maladie peuvent normaliser les taux de NfL, et ceux-ci sont associés à l'efficacité clinique du traitement. Les NfL peuvent donc être considérées dans la MA comme une mesure de l'intensité de la souffrance axonale en cours et apparaissent prometteurs comme futurs marqueurs de réponse thérapeutique.

#### *Marqueurs de neuroinflammation*

Il est maintenant établi que la neuroinflammation est un facteur pathogène dans la MA, perpétuant les dommages neuronaux, via l'activation de la microglie et du système immunitaire inné. Au cours des 25 dernières années, de nombreuses études ont montré des changements persistants des niveaux de cytokines et autres protéines pro- et anti-inflammatoires dans le LCS des patients avec une MA [12]. La protéine acidique fibrillaire gliale (GFAP) est un marqueur d'activation astrogliale. La progression des lésions de la MA est associée à une augmentation des niveaux de GFAP dans le LCS. Le récepteur *Triggering receptor expressed on myeloid cells 2* (TREM-2) est une glycoprotéine exprimée par les microglies, de fonctions multiples (signalisation inflammatoire, phagocytose). Des mutations dans ce gène sont associées à un risque accru de MA. La mesure de TREM-2 dans le LCS a montré des taux augmentés dès le stade préclinique de la maladie. YKL-40 (ou *human chitinase 3*),

glycoprotéine sécrétée par la microglie et les astrocytes dans le LCS, est également augmentée dans la MA, même si cette augmentation est modérée.

Ces différents marqueurs ne sont pas spécifiques de la MA et on observe leur augmentation dans les autres pathologies neurodégénératives ou neuro-inflammatoires, ils ne peuvent donc être de bons biomarqueurs diagnostiques. Toutefois, ils nous apportent des informations physiopathologiques non accessibles par d'autres examens, et ce, du vivant du patient.

### *Biomarqueurs synaptiques*

Le dysfonctionnement et la perte synaptiques sont des événements pathologiques diffus et précoces dans les troubles neurodégénératifs. La réduction des niveaux de protéines pré- et postsynaptiques est reconnue comme une caractéristique physiopathologique essentielle de la MA. L'exploration des protéines synaptiques dans le LCS est maintenant réalisable et leur taux reflète la perte synaptique chez les patients [13].

Parmi ces protéines, la neurogranine est une protéine intracellulaire concentrée dans le compartiment dendritique et postsynaptique des épines synaptiques des neurones. La neurogranine montre de manière consistante des taux accrus dans le LCS des patients avec une MA et prédit la dégradation cognitive [14].

GAP-43 est une protéine présynaptique impliquée dans la mémoire et le stockage des informations. Les niveaux de GAP-43 dans le LCS sont augmentés chez les patients avec une MA que chez des sujets témoins et dans d'autres troubles neurodégénératifs [15]. Les protéines SNAP et Synaptotagmin, pré-synaptiques, étaient également augmentées spécifiquement dans la MA par rapport aux autres pathologies neurodégénératives [13]. Ainsi, la perte synaptique semble plus importante dans la MA, peut-être due à une double toxicité Aß et tau.

En parallèle, de façon contre-intuitive au regard des autres protéines synaptiques, une étude préliminaire démontre que la protéine SV2a, exprimée dans les vésicules synaptiques, était, quant à elle, diminuée dans le LCS dans la MA [16], ce qui est concordant avec l'imagerie en TEP SV2a.

### *Autres biomarqueurs*

D'autres biomarqueurs du LCS ont été publiés et sont en cours de développement. Ainsi, il est possible d'avoir un reflet de l'activité cholinergique en mesurant les niveau d'acétylcholine et/ou de l'activité acetylcholinestérasique [17]. Plusieurs autres protéines, impliquées dans la physiopathologie de la MA, par un rôle à la fois pro-apoptotique et pro-infammatoire, sont mesurables et pourraient être à termes des cibles thérapeutiques. Ces biomarqueurs constitueraient alors des biomarqueurs « compagnons », permettant d'identifier la cible et de mesurer la réponse thérapeutique [18].

## Biomarqueurs sanguins

L'utilisation diagnostique et pronostique des biomarqueurs du LCS est limitée en raison du caractère invasif de la ponction lombaire et de son accès limité. Dans ce contexte, les efforts se sont dirigés vers l'établissement de biomarqueurs sanguins fiables. L'utilisation de biomarqueurs sanguins serait optimale dans la pratique clinique, car peu invasifs et, par conséquent, faciles à collecter et à traiter et permettant le suivi.

Cependant, le développement de biomarqueurs sanguins sensibles et spécifiques s'est révélé difficile pour plusieurs raisons. Seule une faible fraction des protéines cérébrales passe dans le sang et celles-ci doivent être mesurées dans une matrice contenant des niveaux élevés de protéines plasmatiques, avec un risque important d'interférence dans les dosages. Les protéines cérébrales libérées dans le sang peuvent également être dégradées par des protéases, ce qui introduit une variabilité qui ne reflète pas l'état cérébral. Enfin, certaines protéines ne sont pas spécifiques au SNC et peuvent être relarguées par des organes périphériques. La dimension dans laquelle les changements moléculaires périphériques reflètent précisément la dynamique du SNC demeure à mieux caractériser.

Néanmoins, ces dernières années, des améliorations majeures ont été apportées aux techniques de dosage avec le développement, entre autres, du *Single MOlecule Array* (SIMOA), de l'immunoprécipitation – spectrométrie de masse, de la *mesoscale discovery* (MDS) ou de l'électrochimiluminescence, qui ont permis une augmentation significative de la sensibilité par rapport à la technique ELISA. Le développement de biomarqueurs sanguins s'est concentré sur l'étude de marqueurs des processus amyloïde et tau, mais également sur des marqueurs reflétant les autres processus physiopathologiques. Ces derniers seront également utiles dans les autres pathologies affectant le SNC. Les premiers résultats sont prometteurs et on peut penser qu'à moyen terme, ils permettront de sélectionner les patients nécessitant une analyse du LCS ou de faire du *screening* en soin primaire. À long terme, on peut maintenant avoir l'espoir que certains marqueurs, notamment les formes phosphorylées de la protéine tau, permettent, seul ou en combinaison, le diagnostic sans passer par un examen du LCS.

### *Biomarqueurs plasmatiques de la pathologie amyloïde et tau*

L'amyloïdopathie constituant un processus physiopathologique central dans la MA, des tentatives ont été faites pour identifier des marqueurs d'amyloïdopathie dans le sang périphérique. Les marqueurs amyloïdes (Aβ42 ou ratio plasmatique Aβ42/40) corrèlent avec leur mesure dans le LCS par spectrométrie de masse. Ces même marqueurs plasmatiques corrèlent avec la charge amyloïde mesurée par TEP amyloïde, quels que soient les radiotraceurs et permettent de prédire son évolution [19]. Les mesures automatisées en SiMoA ou Elecsys, plus accessibles, présentent une sensibilité et une spécificité moindres pour identifier les individus amyloïde-positifs.

L'avancée majeure dans la validation de biomarqueurs plasmatiques dans le diagnostic de la MA est venue des dosages de la

protéine p-tau dans plusieurs de ses formes : p-tau181, p-tau217 et p-tau 231 [6,7,20]. Plusieurs études ont montré que les taux plasmatiques de p-tau181, de p-tau217 ou p-tau231 peuvent différencier avec précision les individus présentant des lésions neuropathologiques de la MA de ceux qui n'en présentent pas, dont certains avec des pathologies autres (TDP-43, alpha-synucléine.). Dans des études de cohorte, p-tau plasmatique discrimine également, avec une grande précision, la MA des autres maladies neurodégénératives. P-tau217 a une performance diagnostique similaire à celle des biomarqueurs de la MA dans le LCS et de l'imagerie TEP-tau, avec une précision d'environ 90 % [20]. Chez les patients atteints de MCI, des taux de base plus élevés de p-tau plasmatique sont associés à un déclin cognitif ultérieur. Des algorithmes basés sur p-tau plasmatique, pour évaluer le pronostic individualisé des patients atteints de MCI, apparaissent aussi performants que les algorithmes basés sur le LCS [3]. Enfin, des études longitudinales ont montré que p-tau plasmatique (en particulier, p-tau217) présente une augmentation nette au cours du temps dans les phases préclinique et très débutante de la MA et pourrait potentiellement être utilisé pour le *screening* pour les essais thérapeutiques et l'évaluation de leur efficacité.

Concernant t-tau, les taux cérébrospinaux et plasmatiques présentent une faible corrélation. En effet, la protéine tau provient non seulement du cerveau, mais aussi d'autres organes et tissus. Plusieurs études ont rapporté des niveaux plasmatiques élevés de t-tau, chez des individus avec une MA, néanmoins, avec un chevauchement important entre les groupes diagnostiques (sujets sains, MCI, MA) [21] ne permettant de l'utiliser comme biomarqueur diagnostique.

#### *Biomarqueurs plasmatiques de lésions axonales et neuronales*

Le développement de techniques ultrasensibles a également rendu possible le dosage des NfL dans le plasma. La corrélation entre les taux de NfL centraux et périphériques est élevée. Bien qu'il ne s'agisse pas d'un marqueur spécifique de la MA, les NfL sanguins ont le potentiel de suivre ou de prédire de nombreux aspects de la neurodégénérescence dans la MA [22]. Les taux de NfL sont associés à l'atrophie et au degré d'hypométabolisme observé à la TEP-FDG. Les NfL prédisent le déclin cognitif au cours du temps. Leur dynamique discrimine, notamment, les porteurs et les non-porteurs de mutation autosomique dominante 10 ans avant le début présumé de leurs symptômes.

#### *Biomarqueurs plasmatiques de neuroinflammation*

La protéine GFAP, marqueur astroglial, est mesurable dans le plasma et augmentée dans la MA. Notablement, ses performances diagnostiques, mesurées dans le plasma, apparaissent supérieures à celles de son dosage dans le LCS. Elle permet l'identification des sujets amyloïdes positifs avec une sensibilité et une spécificité prometteuses. YKL40 plasmatique est également augmentée de manière précoce.

Les prochaines étapes pour la validation de ces nouveaux marqueurs sanguins seront l'évaluation de leur performance diagnostique dans des cohortes de patients plus représentatives des soins primaires ou des centres mémoires et la standardisation des conditions et de méthodes de prélèvements.

### Autres fluides

La salive est une source périphérique alternative de biomarqueurs de maladies accessibles et non invasifs. Les protéines Aβ, p-Tau et t-Tau, NfL et alpha-synucléine sont toutes détectables dans la salive et des études préliminaires ont montré une utilité diagnostique potentielle [23]. Notamment, Aß42 et p-tau apparaissent significativement augmentées. En revanche, le dosage de NFL dans la salive ne permettait pas de différencier des sujets sains des patients avec un trouble cognitif. L'analyse des larmes a été effectuée dans plusieurs études préliminaires, mais les mesures d'Ab42 et tau montraient un faible pouvoir diagnostique. Plusieurs métabolites urinaires (associés notamment au stress oxydatif), qui pourraient constituer des biomarqueurs potentiels de la MA, ont été identifiés sur la base des changements significatifs observés entre patients et individus témoins.

## Imagerie fonctionnelle

### La TEP amyloïde et la TEP tau

La TEP amyloïde fournit des informations topographiques et quantitatives sur la charge amyloïde cérébrale. Elle permet de retenir l'existence d'un processus amyloide (statut A+) dans les critères diagnostiques NIA-AA de 2011 et une TEP amyloïde négatif permet d'éliminer le diagnostic de MA. Plusieurs ligands existent et permettent la détection in vivo des plaques fibrillaires amyloïdes Aβ riche en fibres ß plissées dans le cortex frontal, pariéto-temporal et cingulaire postérieur [24]. La spécificité de l'Aβ-PET diminue, toutefois, avec l'âge, car des dépôts d'Aβ corticaux sont mis en évidence chez près de 15 % des personnes ayant une cognition normale à 60 ans et chez environ 40 % à 90 ans. La TEP amyloïde permet un diagnostic à un stade très précoce de l'amyloïdopathie, en préclinique. Néanmoins, sa corrélation aux symptômes cliniques reste faible et elle n'est pas de valeur pronostique. Sa place dans la prise en charge en soin courant n'est pas encore entièrement établie du fait de son coût élevé et du manque d'accessibilité.

La TEP-Tau est un biomarqueur permettant de définir le statut T dans la classification ATN. Malgré les résultats préliminaires prometteurs obtenus au cours des cinq dernières années, sa place pour une utilisation en pratique clinique reste à définir. Plusieurs générations de traceurs TEP permettant d'observer la pathologie neurofibrillaire in vivo dans la MA. Aucune liaison significative au ligand n'est observée dans les autres maladies neurodégénérative, même dans les autres taupathies (démence fronto-temporale, paralysie supranucléaire progressive). La charge lésionnelle mesurée par la TEP tau corrèle significativement avec les stades de Braak en post-mortem

[25]. La TEP démontre une association plus importante avec l'état cognitif et son évolution que la TEP amyloïde.

### TEP au fluorodesoxyglucose

La TEP FDG mesure la consommation cérébrale régionale de glucose directement liée à l'intensité régionale de l'activité synaptique glutamatergique et permet l'évaluation de la localisation et du degré de dysfonction neuronale [26]. Elle permet l'évaluation de la localisation et du dégré de dysfonction neuronale et est donc un marqueur indirect de neurodégénérescence qui permet d'aider au diagnostic de la MA et des autres maladies neurodégénératives. La TEP-FDG identifie des *patterns* compatibles avec la MA dès le stade de MCI caractérisé par un hypométabolisme du cortex cingulaire postérieur et du cortex temporo-pariétal, même en l'absence d'atrophie. Dans les cohortes, il permet la prédiction de l'évolution d'un sujet avec un MCI vers des troubles cognitifs majeurs ou vers la stabilité ou la régression des troubles [27]. Il est important de préciser que la TEP FDG ne confirme pas la présence ou non de lésions amyloïdes et tau cérébrales et reste donc un marqueur indirect.

### Autres imageries TEP

#### *TEP synaptique*

La quantification in vivo de la densité synaptique cérébrale est en cours de développement en imagerie TEP avec des radioligands spécifiques de la synapse. Tout récemment, plusieurs radioligands ont été développés dans l'imagerie de la glycoprotéine SV2a qui est la cible moléculaire du Lévétiracétam. Des études utilisant la TEP au [11C]UCB-J ou [18F]UCB-H ont mis en évidence une perte synaptique étendue dans la MA, prédominant dans le lobe temporal et dans le néocortex [28].

#### *TEP microglial*

Le développement de nouveaux radiotraceurs pour évaluer l'activation microgliale par TEP a permis de visualiser une activation du système immunitaire inné de chez les patients avec une MA [29]. Les premières études au 11C-PK11195 (ligand du TSPO, une protéine mitochondriale associée à la microglie) ont montré une augmentation significative de la fixation du ligand dans le cortex entorhinal, le cortex temporo-pariétal et le cortex cingulaire chez les patients avec MA. Néanmoins, le rôle de l'activation microgliale dans la MA, protecteur ou délétère, est toujours débattu. Le phénotype inflammatoire est probablement variable au cours de la maladie et difficilement caractérisé par les biomarqueurs actuellement disponibles.

#### *TEP cholinergique*

La dysfonction cholinergique a été l'une des premières hypothèses physiopathologiques dans la MA et, jusqu'à ce jour, les inhibiteurs de l'acétylcholinestérase sont un des seuls traitements symptomatiques disponibles dans la MA. Plusieurs ligands substrats sélectifs de l'Acétylcholinesterase (AChE) ont été utilisés en imagerie PET : l'activité de l'AChE était significativement réduite dans les régions hippocampiques et néocorticales chez les patients avec une MA sévère [30].

## Autres marqueurs

### Électroencéphalogramme

L'analyse EEG fournit une représentation physiologique directe et dynamique de la fonction neuronale et synaptique. Des altérations des mesures quantitatives dérivées des données EEG chez les patients avec une MA ont été décrites et sont associées à la progression de la maladie et corrèlent avec les mesures des biomarqueurs du LCS [31] : on observe généralement un ralentissement de l'activité cérébrale avec une diminution du rythme alpha, une diminution des rythmes complexes et une synchronisation réduite. La diminution du rythme alpha est notamment associée au degré d'atrophie hippocampique et au niveau de déclin cognitif. De plus, ces anomalies sont détectables dès le stade préclinique de la maladie et apparaissent corrélées au dégré de la charge amyloïde mesurée en TEP. L'hypothèse est qu'elles sont le reflet de mécanismes compensatoires de l'altération synaptique [32].

### Biomarqueurs rétiniens

Sur le plan embryologie, la rétine, le nerf optique et leur vascularisation naissent d'une expansion latérale du cerveau qui restera attachée au diencéphale tout en s'allongeant pour donner le pédicule optique. Ainsi, la rétine et le nerf optique sont des expansions du cerveau et la myéline du nerf optique est similaire à celle du système nerveux central et non à celle du système nerveux périphérique. Des changements rétiniens peuvent donc refléter des processus survenant dans le système nerveux central. En 2007, pour la première fois, Paquet et al. ont démontré une diminution de l'épaisseur de la couche de fibres nerveuses rétiniennes péripapillaires mesurées par OCT chez des sujets souffrants de troubles cognitifs légers liés à une MA, comparativement aux sujets contrôles [33]. Depuis, ces données ont été confirmées et d'autres biomarqueurs sont en cours d'exploration incluant : la morphologie de la tête du nerf optique, la mesure des dépôts amyloïdes rétinien, la morphologie et les densités microvasculaires, le flux sanguin et les marqueurs électrophysiologiques.

## Conclusion et perspectives

Actuellement, il existe un large consensus sur la nécessité de débuter les interventions thérapeutiques dans la MA aux stades précoces, voire dans la phase préclinique. L'accumulation des protéines Aβ et tau indique l'existence d'une fenêtre temporelle réelle pour enrayer les processus pathologiques, mais l'identification clinique de ce processus nécessite l'utilisation de biomarqueurs. Les biomarqueurs sanguins constituent des biomarqueurs idéaux, accessibles et non invasifs. Historiquement, la sensibilité et la spécificité des biomarqueurs sanguins ont été plus faibles que celles des biomarqueurs du LCS.

Néanmoins, durant la dernière décade, plusieurs marqueurs plasmatiques fiables, spécifiques (phospho-tau) et non spécifiques (NfL) ont été développés sur des plateformes de dosage innovantes et apparaissent extrêmement prometteurs pour le diagnostic positif et différentiel, ainsi que le suivi de la MA. Leur translation vers la pratique clinique nécessitera des études cliniques prospectives pour évaluer leur performance diagnostique et pronostique et les comparer aux biomarqueurs validés. L'adjonction des biomarqueurs caractérisant les autres dimensions de la maladie (atteinte synaptique, neuroinflammation, atrophie) permettront d'établir une signature spécifique de la MA chez chaque patient.

**Déclaration de liens d'intérêts :** les auteurs déclarent ne pas avoir de liens d'intérêts.

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# 9. PAPERS

Alzheimer's Research & Therapy

RESEARCH

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# Plasma neuregulin 1 as a synaptic biomarker in Alzheimer's disease: a discovery cohort study

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## Abstract

**Background:** Synaptic dysfunction is an early core feature of Alzheimer's disease (AD), closely associated with cognitive symptoms. Neuregulin 1 (NRG1) is a growth and differentiation factor with a key role in the development and maintenance of synaptic transmission. Previous reports have shown that changes in cerebrospinal fluid (CSF) NRG1 concentration are associated with cognitive status and biomarker evidence of AD pathology. Plasma biomarkers reflecting synaptic impairment would be of great clinical interest.

**Objective:** To measure plasma NRG1 concentration in AD patients in comparison with other neurodegenerative disorders and neurological controls (NC) and to study its association with cerebrospinal fluid (CSF) core AD and synaptic biomarkers.

**Methods:** This retrospective study enrolled 127 participants including patients with AD at mild cognitive impairment stage (AD-MCI, $n = 27$) and at dementia stage ($n = 35$), non-AD dementia ($n = 26$, Aβ-negative), non-AD MCI ($n = 19$), and neurological controls ($n$=20). Plasma and CSF NRG1, as well as CSF core AD biomarkers (Aβ 42/Aβ 40 ratio, phospho-tau, and total tau), were measured using ELISA. CSF synaptic markers were measured using ELISA for GAP-43 and neurogranin and through immunoprecipitation mass spectrometry for SNAP-25.

**Results:** Plasma NRG1 concentration was higher in AD-MCI and AD dementia patients compared with neurological controls (respectively $P = 0.005$ and $P < 0.001$). Plasma NRG1 differentiated AD MCI patients from neurological controls with an area under the curve of 88.3%, and AD dementia patients from NC with an area under the curve of 87.3%. Plasma NRG1 correlated with CSF NRG1 ($\beta = 0.372$, $P = 0.0056$, adjusted on age and sex). Plasma NRG1 was associated with AD CSF core biomarkers in the whole cohort and in Aβ-positive patients ($\beta = -0.197$–$0.423$). Plasma NRG1 correlated with CSF GAP-43, neurogranin, and SNAP-25 ($\beta = 0.278$–$0.355$). Plasma NRG1 concentration correlated inversely with MMSE in the whole cohort and in Aβ-positive patients (all, $\beta = -0.188$, $P = 0.038$; Aβ+: $\beta = -0.255$, $P = 0.038$).

**Conclusion:** Plasma NRG1 concentration is increased in AD patients and correlates with CSF core AD and synaptic biomarkers and cognitive status. Thus, plasma NRG1 is a promising non-invasive biomarker to monitor synaptic impairment in AD.

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**Keywords:** Alzheimer's disease, NRG1, Synaptic pathology, Plasma biomarker

## Introduction

Synaptic impairment is a core feature of Alzheimer's disease (AD) and one of the earliest detectable changes [1, 2]. Neuropathological examination has demonstrated that synaptic demise shows a higher association to cognitive decline than amyloid plaque load or neurofibrillary tangle pathology [3, 4]. Positron emission tomography (PET) imaging using synaptic tracers indicates that synaptic density is significantly reduced in the hippocampus in AD patients, especially in its early symptomatic stages [5, 6]. The evaluation of several synaptic proteins has been achieved in the cerebrospinal fluid (CSF) [7–10]. Presynaptic synaptosomal-associated protein 25 (SNAP-25), synaptotagmin, or growth-associated protein-43 (GAP-43) as well as post-synaptic neurogranin levels are altered in AD CSF and are reliable biomarkers of synaptic impairment, as early as in the preclinical stage of the disease [11, 12]. Those synaptic biomarkers also contribute to the understanding of the underlying pathological processes of the cognitive decline. Alteration of current CSF synaptic biomarkers appears to show specificity to AD, indicating AD as the pathology with the highest synaptic involvement [11, 12]. Moreover, the different synaptic proteins involved in various processes in pre- and post-synaptic compartments most likely reflect different mechanisms at play at the synapse [13]. Thus, synaptic biomarker investigation appears as a key tool to investigate the pathological mechanisms responsible for synaptic damage.

Regarding blood, the presynaptic betasynuclein measured using quantitative mass spectrometry could discriminate AD and CJD from controls and other neurodegenerative disorders [14]. Other synaptic markers have been explored in blood but so far, due to the existence of peripheral expression or of other factors of variability, there is no validated reliable biomarker of synaptic pathology [13]. Plasma markers allow for easy, cost-effective, and repeated measurements both in research and in clinical settings. Synaptic impairment markers are a category of biomarkers expected to be most closely correlated with cognitive function. It would make a synaptic plasma biomarker of high interest for monitoring AD progression, as well as for screening and inclusion, and measure of the therapeutic response in clinical trials.

Neuregulin 1 (NRG1), a protein encoded by the *NRG1* gene, is a member of the epithelial growth factor (EGF) family. They constitute ligands with a high affinity for ErbB tyrosine kinase receptors. NRG1 is implicated in many processes during neural development including the proliferation of neuronal progenitors, neuron migration and survival, axon guidance, glial development, and myelination, as well as synaptogenesis [15, 16]. In the adult brain, NRG1 is expressed in multiple regions and regulates neurotransmission and synaptic plasticity [17]. Membrane-bound, NRG1 requires processing by a protease to initiate release and signaling. Among implicated proteases, NRG1 can undergo cleavage by the β-site amyloid precursor protein cleaving enzyme 1 (BACE1) at multiple sites [18–20]. Proteolytic processing results in the secretion of soluble forms that will further activate ErbB receptors, mainly at the post-synaptic level. NRG1 and its receptor ErbB4 levels have been found altered in the human AD brain, both in the hippocampus and cortex [21, 22]. In CSF, two studies including our prior work have reported modified NRG1 levels in AD patients compared with controls and to patients with non-AD-related cognitive decline [23, 24].

The purpose of our study was to investigate plasma NRG1 levels in a cohort of patients with cognitive decline due to AD, non-AD-related cognitive decline, and neurological controls and to assess its association with core AD CSF biomarkers, CSF synaptic markers, and cognitive status.

## Methods

### Cohort

A total of 127 patients from the Cognitive Neurology Center, Lariboisière Fernand Widal Hospital, Université Paris Cité, was retrospectively included in our study comprising patients with AD at the stage of mild cognitive impairment (AD-MCI, $n = 27$) and at the stage of dementia ($n = 35$), non-AD-related mild cognitive decline (non-AD MCI, $n = 19$), non-AD dementia ($n = 26$), and neurological controls (NC, $n = 20$).

Patients had undergone CSF biomarker analysis from 2012 to 2015 including Aβ 42/Aβ 40 ratio, tau phosphorylated on threonine 181 (p-tau), and total-tau (t-tau) measurements, in the context of the diagnostic workup of a cognitive complaint. Consensus diagnoses were made by neurologists, geriatricians, neuropsychologists, neuroradiologists, and biologists after comprehensive neurological examination, neuropsychological assessment, brain imaging, and CSF biomarker analysis, according to current diagnostic criteria [25–29].

All AD patients met the NIA-AA research framework criteria and displayed a CSF profile on the AD *continuum* [26]. AD-MCI patients followed Albert et al. definition of MCI due to AD [25]. The non-AD MCI group comprised

subjects with cognitive decline unrelated to AD, encompassing diagnosis of psychiatric disorders, systemic disorders, or non-neurodegenerative disorders. The non-AD dementia group included patients with dementia with Lewy bodies (DLB, $n=6$), behavioral variant frontotemporal dementia (FTD, $n=9$), and vascular dementia (VD, $n=7$). Non-AD MCI and non-AD dementia patients had normal amyloid ratio Aβ 42/40 and normal or abnormal p-tau and t-tau. NC included patients with subjective cognitive decline, anxiety, depression, or sleep apnea syndrome, presenting with normative or sub-normative cognitive scores, normal CSF biomarkers, and an absence of cognitive decline at follow-up.

This study was approved by the Bichat Hospital Ethics Committee of Paris Diderot University (N°10–037, 18/03/2010) and all the participants have given their written consent.

#### CSF biomarkers

CSF was obtained through a lumbar puncture; the second and third milliliters were collected and centrifuged to prevent blood contamination. The supernatant was stored at − 80 °C until further analysis.

CSF core AD biomarkers (Aβ 42, Aβ 40, p-tau, and t-tau) were analyzed at the Department of Biochemistry at Lariboisiere University Hospital Paris, France, using commercially available INNOTEST® kits (Fujirebio Europe NV, Gent, Belgium) in a delay of 1 month after collection. CSF profiles were analyzed according to the following cut-offs: A+: Aβ42/Aβ40 ratio < 0.076; T+: p-tau > 58 pg/mL; N+: t-tau > 340 pg/mL [26]. Patients were classified as Aβ-positive and Aβ-negative according to the Aβ42/Aβ40 ratio.

CSF NRG1 concentration was measured using the Human NRG1 DuoSet ELISA kit (R&D Systems, Minneapolis, MN) as reported in Mouton-Liger et al. [24].

All the CSF synaptic markers were assessed at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital (Mölndal, Sweden). CSF neurogranin and CSF GAP-43 concentrations were measured using in-house developed ELISAs [8, 10]. CSF SNAP-25 concentration was measured by immunoprecipitation mass spectrometry according to a validated method [9, 11].

#### Plasma NRG1 measurement

Blood samples were obtained through venipuncture under fasting condition and collected into ethylenediaminetetraacetic acid (EDTA) tubes. Samples were centrifuged at 2000×*g* for 20 min at 4°C. Plasma supernatant was collected and frozen at −80°C until further use. Prior to analysis, samples were centrifuged at 2000*g* for 10 min after thawing at room temperature. Plasma NRG1 was assessed using the Human NRG1 DuoSet ELISA kit (R&D Systems) in Mölndal, Sweden, following the manufacturer's protocol. This assay has been shown to be highly sensitive to human NRG1 alpha-subunit with a sensitivity of 125–4000 pg/mL [24, 30, 31]. Plasma samples from study participants were analyzed in duplicates. Intra-plate and inter-plate coefficients of variation were respectively 5.9% and 7.4%. Ten samples (7.9% of samples total) were below the detection limit of the assays, including 4 NC, 3 AD, 2 non-AD dementia, and one non-AD MCI other patient. For those samples, plasma NRG1 levels were interpolated from the standard curve or if this was not possible due to the very low signal the values were imputed to the lowest interpolated value. One outlier sample (plasma NRG1 value > mean+5SD) was excluded from the analysis.

#### Statistical analysis

Participants' characteristics were examined in 5 groups: NC, AD-MCI patients, AD dementia, non-AD MCI, and non-AD dementia. Patients were also divided into Aβ-positive and Aβ-negative groups according to their CSF Aβ42/Aβ40 ratio using the clinically validated cut-off. Data are expressed as mean (standard deviation) for continuous variables or percentage (%) for categorical variables. We used the Kruskal-Wallis test to compare age and Mini-Mental State Examination (MMSE) scores between groups and Pearson's chi-square for sex. Fluid biomarker levels were log-transformed prior to analysis and compared using a one-way ANCOVA adjusted on age and sex followed by a post hoc least significant difference (LSD) test for pairwise group comparisons, adjusted for multiple comparisons (Bonferroni). Delay between sample collection and analysis was added in the model to test for association with biomarker levels. Linear regression adjusted on age and sex was used to explore the association between CSF and plasma NRG1. A receiver operator curve (ROC) analysis was performed to study the accuracy of plasma NRG1 in differentiating the different groups. The association of plasma NRG1 with core AD CSF biomarkers and CSF synaptic markers and with MMSE was explored by linear regression adjusted on age and sex in the whole cohort and in regard to Aβ status.

A *p-value* (*P*) < 0.05 was overall considered significant. Statistical analyses were performed using SPSS IBM 27.0 (IBM, Armonk, NY) and GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

#### Data availability

The datasets analyzed during the current study are available from the corresponding authors on a reasonable request.

## Results

### Cohort

Demographics and biomarker values in our cohort are reported in Table 1. A detailed description of non-AD dementia patients is reported in Supplemental Table 1. AD-MCI, AD dementia, and non-AD dementia patients were older than NC and non-AD MCI patients ($P = 0.003 - P < 0.001$). There was no difference regarding sex between groups ($P = 0.155$). All further analysis was adjusted on age and sex, unless otherwise specified. AD-MCI and AD dementia patients displayed decreased CSF Aβ42/Aβ40 ratio and increased p-tau and t-tau levels compared with other groups. CSF synaptic markers neurogranin, GAP-43, and SNAP-25 were significantly higher in AD patients compared to NC and displayed high accuracies in identifying AD (Supplemental Fig. 1). AD-MCI, non-AD MCI, AD dementia, and non-AD dementia patients had decreased MMSE compared to NC. Delay between the collection of samples and analysis was not associated with biomarker levels in uni- and multivariate analysis (results not presented); thus, it was not further added as a covariate.

### Plasma NRG1 levels across groups

A higher concentration of plasma NRG1 was found in AD dementia patients compared to NC after adjustment on age and sex (940.3 versus 378.9 pg/mL, $P < 0.001$, Fig. 1A). AD-MCI patients also displayed higher concentrations compared to NC (707.6 versus 378.9 pg/mL, $P = 0.005$). Non-AD dementia had higher levels compared to NC (615.5 versus 378.9 pg/mL, $P = 0.014$). Plasma NRG1 concentration did not differ significantly between NC and non-AD MCI patients. Participants were then dichotomized according to their Aβ status defined by CSF Aβ42/Aβ40 ratio (Supplemental Table 2). Aβ-positive patients displayed higher plasma NRG1 concentration than Aβ-negative patients (774.0 versus 538.4 pg/mL, $P = 0.023$, Fig. 1B).

### Plasma NRG1 accuracy in identifying AD

We studied plasma NRG1 accuracy in discriminating AD patients from other diagnosis groups (Fig. 1C). Plasma NRG1 showed good performance in differentiating AD patients from NC both at MCI stage (AUC = 88.3%, 95% CI: 77.2–0.99.6%) and at dementia stage (AUC = 87.6%, 95% CI: 76.9–98.2%). When comparing AD-MCI to non-AD MCI patients, plasma NRG1 showed similar accuracy (AUC = 86.4%, 95% CI: 74.7–98.3%). However, its discriminating power was lower between AD patients and non-AD dementia patients (AUC = 69.3%, 95% CI: 55.7–82.3%).

**Table 1** Demographics and biomarker values

| ***n* = 127** | **Neurological controls *n* = 20** | **Non-AD MCI *n* = 19** | **AD-MCI *n* = 25** | **AD dementia *n* = 37** | **Non-AD dementia *n* = 26** | ***P-value*** |
|---|---|---|---|---|---|---|
| Age, years | 60.6 (9.6) | 61.1 (8.4) | 70.3 (5.8)[#] | 67.7 (7.9)[#] | 68.1 (7.0)[#] | **<0.001** |
| Female, *n* (%) | 70% (14) | 63% (12) | 68% (17) | 62% (23) | 38% (10) | 0.155 |
| LoE, years | 11.6 (3.8) | 9.7 (2.5) | 11.0 (3.9) | 9.0 (3.5)[#] | 11.2 (3.4) | **0.050** |
| MMSE | 27.42 (1.6) | 25.0 (2.3)[#] | 25.1 (2.4)[#] | 18.2 (4.3)[#] | 22.8 (5.4)[#] | **<0.001** |
| **CSF biomarkers** | | | | | | |
| CSF Aβ42, pg/mL | 1041.6 (264.4) | 987.2 (326.0) | 516.4 (122.5)[#] | 548.3 (135.9)[#] | 919.3 (428.7) | **<0.001** |
| CSF Aβ42/Aβ40 ratio | 0.129 (0.045) | 0.092 (0.029) | 0.051 (0.027)[#] | 0.045 (0.017)[#] | 0.104 (0.033) | **<0.001** |
| CSF p-tau, pg/mL | 33.7 (10.7) | 40.7 (17.2) | 79.2 (22.9)[#] | 95.7 (32.5)[#] | 45.2 (20.5)[#] | **<0.001** |
| CSF t-tau, pg/mL | 196.0 (66.7) | 223.0 (100.6) | 501.7 (203.4)[#] | 703.9 (285.2)[#] | 305.2 (152.1)[#] | **<0.001** |
| CSF NRG1, pg/mL | 295.8 (107.1) | 324.4 (137.5) | 312.8 (157.8) | 403.9 (155.1)[#] | 315.0 (134.4) | **0.044** |
| CSF neurogranin, pg/mL | 208.1 (69.5) | 213.6 (84.7) | 364.4 (83.1)[#] | 351.4 (91.9)[#] | 230.2 (107.9) | **<0.001** |
| CSF GAP-43, pg/mL | 1677.2 (616.2) | 2004.8 (987.9) | 3422.9 (1087.9)[#] | 3787.6 (1388.7)[#] | 2348.8 (1267.8)[#] | **<0.001** |
| CSF SNAP-25, pg/mL | 6.7 (2.2) | 8.2 (3.5) | 13.1 (3.7)[#] | 17.1 (5.3)[#] | 7.8 (3.9) | **<0.001** |
| **Plasma biomarker** | | | | | | |
| Plasma NRG1, pg/mL | 378.9 (400.7) | 488.4 (392.2) | 707.6 (562.7)[#] | 940.3 (737.5)[#] | 615.5 (486.3)[#] | **<0.001** |

Data are shown as mean (SD) or *n* (%), as appropriate. The Kruskal-Wallis test was used to compare age between groups and Pearson's chi-square to compare sex. Fluid biomarker levels and MMSE were compared with a one-way ANCOVA adjusted by age and sex followed by the least square difference test adjusted for multiple comparisons

*Abbreviations*: *Aβ42* amyloid-beta 42, *Aβ40* amyloid-beta 40, *AD* Alzheimer's disease, *AD-MCI* MCI due to Alzheimer's disease, *CSF* cerebrospinal flui , *GAP-43* growth-associated protein 43, *LoE* level of education, *MCI* mild cognitive impairment, *MMSE* Mini-Mental State Examination, *NRG1* neuregulin 1, *p-tau* phosphorylated tau, *SNAP-25* synaptosomal-associated protein 25, *t-tau* total tau

[#] $P < 0.05$ compared to neurological controls

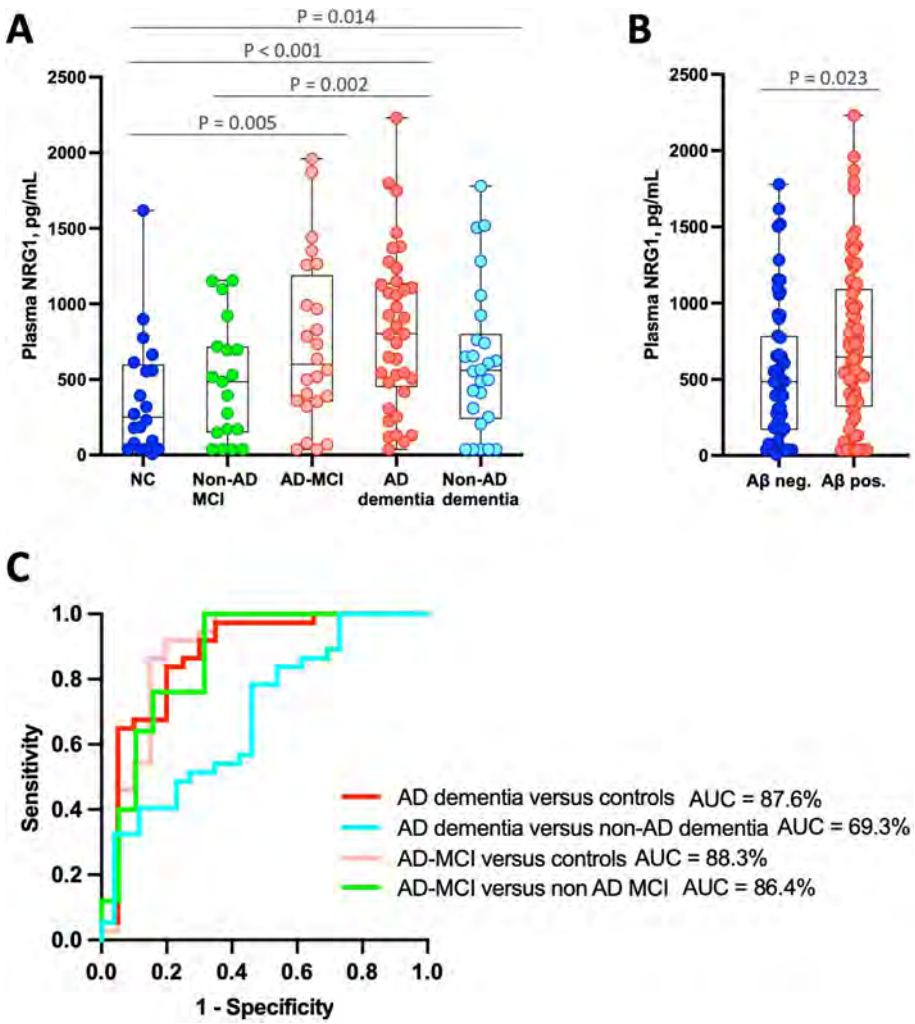


**Fig. 1** Plasma NRG1 levels across groups and correlation to CSF NRG1. **A** Box plots displaying plasma NRG1 levels across diagnosis groups. Levels were compared using a one-way ANCOVA adjusted on age and sex followed by a post hoc LSD test adjusted for multiple comparisons. **B** Box plot displaying plasma NRG1 levels in Aβ-positive patients ($n = 62$) and Aβ-negative patients ($n = 55$). Levels were compared using a one-way ANCOVA adjusted on age and sex followed by a post hoc LSD test adjusted for multiple comparisons. **C** ROC curves displaying plasma NRG1 accuracy in differentiating AD patients from neurological controls (AUC = 87.6%, 95% CI: 76.9–98.2%), AD dementia from non-AD dementia patients (AUC = 69.3%, 95% CI: 55.7–82.3%), AD-MCI from NC (AUC = 88.3%, 95% CI: 77.2–0.99.6%), and AD-MCI from non-AD MCI patients (AUC = 86.4%, 95% CI: 74.7–98.3%). Abbreviations: Aβ, amyloid-beta; AD, Alzheimer's disease; AUC, area under the curve; MCI mild cognitive impairment; LSD test, least square difference test; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; NRG1, neuregulin 1; ROC, receiver operator characteristics

### Correlation to CSF NRG1

Plasma and CSF NRG1 concentrations correlated in the overall cohort ($\beta = 0.372$, $P = 0.0056$, adjusted on age and sex) (Fig. 2A). This correlation was also detected in the Aβ-positive group ($\beta = 0.292$, $P = 0.034$). No correlation was observed between plasma and CSF NRG1 in the Aβ-negative group ($\beta = 0.156$, $P = 0.305$).

### Correlation to AD biomarkers

Plasma NRG1 displayed a weak inverse correlation with CSF Aβ42/Aβ40 ratio in the whole cohort ($\beta = -0.197$, $P = 0.043$, adjusted on age and sex, Fig. 2B). This correlation was stronger in the Aβ-positive group ($\beta = -0.372$, $P = 0.003$). CSF p-tau and CSF t-tau displayed a stronger correlation with plasma NRG1 in the whole population (respectively: $\beta = 0.361$, $P < 0.001$ and $\beta = 0.423$, $P$

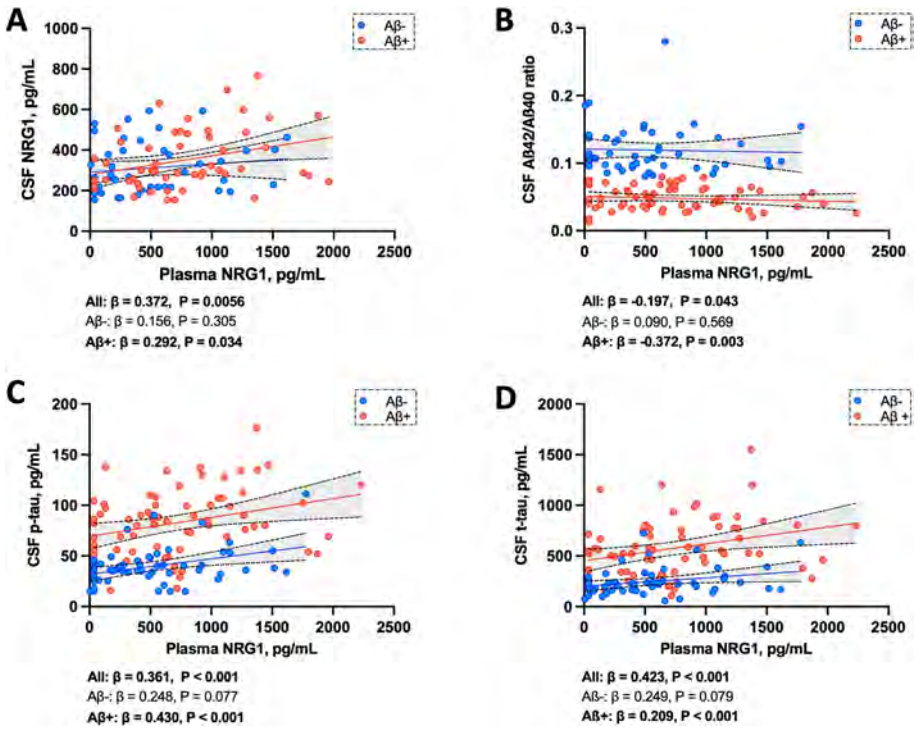


**Fig. 2** Association to AD CSF biomarkers. Association of plasma NRG1 with **A** CSF NRG1 **B** CSF Aβ42/Aβ40 ratio, **C** CSF p-tau, and **D** CSF t-tau, studied using linear regression adjusted on age and sex, in the whole cohort and in regard to Aβ status. Solid lines indicate the regression line and dashed lines, the 95% CI in Aβ-positive and Aβ-negative groups. Abbreviations: Aβ+, amyloid-beta positive; Aβ-, amyloid-beta negative; Aβ42, β-amyloid 42; Aβ40, β-amyloid 40; AD, Alzheimer's disease; 95% CI, confidence interval; CSF, cerebrospinal fluid; NRG1, neuregulin 1; p-tau, phosphorylated tau; t-tau, total tau

< 0.001) (Fig. 2C, D). These correlations were both sustained in the Aβ-positive patients (CSF p-tau: $\beta = 0.430$, $P < 0.001$; CSF t-tau: $\beta = 0.209$, $P < 0.001$). There was no correlation between plasma NRG1 and CSF Aβ42/Aβ40 ratio, p-tau, and t-tau in Aβ-negative patients.

### Association to synaptic biomarkers and to cognition

We studied the association of plasma NRG1 with three CSF synaptic biomarkers, neurogranin, GAP-43, and SNAP-25, after adjustment on age and sex (Fig. 3). Plasma NRG1 levels were overall associated with CSF GAP-43 levels ($\beta = 0.355$, $P < 0.001$) (Fig. 3A). This association remained significant in Aβ-positive patients ($\beta = 0.434$, $P < 0.001$) but not in the Aβ-negative group. Similarly, plasma NRG1 levels were associated with CSF neurogranin levels, in the whole cohort ($\beta = 0.278$, $P = 0.002$) and in the Aβ-positive patients ($\beta = 0.322$, $P = 0.007$) (Fig. 3B). CSF SNAP-25 levels were associated with plasma NRG1 in the whole cohort ($\beta = 0.327$, $P = 0.001$) as in the Aβ-positive ($\beta = 0.375$, $P = 0.004$) and in Aβ-negative group ($\beta = 0.339$, $P = 0.026$, Fig. 3C).

MMSE scores were significantly associated with plasma NRG1 levels after adjustment on age and sex ($\beta = -0.188$, $P = 0.038$, Fig. 3D). This association was sustained in the Aβ-positive group ($\beta = -0.255$, $P = 0.037$) but not in the Aβ-negative group.

## Discussion

Accessible biomarker monitoring synaptic dysfunction and loss would be of great clinical use in AD for early diagnosis, prediction, and monitoring of cognitive decline and for drug evaluation. In this study, we report that plasma synaptic marker NRG1 (i) was increased in AD patients already at the MCI stage; (ii) had a promising AUC to discriminate AD patients both at MCI and dementia stage, from NC; (iii) was associated with CSF AD biomarkers in Aβ-positive individuals; (iv) correlated with CSF synaptic markers; and (v) was inversely correlated with cognition.

NRG1 is expressed at the synapse in multiple brain regions, including those preferentially affected in AD, as the hippocampus and entorhinal cortex [32–34]. Postmortem studies have reported NRG1 accumulation in neuritic plaques in association with dystrophic neurites, activated astrocytes, and microglia in human AD brains [21, 22]. NRG1- and ErbB4-directed immunoreactivity

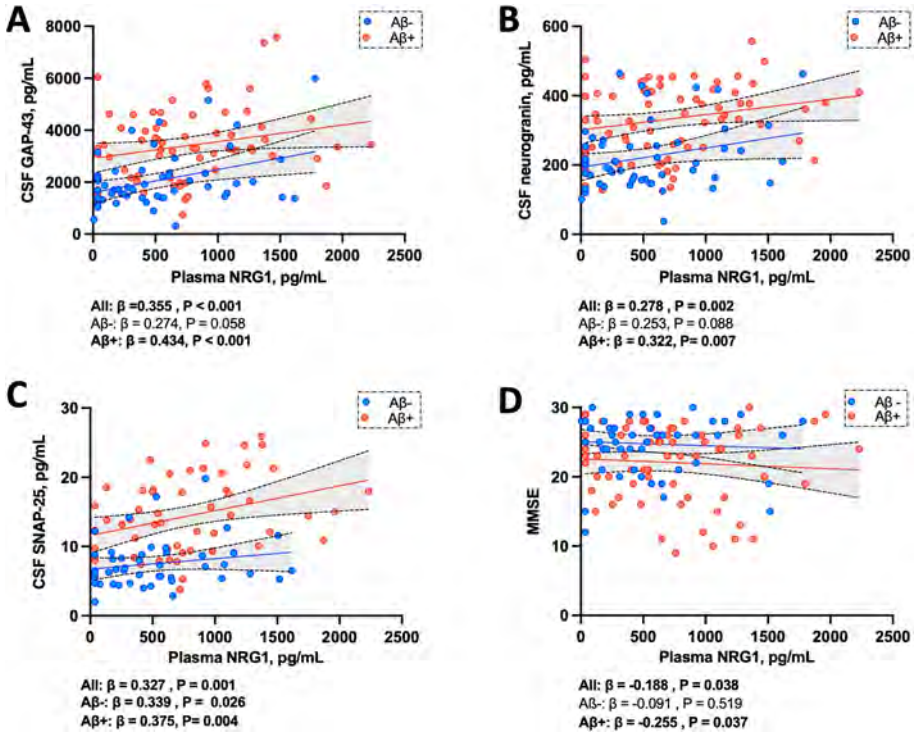


**Fig. 3** Association to CSF synaptic biomarkers and cognition. Association of plasma NRG1 with **A** CSF GAP-43, **B** CSF neurogranin, **C** CSF SNAP-25, and **D** MMSE, studied using linear regression adjusted on age and sex, in the whole cohort and in regard to Aβ status. Solid lines indicate the regression line and dashed lines, the 95% CI in Aβ-positive and Aβ-negative groups. Abbreviations: Aβ+, amyloid-beta positive; Aβ-, amyloid-beta negative; 95% CI, 95% confidence interval; CSF, cerebrospinal fluid; GAP-43, growth-associated protein 43; MMSE, Mini-Mental State Examination; NRG1, neuregulin 1; SNAP-25, synaptosomal associated protein 25

was observed in the hippocampus located in neuronal cell bodies and dendrites [22]. Interestingly, NRG1 can be measured in human fluids [17, 23, 24, 30, 31, 35, 36]. Increased levels of CSF NRG1 in AD compared with controls and with non-AD-related cognitive decline have been reported in the literature, including our prior work [23, 24]. In Pankonin et al., CSF NRG1 was increased in AD patients from an early stage of the disease. More recently, in a larger cohort using the most recent AD diagnosis criteria including CSF biomarkers, we have confirmed those results [24]. CSF NRG1 was significantly associated with CSF AD core biomarkers, suggesting a possible implication in AD pathophysiological processes. Moreover, CSF NRG1 levels correlated with other CSF synaptic markers, also suggesting that NRG1 was mainly originating from the synapse.

A previous study has already reported increased levels of plasma NRG1 in AD patients, with higher levels in advanced disease [35]. However, in this work, AD was clinically diagnosed with no biomarker to confirm the underlying AD pathophysiological process and correlation with CSF NRG1 levels was not studied. Our study brings evidence that plasma NRG1 is increased in patients with confirmed underlying amyloid pathology, already at the MCI stage. It is interesting to note as APP, at the origin of Aβ, and NRG1 are both cleaved by BACE1 in the brain [18, 20].

Plasma NRG1 levels were significantly correlated with CSF levels in the whole cohort and this association was sustained in the Aβ-positive patient group. The existence of extracerebral expression of NRG1 is known but the significant correlation between plasma and CSF levels indicates that plasma level modifications substantially arise from the central nervous system [37]. Thus, this flags plasma NRG1 levels as a potential surrogate for brain NRG1 modifications in AD. Our cohort was phenotyped using the measure of validated CSF biomarkers: GAP-43, neurogranin, and SNAP-25. Consistently with the existing literature, CSF synaptic biomarker levels were found to be altered in the AD group at MCI and dementia stages and they displayed interesting performance in separating the AD group from the control group. Significant correlation of plasma NRG1 with CSF synaptic markers in the Aβ-positive patients also supports that detected NRG1 changes are related to synaptic modifications.

There was a significant association between plasma NRG1 levels and MMSE in our whole cohort as well as in the Aβ-positive patients. This finding is in agreement with the previous studies in plasma and CSF again showing that NRG1 levels associate with cognition already at early stages of the disease [23, 24, 35].

Plasma NRG1 also displayed increased levels in non-AD dementia compared to NC and its accuracy in identifying AD at the dementia stage was moderate. In a study on vascular dementia, plasma NRG1 levels were found to be increased and inversely correlated to cognitive severity [38]. Neuropathological studies and synaptic CSF biomarker results have highlighted the fact that synapse dysfunction is a prominent feature in AD but that it is not entirely specific to it [39, 40]. It can also be observed in non-AD dementia, although to a much lesser extent than in AD, a finding in line with our results [41].

An underlying mechanistic question to this marker is whether alterations in NRG1 levels are related to a general process of synaptic degeneration and clearance or whether these changes occur as a response, positive or negative, to the development of AD pathology or to an increase in synaptic synthesis and release.

NRG1-ErbB4 signaling is important in regulating synaptic function at both excitatory and inhibitory synapses in the adult brain under physiological conditions [42, 43]. NRG1–ErbB4 signaling appears implicated in short-term synaptic plasticity through modulation of glutamatergic transmission. ErbB4 co-localizes and interacts with PSD95, a postsynaptic scaffold protein essential for the assembly and function of glutamatergic synapses [44]. Studies have shown that the pair can both suppress the induction and the expression of LTP [45, 46]. However, the NRG1 effect on neurotransmission might vary between brain regions. NRG1 administration decreased NMDA-receptor-mediated excitatory postsynaptic potentials in slices of the prefrontal cortex [47]. NRG1 decreased synaptic transmission in entorhinal CA1 but increased in response to entorhinal cortical stimulation in rats [48]. The levels of NRG1–ErbB4 signaling also impact GABAergic transmission and regulate signal integration by pyramidal neurons [43]. There is also evidence to support that NRG1 regulates long-term plasticity in the brain and NRG1 has been shown to stimulate the expression of receptors for key neurotransmitters, including glutamate, GABA, and ACh [49, 50]. Finally, NRG1–ErbB4 pathway is also implicated in neuron survival in different cellular populations including cortical neurons, dopaminergic neurons, motor neurons, and cochlear sensory neurons [42]. NRG1 was first identified as a major susceptibility gene in schizophrenia [51, 52]. Mutant NRG1 mice display both excitatory and inhibitory synaptic impairment and schizophrenia-like behavioral disorder [53]. While loss of NRG1 signaling has been shown to be pejorative to synaptic transmission, excessive NRG1 activity is also associated with synaptic dysfunction resulting from alteration of LTP at glutamatergic synapses [34, 54]. In line with those findings, evidence suggests that NRG1 increase may specifically influence cognitive function and neuropathology in AD [55–57]. Although not formally established, the mechanism of the increase of NRG1 could be explained by the increased levels and activity of BACE1 observed in AD [55]. Yet, its beneficial or detrimental effect is not solved. In experimental works, NRG1 overexpression could rescue APP-induced toxicity in primary cortical neurons [56]. In an AD mouse model, NRG1 treatment prevented amyloid β-induced impairment of long-term potentiation in hippocampal slices via its receptor ErbB4 [58]. Conversely, other experimental works have suggested a negative effect of the NRG1-ErbB4 signaling in AD. Perfusion of NRG1 in the hippocampus decreased LTP in the AD mouse model as well as in control mice [59]. Further understandings of NRG1 response upon amyloid pathology will allow to specify the exact synaptic events associated with CSF and plasma NRG1 modifications observed in AD patients.

In addition to contributing to better understanding of AD mechanisms, our finding that plasma NRG1 levels could reflect synaptic impairment in AD may have major practical utility. The development of blood biomarkers measuring Aβ, tau, and neurodegeneration processes has known great advancement recently, but, to date, there are no validated blood biomarkers reflecting synaptic pathology [13]. Recent studies have reported that the measure of markers of AD hallmarks in plasma such as Aβ42, p-tau 181, p-tau217, and p-231 can identify and monitor AD brain pathology with high accuracy, demonstrating that they can be used as non-invasive tools in AD diagnosis [60–62]. As synaptic impairment is one of the earliest abnormal features in AD, already present at the preclinical phase, an accessible non-invasive synaptic marker would be of high added value for early diagnosis [12].

Moreover, synaptic markers hold important promise for monitoring the effects of disease-modifying treatments on synaptic degeneration. Compared with CSF markers, validated blood-based synaptic AD biomarkers would provide a fast, acceptable, and cost-effective method of early detection, diagnosis, and follow-up as well as a screening and follow-up tool in therapeutic trials. Our work shows that plasma NRG1 levels could be one of these potential biomarkers.

#### Limitations

This study has several limitations. The correlation between plasma and CSF NRG1 remained moderate.

Further studies will be needed to understand if this variability is related to the blood-brain barrier's passage, NRG1 metabolism in plasma, matrix effects, or interaction with peripheral NRG1. In our cohort, cognition was evaluated using MMSE, a general test. A study of plasma NRG1 relation to cognition using neuropsychological assessment with tests evaluating specifically episodic memory should give more robust evidence. We could not include measurements of AD-specific blood biomarkers such as p-tau or Aβ. Finally, confirmation of our results in larger cohorts is needed, including larger samples of non-AD dementia patients. A study of plasma NRG1 at the preclinical phase will also be needed to better characterize its kinetic on the whole AD spectrum.

## Conclusion

Our results suggest that plasma NRG1 is a novel biomarker for synaptic dysfunction/degeneration in AD. Plasma NRG1 showed a significant increase in AD patients already at the MCI stage and correlated with biomarkers for AD pathology, as well as with established CSF biomarkers for synaptic dysfunction in AD. As a novel blood synaptic marker, plasma NRG1 may improve the diagnosis of neurodegenerative disorders and may also be useful to monitor clinical disease progression and therapeutic response in clinical trials of novel disease-modifying drug candidates.

#### Abbreviations

AD: Alzheimer's disease; Aβ40: Amyloid beta 1–40; Aβ42: Amyloid beta 1–42; APP: Amyloid precursor protein; AUC: Area under the curve; BACE1: Beta-site amyloid precursor protein cleaving enzyme 1; CSF: Cerebrospinal fluid; erbB4: Receptor tyrosine-protein kinase erbB-4; GAP-43: Growth-associated protein 43; MCI: Mild cognitive impairment; MMSE: Mini-Mental State Examination; NRG1: Neuregulin 1; PET: Positron emission tomography; p-tau: Phosphorylated tau; SNAP-25: Synaptosomal associated protein 25; t-tau: Total tau.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13195-022-01014-7.

**Additional file 1: Supplementary Table 1.** Demographics and biomarkers values of patients with non-AD dementia subgroups. **Supplementary Table 2.** Demographics and biomarkers values according to Aβ status. **Supplementary Figure 1.** CSF synaptic markers levels across groups and diagnostic accuracies.

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#### Authors' contributions

Concept and design: CP, FML, and JH. Acquisition, analysis, and interpretation of the data: all authors. Statistical analysis: AV. Drafting the manuscript: AV, CP, FML, and JH. Review and editing: all authors. All authors have read and agreed to the published version of the manuscript. Obtained funding: CP, JH, HZ, KB. AV had full access to all the data in the study and takes full responsibility for the integrity of the data and the accuracy of the data analysis.

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#### Availability of data and materials

The datasets analyzed during the current study are available from the corresponding authors on a reasonable request.

## Declarations

#### Ethics approval and consent to participate

This study was approved by the Bichat Hospital Ethics Committee of Paris Diderot University (N°10–037, 18/03/2010) and all the participants have given their written consent.

#### Consent for publication

Not applicable.

#### Competing interests

KB has served as a consultant or at advisory boards for Abcam, Axon, Biogen, JOMDD/Shimadzu, Lilly, MagQu, Prothena, Roche Diagnostics, and Siemens Healthineers, and as data monitoring committee for Julius Clinical and Novartis. KB is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures. HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Alector, Annexon, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Pinteon Therapeutics, Red Abbey Labs, Passage Bio, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave; has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure, Biogen, and Roche; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. CP is a member of the International Advisory Boards of Lilly; is a consultant for Fujirebio, Alzhois, Euroimmune, Ads Neuroscience, Roche, AgenT, and Gilead; and is involved as an investigator in several clinical trials for Roche, Esai, Lilly, Biogen, Astra-Zeneca, Lundbeck, and Neuroimmune. The other authors declare no competing interests.

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## Publisher's Note

**Comparison of CSF and plasma NfL and pNfH for Alzheimer's disease diagnosis. A memory clinic study.**

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## ABSTRACT

### Introduction

Plasma neurofilament light chain (NfL) is a promising biomarker of axonal and neuronal damage in central nervous system disorders. Plasma NfL displays high potential to contribute to the differential diagnosis of neurodegenerative diseases, with similar performance as its cerebrospinal fluid (CSF) counterpart. The heavy chain of the neurofilaments, and specifically the phosphorylated form (pNfH), has been mostly studied in amyotrophic lateral sclerosis (ALS) where plasma pNfH appears promising.

### Aim

To compare the positive and differential diagnosis performance in Alzheimer's disease (AD) of NfL and pNfH, in plasma and in CSF, in a cohort of patients from a memory clinic setting.

### Methods

In a cross-sectional retrospective study, we compared NfL and pNfH levels in CSF and plasma for Alzheimer's Disease (AD) diagnosis in a clinical cohort (*n*=188) from the Center of Cognitive Neurology, APHP, Paris, France including neurological controls (*n*=22), AD at MCI stage (AD-MCI, *n*=36) and at dementia stage (*n*=64), non-AD MCI (*n*=38) and non-AD dementia patients (*n*=28). NfL and pNfH were quantified using single molecule array (Simoa).

### Results

NfL and pNfH, in plasma and CSF, were associated with age (*rho*= 0.259-0.451, P<0.003). The correlation between CSF and plasma levels were stronger for NfL than pNfH (respectively, rho=0.77 and rho=0.52). Both CSF and plasma NfL and CSF pNfH were associated with CSF p-tau levels in AD patients. This was not observed for plasma NfL. All neurofilament markers were increased in AD-MCI, AD dementia and non-AD dementia compared to NC. CSF NfL, CSF pNfH and plasma NfL showed high performance to discriminate AD at both MCI and dementia stage from control subjects (AUC=0.82-0.91). Conversely, plasma pNfH displayed overall lower AUCs for discrimination between groups compared to CSF pNfH. Nfs markers showed moderate association to cognition in the whole cohort. NfL displayed significant association with mediotemporal lobe atrophy and white matter lesions and in the whole cohort and in the AD subgroup.

**Conclusion**

CSF NfL and pNfH as well as plasma NfL appear equally performant in positive and differential AD diagnosis in a memory clinic setting. Contrarily as for motoneuron disorders, plasma pNfH did not demonstrate added value compared to plasma NfL.

**Keywords:** Alzheimer's disease; fluid biomarkers; cerebrospinal fluid; plasma biomarkers; neurofilaments; neurofilament light; phospho neurofilament heavy; memory clinic

**Abbreviations:** AD, Alzheimer's disease; Aβ, amyloid beta; ALS, amyotrophic lateral sclerosis; AUC, Area under the curve; CSF, cerebrospinal fluid; FTD, frontotemporal dementia; DLB, dementia with Lewy bodies; MMSE, mini mental state examination; NfL, neurofilament light chain; NC, neurological controls; Nf, neurofilament; MCI, mild cognitive impairment; MRI, magnetic resonnance imaging; PET, positron emission tomography; pNfH, phosphorylated neurofilament heavy chain; ROC, Receiver operator characteristic; Simoa, Single molecule array;VaD, vascular dementia.

## INTRODUCTION

The hallmark lesions of Alzheimer's disease (AD) include aggregated amyloid beta (Aβ) deposition in plaques, intraneuronal neurofibrillary tangles composed of hyperphosphorylated tau (p-tau) leading to neuronal loss. Those features can now be detected in patients using cerebrospinal fluid (CSF) biomarkers which have now been validated for AD diagnosis in clinical practice [1,2]. Nevertheless given the invasive character of lumbar puncture and the cost and limited availability of alternative imaging biomarkers (positron emission tomography, PET), more accessible biomarkers are needed. Moreover, if core AD biomarkers can be used for positive diagnosis, new biomarkers are needed to measure other underlying pathophysiological processes including axonal and synaptic damages, to improve differential diagnosis and to follow up and monitor the therapeutic response in trials.

Blood-based biomarkers as non-invasive would constitute the ideal biomarker for early diagnosis and monitoring of disease progression, in AD and other neurodegenerative diseases [3–6]. Among blood-based biomarkers, neurofilaments chains (Nfs) have emerged as a robust candidate biomarker for axonal damage. Nfs are specific neuronal cytosqueleton intermediate proteins that are released from neurons and axons upon injury [7]. Measurements in plasma and CSF of neurofilament light chain (NfL) and phosphorylated neurofilament heavy chains (pNfH) have been achieved [4,5,8–13]. NfL has been the most extensively studied and displays potential as a biomarker of neuronal damage and loss in various acute and chronic neurological disorders [14]. Under physiological and pathological conditions, NfL plasma levels show robust association with their CSF levels [5]. Plasma NfL levels are increased in a wide range of neurodegenerative disorders including AD, frontotemporal dementia (FTD) and other taupathies, and predict the progression of clinical decline and brain atrophy [3,6]. NfL is consistently increased in AD from preclinical stage and correlates with as well as predicts cognitive decline and cerebral atrophy [4,6,9,15,16]. Additionally, plasma NfL seems to have added value for etiological diagnosis of cognitive disorders in situations frequently encountered in clinical practice. Previous studies have suggested that it could accurately distinguish neurodegenerative disorder from depression in older adults [5]. Even if they are not disease-specific, the higher plasma NfL levels observed in FTD can also contribute to the diagnosis of this condition among other causes of cognitive impairments: they accurately discriminate FTD from primary psychiatric disorders and from FTD phenocopies [17,18]. Moreover, plasma NfL is a highly dynamic marker, and the first studies in multiple sclerosis and spinal muscular atrophy report that it can be used efficiently to monitor the effect of

disease-modifying therapies [19,20]. Large efforts are currently being made to introduce plasma NfL in clinical settings, including defining cut-offs to identify neurodegenerative disorders and reference values for each age group [5,8].

Although less studied, CSF and plasma pNfH levels have been examined in neurocognitive disorders, and high levels are reported in AD, in taupathies as well as in motoneuron diseases [10,21]. In amyotrophic lateral sclerosis (ALS), levels of pNfH are consistently elevated in CSF and blood and some studies show that pNfH levels were associated with survival and/or with indicators of disease progression [22]. However, associations of pNfH concentrations in CSF with disease progression and survival have not been consistently reproduced in blood. Previous studies provided evidence of a rather similar diagnostic performance of pNfH compared with NfL in ALS [23–25]. However, studies across other neurodegenerative disorders, including AD, are scarce [21,26].

In this work, we retrospectively investigated the differential diagnosis performance of CSF and plasma pNfH and NfL in a cohort of patients from a memory clinic setting. We also examined their association with AD core CSF biomarkers as well as with cognitive status and brain atrophy and white matter lesions measured using clinical tools.

## METHODS

### Cohort

We conducted a retrospective monocentric cross-sectional biomarker study in a memory clinic setting. Patients were recruited from January 22, 2015, to and November 22, 2019, in a tertiary memory center (Center of Cognitive Neurology, University Hospital Lariboisière Fernand Widal, Paris, France). We systematically included all patients that had undergone lumbar puncture for CSF AD biomarkers analysis, and with available plasma and CSF samples at the time of the study. All patients underwent an extensive neurological assessment, including clinical and neuropsychological evaluations, biological measurements, and brain imaging. Patients with AD at the stage of mild cognitive impairment (AD-MCI) and dementia fulfilled the criteria for probable AD as defined by NIA-AA, with CSF evidence of AD pathophysiological process. Patients with AD-MCI meet the MCI criteria established by Albert *et al.* [2]. Non-AD dementia patients also had other causes of dementia, including frontotemporal dementia (FTD), vascular dementia and dementia with Lewy bodies (DLB) and vascular dementia (**Supp. Table 1**) [27–29]. Patients with MCI of other causes (non-AD MCI) included patients with psychiatric disorders, sleep apnea or systemic disease. Patients with no

argument for a cognitive disorder, with normal or subnormal scores on neuropsychological assessment and normal CSF core biomarkers were included as neurological controls.
Mini-mental score examination (MMSE) score at the time of the samples collection was collected. Mediotemporal atrophy was rated visually on morphological brain MRI using Scheltens visual scale [30].
All subjects or caregivers signed a written informed consent for CSF assessments and analysis. This research study was approved by the local ethics committee of Bichat Hospital, Paris, France (CEERB GHU Nord n°10-037).

**Sample collection**

Paired CSF and blood samples were collected within a time interval of less than 4 hours. All patients included in this study underwent CSF examination in the context of the diagnosis work up for a cognitive complaint. CSF was obtained by a lumbar puncture and samples were immediately centrifuged at 1800xg for 10 min at +4°C and stored at 80 °C pending analysis. Blood samples were obtained through venipuncture in fasting condition and collected into ethylenediaminetetraacetic acid (EDTA) tubes. All samples were centrifuged at 2000g for 20 minutes at 4° C. Plasma supernatant was collected and frozen at −80◦C until further use. Samples were shortly thawed at room temperature and centrifuged at 4,000g prior to analyses.
All samples were analyzed at the Department of Psychiatry and Neurochemistry, University of Gothenburg, Sweden.

**Core AD biomarkers measurements**

CSF Aβ42/Aβ40 ratio, phospho-tau (p-tau) and total tau (t-tau) were measured using the Lumipulse® G1200 platform (Fujirebio) with the following cut-offs validated for AD diagnosis: Aβ42/Aβ40 <0.61, p-tau>61 ng/L and t-tau> 479 ng/L [31].

**pNfH and NfL measurements**

CSF and plasma pNfH were measured using the SIMOA pNF-Heavy Discovery Kit on a Quanterix HD-X analyzer (Quanterix, Lexington, MA) [32]. Samples were measured over three analytical runs each, for CSF and plasma. All the samples were above the lower limit of quantification reported for this assay (LLOQ, 0.174 pg/mL). Three plasma control samples were run at the beginning and at the end of the plate to assess variability. Intra- and inter-assays coefficients of variation (CV) were respectively of 4.0% and 3.6% for CSF and 3.7% and 4.0% for plasma.

CSF NfL levels were measured using an in-house sandwich Elisa, developed at the University of Gothenburg, Sweden, as described previously [33]. The CVs of intra- and inter-plate variations were respectively 6.7% and 8.4%. Plasma NfL levels were measured using a commercial kit by Quanterix on the SIMOA platform. Intra- and inter-plate CVs were respectively of 7.2% and 9.7%.

**Statistical analysis**

Analyses were performed using SPSS IBM 26.0 (IBM, Armonk, NY), GraphPad Prism 9 (GraphPad Inc., San Diego, CA, USA) and MedCalc Software (Ostend, Belgium).

Patients' data was analyzed in 5 groups: neurological controls, non-AD MCI, AD-MCI, AD dementia and non-AD dementia. Data are reported as mean (standard deviation) for continuous variables and as a percentage (number of subjects) for categorical variables. Categorical data (sex, APOE ε4 positivity) were compared in between groups using the $\chi 2$ test, and numerical data (age, MMSE score) using Kruskal-Wallis test. Biomarkers levels were log-transformed prior analysis and compared between the groups using one-way ancova adjusted on age and sex followed by a post hoc least significant difference (LSD) test for pairwise group comparisons, adjusted for multiple comparisons. Spearman's rank correlation test was used to measure correlations between biomarkers. Comparison of correlation coefficients between NfL and pNfH in CSF and plasma as well as between plasma and CSF for each biomarker was performed after Eid *et al* after *rho*'s Fischer's transformation in z-score [34]. Diagnostic accuracy of our biomarkers to discriminate between the groups were studied using receiver operator characteristics (ROC) curves, adjusted on age and sex. The areas under the curve (AUC) of the different biomarkers were compared using DeLong test. Association with MMSE score and Scheltens score were studied using linear regression to adjust on age and sex. MMSE was transformed into the square root of the number of errors as already described by Jacqmin-Gadda et al, to ensure a Gaussian distribution [35]. A P-value $<0.05$ was overall considered significant.

**RESULTS**

**Cohort description**

A total of n=188 patients was recruited, including neurological controls (NC, n=22), non-AD MCI (n=38), AD-MCI (n=36), AD dementia (n=64) and non-AD dementia patients (n=28, including FTD [n=12], DLB [n=13] and vascular dementia, [n=3]). Demographics and

biomarkers values are displayed in **Table 1;** detailed information about non-AD dementia groups is reported in **Supp. Table 1**. Patients in non-AD MCI, AD-MCI and AD dementia groups were older than NC (overall, P<0.05). MMSE was decreased in all groups compared with NC (P<0.001). ApoE4 carriers were more frequent in the AD-MCI and AD dementia groups compared with all other groups (P<0.0001). CSF and plasma pNfH and NfL all correlated with age (CSF pNfH: rho=0.451, P<0.0001; plasma pNfH: rho=0.259, P=0.003; CSF NfL: rho=0.409, P<0.001; plasma NfL: rho=0.428, P<0.0001). CSF NfL and CSF pNfH were significantly associated with sex in univariate analysis, with higher levels in males. This association remained significant after adjustment on age and diagnosis for CSF pNfH (ß=0.329, P=0.004) but not for CSF NfL (ß=0.157, P=0.081). Subsequently, all further analysis was adjusted on age and sex. ’

**Correlation between CSF and plasma levels**

CSF and plasma NfL displayed stronger correlation (rho=0.77, P<0.0001) than plasma and CSF pNfH (rho=0.52, P<0.001). NfL and pNfH were correlated in CSF as well as in plasma (CSF pNfH versus CSF NfL, rho=0.71, P<0.0001; plasma pNfH versus plasma NfL: rho=0.52, P<0.001).

**CSF NfL and pNfH levels across clinical groups**

CSF pNfH and CSF NfL were significantly increased in all groups (non-AD MCI, AD-MCI, AD dementia and non-AD dementia) in comparison with NC (overall, P <0.05, **Fig. 1**). AD dementia and non-AD dementia patients displayed the highest increase compared with NC (CSF pNfH, AD dementia: +108%, non-AD dementia: +131%; CSF NfL: AD dementia: +165%, non-AD dementia: +193%). Both CSF markers displayed a stepwise increase between AD-MCI and AD dementia (CSF pNfH: +25%; CSF NfL: +24%) but without reaching significance. Regarding, non-AD-dementia, DLB patients had similar levels to controls (CSF pNfH, P=0.782; CSF NfL, P=0.980). FTD showed FTD patients displayed higher levels of both CSF pNfH and CSF NfL compared to all groups and the highest fold changes compared with NC (CSF pNfH: +270%, P <0.001; CSF NfL: +155%, P<0.001; **Supp. Fig. 1**).

**Plasma NfL and pNfH biomarkers levels across clinical groups**

Plasma pNfH levels showed higher levels in all diagnosis groups compared with NC (overall P<0.05), except for non-AD MCI. Plasma NfL levels were higher in all groups including non-AD MCI compared with NC. The increase for both plasma markers in the AD and non-AD

dementia groups were in the same range as their CSF counterparts (plasma pNfH, AD dementia: +121%, non-AD dementia: +155%; plasma NfL: AD dementia: +93%, non-AD dementia: +131%). Similarly to CSF, DLB patients displayed plasma NfL and pNfH levels similar to NC (**Supp. Fig.1**). Conversely, FTD displayed the highest increase compared to NC for both plasma NfL and pNfH. FTD patients displayed higher plasma NfL levels than AD-MCI (P=0.032) but similar plasma pNfH levels (P=0.992). Neither plasma NfL nor plasma pNfH differed between FTD and AD-dementia groups.

**Correlation to amyloid and tau CSF biomarkers**

We then studied the association of CSF and plasma Nfs with AD core CSF biomarkers in the whole cohort (**Fig. 2**) and in subgroups (**Supp. Table 2**).

CSF and plasma Nfs displayed a weak correlation with CSF Aβ42/Aβ40 ratio in the whole cohort (rho=-0.198- -0.2825, P<0.05, **Fig. 2, a&d**). The strength of the association did not differ statistically between any marker (Rho=0.105- P=0.457). There was no association in the subgroups.

CSF pNfH and CSF NfL correlated with CSF p-tau with no significant difference in the association (respectively rho=0.360 and rho=0.453, both P<0.001, **Fig. 2 b**). Plasma pNfH and plasma NfL also did associate with CSF p-tau (rho=0.257-0.309, both P<0.001, **Fig. 2 e**); plasma NfL displayed a weaker association with CSF p-tau than CSF NfL (P=0.028). CSF pNfH was more strongly associated with CSF t-tau than CSF NfL (respectively, rho=0.4232 and rho=0.5334, both P<0.0001; P=0.028, **Fig. 2 c)**. Plasma pNfH and plasma pNfL were associated with CSF t-tau (respectively rho=0.263, P=0.0003 and rho=0.3193, P<0.0001, **Fig. 2 d**). Plasma NfL association to CSF t-tau was weaker than CSF NfL (P=0.001). Regarding the diagnosis subgroups, the association between CSF pNfH and CSF NfL, and CSF p-tau and t-tau were maintained in the AD group. Plasma NfL also correlated with both CSF p-tau and t-tau in AD while plasma pNfH was only associated with CSF t-tau.

**Diagnosis performance of CSF and plasma NfL and pNfH**

Using ROC analysis, we studied the accuracy of our markers to distinguish between the diagnosis groups (**Fig. 3 & 4**). CSF and plasma pNfH and NfL showed good accuracy to discriminate AD dementia and AD-MCI from NC with AUCs>0.80 overall (AD dementia versus NC: AUC=0.82 -0.91; AD-MCI versus controls: AUC=0.82-0.86). There was no difference between CSF NfL and pNfH performance (Delong test between AUCs: P>0.05, **Fig.**

**3**). Plasma pNfH displayed a lower AUC to differentiate AD dementia from NC compared to its CSF counterpart (DeLong: P=0.038); CSF and plasma NfL did not differ to that regard.
The accuracy of both pNfH and NfL in differentiating non-AD MCI either with NC or with AD-MCI was overall weak (AUCs=0.61-0.77). Regarding non-AD dementia, diagnosis accuracy of markers was moderate looking at the group at a whole (AUC=0.58-0.81, **Fig. 3**). However, CSF pNfH and NfL discriminated FTD from NC with high accuracy (respectively AUC=0.98 and AUC=0.96, **Fig. 4**). Plasma NfL had the same performance as CSF (AUC=0.90) but plasma pNfH showed lower AUC than CSF pNfH (AUC=0.81; Delong vs CSF pNfH, P=0.0139). Both CSF markers also had high AUCs for discriminating DLB and FTD (CSF pNfH, AUC=0.83; CSF NfL, AUC=0.93) whereas plasma displayed lower performance (plasma pNfH, AUC=0.58; plasma NfL, AUC=0.79; Delong test plasma versus CSF, P <0.05). For distinguishing AD dementia from FTD, all our markers displayed moderate performance (AUCs=0.69-0.79). Similarly, accuracy in discriminating AD dementia from DLB remained overall moderate (AUCs=0.66-0.83).

**Association of neurofilament levels with cognition and imaging**

We next examined the association of NfL and pNfH levels with cognition (**Fig. 5, a&d**). CSF NfL showed a significant negative association with MMSE score (β=-0.219, P=0.0088) in linear regression adjusted for age and sex, whereas CSF pNfH did not (β=0.102, P=0.2001). Both plasma markers were associated with the MMSE score. Plasma NfL showed similar association with MMSE as CSF NfL (β=-0.213, P=0.0093). Plasma pNfH was significantly associated with MMSE contrarily to CSF pNfH (β=-0.283, P=0.0003). Looking at subgroups, only CSF NfL remained associated with MMSE in the non-AD-MCI group (β=0.721, P=0.004, **Supp. Table 3**).
We then investigated the association of our markers with medio-temporal atrophy, rated visually on morphological MRI using Scheltens score and with white matter lesions evaluated with Fazekas scale (**Fig. 5, b-c, e-f**). All explored Nf markers significantly correlated with Scheltens score. CSF NfL showed higher association with Scheltens score compared with CSF pNfH (β =0.368, P <0.0001 versus β =0.242, P=0.007). Similarly, plasma NfL displayed higher association compared with plasma pNfH (β=0.358, P <0.0001 versus β=0.268, P=0.0002). CSF and plasma NfL were both associated with Scheltens in the AD patients (CSF: β=0.265, P=0.012; plasma: β=0.246, P=0.022 **Supp. Table 3**). Conversely, pNfH levels were not associated with Scheltens score in any subgroup. Regarding white matter lesions, only NfL measured in CSF and plasma correlated with Fazekas score (CSF NfL, β=0.216 P=0.017;

plasma NfL, β=0.313, P=0.0014). In the AD group, a weak correlation was sustained between plasma NfL and Fazekas score (β =0.245, P=0.048). No association was found between pNfH and Fazekas score in the whole or in subgroups.

## DISCUSSION

In this work, we explored the differential performance of NfL and pNfH in clinical practice. Using ultrasensitive assays developed on the same platform, we measured our biomarkers of interest in paired CSF and plasma samples from well-phenotyped patients representative of a memory clinic setting. We observed that both pNfH and NfL levels were strongly associated in CSF and plasma. CSF levels displayed similar accuracy in differentiating clinical groups. Plasma NfL performed similarly as CSF NfL to distinguish AD and non-AD dementia and displayed slightly higher AUCs than plasma pNfH. NfL levels also displayed a stronger association to cognitive score and to mediotemporal lobe atrophy.

Regarding the translation from CSF to blood assessment in clinical practice, we observed a significant correlation for both NfL and pNfH. However, this correlation was stronger for NfL than pNfH, as previously reported in three other comparative studies focusing on ALS. Added to the large evidence on CSF/plasma association regarding NfL in the literature, it suggests that NfL could be more robust for translation to blood, given that these proteins have similar diagnostic performances in the CSF [36]. NfL is the most abundant and soluble subunit of Nfs, most likely making it the easiest Nf to analyze in plasma [7]. The quantification of pNfH levels in plasma using immunoassays could be influenced by several factors, inducing higher variability in blood-based measurements than for NfL. The chemical properties of Nf aggregates assembly and dissociation have been intensively investigated and phosphorylation of NfH was identified as a key feature facilitating Nf formation [37]. The formation of pNfH aggregates, increased by the phosphorylation in comparison to NfL, could mask the epitope targeted by the immunoassay and also bind to soluble pNfH forms. A study using mice models could detect the existence of high molecular weight of hyperphosphorylated NfH aggregates in plasma due to a so-called Nf ’hook-effect’ [38]. Other possible explanations include differential brain blood barrier passage of pNfH or higher levels of post-translational modifications in blood.

We observed weak-to-moderate correlations of all explored markers with age that are concordant with previous published data. Age-related changes in NfL levels, in link with the neuronal loss occurring in normal aging, have been robustly documented and this factor is fundamental to take into account to correctly interpret NfL levels [5,8,39]. Our results bring further evidence that age has to be considered in all interpretations of Nf assessments for clinical use.

CSF pNfH and NfL levels were higher in all groups compared with control subjects. The highest levels were observed in the samples of patients with AD and FTD, as already established in the literature [5,26,40]. In plasma, NfL levels were also increased in all groups of cognitively impaired subjects compared to controls. Conversely, plasma NfH did not differ between non-AD MCI and controls. This difference could be accounted for by variability in plasma measurement. However, a study on 35 patients with MCI and 85 healthy controls, there was no significant increase of serum pNfH in the MCI group over a one-year period, indicating that pNfH measured in blood might not be a sensitive marker for early cognitive impairment [11]. Similarly, in a cohort of genetic FTD mutations carriers, serum NfL increase preceded clinical onset by around 15 years whereas plasma pNfH increase occurred later, close to symptom onset [21].

Regarding diagnosis performance, the accuracy of NfL and pNfH was highly dependent on the diagnostic groups that were compared. While they displayed a high accuracy discriminating dementia from NC, their performance in discriminating AD from non-AD, at both MCI and dementia levels, was positive but less performant. Indeed, Nfs are established as general markers of neurodegeneration and are of limited value from a differential diagnostic perspective. A notable exception is that plasma NfL has shown interesting value to differentiate atypical parkinsonism from idiopathic PD [5].

Prior evidence on pNfH potential for diagnosis in a larger range of neurodegenerative disorders is scarce [26,41]. For differential diagnosis of ALS with other neurodegenerative diseases, including FTD without motor impairment and AD, good discriminating potential was found for CSF NfH and slightly less power for serum NfH compared to NfL.

P-NfH levels in CSF and plasma have been extensively studied in motor neuron disease [10,12,13,22,42]. Nfs have been implicated in the pathogenesis of ALS, suggesting that it has specific potential as a biomarker [43]. Animal studies suggested that an imbalance between the relative expression levels of the different neurofilament subunits may be a contributing

mechanism to physiopathology [44]. At neuropathological examination, accumulations of Nfs were reported in the spinal cord in ALS patients, in the perikaryon and axons of motor neurons [45]. Whereas pNfH presents an added value in ALS diagnosis as it appears directly related to underlying physiopathological processes, it rather constitutes a general marker of neurodegeneration in AD.

In our clinical cohort, all studied Nf biomarkers were similarly associated MMSE results that are concordant with results from research cohorts. Plasma NfL has already been reported to significantly associate with cognitive decline in longitudinal research cohorts but to be inconsistently associated with cognitive status cross sectionally [46,47]. Plasma NfL showed a higher association with Scheltens score, both in the whole cohort and in AD subgroups, while pNfH did not. Plasma NfL levels were reported to associate with gray/white matter volume in voxel-wise analyses in AD in cross-sectional and longitudinal studies [9,48]. Our results suggest that this association can be observed with simpler clinical imaging tools.

**Limitations**

The number of included patients in non-AD dementia subgroups, as FTD or DLB, was limited, which may have impaired the detection of difference in the 4 markers in regards to discriminating between the different diagnosis. Cognitive evaluation was based on MMSE, a general screening test. Detailed neuropsychological assessment of memory, executive functions and language could bring further insights on the association of each marker with cognitive decline and its profile. Lastly, we did not explore the effect of comorbidities, as renal or liver failure, that are known to potentially affect neurofilament levels.

**Conclusion**

Based on a cohort from clinical practice, we conclude that CSF and plasma pNfH and NfL all displayed potential in the identification of dementia and MCI related to AD. Plasma NfL had similar performance than CSF measures to differentiate between diagnosis and associated both to cognition and mediotemporal atrophy. CSF and plasma pNfH did not outperform or add significant value to NfL for identification of AD.

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**Disclosures**

HZ has served at scientific advisory boards for Alector, Eisai, Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, Nervgen, AZTherapies and CogRx, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work); CP is a member of the International Advisory Boards of Lilly; is a consultant for Fujiribio, Alzhois, Neuroimmune, Ads Neuroscience, Roche, AgenT, and Gilead; and is involved as an investigator in several clinical trials for Roche, Esai, Lilly, Biogen, Astra-Zeneca, Lundbeck, and Neuroimmune; KB has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Prothena, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).

**Author's contribution**

AV: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content, study concept or design, analysis or interpretation of data. NJA: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content, study concept or design, analysis or interpretation of data. TKK: Drafting/revision of the manuscript for content, including medical writing for content, study concept or design, analysis or interpretation of data. JLR: Major role in the acquisition of data Drafting/revision of the manuscript for content, including medical writing for content, study concept or design, analysis or interpretation of data. KG: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content. JD: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content. EC: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content. HZ: Drafting/revision of the manuscript for content, including medical writing for content, study concept or design. CP: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content, study concept or design, analysis or interpretation of data. KB: Drafting/revision of the manuscript for content, including medical writing for content study concept or design, analysis or interpretation of data

| N = 188 | Controls | Non-AD MCI | AD-MCI | AD | Non-AD dementia | P-value |
|---|---|---|---|---|---|---|
| | n=22 | n=38 | n=36 | n=64 | n=28 | |
| Age, years | 63.4 (10.1) | 68.7 (9.7) | 72.1 (7.4) | 71.8 (8.4) | 67.4 (7.6) | <0.001 |
| Females, n (%) | 15 (68%) | 25 (66%) | 19 (52.8) | 41 (64%) | 15 (54%) | 0.597 |
| MMSE | 27.1 (2.4) | 24.6 (3.2) | 25.1 (2.9) | 18.2 (5.2) | 23.0 (5.2) | <0.001 |
| ApoE4 carriership, n (%) | 6 (27%) | 6 (16%) | 20 (57%) | 44 (69%) | 8 (29%) | <0.001 |
| **CSF biomarkers** | | | | | | |
| CSF Aβ42, pg/mL | 1136.5 (270.8) | 1040.3 (385.8) | 548.1 (346.3) | 513.4 (166.4) | 947.4 (407.8) | <0.001 |
| CSF Aβ42/Aβ40 ratio | 0.095 (0.007) | 0.087 (0.015) | 0.043 (0.010) | 0.042 (0.009) | 0.085 (0.018) | <0.001 |
| CSF p-tau, pg/mL | 32.0 (8.4) | 38.9 (16.9) | 87.5 (40.6) | 120.7 (62.9) | 38.1 (15.0) | <0.001 |
| CSF t-tau, pg/mL | 233.05 (65.7) | 313.7 (140.0) | 553.5 (249.9) | 769.1 (411.3) | 351.3 (312.0) | <0.001 |
| CSF NfL, pg/mL | 847.4 (371.4) | 1535.0 (1257.6) | 1501.2 (541.8) | 1872.6 (1136.5) | 2159.1 (1869.2) | <0.001 |
| CSF pNfH, pg/mL | 254.1 (68.6) | 410.7 (216.7) | 403.7 (187.8) | 529.9 (640.6) | 588.1 (927.8) | 0.002 |
| **Plasma biomarkers** | | | | | | |
| Plasma NfL, pg/mL | 13.0 (6.8) | 22.2 (17.9) | 23.3 (7.6) | 25.1 (10.8) | 31.1 (26.8) | <0.001 |
| Plasma pNfH, pg/mL | 35.8 (45.0) | 54.5 (59.7) | 104.2 (98.5) | 95.0 (99.9) | 105.1 (116.5) | 0.001 |

**Table 1, Demographics and biomarkers values across diagnosis groups**

Data is shown as mean (SD) or n (%), as appropriate. We used one-way ANOVA to compare age and MMSE between groups and Pearson's chi-square to compare sex and APOE ε4 frequencies between groups. Fluid biomarkers levels were compared with a one-way ANCOVA adjusted on age and sex, followed by a *post hoc* Tukey's HSD test.

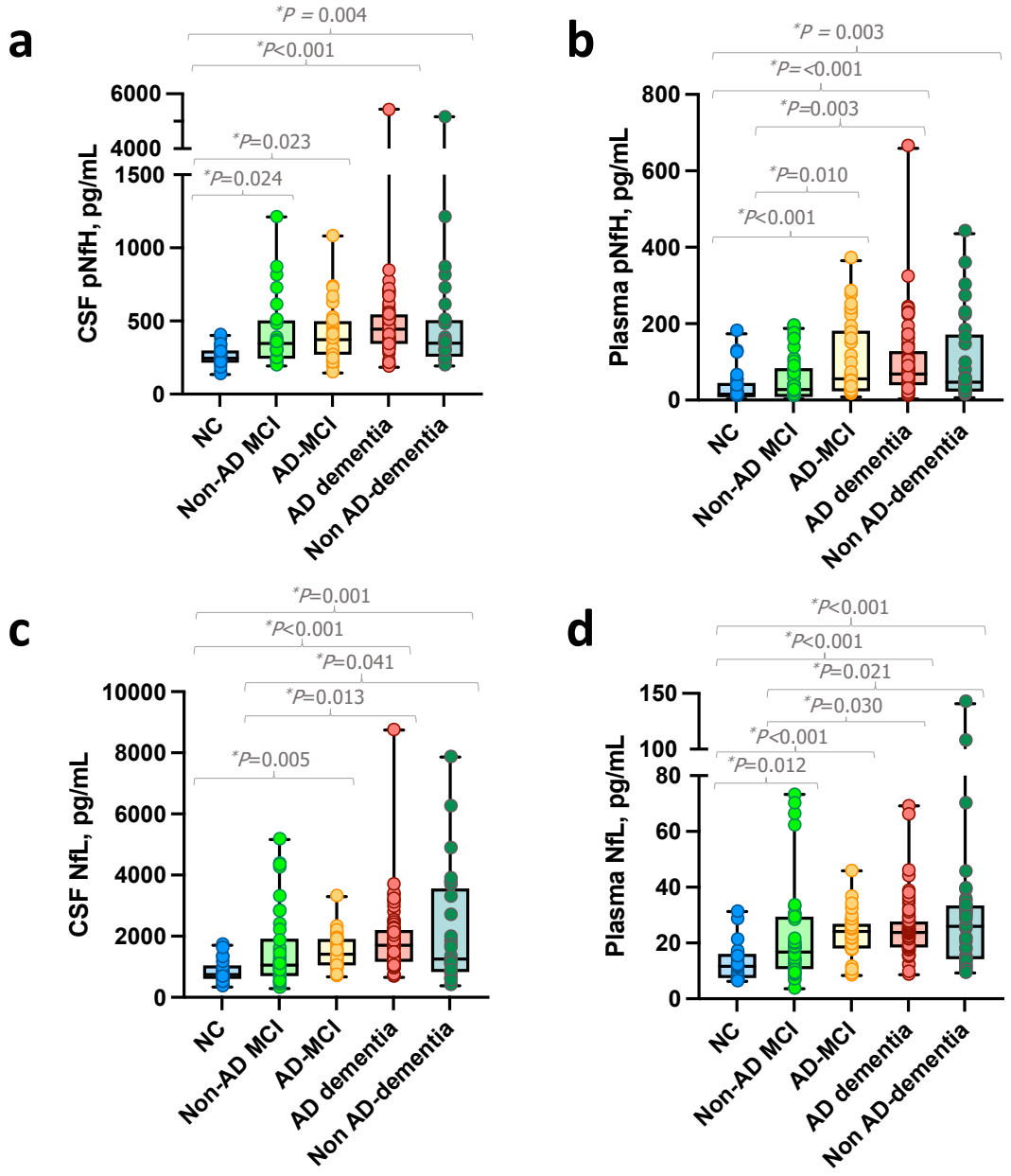



**Figure 1, Biomarkers values across groups**

Box plots comparing: **a,** CSF pNfH levels; **b,** plasma pNfH levels; **c,** CSF NfL levels; **d,** plasma NfL levels across clinical groups. The box plots depict the median (horizontal bar), interquartile range (IQR, hinges) and minimal and maximal values (whiskers). Group comparisons were computed with a one-way ANCOVA adjusting for age and sex followed by post Tukey's HSD test for the pairwise comparisons adjusting for multiple comparisons.

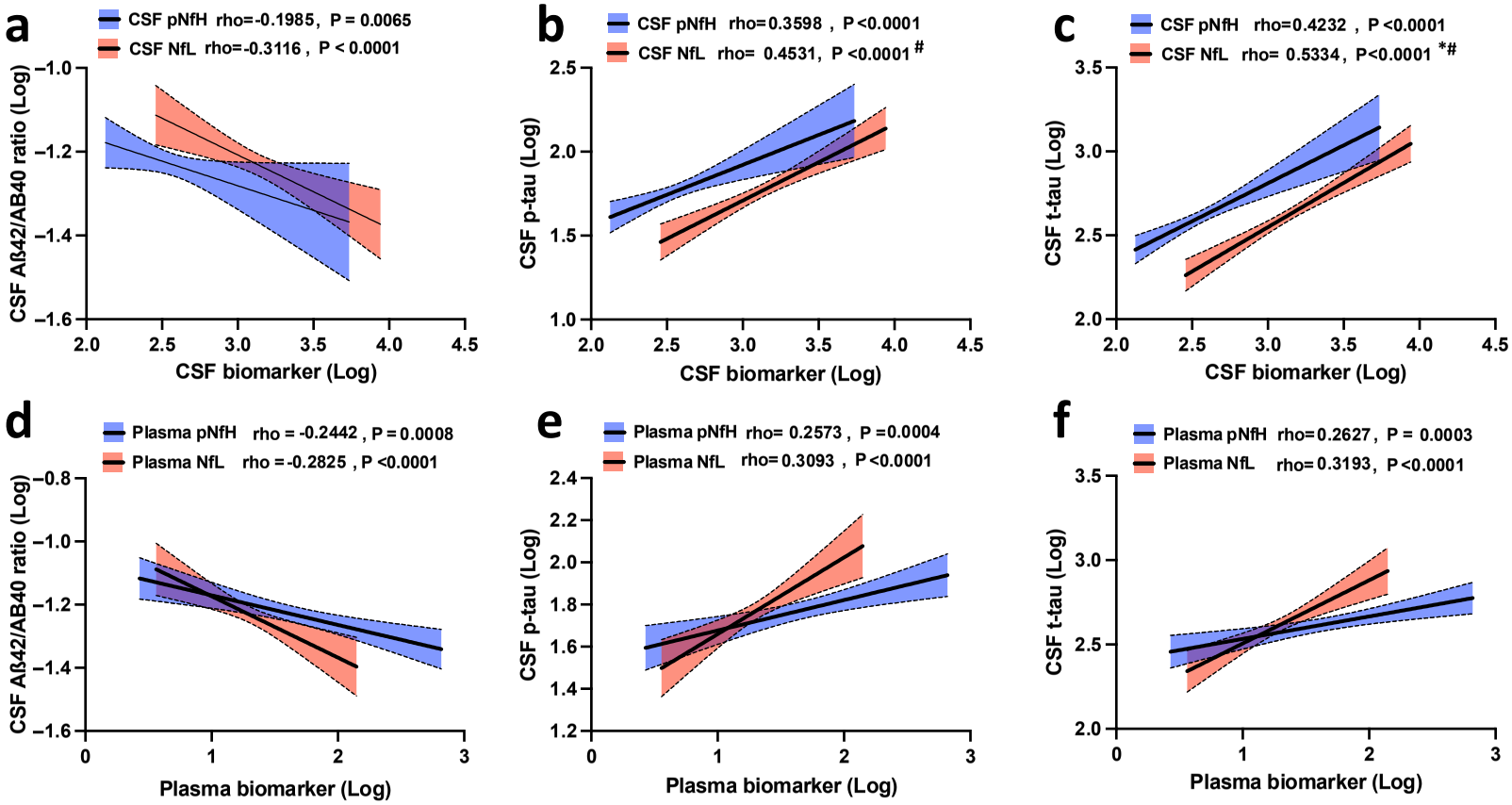



**Figure 2, Association of markers with core AD CSF biomarkers**

Correlation of CSF pNfH and CSF NfL with **a** CSF Aß42/40 ratio; **b,** with CSF p-tau; **c**, with CSF t-tau. Correlation of plasma pNfH and plasma NfL **d,** with CSF Aß42/40 ratio; **e,** with CSF p-tau; **f,** with CSF t-tau. The association between our markers and CSF core AD biomarkers was studied using Spearman rank correlation. Biomarkers levels are presented after log-transformation. Solid lines indicate the regression line and 95% CIs. Strength of correlation with given AD CSF biomarkers with Nf markers were studied using Eid et *al.* method, after Fisher's transformation of rho coefficients in z-score. * CSF NfL showed stronger association with CSF t-tau than CSF pNfH (P=0.028). # CSF NfL showed higher association than plasma NfL with both CSF p-tau (P=0.025) and CSF t-tau (P=0.001).

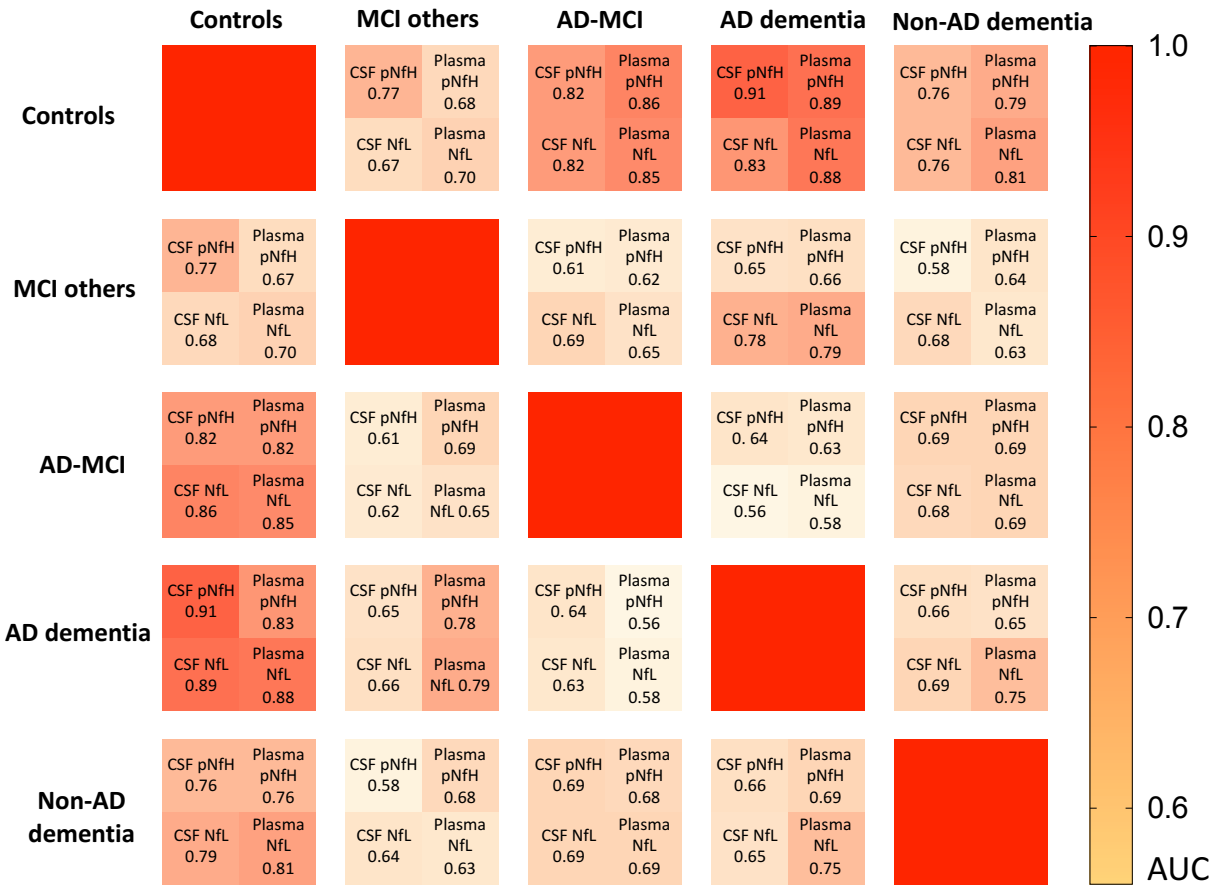



**Figure 3, Accuracy of biomarker in discriminating between groups**

Heat map displaying the AUC of each biomarker, CSF pNfH, CSF NfL, plasma pfNfH and plasma NfL in discriminating between neurological controls, non-AD MCI, AD-MCI, AD dementia and non-AD dementia. AUCs of the different Nf markers to discriminate between given groups were compared using Delong test. [#]Plasma pNfH showed lower AUC than CSF pNfH (Delong vs CSF pNfH, P=0.038).

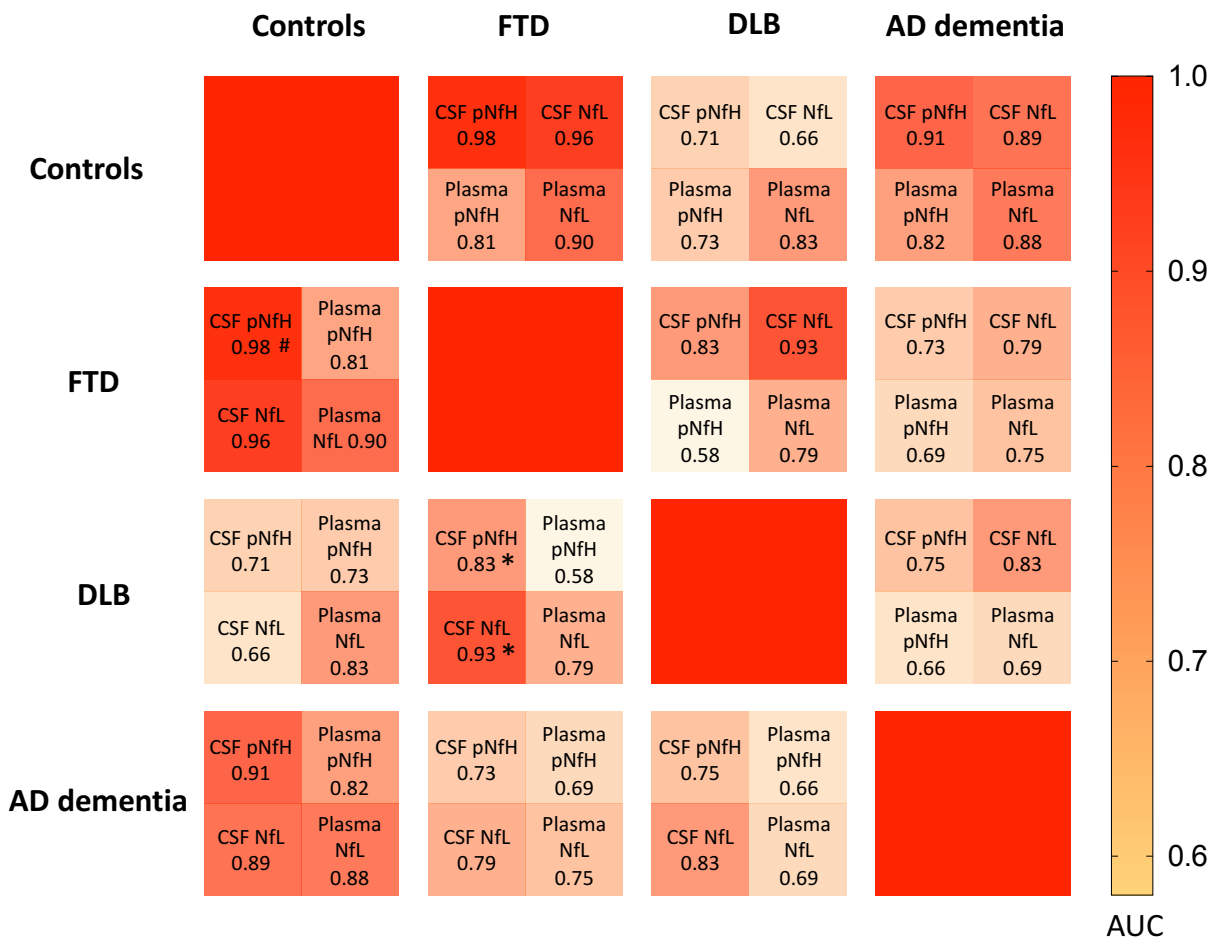



**Figure 4, Accuracy of biomarker in identifying non-AD dementia**

Heat map displaying the AUC of each biomarker, CSF pNfH, CSF NfL, plasma pfNfH and plasma NfL in discriminating between neurological controls, AD dementia, DLB and FTD. AUCs of the different Nf markers to discriminate between given groups were compared using Delong test. #Plasma pNfH showed lower AUC than CSF pNfH (Delong *vs* CSF pNfH, P=0.0139). *CSF Nf had higher AUC to discriminate DLB and FTD than their plasma counterpart (CSF pNfH, AUC=0.83; CSF NfL, AUC=0.93 versus plasma pNfH, AUC=0.58 ; plasma NfL, AUC=0.79; Delong test: P<0.05).

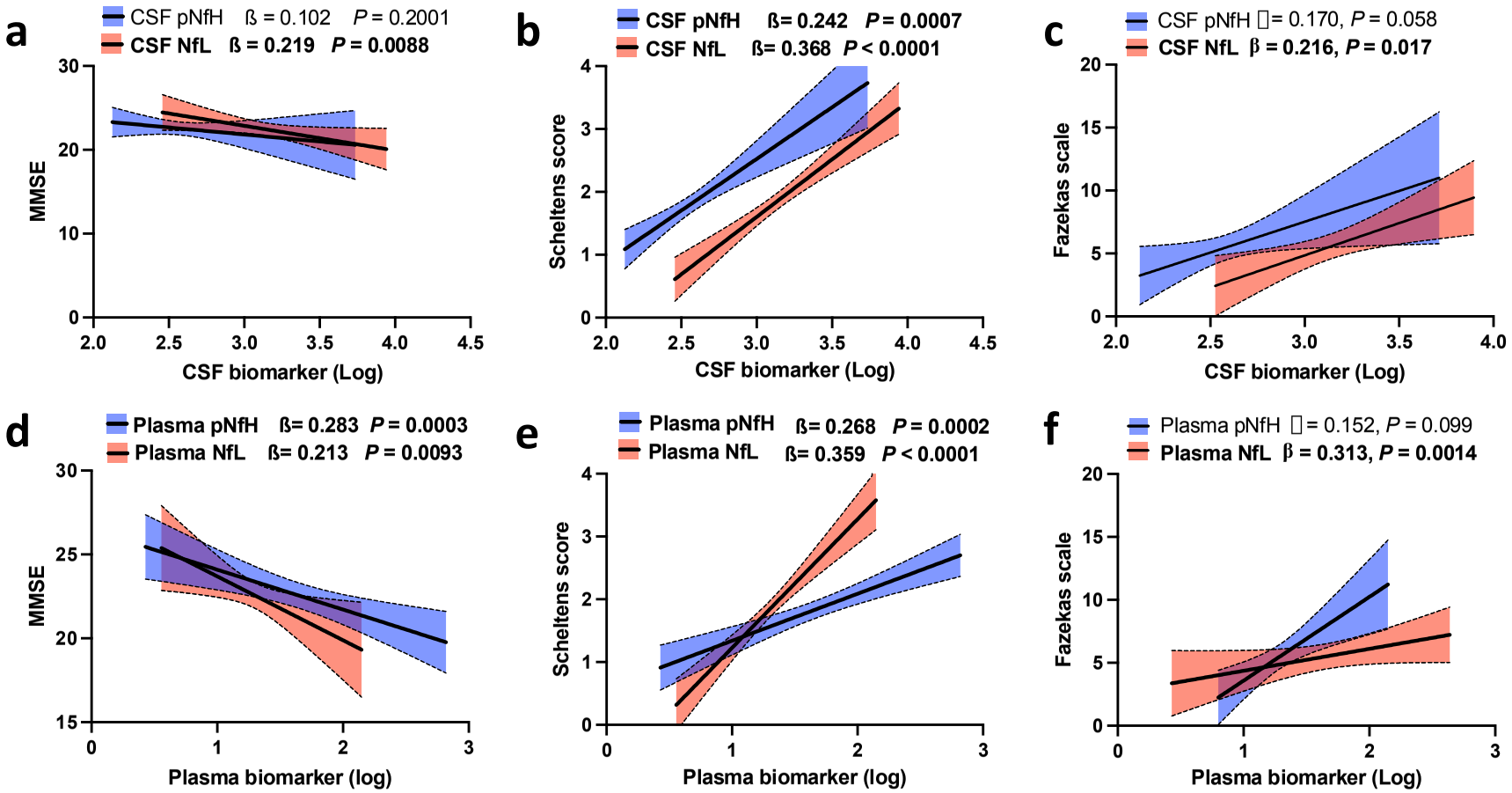



**Figure 5, Association to MMSE and imaging**

Association of MMSE with: **a,** CSF biomarkers; **d,** Plasma biomarkers and of Scheltens score with: **b,** CSF biomarkers; **e,** plasma biomarkers, and Fazekas score with **c,** CSF biomarkers; **f,** plasma biomarkers. Association of our biomarkers with the different scores was studied using linear regression adjusted on age, sex and level of education for MMSE, and age and sex for Scheltens and Fazekas scores.

| **N = 188** | **Controls** | **MCI others** | **AD-MCI** | **AD** | **FTD** | **DLB** | **VaD** |
|---|---|---|---|---|---|---|---|
| | *n* = 22 | *n* = 38 | *n* = 36 | *n* = 64 | *n* = 12 | *n* = 13 | *n* = 3 |
| Age, years | 63.4 (10.1) | 68.7 (9.7) | 72.1 (7.4) | 71.8 (8.4) | 66.7 (6.1) | 66.6 (8.4) | 73.3 (8.7) |
| Females, n (%) | 15 (68%) | 25 (66%) | 19 (52.8) | 41 (64%) | 7 (58%) | 6 (46%) | 1 (33%) |
| MMSE | 27.1 (2.4) | 24.6 (3.2) | 25.1 (2.9) | 18.2 (5.2) | 22.6 (5.4) | 23.9 (5.5) | 20. 3 (4.9) |
| ApoE4 carriership, n | 6 (27%) | 6 (16%) | 20 (57%) | 44 (69%) | 2 (17%) | 4 (31%) | 2 (66%) |
| **CSF biomarkers** | | | | | | | |
| CSF Aß42, pg/mL | 1136.5 (270.8) | 1040.3 (385.8) | 548.1 (346.3) | 513.4 (166.4) | 1142 (448) | 794.8 (348.7) | 827.3 (84.3) |
| CSF Aß42/Aß40 ratio | 0.095 (0.007) | 0.087 (0.015) | 0.043 (0.010) | 0.042 (0.009) | 0.089 (0.019) | 0.080 (0.017) | 0.087 (0.003) |
| CSF p-tau, pg/mL | 32.0 (8.4) | 38.9 (16.9) | 87.5 (40.6) | 120.7 (62.9) | 43.9 (15.6) | 35.0 (14.3) | 27.8 (5.8) |
| CSF t-tau, pg/mL | 233.05 (65.7) | 313.7 (140.0) | 553.5 (249.9) | 769.1 (411.3) | 504.1 (429.3) | 239.6 (92.6) | 224.0 (55.8) |
| CSF NfL, pg/mL | 847.4 (371.4) | 1535.0 (1257.6) | 1501.2 (541.8) | 1872.6 (1136.5) | 3546.4 (2099.3) | 968.1 (422.7) | 1770.6 (930.4) |
| CSF pNfH, pg/mL | 254.1 (68.6) | 410.7 (216.7) | 403.7 (187.8) | 529.9 (640.6) | 938.4 (1361.1) | 329.4 (147.5) | 308.1 (70.6) |
| **Plasma biomarkers** | | | | | | | |
| Plasma NfL, pg/mL | 13.0 (6.8) | 22.2 (17.9) | 23.3 (7.6) | 25.1 (10.8) | 44.6 (36.5) | 19.5 (7.5) | 27,38 (8.5) |
| Plasma pNfH, pg/mL | 35.8 (45.0) | 54.5 (59.7) | 104.2 (98.5) | 95.0 (99.9) | 136.4 (149.2) | 89.6 (89.0) | 47,45 (24.6) |

**Supplementary Table 1, Demographics and biomarkers levels across clinical groups**

Data shown as mean (SD) or n (%), as appropriate. We used one-way ANOVA to compare age and MMSE between groups and Pearson's chi-square to compare sex and APOE ε4 frequencies between groups. Fluid biomarkers levels were compared with a one-way ANCOVA adjusted by age and sex.

| | **CSF Aß42/40 ratio** | | **CSF p-tau** | | **CSF t-tau** | |
|---|---|---|---|---|---|---|
| | rho | P-value | rho | P-value | rho | P-value |
| **CSF pNfH** | | | | | | |
| Whole cohort | **-0.198** | **0.0065** | **0.360** | **<0.0001** | **0.423** | **<0.0001** |
| Controls | 0.122 | 0.588 | -0.027 | 0.907 | -0.062 | 0.786 |
| MCI other | 0.005 | 0.977 | 0.273 | 0.097 | 0.252 | 0.126 |
| AD[#] | 0.107 | 0.292 | **0.213*** | **0.033** | **0.262**** | **0.008** |
| Non-AD dementia | 0.071 | 0.721 | 0.099 | 0.618 | 0.178 | 0.366 |
| **CSF NfL** | | | | | | |
| Whole cohort | **-0.312** | **<0.0001** | **0.453** | **<0.0001** | **0.533** | **<0.0001** |
| Controls | -0.088 | 0.706 | 0.312 | 0.169 | 0.351 | 0.119 |
| MCI other | -0.197 | 0.235 | 0.389* | 0.016 | **0.325*** | **0.046** |
| AD[#] | -0.135 | 0.185 | **0.424**** | **0.000** | **0.483**** | **0.000** |
| Non-AD dementia | 0.010 | 0.962 | 0.260 | 0.182 | **0.380*** | **0.046** |
| **Plasma pNfH** | | | | | | |
| Whole cohort | **-0.244** | **0.008** | **0.257** | **0.0004** | **0.262** | **0.0003** |
| Controls | -0.156 | 0.487 | 0.093 | 0.680 | 0.040 | 0.859 |
| MCI other | 0.091 | 0.586 | 0.197 | 0.236 | 0.065 | 0.698 |
| AD[#] | 0.022 | 0.832 | 0.018 | 0.863 | 0.045 | 0.656 |
| Non-AD dementia | -0.299 | 0.122 | 0.413* | 0.029 | 0.149 | 0.448 |
| **Plasma NfL** | | | | | | |
| Whole cohort | **-0.282** | **<0.0001** | **0.309** | **<0.0001** | **0.190** | **<0.0001** |
| Controls | -0.282 | 0.204 | 0.029 | 0.899 | 0.077 | 0.732 |
| MCI other | -0.178 | 0.284 | 0.138 | 0.409 | 0.074 | 0.660 |
| AD[#] | -0.182 | 0.072 | **0.221*** | **0.027** | **0.225*** | **0.024** |
| Non-AD dementia | -0.071 | 0.719 | 0.200 | 0.307 | 0.129 | 0.514 |

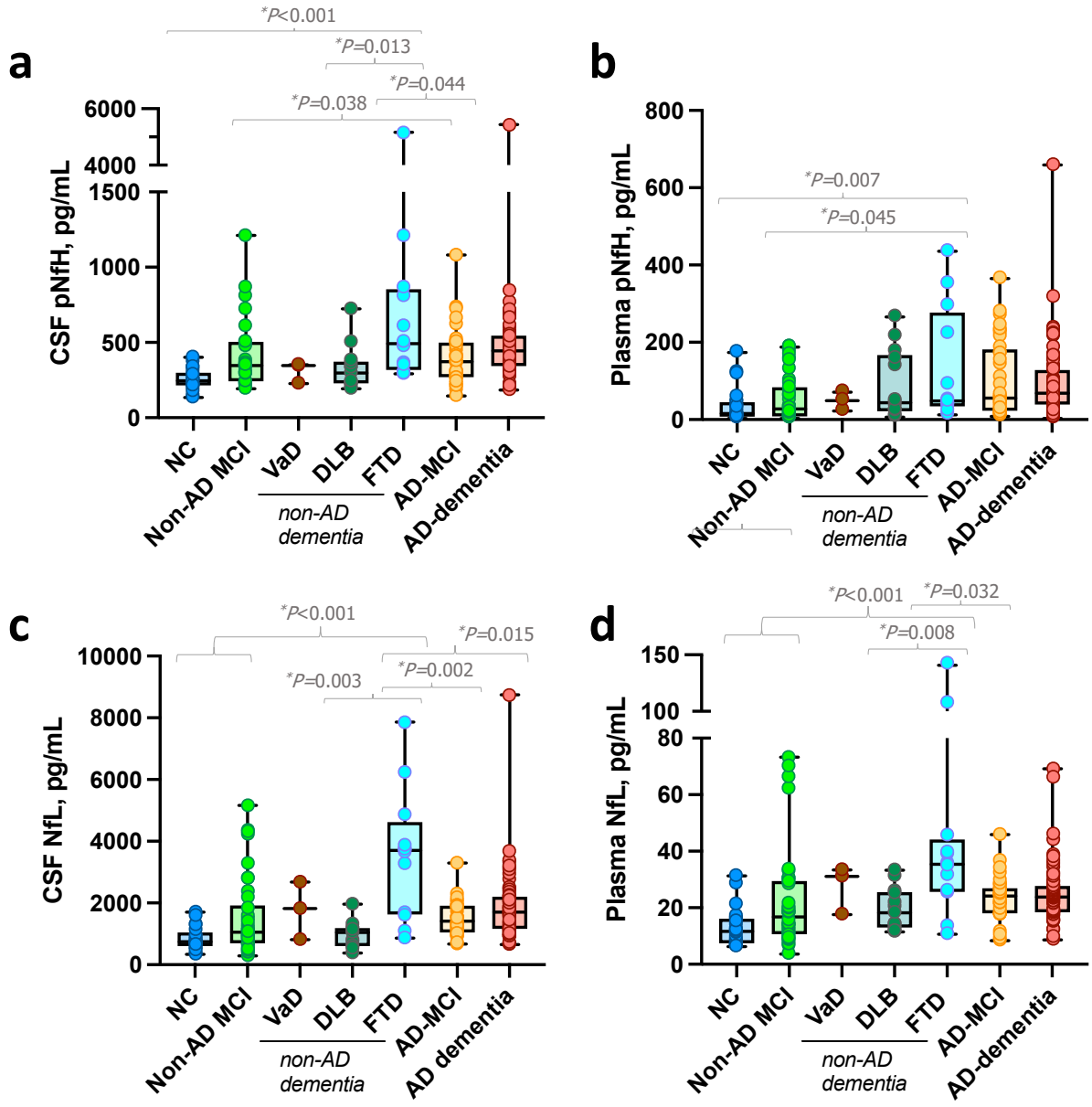
**Supplementary Table 2, Correlation of biomarkers with CSF AD core biomarkers**

Spearman rank correlations of plasma and CSF pNfH and NfL with CSF AD core biomarkers in clinical subgroups. AD-MCI and AD dementia were combined for the analysis A. P<0.005 was considered overall significant.

| | **MMSE** | | **Scheltens score** | | **Fazekas score** | |
|---|---|---|---|---|---|---|
| | ß | P-value | ß | P-value | ß | P-value |
| **CSF pNfH** | | | | | | |
| Whole cohort | 0.102 | 0.200 | **0.242** | **0.0007** | 0.170 | 0.058 |
| Controls | -0.322 | 0.225 | **0.501** | **0.037** | -0.099 | 0.724 |
| MCI other | 0.155 | 0.448 | 0.113 | 0.487 | 0.270 | 0.193 |
| AD | -0.010 | 0.929 | 0.191 | 0.080 | 0.082 | 0.513 |
| Non-AD dementia | -0.036 | 0.859 | 0.161 | 0.462 | -0.028 | 0.912 |
| **CSF NfL** | | | | | | |
| Whole cohort | **0.219** | **0.0088** | **0.368** | **<0.0001** | **0.216** | **0.017** |
| Controls | -0.063 | 0.825 | 0.245 | 0.505 | -0.012 | 0.970 |
| MCI other | **0.721** | **0.004** | 0.304 | 0.206 | 0.263 | 0.205 |
| AD | -0.003 | 0.978 | **0.265** | **0.012** | 0.035 | 0.780 |
| Non-AD dementia | -0.097 | 0.637 | 0.278 | 0.202 | 0.083 | 0.743 |
| **Plasma pNfH** | | | | | | |
| Whole cohort | **0.283** | **0.0003** | **0.268** | **0.0002** | 0.152 | 0.099 |
| Controls | 0.093 | 0.690 | 0.261 | 0.267 | -0.179 | 0.519 |
| MCI other | 0.327 | 0.075 | 0.117 | 0.467 | 0.073 | 0.727 |
| AD | 0.090 | 0.371 | 0.191 | 0.065 | 0.131 | 0.295 |
| Non-AD dementia | 0.166 | 0.426 | 0.154 | 0.495 | 0.173 | 0.492 |
| **Plasma NfL** | | | | | | |
| Whole cohort | **0.213** | **0.0093** | **0.359** | **<0.0001** | **0.313** | **0.014** |
| Controls | -0.190 | 0.566 | -0.031 | 0.926 | 0.263 | 0.3397 |
| MCI other | 0.407 | 0.096 | 0.198 | 0.365 | 0.311 | 0.131 |
| AD | 0.026 | 0.805 | **0.246** | **0.022** | **0.245** | **0.048** |
| Non-AD dementia | 0.044 | 0.749 | 0.322 | 0.134 | -0.077 | 0.762 |

**Supplementary Table 3, Association of neurofilament biomarkers with cognition and imaging score in diagnosis subgroups**

Association of plasma and CSF pNfH and NfL with MMSE, Scheltens and Fazekas scores, studied using linear regression adjusted on age and sex, and the level of education for MMSE. AD and AD-MCI were combined in an AD group. A $P<0.05$ was considered overall significant (bold).




**Supplementary Figure 1, Biomarkers values across groups, including non-AD dementia groups**

Box plots displaying: **a,** CSF pNfH levels; **b,** plasma pNfH levels; **c,** CSF NfL levels; **d,** plasma NfL levels across clinical groups. The box plots depict the median (horizontal bar), interquartile range (IQR, hinges) and minimal and maximal values (whiskers). VaD group was excluded of analysis because of small size. Group comparisons with DLB and FTD were computed with a one-way ANCOVA adjusting for age and sex followed by Tukey's HSD test.

JAMA Neurology | Original Investigation

# Differences Between Plasma and Cerebrospinal Fluid Glial Fibrillary Acidic Protein Levels Across the Alzheimer Disease Continuum

Andréa L. Benedet, PhD; Marta Milà-Alomà, MSc; Agathe Vrillon, MD, MSc; Nicholas J. Ashton, PhD; Tharick A. Pascoal, MD, PhD; Firoza Lussier, BSc; Thomas K. Karikari, PhD; Claire Hourregue, MD; Emmanuel Cognat, MD, PhD; Julien Dumurgier, MD, PhD; Jenna Stevenson, BSc; Nesrine Rahmouni, BSc; Vanessa Pallen, BSc; Nina M. Poltronetti, MSc; Gemma Salvadó, PhD; Mahnaz Shekari, MSc; Gregory Operto, PhD; Juan Domingo Gispert, PhD; Carolina Minguillon, PhD; Karine Fauria, PhD; Gwendlyn Kollmorgen, PhD; Ivonne Suridjan, PhD; Eduardo R. Zimmer, PhD; Henrik Zetterberg, MD, PhD; José Luis Molinuevo, MD, PhD; Claire Paquet, MD, PhD; Pedro Rosa-Neto, MD, PhD; Kaj Blennow, MD, PhD; Marc Suárez-Calvet, MD, PhD; for the Translational Biomarkers in Aging and Dementia (TRIAD) study, Alzheimer's and Families (ALFA) study, and BioCogBank Paris Lariboisière cohort

**IMPORTANCE** Glial fibrillary acidic protein (GFAP) is a marker of reactive astrogliosis that increases in the cerebrospinal fluid (CSF) and blood of individuals with Alzheimer disease (AD). However, it is not known whether there are differences in blood GFAP levels across the entire AD continuum and whether its performance is similar to that of CSF GFAP.

**OBJECTIVE** To evaluate plasma GFAP levels throughout the entire AD continuum, from preclinical AD to AD dementia, compared with CSF GFAP.

**DESIGN, SETTING, AND PARTICIPANTS** This observational, cross-sectional study collected data from July 29, 2014, to January 31, 2020, from 3 centers. The Translational Biomarkers in Aging and Dementia (TRIAD) cohort (Montreal, Canada) included individuals in the entire AD continuum. Results were confirmed in the Alzheimer's and Families (ALFA+) study (Barcelona, Spain), which included individuals with preclinical AD, and the BioCogBank Paris Lariboisière cohort (Paris, France), which included individuals with symptomatic AD.

**MAIN OUTCOMES AND MEASURES** Plasma and CSF GFAP levels measured with a Simoa assay were the main outcome. Other measurements included levels of CSF amyloid-β 42/40 (Aβ42/40), phosphorylated tau181 (p-tau181), neurofilament light (NfL), Chitinase-3-like protein 1 (YKL40), and soluble triggering receptor expressed on myeloid cells 2 (sTREM2) and levels of plasma p-tau181 and NfL. Results of amyloid positron emission tomography (PET) were available in TRIAD and ALFA+, and results of tau PET were available in TRIAD.

**RESULTS** A total of 300 TRIAD participants (177 women [59.0%]; mean [SD] age, 64.6 [17.6] years), 384 ALFA+ participants (234 women [60.9%]; mean [SD] age, 61.1 [4.7] years), and 187 BioCogBank Paris Lariboisière participants (116 women [62.0%]; mean [SD] age, 69.9 [9.2] years) were included. Plasma GFAP levels were significantly higher in individuals with preclinical AD in comparison with cognitively unimpaired (CU) Aβ-negative individuals (TRIAD: Aβ-negative mean [SD], 185.1 [93.5] pg/mL, Aβ-positive mean [SD], 285.0 [142.6] pg/mL; ALFA+: Aβ-negative mean [SD], 121.9 [42.4] pg/mL, Aβ-positive mean [SD], 169.9 [78.5] pg/mL). Plasma GFAP levels were also higher among individuals in symptomatic stages of the AD continuum (TRIAD: CU Aβ-positive mean [SD], 285.0 [142.6] pg/mL, mild cognitive impairment [MCI] Aβ-positive mean [SD], 332.5 [153.6] pg/mL; AD mean [SD], 388.1 [152.8] pg/mL vs CU Aβ-negative mean [SD], 185.1 [93.5] pg/mL; Paris: MCI Aβ-positive, mean [SD], 368.6 [158.5] pg/mL; AD dementia, mean [SD], 376.4 [179.6] pg/mL vs CU Aβ-negative mean [SD], 161.2 [67.1] pg/mL). Plasma GFAP magnitude changes were consistently higher than those of CSF GFAP. Plasma GFAP more accurately discriminated Aβ-positive from Aβ-negative individuals than CSF GFAP (area under the curve for plasma GFAP, 0.69-0.86; area under the curve for CSF GFAP, 0.59-0.76). Moreover, plasma GFAP levels were positively associated with tau pathology only among individuals with concomitant Aβ pathology.

**CONCLUSIONS AND RELEVANCE** This study suggests that plasma GFAP is a sensitive biomarker for detecting and tracking reactive astrogliosis and Aβ pathology even among individuals in the early stages of AD.

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Supplemental content

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**Group Information:** The members of the Translational Biomarkers in Aging and Dementia (TRIAD) study, Alzheimer's and Families (ALFA) study, and BioCogBank Paris Lariboisière cohort are listed in Supplement 2.

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The rapid advancements in the development of blood biomarkers to accurately detect Alzheimer disease (AD) point to a prompt application of these biomarkers in clinical routine and clinical trials. This application is especially true for individuals with preclinical AD, as scalable and less invasive biomarkers are needed to screen large populations of cognitively unimpaired (CU) individuals to test innovative interventions.

Currently, the most promising blood biomarkers for detecting AD are the phosphorylated tau species (p-tau)[1-6] and amyloid-β 42/40 (Aβ42/40) ratio.[7-12] However, it is still desirable to have more sensitive blood biomarkers for preclinical AD. Alzheimer disease pathology is associated with morphologic, molecular, and functional remodeling of astrocytes, a process termed *reactive astrogliosis*.[13,14] However, few astrocyte imaging and fluid biomarkers have been investigated.[15] Blood levels of glial fibrillary acidic protein (GFAP), a reactive astrogliosis biomarker, are higher in individuals with preclinical AD, constituting a promising candidate biomarker for this early stage of the disease.[16] A recent meta-analysis demonstrated that GFAP levels were consistently altered in the cerebrospinal fluid (CSF) of symptomatic patients with AD, but studies of blood GFAP present relatively high variability.[17]

It is not yet well known how plasma GFAP levels change across the overall AD continuum and whether GFAP concentrations in CSF and blood reflect the same pathologic processes because reactive astrocytes assume multiple states—the so-called astrocyte heterogeneity. Thus, our main aim was to evaluate the levels of plasma GFAP throughout the AD continuum and compare them with the levels of CSF GFAP, with particular attention to preclinical AD. We hypothesized that plasma GFAP levels are already higher early in the preclinical stage and further elevated in symptomatic stages.

### Key Points

**Question** What are the levels of plasma glial fibrillary acidic protein (GFAP) throughout the Alzheimer disease (AD) continuum, and how do they compare with the levels of cerebrospinal fluid (CSF) GFAP?

**Findings** In this cross-sectional study, plasma GFAP levels were elevated in the preclinical and symptomatic stages of AD, with levels higher than those of CSF GFAP. Plasma GFAP had a higher accuracy than CSF GFAP to discriminate between amyloid-β (Aβ)–positive and Aβ-negative individuals, also at the preclinical stage.

**Meaning** This study suggests that plasma GFAP is a sensitive biomarker that significantly outperforms CSF GFAP in indicating Aβ pathology in the early stages of AD.

## Methods

### Study Population

This cross-sectional study, which included participants from 3 cohorts, collected data from July 29, 2014, to January 31, 2020. The Translational Biomarkers in Aging and Dementia (TRIAD) cohort (Montreal, Canada)[18] comprised 300 individuals (177 women [59.0%]; mean [SD] age, 64.6 [17.6] years), including young CU adults, elderly CU adults, individuals with mild cognitive impairment (MCI), and patients with AD dementia. The ALFA+ cohort (Barcelona, Spain),[19] which is a nested study of the ALFA (for Alzheimer's and Families) study, included 384 middle-aged CU individuals (234 women [60.9%]; mean [SD] age, 61.1 [4.7] years) at elevated risk for AD. The BioCogBank Paris Lariboisière cohort (Paris, France)[20] included 166 patients with cognitive disorders from the Center of Cognitive Neurology, Lariboisière Hospital, as well as 21 CU individuals. In addition to clinical classification (CU, MCI, and dementia), participants were categorized according to Aβ status (Aβ-positive [Aβ+] and Aβ-negative [Aβ-]), defined by results of Aβ positron emission tomography (PET) in TRIAD and the CSF Aβ42/40 ratio in ALFA+ and Paris, if not otherwise specified. ALFA+ participants were also classified using the AT (Aβ and tau pathology) classification.[21,22] Participants with non–AD dementia (frontotemporal dementia [FTD] or dementia with Lewy bodies) from the TRIAD and Paris cohorts were included for supplementary analysis. All studies have been approved by their regional ethical committees (TRIAD: McGill University and Douglas Hospital Research Centre institutional review boards; ALFA+: Independent Ethics Committee "Parc de Salut Mar," Barcelona; and Paris Cohort: Bichat Ethics Comittee), and all participants provided written informed consent. Additional details of the 3 cohorts are reported in the eMethods in Supplement 1.

### Fluid and Neuroimaging Biomarkers

Plasma and CSF samples from the 3 cohorts were independently analyzed at the Clinical Neurochemistry Laboratory, University of Gothenburg, Gothenburg, Sweden. Plasma and CSF GFAP levels were quantified for all cohorts on the Simoa HD-X (Quanterix) using the commercial single-plex assay (No. 102336). A comprehensive description of the fluid and neuroimaging biomarker measurements can be found in the eMethods in Supplement 1.

### Statistical Analysis

We used linear regression models to assess the association between plasma or CSF GFAP levels and the other biomarkers. Similar models were applied to evaluate group differences and associations with age and sex; the Tukey honestly significant difference test was used for post hoc pairwise comparisons. Fold changes and the effect size of the differences (estimated with Cohen *d*) were calculated using Aβ- CU (CU-) individuals (TRIAD and Paris) and Aβ- and tau- (A-T-) individuals or Aβ– individuals (ALFA+) as reference groups. All analyses were adjusted for age and sex if not otherwise specified. The Spearman rank test was used for correlations using raw biomarker values. Receiver operating curve (ROC) analyses provided the area under the curve (AUC) for Aβ positivity or diagnostic groups. The "pROC" package in R, version 3.6.3 (R Group for Statistical Computing) was used to compare AUCs, and the false discovery rate was used to correct *P* values for multiple comparisons. Mediation analyses were performed with the R package "mediation." All tests were 2-tailed, with a significance level

of α = .05. All statistical analyses and figures were performed with R, version 3.6.3. Further details are provided in the eMethods in Supplement 1.

## Results

### Participants' Characteristics and Correlations Between Biomarkers

Demographic and clinical data from the 3 studies are summarized in **Table 1** and eTable 1 in Supplement 1. There was a positive association between age and both plasma and CSF GFAP levels in the 3 cohorts (TRIAD: plasma, β [SE] = 0.64 [0.13]; $P < .001$; CSF, β [SE] = 0.35 [0.15]; $P = .02$); ALFA+: plasma, β [SE] = 0.38 [0.048]; $P < .001$; CSF, β [SE] = 0.26 [0.049]; $P < .001$; and Paris: plasma, β [SE] = 0.26 [0.06]; $P < .001$; CSF, β [SE] = 0.32 [0.07]; $P < .001$), which can also be evidenced when comparing plasma or CSF GFAP mean levels between young CU participants and elderly CU- individuals (TRIAD: plasma, CU- mean [SD], 185.1 [93.5] pg/mL; young CU mean [SD], 95.1 [62.1] pg/mL; $P = .001$; CSF, CU- mean [SD], 12 506 [5148] pg/mL; young CU mean [SD], 4134 [1483] pg/mL; $P < .001$). Plasma GFAP levels were higher in CU women than in CU men (TRIAD: mean [SD], 161.0 [81.7] pg/mL in men vs 239.01 [123.84] pg/mL in women; $P < .001$; ALFA+: mean [SD], 128.9 [59.7] pg/mL in men vs 145.6 [63.1] pg/mL in women; $P < .001$) and were also higher specifically in CU- women compared with CU- men (TRIAD: mean [SD], 142.5 [63.2] pg/mL in men vs 209.1 [99.5] pg/mL in women; $P < .001$; ALFA+: mean [SD], 117.0 [43.9] pg/mL in men vs 125.1 [41.2] pg/mL in women; $P = .01$; and Paris cohort: mean [SD], 118.9 [34.6] pg/mL in men vs 179.34 [68.26] pg/mL in women; $P = .03$). The same sex differences were also observed when all participants were included (adjusting for age and diagnosis, TRIAD: mean [SD], 224.7 [153.2] pg/mL in men vs 248.1 [146.1] pg/mL in women; $P = .002$; Paris: mean [SD], 262.7 [138.4] pg/mL in men vs 326.7 [189.6] pg/mL in women; $P < .001$). *APOE* ε4 carriership (NCBI Gene ID: 348) was not associated with plasma or CSF GFAP levels in any of the cohorts when models accounted for Aβ status or clinical diagnosis.

There was a positive correlation between plasma and CSF GFAP levels in the 3 cohorts (eFigure 1 in Supplement 1). Spearman rank correlations between plasma and CSF GFAP levels and other biomarkers are presented in eFigure 2 in Supplement 1.

### Plasma GFAP Levels Throughout the AD Continuum

In the TRIAD cohort, levels of plasma and CSF GFAP were higher across the AD continuum, namely, in Aβ+ CU (CU+) individuals (ie, preclinical AD), individuals with Aβ+ MCI (MCI+; ie, MCI due to AD), and individuals with AD dementia (**Figure 1**A). Compared with the CU- group, plasma GFAP levels were higher in the CU+ group (54% increase; $P = .001$; $d = 0.66$), in the MCI+ group (79% increase; $P < .001$; $d = 1.35$), and in the AD dementia group (107% increase; $P < .001$; $d = 2.10$). Patients with FTD had plasma GFAP levels as low as CU- individuals (eFigure 3A in Supplement 1). Levels of CSF GFAP were also higher in the AD continuum groups compared with CU- individuals (Figure 1B), but the group differences were not significant after correction for multiple comparisons. The magnitude of the CSF GFAP changes was not as large as that of the plasma GFAP changes (the CSF GFAP level increases with CU- individuals as the reference group: CU+ individuals, 24% increase; $P = .24$; $d = 0.56$; individuals with MCI+, 35% increase; $P = .06$; $d = 0.82$; and individuals with AD dementia, 30% increase; $P = .03$; $d = 0.86$). Similar to plasma GFAP levels, patients with FTD had lower CSF GFAP levels than patients on the AD continuum (eFigure 3B in Supplement 1).

In ALFA+, we used the biomarker-based AT classification[21,22] to study 2 stages in preclinical AD: Aβ+ but tau- (A+T-) and Aβ+ and tau+ (A+T+) and compared it with the A-T- stage. Plasma GFAP levels were significantly higher in the A+T- group compared with the A-T- group (32% increase; $P < .001$; $d = 0.55$) (Figure 1C), whereas CSF GFAP levels were not (1% increase; $P = .99$; $d = 0.01$; Figure 1D). Both plasma and CSF GFAP were significantly higher in the A+T+ group compared with the A-T- group (plasma: 60% increase; $P < .001$; $d = 1.09$; CSF: 77% increase; $P < .001$; $d = 1.18$). Participants in the Aβ- and tau+ (A-T+ group) did not have higher plasma or CSF GFAP levels compared with the A-T- group. To further test whether plasma and CSF GFAP levels were increased in the earliest stage of the preclinical AD continuum, we analyzed a group of individuals with a low burden of Aβ pathology, namely, a positive CSF Aβ42/40 ratio but Aβ PET centiloids lower than 30[23] (eMethods in Supplement 1). We observed that plasma GFAP levels were significantly higher in this group compared with Aβ- participants (28% increase; $P < .001$; $d = 0.57$; eFigure 4A in Supplement 1) while CSF GFAP levels were not (8% increase; $P = .37$; $d = 0.16$; eFigure 4B in Supplement 1).

In the Paris cohort, plasma and CSF GFAP levels followed similar patterns to those described for TRIAD. Plasma GFAP levels were higher in individuals with MCI+ (128% increase; $P < .001$; $d = 1.40$) and in those with AD dementia (133% increase; $P < .001$; $d = 1.37$) compared with the CU- group, and no difference was found between the CU- group and non-AD group (Figure 1E). Levels of CSF GFAP were higher in individuals with MCI+ (72% increase; $d = 0.44$) and AD dementia (89% increase; $d = 0.64$) compared with CU- individuals, but differences were not statistically significant after correction for multiple comparisons (Figure 1F). Similar to TRIAD, patients with FTD and dementia with Lewy bodies had plasma and CSF GFAP levels comparable to CU- individuals (eFigure 3C and 3D in Supplement 1).

### Association of Plasma GFAP Levels With Aβ Pathology and Discrimination of Aβ Status

We evaluated the association of plasma and CSF GFAP levels with Aβ pathology as measured with CSF Aβ42/40 or Aβ PET. Because our aim was to study the AD continuum, for all subsequent analyses, we included only CU individuals, those with MCI, and those with AD dementia (for TRIAD and Paris cohorts). In the ALFA+ cohort, we excluded individuals with an A-T+ (non-AD pathologic change) biomarker profile. In TRIAD, both plasma and CSF GFAP levels were negatively associated with CSF Aβ42/40 (plasma GFAP, $P < .001$; $\eta_p^2 = 0.26$; CSF GFAP, $P = .01$; $\eta_p^2 = 0.11$; **Figure 2**A and B) and positively

Table 1. Demographic Characteristics and Biomarker Levels of the Study Cohorts by Clinical and Biomarker-Defined Groups[a]

| Characteristic | TRIAD cohort (n = 300) | | | | | | | ALFA+ cohort (n = 384) | | | BioCogBank Paris Lariboisière cohort (n = 187) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (SD) | | | | | | | Mean (SD) | | | Mean (SD) | | | | |
| | Young CU (n = 35) | CU− (n = 114) | CU+ (n = 42) | MCI+ (n = 39) | AD dementia (n = 45) | Non-AD (n = 25)[b] | *P* value | CU− (n = 249)[c] | CU+ (n = 135)[d] | *P* value | CU− (n = 21) | MCI+ (n = 42) | AD dementia (n = 76) | Non-AD (n = 48)[e] | *P* value |
| Age, y | 23.1 (1.8) | 69.9 (9.4) | 74.1 (7.7) | 71.2 (7.7) | 66.1 (9.7) | 70.8 (11.0) | <.001 | 60.5 (4.5) | 62.2 (4.9) | <.001 | 64.4 (9.5) | 72.4 (7.9) | 72.2 (8.4) | 66.6 (9.7) | .001 |
| Female, No. (%) | 22 (62.9) | 73 (64.0) | 29 (69.0) | 21 (53.8) | 21 (46.7) | 11 (44.0) | .12 | 153 (61.4) | 81 (60.0) | .87 | 14 (66.7) | 26 (61.9) | 47 (61.8) | 29 (60.4) | .97 |
| Educational level, y | 16.6 (1.5) | 15.6 (3.9) | 14.8 (3.2) | 15.2 (3.2) | 14.6 (3.6) | 13.8 (3.9) | .02 | 13.6 (3.5) | 13.3 (3.6) | .49 | 11.2 (1.6) | 10.7 (1.8) | 9.7 (2.0) | 10.7 (1.9) | .004 |
| *APOE* ε4 carriers, No. (%) | 8 (22.9) | 29 (26.9) | 12 (28.6) | 23 (62.2) | 24 (55.8) | 5 (22.7) | <.001 | 106 (42.6) | 103 (76.3) | <.001 | 6 (28.6) | 24 (57.1) | 49 (64.5) | 7 (14.6) | <.001 |
| MMSE score | 30 (0) | 29 (1.0) | 29 (1.0) | 28 (2.0) | 19 (6.0) | 27 (2.0) | <.001 | 29.1 (0.9) | 29.1 (1.0) | .93 | 27.4 (2.5) | 23.5 (4.4) | 19.3 (5.6) | 24.6 (3.7) | <.001 |
| Centiloids | −11.6 (6.6) | −3.12 (8.6) | 52.5 (31.2) | 91.1 (36.0) | 91.8 (40.0) | 1.10 (12.3) | <.001 | −4.54 (6.6) | 16.8 (21.1) | <.001 | NA | NA | NA | NA | NA |
| **CSF biomarkers, pg/mL** | | | | | | | | | | | | | | | |
| Aβ42/40 | 0.091 (0.006) | 0.087 (0.017) | 0.055 (0.015) | 0.043 (0.010) | 0.045 (0.011) | 0.082 (0.026) | <.001 | 0.087 (0.009) | 0.051 (0.012) | <.001 | 0.095 (0.007) | 0.044 (0.009) | 0.042 (0.009) | 0.089 (0.012) | <.001 |
| p-tau181 | 22.6 (7.1) | 36.2 (14.4) | 59.3 (35.2) | 89.4 (34.6) | 99.9 (55.8) | 59.7 (63.5) | <.001 | 13.9 (4.2) | 18.4 (7.2) | <.001 | 32.8 (8.6) | 93.0 (46.9) | 115.4 (59.3) | 37.7 (16.4) | <.001 |
| t-tau | 195.3 (48.1) | 311.0 (126.8) | 396.4 (197.0) | 539.4 (210.1) | 659.6 (331.7) | 448.4 (398.6) | .001 | 174.8 (48.0) | 222.6 (76.9) | <.001 | 243.1 (70.9) | 587.6 (280.3) | 732.6 (390.7) | 305.6 (148.6) | <.001 |
| NfL | 184.6 (57.7) | 1132.3 (1038.3) | 862.5 (268.7) | 1126.8 (257.7) | 1646.2 (965.0) | 1783.0 (1662.5) | .07 | 76.3 (23.6) | 89.2 (27.5) | <.001 | 889.3 (352.1) | 1532 (643.4) | 1695 (673.0) | 1456 (1214) | .03 |
| GFAP | 4134 (1483) | 12 506 (5148) | 15 669 (6771) | 17 114 (5890) | 16 314 (8513) | 14 074 (7497) | .02 | 4090 (2018) | 4859 (2333) | .01 | 2423 (2194) | 4189 (3313) | 4601 (3759) | 2872 (2356) | .14 |
| **Plasma biomarkers, pg/mL** | | | | | | | | | | | | | | | |
| NfL | 6.5 (2.7) | 22.1 (9.8) | 27.9 (24.8) | 25.7 (14.4) | 33.6 (13.5) | 28.6 (11.4) | <.001 | 9.8 (3.3) | 11.6 (4.2) | <.001 | 13.1 (6.8) | 24.2 (10.4) | 24.4 (8.7) | 21.2 (16.7) | .06 |
| p-tau181 | 7.9 (3.6) | 9.9 (4.4) | 14.8 (11.0) | 18.1 (8.1) | 24.1 (9.6) | 11.8 (12.3) | <.001 | 8.8 (3.2) | 11.0 (4.6) | <.001 | 3.0 (1.8) | 11.5 (6.2) | 12.8 (3.6) | 9.5 (6.7) | <.001 |
| GFAP | 95.1 (62.1) | 185.1 (93.5) | 285.0 (142.6) | 332.5 (153.6) | 388.1 (152.8) | 188.9 (105.9) | <.001 | 121.9 (42.4) | 169.9 (78.5) | <.001 | 161.2 (67.1) | 368.6 (158.5) | 376.4 (179.6) | 185.0 (96.0) | <.001 |

Abbreviations: Aβ, amyloid-β; AD, Alzheimer disease; ALFA, Alzheimer's and Families; CSF, cerebrospinal fluid; CU−, Aβ-negative cognitively unimpaired; CU+, Aβ-positive cognitively unimpaired; GFAP, glial fibrillary acidic protein, MCI+, Aβ-positive mild cognitive impairment; MMSE, Mini-Mental State Examination; NA, not available; NfL, neurofilament light chain; p-tau181, tau phosphorylated at threonine 181; t-tau, total tau; TRIAD, Translational Biomarkers in Aging and Dementia.

[a] Within each cohort, we used *t* test or 1-way analysis of variance to compare age, educational level, and MMSE between groups and Pearson $\chi^2$ to compare sex and *APOE* ε4 frequencies between groups. Centiloids and fluid biomarker levels were compared with a 1-way analysis of covariance adjusted by age and sex and followed by false discovery rate multiple comparison correction. Aβ status for group definition was based on positron emission tomography visual result in the TRIAD cohort and on CSF Aβ42/40 for the ALFA+ and BioCogBank Paris Lariboisière cohorts.

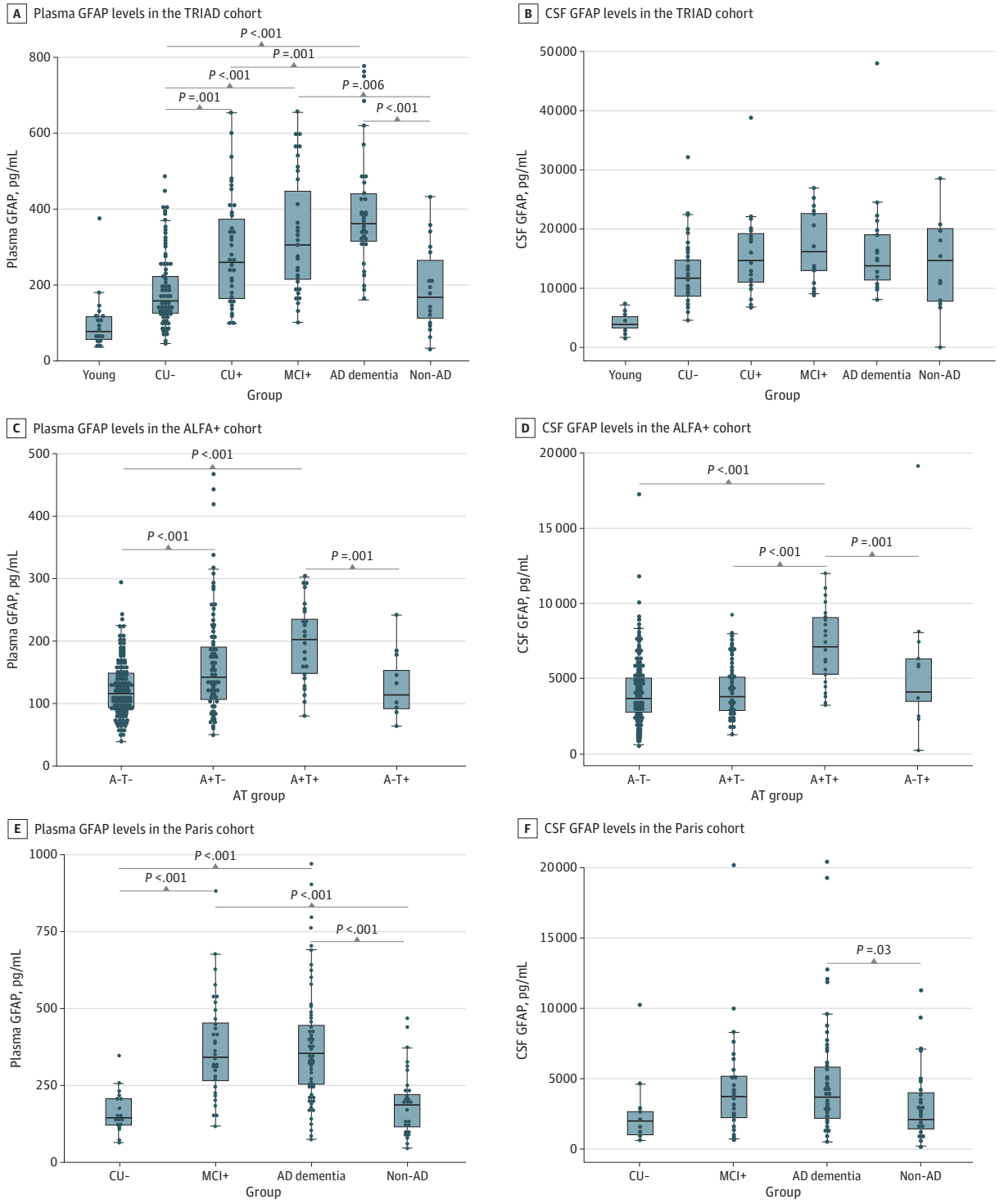
[b] Among the non-AD group, there were 21 individuals with MCI with a negative Aβ positron emission tomography visual result and 4 participants with a clinical diagnosis of AD dementia syndrome with a negative Aβ positron emission tomography visual result.

[c] A total of 68 of 248 participants (27.4%) had subjective cognitive decline.

[d] A total of 39 of 135 participants (28.9%) had subjective cognitive decline.

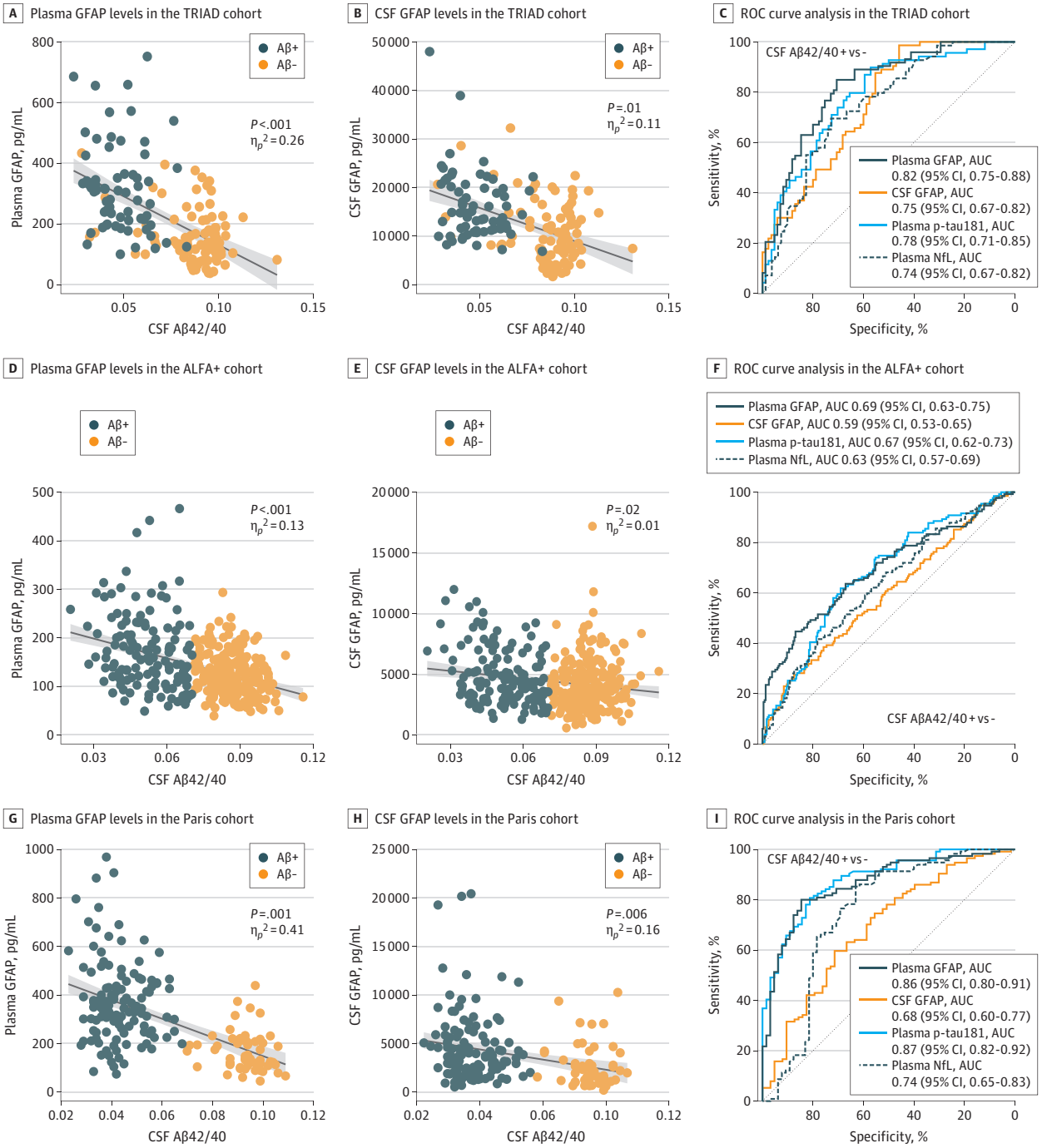
[e] In the non-AD group all participants had MCI with normal CSF Aβ42/40 levels.

Figure 1. Plasma and Cerebrospinal Fluid (CSF) Glial Fibrillary Acidic Protein (GFAP) Group Comparisons




Box plots depict median (horizontal bar), IQR (hinges), and 1.5 × IQR (whiskers). Group comparisons were computed with a 1-way analysis of covariance adjusting for age and sex. The Tukey honestly significant difference test was used for post hoc pairwise comparisons in all cohorts. Fold changes are depicted for the Alzheimer disease (AD) continuum groups and were calculated using amyloid-β (Aβ)–negative cognitively unimpaired (CU−) individuals (Translational Biomarkers in Aging and Dementia [TRIAD] and BioCogBank Paris Lariboisière [Paris] cohorts) or Aβ-negative and tau-negative (A−T−) individuals (Alzheimer's and Families [ALFA+] cohort) as the reference group. Aβ status was defined by Aβ positron emission tomography in the TRIAD cohort and CSF Aβ42/40 ratio in the ALFA+ and Paris cohorts. The non-AD group included 21 individuals with Aβ-negative mild cognitive impairment (MCI), 4 individuals with Aβ-negative AD dementia syndrome in the TRIAD cohort, and 48 individuals with MCI− in the Paris cohort. A+T− indicates Aβ-positive and tau-negative; A+T+, Aβ-positive and tau-positive; A−T+, Aβ-negative and tau-positive; CU+, Aβ-positive cognitively unimpaired; MCI+, Aβ-positive MCI.

Figure 2. Associations of Plasma and Cerebrospinal Fluid Glial Fibrillary Acidic Protein Levels With Aβ Pathology and Discriminative Accuracy




Individuals are color coded by amyloid-β (Aβ) status (as defined by Aβ positron emission tomography in the Translational Biomarkers in Aging and Dementia [TRIAD] cohort and cerebrospinal fluid (CSF) Aβ42/40 ratio in the Alzheimer's and Families [ALFA+] and BioCogBank Paris Lariboisière [Paris] cohorts). Solid lines indicate the regression line and 95% CIs. *P* values were computed with linear models adjusted by age, sex, and clinical diagnosis (the latter only for the TRIAD and Paris cohorts). Sizes of the associations between variables are shown by the partial $\eta^2$ ($\eta_p^2$). For comparative purposes, we also included plasma tau phosphorylated at threonine 181 (p-tau181) and plasma neurofilament light chain (NfL) in these analyses. AUC indicates area under the curve; GFAP, glial fibrillary acidic protein; ROC, receiver operating characteristic.

associated with Aβ PET (plasma GFAP, $P < .001$; $\eta_p^2 = 0.32$; CSF GFAP, $P < .001$; $\eta_p^2 = 0.10$; eFigure 5A and 5B in Supplement 1). The sizes of the associations of Aβ pathology (either CSF Aβ42/40 or Aβ PET) with plasma GFAP levels were larger than those with CSF GFAP levels. We performed the same analyses within the CU individuals, and plasma GFAP levels

Table 2. ROC Curve Analyses to Discriminate Aβ-Positive From Aβ-Negative Individuals

| | Aβ+ vs Aβ−, AUC (95% CI)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | CSF Aβ42/40 | | | Aβ PET | | | |
| | | | | Visual result | | Centiloid cutoff | |
| Biomarker | TRIAD cohort | ALFA+ cohort | BioCogBank Paris Lariboisière cohort | TRIAD cohort | ALFA+ cohort | TRIAD cohort | ALFA+ cohort |
| GFAP | | | | | | | |
| Plasma | 0.82 (0.75-0.88) | 0.69 (0.63-0.75) | 0.86 (0.80-0.91) | 0.85 (0.79-0.91) | 0.75 (0.67-0.84) | 0.83 (0.77-0.89) | 0.82 (0.72-0.92) |
| CSF | 0.75 (0.67-0.82)[b] | 0.59 (0.53-0.65)[c] | 0.68 (0.60-0.77)[c] | 0.75 (0.69-0.82)[c] | 0.68 (0.59-0.77) | 0.75 (0.68-0.84)[d] | 0.76 (0.64-0.87) |
| Other plasma biomarkers | | | | | | | |
| p-tau181 | 0.78 (0.71-0.85) | 0.67 (0.62-0.73)[e] | 0.87 (0.82-0.92)[e] | 0.77 (0.70-0.85) | 0.67 (0.58-0.76) | 0.79 (0.71-0.86) | 0.76 (0.67-0.86) |
| NfL | 0.74 (0.67-0.82) | 0.63 (0.57-0.69) | 0.74 (0.65-0.83)[c] | 0.67 (0.59-0.76)[c] | 0.66 (0.58-0.75) | 0.68 (0.59-0.76)[c] | 0.73 (0.63-0.83) |

Abbreviations: Aβ, amyloid-β; ALFA, Alzheimer's and Families; AUC, area under the curve; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; NfL, neurofilament light chain; p-tau181, tau phosphorylated at threonine 181; PET, positron emission tomography; ROC, receiver operating characteristic; TRIAD, Translational Biomarkers in Aging and Dementia.

[a] ROC curve analyses to test whether plasma GFAP discriminates between Aβ-positive (Aβ+) and Aβ-negative (Aβ−) individuals, as defined by the CSF Aβ42/40 ratio, Aβ PET visual result, or Aβ PET using a cutoff of 24 (TRIAD) or 30 (ALFA) centiloids. We also included CSF GFAP, plasma p-tau181, and plasma NfL for comparison. AUC differences were tested using the DeLong test followed by false discovery rate multiple comparison correction.

[b] $P = .06$ vs plasma GFAP (before correction for multiple comparisons).

[c] $P < .05$ vs plasma GFAP.

[d] $P = .03$ vs plasma GFAP (before correction for multiple comparisons).

[e] $P < .05$ vs CSF GFAP.

were significantly associated with both Aβ biomarkers (CSF Aβ42/40: $P = .008$; $\eta_p^2 = .07$; Aβ PET: $P < .001$; $\eta_p^2 = .06$). In contrast, CSF GFAP levels were not significantly associated with CSF Aβ42/40 ($P = .18$) or Aβ PET ($P = .07$) within the CU individuals.

In ALFA+, plasma GFAP levels were positively associated with Aβ pathology as shown by a significant negative association with CSF Aβ42/40 in the whole sample ($P < .001$; $\eta_p^2 = 0.13$) but also in the CU- group ($P = .002$; $\eta_p^2 = 0.04$) and CU+ group ($P = .03$; $\eta_p^2 = 0.04$) (Figure 2D). Levels of CSF GFAP also showed a negative association with CSF Aβ42/40 in the whole sample ($P = .02$; $\eta_p^2 = 0.01$; Figure 2E) and in the CU+ group ($P = .005$; $\eta_p^2 = 0.06$). Conversely, a positive association between CSF GFAP levels and CSF Aβ42/40 was observed in CU- participants ($P = .02$; $\eta_p^2 = 0.02$). Both plasma and CSF GFAP levels were associated with Aβ deposition as quantified by Aβ PET (eFigure 5C and D in Supplement 1) in the whole sample (plasma GFAP, $P < .001$; $\eta_p^2 = 0.10$; CSF GFAP, $P = .001$; $\eta_p^2 = 0.04$).

The same analysis was repeated in the Paris cohort, and the size of the association of CSF Aβ42/40 with plasma GFAP levels (plasma, $P < .001$; $\eta_p^2 = 0.41$) was greater than that with CSF GFAP levels (CSF, $P = .006$; $\eta_p^2 = 0.16$; Figure 2G and H).

We next investigated how plasma and CSF GFAP levels discriminate Aβ status using ROC analysis (**Table 2** and Figure 2). Aβ statuses were defined by CSF Aβ42/40, Aβ PET visual read, or the Aβ PET centiloids cutoffs used in each cohort (Table 2). In the entire TRIAD cohort, plasma GFAP as a biomarker accurately discriminated Aβ+ from Aβ− individuals, with an AUC ranging from 0.82 to 0.85. In contrast, CSF GFAP as a biomarker had an AUC of 0.75. In CU individuals, plasma GFAP as a biomarker distinguished Aβ status with an AUC of 0.75 to 0.79, whereas CSF GFAP as a biomarker had AUCs of 0.74 to 0.76. In ALFA+, plasma GFAP as a biomarker discriminated with an AUC of 0.69 to 0.82, while for CSF GFAP as a biomarker, AUCs were 0.59 to 0.76. In the Paris cohort, plasma GFAP as a biomarker accurately differentiated CSF Aβ42/40 status with an AUC of 0.86, while CSF GFAP as a biomarker had an AUC of 0.68. In addition, ROCs were performed contrasting CU- individuals with those with MCI+, individuals with Aβ- MCI (MCI-) with those with MCI+, and CU- individuals with those with AD (eTable 2 in Supplement 1). For comparison purposes, we also performed ROC analyses with plasma tau phosphorylated at threonine 181 (p-tau181) and neurofilament light chain (NfL), and none of them performed better than plasma GFAP.

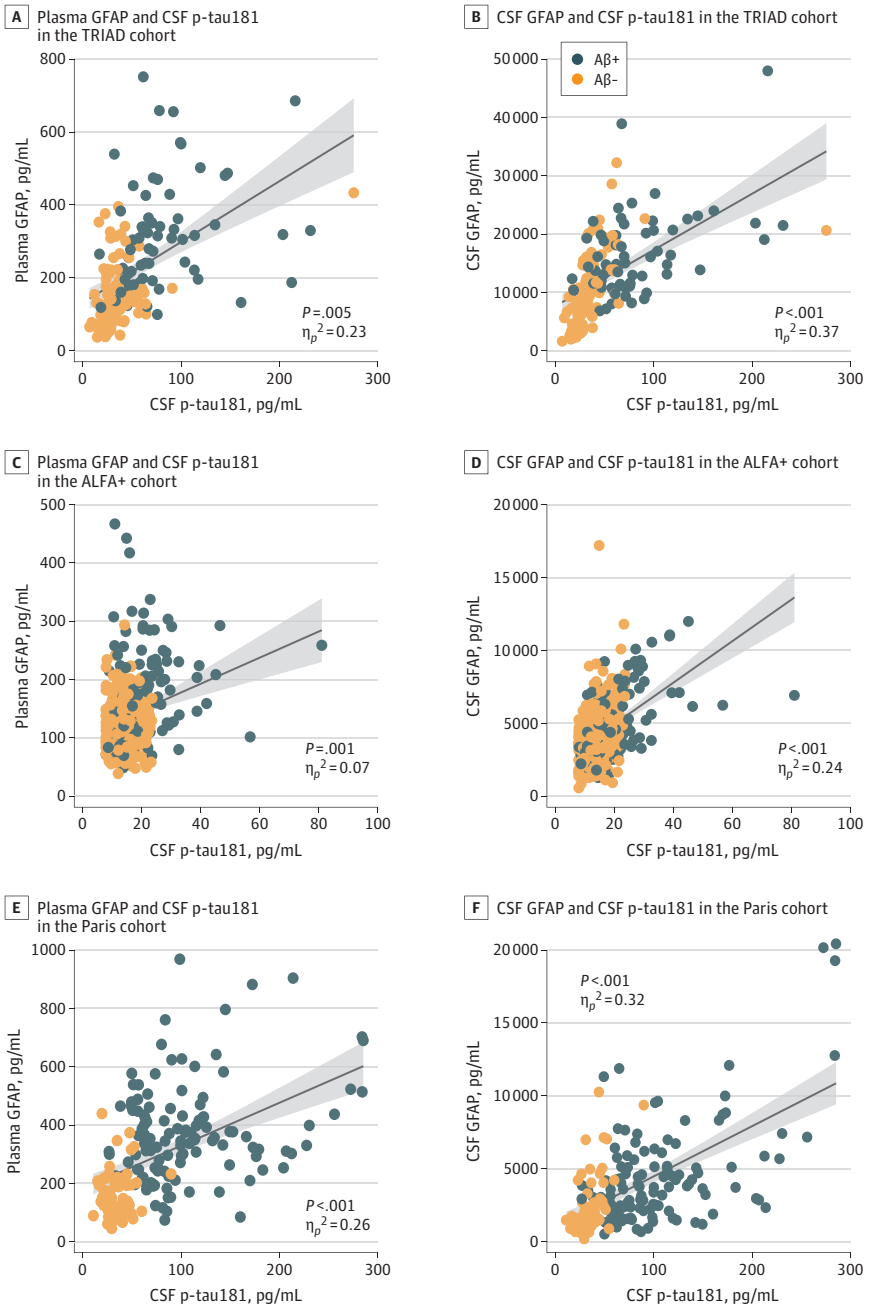
We also performed analyses comparing different combinations of plasma biomarkers (eTable 3 in Supplement 1). We found that adding plasma GFAP to any of the other plasma biomarkers (either p-tau181 or NfL) was associated with improved accuracy to discriminate Aβ status (as measured by CSF Aβ42/40) in the 3 cohorts.

## Association of Plasma GFAP Levels With Tau Pathology Among Individuals With Concomitant Aβ Pathology

We evaluated the associations between GFAP levels and tau biomarkers (CSF p-tau181 and tau PET). In TRIAD, higher plasma and CSF GFAP levels were associated with increased tau PET burden (plasma GFAP, $P < .001$; $\eta_p^2 = 0.29$; CSF GFAP, $P = .005$; $\eta_p^2 = 0.08$; eFigure 6A and B in Supplement 1). Both plasma and CSF GFAP levels were significantly associated with CSF p-tau181 levels in the 3 cohorts (**Figure 3**A-F).

We conducted a mediation analysis to assess whether the associations between GFAP levels and tau biomarkers were mediated by Aβ status. Results in TRIAD indicated that the association of plasma GFAP levels with tau was mediated by Aβ (eFigure 7A in Supplement 1), with a significant indirect association corresponding to 60% of the total association of tau with plasma GFAP levels. These findings were replicated using PET biomarkers (eFigure 7A in Supplement 1). A similar analysis was performed with CSF GFAP levels as the response variable, and tau had both a direct and an indirect association with CSF GFAP levels.

Figure 3. Association of Plasma and Cerebrospinal Fluid (CSF) Glial Fibrillary Acidic Protein (GFAP) Levels With Tau Phosphorylated at Threonine 181 (p-tau181)




A, Association of plasma GFAP with CSF p-tau181 in the Translational Biomarkers in Aging and Dementia (TRIAD) cohort. B, Association of CSF GFAP with CSF p-tau181 in the TRIAD cohort. C, Association of plasma GFAP with CSF p-tau181 in the Alzheimer's and Families (ALFA+) cohort. D, Association of CSF GFAP with CSF p-tau181 in the ALFA+ cohort. E, Association of plasma GFAP with CSF p-tau181 in the BioCogBank Paris Lariboisière (Paris) cohort. F, Association of CSF GFAP with CSF p-tau181 in the Paris cohort. Individuals are colored by amyloid-β (Aβ) status (as defined by Aβ positron emission tomography in the TRIAD cohort or CSF Aβ42/40 in the ALFA+ and Paris cohorts). The solid lines indicate the regression line and the 95% CIs. *P* values were computed with linear models adjusted by age, sex, and clinical diagnosis (the latter only for the TRIAD and Paris cohorts). The sizes of the associations between variables are shown by the partial $\eta^2$ ($\eta_p^2$).

Results were consistent across cohorts (eFigure 7B and C in Supplement 1). In the ALFA+ and Paris cohorts, the association of CSF p-tau181 with plasma GFAP levels was mediated by CSF Aβ42/40, with a significant indirect association corresponding to 62% and 63% of the total association of CSF p-tau181 with plasma GFAP levels, respectively. Conversely, CSF p-tau181 did not show a significant indirect association with CSF GFAP levels, suggesting Aβ-independent effects.

## Association of CSF and Plasma GFAP Levels With Neuroinflammation

Finally, we explored how plasma and CSF GFAP levels are associated with other glial biomarkers. In TRIAD, levels of CSF GFAP, but not plasma GFAP, showed a positive association with CSF soluble triggering receptor expressed on myeloid cells 2 (sTREM2) and Chitinase-3-like protein 1 (YKL40) (TRIAD: plasma GFAP association with sTREM2, β [SE] = 0.11 [0.08]; *P* = .17; YKL40, β [SE] = 0.02 [0.06]; *P* = .67; CSF GFAP

association with sTREM2, β [SE] = 0.25 [0.09]; $P < .001$; YKL40, β [SE] = 0.32 [0.07]; $P < .001$) (eFigure 8A and B in Supplement 1). Similar results were observed in the ALFA+ and Paris cohorts (ALFA+: plasma GFAP association with sTREM2, β [SE] = 0.083 [0.086]; $P = .14$; YKL40, β [SE] = 0.075 [0.051]; $P = .14$; CSF GFAP association with sTREM2, β [SE] = 0.41 [0.048]; $P < .001$; YKL40, β [SE] = 0.40 [0.045]; $P < .001$; and Paris: plasma GFAP association with YKL40, β [SE] = 0.06 [0.09]; $P = .49$; CSF GFAP association with YKL40, β [SE] = 0.52 [0.12]; $P < .001$) (eFigure 8C-E in Supplement 1).

## Discussion

In this study, which includes 3 thoroughly characterized cohorts, we showed that plasma GFAP levels were significantly higher among individuals with preclinical AD and reached their higher levels at symptomatic stages of AD. The effect sizes of the increases of plasma GFAP levels were always larger than those of CSF GFAP levels. Therefore, plasma GFAP levels appear to be a superior biomarker tracking Aβ pathology than its CSF counterpart. This finding is particularly evident for individuals with preclinical AD; plasma GFAP levels were significantly higher in CU+ individuals and significantly discriminated them from CU- individuals, whereas CSF did not.

Previous studies showed that plasma and serum GFAP levels are higher in those with symptomatic AD,[9,24-27] results that are in line with those reported for CSF GFAP levels.[24,28-31] However, less is known about plasma GFAP levels among individuals along the whole AD continuum and, particularly, in those with preclinical AD. A recent study demonstrated that plasma GFAP levels were higher in a group of 33 CU+ individuals compared with 63 CU- individuals (AUC = 0.795).[16] Preceding studies showed that plasma GFAP levels were associated with both clinical diagnosis and Aβ status.[25] Another study revealed a quadratic (inverted U-shape) association between plasma GFAP levels and Aβ deposition.[26] To our knowledge, no other studies investigated the whole AD continuum or included participants with preclinical AD, and no other studies compared plasma and CSF compartments in the same individuals.

We also analyzed the association of plasma GFAP levels with Aβ pathology (either CSF Aβ42/40 ratio or Aβ PET), and we found a positive association between plasma GFAP levels and Aβ pathology in all cohorts and high rates of accuracy to discriminate Aβ+ from Aβ- individuals (AUC = 0.82-0.86). It was also apparent when assessing the whole AD continuum that plasma GFAP levels were higher in individuals with a more advanced clinical diagnosis (CU+ less than MCI+, which was less than AD dementia). In contrast, CSF GFAP levels showed no significant difference across the AD continuum groups. Consistent with this finding, we observed a significant association between plasma GFAP levels and tau PET findings.

We included many individuals with preclinical AD: 42 in TRIAD and 135 in ALFA+. Plasma GFAP discriminated CU+ individuals from CU- individuals with an AUC of 0.75 to 0.79 in TRIAD, similar to the AUC of 0.795 previously described.[16] Furthermore, in ALFA+, we studied the earliest phase of preclinical AD. We assessed 104 individuals who were A+T- (ie, had Aβ pathology but not yet tau pathology) and 89 individuals with a low Aβ burden (ie, they had decreased CSF Aβ42/40 but not yet a positive Aβ PET result). Both groups had significantly higher plasma GFAP levels but not CSF GFAP levels, reinforcing the idea that plasma GFAP may be an early biomarker of AD pathologic changes. Levels of CSF GFAP only become significantly higher in the A+T+ group when there is biomarker evidence of both Aβ and tau pathology. Data from cellular models indicate that astrocytes react to early preplaque-insoluble Aβ oligomeric species.[32] Our results can be contextualized with findings using other fluid or imaging biomarkers of reactive astrogliosis. Studies using the PET tracer $^{11}$C-deuterium-L-deprenyl ([$^{11}$C]DED), which binds to monoamine oxidase-B, mainly expressed in reactive astrocytes, support fluctuations during the AD continuum in reactive astrocyte states. More specifically, [$^{11}$C]DED binding in the frontal and parietal cortices is significantly increased in those with prodromal AD compared with CU individuals.[33] Early increases in [$^{11}$C]DED binding have also been found in autosomal carriers of a dominant AD variation almost 30 years before the emergence of symptoms.[34] In a transgenic mouse model that overexpresses the human *APPswe* variation, increased [$^{11}$C]DED binding precedes detectable Aβ pathology.[35] Moreover, CSF YKL40, a biomarker of a subset of reactive astrocytes, is also elevated in those with preclinical AD.[36,37] Recently, a model of reactive astrogliosis in the AD continuum[15] has been proposed that would encompass early reactive astrocytes in the preclinical stage (supported by in vivo evidence of higher monoamine oxidase-B expression), followed by more widespread reactivity (supported by increases in CSF YKL40, GFAP, and S100b) and, finally, the end-stage reactive astrocytes, in which their physiological function may be lost. Our findings situate plasma GFAP levels as a marker of early reactive astrocytes.

Our results point to plasma GFAP as a possible biomarker specific for Aβ pathology. First, plasma GFAP levels were not higher among individuals with non-AD neurodegenerative diseases in the TRIAD and Paris cohorts. Plasma GFAP levels were normal in those with FTD despite gliosis being a characteristic of FTD.[38,39] Second, in ALFA+, the A-T+ group did not have high plasma GFAP levels; this finding may suggest that plasma GFAP levels specifically reflect Aβ pathology in preclinical stages, but a direct comparison with the preclinical stage of other neurodegenerative diseases should be performed. Third, the association between plasma GFAP levels and tau pathology was mediated by Aβ pathology. These results are consistent with the increased expression of GFAP surrounding Aβ plaques.[40-43] Although CSF GFAP levels were associated with other glial biomarkers (YKL40 and sTREM2), plasma GFAP levels were not. It is possible that CSF GFAP better reflects reactive astrocytes in response to neuroinflammatory changes, such as microglial activation, while plasma GFAP is more closely associated with reactive astrogliosis because of Aβ burden. High levels of blood GFAP can be found in individuals with other neurodegenerative diseases,[24,44,45] but this finding occurs at the symptomatic, and thus advanced, stages of the disease. The increase in blood GFAP levels after acute brain conditions, such as subarachnoid hemorrhage and traumatic and hypoxic brain

injury, has been extensively documented,[46-50] but this increase may come through other mechanisms, such as a trauma-induced temporary opening of the blood-brain barrier. Based on these findings, it would seem that GFAP responds to acute neuronal injury; however, in a chronic neurodegenerative disease, and unlike NfL, plasma GFAP may principally (but not exclusively) reflect Aβ pathology.

A unique feature of our study is that we measured both plasma and CSF GFAP levels in the same participants. This feature allowed us to draw one of the main conclusions of this study, namely, that differences in plasma GFAP levels are larger than those of CSF GFAP levels between the groups, and the effect sizes of the associations between plasma GFAP levels and biomarkers of Aβ are greater than those of CSF GFAP levels. Moreover, the AUCs to discriminate Aβ status are higher for plasma GFAP than CSF GFAP, especially when Aβ pathology is defined by CSF Aβ42/40, suggesting an early increase of plasma GFAP levels. This result is surprising because neurologically associated blood biomarkers have usually been considered a proxy of the CSF biomarkers. A possible explanation of why plasma GFAP outperforms CSF GFAP would be the different clearance mechanisms into the biofluids. Astrocytes are part of the neurovascular unit and the blood-brain barrier, which is altered in individuals with AD.[51] Astrocytic end-feet cover brain capillaries, which may be a direct route for the release of GFAP from reactive astrocytes to the bloodstream.[52] It could be speculated that blood-brain barrier dysfunction facilitates the release of GFAP into the bloodstream; this may also explain the elevations of plasma GFAP in individuals with acute neurologic injuries. Astrocytes are also part of the glymphatic system, which is a highly organized system that clears the brain of insoluble proteins and metabolites by draining them into the venous system.[53] GFAP may also reach the bloodstream via the meningeal lymphatic system.[54] Finally, preanalytical and analytical factors that need to be further studied may also account for these differences. A previous study described that plasma GFAP is very stable to freeze-thaw cycles,[55] whereas CSF GFAP is far more sensitive over time.[56] The fact that plasma GFAP has a wider range of values than CSF GFAP may also be associated with the higher accuracy of the former.

It remains unanswered which plasma biomarker (GFAP, Aβ42/40, or forms of p-tau) is more accurately associated with Aβ pathology in particular in the preclinical stage. A head-to-head comparison of these biomarkers in several independent cohorts is needed. However, GFAP is an additional tool that has shown consistent results across multiple cohorts and is easily detectable using commercially available immunoassays. Moreover, we show that adding plasma GFAP to models with other plasma biomarkers (p-tau181 and/or NfL) improves their accuracy. All of these biomarkers perform satisfactorily, but a combination of some will probably render the highest accuracy for Aβ pathology. This is particularly true in preclinical AD, when the individual increases of these biomarkers may be statistically significant, but the effect sizes of these increases are not large.

### Limitations

This study has some limitations. It is a cross-sectional study, and findings need to be confirmed with longitudinal data. The 3 cohorts have differences in the design and goals, and not all of them had the same data available. Also, the definitions of Aβ pathology differed between cohorts, which may limit comparability between them; however, the fact that the main results are validated in diverse studies confirms the robustness of our results. Finally, we did not include measurements of Aβ in blood.

## Conclusions

Altogether, these results suggest that high plasma GFAP levels are found early in the AD continuum and become greater during disease progression, in parallel with clinical syndrome severity and markers of tau pathology. Our findings have important implications in facilitating the detection of AD, particularly in its preclinical stage. This earlier detection may accelerate primary and secondary prevention trials and the design of interventional studies at early stages of AD. Plasma GFAP, alone or in combination with other biomarkers, could be used to screen for Aβ+ individuals at any stage across the AD continuum.

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ORIGINAL PAPER

# Galectin-3 is elevated in CSF and is associated with Aβ deposits and tau aggregates in brain tissue in Alzheimer's disease

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**Abstract**

Galectin-3 (Gal-3) is a beta-galactosidase binding protein involved in microglial activation in the central nervous system (CNS). We previously demonstrated the crucial deleterious role of Gal-3 in microglial activation in Alzheimer's disease (AD). Under AD conditions, Gal-3 is primarily expressed by microglial cells clustered around Aβ plaques in both human and mouse brain, and knocking out Gal-3 reduces AD pathology in AD-model mice. To further unravel the importance of Gal-3-associated inflammation in AD, we aimed to investigate the Gal-3 inflammatory response in the AD continuum. First, we measured Gal-3 levels in neocortical and hippocampal tissue from early-onset AD patients, including genetic and sporadic cases. We found that Gal-3 levels were significantly higher in both cortex and hippocampus in AD subjects. Immunohistochemistry revealed that Gal-3+ microglial cells were associated with amyloid plaques of a larger size and more irregular shape and with neurons containing tau-inclusions. We then analyzed the levels of Gal-3 in cerebrospinal fluid (CSF) from AD patients ($n=119$) compared to control individuals ($n=36$). CSF Gal-3 levels were elevated in AD patients compared to controls and more strongly correlated with tau (p-Tau181 and t-tau) and synaptic markers (GAP-43 and neurogranin) than with amyloid-β. Lastly, principal component analysis (PCA) of AD biomarkers revealed that CSF Gal-3 clustered and associated with other CSF neuroinflammatory markers, including sTREM-2, GFAP, and YKL-40. This neuroinflammatory component was more highly expressed in the CSF from amyloid-β positive (A+), CSF p-Tau181 positive (T+), and biomarker neurodegeneration positive/negative (N+/−) (A + T + N+/−) groups compared to the A + T−N− group. Overall, Gal-3 stands out as a key pathological biomarker of AD pathology that is measurable in CSF and, therefore, a potential target for disease-modifying therapies involving the neuroinflammatory response.

## Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the formation of amyloid-β (Aβ) deposits and intraneuronal tau aggregates called neurofibrillary tangles. Recent transcriptomic data highlight a critical role of the innate immune system in AD pathology [28–30]. Genome-wide association studies have also identified innate immunity-related variants in genes such as *TREM-2*, *CD33*, *CR1* and *MEF2C* [14, 15, 20, 56] that are associated with a significant risk of developing AD. Microglial cells are central nervous system (CNS) resident macrophages. They play diverse roles, including brain parenchyma surveillance, phagocytosis, synaptic remodeling, and inflammatory response [11]. Indeed, reactive microglial cells surrounding Aβ plaques are a pathological hallmark of AD. A specific AD microglial phenotype was recently discovered, the so-called disease-associated microglia (DAM) [2]. DAM is characterized by the upregulation of *TREM-2*, *ApoE*, *Spp1*, *Itgax* and *Axl* and seems to play a beneficial role in AD [29]. On the other hand, a more detrimental microglial subtype

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has recently been characterized, the so-called microglia neurodegenerative (MGnD) [30]. This phenotype is associated, among others, with *Cecl7a*, *Lgals3*, *TREM2* and *Ccl2* gene upregulation.

Galectin-3 (Gal-3) is a beta-galactosidase binding protein encoded by *LGALS3* and is mainly expressed by microglial cells in the CNS. Gal-3 consists of one N-terminal domain and one carbohydrate recognition domain [48] and is released into the extracellular space by activated microglia. Once in the extracellular space, Gal-3 can act autocrine or paracrine by binding to different membrane receptors, such as TLR4 and TREM-2 [8, 10]. Previously, we demonstrated that Gal-3 plays a detrimental role in microglial activation in AD [8]. First, we found that Gal-3 was highly upregulated in cortical tissue from AD patients compared to age-matched controls. There, Gal-3 was found primarily in microglia clustered around Aβ plaques. Next, we generated a mouse model based on the 5xFAD model of AD but lacking Gal-3. The lack of Gal-3 in 5xFAD mice lessened the Aβ burden and improved cognitive performance [8]. Our study also confirmed that Gal-3 was linked to TREM-2 activity by employing STORM microscopy, fluorescence anisotropy, and a TREM-2–DAP12 reporter cell line. The role of Gal-3 in AD was later supported by Chih-Chieh Tao et al. [53], who found that Gal-3 is involved in disease progression and Aβ oligomerization using amyloid precursor protein (APP) transgenic mice lacking Gal-3.

Further, a recent large-scale proteomic analysis using human AD brain tissue (> 2000 brains) highlighted a microglia module as one of the most affected processes in AD brain. An astrocytic/microglial metabolism module was significantly enriched in gene products connected to AD risk factor loci [28]. The study pointed out the top 30 most differentially abundant microglial transcripts in an AD mouse model that correspond with proteins in the human microglia module related to AD pathology. Remarkably, within this list, Gal3 ranked fifth, supporting its role as one of the key molecules related to AD pathology [28].

Cerebrospinal fluid (CSF) plays a key role in brain metabolism and can be used to measure the concentration of pathological hallmarks related to AD pathology progression [4, 25, 46]. So far, measures of the Aβ42/Aβ40 ratio, total tau (t-tau) and hyperphosphorylated tau (p-Tau) are the most reliable markers for disease diagnosis. As the AD process initiates, CSF Aβ42 levels drop and the levels of different isoforms of tau increase, which is associated with neurodegeneration [4]. Similarly, inflammatory molecules can be detected in the CSF of AD patients [37]. For instance, markers of astrocytic activation, namely GFAP and YKL-40, are elevated in the CSF of AD patients and linked to pathology progression [2, 5, 24]. A key microglial marker, TREM-2, has also been noted as being elevated in AD patient CSF [18, 51] and has recently been linked to slower Aβ deposition [17], reduced cognitive decline [18] and tau-related neurodegeneration [52]. Interestingly, Gal-3 has been detected in the CSF of AD patients and its measures suggest that it could be elevated in AD [21, 59].

Considering our previous findings on the role of Gal-3 in AD progression and the interaction between Gal-3 and TREM-2, we: (i) further explored Gal-3 expression in AD brain and (ii) explored whether CSF Gal-3 levels correlate with levels of Aβ, inflammatory markers, including TREM-2, and neurodegenerative biomarkers in CSF in AD. First, we studied Gal-3 levels in frontal cortex and hippocampal tissue from genetic and sporadic AD cases from the Neurological Tissue Bank of the Hospital Clinic de Barcelona-IDIBAPS. We wanted to expand our previous findings by measuring Gal-3 levels in different brain areas and different AD phenotypes. We could determine whether AD-related genetic mutations alter Gal-3 microglial expression in AD by comparing genetic cases with sporadic cases. To further elucidate the role of Gal-3-dependent microglial activation in AD, we performed immunohistochemistry on human brain sections from AD patients. We also analyzed the association between Gal-3, amyloid plaques and tau neurofibrillary tangles.

The second main goal of this study was to determine whether Gal-3 in CSF could be an AD biomarker. Therefore, we studied CSF Gal-3 levels in patients with evidence of AD physiopathology compared to control subjects. In CSF, we evaluated the association between Gal-3 and the main pathological hallmarks of the disease, Aβ and Tau. Moreover, we studied the relationship between levels of Gal-3 and two synaptic markers, Neurogranin and GAP-43, in addition to inflammatory markers, including GFAP and TREM-2. In addition, we evaluated whether Gal-3 levels were linked to cognitive decline in AD. Lastly, principal component analysis (PCA) was performed to determine whether we could detect distinct populations in the cohort defined by biomarker composition, i.e., inflammation vs. classic AD biomarkers.

## Materials and methods

### CSF cohort

A total of 155 participants were recruited from the Cognitive Neurology Center, APHP Université de Paris Lariboisière Fernand-Widal Hospital in Paris, France, including $n = 36$ neurological controls (NC) and $n = 119$ AD patients. All patients who had undergone a lumbar puncture to explore a cognitive complaint had a CSF sample collected at the BioCogBank Lariboisière Paris. Patients underwent a comprehensive neurological examination, neuropsychological assessment, morphological brain imaging and lumbar

puncture. AD diagnosis was made according to the NIA-AAA's most recent diagnostic research criteria [25]. CSF profile was classified according to the ATN classification: A+/− indicating biomarker evidence of Aβ deposition; T ± indicating biomarker evidence of pathologic tau; and N+/− indicating biomarker evidence of neurodegeneration [26]. CSF biomarker profiles of AD patients fell onto a continuum defined by decreasing $A\beta_{42/40}$ ratio. NC had normative or sub-normative cognitive scores, normal brain morphology and a normal CSF biomarker profile (A−T−N−). Included subjects gave informed consent to participate, and the study was approved by the Bichat Ethics Committee (*n*°10-037 18/03/2010) and followed the principles of the Declaration of Helsinki. Demographic data from the CSF cohorts is described in Table 1.

## Human brain tissue

Frozen hippocampal and cerebral cortical tissue from non-demented controls ($n=10$), EOAD cases ($n=11$) and genetic AD cases ($n=9$) (Neurological Tissue Bank, Biobanc-Hospital Clínic-IDIBAPS, Barcelona, Spain) and sections of formalin-fixed and glucose-immersed hippocampal tissue from non-demented controls ($n=3$) and AD cases ($n=8$) (The Netherlands Brain Bank) were analyzed. Detailed neurological assessment of the samples is available in Supp. Table 1 (online resources). Written informed consent for the use of brain tissue and clinical data for research purposes was obtained from all patients or their next of kin following the International Declaration of Helsinki and Europe's Code of Conduct for Brain Banking. The medical ethics committee of VU medical center in Amsterdam and the IRB of Clinic Hospital in Barcelona approved the procedures for brain tissue collection. The regional ethical review board in Sweden approved the study.

**Table 1** Demographics and biomarkers values

| $N=155$ | Neurological controls $N=36$ | AD $N=119$ | *P* value |
|---|---|---|---|
| Female sex | 72% (26) | 60% (72) | 0.140 |
| Age | 62.7 (9.67) | 72.4 (8.0) | <0.001 |
| LoE | 12.48 (3.472) | 11.4 (3.6) | 0.060 |
| ApoE4 carriership | 25% (9) | 60% (72) | <0.001 |
| MMSE | 27. 0 (2.1) | 21.2 (5.5) | <0.001 |
| CSF markers | | | |
| Aβ42, pg/mL | 1059.3 (311.5) | 557.9 (299.1) | <0.001 |
| Aβ42/Aß40 ratio | 0.099 (0.034) | 0.044 (0.011) | <0.001 |
| CSF t-tau, pg/mL | 223.6 (65.9) | 667.7 (350.4) | <0.001 |
| CSF p-Tau, pg/mL | 32.9 (8.1) | 104.3 (55.8) | <0.001 |
| CSF GAL-3, pg/mL | 960.5 (177.4) | 1168.8 (332.2) | 0.037 |
| CSF sTREM-2, pg/mL | 3523.7 (1411.7) | 4582.0 (1924.4) | 0.124 |
| CSF YKL-40, pg/mL | 1625.5 (632.3) | 2392.9 (2230.4) | 0.034 |
| CSF GFAP, pg/mL | 1913.3 (1247.3) | 4633.0 (3660.2) | <0.001 |
| CSF neurogranin, pg/mL | 144.9 (73.4) | 259.2 (82.4) | <0.001 |
| CSF GAP-43, pg/mL | 2416.4 (706.2) | 4306.9 (1880.1) | <0.001 |

## Protein extraction

RIPA solution was prepared with a protease inhibitor (cOmplete Protease Inhibitor Cocktail, Roche) and phosphatase inhibitor (PhoSStop, Roche). Frozen human tissue samples of hippocampus and cerebral cortex were homogenized in RIPA buffer (1 mL/100 µg of tissue, Sigma-Aldrich, Germany) and briefly sonicated. The pellet was subsequently ultracentrifuged at 25,000*g* for 25 min. The supernatant was later used for analysis.

## Western blotting

Protein extracts in RIPA were separated by SDS-PAGE using pre-cast gels (4–20%, Bio-Rad) in Tris–Glycine-SDS buffer (Bio-Rad, Spain). Protein was transferred to nitrocellulose membranes (Bio-Rad) using the TransBlot Turbo system from Bio-Rad. Membranes were subsequently blocked for 1 h with skim milk at 5% (w/v) in PBS and then washed three times for 10 min each in PBS supplemented with 0.1% (v/v) Tween 20 (PBS-T). Membranes were then incubated with primary antibodies in PBS-T overnight. Following this, the membranes were incubated with secondary antibodies for 2 h. After the secondary antibody incubation, membranes were washed three times with PBS-T. According to the manufacturer's protocol, the membranes were developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoScientific, Spain) and imaged using a ImageQuant LAS-4000 biomolecular imager (GE Healthcare).

## MSD ELISA

Meso Scale Discovery (MSD) kits were used to measure the levels of Aβ42, p-Tau, and t-tau in the RIPA fraction of the human brain. Serial dilutions of the RIPA fractions were tested to measure protein levels accurately. Before performing the assay, protein levels were measured using a Thermofisher BCA Assay Kit. With those results, 1 µg of protein from the soluble fraction was diluted to evaluate Aβ42, t-tau and p-Tau levels. The plates were developed and read using a QuickPlex Q120 reader (Meso Scale Diagnostics). ELISA plates from Abcam (ab269555) were used to measure the levels of Gal-3 (detection range 58.8–2000 pg/ml) in tissue homogenates. The protocol was carried out according to the manufacturer's instructions. A Biotek Synergy 2 was used to read the ELISA Gal-3 assay.

## Immunohistochemistry

Hippocampal sections 40-µm-thick were washed (3 × 15 min) in 0.1 M KPBS and then incubated in 0.1 M KPBS, Tween 20 0.25% and normal donkey serum 5% for one hour at room temperature. For immunofluorescence labeling (Iba1, Gal-3, Aβ, or p-Tau), sections were first incubated with the primary antibodies followed by the corresponding Alexa 488/555/647 secondary antibodies (1:1000 dilution, AlexaFluor, Life Technologies). After mounting and drying on slides, the sections were incubated in 0.6 g Sudan Black (Sigma) dissolved in 70% ethanol for 5 min. Subsequently, the sections were washed in PBS and mounted with the mounting medium. When imaging, the camera settings were adjusted at the start of the experiment and maintained for uniformity. A Nikon Eclipse Ti confocal microscope (Nikon, Japan) and NIS elements software (Nikon, Japan) were used to take 20 × magnification pictures and for the final collage. Primary antibodies included anti-Iba1 (Wako, 019-19741), Gal-3 (R&D, AF1197), Aβ (6E10, Covance), and p-Tau (Thermofisher, MN120). NIS Element Analysis software (Nikon, Japan) was used to evaluate plaque size and shape. To create the 3D model, we used Imaris version 9.8 (Oxford Instruments). For the model, image stacks were taken using a Leica TCS SP8 laser scanning confocal microscope (Leica Microsystems) with the Leica Application Suite X software version 3.5.7 (Leica Microsystems).

## CSF biomarker analysis

Lumbar puncture was performed after overnight fasting. CSF samples were centrifuged at 1000 × *g* for 10 min at 4 °C within 2 h of collection and then aliquoted into 0.5 mL polypropylene tubes before being stored at − 80 °C for further analysis. All biomarkers assessed in this study were measured at the Clinical Neurochemistry Laboratory at the University of Gothenburg in Mölndal, Sweden. Gal-3 was measured in CSF using commercially available sandwich ELISA kits (Abcam ab269555, Cambridge, UK) following the manufacturer's instructions. Samples were analyzed in singlicate with standards run in duplicate. Duplicates of CSF pool quality controls were placed at the beginning and end of each plate. In a polystyrene 96-well plate, pre-diluted 1:2 CSF samples, CSF pool quality controls and standards were incubated with a solution containing an affinity tag-labeled capture Gal-3 antibody and a reporter-conjugated detection Gal-3 antibody. The entire sandwich complex was immobilized to the well via immunoaffinity of an anti-tag antibody. Following the washing procedure, wells were incubated with 3,3′,5,5′-Tetramethylbenzidine TMB. The addition of a stop solution terminated the reaction, and the absorbance was read in a Sunrise microplate absorbance reader (Tecan, Männedorf, Switzerland) at 450 nm. Four-parameter logistic regression was used for standard curve-fitting analysis. All samples were within detection range and were quantified. The sensibility indicated by the manufacturer was 13.3 pg/mL. The coefficients of variation (CVs) for intra- and inter-plate variations were 4.9% and 5.9%, respectively.

CSF soluble TREM-2 (sTREM-2) was measured in-house using an electrochemiluminescence immunoassay with a Meso Scale Discovery (MSD) SECTOR imager 6000 (MSD, Rockville, MD), as previously described by Alosco et al. [1]. In brief, the capture antibody was a biotinylated polyclonal goat anti-human TREM-2 antibody (0.25 µg/mL R&D Systems, Minneapolis, MN), and the detector antibody was a monoclonal mouse anti-human TREM-2 antibody (1 µg/Ml Santa Cruz Biotechnology, Dallas, TX). Recombinant human TREM-2 (4000–62.5 pg/mL) was used to compute a calibration curve. Samples were diluted 1:4 for analysis. The intra- and inter-plate CVs were 3.8% and 4.9%, respectively. CSF levels of Aβ42, Aβ40, p-Tau181 and t-tau were measured with the commercially available LUMIPULSE G1200 automated immunoassay instrument following the manufacturer's instructions (Fujirebio). Diagnostic cutoffs used were the following: Aß42/Aß40 < 0.61 pg/mL; p-Tau181 < 61 pg/mL; t-tau < 450 pg/mL. CSF neurofilament light (NfL) was measured using an in-house sandwich ELISA with capture and detection antibodies directed against the central rod domain of the protein, NfL21 and NfL23, respectively, as described previously [19]. The intra- and inter-plate variation CVs were 6.7% and 8.4%, respectively. CSF YKL-40 was measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN). The intra-plate CV was 8.7%, and the inter-plate CV was 10.8%. CSF GFAP was quantified using the HD-X SIMOA platform using a commercial kit by Quanterix (Billerica, MA, USA). The intra-plate CV was 6.1%, and the inter-plate CV was 6.0%.

## Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA) and SPSS v. 26 (IBM Corp., Armonk, NY, USA) software. Normality was assessed by Kolmogorov–Smirnov test. All AD-related variables from the cortical and hippocampal tissue were analyzed with a non-parametric test. Mann–Whitney test was used to compare independent groups. For multiple comparisons, the Kruskal–Wallis test was performed followed by Dunn's post hoc correction. Chi-square ($\chi^2$) tests were used to investigate the differences between groups with categorical variables. Outlier analysis was performed in GraphPad to identify anomalous observations in the dataset. Brain tissue samples from patients with acute or subacute infarct were excluded from the analysis. CSF biomarker levels were compared between AD and NC with

linear regression adjusted for age, sex and ApoE4 carriership. The correlation of Gal-3 with other biomarkers values was analyzed using Spearman's rank correlation. We calculated the area under receiver-operating characteristic curve (AUROC) to study biomarker performance in differentiating AD patients from neurological controls. The area under the precision–recall curve (AUPRC) was also computed accounting for unbalanced data. Cutoffs for identification of AD were computed using Youden index to maximize sensitivity and specificity for each CSF biomarker. Confusion matrix was set up in order to calculate sensitivity, specificity, and accuracy at established cutoffs.

Principal component analysis (PCA) was performed on the whole cohort and in the amyloid and tau positive (A + T +) sub-group to explore the pattern of association between the different biomarkers. Outlier values, defined by a value > mean ± 3SD, were excluded for each biomarker before analysis. The suitability of the dataset was evaluated by the Kaiser–Meyer–Olkin Measure of Sampling Adequacy test and Bartlett's Test of Sphericity. The number of components was determined by the number of eigenvalues greater than one. Variables with a loading factor > 0.4 or < − 0.4 were regarded as representative of the component. Each component was interpreted according to the current understanding of the physiopathology underlying each biomarker in AD. Component scores obtained were compared between groups using linear regression adjusted on age and sex. A two-sided $p$ value < 0.05 was considered statistically significant.

## Results

### Gal-3 levels are elevated in the neocortex and hippocampus of post-mortem samples from AD patients

First, we evaluated the levels of Gal-3 in cortex and hippocampus from AD cases by ELISA (see Supp. Fig. S 1 for demographics). We found Gal-3 levels significantly elevated in AD samples compared to age-matched controls (Fig. 1a). Further analysis showed higher Gal-3 upregulation in the

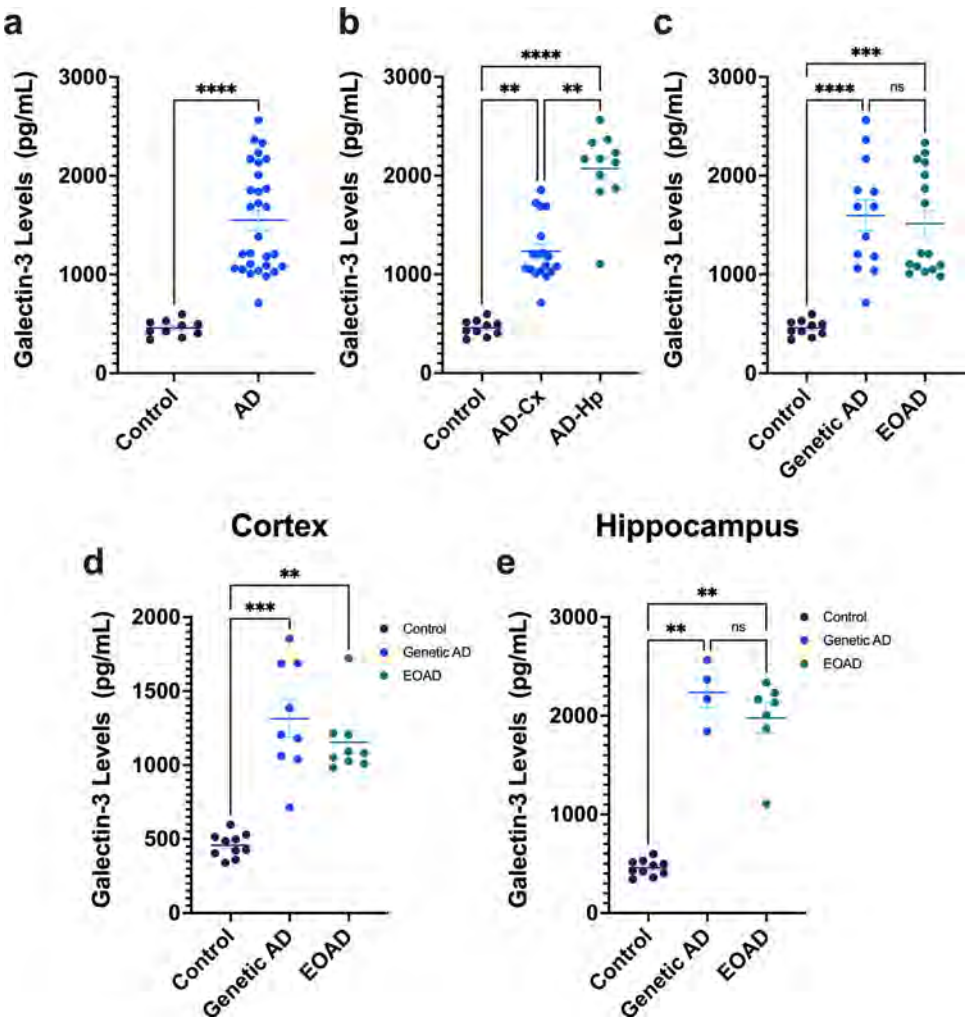



**Fig. 1** Gal-3 levels are increased in the cortex and the hippocampus in AD patients. First, Gal-3 levels were compared between AD samples versus control samples (**a**). Gal-3 levels measured by ELISA are increased in cortical and hippocampal tissue from AD patients compared to controls (**b**). **c** AD patients were divided into sporadic early-onset AD (EOAD) and genetic AD (PSEN1 mutation) cases. Then, Gal-3 levels were compared between AD groups and controls. **d**, **e** Cortical and hippocampal Gal-3 levels were compared between EOAD and genetic AD groups. Differences were found compared to control samples but not between EOAD and genetic AD groups themselves. Non-parametric t-test **a** and Kruskal–Wallis multiple comparisons (b-e) were performed. Data are shown as mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$. $^{****}p < 0.0001$

hippocampus compared to the cortex in AD cases (Fig. 1b). Next, to evaluate whether genetic AD cases varied in Gal-3 levels, we divided our cohort into genetic AD cases (*PSEN1* mutation carriers) and sporadic early-onset (EOAD) cases. Our analysis revealed no differences in Gal-3 protein levels between AD groups (genetic AD vs EOAD) (Fig. 1c). However, Gal-3 levels were significantly increased in genetic AD and EOAD samples compared to control (Fig. 1c). Furthermore, we studied regional differences in Gal-3 protein levels between genetic AD and EOAD cases. Therefore, we compared cortical and hippocampal brain tissue but found no differences between the two AD groups (Fig. 1d, e). However, we found that Gal-3 levels in genetic AD and EOAD samples were significantly upregulated in both cortex and hippocampus compared to control cases, suggesting a similar Gal-3-associated immune response in both AD groups. We also confirmed the increase in cortical Gal-3 levels in AD patients compared to controls by western blot (Supp. Fig. S 1g, online resource). Along with Gal-3 levels, we measured Aβ42, t-tau and p-Tau levels. Aβ42 and p-Tau were significantly increased in the frontal cortex and hippocampal samples from AD patients compared to controls (Supp. Fig. S1a, b, e, f, see online resource). Moreover, Gal-3 levels were not affected by post-mortem time (PMT), age, or sex (Supp. Fig. S2, online resource) in both AD and control samples. Finally, we performed a correlation analysis on Gal-3 levels with Aβ42, t-tau and p-Tau levels. In both control and AD cases, Gal-3 levels in hippocampal or cortical tissue did not correlate with Aβ42, t-tau or p-Tau levels (Supp. Fig. S3, online resource).

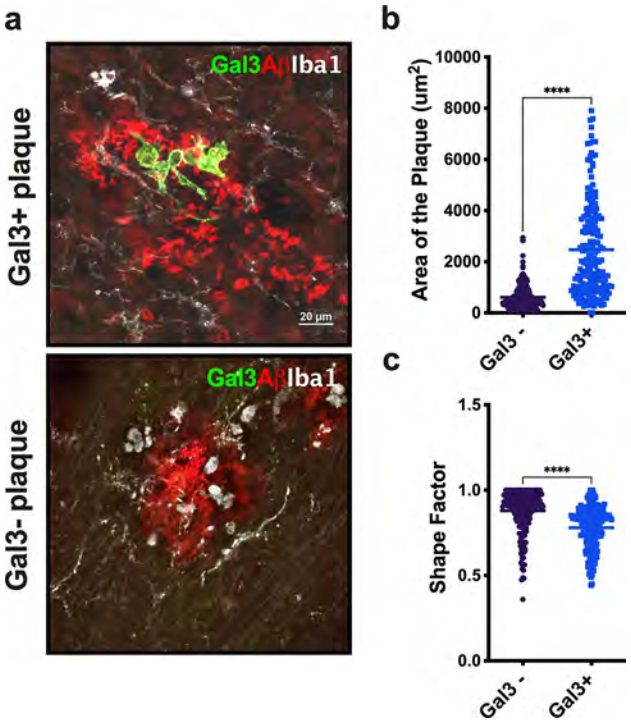



**Fig. 2** Gal-3-positive microglial cells are associated with larger and more irregularly shaped Aβ plaques. **a** Gal-3-positive microglial cells were associated with larger and more irregularly shaped Aβ plaques (Gal3+ plaques) compared to Gal-3-negative Aβ plaques (Gal3− plaques). **b**, **c** Gal3+ plaques were larger and more irregularly shaped than Gal-3− plaques. Aβ (red), Galectin-3 (green), Iba1 (white). Data are shown as mean ± SEM. Non-parametric t-tests were performed. ****$p < 0.0001$. ($n = 3$ (HC), $n = 8$ (AD). Gal-3-negative plaques, $n = 212$; Gal-3-positive plaques, $n = 197$)

## Gal-3-positive microglia is associated with Aβ plaques and tau tangles in AD

We demonstrated a subpopulation of microglia clustered around Aβ plaques identified by high expression of Gal-3[8]. However, we also observed another subset of plaques that were not surrounded by Gal-3-positive microglial cells. Here, we sought to evaluate the main morphological features of Aβ plaques (size and shape) and their association with microglia expressing Gal-3. Plaque size referred to the plaque area as measured in square micrometers based on 6E10 (APP/Aβ) immunolabeling. Plaque shape was defined as a value between 0 and 1. Values closer to 1 indicated a more rounded and regular plaque shape whereas values closer to zero indicated a more irregular plaque shape. To study plaque morphology, we performed triple immunolabeling using the antibodies against Iba1 (microglial marker), 6E10 (APP/Aβ), and Gal-3 on human AD cortical tissue (Fig. 2a). Notably, clear morphological differences were observed between Gal-3-positive and Gal-3-negative plaques. We defined Gal-3-positive and Gal-3-negative plaques as those surrounded by microglial cells expressing or lacking Gal-3, respectively. Gal-3-positive plaques covered a larger area (Fig. 2b) and had a more irregular shape (Fig. 2c) compared to Gal-3-negative plaques. Our analysis revealed that microglial cells expressing Gal-3 were associated with Aβ plaques (Fig. 3a–g), which may indicate plaque phagocytosis by the microglia [13, 23, 60, 62]. Indeed, microglial cells expressing Gal-3 near plaques presented numerous Aβ inclusions (Fig. 3, white arrows). Moreover, we also found Gal-3-positive microglial cells close to p-Tau aggregates in senile plaques (Fig. 4a–g). Notably, 3D modeling revealed p-Tau interacting with Gal-3-positive microglial cells in what is likely a senile plaque containing p-Tau-aggregates (Fig. 4b, white arrows).

## CSF Gal-3 levels are increased in AD and are associated with neuroinflammatory alterations

We then analyzed CSF samples from a cohort including 119 patients with AD and 36 neurological controls (NC). The cohorts and CSF biomarkers levels are described in Table 1. AD patients were significantly older than NC ($P < 0.001$) and displayed higher ApoE4 carriership

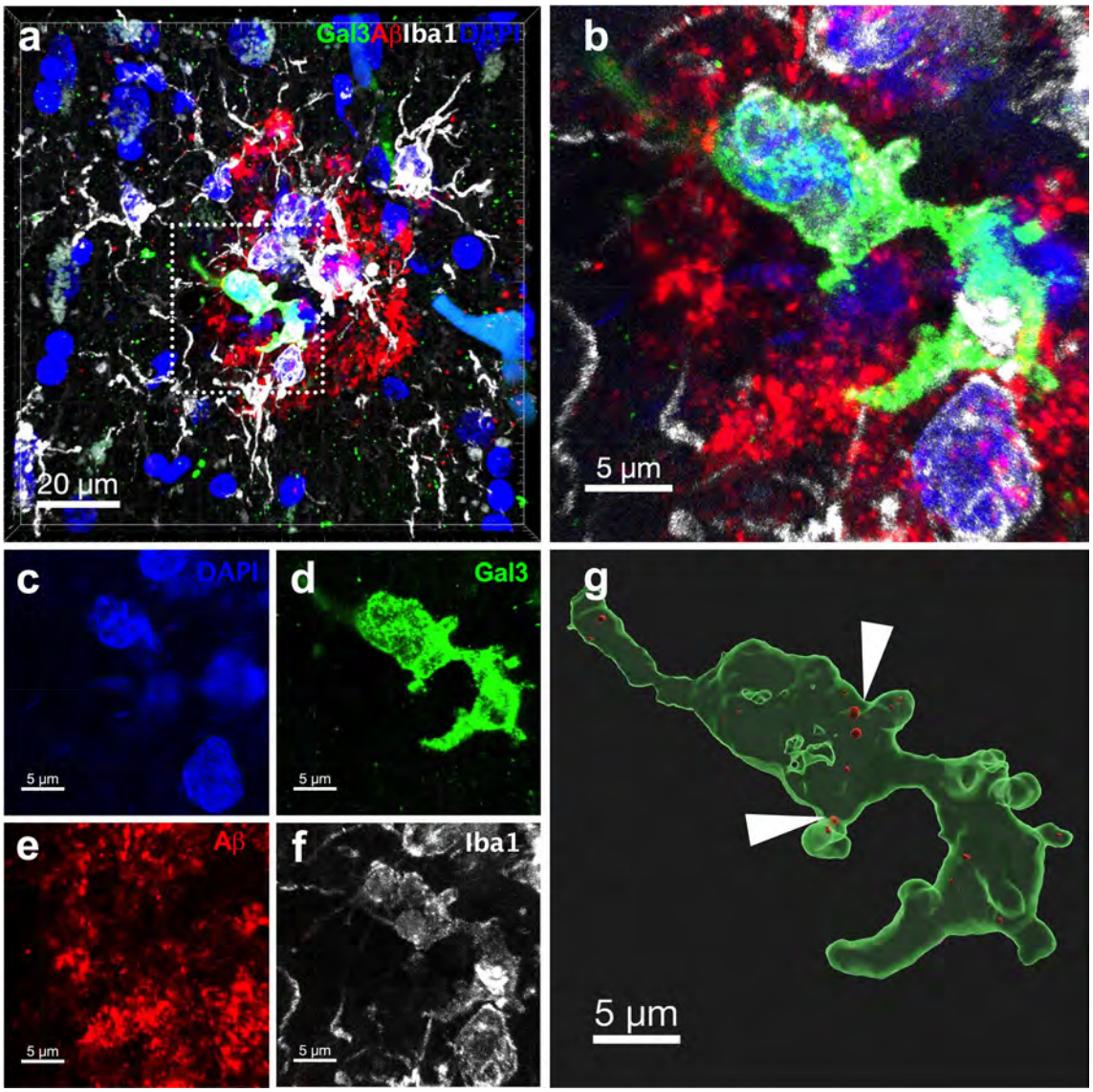



**Fig. 3** Reactive microglial cells expressing Gal-3 presented Aβ inclusions in human tissue samples. **a**–**f** Gal-3-positive microglial cell associated with Aβ plaques. **g** 3D reconstruction of microglial cells with multiple Aβ inclusions inside. Gal3 (green), Aβ (red), Iba1 (white), DAPI (blue). White arrows are pointing to Aβ inclusions (in red) ($n = 3$ (HC), $n = 8$ (AD)

($P < 0.001$). First, we focused our investigation on determining the relationship between CSF Gal-3 and AD characteristics. We found that CSF Gal-3 levels correlated positively with age (rho = 0.402, $P < 0.001$). However, Gal-3 did not associate significantly with sex ($P = 0.079$) or *APOE*-ε4 carrier status ($P = 0.432$). Overall, AD patients displayed higher CSF Gal-3 levels compared to NC (1168.8 pg/mL versus 960.5 pg/mL, $P = 0.030$ adjusted for age, sex, and ApoE4 carriership Fig. 5a). The CSF profile in relation to the AD continuum showed that Gal-3 levels did not differ between A + T−N−, A + T + N− and A + T + N + groups ($P = 0.440$).

We next analyzed neuroinflammatory-related markers in CSF and considered their relationship with CSF Gal-3. We found that sTREM-2 levels did not significantly differ between NC and AD groups ($P = 0.217$, Fig. 5b). To distinguish AD patients from NC, CSF Gal-3 and sTREM-2 levels were moderately good predictors (AUROC = 0.80 and AUROC = 0.78, respectively, Fig. 5c). However, their performance remained lower than CSF p-Tau and t-tau, the gold standard for diagnosis (respectively, AUROC = 0.95 and AUROC = 0.92). Detailed comparison of sensitivity, specificity and accuracy at optimal cutoff designed through ROC analysis are available for each biomarker in Supp. Table 2 (online resources) and Supp. Fig. S4 (online resources).

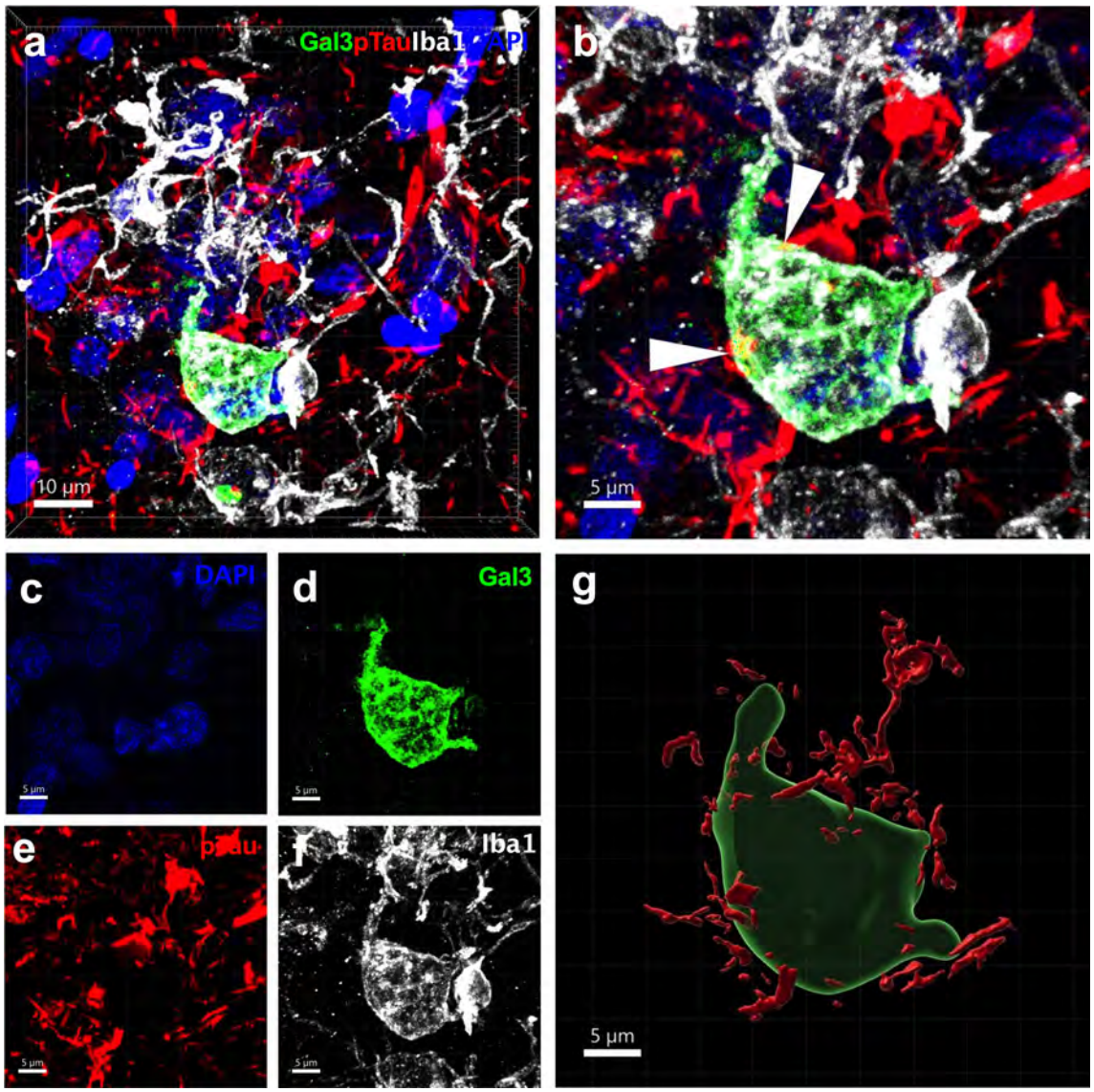



**Fig. 4** Reactive microglial cells expressing Gal-3 interact with p-Tau in senile plaques from human tissue samples. **a**–**f** Gal-3-positive microglial cell associated with p-Tau plaques. **g** 3D reconstruction of microglial cell with multiple p-Tau interactions. Gal3 (green), p-Tau (red), Iba1 (white) and DAPI (blue). White arrows are pointing p-Tau Gal-3 interactions (in orange). $n=3$ (HC), $n=8$ (AD)

Relating these two factors, we observed that Gal-3 weakly correlated with sTREM-2 (rho = 0.326, $P<0.0001$, Fig. 5e). This correlation held when looking specifically in AD patients and the NC group (rho = 0.269, $P=0.0033$ and rho = 0.405, $P=0.0142$, respectively). In addition, CSF Gal-3 weakly correlated with GFAP (rho = 0.378, $P<0.0001$, Fig. 5f) and YKL-40 (rho = 0.339, $P<0.0001$, Fig. 5g) when looking at the whole cohort. These correlations were sustained for both markers in the AD patient group (GFAP: AD, rho = 0. 410, $P<0.0001$; YKL-40: AD, rho = 0.354, $P=0.0002$) but not in the NC group. We used the CSF/plasma albumin quotient to indirectly study the relationship between Gal-3 and brain blood barrier integrity. CSF Gal-3 levels correlated very moderately with the CSF/plasma albumin quotient in the whole cohort (rho = 0.255, $P=0.0043$) and in the AD group (rho = 0.263, $P=0.0067$). Regarding the other CSF biomarkers, only CSF GFAP was associated with the CSF/plasma albumin quotient in the AD group (Supp. Table S3, online resources).

## Gal-3 levels are associated with tau and synaptic marker levels in CSF in AD

We next looked at the relationship between CSF Gal-3 levels and other CSF biomarkers of AD. Gal-3 levels were negatively correlated with Aβ42/Aβ40 ratio (rho = − 0.285, $P=0.0004$, Fig. 6a) in the whole cohort

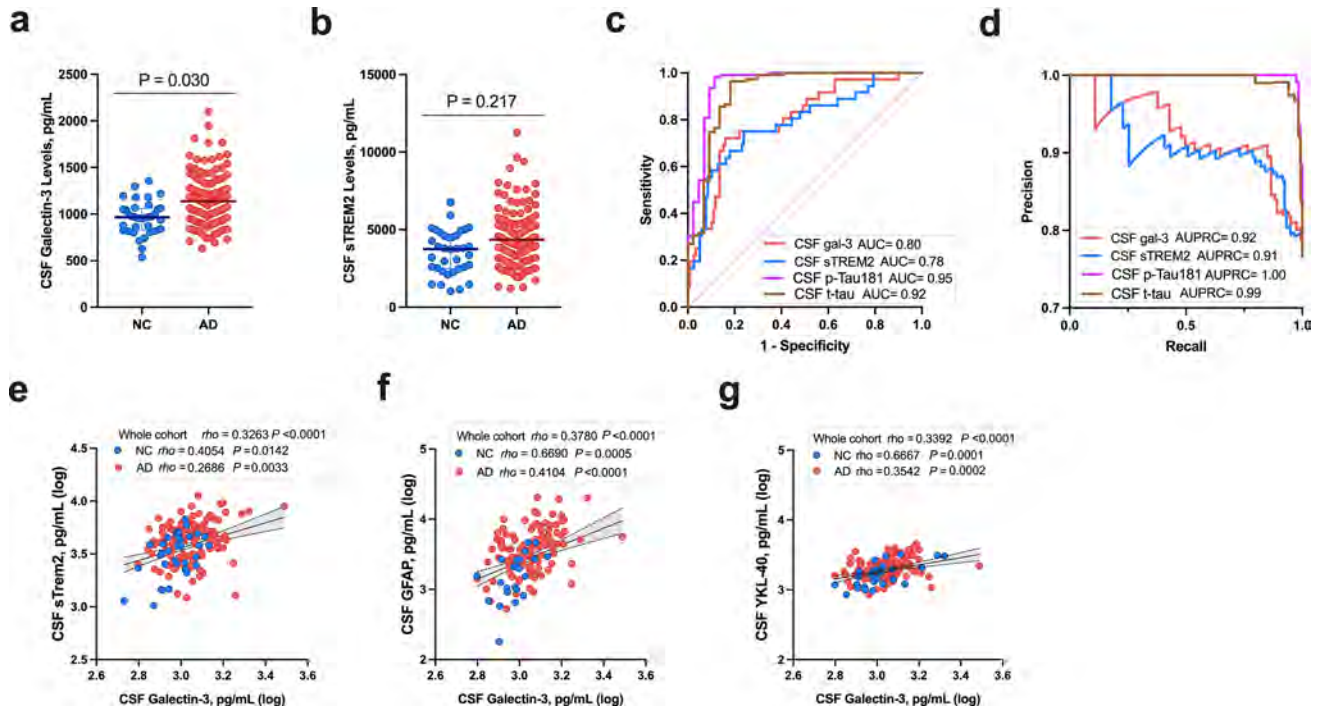
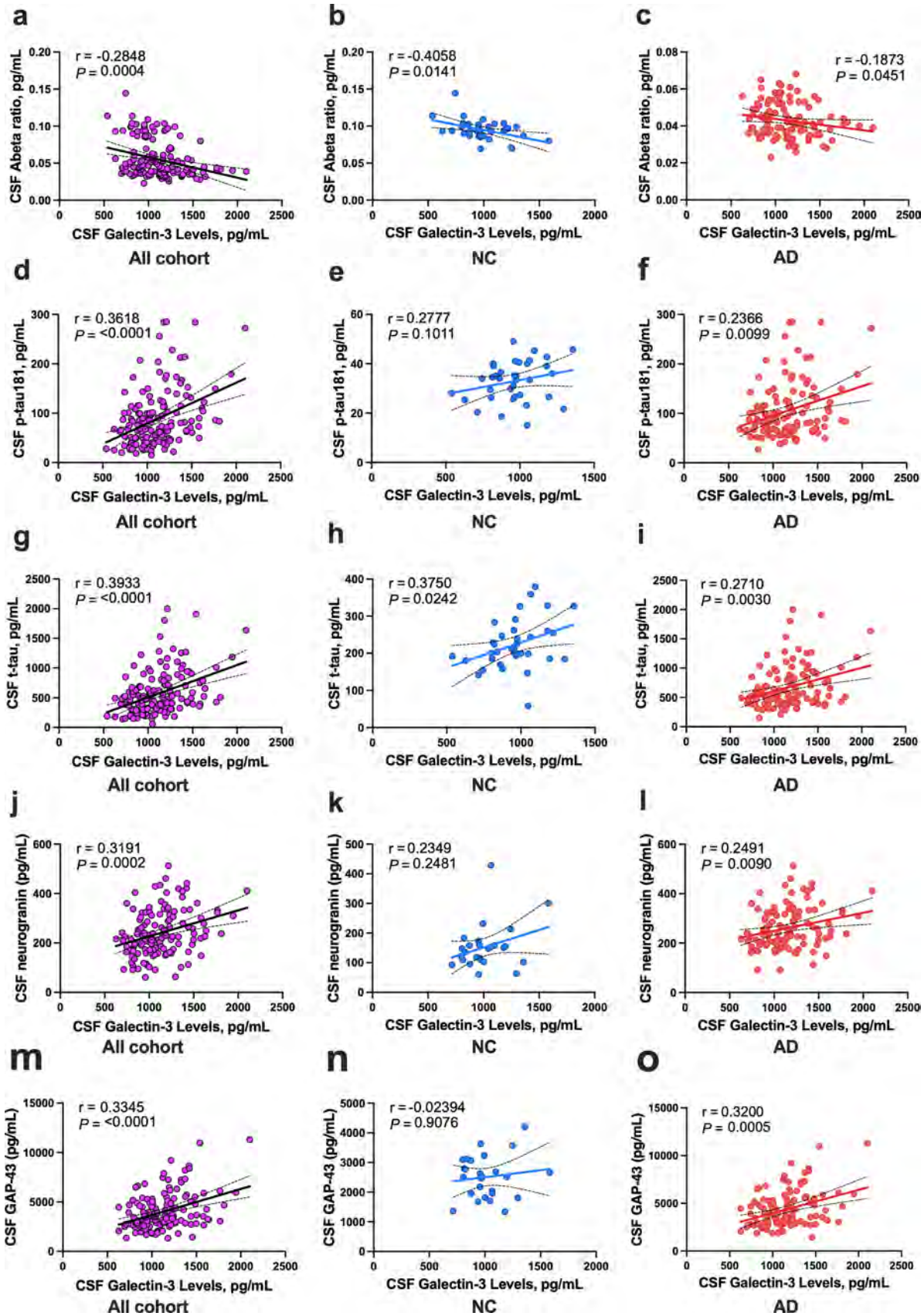



**Fig. 5** CSF Gal-3 levels are increased in AD patients and correlate with other CSF neuroinflammatory biomarkers. **a** CSF Gal-3 levels were measured by ELISA in control subjects and AD patients. Gal-3 levels were significantly elevated in AD patients compared to controls (*$P=0.030$) after adjustment on age, sex and ApoE4 carriership. **b** CSF sTREM-2 levels were measured in our cohort. No difference was found between the AD and control groups in our analysis adjusted for age, sex and ApoE4 carriership ($P=0.217$). **c**, **d** Analysis of ROC curves revealed moderate performance of CSF Gal-3 and sTREM-2 levels for differentiating AD patients from neurological controls (**c** Gal-3 AUROC = 0.80 [95% CI = 0.72–0.88], sTREM-2 AUROC = 0.78 [95% CI = 0.69–0.88]; **d** Gal-3 AUPRC = 0.92, sTREM-2 AUPRC = 0.91). For comparison, CSF markers p-tau and t-tau demonstrated high discriminating performance between AD and controls (c: p-tau AUROC = 0.95 [95% CI = 0.91–1.00], t-tau AUROC = 0.92 [95% CI = 0.86–0.98]; 5d: p-tau AUPRC = 1.00, t-tau AUPRC = 0.99). **e**, **f**, **g** The relationships between CSF Gal-3 and other CSF neuroinflammatory biomarkers—sTREM-2, GFAP and YKL-40—were studied using Spearman's rank correlation in the whole cohort as well as in AD and neurological control (NC) subgroups

as well as in the NC (rho = − 0.406, $P=0.0141$) and AD patient (rho = − 0.187, $P=0.0451$) sub-groups (Fig. 6b, c). In contrast, Gal-3 levels were positively correlated with p-Tau181 in the whole cohort (rho = 0.362, $P<0.0001$), but of the sub-groups, only the AD cohort maintained a statistically significant correlation (rho = 0.237, $P=0.0099$, Fig. 6d–f). Similarly, CSF Gal-3 positively correlated with t-tau (rho = 0.393, $P<0.0001$) in the whole cohort and separately in the AD cohort (rho = 0.271, $P=0.003$) and the NC group (rho = 0.375, $P=0.024$) (Fig. 6g–i). Looking at the relationship between CSF Gal-3 and CSF synaptic biomarkers, we found that Gal-3 positively correlated with neurogranin and GAP-43 when including the whole cohort (rho = 0.319, $P=0.0002$ and rho = 0.334, $P<0.0001$, respectively, Fig. 6j, m). This statistically significant correlation was reflected in the AD group for both neurogranin (rho = 0.249, $P=0.0090$, Fig. 6l) and GAP-43 (rho = 0.320, $P=0.0005$, Fig. 6o) but not in the NC group.

## CSF Gal-3 levels correlate with cognitive decline in AD

We studied the association between CSF Gal-3 levels and general cognition via Mini-Mental State Exam (MMSE) scores (Supp. Fig. S5a, online resource). Gal-3 levels were associated with MMSE scores in AD patients, independently of sex, age and level of education ($\beta=0.176$, 95% CI = 0.010 to 0.341, $P=0.0217$). However, Gal-3 levels were not associated with MMSE scores after adjusting for age, sex and level of education when looking at the whole cohort ($\beta=-0.042$, 95% CI = − 0.202–0.118, $P=0.605$) nor in the NC cohort ($\beta=0.010$, 95% CI = − 0.371–0.391, $P=0.957$). We performed regression analysis to study the relationship between CSF Gal-3 levels with MMSE scores in the AD cohort (Supp. Fig. S5b, online resource). A significant quadratic function was found for the relationship between CSF Gal-3 levels and MMSE scores in AD patients ($P=0.039$). This could be depicted as an inverse U-shaped


a
r = -0.2848
P = 0.0004
CSF Abeta ratio, pg/mL
CSF Galectin-3 Levels, pg/mL
All cohort
b
r = -0.4058
P = 0.0141
CSF Abeta ratio, pg/mL
CSF Galectin-3 Levels, pg/mL
NC
c
r = -0.1873
P = 0.0451
CSF Abeta ratio, pg/mL
CSF Galectin-3 Levels, pg/mL
AD
d
r = 0.3618
P = <0.0001
CSF p-tau181, pg/mL
CSF Galectin-3 Levels, pg/mL
All cohort
e
r = 0.2777
P = 0.1011
CSF p-tau181, pg/mL
CSF Galectin-3 Levels, pg/mL
NC
f
r = 0.2366
P = 0.0099
CSF p-tau181, pg/mL
CSF Galectin-3 Levels, pg/mL
AD
g
r = 0.3933
P = <0.0001
CSF t-tau, pg/mL
CSF Galectin-3 Levels, pg/mL
All cohort
h
r = 0.3750
P = 0.0242
CSF t-tau, pg/mL
CSF Galectin-3 Levels, pg/mL
NC
i
r = 0.2710
P = 0.0030
CSF t-tau, pg/mL
CSF Galectin-3 Levels, pg/mL
AD
j
r = 0.3191
P = 0.0002
CSF neurogranin (pg/mL)
CSF Galectin-3 Levels, pg/mL
All cohort
k
r = 0.2349
P = 0.2481
CSF neurogranin (pg/mL)
CSF Galectin-3 Levels, pg/mL
NC
l
r = 0.2491
P = 0.0090
CSF neurogranin (pg/mL)
CSF Galectin-3 Levels, pg/mL
AD
m
r = 0.3345
P = <0.0001
CSF GAP-43 (pg/mL)
CSF Galectin-3 Levels, pg/mL
All cohort
n
r = -0.02394
P = 0.9076
CSF GAP-43 (pg/mL)
CSF Galectin-3 Levels, pg/mL
NC
o
r = 0.3200
P = 0.0005
CSF GAP-43 (pg/mL)
CSF Galectin-3 Levels, pg/mL
AD

◂**Fig. 6** CSF Gal-3 levels correlate with CSF tau and synaptic markers. **a–o** Scatter plots depicting the association between CSF Gal-3 levels with other CSF AD and synaptic markers in the whole cohort and in the sub-groups (neurological controls [NC] $n=36$ and AD $n=119$). **a–c** CSF Aβ ratio weakly correlated with CSF Gal-3 in the whole cohort and sub-groups. **d–i** CSF Gal-3 significantly correlated with CSF p-tau181 and t-tau in the whole cohort and some sub-groups. **j–o** CSF Gal-3 levels correlated with CSF synaptic markers neurogranin **j–l** and GAP-43 **m–o** in the whole cohort and in the AD patient sub-group. Associations were assessed using Spearman's rank correlation. Solid line indicates regression line, and dotted lines border the 95% confidence interval

curve showing that higher levels of CSF Gal-3 were associated with intermediate MMSE scores. However, lower Gal-3 levels were associated with the highest and lowest MMSE scores in AD patients.

### CSF Gal-3 clusters with neuroinflammatory CSF biomarkers in PCA analysis

Lastly, we performed PCA to investigate the relationship between the different biomarkers. We identified 2 principal components that explained 71% of the total variance in the dataset (Fig. 7a, b). Component 1 (PC1) accounted for 57% of the variance and was associated with core AD biomarkers: Aβ40/Aβ42, p-Tau181, t-tau, and the synaptic markers neurogranin and GAP-43. Component 2 (PC2) captured 14% of the variance and was associated with neuroinflammatory markers Gal-3, sTREM-2, YKL-40 and GFAP. Notably, in the neuroinflammatory component PC2, the marker with the highest weight was Gal-3. In the core AD component PC1, p-Tau181 was the leading marker. After adjusting for age and sex, PC1 was significantly increased in the AD group compared to NC ($P<0.0001$, Fig. 7c). Neuroinflammation PC2 did not differ between groups after adjusting for multiple comparisons (Fig. 7d). However, of all AD patients, PC2 was significantly higher in the A + T + N + group compared to the A + T−N− group ($P=0.002$, Fig. 7e). PCA analysis was further performed on the A + T + AD patient group, and the same two components, core AD PC1 and neuroinflammation PC2 could be detected (Supp. Fig. S6, online resources). Interestingly, in A + T + subjects, PC1 and PC2 had a quadratic relationship, wherein PC2 had a U-shaped form following increasing levels of core AD PC1 ($P=0.031$) (Supp. Fig. S6c, online resource).

## Discussion

In the present study, we assessed the microglial activity marker Gal-3 in clinically diagnosed and neuropathologically confirmed AD patients and analyzed the relationship between CSF levels of Gal-3 and AD markers and characteristics in a clinical cohort. Our data showed an upregulation of Gal-3 in cortical and hippocampal tissue from sporadic EOAD and genetic AD cases compared to controls, further highlighting Gal-3-specific microglial activation in AD brain related to Aβ plaque deposits. Gal-3 levels were not associated with age or post-mortem time delay. A detailed analysis demonstrated that Gal-3-positive microglia associated more frequently with Aβ plaques that were large and irregular and associated with neurons with p-Tau inclusions in human brain tissue samples. Complementing this, we measured CSF Gal-3 levels and other CSF AD biomarkers in AD patients. Like in tissue, we found higher CSF Gal-3 levels in AD patients compared to control subjects. CSF Gal-3 levels correlated with markers of neuronal degeneration (tau and p-Tau181), synaptic dysfunction (neurogranin and GAP-43), and to an even greater extent, inflammatory markers (GFAP, YKL-40 and sTREM-2). Lastly, we performed PCA and found that neuroinflammatory markers cluster together separately from the traditional hallmarks of the pathology. With this study, we expanded on our work demonstrating the role of Gal-3 on the detrimental inflammatory response in AD and show, in human CSF, that Gal-3 is associated with core biomarkers of AD and with neuroinflammatory markers.

In AD, Gal-3 is preferentially expressed by activated microglia and is released into the extracellular space. Because of this, Gal-3 can be detected in CSF and serum [3, 8, 10, 55, 59]. We sought to explore the potential of Gal-3 as an AD biomarker by analyzing the relationship of Gal-3 with markers of pathology progression and cognitive decline in CSF. Compared to controls, we have observed that AD patients have significantly higher Gal-3 levels in the brain, both in this study and our previous one [10]. Moreover, Gal-3 levels were higher in hippocampus compared to frontal cortex. This may be related to the larger size of Aβ deposits in hippocampus and, therefore, more robust microglial activation. Indeed, in our previous study, we demonstrated Gal-3-dependent microglial activation that took place only in the vicinity of Aβ plaques [8]. An increase in Gal-3 makes sense given its source and the findings demonstrating an increase in microglial activity in AD [58]. In our study, the amyloid plaque morphology associated with Gal-3-positive plaques resembled the recently named coarse-grained plaques [7]. Notably, coarse-grained plaques are associated with intense neuroinflammation (CD68 and MHCII positive), ApoE4, and vascular pathology [16].

Interestingly, Gal-3+ microglia contained a notable number of Aβ inclusions, highlighting their phagocytic capacity associated with the pathology [62]. The receptors involved in microglial cells phagocytosis of Aβ plaques are not fully understood. Recently, the importance of two specific TAM receptors, Axl and MerTK, has been highlighted in the detection and engulfment of Aβ plaques [23]. This was demonstrated in an APP mouse model lacking Axl

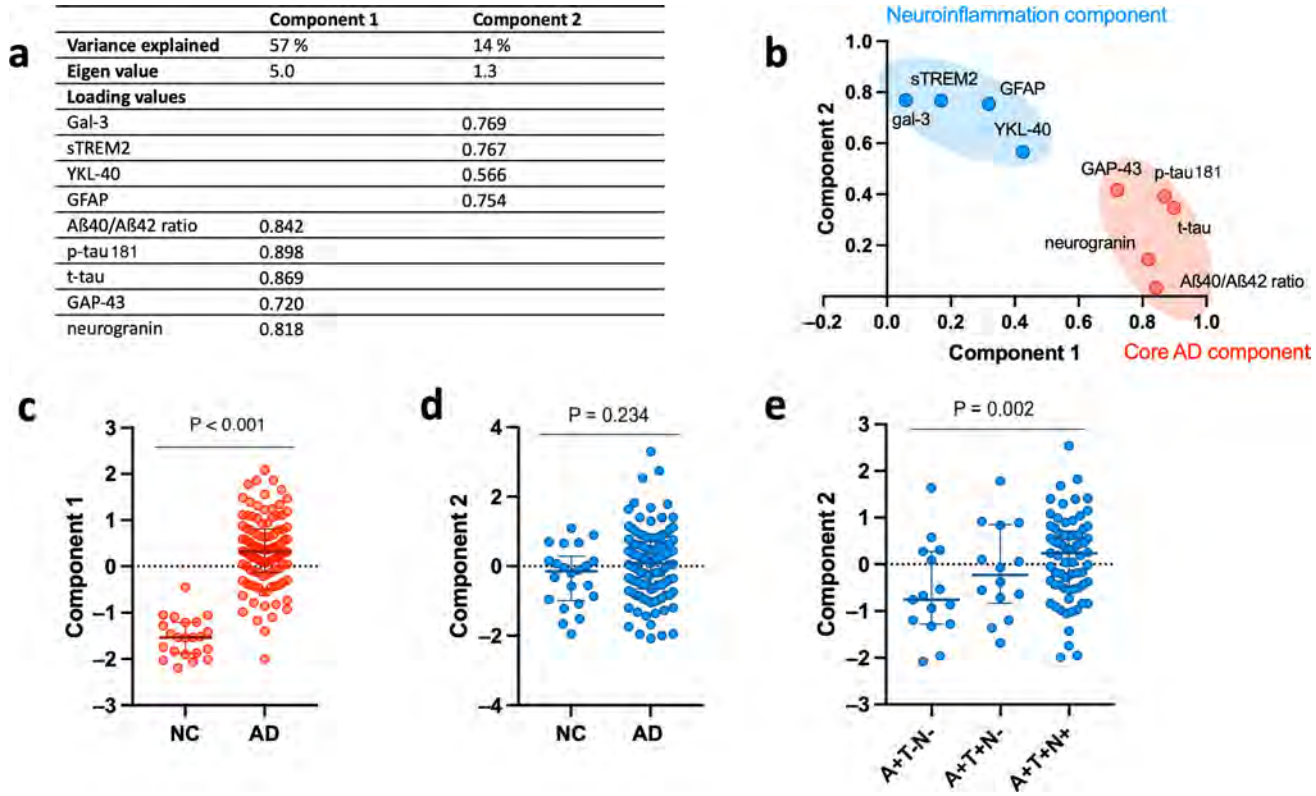



**Fig. 7** CSF Gal-3 clusters with a neuroinflammatory component in principal component analysis. **a**, **b** Principal component analysis (PCA) in the whole cohort revealed clustering of the CSF biomarkers in two principal components (**a** loading values of each CSF biomarker, eigenvalues, and variance explained for each component identified; **b**: scree-plot in Varimax rotation). Component 1 is associated with CSF core AD biomarkers (Aß ratio, p-Tau181, and t-tau) and CSF synaptic markers (neurogranin, GAP-43). Component 2 included the CSF neuroinflammation markers (Gal-3, sTREM-2, GFAP, and YKL-40). Component 1 and Component 2 accounted for 57% and 14% of the variance. **c**–**e** Identified components were compared between groups using one-way ANCOVA-adjusted on age and sex followed by post hoc Least square test, adjusted with Bonferroni for multiple comparisons. **c** Core AD Component 1 was significantly higher in AD patients than in all other groups (****$P < 0.0001$ versus all other groups). **d** Neuroinflammation Component 2 did not differ between the groups. **e** Focusing on the patients on the AD continuum, neuroinflammatory Component 2 was significantly higher in patients with a [A+T+N+] CSF profile compared to patients with an [A+T−N−] profile ($P = 0.002$)

and Mer which had impaired detection and engulfment of Aβ plaques. Importantly, Gal-3 is a ligand for MerTk and thereby stimulates phagocytosis. Therefore, the Aβ inclusion observed inside microglial cells expressing Gal-3 may be partially mediated by Axl, MerTK and Gal-3 interaction [12]. We also describe Gal-3+ microglia close to neurons with p-Tau aggregates in human senile plaques, suggesting a relation between microglial activation and p-Tau aggregates. Recently, Pascoal et al. demonstrated the joint propagation of tau-pathology and microglial activation in AD [40]. Indeed, this study revealed that tau propagation is more dependent on microglial networks than tau network circuits [40]. A key regulator of tau propagation is Low-Density Lipoprotein 1 receptor (LRP1), which has been related to tau uptake and spreading [43] and also to the microglial inflammatory response [57]. Notably, galectin-3 binding studies performed on mesenchymal retinal cells have demonstrated a high binding affinity between galectin-3 and LRP1 [38]. In regards to tau-dependent microglial activation, Jin et al. discovered a role of polyglutamine binding protein 1 (PQBP1) in sensing extracellular tau and an associated microglial pro-inflammatory response. This microglial inflammatory response is dependent on NFκB-dependent transcription of inflammatory genes, leading to brain inflammation [27]. Despite all the progress, little is known about the mechanisms involving extracellular tau-dependent microglial activation and further experiments are needed to address this question.

Traditionally, Aβ, p-Tau and tau are the main diagnostic biomarkers for AD used in clinical practice [16]. However, less is known whether these biomarkers are associated with other key neuropathological features, including neuroinflammation, vascular impairment and blood–brain barrier disruption. Our data looking at CSF consistently underline the relation of Gal-3 with other key CSF biomarkers in AD progression. Higher Gal-3 levels correlated with tau and p-Tau181 levels, two indicators of pathology progression

in AD. Indeed, microglial activation progresses along with tau deposition across the different Braak stages, indicating the cooperation of both phenomena [40]. Indeed, the combination of neuroinflammatory microglial activity and tau deposition measured by PET predicts cognitive decline in AD [35]. The latest is consistent with the pathological presentation of microglial cells expressing Gal-3.

Together with tau-pathology biomarkers, Gal-3 levels strongly correlated with CSF markers of synaptic dysfunction, GAP-43, and neurogranin. Synaptic failure is an early process of AD, and synapse loss is a neuropathological hallmark in connection with cognitive decline. Physiologically, microglia have been implicated in maintaining functional synaptic connections and plasticity [36]. Synaptic dysfunction is an early feature in AD, and recent studies suggest that microglia-mediated synapse removal could be a contributing factor [22]. In AD, microglia constitute a cellular mediator of synapse loss through phagocytosis or release of synaptotoxic factors [9, 22, 36, 45]. In our work, CSF Gal-3 correlated with pre-synaptic protein GAP-43, which displays a significant increase in AD and correlates with the magnitude of neurofibrillary tangles and Aβ plaques [44]. CSF Gal-3 also correlates with levels of neurogranin, a post-synaptic protein consistently increased in CSF in AD [41]. Neurogranin levels are positively correlated with increased neuritic plaques [41].

In our study, Gal-3 levels weakly correlated with CSF neuroinflammatory markers YKL-40, GFAP and sTREM-2. Notably, in our cohort, Gal-3 demonstrated better sensitivity and specificity than sTREM-2 to differentiate AD patients from neurological controls. In AD, YKL-40 has been found to be elevated and associated with astrocyte reactivity [31, 34, 42]. Like YKL-40, GFAP has been linked to astrocyte activity and found to be consistently elevated in CSF and serum of patients in preclinical and symptomatic AD stages [6, 24, 39]. The correlations with the neuroinflammatory markers were weaker than neuronal or synaptic markers, suggesting that Gal-3 monitors complementary inflammatory processes differently than those monitored by GFAP, sTREM-2 or YKL-40. sTREM-2 is considered a microglial marker in AD [51]. In our study, sTREM-2 was slightly but not significantly upregulated in AD samples compared to cognitively normal samples. Other studies have pointed out significantly elevated levels of sTREM-2 in CSF from AD samples compared to control. It is important to note that these studies were longitudinal studies on genetic-case cohorts (DIAN) or studies focused on the early stages of AD pathology [17, 18, 50, 51]. In this translational work, we studied CSF Gal-3 in a discovery cohort to explore how it could translate as a CSF biomarker of the microglial response. Brain expression of TREM-2 has also been linked to disease-associated microglial phenotype and plaque growth dynamics [29, 32, 54, 61]. The lack of TREM-2 has been linked to a more diffuse amyloid plaque growth, leading to increased formation of dystrophic neurites [61]. In clinical studies, higher levels of sTREM-2 have been linked to reducing cognitive decline and clinical decline [18] and lower ratios of amyloid-beta [17], which might be linked to its role in plaque formation as described by Yuan et al. [61]

Our PCA analysis displayed a U-shape relation between PC1 (Core AD biomarkers) and PC2 (Neuroinflammatory biomarkers) that might indicate two different stages of inflammatory response throughout the pathology. Indeed, neuroinflammation and microglia have been shown to be increasingly important in AD progression. However, whether microglia and the inflammatory process can and should be judged as beneficial or harmful is often debated. These debates have been fueled by the discovery of specific microglial phenotypes, namely disease-associated microglia (DAM) and neurodegenerative microglia (MGnD) [29, 30]. The DAM phenotype involves TREM-2 signaling and critical genes, such as *Axl*, *cst7*, *lpl or cd9*. On the other hand, the MGnD phenotype, discovered by Krasemann et al. [30], depends on TREM-2-ApoE signaling, shares similarities with the DAM phenotype, and involves key genes such as *Gal-3*, *Clec7a*, *Itgax and Spp1*. Therefore, we can distinguish two stages linked to pathology progression: a primary stage when the neuroinflammatory response occurs in patients with lower levels of amyloid-beta and tau relying on TREM-2 expression, and a second stage when a patient presents with a more advanced stage of the pathology with Gal-3 playing a prominent role. Indeed, Gal-3-positive microglia lead to a pro-inflammatory microglial phenotype that might be unrelated to the TREM-2-dependent phenotype (DAM) but closer to the ApoE-dependent MGnD phenotype [30]. Therefore, TREM-2-dependent microglial activation and Gal-3-dependent microglial activation might represent a sequential process initiated by TREM-2 to counteract the progression of the pathology followed by a Gal-3-dependent secondary response leading to a pro-inflammatory and more detrimental microglial phenotype. Nevertheless, some of the genes involved in each phenotype are shared, and the different phenotypes may co-exist. Understanding the precise role of Gal-3 in these phenotypes as well as in AD pathology is important, especially so if Gal-3 is considered as a potential neuroinflammatory biomarker and a druggable target for AD. Indeed, clinical trials are underway targeting Gal-3 with an antibody-based treatment (Clinicaltrials.gov (NCT05156827)).

Lastly, the cognitive evaluation revealed a quadratic relation between Gal-3 levels and MMSE score. This quadratic relation might reflect the evolution of microglial activation over different stages of the pathology [33, 37]. This kinetic could indicate the progression of microglial activity over pathology progression: a primary phase wherein Gal-3 levels increase and correspond with MMSE scores in all the way

into the intermediate stage of AD pathology. However, Gal-3 levels are reduced in the second phase and correspond to the lowest MMSE scores. This may indicate that in the latest stage of AD pathology, microglial cells become dystrophic [47, 49] with impaired functionality, activation capacity and Gal-3 production and release.

We note several limitations in our work. Regarding the CSF study, we recognize that due to a limited number of subjects, we lacked the power needed to explore subtler differences between groups with certain biomarkers, notably sTREM-2. Moreover, there was a significant age gap between AD patients and NC, which could constitute a confounding factor even though the analysis was adjusted for the age difference. Finally, due to the cross-sectional nature of this study, we were unable to depict CSF Gal-3 changes within individuals as they progressed through the AD continuum.

Finding new biomarkers to complement current methods is needed not only for early diagnosis, but also for improving the design of clinical trials and monitoring the effectiveness of disease-modifying therapies. Microglial activation, Aβ plaques and tau aggregation are key in neuronal dysfunction. We demonstrate that Gal-3 is strongly associated with the core biomarkers in AD pathology, and, like others, that Gal-3 is a key mediator of the microglial pro-inflammatory phenotype in AD [28, 30].

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**Author contributions** ABS, AV, KB, JLV, and TD designed the study. ABS, AV, and KM performed the experiments. ABS and AV analyzed the experiments and wrote the manuscript. AP contributed to the plaque analysis. LMP selected and prepared the brain tissue samples. MW performed the immunofluorescence staining. All the authors discussed the results and commented on the manuscript.

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## Declarations

**Conflict of interest** The authors do not declare any conflict of interest.



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**Plasma biomarkers for diagnosis of Alzheimer's disease in clinical settings: a memory clinic cohort study.**

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## ABSTRACT

### Objective

To study the diagnostic performance of a series of plasma biomarkers to identify Alzheimer's disease (AD) at mild cognitive impairment (MCI) and dementia stages in a memory clinic setting.

### Method

We examined plasma and CSF samples of 204 memory clinic patients, issued from the Center of Cognitive Neurology, Lariboisière Hospital Paris France, including neurological controls (NC, n=22), patients with AD at MCI stage (AD-MCI, n=42) and dementia stage (n=71), non-AD dementia (n=31), non-AD MCI (n=37). Plasma amyloid beta 1-40/1-42 ratio (Aβ42/Aβ40), phospho-tau181 (p-tau181) and phospho-tau 231 (p-tau231), total tau (t-tau), neurofilament-light (NfL) and glial fibrillary acidic protein (GFAP) were measured using Simoa platform. Correlations to CSF markers, diagnostic performance and association to cognitive measures and morphological imaging were also analyzed.

### Results

Plasma p-tau181, p-tau181 and NfL levels displayed strong correlations with their CSF levels. Plasma p-tau231, p-tau181 and GFAP measured in plasma were specifically increased in AD-MCI and AD-dementia and displayed interesting potential as standalone biomarkers. Combination of biomarkers associating mainly p-tau231, p-tau181, GFAP and NfL improved AD positive and differential diagnosis. Mostly, the association of a plasma p-tau measurement (p-tau231 or p-tau181) with plasma NfL could differentiate the AD and non-AD related etiologies underlying MCI and dementia. Moreover, the same markers associated with cognition and medio-temporal atrophy measured with tools available in clinical routine.

### Conclusion

Plasma p-tau181, p-tau231 and GFAP display high AD diagnosis performance in unselected patients from a memory clinic setting. Combination of plasma biomarkers improve diagnosis accuracy.

**Classification of evidence**

This study provides Class III evidence that plasma p-tau measurements and GFAP have superior positive and differential diagnostic performance for AD compared with plasma amyloid ratio, total tau and NfL, used as single biomarkers in a memory clinic setting and that combinations of biomarkers increased the diagnostic performance.

**Keywords:** Alzheimer's disease; dementia; fluid biomarkers; plasma biomarkers; phospho-tau; memory clinic

**Abbreviations:** AD, Alzheimer's disease; Aβ, amyloid beta; AUC, Area under the curve; CSF, cerebrospinal fluid; FTD, frontotemporal dementia; DLB, dementia with Lewy bodies; GFAP, glial acid fibrillary protein; MMSE, mini mental state examination; NfL, neurofilament light chain; NC, neurological controls; Nf, neurofilament; MCI, mild cognitive impairment; MRI, magnetic resonance imaging; p-tau; phosphorylated tau; PET, positron emission tomography; ROC, Receiver operator characteristic; Simoa, Single molecule array; t-tau, total tau; VaD, vascular dementia.

## INTRODUCTION

Alzheimer's disease (AD) is the commonest neurodegenerative disease and the most frequent cause of dementia. The main clinical phenotype at presentation is the amnestic phenotype with episodic memory impairment. Beyond this typical AD profile, rarer phenotypes such as posterior cortical atrophy, logopenic variant of primary progressive aphasia, frontal variant AD have been identified. Differential diagnosis can also present with close clinical presentation (Encephalopathy, dementia with Lewy bodies, frontotemporal dementia, , vascular dementia Limbic-predominant Age-Related TDP-43…). The overlap in the clinical presentation of different underlying pathologies constitutes a diagnosis for the physician in clinical practice. Additionally, the clinical symptoms can be confounded with non-neurodegenerative causes of cognitive impairment including psychiatric diseases, systemic conditions, sleep apnea... especially at mild cognitive impairment (MCI) stage.

Cerebrospinal fluid (CSF) and positron emission tomography imaging have significantly improved diagnosis and significantly increase the physician's confidence (1,2). However, despite their high accuracy, their utility is limited by cost, invasiveness and accessibility, and therefore restricts their potential as first-line tools for diagnosis and disease monitoring in primary care or memory clinics. However, these limitations would be countered by use of blood-based biomarkers if proven to be comparable to CSF/PET biomarkers and/or if they vastly improve the speed in which an accurate diagnosis can be made. In recent years, the development of ultrasensitive immunoassays and targeted mass spectrometry techniques have allowed for the detection of plasma Aβ (3,4), phosphorylated forms of tau (p-tau), total tau (t-tau) (5–7) as well as neuronal damage (8,9) and astrogliosis (10,11) biomarkers. The accurate quantification of plasma p-tau shows the most potential, as an early and specific biomarker of both amyloid and tau processes in AD (12–14). However, the evaluation of these plasma biomarkers for diagnosis has only been performed in selected research populations, with strict exclusion criteria, and its performance in a heterogeneous sample set is currently unknown. The general population in neurological and memory clinics is typically more complex regarding presentation, comorbidities, and can present with clinically- and biologically divergent and confusing factors that can differ greatly from the well-characterized populations included in research cohorts (15–17). Moreover, diagnosis now tends to be made at the MCI stage or even upon subjective cognitive complaints (18).

Our aim was to compare a series of blood biomarkers for diagnosis of AD in an unselected memory clinic cohort.

## METHOD

### Cohort

To evaluate the potential of these biomarkers for diagnostic use in a routine clinical setting, we conducted this work in a heterogeneous memory-clinic population. This cross-sectional observational study retrospectively included all patients followed for a cognitive complaint who had undergone CSF analysis and CSF and plasma biobanking at the Centre of Cognitive Neurology at Lariboisière Fernand-Widal University Hospital, APHP, Université de Paris, between 17 March 2014 and 19 December 2019. Patients underwent a thorough clinical examination involving personal medical and family histories, neurological examination, neuropsychological assessment, CSF biomarker analysis, magnetic resonance imaging (MRI) of brain structure as well as plasma and CSF biobanking.

Mini-mental state examination (MMSE) was used as a general measure of cognition MRI was obtained during the diagnosis workup, within <1 year of plasma collection. Medio-temporal atrophy was visually rated using the Scheltens visual scale independently by two physicians. Fazekas score for white matter lesions was rated by a single operator (19). All patients were genotyped for *APOE* using standard polymerase chain reaction.

The diagnosis for each patient was made during multidisciplinary consensus meetings (by neurologists, neuropsychologists, gerontologist and biochemist) considering CSF results and according to validated clinical diagnostic criteria for AD dementia, MCI due to AD (AD-MCI), dementia with Lewy Bodies (DLB) or frontotemporal dementia (FTD) (20–23). MCI of other causes (non-AD MCI) included patients with psychiatric disorder, sleep apnea or systemic disease. The individuals with no underlying neurocognitive disease and normal CSF biomarkers profile were enrolled as neurological controls.

### Samples collection

All patients included in this study underwent CSF and plasma examination as part of diagnosis workup. CSF was obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space; samples were immediately centrifuged at 1800xg for 10 min at +4 C, and stored at -80°C pending analysis. Blood samples were collected through venipuncture under fasting condition and collected into ethylenediaminetetraacetic acid (EDTA) tubes. Samples were centrifuged at 2000x*g* for 20 minutes at 4°C. Plasma supernatant was collected and frozen at

−80◦C until further use. Prior to analysis, samples were centrifuged at 2000 × *g* for 10 minutes after thawing at room temperature.

**Biomarkers measurements**

All our biomarkers were measured blinded and randomized in Neurokemi Lab, University of Gothenburg, Sweden. All plasma biomarkers were measured on the Simoa platform, using a Simoa HD-1 analyzer. Plasma Aβ40, Aβ42 and t-tau levels were measured using a commercial Quanterix Triplex kit (Neurology 3-plex A No. 101995).

Plasma p-tau231 and plasma p-tau181 were measured using in-house assays developed on the Simoa platform, whose performances have been already published (12,13). CSF p-tau231 and p-tau181 were measured with the same in-house Simoa assays to assess plasma and CSF correlations. Plasma NfL and GFAP levels were measured using a commercial kit from Quanterix® (2-plex B, No. 103520). CSF GFAP levels were measured with a commercial kit (Quanterix®No. 102336). CSF NfL was measured in CSF using a in-house Elisa from the Neurokemi Lab, University of Gothenburg, as reported by Alcoso *et al.*, (24).

CSF core biomarkers (Aβ42/Aß40 ratio, p-tau181 and t-tau) were measured using the clinically validated Lumipulse® G1200 (Fujirebio). CSF profile was defined according to the following clinically validated cut-offs: Aβ42/Aβ40 >0.61, p-tau <61 ng/L, t-tau< 479 ng/L (25).

**Statistical analysis**

Statistical analysis was performed using SPSS IBM 26.0 (IBM, Armonk, NY), GraphPad Prism 9 (GraphPad Inc., San Diego, CA, USA) and MedCalc Software (Ostend, Belgium). Categorical data (sex, *APOE* ε4 positivity) was analyzed using the χ2 test, and numerical data (age, MMSE score) using Kruskal-Wallis test. Biomarkers were log-transformed prior to analysis. Group differences for biomarkers results were assessed with one-way ANCOVA adjusted for age and sex, with post-hoc Least Signant differenceTukey's test. Correlations between CSF and plasma biomarkers were assessed using Spearman correlation.

Diagnostic accuracy of studied blood biomarkers was assessed with receiver operating characteristic curves (ROC) and binary logistic regression models. Binary logistic regression was performed with diagnosis as the dependent variable, including blood biomarkers and clinical parameters as explicative variables. All models were adjusted for age and sex. Probabilities from the models were saved as variables. ROC analyses were then used to evaluate the performance of each single blood biomarker and of combinations of markers. AUCs were statistically compared using the DeLong Test. Linear regression was used to

examine the association of each biomarker with MMSE score as well as with mediotamporal atrophy Scheltens score. Models were adjusted for age, sex and years of education. An unpaired, two-tailed P-value <0.05 was overall considered to indicate statistical significance.

## RESULTS

### Characteristics of the cohort

Demographics and biomarkers values of our cohort are presented in **Table 1**.

We included 203 participants: 22 neurological controls, 37 patients with non-AD MCI, 42 with AD-MCI and 71 with AD dementia as well as 31 patients with non-AD dementia (**Table 1)**. The other dementia group (n=31) included subjects with FTD (n=X), DLB (n=1X), VaD (n=4) and Creutzfeldt Jakob disease (n=1). Patients with AD-MCI and AD dementia were older than neurological controls and non-AD dementia patients (p<0.001). All biomarkers correlated with age but plasma t-tau. Spearman's rho correlation coefficient with age in the whole sample was: -0.229 for plasma Aβ ratio, 0.296 for plasma p-tau181, 0.260 for plasma p-tau231, 0.430 for plasma NfL and 0.371 for plasma GFAP (all, P<0.001). Regarding association to sex, after adjustment for age, plasma GFAP levels were significantly higher in women (P<0.001). Other CSF or plasma biomarkers, including CSF GFAP, did not show association with sex. Subsequently, all further analysis was adjusted for age and sex.

Plasma Aβ ratio, p-tau181, p-tau231 and GFAP levels were higher in ApoE4 carriers compared to non ApoE4 carriers (all, P<0.001) whereas plasma t-tau and NfL levels were not associated with ApoE4 carriership.

### Association between CSF and plasma biomarkers levels

In the whole cohort, the Spearman *rho* correlation coefficients between the corresponding plasma and CSF biomarkers levels were: 0.36 for Aβ42/Aβ40 ratio, 0.73 for p-tau181, 0.59 for p-tau231, 0.15 for t-tau, 0.67 for NfL and 0.42 for GFAP. No association remained for Aβ42/Aβ40, t-tau and GFAP looking at subgroups. Significant correlations remained between CSF and plasma in both AD-MCI and AD-dementia groups for p-tau181, p-tau231 and NfL.

### Biomarkers levels across clinical groups

Plasma Aβ42/Aβ40 ratio was lower in AD-MCI and AD-dementia compared to NC (P<0.05, Fi. **1**). It was lower in AD-MCI *vs* non-AD MCI (P=0.001). However it did not differ between AD-dementia and non-AD dementia. Plasma p-tau181 and plasma p-tau231 displayed similar

patterns: levels were significantly higher in AD-MCI and in AD-dementia compared to all other groups (p-tau181: P<0.001; p-tau231, P<0.002). Levels did not significantly differ between NC, non-AD-MCI and non-AD dementia. Plasma t-tau levels were higher at dementia stages, in both AD and non-AD dementia, compared to NC (P<0.020). Plasma NfL levels were higher in AD-MCI, AD-dementia and non-AD dementia compared to NC (overall P<0.001). Both AD-MCI and AD-dementia groups displayed higher levels compared to non-AD MCI (P<0.015). Plasma GFAP levels were higher in AD-MCI and AD-dementia compared to all non-AD groups (overall P<0.001). Levels did not significantly differ between non-AD groups.

**Biomarkers diagnosis performance**

We first studied our plasma biomarkers performance as stand-alone tests to differentiate between groups.

To distinguish between AD and controls, the highest AUCs were obtained for plasma p-tau181 (0.95), plasma p-tau231 (0.92) and GFAP (0.91). More moderate AUCs were yielded by plasma Aβ42/Aβ40 (0.80), t-tau (0.83) and NfL (0.87). To differentiate AD-dementia from non-AD dementia, plasma p-tau181 (0.85), p-tau231 (0.81) and GFAP (0.81) still performed the best compared to plasma Aβ42/Aβ40 ratio, t-tau and NfL (0.69-0.72). The same plasma markers performed the best to differentiate AD-MCI from controls, as in dementia stages: p-tau 181 (0.92), p-tau231 (0.89) and GFAP (0.92). Plasma markers displayed overall lower AUCs to distinguish AD-MCI from non-AD-MCI: p-tau181 (0.84), p-tau231 (0.82) and GFAP (0.85) and Aβ42/Aβ40 ratio, t-tau and NfL (0.66-0.73).

The combination of plasma biomarkers increased their diagnosis value. We sought the best performing and parsimonious marker combination using linear regression with stepwise backward elimination. As plasma p-tau181 and p-tau231 showed high correlation to each another (Spearman *rho*=0.72), only one plasma p-tau measure was included in the models. The p-tau markers revealed interchangeable, yielding similar AUCs.

To distinguish AD from NC, the association of p-tau181 and NfL yielded a high performance (AUC=0.97). Adding plasma Aβ ratio, t-tau or GFAP did not improve diagnosis performance. To distinguish AD-dementia from non-AD dementia, the association of p-tau181 and NfL (AUC=0.86) did not differ statistically from the association of p-tau181, NfL and GFAP (AUC=0.91). To differentiate AD-MCI from NC, the association of plasma p-tau181 and NfL showed a high performance (AUC=0.93). Adding plasma GFAP and t-tau increased the AUC to 0.99, but it was not a statistically different from the simpler model combining plasma p-tau181 and NfL.

To discriminate AD-MCI from non AD-MCI, it was the combination of plasma p-tau181, GFAP and NFL that showed good potential (AUC=0.91). The association of p-tau181 and NfL yielded a significantly lower AUC (AUC=0.83). All these combinations significantly outperformed individual biomarkers.

**Association to cognition and medio-temporal atrophy**

Lastly, we investigated the association of our biomarkers with cognition and medio-temporal lobe atrophy. In plasma, the following plasma markers showed an association with MMSE: GFAP (β=-0.249), t-tau (β=-0.248), p-tau231 (β=-0.163) and p-tau181 (β=-0.138). In the AD subgroup, a significant association remained only between MMSE and plasma p-tau231 levels (β=-0.357).

Regarding mediotemporal atrophy, several plasma markers were associated with Scheltens score: Nfl (β=0.321), GFAP (β=0.284), p-tau181 (β=0.155) and p-tau231 (β=0.150). In the AD subgroup, a significant association remained between the Scheltens score and plasma NfL (β=0.272) and plasma GFAP (β=0.0.221).

## DISCUSSION

In the present study, we investigated the diagnostic potential of a series of plasma biomarkers in a real-world clinical setting. While recent advancements in PET and CSF biomarker analysis have considerably improved the accuracy within the AD diagnostic process, these methods have some limitations in a routine clinical setting including high cost, limited availability and invasiveness (26,27). In response to the aforementioned limitations, blood biomarkers appear as highly promising alternatives (12–14,28,29). In this work, we investigated a series of plasma and CSF biomarkers of different AD pathophysiological processes and their relationship with cognition in our memory-clinic based cohort. We observed that p-tau231, p-tau181 and GFAP measured in plasma displayed high specificity to AD in unselected patients. Combination of biomarkers associating mainly p-tau231, p-tau18, GFAP and NfL improved AD positive and differential diagnosis. Mostly, the association of a plasma p-tau measurement (p-tau231 or p-tau181) with plasma NfL could differentiate the AD and non-AD related etiologies underlying MCI and dementia. Moreover, the same markers associated with cognition and imaging measured with tools available in clinical routine.

The pressing need of additional biomarkers in neurocognitive disorders led us to move from CSF to blood biomarkers offering better accessibility. However, to what extent blood biomarkers constitute an accurate proxy for CSF biomarkers is not entirely established. Plasma Aβ measurements, t-tau and GFAP displayed a weak association with their CSF levels while plasma p-tau measurements (p-tau181, p-tau231), plasma Nfl and NFh, had a higher one. Plasma amyloid is known to be sensitive to peripheral processing and to confounding Abeta coming from the periphery (30,31). Similarly, plasma t-tau only reflects very partially CNS changes as most plasma tau measured is issued from non-neurological organs (heart, testis)(32,33). Plasma GFAP also showed weak correlation to its CSF counterpart, but surprisingly it seemed to have more discriminative power in NDD than CSF. This was already reported and it is suspected to be multifactorial, including mostly AD-related BBB alterations, direct glymphatic release and higher sensibility of CSF GFAP to pre-analytical conditions (34–36). Conversely p-tau measurements and NfL appear to reflect robustly CSF alterations. Tau fragments can be detected in CSF and plasma using mass spectrometry and among these forms, p-tau species including p-tau181 and p-tau217 showed a high correlation between CSF and plasma (33,37). Similarly, plasma NfL levels showed a significant correlation with their CSF levels in line with numerous existing studies, despite being several fold lower than their CSF levels.

As for diagnosis purpose, plasma p-tau measurements could best identify AD, both at MCI and AD stage, as stand-alone markers. They showed high AUCs to discriminate AD vs non-AD cause of cognitive impairment, which is the question to which physicians are actually facing in clinical practice. Increased levels of plasma p-tau in AD have been demonstrated in numerous studies, across multiple analytical platforms (12–14,38,39). Blood p-tau measurements also appear specific to AD: p-tau181, p-tau217 and p-tau231 have been shown to accurately discriminate between AD and non-AD cases, not only when diagnosed clinically but also when neuropathologically confirmed (40,41). In a neuropathological study on 312 individuals, plasma p-tau181 and plasma p-tau231 showed the strongest overall sensitivity and specificity for AD neuropathological changes compared with plasma Aβ42/Aβ40, t-tau and NfL (40). Additionally, p-tau181 and p-tau231 did not significantly differ in performance and displayed similar results in biomarkers combination. This is supported by the existing literature, showing that p-tau181, p-tau217 and p-tau231 could be used interchangeably in symptomatic AD (39). In comparative studies, p-tau231 seems to change the earliest, followed by p-tau217 and then p-tau181. However, these changes occur already at preclinical stage,

whereas currently in the absence of validated treatment, diagnosis in clinical settings is made in presence of overt symptoms (42). The second-best performing biomarker studied in paper V was plasma GFAP, coherently with our finding in paper III. It displayed better performance than its CSF levels. In a recent study, GFAP had the highest AUC in differentiating between Aβ+ and Aβ – cognitively impaired older adults, compared to other plasma biomarkers (43). Its strong association to CSF AD core biomarkers could suggest that it is a biomarker of astrocytic response due to Aβ rather than a general marker of astrogliosis in a context of neuroinflammation (28,44). Plasma Aβ42/Aβ40 measures showed significant diagnostic performance but were outperformed by p-tau measurements as already reported in the literature. We observed a small fold change between Aβ-positive and Aβ-negative patients in our clinical cohort, resulting in large overlap between the groups. This also seems to align with the AD pathophysiology, with Aβ proteins starting to change and plateauing early, making them less informative at later stages of overt cognitive change, while p-tau continues to increase through the AD continuum to the dementia (45). Plasma NfL, as a general marker of neurodegeneration, differentiated well AD-MCI and AD-dementia from control subjects, but not from non-AD dementia.

We explored whether a combination of blood biomarkers could be useful in AD diagnosis for clinical use. In paper V, the association of biomarkers increased diagnosis accuracy. To differentiate AD-dementia from non-AD dementia, a promising AUC was found of 0.86 for the combination of p-tau181 and NfL. At MCI stage, the association of three biomarkers reached an AUC of 0.90 to identify AD (p-tau181, NfL and GFAP). There is prior evidence in the literature that combination of plasma biomarkers, generally reflecting different pathophysiological processes of AD, could accurately predict cognitive decline and conversion to dementia (46–48).

However, our plasma biomarkers performance remained overall slightly lower than their demonstrated performance in research cohorts. Plasma p-tau measurements used as standalone markers yielded AUCs between 0.90-0.96 in differentiating AD-dementia vs other dementia in selected research cohorts (12,14,38). Similarly, the performance of plasma Aβ and NfL were lower than previously reported (29). Several hypotheses can be made on these findings. Most research has been conducted in relatively healthy individuals, apart from their neurocognitive disorder. Conversely, evidence exists that there is a high prevalence of comorbid medical conditions and of somatic complaints among people with dementia (49,50). Pre-existent neuropsychiatric conditions such as depression, bipolar disorder, neurovascular events or

alcohol misuse are highly frequent comorbidities encountered in clinical (50). It has been shown that those conditions can affect biomarkers measurements. Heart failure and kidney or liver failures have been shown to alter plasma p-tau levels through modification of protein metabolism in plasma (51,52). A capital point will be, for each marker, to understand if comorbidities confound the interpretation of the biomarker levels or if comorbidities affect the underlying process measured by the biomarker. Additionally, variability in pre-analytical conditions in clinical routine could affect the biomarker performances, in comparison to highly controlled research settings.

Exploration of plasma biomarkers association with cognitive status can inform on their potential value in monitoring cognitive decline and therapeutical response in clinical trial.
High GFAP, p-tau and t-tau plasma concentrations were independently associated with worse cognitive performance at a cross-sectional level. Plasma p-tau measurements showed a significant association with cognition, adding to the existing evidence of their clinical relevance. Plasma p-tau concentrations increase with clinical disease severity in large cohort studies (12,13). Longitudinal measurements of plasma p-tau have also shown associations with cognition (47,53). All in all, our results and the literature point out to a probable high utility of p-tau biomarkers to monitor and predict cognitive decline in clinical settings.

Regarding imaging, plasma NfL showed a significant association with medio-temporal atrophy. There is significant evidence in the literature that NfL levels, in CSF and plasma, associate with global brain atrophy (8,54). Higher NfL levels are associated to the progression of brain atrophy in AD but also in other dementia (55,56). Plasma p-tau measurements showed significant association with Scheltens score. Plasma p-tau231 and p-tau181 levels have previously been reported to associate with hippocampal atrophy (12,57,58). Plasma GFAP levels correlated positively with mediotemporal atrophy. In line with those findings, plasma GFAP associated with higher gray matter volumes at the earliest stages of the AD continuum, which reverted later during the course of the disease (59). Overall, these findings indicate that, despite not providing structural information, plasma p-tau measurements, NfL and GFAP levels associate, at least to some extent, with neurodegeneration. Therefore, they can be useful and cost-effective biomarkers to predict AD-related neurodegeneration.

The strengths of this study include a large, well-characterized cohort which is representative of a memory clinic population. In addition, all included patients were diagnosed using

established CSF biomarkers for AD, confirming that cognitive impairment was related to AD disorder and allowing addressing relationship of plasma biomarkers to cerebral pathology. A high variability is often observed between the results from specific research cohorts and their utility in clinical practice, possibly related to inconsistencies in clinical diagnosis criteria and practice, sample availability, and pre-analytical as well as analytical conditions. This study, performing a head to head comparison of plasma biomarkers in a 'real-life' unselected clinical cohort, could contribute to the translation of this biomarker from bench side and research cohorts to clinical settings.

Regarding limitations, some diagnostic groups were relatively small, especially the neurological controls groups non-AD neurodegenerative disease groups. Future studies are needed to compare plasma biomarkers for differential diagnosis of AD (for example, distinguishing AD from FTD or DLB). Another limitation was that we did not include longitudinal samples, which are necessary to determine the usability in clinical settings for screening in primary care, prediction of cognitive evolution and evaluation of therapeutic answer in clinical trials. Nevertheless, some of our biomarkers showed association with cognitive score encouraging its evaluation in longitudinal studies. Further studies using larger sample sizes are needed to properly characterize the relationship between plasma markers and cognitive and imaging findings across time. Finally, our findings should also be replicated in other clinical settings, including a primary care.

A number of recent studies have demonstrated that blood-based biomarkers have the potential to improve detection and diagnosis of AD by increasing convenience, acceptability and ease of testing, as well as reducing costs. The differential diagnosis of AD is highly clinically important to administer suitable symptomatic treatment at the earliest stage but also to indicate a non-AD dementia which would require further investigation (e.g., FDG-PET). The importance of an accurate differential diagnosis is also crucially important for caregiver counseling and prognosis. Our data indicates that plasma p-tau measurements and GFAP demonstrate high accuracy as a standalone test alone for to differentiate patients with MCI-AD and AD dementia from those with other cognitive disorders. Additionally, combination of markers increased diagnosis performance. Thus, blood biomarkers have the potential to be used in screening in primary care or in patients not suitable for CSF testing in specialist memory clinics.

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HZ has served at scientific advisory boards for Alector, Eisai, Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, Nervgen, AZTherapies and CogRx, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work); CP is a member of the International Advisory Boards of Lilly; is a consultant for Fujiribio, Alzhois, Neuroimmune, Ads Neuroscience, Roche, AgenT, and Gilead; and is involved as an investigator in several clinical trials for Roche, Esai, Lilly, Biogen, Astra-Zeneca, Lundbeck, and Neuroimmune; KB has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Prothena, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).

**Author's contribution**

AV: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content, study concept or design, analysis or interpretation of data. NJA: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content, study concept or design, analysis or interpretation of data. TKK: Drafting/revision of the manuscript for content, including medical writing for content, study concept or design, analysis or interpretation of data. JLR: Major role in the acquisition of data Drafting/revision of the manuscript for content, including medical writing for content, study concept or design, analysis or interpretation of data. KG: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content. EC: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content. ML: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content. CH: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content. JD: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including medical writing for content. HZ: Drafting/revision of the manuscript for content, including medical writing for content, study concept or design. CP: Major role in the acquisition of data, Drafting/revision of the manuscript for content, including

medical writing for content, study concept or design, analysis or interpretation of data. KB: Drafting/revision of the manuscript for content, including medical writing for content study concept or design, analysis or interpretation of data.

| n=203 | **Controls** n=22 | **Non-AD MCI** n=37 | **AD-MCI** n=42 | **AD dementia** n=71 | **Non-AD dementia** n=31 | **P-value** |
|---|---|---|---|---|---|---|
| Age, years | 63.8 (9.8) | 67.8 (9.1) | 72.3 (8.0) | 72.4 (8.3) | 65.0 (7.3) | P<0.001 |
| Male, % (n) | 36% (8) | 35% (13) | 38% (16) | 38% (27) | 48% (15) | 0.822 |
| APOE ε4 carriership, % (n) | 36% (8) | 11% (4) | 60% (25) | 66% (47) | 26% (8) | P<0.001 |
| Level of education, years | 13.0 (3.0) | 11.6 (3.1) | 13.2 (2.3) | 10.5 (3.7) | 12.4 (3.4) | P<0.001 |
| MMSE score | 27.4 (2.4) | 24.0 (3.5) | 23.4 (4.6) | 19.3 (5.6) | 23.9 (5.1) | P<0.001 |
| **CSF and plasma biomarkers** | | | | | | |
| CSF Aβ42, pg/mL, Lumipule® | 0.095 (0.010) | 0.0874 (0.015) | 0.047 (0.014) | 0.0423 (0.009) | 0.089 (0.014) | P<0.001 |
| CSF Aβ42/Aβ40 , Lumipulse® | 1145.0 (300.4) | 1068.3 (464.0) | 590.1 (343.9) | 507.1 (165.5) | 973.3 (391.2) | P<0.001 |
| CSF p-tau, pg/mL, Lumipulse® | 33.7(9.2) | 38.8 (17.8) | 91.1 (48.5) | 114.4 (60.1) | 34.2 (11.1) | P<0.001 |
| CSF t-tau, pg/mL, Lumipulse® | 255.6 (78.2) | 306.0 (144.8) | 589.4 (286.12) | 727.5 (394.1) | 359.0 (343.1) | P<0.001 |
| CSF N-term p-tau181, pg/mL, Simoa | 225.5 (65.4) | 273.6 (167.4) | 918.8 (635.4) | 1232.1 (813.6) | 261.5 (122.8) | P<0.001 |
| CSF N-term p-tau231, pg/mL, Simoa | 273.1 (63.8) | 311.8 (143.0) | 637.8 (215.0) | 811.8 (651.6) | 299.3 (118.8) | P<0.001 |
| CSF NfL, pg/mL | 13.4 (6.2) | 22.1 (18.1) | 26.2 (11.9) | 25.3 (11.6) | 28.6 (26.8) | P<0.001 |
| CSF GFAP, pg/mL | 2281.7 (2142.1) | 2885.8 (2265.9) | 4549.4 (3450.9) | 4676.7 (3797.3) | 1988. 0 (1380.6) | P<0.001 |
| Plasma Aβ42/Aβ40, pg/mL | 0.625 (0.088) | 0.601 (0.095) | 0.515 (0.121) | 0.544 (0.085) | 0.581 (0.122) | P<0.001 |
| Plasma p-tau181, pg/mL | 3.3 (1.9) | 5.6 (3.6) | 11.4 (6.2) | 13.0 (7.1) | 4.8 (3.6) | P<0.001 |
| Plasma p-tau231, pg/mL | 8.0 (3.8) | 11.4 (6.6) | 18.6 (8.5) | 21.4 (9.1) | 11.6 (6.9) | P<0.001 |
| Plasma t-tau, pg/mL | 0.243 (0.109) | 0.321 (0.137) | 0.297 (0.111) | 0.358 (0.179) | 0.357 (0.252) | P<0.001 |
| Plasma GFAP, pg/mL | 13.4 (6.2) | 22.1 (18.1) | 26.2 (11.9) | 25.3 (11.6) | 28.6 (26.8) | P<0.001 |
| Plasma NFL, pg/mL | 158.3 (73.4) | 189.4(86.2) | 366.0(158.8) | 377.1 (180.9) | 187.4 (109.2) | P<0.001 |

**Table 1, Demographics and biomarkers values across groups**

Data is shown as mean (SD) or n (%), as appropriate. Kruskall Wallis test was used to compare age, education years and MMSE between groups and Pearson's chi-square to compare sex and *APOE*-ε4 frequencies between groups. Fluid biomarkers levels were compared with a one-way ANCOVA adjusted by age and sex and followed by false discovery rate (FDR) multiple comparison correction.

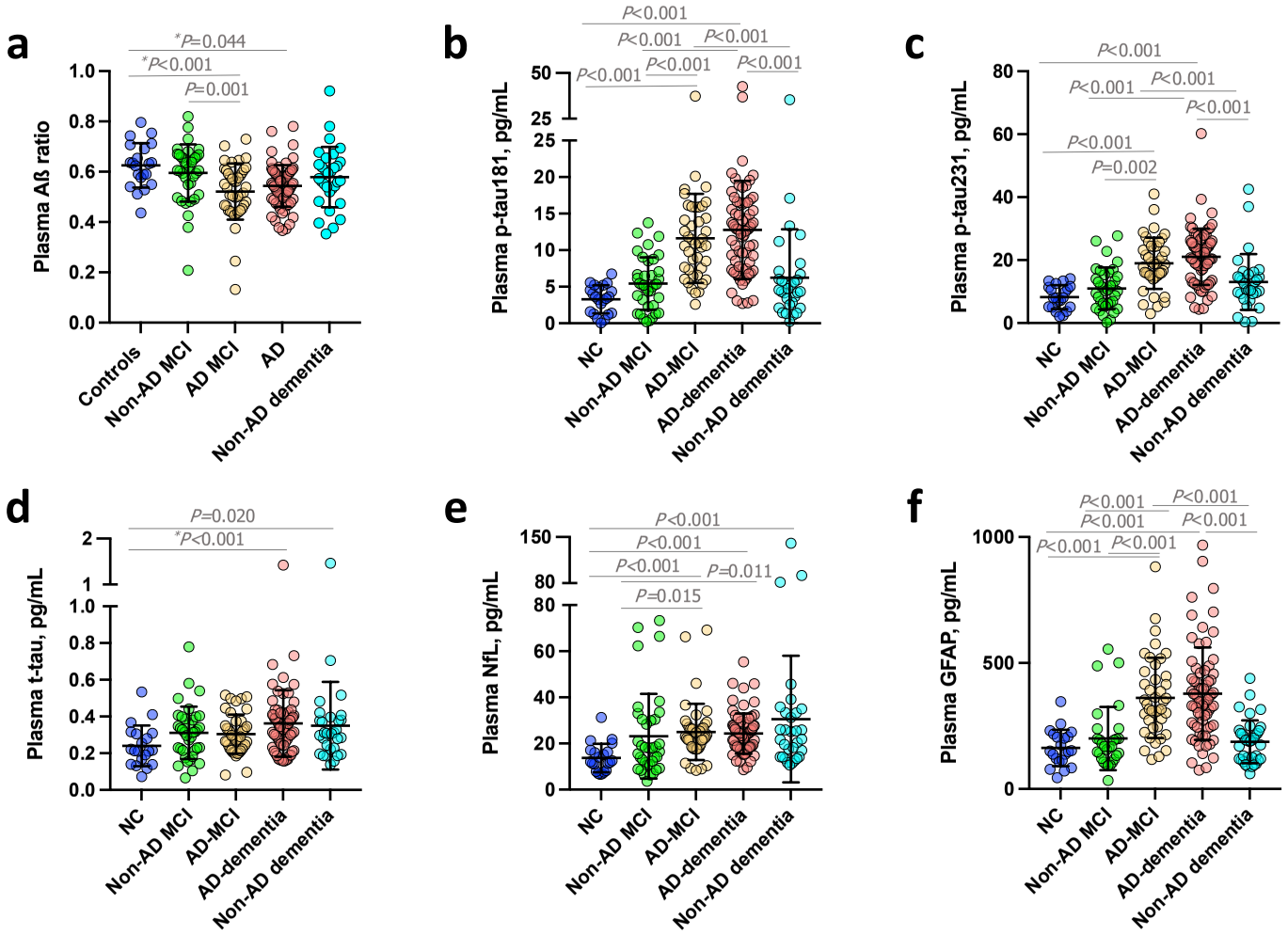


**Figure 1, Levels of plasma biomarkers across clinical groups**

**a**) Plasma Aβ42/Aβ40 ratio levels; **b)** Plasma p-tau181 levels; **c**) Plasma p-tau231 levels; **d**) Plasma t-tau levels; **e**) Plasma NfL levels; **f**) Plasma GFAP levels across diagnosis groups. The box-plots depict the median (horizontal bar), interquartile range (hinges) and maximum value (whiskers). Differences across groups were analyzed using one-way oncova adjusted on age and sex with post hoc analysis using Tukeys, adjusted for multiple comparisons. A P<0.05 was considered overall significant.

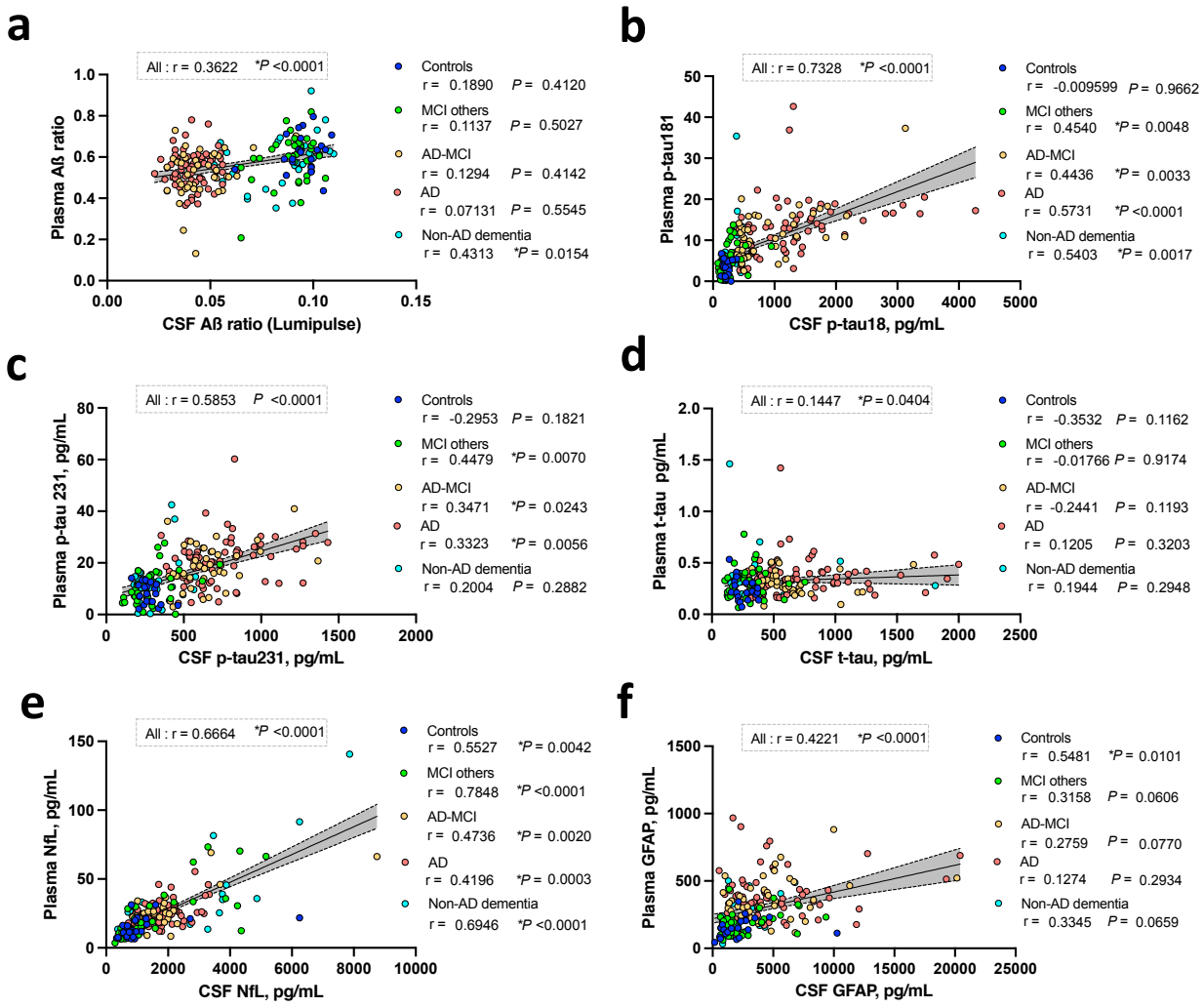


**Figure 2, Correlation between CSF and plasma markers**

Scatter plots depicting the correlations between CSF and plasma levels for: **a)** Aβ42/Aβ40 ratio; **b)** p-tau181; **c)** p-tau231; **d)** t-tau; **e)** NfL; **f)** GFAP, in the whole cohorts.
Correlations were assessed with Spearman rank test correlation coefficient (r) in the whole population and for each diagnostic groups (controls, non-AD MCI, AD-MCI, AD dementia and non-AD dementia).

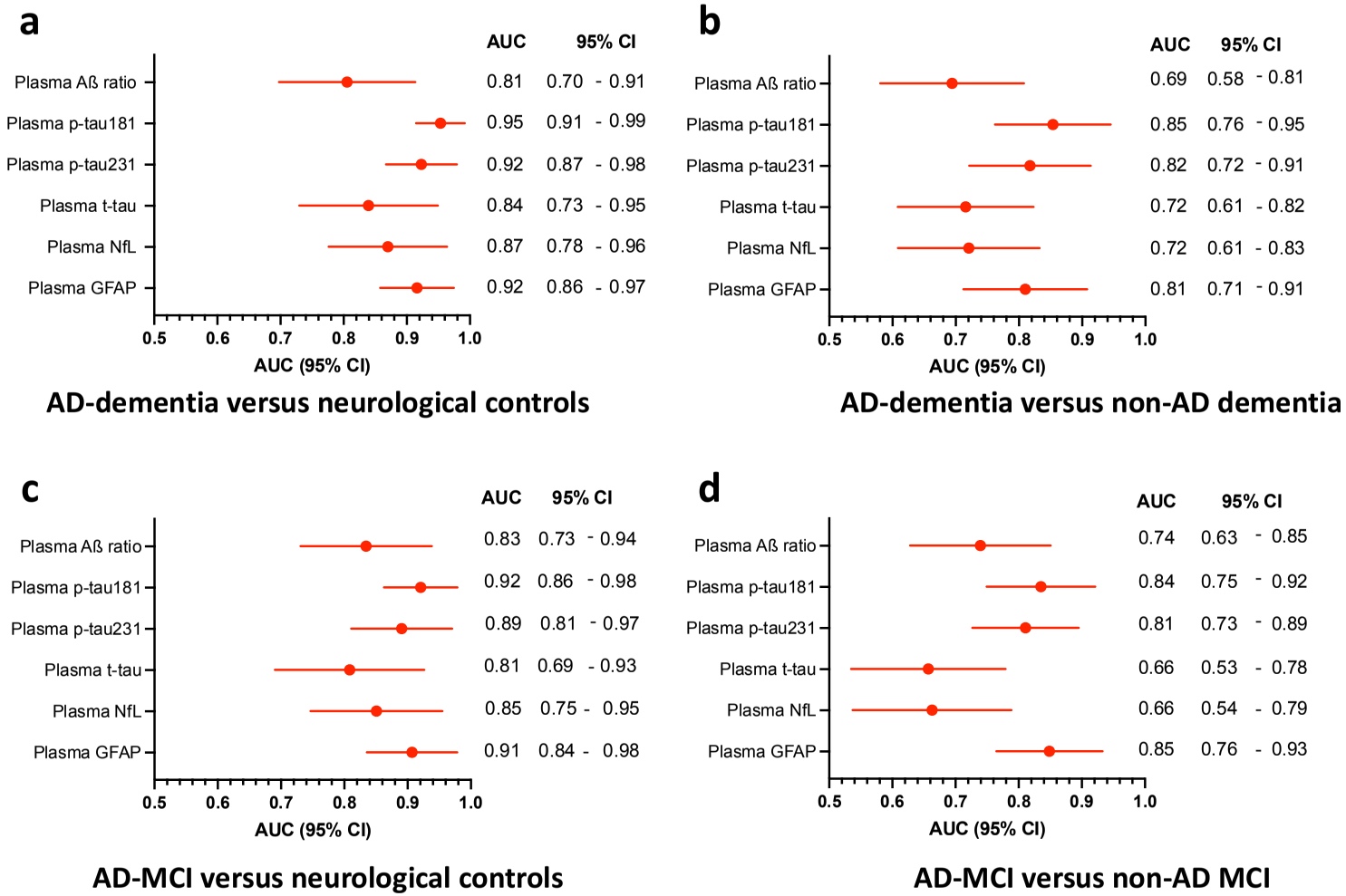


**Figure 3, Diagnostic performance of plasma biomarkers**

Forest plot of AUCs of plasma biomarkers in **a)** differentiating patients with AD from neurological controls; **b)** differentiating patients with AD dementia from patients with non-AD dementia; **c)** differentiating AD-MCI from neurological controls; **d)** differentiating AD-MCI from non-AD MCI. AUCs were computed in ROC analysis, after adjustment for age and sex.

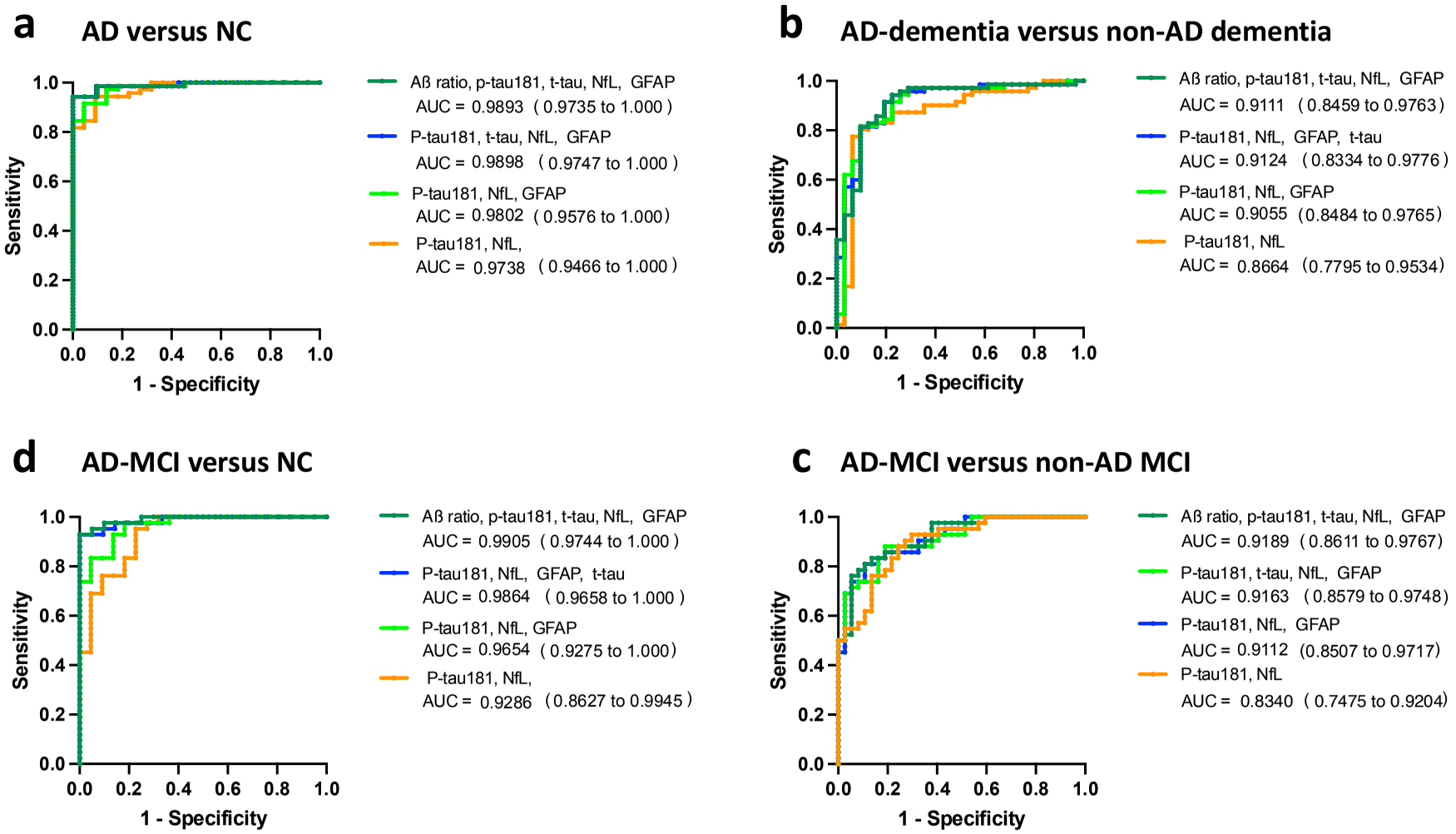


**Figure 4, Performance of combinations of plasma biomarkers for AD diagnosis**

Diagnostic accuracies of combinations of markers in **a)** separating between patients with AD dementia and neurological controls; **b)** separating between patient with AD dementia and patients with non-AD dementia (including DLB, FTD and VaD); **c)** separating between patients with AD-MCI and neurological controls; **d)** separating between patients with AD-MCI and patients with non-AD MCI. Models were computed using backward stepwise elimination in linear regression, adjusted for age and sex.

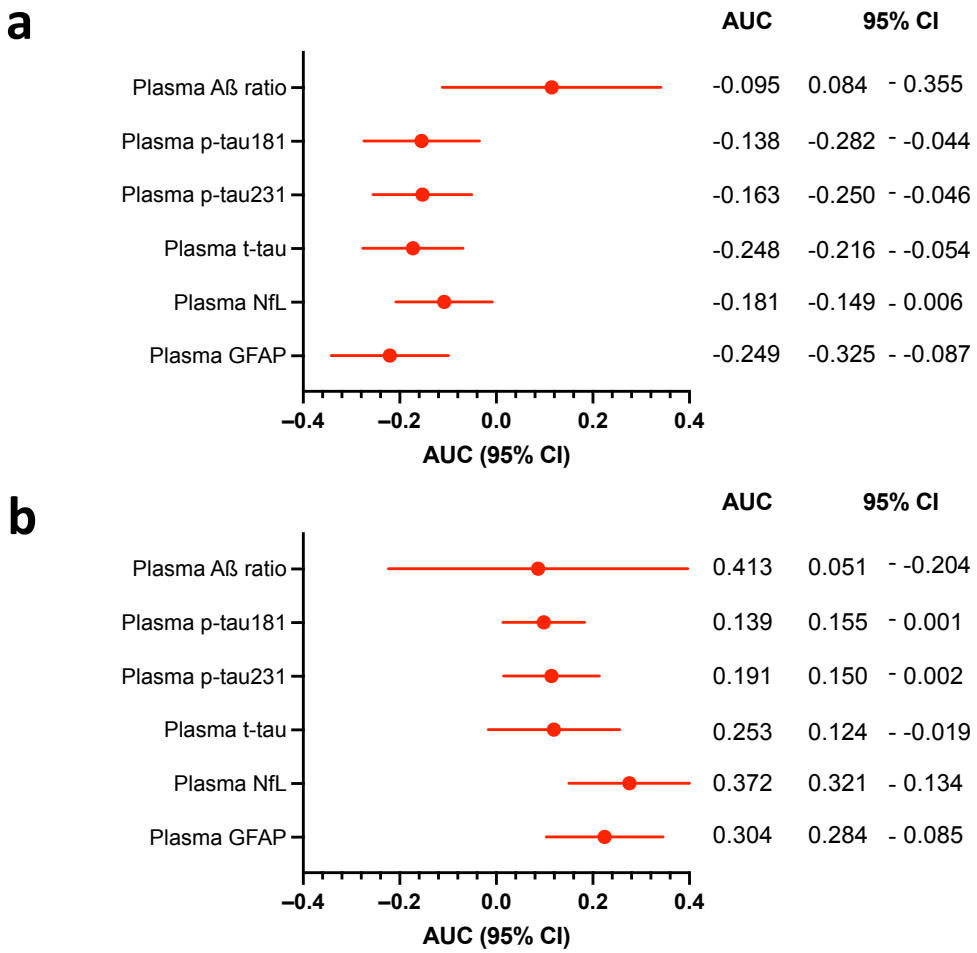


**Figure 5, Association of CSF and plasma biomarkers with MMSE and Scheltens scores**

**a)** Forest plot of standardized ß estimates of the association of CSF and plasma biomarkers with MMSE score in the whole sample; **b)** Forest plot of standardized ß estimates of the association of plasma biomarkers with Scheltens score in the whole sample.
Association between MME and Scheltens score with plasma biomarkers was studied using linear regression adjusted on age and sex. CI, 95 confidence interval; stand. ß: standardized beta estimates.