

**PLASMA AND CSF BIOMARKERS IN SPORADIC
AND DOWN SYNDROME–ASSOCIATED ALZHEIMER'S DISEASE:
FROM DIAGNOSTIC VALIDATION TO CLINICAL IMPLEMENTATION**

Javier Arranz Martínez

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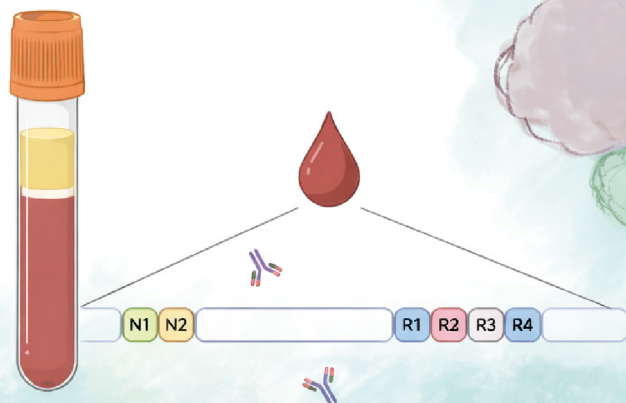
Doctoral Program in Medicine
Department of Medicine
Universitat Autònoma de Barcelona

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Barcelona, 2026

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Barcelona, 2026

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ACKNOWLEDGEMENTS

A María, por estos once años.

A Carmen, por darle color a todo. Vas a ser una hermana mayor maravillosa.

A mis padres, Irene y Julio, y a mi hermano, Álvaro. Por no entender qué hacía y aún así apoyarme.

A mis suegros, Maritere y a Bernardino, por su apoyo (y la comida). Los pajaritos también cantarán en Alicante.

A Tofu, por salvarnos, y por ser mi estufa durante mis batallas con R.

A la Unidad de Memoria del Hospital de la Santa Creu i Sant Pau y a la Unidad Alzheimer Down. Vais a ser siempre mi referencia.

A l@s *fellows*. Habéis sido como mi familia. Sois la secret sauce de la unidad.

A Mireia y a Rosa. A Nick y a Hanna. Esta tesis es gracias a vosotros/as.

A Dani y a Juan por haberme dirigido esta tesis. Habría necesitado cinco vidas para aprender todo lo que quería de vosotros. Seguiré haciendo todo lo que pueda en esta.

A Eva y a todas las personas que me han ayudado a llevar este frenético depósito a término.

Un abrazo a todos/as, y muchas gracias.

Nos vemos por Alicante.

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
DSAD	Down Syndrome-associated Alzheimer's disease
FTD	Frontotemporal dementia
LBD	Lewy body disease
CSF	Cerebrospinal Fluid
Aβ	Amyloid Beta
AA	Alzheimer's Association
IWG	International Working Group
pTau	plasma phosphorylated tau
PET	Positron Emission Tomography
FDG	Fluorodeoxyglucose
BMI	body mass index
NfL	Neurofilament Light
GFAP	Glial fibrillary acid protein
GBSC	Global Biomarker Standardization Consortium
Simoa	Single molecule array
IP-MS	Immunoprecipitation Mass Spectrometry
A	Amyloid
T	Tau
N	Neurodegeneration
BBB	Blood-brain barrier
DS	Down Syndrome

APP	Amyloid Precursor Protein
CDR-SoB	Clinical Dementia Rating Scale Sum of Boxes
iADRS	integrated Alzheimer's Disease Rating Scale
DMT	Disease Modifying Therapies
MRI	Magnetic Resonance Imaging
CV	Coefficient of variation
ECLIA	Electrochemiluminescence Immunoassay
CLEIA	Chemiluminescent Enzyme Immunoassay
LC-MS/MS	Liquid Chromatography - Tandem Mass Spectrometry
NULISA	Nucleic Acid Linked Immuno-Sandwich Assay
ALS	Amyotrophic Lateral Sclerosis
ELISA	Enzyme-Linked Immunosorbent Assay
HISCL	High-Sensitivity Chemiluminescent Immunoassay
MTBR	Microtubule-Binding Region
CNS	Central Nervous System
ARIA	Amyloid-related imaging abnormalities
AUC	Area Under the ROC Curve
UGOT	University of Gothenburg

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ABSTRACT

Blood-based biomarkers are transforming the diagnostic landscape of Alzheimer's disease (AD), providing a minimally invasive and scalable alternative to cerebrospinal fluid (CSF) analysis and positron emission tomography. Among these biomarkers, plasma phosphorylated tau at threonine 217 (pTau217) has consistently demonstrated high diagnostic accuracy. Nevertheless, its translation into routine clinical practice requires validation of both its diagnostic performance and its analytical robustness under real-world conditions.

This doctoral thesis evaluates the clinical utility of plasma pTau217 across complementary methodological and translational settings, with a particular focus on its measurement using fully automated immunoassay platforms.

First, we assessed the diagnostic performance of plasma pTau217, pTau181, and amyloid- β ratios measured on the LUMIPULSE automated platform in a consecutive memory clinic cohort undergoing lumbar puncture. Plasma pTau217 showed the highest accuracy for identifying CSF-defined amyloid pathology, outperforming other plasma biomarkers and achieving robust global accuracy using a two-threshold approach, particularly in symptomatic stages of the disease

Second, we prospectively evaluated the analytical and pre-analytical stability of plasma pTau217 in a tertiary memory clinic setting. Plasma pTau217 demonstrated low lot-to-lot analytical variability and minimal sensitivity to short-term storage conditions. Importantly, previously established diagnostic cut-offs maintained high accuracy when applied prospectively, supporting the reliability of this biomarker for routine clinical implementation

Finally, we examined the diagnostic performance of plasma pTau217 in individuals with Down syndrome, a population with a genetically determined form of AD and unique diagnostic challenges. In a large cohort including

individuals with Down syndrome and euploid controls, plasma pTau217 accurately discriminated amyloid-positive from amyloid-negative individuals and differentiated asymptomatic, prodromal, and dementia stages with high precision, demonstrating comparable or superior performance to other plasma biomarkers across the AD continuum.

Overall, this thesis provides comprehensive evidence supporting plasma pTau217 as a reliable, accurate, and clinically feasible biomarker for the detection of AD pathology across diverse populations and real-world settings, paving the way for its integration into routine diagnostic algorithms and preventive clinical trials.

RESUMEN

Los biomarcadores en sangre están transformando el panorama diagnóstico de la enfermedad de Alzheimer (EA), al proporcionar una alternativa mínimamente invasiva y escalable al análisis del líquido cefalorraquídeo (LCR) y a la tomografía por emisión de positrones. Entre estos biomarcadores, la tau fosforilada en treonina 217 en plasma (pTau217) ha demostrado de forma consistente una elevada precisión diagnóstica. No obstante, su traslación a la práctica clínica rutinaria requiere la validación tanto de su rendimiento diagnóstico como de su robustez analítica en condiciones reales.

Esta tesis doctoral evalúa la utilidad clínica de pTau217 plasmática en distintos contextos metodológicos y traslacionales complementarios, con especial énfasis en su medición mediante plataformas de inmunoensayo totalmente automatizadas.

En primer lugar, se evaluó el rendimiento diagnóstico de pTau217, pTau181 y la ratio de beta-amiloide en plasma medidos en la plataforma automatizada LUMIPULSE en una cohorte consecutiva de una unidad de memoria. La pTau217 plasmática mostró la mayor precisión para identificar patología amiloide definida por LCR, superando a otros biomarcadores plasmáticos y alcanzando una elevada precisión global mediante el empleo de dos puntos de corte, especialmente en las fases sintomáticas de la enfermedad.

En segundo lugar, se evaluó de forma prospectiva la estabilidad analítica y preanalítica de la pTau217 plasmática en un entorno de unidad de memoria de tercer nivel. La pTau217 plasmática mostró una baja variabilidad analítica entre lotes de reactivos y una sensibilidad mínima a las condiciones de almacenamiento a corto plazo. De forma relevante, los puntos de corte diagnósticos previamente establecidos mantuvieron una alta precisión cuando se aplicaron de manera prospectiva, lo que respalda la fiabilidad de este biomarcador para su implementación en la práctica clínica habitual.

Por último, se examinó el rendimiento diagnóstico de pTau217 en plasma en personas con síndrome de Down, una población con alto riesgo genéticamente determinado de enfermedad de Alzheimer y con desafíos diagnósticos específicos. En una amplia cohorte que incluyó personas con síndrome de Down y controles euploides, pTau217 en plasma discriminó con precisión entre individuos amiloide-positivos y amiloide-negativos, y diferenció con alta exactitud las fases asintomática, prodrómica y de demencia, mostrando un rendimiento comparable o superior al de otros biomarcadores plasmáticos a lo largo del continuo de la enfermedad de Alzheimer.

En conjunto, esta tesis aporta evidencia sólida que respalda la pTau217 plasmática como un biomarcador fiable, preciso y clínicamente factible para la detección de la patología de la enfermedad de Alzheimer en poblaciones diversas y en contextos reales, allanando el camino para su integración en los algoritmos diagnósticos de práctica clínica rutinaria y en ensayos clínicos preventivos.

INTRODUCTION

1. INTRODUCTION

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder. With the advent of disease-modifying therapies, early identification of AD has become essential, as patients who receive treatment at the earliest stages are expected to obtain the greatest clinical benefit(1). For this reason, blood biomarkers, due to their accessibility and diagnostic performance, in some cases comparable to cerebrospinal fluid (CSF)(2), are emerging as a highly valuable tool for identifying patients eligible for treatment in the early phases of the disease.

1.1. History of Alzheimer's Disease

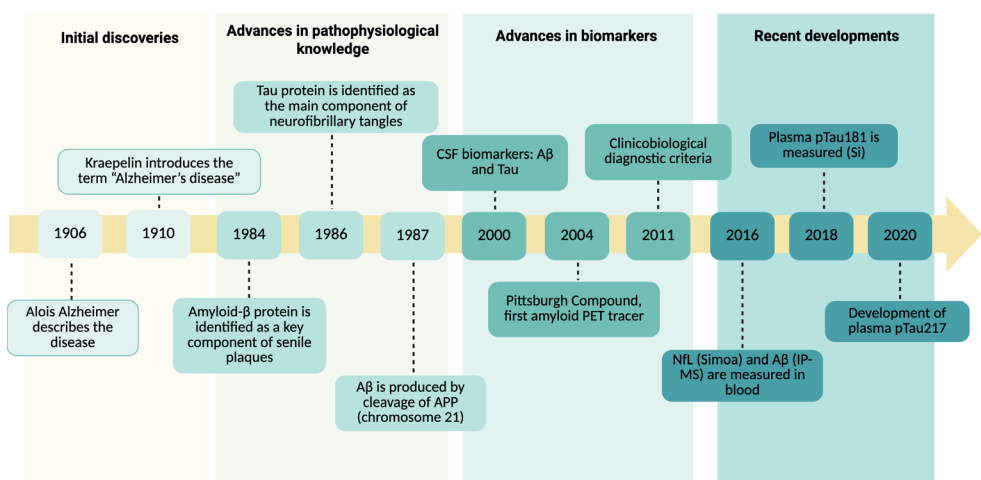


Figure 1. Key milestones in Alzheimer's disease biomarker development. Image created with BioRender.com

Alzheimer's disease was first described in 1907 by Alois Alzheimer, who identified amyloid plaques and neurofibrillary tangles in the brain of a patient with cognitive impairment. Although it was initially considered a rare form of presenile dementia, in the 1960s and 1970s it was demonstrated that it shared features with senile dementia, consolidating it as the most common

cause of dementia(3). In the following decades, the major pathological proteins constituting neuritic plaques and neurofibrillary tangles (beta-amyloid and tau, respectively) were identified(4,5), genetic mutations were discovered(6,7), and the amyloid cascade hypothesis was formulated(8). From the 1990s onward, the development of CSF biomarkers(9) and PET imaging(10) enabled the in-vivo detection of these aggregates. Since 2018(11), there has been ongoing debate about whether AD should be considered a clinical-biological construct (International Working Group diagnostic criteria 2024)(12) or a biological process identifiable through biomarkers even in the absence of clinical symptoms (Alzheimer's Association diagnostic criteria 2024)(13). Figure 1 shows an infographic of the key milestones in AD biomarker development.

1.2. Epidemiology and economic burden

AD is highly prevalent, and its prevalence increases exponentially with age. In Spain, more than 700,000 individuals are currently affected, and this number is projected to double by 2050, surpassing 1.5 million cases(14). Globally, AD and other dementias affect 57 million people (2021), with estimates exceeding 130 million by 2050, particularly impacting low- and middle-income countries (World Health Organization).

The economic burden associated with AD is substantial and among the highest of all chronic diseases, largely driven by informal care and the loss of productivity experienced by both patients and caregivers(15). The continued rise in AD prevalence therefore represents a significant healthcare and economic challenge.

In this setting, plasma biomarkers are gaining importance as they offer more accessible, less invasive, and potentially cost-effective approaches for early detection, complementing current diagnostic techniques and supporting improved clinical planning.

1.3. Established Alzheimer's Disease Biomarkers: PET and CSF

Until the 2024 update of the diagnostic criteria by the Alzheimer's Association (AA) and the International Working Group (IWG), the primary biomarkers of AD pathophysiology were amyloid positron emission tomography (PET) and CSF, which had been integrated within the AT(N) framework as markers of amyloid pathology (A) and tau pathology/neurodegeneration (T/N).

Regarding CSF, A β peptides and pTau were identified in this matrix in the year 2000(16,17). Initially, they were measured using immunoassay techniques (sandwich ELISA), which have been progressively refined and are now quantified on automated laboratory platforms, substantially reducing analytical variability and enabling their implementation in clinical practice(18). Their main limitation is the requirement of a lumbar puncture, which is a (minimally) invasive procedure. Other specific markers of AD are PET with specific tracers that track amyloid and tau pathology.

1.4. The path toward blood biomarkers

In 2018, plasma phosphorylated tau 181 (pTau181) was identified as a useful blood biomarker for detecting AD pathology(19). In 2020, studies showed that plasma pTau181 levels can predict long-term cognitive decline in cognitively unimpaired older adults and are significantly elevated in patients with Alzheimer's disease(20).

In 2020, pTau217 was also established as a highly specific blood biomarker for Alzheimer's disease(21–23), showing stronger correlations with amyloid PET and CSF A β 42 than pTau181.

1.5. From CSF to blood biomarkers

Reaching the point where blood biomarkers could be reliably measured has not been straightforward, as these proteins or protein fragments are present at much lower concentrations than those derived from the central nervous

system. Moreover, they are diluted in a matrix with a very high concentration of other proteins, may be affected by peripheral expression, exhibit variability related to blood–brain barrier permeability, undergo degradation by plasma proteases, and are more sensitive to variations in pre-analytical sample processing(24). For these reasons, ultrasensitive analytical techniques had to be developed, more specific targets employed, and strict, standardized protocols implemented to measure them effectively.

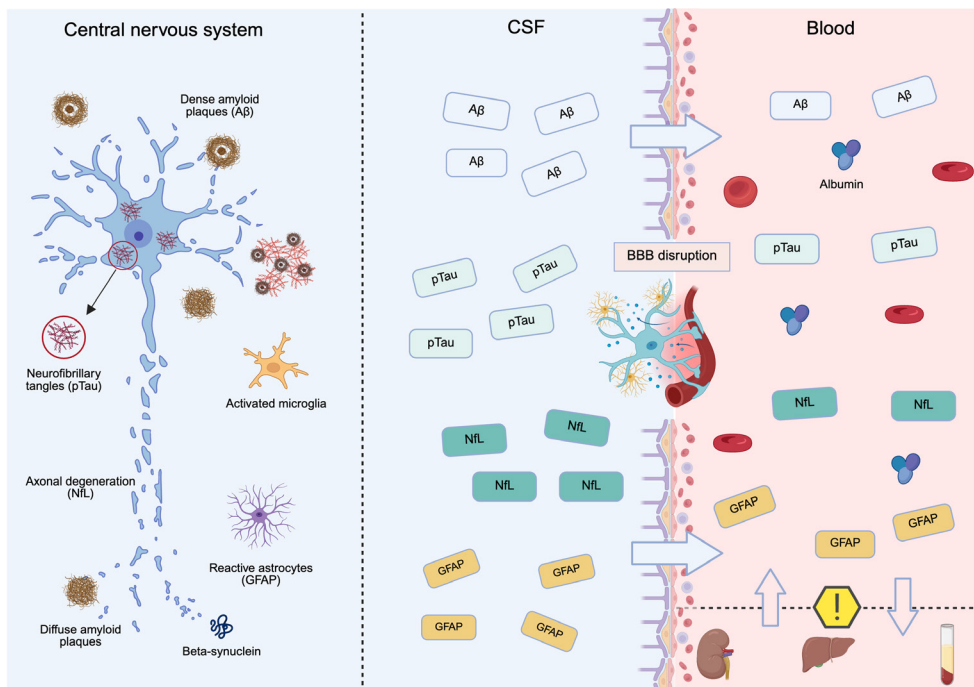


Figure 2. Provides a summary of the pathological mechanisms involved in Alzheimer's disease and their association with biofluid biomarkers (CSF and plasma). Adapted from Teunissen et al. 2020(24).

The figure illustrates how pathological lesions can be measured through biomarkers in both CSF and blood, the latter being a more complex matrix given that biomarker concentrations are much lower (with the exception of GFAP) and that it contains a higher amount of cells and proteins, which hinders biomarker quantification (higher signal to noise ratio than CSF). In addition, in this matrix (blood), peripheral synthesis of certain biomarkers may occur, and their concentrations can be affected by various systemic conditions (such as renal or hepatic insufficiency). Image created with BioRender.com.

Abbreviations: pTau, phosphorylated tau. Aβ, amyloid beta. NfL, neurofilament light chain. GFAP, glial fibrillary acidic protein. BBB, blood–brain barrier.

1.6. Alzheimer's Disease in persons with Down Syndrome

Down syndrome (DS) is the most common genetic cause of intellectual disability. Thanks to advances in medical care, the life expectancy of this population now approaches 60 years, which has led to an increase in the prevalence of age-related comorbidities. Among these, Alzheimer's disease (AD) is particularly prominent: it is estimated that more than 90% of adults with DS over the age of 65 will develop dementia due to AD, which has become the leading cause of mortality in this group(25).

The close relationship between DS and AD is explained by trisomy 21, which results in triplication of the gene encoding the amyloid precursor protein (APP). As in autosomal dominant forms of AD, this genetic alteration induces a pathogenic cascade characterized by: (i) overexpression of the APP gene, (ii) overproduction of β -amyloid ($A\beta$) peptides, and (iii) accumulation of $A\beta$ in neuritic plaques. For this reason, DS is considered a genetic form of AD(26).

The clinical diagnosis of cognitive impairment in this population can be challenging. Therefore, the diagnosis of AD increasingly relies on clinically validated biomarkers in cerebrospinal fluid (CSF) and neuroimaging (positron emission tomography [PET] and magnetic resonance imaging [MRI]).

Given the specific characteristics of this genetically determined form of AD, it is essential to evaluate the diagnostic performance and clinical applicability of plasma biomarkers in this population.

1.7. Clinical manifestations of Alzheimer's Disease

The pathophysiological changes underlying the disease begin decades before the onset of the symptomatic phase, in individuals who are cognitively unimpaired. Cognitive deficits typically start as episodic memory loss(27), enabling the diagnosis of mild cognitive impairment or prodromal AD. As these deficits progress, patients enter a stage of functional dependence that defines the clinical syndrome of dementia.

Therefore, the disease spans a continuum that includes a preclinical phase lasting approximately two decades, a mild cognitive impairment phase lasting 4 to 7 years, and a dementia phase that typically lasts 6 to 8 years(28). These temporal dynamics are shorter in individuals with Down syndrome, where prodromal phases may last on average 2.5 years and dementia stages approximately 4 years(25,26). It is important to note, however, that there is substantial variability in the duration of these phases, and at present we are unable to predict them accurately for each individual.

1.8. Disease Modifying Therapies (DMT)

Two disease-modifying therapies for early-stage Alzheimer's disease have now been approved in Europe: Lecanemab (Clarity-AD), which demonstrated a 27% reduction in cognitive decline measured by CDR-Sum of Boxes(29), and Donanemab (Trailblazer-ALZ 2), which showed a 22% reduction in the iADRS score, increasing to 35% in the subgroup with low/medium Tau burden on PET(1). These results indicate that we now have effective therapies capable of slowing the progression of a disease with a profound personal, familial, and societal impact.

It is essential to have tools that allow us to identify the disease at these early stages, as this is when patients derive the greatest benefit from treatment. Having blood biomarkers with high diagnostic accuracy, that are accessible, minimally invasive at low-cost, will greatly facilitate the identification of individuals with Alzheimer's disease and their access to disease-modifying therapies.

1.9. The Role of Biomarkers in the Diagnosis of Alzheimer's Disease

The conceptualization of Alzheimer's disease has evolved from an initial anatomopathological definition to a clinical–biological one (IWG 2024), and even to a purely biological framework (AA 2024). Following Alois Alzheimer's original description in 1907, the disease was defined for decades by its characteristic neuropathological lesions (β -amyloid plaques and tau tangles).

In 1984, clinical–pathological criteria were established, requiring post-mortem confirmation(30). The development of CSF biomarkers in the 1990s and PET tracers in the 2000s enabled in-vivo demonstration of the underlying pathophysiology and led to the 2007 and 2011 clinical–biological criteria, which required a compatible clinical syndrome and biomarker confirmation of an AD pathophysiology(31).

Since 2018, and in the 2024 criteria, the Alzheimer’s Association proposed that AD should be defined as a strictly biological construct, diagnosable through biomarkers even in the absence of symptoms(13). However, this remains controversial, as the 2024 IWG criteria continue to require clinical symptoms for the diagnosis of the disease, whereby an asymptomatic amyloid-positive individual would be considered “at risk” for Alzheimer’s disease, rather than having preclinical AD(12).

Biomarkers have been essential in the evolution of the AD diagnostic criteria and are becoming even more critical with the advent of disease-modifying therapies, which use biomarkers for multiple purposes: diagnosis, risk stratification, staging, target engagement, safety monitoring, and, in the future, potentially for assessing therapeutic efficacy.

We first summarize the most commonly used classification framework for AD biomarkers, the A/T(N) system(11), in which each letter reflects a distinct biological process: amyloid- β deposition, tau pathology, and neurodegeneration, respectively.

The AT(N) framework

- A (Amyloid pathology): Detectable by amyloid PET tracers, or in plasma and CSF through immunoassays or mass spectrometry techniques.
- T (Tau pathology): Detectable by tau PET tracers, or in plasma and CSF through immunoassays or mass spectrometry techniques.
- N (Neurodegeneration): Detectable by MRI, FDG-PET, or in plasma and CSF through immunoassays or mass spectrometry techniques.

The A and T biomarkers achieve diagnostic accuracies greater than 90% in both CSF and plasma when compared with PET, and even higher accuracies in the case of PET when validated against neuropathology. Consequently, PET is commonly used as a surrogate reference standard to accelerate the validation of biochemical biomarkers and to avoid reliance on post-mortem pathological confirmation(32–35).

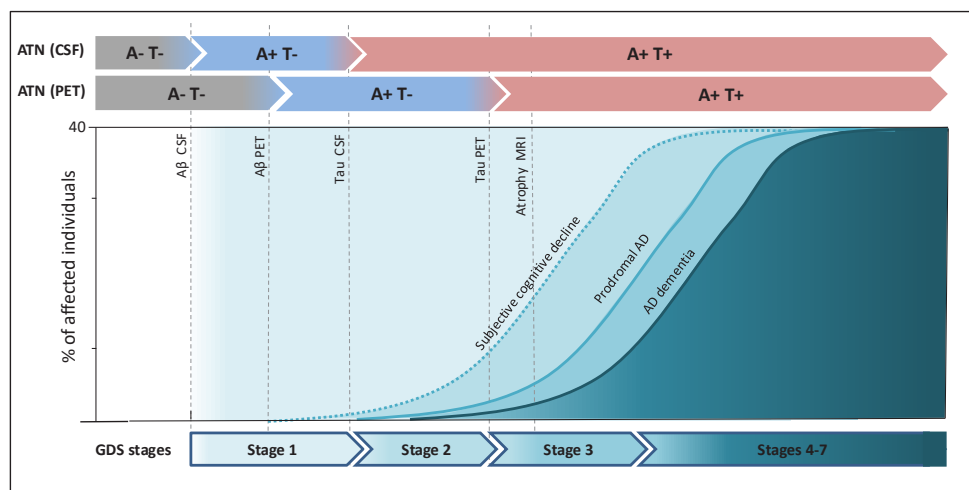


Figure 3. Pathophysiological changes across the Alzheimer's disease continuum (image adapted from Fortea & Carmona-Iragui et al., Lancet Neurology 2021(26))

The 2024 Alzheimer's Association Diagnostic Criteria classify biomarkers of Alzheimer's disease (AD) neuropathological change as core biomarkers, stratified into two main groups(13):

- Core 1 biomarkers (diagnostic), reflecting the characteristic pathology of AD, include:
 - i) A (Aβ pathology): CSF or plasma Aβ42 levels, the CSF Aβ42/40 ratio, and Aβ positron emission tomography (PET);
 - ii) T1 (phosphorylated and secreted tau): pTau217, pTau181, and pTau231 in CSF or plasma.

- Core 2 biomarkers (staging), reflecting more advanced tau-related processes (T2, tau proteinopathy), include:
 - iii) MTBR-tau243, other phosphorylated tau isoforms such as pTau205, mid-region non-phosphorylated tau fragments, and tau-PET.

In addition, the criteria incorporate biomarkers of nonspecific mechanisms involved in AD pathophysiology, including N (neurodegeneration) and I (inflammation), as well as biomarkers of copathologies: V (vascular injury) and S (α -synuclein).

For diagnostic purposes, Core 1 biomarkers enable a diagnosis of AD. Notably, these criteria incorporate, for the first time, the possibility of using plasma biomarkers, provided that they demonstrate diagnostic accuracy comparable to cerebrospinal fluid measures. Importantly, this new framework also expands the role of fluid biomarkers in disease staging, explicitly including plasma biomarkers for this purpose.

In this thesis, we will use the AA criteria and terminology when studying the disease's pathophysiology, although the objective is not to evaluate biomarker performance in preclinical or at-risk Alzheimer's disease, but rather in symptomatic patients seen in routine clinical practice in a memory clinic, where the two diagnostic frameworks do not differ.

1.10. Platforms and assays

Next, we will describe the different analytical platforms, their advantages and disadvantages, and the specific analytes they are able to measure. Figure 4 presents an infographic summarizing some of these platforms.

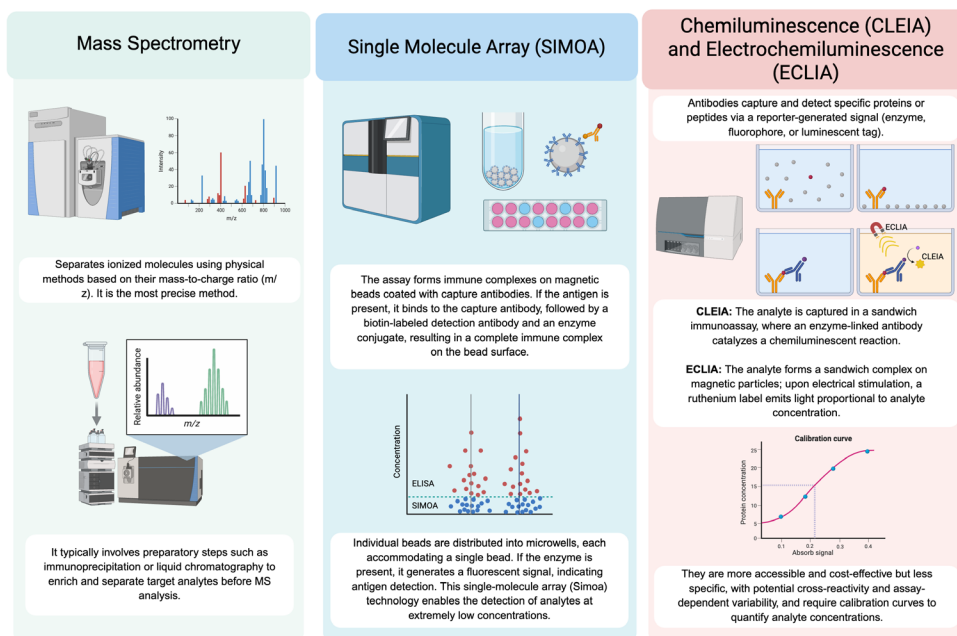


Figure 4. Infographic illustrating the different analytical platforms and assay types. Image created with BioRender.com

1.10.1. Mass spectrometry–based assays

Mass spectrometry provides the most reliable analytical performance, as it separates ionized molecules by physical methods based on their mass-to-charge ratio, yielding highly accurate measurements of their molecular weight. It frequently incorporates immunoprecipitation (IP), in which specific antibodies capture and enrich target analytes from complex matrices such as plasma, removing unrelated proteins and increasing the specific signal of the peptide of interest. It may also employ liquid chromatography (LC-MS/MS) to separate peptides prior to MS analysis, and enzymatic digestion to measure defined peptide fragments.

Mass spectrometry offers high analytical precision at low concentrations in complex matrices like plasma and enables absolute quantification using isotopically labelled internal standards. It avoids the calibration-curve variability inherent to immunoassays, which rely on recombinant standards, and provides high specificity, eliminating cross-reactivity. MS-based workflows

also allow multiplexing (simultaneous quantification of multiple analytes), show lower inter-run variability, and do not suffer from batch-to-batch differences because they do not depend on antibody lots, resulting in low intra- and inter-assay coefficients of variation (CV% <5–8%).

The main limitations of mass spectrometry are its limited accessibility, as it requires expensive instrumentation and specialized technical expertise, which makes widespread clinical implementation challenging. MS methods are also more time-consuming and have a lower analytical throughput (fewer samples processed simultaneously) compared with immunoassays.

1.10.2. Immunoassay-based techniques

These methods use antibodies to capture and detect specific proteins or peptides in a sample. Detection typically relies on a signal generated by a marker molecule attached to the antibody (e.g., an enzyme, fluorophore, or luminescent label). Immunoassays are the most widely used techniques due to their accessibility and analytical performance. They are more affordable, easier to implement, and allow automation, which facilitates more robust and reproducible results across laboratories and over time.

Ultrasensitive platforms have been developed, including Simoa, electrochemiluminescence, chemiluminescence and immunomagnetic reduction, which have further increased their sensitivity.

Because they rely on antibodies, issues related to specificity may arise, such as cross-reactivity with structurally similar proteins, different isoforms, or alternative phosphorylation sites of the target protein. Immunoassays developed to measure the same analyte can show substantial differences in performance due to variations in antibodies (capture or detection), reagents, or the specific assay platform used. Quantification depends on calibration curves, which may vary between runs. Additional drawbacks include antibody reproducibility and batch-to-batch variability. Analytical variability may also be influenced by the presence of other molecules in the matrix (e.g., lipids, other proteins) or by interference from heterophilic or rheumatoid-factor antibodies.

Immunoassays may detect non-specific regions of the molecule or may lack the precision required to accurately measure very low concentrations. Nevertheless, their accessibility, lower cost, and reduced technical complexity make them valuable tools in clinical practice. Concentrations may vary depending on reagent lots, which is why each run is calibrated using controls to monitor variability.

More recently, immunoassays have been refined through improved detection systems such as Nucleic acid Linked Immuno-Sandwich Assay (NULISA), which integrates traditional immunocapture with nucleic-acid-based signal amplification, providing superior sensitivity and enabling multiplexed analyses in peripheral matrices(36).

Table 1. Examples of platforms/types (adapted from Alcolea, Neurology 2023(37))

Type of platform	Commercial name / manufacturer	Detection principle	Commonly measured biomarkers	Key features
ELISA (Enzyme-Linked Immunosorbent Assay)	Various (manual assays or laboratory kits)	Antigen – antibody binding + enzyme generating a colorimetric signal	NfL, GFAP, total tau, Aβ42/40	Widely used basic technique. Low cost. Lower sensitivity. Limited for low-concentration analytes (e.g., plasma NfL).
ECLIA (Electrochemiluminescence Immunoassay)	Elecsys (Roche), MSD (Meso Scale Discovery)	Immunoassay sandwich with light emission via electrochemical reaction	pTau, GFAP, NfL, tTau	High sensitivity and precision. Automatable. Broad clinical use. Elecsys commercial kits; Fully-automated. MSD allows limited multiplexing.
CLEIA (Chemiluminescent Enzymatic Immunoassay)	Lumipulse (Fujirebio), HISCL (Sysmex)	Sandwich immunoassay; enzyme generates luminescent signal	Aβ42/40, pTau, NfL, GFAP	Automated, good sensitivity. Lumipulse and HISCL have commercial plasma kits. Fully-automated. HISCL used in early screening (Japan, EU).

Table 1. Examples of platforms/types. *Continuation*

Type of platform	Commercial name / manufacturer	Detection principle	Commonly measured biomarkers	Key features
Simoa (<i>Single Molecule Array</i>)	Quanterix Simoa	Detection of individual molecules in microwells via fluorescence	pTau, GFAP, NfL, tTau	Very high sensitivity (fg/mL), ideal for plasma. Enables multiplexing. Widely used in research. Commercial kits available.
NULISA (<i>Nucleic Acid Linked Immuno-Sandwich Assay</i>)	NULISAseq (Alamar Biosciences)	Sandwich immunoassay in which detection is amplified through nucleic-acid tags linked to antibodies	pTau, GFAP, NfL, Aβ42/40, >100 other proteins	Next-generation platform. Sensitivity 10–20× higher than Simoa. High multiplexing (up to 120 analytes). Intra/inter-plate CV <10%. Requires specific instrumentation and technical expertise. Under clinical validation.
Bead-based multiplex immunoassay	Luminex xMAP	Capture using fluorescent microbeads + detection by flow cytometry or microscopy	Multiple proteins in parallel	Efficient multiplexing. Variable sensitivity. Requires specialized equipment (flow cytometer).
IMR (<i>Immunomagnetic reduction</i>)	MagQu	Magnetic signal changes when antibody-coated nanoparticles bind the analyte	Aβ42, total tau, pTau	Emerging technique. High sensitivity. No washing required. Clinical validation in progress.
MS (<i>Mass Spectrometry</i>)	Advanced academic or clinical platforms	Direct analysis of ionized molecules, measuring mass-to-charge (m/z) with very high precision and specificity	Small peptides, lipids, amino acids	Absolute quantification, isoform detection. Very precise but requires clean samples and high specialization.

Table 1. Examples of platforms/types. *Continuation*

Type of platform	Commercial name / manufacturer	Detection principle	Commonly measured biomarkers	Key features
IP-MS <i>(Immunoprecipitation + Mass Spectrometry)</i>	PrecivityAD (C2N Diagnostics), others	Immunological purification of the biomarker (IP) followed by MS analysis. IP increases specificity	Aβ42, Aβ40, pTau217, ApoE, α-synuclein	Ultrasensitive detection of specific proteins. Very high specificity. Ideal for ratios such as Aβ42/40 or %pTau217 (phosphorylation index).
LC-MS/MS <i>(Liquid Chromatography + Tandem Mass Spectrometry)</i>	Academic or reference platforms	LC separates compounds; tandem MS discriminates between structurally similar analytes	pTau217, tTau, synaptic markers, Aβ42	Robust technique, ideal for validation. High precision. Less automatable; requires expert personnel.

The table provides an overview of the main analytical platforms used for biomarker detection, outlining their detection principles, representative biomarkers, and key features that differentiate them. Immunoassays are generally more accessible and cost-effective, offer higher analytical throughput, and are easily automated, but they may be limited by antibody specificity, cross-reactivity, and batch-to-batch variability, and rely on calibration curves for quantification. In contrast, mass spectrometry offers greater accuracy, analytical precision, and specificity, including absolute and multiplexed quantification, although it requires more specialized infrastructure and typically has lower throughput. As this is a rapidly evolving field, ongoing technological advances are expected to expand biomarker panels and improve analytical performance, progressively narrowing the current differences between platforms. In this thesis, biomarker measurements were performed using the LUMIPULSE fully-automated platform and the Simoa platform.

1.11. Main blood biomarkers in AD

Table 2. Summary of plasma biomarkers (adapted from M. Schöll & Alcolea et al., 2024(38))

Category	Biomarker	Characteristics
Core 1 AD biomarkers	A β 42/40	A decrease in blood that correlates inversely with cerebral β -amyloid pathology (PET or CSF), particularly in the early stages of the disease. It shows good discriminatory ability compared with other neurodegenerative disorders. However, the difference in levels between A β ⁺ and A β ⁻ individuals is relatively small (~10–15%), and minimal variation in measurement can substantially affect its discriminative capacity.
	Phosphorylated Tau (pTau)	Different isoforms such as pTau181, pTau231, pTau217, and others show high performance for detecting Alzheimer's pathology. pTau217 is the most promising due to its wide dynamic range and high sensitivity, with performance comparable to CSF for identifying amyloid positivity. pTau217 and pTau231 may be useful in very early stages, whereas pTau205 may improve the identification of more advanced stages. Differences between isoforms may depend more on the analytical platform than on the isoform itself.
Core 2 AD biomarkers	Non-phosphorylated Tau	Non-phosphorylated tau forms such as NTA-Tau and eMTBR-Tau243 may be useful as markers of tau pathology, as they correlate with more advanced Braak stages. Total tau shows low specificity in blood.
Neuronal and synaptic degeneration	Neurofilament light chain (NfL)	A nonspecific biomarker of neurodegeneration (central or peripheral). It is elevated in multiple neurological diseases. High baseline concentrations and longitudinal increases are associated with a greater risk of dementia.
	Brain derived Tau	Proposed as a specific marker of neurodegeneration in Alzheimer's disease.

Table 2. Summary of plasma biomarkers. *Continuation*

Category	Biomarker	Characteristics
Neuronal and synaptic degeneration	β -synuclein	A presynaptic protein that reflects synaptic degeneration. It is elevated in Alzheimer's disease and in Down syndrome. It has been suggested to have diagnostic and prognostic value in preclinical Alzheimer's disease and shows correlations with cognitive measures and brain atrophy.
Neuroinflammation	Glial Fibrillar Acid Protein (GFAP)	An astrocytic marker associated with early cerebral amyloidosis and the onset of tau aggregation. It is elevated in Alzheimer's disease, mild traumatic brain injury, spinal cord injury, multiple sclerosis, neuromyelitis optica spectrum disorders, and other neurodegenerative diseases (including FTD, Parkinson's disease, and ALS). It shows high accuracy for distinguishing AD from controls, although its specificity is low for determining the underlying cause of cognitive impairment.
	Other inflammatory molecules	CX3CL1, metalloproteinases, and TREM2 have been studied as potential blood biomarkers for Alzheimer's disease.

1.11.1. A β peptides

Plasma A β 42 and A β 40 peptides can be measured using several methods: Enzyme-Linked Immunosorbent Assay (ELISA), Simoa, electrochemiluminescence (ECLIA) and chemiluminescence (CLEIA), multiplex bead-based immunoassays, immunomagnetic reduction (IMR), and immunoprecipitation–mass spectrometry (IP-MS)(34,39–44).

One of the main limitations for their clinical use is that the differences between patients with Alzheimer's disease and controls are smaller in plasma (~10–15%) compared with CSF (~40–50%)(38,45–47). As a result, small variations in measurement (inherent to immunoassays) can markedly reduce their discriminative ability (Figure 5). This impact can be mitigated by using the A β 42/40 ratio, which shows a stronger (inverse) correlation with cerebral amyloid burden (PET) than A β 42 alone.

IP-MS and HISCL (High-Sensitivity Chemiluminescent Immunoassay) have demonstrated higher diagnostic performance than other platforms, achieving an AUC between 0.85 and 0.95 for discriminating Alzheimer's disease from healthy controls or other neurodegenerative conditions(42,48). In addition, plasma A β 42 measurements across different platforms show lower inter-platform correlation than in CSF and are more sensitive to pre-analytical conditions and to the effects of certain medications(49,50).

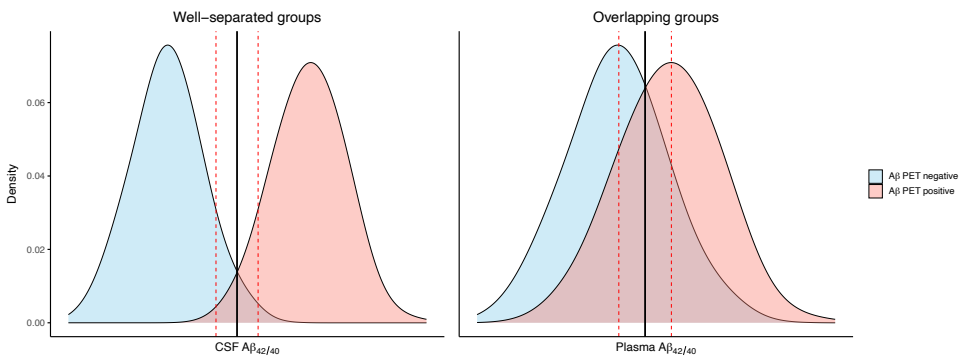


Figure 5. Comparison of the diagnostic performance of the A β 42/40 ratio in CSF and plasma

The figure illustrates how the A β 42/40 ratio shows less separation between groups (A β -positive vs. A β -negative) in plasma compared with CSF, thereby resulting in poorer diagnostic performance. The black line represents the cut-off, and the dashed red lines indicate how small variations in measurement can lead to patient misclassification. This demonstrates that, for this biomarker, CSF is more reliable than plasma. To classify patients robustly, a biomarker must exhibit a percentage change greater than the total analytical error.

1.11.2. Tau Protein

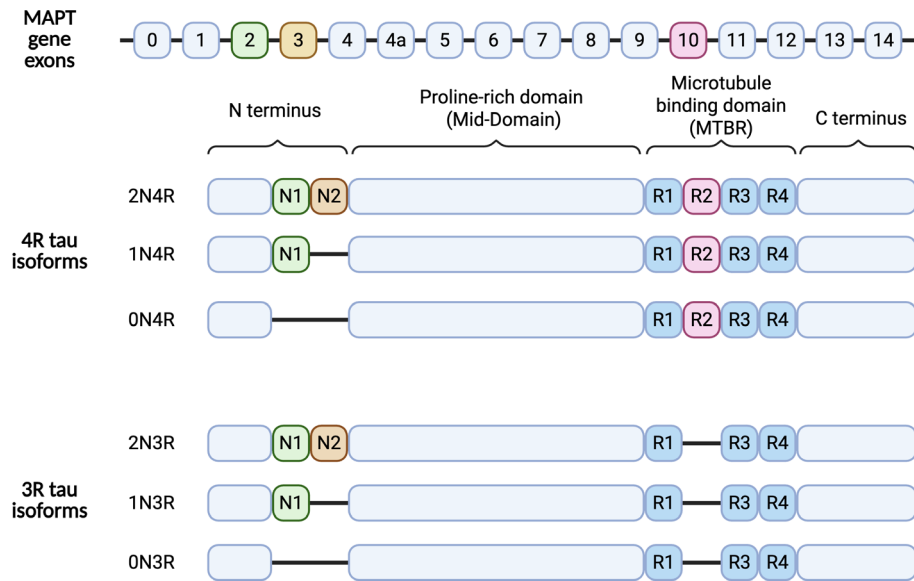


Figure 6. The figure illustrates the 6 isoforms of tau protein in CNS. Created with Biorender.org. Adapted from Holper et al. IJMS 2022(51)

The Tau protein is encoded by the MAPT gene, which generates six different Tau isoforms through alternative splicing of exons 2, 3, and 10. These isoforms range in size from 352 to 441 amino acids. They differ according to the presence of 0, 1, or 2 N-terminal insertions (encoded by exons 2 and 3) and by containing either 3 (3R) or 4 (4R) microtubule-binding repeats (regulated by exon 10). Tau is a microtubule-associated protein localized predominantly in axons of the central nervous system, where it supports microtubule assembly and stabilization. However, it also fulfills multiple additional functions in other neuronal compartments, including dendritic maturation, DNA and RNA protection, and synapse formation, among others(52).

Tau undergoes post-translational modifications, including hyperphosphorylation, which can suppress its physiological activity. Hyperphosphorylated Tau occurs in neurodegenerative diseases such as Alzheimer's disease (AD)(53), but has also been reported in other disorders including Niemann–Pick disease(54) and amyotrophic lateral sclerosis(55). Tau can be phosphorylated at up to 40 distinct sites in AD. The most abundantly phosphorylated residues cluster in

the P1 and P2 regions of the proline-rich domain (PRD), between residues T181 and S235, predominantly on threonine and serine(56).

Studies in large cohorts have shown that blood pTau strongly correlates with amyloid and tau PET and detects Alzheimer's pathology throughout the entire course of the disease(33,57–64).

The most extensively studied Tau forms are pTau181, pTau217, pTau231, pTau205, and pTau212, although non-phosphorylated forms such as eMTBR-Tau243 and NTA-Tau have also been investigated, as well as brain-derived Tau (BD-Tau). Brain-derived Tau “escapes” the peripheral synthesis that affects total Tau, making it highly specific to the central nervous system.

These Tau species show moderate-to-strong correlations. pTau181 was the first phosphorylated Tau form measured in blood and demonstrated good diagnostic discrimination ($AUC > 0.90$)(19). A few years later, pTau217 assays were developed, outperforming pTau181 in all domains because phosphorylation at this site provides a wider dynamic range, and therefore greater discriminative power, for Alzheimer's pathology. For this reason, pTau217 has emerged as the most promising biomarker for clinical implementation(2,21,45,47,65). It discriminates Alzheimer's disease across clinical stages from controls, other neurodegenerative, and non-degenerative conditions, achieving diagnostic accuracies above 90% using a dual-cut-off approach(60).

pTau231 has likewise shown high diagnostic accuracy in distinguishing AD from other neurodegenerative disorders at very early stages, potentially preceding amyloid PET changes. It is therefore a promising candidate for identifying cognitively unimpaired individuals at increased risk of progressing to MCI due to AD, enabling early preventive strategies(66–68). Diagnostic performance results for pTau181, pTau217, and pTau231 have been validated against post-mortem neuropathology (the gold standard). In some contexts, plasma performance is similar or slightly inferior to CSF and amyloid PET (2). All three biomarkers become abnormal prior to detectable Tau PET deposition, suggesting that they reflect early changes in soluble Tau levels triggered by amyloid pathology.

Total tau in plasma has limited discriminatory capacity for detecting Alzheimer's disease, likely due to substantial peripheral sources of tau. To overcome this limitation, a brain-derived tau (BD-tau) assay was recently developed to provide CNS-specific measurement of neurodegeneration(69). BD-tau uses a barcoding antibody strategy that selectively captures tau originating from the brain. Its antibody combination binds exclusively to the six adult CNS tau isoforms while excluding the higher-molecular-weight peripheral isoforms containing the exon-4a insert ("big tau"). Compared with plasma total tau, BD-tau offers true brain specificity, shows stronger associations with neurodegeneration and Alzheimer's pathology, and markedly improves diagnostic discrimination by eliminating peripheral tau contamination.

The IP-MS technique can determine not only pTau concentrations but also the percentage of phosphorylation(70–72). For example, the proportion of tau phosphorylated at threonine 217 relative to all tau fragments containing the Tau217 region, both phosphorylated and non-phosphorylated (pTau217/npTau217), may allow discrimination between individuals with similar total pTau217 levels but different phosphorylation occupancy, potentially reflecting distinct disease trajectories.

Despite major advances in characterizing tau phosphorylation in recent years, much remains unknown. Elevated pTau levels have also been observed in other conditions, such as Nieman-Pick disease, ALS(73) and in non-pathological scenarios, including newborns(74) and hibernating bears (unpublished), where increases appear transient and reversible.

1.11.3. Neurofilament light chain

The neurofilament light chain (NfL) was the first blood-based biomarker to demonstrate diagnostic utility (SIMOA)(75). It is an axonal protein specific to neurons, and its plasma concentration increases across a wide range of neurological conditions affecting both the central and peripheral nervous systems. These include neurodegenerative diseases, stroke, multiple sclerosis, traumatic brain injury, hypoxia, and polyneuropathies, among others(76–78).

Plasma or serum NfL levels can be measured using several immunoassay-based platforms, and they correlate strongly with cerebrospinal fluid concentrations.

Although not specific to any particular disorder, NfL is considered a general biomarker of neuronal injury and a useful tool for monitoring progression in various neurodegenerative diseases. Higher plasma NfL concentrations have been consistently reported in ALS, followed by Alzheimer's disease in Down syndrome and 4R tauopathies(79). In multiple sclerosis, NfL can help monitor treatment response(80). It may also assist in distinguishing controls or primary psychiatric disorders from frontotemporal dementia (FTD). However, for differentiating Alzheimer's disease from FTD, NfL lacks specificity, and pTau biomarkers outperform NfL(81,82).

NfL concentration in blood increases with age, so age-adjusted thresholds are needed(83). Levels may also be influenced by body mass index (BMI) and renal function. Standardized cut-offs are not yet available, as values vary across measurement platforms, clinical contexts, and physiological factors.

Interestingly, elevated blood NfL levels have also been observed in astronauts upon returning from space missions.

1.11.4. GFAP

Glial fibrillary acidic protein (GFAP) is expressed in astrocytes and serves as a marker of astrocytic reactivity. Its blood levels increase during the preclinical stages of Alzheimer's disease (AD) and continue to rise with symptomatic progression(84). Higher plasma GFAP concentrations have been observed in individuals with AD compared with controls and show strong associations with cerebral amyloid burden. Elevated levels have also been reported in frontotemporal lobar degeneration-related disorders, although to a lesser extent than in AD(85). GFAP can also rise in other neurodegenerative conditions such as Creutzfeldt–Jakob disease and dementia with Lewy bodies. Despite its lack of disease specificity, it retains clinical utility for distinguishing AD from other neurodegenerative disorders.

In AD, the magnitude of increase is greater in blood than in CSF, possibly reflecting blood–brain barrier disruption associated with neurodegeneration(84). GFAP is typically found at higher concentrations in plasma than in cerebrospinal fluid for several biological and analytical reasons. As an intracellular filament protein, only small quantities of GFAP are released into CSF even during astrocytic injury(86), and low-level peripheral expression contributes to baseline circulating levels(87). Plasma GFAP also reaches higher steady-state concentrations due to slower clearance compared with CSF and ultrasensitive platforms such as Simoa detect GFAP with greater analytical sensitivity in plasma(88).

GFAP levels in blood also rise in other clinical scenarios; for instance, several studies have shown increased GFAP concentrations in patients with mild traumatic brain injury, supporting its role as an early marker of brain injury(89,90).

1.12. Diagnostic performance considerations

The performance of the assays is typically evaluated using Receiver Operating Characteristic (ROC) analyses, including the area under the curve (AUC), sensitivity, specificity, overall accuracy, and the Youden index. Positive predictive value (PPV) and negative predictive value (NPV) are also calculated. Importantly, for a given sensitivity and specificity, changes in disease prevalence directly affect PPV and NPV, meaning that the interpretation of positive or negative results will vary depending on how common the disease is in the tested population(91).

Using plasma pTau217 as an example, caution is warranted when interpreting a positive result in a younger individual (<65 years) in a primary care setting, where a low disease prevalence would translate into a lower PPV (Figure 7). Conversely, a negative result should be interpreted carefully in an older adult (>80 years) with symptoms compatible with Alzheimer's disease evaluated in a memory clinic, where disease prevalence is high, lowering the NPV. Thus, the intended use in primary care will likely differ (screening requiring confirmatory testing) compared with a memory clinic setting (diagnosis and prognosis).

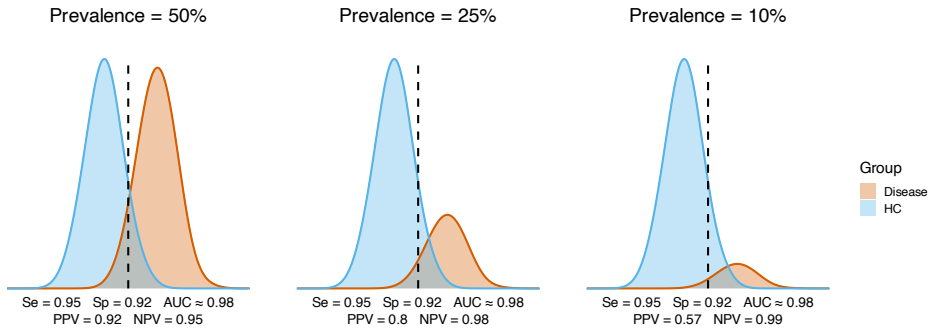


Figure 7. Influence of disease prevalence on predictive values for a given diagnostic performance

The figure illustrates how the prevalence of a disease (“Disease”, shown in red) influences predictive values when sensitivity, specificity, and the area under the curve are held constant for a given biomarker cut-off (indicated by the red dashed line). As shown, negative predictive values (NPV) decrease as prevalence increases, while positive predictive values (PPV) drop substantially as prevalence declines (PPV falling from 0.93 to 0.6). Therefore, interpreting the result of a diagnostic test with good analytical performance must always take into account the clinical context in which it is applied.

Abbreviations: Prev, prevalence. HC, healthy controls. Se, sensitivity. Sp, specificity. AUC, area under the curve. PPV, positive predictive value. NPV, negative predictive value.

1.12.1. Definition of the gold standard (reference marker)

The gold standard (or reference standard) in medical diagnosis is the benchmark method considered the most accurate and reliable for determining whether a patient does or does not have a disease. It serves as the point of comparison to evaluate the validity of new diagnostic tests. In neurodegenerative diseases, the gold standard is neuropathology. Since in many cases the goal is to achieve the highest possible diagnostic certainty in vivo, amyloid PET has been shown to provide results comparable to pathology in Alzheimer’s disease, and this PET imaging has subsequently been validated against CSF. Plasma biomarkers have been validated against both CSF and PET, achieving high diagnostic accuracy comparable to CSF(2).

It is important to note that when measuring the same protein with different analytical tools or modalities, perfect concordance between methods is not expected. For instance, CSF detects A β pathology at earlier stages (higher sensitivity)(92,93), whereas amyloid PET reflects more advanced amyloid

deposition(94). Although both measure the same protein, CSF captures it in its soluble or fibrillar state, while PET detects diffuse or dense plaque deposition at later disease stages. This can lead to discordant results in the same patient (CSF positive / PET negative). A similar phenomenon may occur with plasma pTau217, which could reflect pathology later than CSF A β 42/A β 40 and more closely aligned with amyloid PET (92).

1.12.2. Pre-analytical and analytical variables

Diagnostic performance will depend on multiple factors. The most important, and the one that conditions all subsequent tests, is the underlying clinical diagnostic suspicion, as a well-phenotyped patient allows for a more accurate interpretation of confirmatory tests. Next in importance are the biological and pre-analytical variables, given that several blood biomarkers are known to vary according to age, comorbidities (renal or hepatic dysfunction), blood draw characteristics (e.g., hemolysis), and initial sample-handling protocols, such as time at room temperature, centrifugation conditions, collection materials (EDTA), aliquoting containers, aliquot volume, time to freezing, temperature during collection, storage, and freeze–thaw cycles(95).

Some biomarkers are highly sensitive to these processes, particularly A β peptides, while others, such as NfL and pTau, show greater robustness. Biomarker concentrations can be altered by one or several of these variables, potentially leading to invalid or uninterpretable results or, worse, contributing to diagnostic misclassification.

Additionally, the biological matrix (plasma, serum, dried blood spots) influences the ability to accurately measure biomarkers (for instance, plasma collected in EDTA tubes avoids coagulation-related interference).

After all pre-analytical procedures have been completed, from blood collection to sample processing and storage prior to analysis, analytical variations must be considered. It is important to note that each analytical platform may use different measurement methods (physical, immunochemical, etc.) and may quantify one or multiple biomarkers (some platforms allow multiplexing in a single run). In the absence of universal standardization, the values obtained across different platforms are not directly comparable.

Furthermore, each platform operates under specific conditions, and its precision may vary depending on whether the process is fully automated (minimal involvement of laboratory staff) or manual/semi-automated (where inter-operator variability may affect performance). Immunoassays may also present variability related to reagent lot differences, specificity of the antibodies, or run-to-run fluctuations, and they require calibration curves to derive quantitative measurements.

1.13. Minimum acceptable requirements for blood biomarkers for use in clinical practice. Challenges and obstacles(38,45,47)

1.13.1. Technical requirements

The first essential step in evaluating a biomarker is its analytical validation within the intended context of use, as blood-based biomarkers may serve multiple purposes, including screening, diagnosis, prognosis, monitoring disease progression, treatment response, and even detecting complications (e.g., ARIA in AD). In addition, the biomarker must be specific to its target analyte, and its precision, sensitivity, dynamic range, and robustness must be assessed. It is also necessary to determine the pre-analytical factors related to sample collection, processing, and storage prior to analysis, as these can influence biomarker measurements.

For Alzheimer's disease (AD) pathology, analytes can be measured in either plasma or serum, although plasma (EDTA) is generally recommended because it is the matrix with the strongest supporting evidence. The use of automated assays improves reproducibility, and robustness across different reagent lots is a key requirement for reliable implementation.

There are ongoing initiatives aimed at standardizing the different assays; however, until these efforts are completed, blood biomarker results must be interpreted and verified locally, as they will be specific to each assay and each laboratory. It is recommended that the diagnostic performance of the assay be evaluated periodically to ensure that the established cut-offs remain valid.

1.13.2. Roadmap for clinical implementation

Once technical validity has been established, clinical validation must be conducted through prospective cohort studies that are representative of the population in which the biomarker is intended to be used. These studies should include symptomatic patients across different cognitive stages, with heterogeneous clinical presentations and comorbidities (Real World Data, rather than exclusively highly selected research cohorts), in whom Alzheimer's disease is part of the differential diagnosis. Biomarkers must demonstrate good concordance with the gold standard (neuropathology) or with clinically validated biomarkers such as CSF or amyloid PET.

Although high-sensitivity assays have demonstrated consistent performance in research cohorts, their translation into routine clinical practice requires a structured, stepwise process, commonly described through a five-phase implementation roadmap adapted from the Geneva framework(24).

In this roadmap, early phases (1–3) encompass biomarker discovery, assay development, and retrospective or longitudinal validation, which have been largely completed for core biomarkers such as amyloid- β , phosphorylated tau, and neurofilament light chain. Later phases (4–5) focus on prospective real-world validation, definition of clinical cut-offs, regulatory certification of in-vitro diagnostic assays, and assessment of clinical impact, and remain ongoing. Implementation timelines differ by clinical setting: short-term adoption is expected in specialist memory clinics, intermediate-term use in primary care as a screening tool, and population-wide screening is considered unlikely in the near future due to accuracy, cost, and treatment constraints.

Successful implementation is challenged by biological heterogeneity and technical factors, including the influence of age, sex, body mass index, renal function, and pre-analytical handling. Beyond clinical diagnostics, blood-based biomarkers are already being integrated into clinical trials for participant selection, target engagement, and monitoring treatment response, supporting their progressive transition from research tools to routine clinical use.

HYPOTHESIS

2. HYPOTHESIS

2.1. General hypothesis

Blood biomarkers will show high diagnostic performance for discriminating Alzheimer's disease pathology.

2.2. Specific hypotheses

- Blood biomarkers analyzed in retrospective cohorts will allow the discrimination of Alzheimer's disease pathology with high diagnostic accuracy and show good diagnostic agreement with CSF biomarkers.
- The diagnostic performance of blood biomarkers observed in retrospective samples will be reproducible in prospective cohorts, demonstrating robustness, generalizability, and stability against pre-analytical (handling, processing, storage) and analytical (platform, lot) variations.
- Blood biomarkers will reliably discriminate Alzheimer's disease pathology in genetically determined forms such as Down syndrome-associated Alzheimer's disease (DSAD).

OBJECTIVES

3. OBJECTIVES

3.1. General objective

The general objective of this thesis is to demonstrate that blood biomarkers exhibit high diagnostic accuracy for detecting Alzheimer's disease (AD) pathology in both sporadic and genetic forms, including Down syndrome-associated Alzheimer's disease (DSAD).

3.2. Specific objectives

- To assess the diagnostic accuracy of different blood biomarkers, specifically pTau217, pTau181, and the A β 42/40 ratio, determine their agreement with cerebrospinal fluid in discriminating Alzheimer's disease pathology, and examine pre-analytical factors that may affect biomarker concentrations, such as renal insufficiency or vascular risk factors.
- To validate in a prospective cohort the blood biomarker cut-offs derived from a retrospective cohort of patients from a tertiary Memory Unit, and to evaluate their pre-analytical stability (handling, processing, and storage conditions during the first 24 hours) as well as their analytical stability by assessing intra- and inter-lot variability.
- To evaluate the diagnostic accuracy of different blood biomarkers (pTau217, GFAP, and NfL measured using Simoa) and to assess their agreement with the A β 42/40 ratio in CSF in discriminating Alzheimer's disease pathology in Down syndrome-associated AD.

COMPENDIUM OF PUBLICATIONS

4. COMPENDIUM OF PUBLICATIONS

4.1. Publication 1: Diagnostic performance of plasma pTau217, pTau181, A β 1-42 and A β 1-40 in the LUMIPULSE automated platform for the detection of Alzheimer disease

Arranz, Javier. Zhu, Nuole. Rubio-Guerra, Sara. Rodríguez-Baz, Iñigo. Ferrer, Rosa. Carmona-Iragui, María. Barroeta, Isabel. Illán-Gala, Ignacio. Santos-Santos, Miguel. Fortea, Juan. Lleó, Alberto. Tondo, Mireia & Alcolea, Daniel. Diagnostic performance of plasma pTau217, pTau181, A β 1-42 and A β 1-40 in the LUMIPULSE automated platform for the detection of Alzheimer disease. *Alz Res Therapy* 16, 139 (2024).

<https://doi.org/10.1186/s13195-024-01513-9>. IF 7,6. Q1.

RESEARCH

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Diagnostic performance of plasma pTau₂₁₇, pTau₁₈₁, Aβ₁₋₄₂ and Aβ₁₋₄₀ in the LUMIPULSE automated platform for the detection of Alzheimer disease

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Abstract

Background Recently developed blood markers for Alzheimer's disease (AD) detection have high accuracy but usually require ultra-sensitive analytic tools not commonly available in clinical laboratories, and their performance in clinical practice is unknown.

Methods We analyzed plasma samples from 290 consecutive participants that underwent lumbar puncture in routine clinical practice in a specialized memory clinic (66 cognitively unimpaired, 130 participants with mild cognitive impairment, and 94 with dementia). Participants were classified as amyloid positive (A+) or negative (A-) according to CSF Aβ₁₋₄₂/Aβ₁₋₄₀ ratio. Plasma pTau₂₁₇, pTau₁₈₁, Aβ₁₋₄₂ and Aβ₁₋₄₀ were measured in the fully-automated LUMIPULSE platform. We used linear regression to compare plasma biomarkers concentrations between A+ and A- groups, evaluated Spearman's correlation between plasma and CSF and performed ROC analyses to assess their diagnostic accuracy to detect brain amyloidosis as determined by CSF Aβ₁₋₄₂/Aβ₁₋₄₀ ratio. We analyzed the concordance of pTau₂₁₇ with CSF amyloidosis.

Results Plasma pTau₂₁₇ and pTau₁₈₁ concentration were higher in A+ than A- while the plasma Aβ₁₋₄₂/Aβ₁₋₄₀ ratio was lower in A+ compared to A-. pTau₁₈₁ and the Aβ₁₋₄₂/Aβ₁₋₄₀ ratio showed moderate correlation between plasma and CSF (Rho = 0.66 and 0.69, respectively). The areas under the ROC curve to discriminate A+ from A- participants were 0.94 (95% CI 0.92–0.97) for pTau₂₁₇, and 0.88 (95% CI 0.84–0.92) for both pTau₁₈₁ and Aβ₁₋₄₂/Aβ₁₋₄₀. Chronic kidney disease (CKD) was related to increased plasma biomarker concentrations, but ratios were less affected. Plasma pTau₂₁₇ had the highest fold change (× 3.2) and showed high predictive capability in discriminating A+ from A-, having 4–7% misclassification rate. The global accuracy of plasma pTau₂₁₇ using a two-threshold approach was robust in symptomatic groups, exceeding 90%.

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Conclusion The evaluation of blood biomarkers on an automated platform exhibited high diagnostic accuracy for AD pathophysiology, and pTau₂₁₇ showed excellent diagnostic accuracy to identify participants with AD in a consecutive sample representing the routine clinical practice in a specialized memory unit.

Keywords Plasma, Biomarkers, Alzheimer, Blood, Amyloid, Tau

What is already known on this topic

Blood biomarkers have shown high accuracy to detect AD pathophysiology. The feasibility of those biomarkers in different platforms and the influence of comorbidities in their concentrations needs to be further studied.

What this study adds

We analyzed the feasibility and diagnostic performance of blood AD biomarkers using a fully-automated platform in the setting of a memory clinic and assessed the impact of comorbidities on their diagnostic performance.

How this study might affect research, practice, or policy: The measurement of plasma AD biomarkers in an automated platform yields high accuracy to detect AD pathophysiology and would be easy to implement. Plasma pTau₂₁₇ was the single most accurate marker for clinical implementation.

Introduction

Early and accurate diagnosis is becoming an increasing priority with the recent developments of disease-modifying therapies for Alzheimer's Disease (AD). Pathophysiological biomarkers in cerebrospinal fluid (CSF) and positron emission tomography (PET) imaging with amyloid and tau tracers have extensively proven to be useful to detect the disease pathophysiology but are either expensive and/or invasive [1], which can delay the diagnosis and access to treatment.

Measurement of AD biomarkers in blood through reliable high-throughput platforms would simplify the diagnostic process. This is now technically possible thanks to the development of sensitive technologies that can consistently quantify brain-derived molecules that are present in blood in very low concentrations [2–4]. Amyloid- β (A β) peptides and different isoforms of phosphorylated tau (pTau) in blood have shown high accuracy for detecting AD pathophysiology in previous research studies [5–12]. How all these plasma markers are affected by different comorbidities is also starting to be understood thanks to large well-characterized cohorts [13–15]. Thus, blood-based markers have the potential to be of great use in screening, early diagnosis, tracking progression, and ultimately, monitoring the efficacy of treatment [16–20]. Of all the plasma biomarkers evaluated, pTau₂₁₇ has demonstrated a promising profile in identifying amyloidosis,

showing the largest fold changes in symptomatic AD patients and the most predictive ability to identify cognitive decline [21–29]. In previous immunoassay studies CSF pTau₂₁₇ showed better correlation with amyloid-PET and tau-PET than pTau₁₈₁ [30]. Subsequent research has revealed comparable efficacy of pTau₂₁₇ in both plasma and CSF for the identification of AD neuropathology and for distinguishing pathophysiological AD from other neurodegenerative diseases [31–33]. However, most of the existing studies have assessed each of these plasma markers separately, measuring them on different platforms or through techniques not widely available in clinical laboratories, which limits their potential to be widely applied in the clinical routine. The implementation of blood AD markers in a fully-automated platform would facilitate their reproducibility and accessibility in clinical laboratories [34].

The fully-automated platform LUMIPULSE G, extensively used to measure CSF AD biomarkers in clinical laboratories world-wide, has recently launched developed assays to measure pTau₂₁₇, pTau₁₈₁, A β ₁₋₄₂ and A β ₁₋₄₀ in plasma. In this study, our aim was to assess the feasibility and diagnostic performance of pTau₂₁₇, pTau₁₈₁, A β ₁₋₄₂ and A β ₁₋₄₀ in plasma in the LUMIPULSE fully-automated platform in a cohort of well characterized consecutive individuals assessed in a memory clinic.

Methods

Study participants and clinical classification

We included all consecutive individuals who underwent lumbar puncture for the analysis of AD CSF biomarkers assessed at the Sant Pau Memory Unit (SPIN cohort, Barcelona, Spain) as part of their diagnostic work-up [35] between January 2021 and December 2021 (shown in flowchart in Supplementary Material (Fig. 1)). The study was approved by the Sant Pau Ethics Committee (Protocol code: EC/22/202/6880) following the standards for medical research in humans recommended by the Declaration of Helsinki. All participants or their legally authorized representative gave written informed consent to participate in biomarkers research studies.

At the time of CSF and plasma acquisition, participants had a diagnosis of dementia, mild cognitive impairment (MCI), or were cognitively unimpaired (CU). The clinical diagnosis was established after a thorough neurological

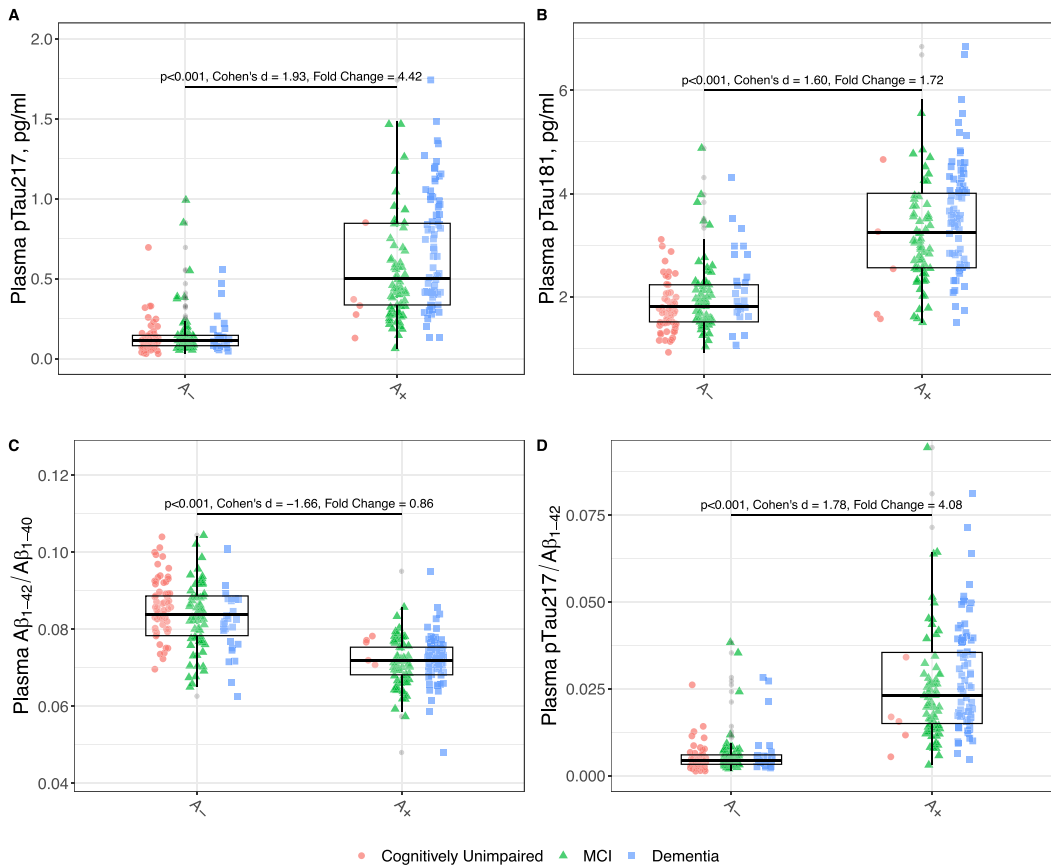


Fig. 1 Levels of plasma biomarkers and their ratios according to the A status in CSF. All p-values are derived from multivariate linear model, adjusted for the effects of age, sex, APOE $\epsilon 4$ status, chronic kidney disease stage, vascular risk factors and clinical stage. For better visualization, two outliers exceeding 2 pg/mL were excluded from the pTau₂₁₇ boxplot for the A+ group in the dementia stages. Not adjusted by other variables effect sizes are shown (Cohen's d). Plasma biomarkers Cohen's d and Fold Change calculated in log-transformed data. pTau₂₁₇: phosphorylated tau 217, pTau₁₈₁: phosphorylated tau 181. A β ₁₋₄₂: Amyloid β ₁₋₄₂. A β ₁₋₄₀: Amyloid β ₁₋₄₀. MCI: Mild cognitive impairment

and neuropsychological evaluation [35]. A more extensive description about how cognitively unimpaired participants were recruited and classified is provided in Supplementary Material (Text 1 & Fig. 1). To assess the impact of vascular risk factors and comorbidities, we collected information about the presence of high blood pressure, dyslipidemia, diabetes, obstructive sleep apnea and history of stroke. Participants were also classified according to the estimated glomerular filtrate rate (eGFR) in different stages (1–5) of chronic kidney disease (CKD) using CKD-EPI formula.

After a full evaluation that included analysis of AD CSF biomarkers, participants were classified according to their etiologic diagnosis, as Alzheimer disease (AD),

other neurodegenerative diseases (OtherDem), not neurodegenerative diseases (OtherNotDeg) or CU. A proportion of participants' diagnosis was classified as "uncertain" as they had an unclear etiological diagnosis after a full initial evaluation and required clinical follow-up.

Cognitively unimpaired participants were patients with no evidence of cognitive impairment after a thorough neuropsychological evaluation and healthy volunteers interested in research.

Sample collection and analysis

Blood samples were collected in EDTA-K2 tubes and subsequently centrifuged (2000 rpm $\times$ 10 min, 4°C)

within 2 h after extraction. Plasma was aliquoted and stored at -80°C until analysis. CSF samples were obtained through lumbar puncture, and were also centrifuged, aliquoted and stored at -80°C until analysis. Blood and CSF samples were collected simultaneously. Full protocol for CSF and blood sample collection in our center has been previously reported [35, 36].

All plasma samples were measured in the Lumipulse fully-automated platform G600II using commercially available kits (Fujirebio Europe, Ghent, Belgium) for pTau_{181} , $\text{A}\beta_{1-42}$ and $\text{A}\beta_{1-40}$ between July and August 2022 with the same lot of reagents. Plasma pTau_{217} was analyzed between August and September 2023 in another aliquot of the same samples using a novel assay recently developed by Fujirebio. On the day of the analysis, plasma samples were brought to room temperature, mixed thoroughly, centrifuged for 5 min at 2000 g, and subsequently transferred to specific cuvettes for analysis in the Lumipulse platform.

CSF markers $\text{A}\beta_{1-42}$, $\text{A}\beta_{1-40}$, pTau_{181} and tTau were used in the diagnostic assessment of patients and measured in routine runs scheduled twice a month throughout 2021 following previously reported methods [37]. According to CSF markers, all participants were classified as amyloid positive (A+, $\text{CSF } \text{A}\beta_{1-42}/\text{A}\beta_{1-40} < 0.062$) or negative (A-), and as tau positive (T+, $\text{pTau}_{181} > 63 \text{ pg/mL}$) or negative (T-). Validation of these cutoff values has been described elsewhere [37]. To validate that our main findings would not be overly influenced by analytical error in CSF measurements, we performed additional sensitivity analyses excluding participants $\pm 10\%$ of the CSF cutoff points (Supplementary Material, Figs. 1–3).

DNA was extracted from full blood using standard procedures, and *APOE* was genotyped following previously reported methods [35]. Briefly, direct DNA sequencing of exon 4 was performed routinely for all participants in the SPIN cohort, followed by visual analysis of the resulting electropherogram to identify the two coding polymorphisms that encode the three possible *APOE* isoforms.

Statistical analysis

Data normality was assessed with the Shapiro–Wilk test. Non-normally distributed variables were log-transformed when necessary. Kruskal–Wallis and Wilcoxon rank sum test were used in continuous variables that were not normal distributed. Linear regression models and ANCOVA adjusted by age and sex were performed for group comparison. We used Chi Square test to assess differences in categorical variables and Fisher's exact test in group comparisons with small number of observations, and Spearman test to assess the correlation between plasma and CSF markers.

Diagnostic accuracy of plasma biomarkers was assessed through receiver operating characteristic (ROC) analysis. We calculated the areas under the curve (AUC) of individual markers and that of logistic regression models that combined them with each other and with clinical variables. A basic model that included Age, Sex and *APOE* $\epsilon 4$ status was used as a reference to assess the added diagnostic value of plasma markers. We compared the accuracy of individual markers and regression models using DeLong's test adjusted by multiple comparisons using Bonferroni method. We evaluated the sensitivity, specificity, and Youden's J index of a range of cutoffs to discriminate A+ from A- participants. We analyzed the concordance of pTau_{217} with CSF amyloidosis. We followed a previously reported approach [38] to stratify our cohort in low, medium, and high risk of having CSF amyloidosis. Using predictive models, according to the risk of the participants of being A+, we selected conservative (97.5% sensitivity/specificity) and more lenient (95% sens/spec) cutoffs. We bootstrapped to assess the cutoff robustness. All tests were performed in R statistical software version 4.2.1. Alpha threshold was set at 0.05 for all analysis.

Results

Study participants and clinical classification

We included 290 participants who were syndromically classified as cognitively unimpaired (CU, $n=66$), having mild cognitive impairment (MCI, $n=130$) or a clinical diagnosis of dementia ($n=94$). Table 1 shows the etiologic diagnoses in each category, main demographic characteristics, and biomarker measures in each group. CU participants were younger than those with MCI ($p < 0.001$) and those with dementia ($p < 0.001$). There were more female participants (62%). The proportion of A+, A+T+ and *APOE* $\epsilon 4$ positive increased according to the clinical stage. More extensive demographics details, including stratification by clinical diagnosis or A status, can be found in Supplementary Material (Tables 1 & 2).

Measures of pTau_{217} , pTau_{181} , $\text{A}\beta_{1-42}$ and $\text{A}\beta_{1-40}$ in plasma

All plasma measures for pTau_{217} , pTau_{181} , $\text{A}\beta_{1-42}$ and $\text{A}\beta_{1-40}$ were above their lower limit of quantification. The plasma concentration ranges in the study were 0.03 to 2.84 for pTau_{217} , 0.92 to 9.63 pg/mL for pTau_{181} , 14.06 to 50.84 pg/mL for $\text{A}\beta_{1-42}$ and 180.41 to 569.89 pg/mL for $\text{A}\beta_{1-40}$. Inter-assay coefficients of variation are shown in Supplementary Material (Table 3).

Correlation between plasma and CSF biomarkers

As per inclusion criteria, all participants had CSF biomarkers measures, and we explored the correlation between

Table 1 Demographics and plasma biomarker concentrations

	Cognitively Unimpaired, N = 66	MCI, N = 130	Dementia, N = 94	P value
Age	57 (12)	73 (11)	75 (9)	<0.001
Sex (Female)	46 (70%)	77 (59%)	57 (61%)	0.3
MMSE	29.0 (1.0)	26.0 (4.0)	22.0 (5.8)	<0.001
APOEϵ4	15 (23%)	32 (25%)	36 (38%)	0.042
Plasma pTau₂₁₇ (pg/mL)	0.11 (0.08)	0.23 (0.35)	0.46 (0.63)	<0.001
Plasma pTau₁₈₁ (pg/mL)	1.71 (0.55)	2.32 (1.28)	3.35 (1.86)	<0.001
Plasma Aβ₁₋₄₂ (pg/mL)	25.0 (4.6)	23.0 (5.0)	24.6 (6.2)	0.028
Plasma Aβ₁₋₄₀ (pg/mL)	290 (65)	301 (66)	319 (72)	<0.001
Plasma Aβ₁₋₄₂/Aβ₁₋₄₀	0.085 (0.010)	0.076 (0.014)	0.074 (0.008)	<0.001
A				<0.001
A +	5 (7.6%)	66 (51%)	69 (73%)	
AT				<0.001
A-T-	61 (92%)	64 (49%)	25 (27%)	
A+T-	2 (3.0%)	17 (13%)	12 (13%)	
A+T+	3 (4.5%)	49 (38%)	57 (61%)	
Etiology				<0.001
CU	56 (85%)	0 (0%)	0 (0%)	
AD	4 (6.1%)	43 (33%)	50 (53%)	
OtherNotDeg	1 (1.5%)	37 (28%)	11 (12%)	
OtherDem	3 (4.5%)	18 (14%)	20 (21%)	
Uncertain	2 (3.0%)	32 (25%)	13 (14%)	
VRF	39 (60%)	102 (82%)	75 (85%)	<0.001
HBP	24 (37%)	71 (57%)	49 (56%)	0.021
DM	11 (17%)	28 (23%)	20 (23%)	0.6
DLP	21 (32%)	64 (52%)	55 (63%)	0.001
eGFR_{1.73m³} (mL/min/1.73m³)				<0.001
> 90	36 (64%)	29 (23%)	13 (14%)	
60–90	18 (32%)	91 (71%)	68 (72%)	
< 60	2 (3.6%)	8 (6.3%)	13 (14%)	

Median (IQR); n (%)

Kruskal–Wallis rank sum test was used to compare continuous variables that were not normally distributed; Pearson's Chi-squared test was used to compare categorical variables; Fisher's exact test was used to compare categorical variables with small number of observations

Unless otherwise specified, values are presented as median (IQR)

CU Cognitively Unimpaired, MCI Mild cognitive impairment, AD Alzheimer disease, OtherNotDeg Other not degenerative, OtherDem Other diseases, VRF one or more vascular risk factors, HBP High blood pressure, DM Diabetes mellitus, DLP Dyslipidemia, eGFR Estimated glomerular filtration rate

both matrices. The correlation between plasma and CSF was moderate for pTau₁₈₁ (Rho=0.66, $p<0.001$) and low for A β ₁₋₄₂ (Rho=0.26, $p=0.007$), and A β ₁₋₄₀ (Rho 0.11, $p=0.06$). When using ratios, the correlation was moderate for A β ₁₋₄₂/A β ₁₋₄₀ (Rho=0.69, $p<0.001$). The correlation between plasma pTau₂₁₇ and CSF pTau₁₈₁ was high (Rho=0.75, $p<0.001$). Both plasma pTau₂₁₇ and pTau₁₈₁ correlated with age (Rho=0.42, $p<0.001$ and Rho=0.45, $p<0.001$ respectively). Detailed correlations within clinical subgroups and partial correlations are shown in Supplementary Material (Figs. 4 and 5).

Association between plasma biomarkers and amyloid status in CSF

We assessed the differences in plasma biomarkers between CSF amyloid positive and amyloid negative individuals considering other variables in a multivariate model. We studied the effect of age, sex, APOE status (APOE ϵ 4+), renal function measured by the estimated glomerular filtration rate (eGFR), vascular risk factors (presence of at least one of the following: high blood pressure, diabetes mellitus, dyslipidemia, history of stroke, obstructive sleep apnea with CPAP) and clinical

status (CU, MCI and Dementia). As shown in Fig. 1, the log transformed multivariate model confirmed that the A+ group had higher plasma concentrations of pTau₂₁₇ (fold-change 4.42, $p < 0.001$) and pTau₁₈₁ (fold-change 1.72, $p < 0.001$) compared to the A- group. Similar results were seen using the ratios pTau₂₁₇/A β ₁₋₄₂ and A β ₁₋₄₂/A β ₁₋₄₀. The plasma pTau₂₁₇/A β ₁₋₄₂ ratio was higher in A+ compared to A- (fold-change 4.08, $p < 0.001$). The plasma A β ₁₋₄₂/A β ₁₋₄₀ ratio was lower in A+ compared to A- (fold-change 0.86, $p < 0.001$). We found lower plasma concentrations of A β ₁₋₄₂ in A+ compared to the A- group (fold-change 0.92, $p < 0.001$) and no differences in A β ₁₋₄₀ concentrations between A status (not shown). To assess the influence of the values close to CSF A β ₁₋₄₂/A β ₁₋₄₀ cutoff, we conducted a sensitivity analysis by excluding participants with values close to the cutoff point ($\pm 10\%$) (Supplementary Material, Figs. 1– 3). We found no significant influence of these observations in our analysis.

Effect of other variables on plasma biomarkers

We assessed whether log transformed plasma pTau₂₁₇, pTau₁₈₁, A β ₁₋₄₂ and A β ₁₋₄₀ were affected by other variables

in the multivariate model. As seen in Fig. 2, amyloid positivity was the variable with the largest effect on all plasma markers. We also observed that decreased renal function was associated with higher concentrations of pTau₂₁₇ ($p = 0.019$) and pTau₁₈₁ ($p < 0.001$) and higher A β ₁₋₄₂/A β ₁₋₄₀ ratio ($p < 0.001$). Male sex was associated with higher pTau₂₁₇ ($p = 0.033$) and pTau₁₈₁ ($p = 0.0009$), and age was associated with lower A β ₁₋₄₂/A β ₁₋₄₀ ratio. Our model had and adjusted R^2 value of 0.62 for pTau₂₁₇, 0.52 for pTau₁₈₁ and 0.48 for A β ₁₋₄₂/A β ₁₋₄₀. All the presented coefficients were obtained from the model in which all the listed variables were included. Complete forest plot shown in Supplementary Material (Fig. 6).

To further investigate the association of pTau₂₁₇ and pTau₁₈₁ with renal function, we performed a subanalysis stratifying by estimated glomerular filtration rate. We found that pTau₁₈₁ concentration in plasma was higher as renal function decreased (< 60 vs $60-90$ mL/min/1.73m² and < 60 vs > 90 mL/min/1.73m², $p < 0.001$). A β ₁₋₄₂ and A β ₁₋₄₀ concentrations in plasma were also higher as renal function decreased ($p < 0.001$). We also observed marginally significant differences in pTau₂₁₇ concentrations in plasma samples from patients with low renal function

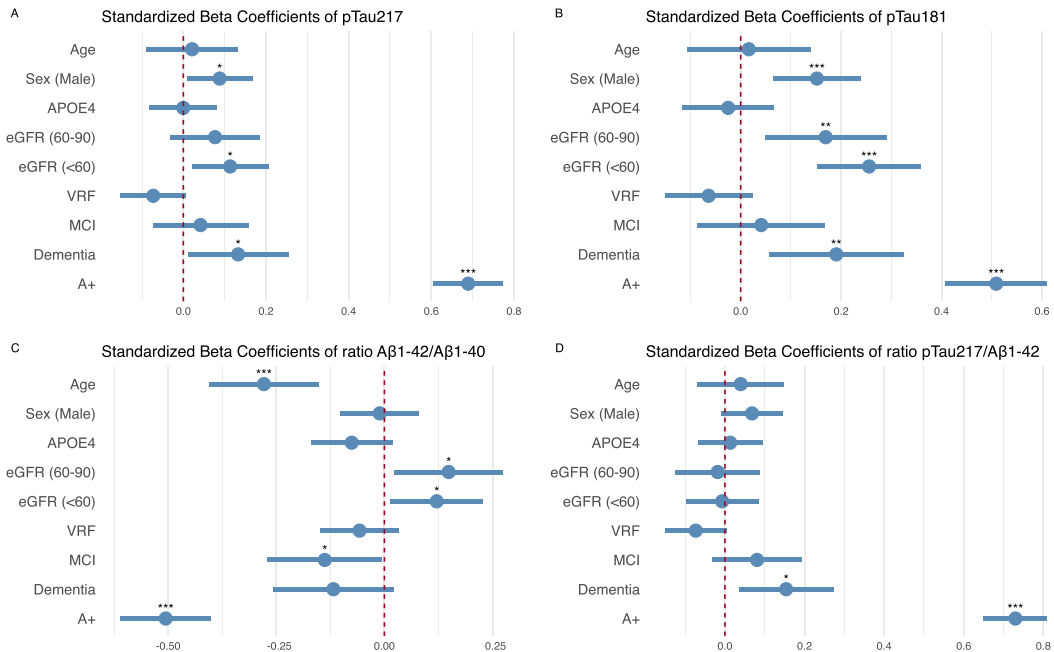


Fig. 2 Effect of different variables on plasma pTau₂₁₇, pTau₁₈₁, A β ₁₋₄₂/A β ₁₋₄₀ and pTau₂₁₇/A β ₁₋₄₂. Dots and bars represent the standardized beta coefficients of each variable in a multivariate regression model. Lines represent the 95% confidence interval for each standardized beta coefficient. Red vertical dashed lines indicate a null effect. We can see the effect size of A positivity adjusted by other variables. pTau₂₁₇: phosphorylated tau 217. pTau₁₈₁: phosphorylated tau 181. A β ₁₋₄₂: Amyloid β ₁₋₄₂. A β ₁₋₄₀: Amyloid β ₁₋₄₀. MCI: mild cognitive impairment. VRF: vascular risk factors. eGFR: estimated glomerular filtration rate

(<60 mL/min/1.73m²) compared to those with normal renal function (>90 mL/min/1.73m², $p=0.047$). However, those differences were lost when using the $\text{A}\beta_{1-42}/\text{A}\beta_{1-40}$ or the $\text{pTau}_{217}/\text{A}\beta_{1-42}$ ratios.

Diagnostic accuracy of plasma biomarkers and their combinations for the discrimination of A+ from A-

In the whole sample, the AUC to discriminate A+ from A- participants were 0.94 (95% CI 0.92–0.97) for pTau_{217} , and 0.88 (95% CI 0.84–0.92) for both pTau_{181} and $\text{A}\beta_{1-42}/\text{A}\beta_{1-40}$ (Fig. 3). The diagnostic accuracy of pTau_{217} to detect amyloid positivity was not outperformed by any other individual plasma biomarker, their ratios or their combinations. $\text{A}\beta_{1-42}$ and $\text{A}\beta_{1-40}$ individually had poor diagnostic accuracy, yielding AUCs below 0.70. Detailed two-by-two comparisons can be found as Supplementary Table 4. Sensitivity, specificity and Youden indices yielded by individual plasma markers are shown in Supplementary Fig. 7.

The diagnostic performance of pTau_{217} was also high across different clinical categories. In the MCI

group, the accuracy of pTau_{217} (AUC=0.92, 95% CI 0.87–0.98) was not significantly different than that pTau_{181} (AUC=0.86, 95% CI 0.79–0.93) or that of the basic model with Age, Sex and $\text{APOE } \epsilon 4$ (AUC=0.81, 95% CI 0.74–0.88). However, the addition of pTau_{217} to Age, Sex and $\text{APOE } \epsilon 4$ increased the accuracy of the model significantly from 0.81 (95% CI 0.74–0.88) to 0.94 (95% CI 0.9–0.99). pTau_{217} also showed very high accuracies (AUC=0.97; 95% CI 0.94–1.00) to discriminate A+ patients with a diagnosis of AD from different A- clinical groups (CN, other dementias and other not degenerative, Supplementary Fig. 8). The performance of plasma biomarkers to discriminate A+T+ from A-T- participants yielded similar results (Supplementary Fig. 9).

Cutoffs application

Table 2 shows the accuracy of different thresholds for pTau_{217} , pTau_{181} and $\text{A}\beta_{1-42}/\text{A}\beta_{1-40}$ to detect amyloid positivity with a sensitivity and specificity of 97.5%, 95% and 90%. As pTau_{217} was the individual biomarker with

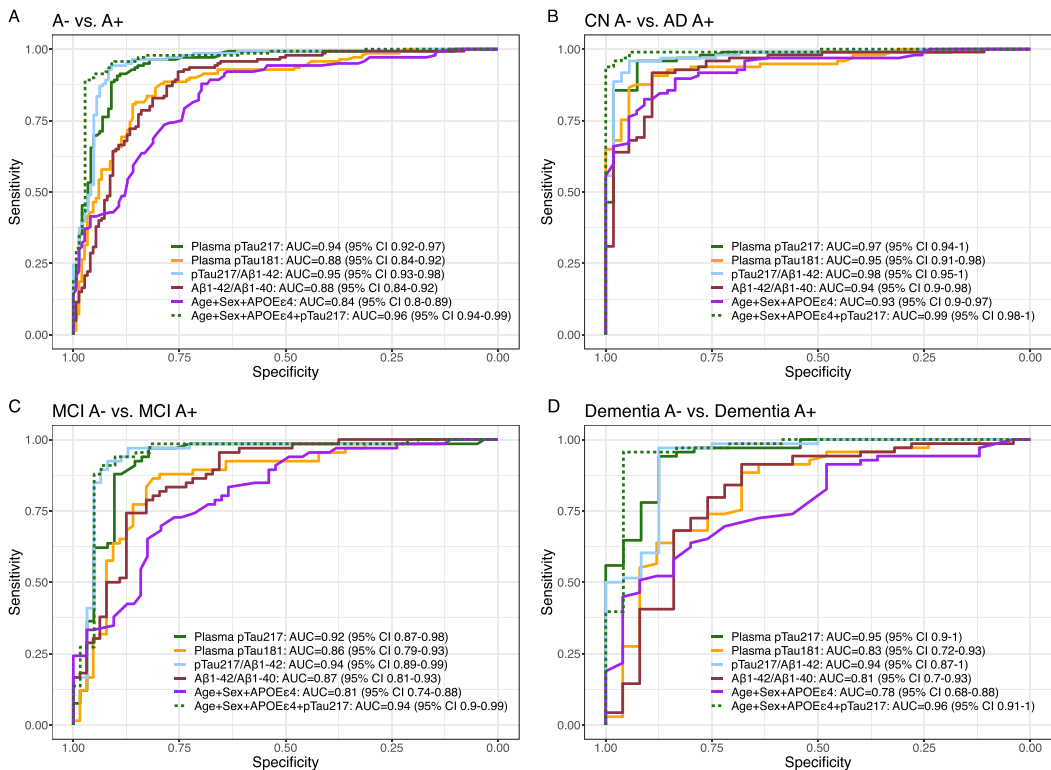


Fig. 3 Diagnostic accuracy of plasma biomarkers for the discrimination of A+ from A- categories. pTau_{217} : phosphorylated tau 217, pTau_{181} : phosphorylated tau 181. $\text{A}\beta_{1-42}$: Amyloid β_{1-42} , $\text{A}\beta_{1-40}$: Amyloid β_{1-40} . CN, cognitively unimpaired. MCI, mild cognitive impairment

Table 2 Thresholds for plasma pTau₂₁₇, pTau₁₈₁ and Aβ₁₋₄₂/Aβ₁₋₄₀ to detect A + participants

Threshold	Sensitivity	Specificity	NPV	PPV	Youden	Accuracy
Accuracy of plasma pTau ₂₁₇						
0.130	97.8%	65.5%	96.9%	73.1%	0.634	81.3%
0.186	95.0%	82.1%	94.4%	83.5%	0.770	88.4%
0.247	90.6%	89.7%	90.9%	89.4%	0.803	90.1%
0.249	89.9%	89.7%	90.3%	89.3%	0.796	89.8%
0.388	69.8%	95.2%	76.7%	93.3%	0.650	82.7%
0.552	45.3%	97.2%	65.0%	94.0%	0.426	71.8%
Accuracy of plasma pTau ₁₈₁						
1.573	97.9%	32.0%	94.1%	57.3%	0.299	63.8%
1.740	95.0%	44.7%	90.5%	61.6%	0.397	69.0%
2.125	90.0%	71.3%	88.4%	74.6%	0.613	80.3%
2.815	64.3%	90.0%	73.0%	85.7%	0.543	77.6%
3.345	47.9%	94.7%	66.0%	89.3%	0.425	72.1%
3.840	31.4%	97.3%	60.3%	91.7%	0.288	65.5%
Accuracy of Aβ ₁₋₄₂ /Aβ ₁₋₄₀						
0.086	97.9%	41.3%	95.4%	60.9%	0.392	68.6%
0.080	95.0%	65.3%	93.3%	71.9%	0.603	79.7%
0.078	90.0%	75.3%	89.0%	77.3%	0.653	82.4%
0.073	65.0%	90.0%	73.4%	85.8%	0.550	77.9%
0.070	37.9%	94.7%	62.0%	86.9%	0.325	67.2%
0.067	20.7%	97.3%	56.8%	87.9%	0.180	60.3%

pTau₂₁₇: phosphorylated tau 217, pTau₁₈₁: phosphorylated tau 181. Aβ₁₋₄₂: Amyloid β₁₋₄₂. Aβ₁₋₄₀: Amyloid β₁₋₄₀. NPV, negative predictive value. PPV, positive predictive value. Threshold units for pTau are in pg/mL.

highest diagnostic accuracy, we assessed the performance of selected cutoffs for this marker in different clinical groups, stratifying by decade. We found that the cutoff that had a global sensitivity of 95% (0.186 pg/mL) yielded accuracies above 84% across all decades and clinical groups when the amyloidosis prevalence was above 20%. In groups with lower amyloidosis prevalence (i.e. CN) the cutoff with specificity of 95% (0.388 pg/mL) showed higher accuracies. We observed that the cutoff that had a specificity of 95% showed a progressive decrease in accuracy with age in the MCI group (accuracy 84% in 60–69, 75% in 70–79 and 62% over 80 years). The negative predictive value of this cutoff was low over 70 years (58%). Detailed information on the accuracy of cutoffs in distinct clinical groups and decades can be found in Supplementary Material (tables 5, 6, 7 & 8).

We conducted a supervised decision tree analysis to determine the potential of various biomarkers and demographic factors in correctly identifying individuals with amyloidosis. This analysis incorporated plasma pTau₂₁₇, pTau₁₈₁, ratio Aβ₁₋₄₂/Aβ₁₋₄₀, Age, Sex, *APOE* ε4 allele presence, and clinical diagnosis group (cognitively unimpaired, mild cognitive impairment, and dementia). The most effective discriminators for amyloidosis were plasma pTau₂₁₇ followed by the Aβ₁₋₄₂/Aβ₁₋₄₀ ratio when

pTau₂₁₇ was high (Supplementary Fig. 10). Other variables were deemed less critical for amyloidosis detection and thus excluded from the decision tree. This algorithm exhibited a misclassification rate of 7.4%, with a sensitivity of 91%, specificity of 94%, overall accuracy of 93%, positive predictive value (PPV) of 94%, and negative predictive value (NPV) of 91%, accompanied by a false-negative rate (FNR) of 9.4% and a false-positive rate (FPR) of 5.6%.

Potential of plasma pTau₂₁₇ to predict Alzheimer disease pathophysiology

We assessed the predictive capability of pTau₂₁₇ to properly classify participants with amyloidosis in our dataset, defined as CSF Aβ₁₋₄₂/Aβ₁₋₄₀ < 0.062. As the model that combined pTau₂₁₇ with Age, Sex and *APOE* ε4 did not perform better than pTau₂₁₇ alone in any comparison, we chose the simplest predictive model with pTau₂₁₇. We bootstrapped to obtain robust predictive cutoffs. We found no differences between the initial prediction and the mean of the 1000 predictive iterations, with a perfect correlation between them (Rho = 1, *p* < 0.001) thus reinforcing the robustness of our initial predictions.

To facilitate the clinical implementation of plasma biomarkers while ensuring accuracy, we applied a

two-threshold approach to classify participants into three groups, those with high, medium, and low likelihood of being CSF amyloid positive. Following this approach, those with a medium risk would benefit from a confirmation with gold standard tests like CSF or Amyloid PET. Figure 4 shows the thresholds in two different scenarios based on two levels of restrictiveness in sensitivity and specificity (97.5% and 95%). Using a highly accurate combination of cutoffs (one for 97.5% Sens and another for 97.5% Spec), only 41.9% of patients in the whole sample would require an additional test, with a global misclassification rate of 4.2%. Using less restrictive cutoffs (95% Sens/Spec), the proportion could be reduced to 19% with a global misclassification rate of 6%. Similar results were found in the subgroup of patients with CU, MCI and Dementia.

We finally assessed the robustness of those cutoffs in the whole sample, considering the increase of prevalence of amyloidosis with age in our population. In Fig. 5, we show the NPV, PPV, and global accuracy of pTau₂₁₇ to detect A positivity by decades using cutoff combinations with 95% and 97.5% sensitivity and specificity in different clinical groups. We found high global accuracy (75%-100%) of the two-threshold application in all the scenarios, with variations of PPV and NPV according to CSF amyloid positive prevalence in our sample.

Discussion

In this study, we found that the concentration of plasma pTau₂₁₇, plasma pTau₁₈₁, and the ratio $A\beta_{1-42}/A\beta_{1-40}$ measured in a fully automated platform, yielded excellent accuracy to detect the AD pathophysiology in the setting of the routine clinical practice of a memory clinic. Of all plasma markers, the recently developed assay that measures pTau₂₁₇ was the most accurate followed by the $A\beta_{1-42}/A\beta_{1-40}$ ratio and pTau₁₈₁. We also found that different comorbidities had a mild significant effect on plasma markers of AD, but the amyloid status was the single variable with the largest effect on their concentration. In patients with advanced chronic kidney disease, the use of ratios could reduce the impact of having higher plasma concentrations associated to low

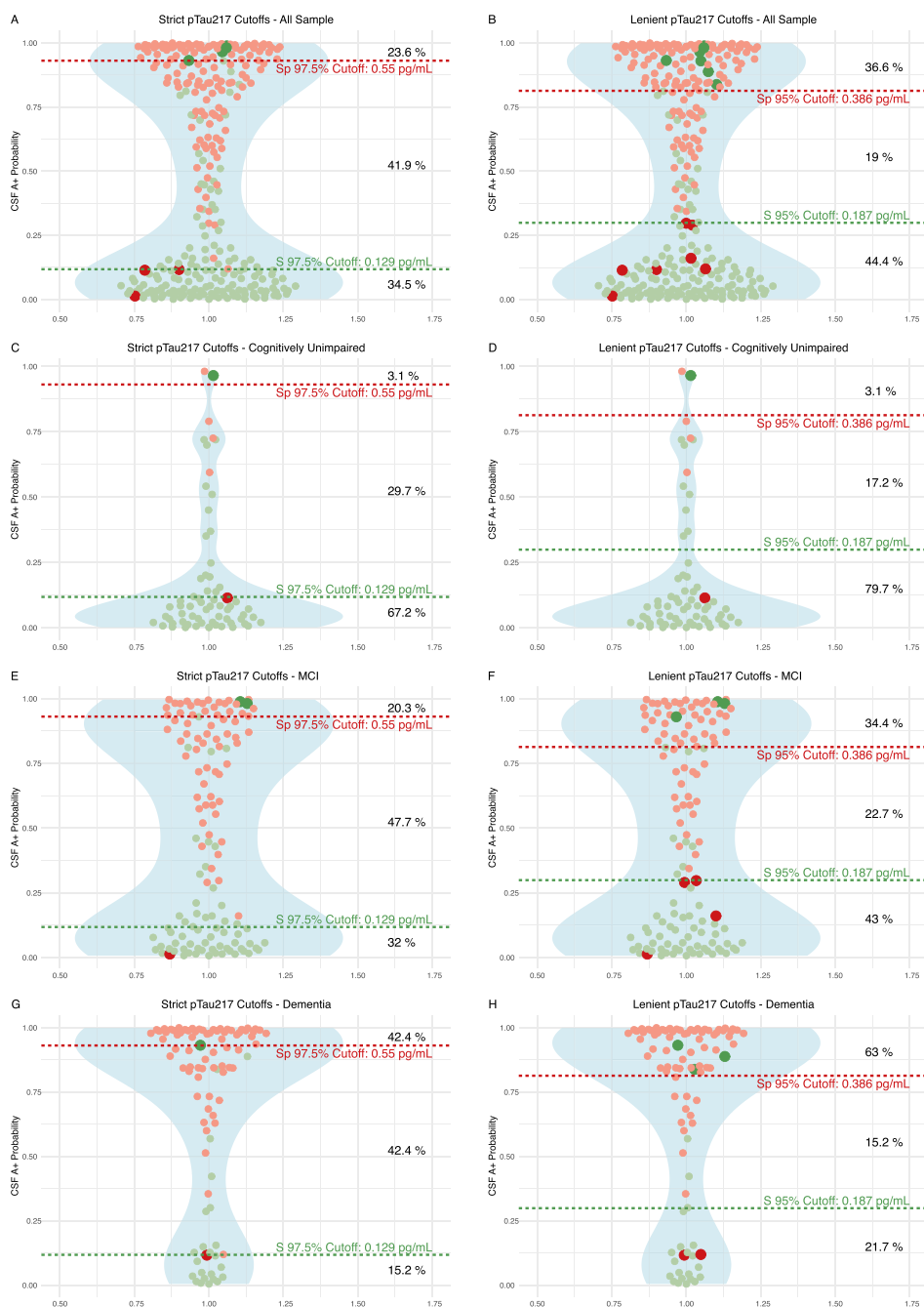
renal function. Furthermore, we applied predictive models to obtain stratification profiles that showed the potential to reduce the need of more costly or invasive procedures by approximately 60 to 80%.

The performance of plasma markers to detect the AD pathophysiology has been assessed in previous studies using different analytical platforms, with AUCs ranging from 0.70 to 0.96 for pTau₁₈₁ [7, 23, 39–43], and from 0.64 to 0.86 for $A\beta_{1-42}/A\beta_{1-40}$ [5, 8, 10]. Plasma pTau₂₃₁ and pTau₂₁₇ have shown to better capture the earliest cerebral $A\beta$ changes in CU, before overt $A\beta$ plaque pathology is present [26]. For plasma pTau₂₁₇, accuracies have varied depending on the platform, yet the performance has consistently been high in discriminating amyloidosis in MCI and predicting progression, typically outperforming that of other plasma pTau isoforms [7, 23, 33, 44]. Most research studies reported better accuracies with the use of composite measures that combined two or more markers and/or clinical or genetic information [45–47]. In our study, plasma pTau₂₁₇, the $A\beta_{1-42}/A\beta_{1-40}$ ratio and pTau₁₈₁ measured with a fully automated platform showed high diagnostic performance to detect amyloid positivity. Of these, pTau₂₁₇ was the marker that showed higher accuracy and, importantly, it was not outperformed by composite measures indicating that it is a good candidate for its implementation as a single biomarker.

Automated platforms have revolutionized CSF analysis by resolving critical analytical factors and, similarly, they hold promise for transforming plasma testing, enhancing both precision and efficiency in biomarker quantification. Recent studies have assessed the diagnostic performance of pTau₁₈₁ and $A\beta_{1-42}/A\beta_{1-40}$ in the Lumipulse platform. Janelidze et al. reported an AUC of 0.7 for pTau₁₈₁ for the identification of CSF amyloidosis in MCI [23] and of 0.74 to detect progression to dementia. However, Wilson et al. reported a higher accuracy of 0.96 [40] for the discrimination between $A\beta^-$ CU and $A\beta^+$ AD patients. Another recent study using the Lumipulse platform analyzed the accuracy to detect AD of plasma pTau₁₈₁ and $A\beta_{1-42}/A\beta_{1-40}$ ratio in cognitively unimpaired participants and found that $A\beta_{1-42}/A\beta_{1-40}$ ratio was the most cost-effective (AUC

(See figure on next page.)

Fig. 4 Plasma pTau₂₁₇ predictive models. Strict and lenient cutoffs in the whole sample and in the distinct clinical groups. We illustrate the implementation of various cutoff thresholds within our sample, denoted by dashed lines in red and green at distinct Y-axis levels, each line representing the associated sensitivity and specificity values. Displayed in red dots are individuals with amyloid positivity in cerebrospinal fluid (CSF) and in green those amyloid negatives. Those participants with pTau₂₁₇ concentrations above dashed red lines are classified as high risk of amyloid CSF positivity. The medium risk category is between dashed red and green lines. Below dashed green line are the participants classified as low risk. Observations that have been incorrectly categorized into high or low risk groups are represented by distinct sizes and colors. To the right, the corresponding percentages of the sample assigned to each risk category are presented. pTau₂₁₇: phosphorylated tau 217, pTau₁₈₁: phosphorylated tau 181. $A\beta_{1-42}$: Amyloid β_{1-42} , $A\beta_{1-40}$: Amyloid β_{1-40} . Model 1 included only plasma pTau₂₁₇ for predictions. S, sensitivity. Sp, specificity

**Fig. 4** (See legend on previous page.)

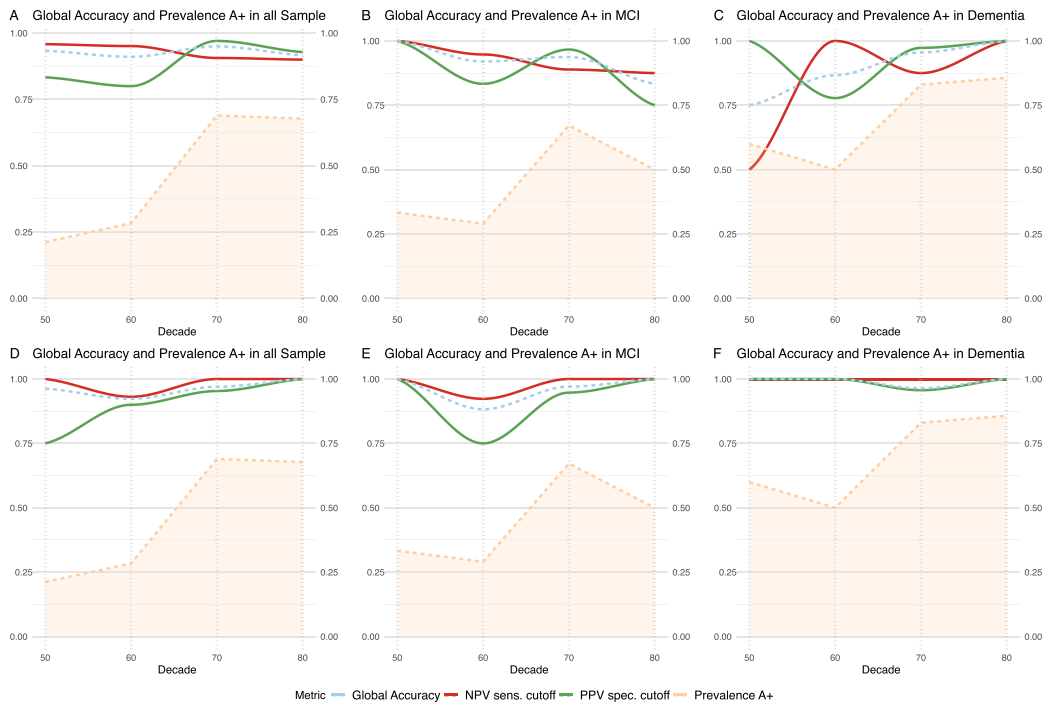


Fig. 5 Negative predictive value, positive predictive value and global accuracy of pTau₂₁₇ for the combination of cutoffs with 95% (A, B, C) and 97.5% (D, E, F) sensitivity and specificity and their relationship with age and prevalence of CSF Amyloidosis. The figure illustrates the association between the prevalence of cerebrospinal fluid (CSF) Amyloidosis (depicted by a dashed orange line), Positive Predictive Value (PPV, red line), Negative Predictive Value (NPV, green line), and global accuracy (dashed blue line) across different decades of life (X-axis) within our study cohort. In panels A-C, the PPV was determined using a cutoff for 95% specificity set at 0.386 pg/mL, while the NPV was ascertained using a 95% sensitivity cutoff at 0.187 pg/mL. In panels D-F, the PPV was determined using a cutoff for 97.5% specificity set at 0.55 pg/mL, while the NPV was ascertained using a 97.5% sensitivity cutoff at 0.129 pg/mL. The term 'global accuracy' in this context refers to the proportion of participants accurately classified as either positive or negative, based on the application of these two cutoffs. It should be noted that this calculation of global accuracy excludes participants categorized as 'indeterminate' (falling within the grey zone), for whom a confirmatory test is recommended. pTau₂₁₇; phosphorylated tau 217, Sens, sensitivity. Spec, specificity. NPV, negative predictive value. PPV, positive predictive value. MCI, mild cognitive impairment

0.9 for A+ and 0.89 for A+ T+), followed by pTau₁₈₁ that showed an AUC of 0.76 for A+ and 0.86 for A+ T+ [48]. Our results are in line with previous studies, and show a global accuracy of 0.88 for plasma pTau₁₈₁ and for the plasma Aβ₁₋₄₂/Aβ₁₋₄₀ ratio. A variety of reasons could explain the minor discrepancies between studies, including differences in preanalytical conditions [49], in the kits that were used, characteristics of the sample and cohorts, and the design of the studies. Our design including consecutive patients that underwent a lumbar puncture for routine diagnostic work-up in a memory clinic setting, provides information about the potential implementation of plasma markers in this context.

Together with the commercially available pTau₁₈₁ and Aβ₁₋₄₂/Aβ₁₋₄₀, we evaluated the performance of plasma

pTau₂₁₇, recently developed for the same automated platform, and found that it outperformed pTau₁₈₁ and Aβ₁₋₄₂/Aβ₁₋₄₀ in the detection of amyloid positivity. In previous research studies, plasma pTau₂₁₇ has consistently shown exceptionally high accuracy across different platforms and has demonstrated strong correlations with other markers of AD (CSF biomarkers, amyloid PET and Tau PET) and with neuropathology [30]. The fact that it has shown greatest fold changes and effect sizes compared to other pTau isoforms, makes it a perfect candidate for its implementation in clinical settings, as small analytical variations (5–10%) would not substantially affect its diagnostic performance. Its implementation on a fully automated platform would not only simplify the process but also enhance accessibility for clinical laboratories.

In our study using a fully automated platform, we found that A+ patients had 4.42 times higher concentrations of plasma pTau₂₁₇. This magnitude of effect, combined with the advantages of automation, makes this assay particularly promising for integration into standard clinical practices.

To facilitate a more rational utilization of plasma biomarkers, we followed the methodology delineated by Brum et al. stratifying the risk of having AD pathophysiology [38]. By implementing this strategy, it is feasible to define threshold values that are highly sensitive and specific to either detect or rule out CSF amyloidosis minimizing the probability of misclassification. This would also allow for selecting those patients that fall within an intermediate likelihood category or 'grey zone' and that would benefit of further etiological investigations (CSF biomarkers, amyloid or Tau PET). This stratification framework represents a pragmatic and flexible approach for the incorporation of plasma biomarkers in clinical settings. An open point of discussion is the optimal context of use—primary care, general neurology, or specialized memory clinics—and the management strategies for participants identified as high or low risk. Our study showed that following this approach, pTau₂₁₇ had an excellent performance in the context of a specialized memory clinic, but these classifications will need to be contextualized within other clinical settings [4, 34]. The effect of comorbidities as CKD on plasma biomarker concentrations points in the same direction as recently published studies [15, 50], in which the use of ratios could attenuate the effect of CKD. Moreover, we found that the effect of renal dysfunction on plasma pTau₂₁₇ concentrations was significantly less than that of the amyloid positivity status, suggesting that the actual impact of CKD on the diagnostic performance of this marker would be minimal. When we assessed the impact of renal dysfunction on different plasma pTau biomarker concentrations, we found that the effect size of eGFR < 60 mL/min/1.73m³ in pTau₁₈₁ was double that observed in pTau₂₁₇, with standardized beta coefficients of 0.25 and 0.11, respectively (Fig. 2), both statistically significant. When we compared the AUC of plasma pTau₂₁₇ and pTau₁₈₁ in the subset of patients with eGFR < 60 mL/min/1.73m³, although higher in pTau₂₁₇ (AUC 0.83, CI 95% 0.64–1) than in pTau₁₈₁ (AUC 0.75, CI 95% 0.53–0.97), accuracy did not differ between both biomarkers, probably because of low statistical power to capture differences in this subset in our cohort, as only 11 patients were A- and 12 patients A+.

One of the strengths of our study is that we included all consecutive participants from routine clinical practice that underwent lumbar puncture throughout one year in our memory clinic including a variety of diagnoses. This

approach reduces the risk of selection biases and ensures a reliable representation of the population assessed in the setting of a specialized memory clinic, also providing relevant information on their potential implementation in the routine diagnostic work-up in this context. Other strengths in our study are the fact that all markers were measured using the same batch of reagents and that the clinical information available allowed us to analyze the potential impact of comorbidities and perform sub analyses within distinct clinical stages.

Limitations

Our study also has some limitations. First, as the inclusion criteria required that participants had received a lumbar puncture for CSF biomarkers, the extrapolation to other contexts of use different than specialized memory units, such as primary care or population screening programs, should be made cautiously. Second, we could not compare plasma pTau₂₁₇ with its counterpart in CSF as the assay was specifically developed for plasma. Another limitation is the lack of Amyloid/Tau PET or neuropathological confirmation in our participants, and although the CSF biomarker cutoffs in our center were validated against amyloid PET [37], we cannot be certain about how using a different gold-standard might affect our results. Finally, this is a single-center study, and even though this increases uniformity and we performed a bootstrapped cross-validation to ensure the predictive robustness of our models, the accuracy and true predictive power of the cutoffs derived from our results need to be verified in diverse datasets with comparable characteristics.

Conclusions

Our study provides evidence that plasma markers can reliably be measured in an automated platform, and highlights plasma pTau₂₁₇ as the most promising plasma biomarker, showing great potential for the detection of AD pathophysiology in the context of a memory clinic. With the arrival of disease-modifying treatments into clinical practice, it is urgent to have easily accessible and efficient diagnostic methods to identify patients that could benefit from these therapies. The implementation of plasma biomarkers in readily accessible fully automated platforms will streamline the diagnosis and enhance the accessibility of disease modifying therapies.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13195-024-01513-9>.

Supplementary Material 1.

Acknowledgements

We are grateful to all participants in the study and their families. We also thank all the clinical team members that were involved in the selection and assessment of participants in the SPIN cohort, and the laboratory teams for sample handling, biomarker analyses, and structural support. We also thank Fujirebio for providing technical support and the reagents necessary to complete the study.

Authors' contribution

DA and AL designed the study. JA, NZ, SR-G, IR-B, RF, MC-I, IB, IL-G, MS-S, AL, JF, MT, DA acquired data relevant for the study. MC-I, IL-G, JF, AL, MT, DA contributed vital reagents/tools/patents. MC-I, IL-G, MS-S, JF, AL, DA obtained funding for the study. DA and JA performed statistical analysis. DA, JA contributed in analysis and interpretation of data. DA, AL, JF participated in study supervision or coordination. JA and DA drafted the first version of the manuscript.

Funding

This study was supported by the Fondo de Investigaciones Sanitarias (FIS), Instituto de Salud Carlos III (PI21/00791 to ILG, PI14/01126, PI17/01019 and PI20/01473 to JF, PI13/01532 and PI16/01825 to RB, PI18/00335 to MCJ, PI19/00882 to MS-S, PI18/00435, PI22/00611 and INT19/00016 to DA, PI17/01896 and AC19/00103 to AL) and the CIBERNED program (Program 1, Alzheimer Disease to AL), jointly funded by Fondo Europeo de Desarrollo Regional, Unión Europea, "Una manera de hacer Europa". This work was also supported by the National Institutes of Health (NIA grants 1R01AG056850-01A1; R21AG056974; and R01AG061566 to JF), by Generalitat de Catalunya (2017-SGR-547, SLT006/17/125 to DA, SLT006/17/119 to JF, SLT002/16/408 to AL) and "Marató TV3" foundation grants 20141210 to JF, 044412 to RB and 20142610 to AL. This work was also supported by a grant from the Fundació Bancaria La Caixa to RB (DABNI project). Fundació Catalana Síndrome de Down and Fundació Víctor Grífol i Lucas partly supported this work. Horizon 21 Consortium is partly funded by Jérôme Lejeune Foundation (Clinical and trial outcome measures for dementia in individuals with Down syndrome). The reagents necessary to complete the study were funded by Fujirebio-Europe. The sponsors of the study did not take part in the design and conduct of the study; collection, management, analysis, and interpretation of the data; writing and review of the report; or the decision to submit the article for publication.

Availability of data and materials

Raw anonymized data and code for statistical analysis are available upon reasonable request. All requests should be sent to the corresponding author detailing the study hypothesis and statistical analysis plan. The steering committee of this study will decide whether data/code sharing is appropriate based on the novelty and scientific rigor of the proposal. All applicants will be asked to sign a data access agreement.

Declarations

Ethics approval and consent to participate

The ethics committee of Hospital Sant Pau approved all procedures included in this study following the standards for medical research in humans recommended by the Declaration of Helsinki. All participants or their legally authorised representative gave written informed consent before enrolment in the study.

Consent for publication

All authors revised the manuscript for content and provided critical feedback.

Competing interests

Daniel Alcolea is employed by Hospital de la Santa Creu i Sant Pau and received research grants from Pla Estratègic de Recerca i Innovació en Salut (PERIS SLT006/17/125), and from Instituto de Salud Carlos III (PI18/00435 and INT19/00016). He participated in advisory boards from Fujirebio-Europe and Roche Diagnostics and received speaker honoraria from Fujirebio-Europe, Roche Diagnostics, Nutricia, Zambon S.A.U., Esteve, and from Krka Farmacéutica S.L. Javier Arranz is employed by Biomedical Research Institute Sant Pau. He is funded by a "Rio Hortega" research grant from the Institute of Health Carlos III. Declarations of interest: none.

Nuole Zhu is employed by Biomedical Research Institute Sant Pau. He is funded by a "Rio Hortega" research grant from the Institute of Health Carlos III. Declarations of interest: none.

Sara Rubio-Guerra is employed by Hospital de la Santa Creu i Sant Pau. Declarations of interest: none.

Iñigo Rodríguez-Baz is employed by Biomedical Research Institute Sant Pau. He is funded by a "Rio Hortega" research grant from the Institute of Health Carlos III. Declarations of interest: none.

Rosa Ferrer is employed by Hospital de la Santa Creu i Sant Pau. Declarations of interest: none.

María Carmona-Iragui is employed by Hospital de la Santa Creu i Sant Pau. Declarations of interest: none.

Isabel Barroeta is employed by Hospital de la Santa Creu i Sant Pau. Declarations of interest: none.

Dr. Illán-Gala is a senior Atlantic Fellows for Equity in Brain Health at the Global Brain Health Institute (GBHI), and is supported with funding from GBHI, Alzheimer's Association, and Alzheimer's Society (GBHI ALZ UK-21-720973 and AACSF-21-850193). Dr Illán-Gala was also supported by the Juan Rodés Contract (JR20/0018) and Fondo de Investigaciones Sanitarias, (PI21/00791) from Instituto de Salud Carlos III.

Ignacio Illán-Gala reported receiving personal fees from Nutricia, Esteve, UCB, and Neuraxpharm Spain outside the submitted work.

Miguel Santos-Santos is employed by Hospital de la Santa Creu i Sant Pau. His research is supported by funding from the Spanish Institute of Health Carlos III (Juan Rodés contract JR18-00018; Fondo de investigación sanitaria grant PI19/00882), the Alzheimer's Association clinician scientist fellowship (AACSF-22-972945), and the National Institutes of Health (R01AG080470).

Juan Fortea is employed by Hospital de la Santa Creu i Sant Pau and received research grants from Institute of Health Carlos III, National Institutes of Health, Fundació La Marató de TV3, and Pla Estratègic de Recerca i Innovació en Salut (PERIS). Dr. Fortea has served as a consultant for Novartis and Lundbeck, has received honoraria for lectures from Roche, NovoNordisk, Esteve and Biogen and served at advisory boards for AC Immune, Zambon and Lundbeck.

Alberto Lleó is employed by Hospital de la Santa Creu i Sant Pau and received research grants from CIBERNED, Instituto de Health Carlos III, Generalitat de Catalunya (PERIS and AGAUR) and Fundación Tatiana and BBVA. He participated in advisory boards from Biogen, Eisai, Fujirebio-Europe, Novartis, NovoNordisk, Nutricia, Otsuka Pharmaceutical, and Zambón, and received speaker honoraria from Lilly, Biogen, KRKA and Zambon.

Mireia Tondo is employed by Hospital de la Santa Creu i Sant Pau and has received research grants from Instituto de Salud Carlos III (PI18/00164; PI21/00140) and has served as consultant in Araclon.

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Received: 8 December 2023 Accepted: 20 June 2024

Published: 26 June 2024

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4.2. Publication 2: Prospective evaluation of plasma pTau217 stability for the detection of Alzheimer's disease in a tertiary memory clinic

Arranz, Javier. Ferrer, Rosa. Zhu, Nuole., Rubio-Guerra, Sara. Rodríguez-Baz, Iñigo. Arriola-Infante, José Enrique. Maure-Blesa, Lucía. García-Castro, Jesús. Selma-González, Judit. Carmona-Iragui, María. Barroeta, Isabel. Illán-Gala, Ignacio. Santos-Santos, Miguel. Fortea, Juan. Lleó, Alberto. Tondo, Mireia & Alcolea, Daniel. Prospective evaluation of plasma pTau217 stability for the detection of Alzheimer's disease in a tertiary memory clinic. *Alz Res Therapy* 17, 150 (2025).

<https://doi.org/10.1186/s13195-025-01779-7>. IF 7,6. Q1.

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Prospective evaluation of plasma pTau₂₁₇ stability for the detection of Alzheimer's disease in a tertiary memory clinic

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Abstract

Background Knowledge on the effect of analytical variability and storage conditions are essential for the successful implementation of plasma pTau₂₁₇ in prospective settings.

Aims To investigate the performance of plasma pTau₂₁₇, measured in consecutive samples with LUMIPULSE, for detecting Alzheimer's disease in a prospective memory clinic setting, along with evaluating its pre-analytical and analytical stability.

Methods We prospectively measured pTau₂₁₇ using the LUMIPULSE automated platform in consecutive patient plasma samples collected between May and November 2024 at the Sant Pau Memory Unit (Barcelona). A subset of participants also underwent paired lumbar puncture for CSF AD biomarkers. We compared biomarker concentrations under different short-term storage conditions (4°C vs -20°C) and different protocol pipelines, and assessed lot-to-lot variability. In the subset with available CSF biomarkers, logistic regression was used to evaluate the association between plasma pTau₂₁₇ and the likelihood of a positive (A+) or a negative (A-) CSF amyloid status. Using ROC analysis, in this prospective cohort we evaluated the accuracy of previously established thresholds derived from historical samples.

Results We included 280 participants, divided into two groups: those with ($n = 109$) and without CSF data ($n = 171$). Among the subset with CSF, 48% were A+, with a plasma pTau₂₁₇ fold-change of 4.5× compared to A-. We found no differences in plasma pTau₂₁₇ concentrations between either short-term storage conditions. The overall coefficients of analytical variation ranged from 1.8% to 3.2%. Plasma pTau₂₁₇ concentrations were slightly higher in paired samples of the clinical protocol. Following a two-threshold approach, the need of confirmatory tests (grey zones) after measuring plasma pTau₂₁₇ ranged between 45.9% and 18.3% using our previously reported strict or lenient cutoffs (overall accuracy 96.6% and 92.1%, respectively).

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Conclusions The robust stability and low lot-to-lot variability of plasma pTau₂₁₇ measurement in an automated platform result in high diagnostic performance of this biomarker in the prospective evaluation of patients in a memory clinic setting. These findings support its implementation into clinical routine, offering clinicians an accessible biomarker for AD diagnosis.

Keywords Stability, Plasma, Biomarkers, Alzheimer, Blood, Amyloid, Tau

Introduction

Early diagnosis of Alzheimer disease enables the timely initiation of social and therapeutic interventions, as well as the opportunity to receive disease-modifying treatments or participate in clinical trials. Gold-standard biomarkers such as Amyloid-PET and CSF are invasive or not widely available in routine clinical practice, whereas blood-based biomarkers provide a more cost-effective and accessible alternative [1–5]. Plasma pTau₂₁₇ has demonstrated high accuracy in differentiating symptomatic Alzheimer's disease (AD) from other neurodegenerative diseases [6, 7] and has positioned as one of the most promising plasma-based tau species [8–19]. Measurement of plasma pTau₂₁₇ is feasible across various platforms [8, 10, 20–26] with the LUMIPULSE platform exhibiting particularly high accuracy in recent studies [27, 28]. Furthermore, as a fully automated system, this platform reduces the risk of analytical variability, thus facilitating safer translation into clinical practice [29–35].

However, the performance of this biomarker in real-world clinical settings remains poorly understood, where slight changes in extraction protocols, storage conditions or reagent variations could impact biomarker levels, thereby influencing its diagnostic performance [33, 34]. Therefore, assessing the stability of pTau₂₁₇ under different real-world conditions is critical, as pre-analytical and analytical variations could lead to misdiagnosis [36–38]. Understanding its performance in routine blood draw scenarios, rather than controlled research laboratory environments, will help determine the impact of standard procedure variations on the biomarker's robustness and reliability.

In a previous study, we assessed the diagnostic performance of plasma pTau₂₁₇ for the detection of amyloid pathology in CSF in a collection of stored samples using a two-threshold approach, finding an overall accuracy of over 90% in symptomatic groups, with the potential to reduce confirmatory testing by 50% to 80% [6]. In this study, we evaluated the performance of these cutoffs in a new, prospective real-world setting, using the routine blood collection pipeline of the hospital. Additionally, we examined the impact of different short-term storage conditions and batch-to-batch analytical variability on biomarker concentrations. These

factors are essential for the successful implementation of this approach in routine laboratory workflows.

Methods

Study participants and clinical classification

We included consecutive patients assessed prospectively in the Sant Pau Memory Unit that underwent blood extraction as part of their diagnostic work-up [39] between May and November 2024. A subset of patients with a consistent clinical diagnosis of mild cognitive impairment (MCI) or early dementia and with no contraindications for the procedure underwent lumbar puncture for the analysis of AD CSF biomarkers and had matched CSF and blood samples collected. Patients in this subset, were further classified as A + or A – based on the CSF A β _{1–42}/A β _{1–40} ratio [40].

The Sant Pau Memory Unit covers a population area of approximately 450.000 inhabitants, and patients are typically referred by primary care physicians or other departments in the hospital due to concerns in cognitive performance. Each year, we provide care to over 3,700 patients, including 700 first-time visits and 3,000 follow-up appointments.

At the time of CSF and/or plasma sample collection, participants had a syndromic clinical diagnosis of subjective cognitive impairment (SCI), mild cognitive impairment (MCI) or dementia. Clinical diagnoses were established following comprehensive neurological, neuroimaging and neuropsychological assessments [39]. Following a comprehensive evaluation that included analysis of AD biomarkers in CSF or plasma, participants were classified based on their etiologic diagnosis into one of the following categories: Alzheimer's Disease (AD), Frontotemporal Lobar Degeneration (FTLD), Lewy Body Disease (LBD) or other non-neurodegenerative diseases (OND). A subset of participants received a classification of "Unknown," as their etiologic diagnosis remained uncertain following the initial evaluation, requiring further clinical follow-up. To evaluate the impact of kidney dysfunction on biomarker concentrations, data on estimated glomerular filtration rate (eGFR) were collected.

Sample collection and analysis

Blood and CSF protocols

Blood samples were collected in EDTA-K2 tubes under fasting conditions before 11 a.m. and then transferred to the clinical laboratory following the routine blood collection pipeline of the hospital. Upon arrival, samples were centrifuged (2000 g for 10 min at 4 °C), aliquoted in 5 mL polystyrene tubes (Falcon™, Ref. 352,052), and stored at –80 °C for subsequent analysis, all within a 4-h timeframe.

In the subset of patients that underwent lumbar puncture, blood was collected immediately after lumbar puncture simultaneously in two EDTA-K2 tubes. One tube (A) was sent to the clinical laboratory as described above, and a second tube (B) was sent to our research laboratory, where it was processed following our previously described research protocol [39]. Briefly, blood samples were centrifuged (2000 g for 10 min at 4 °C) within 2 h of collection, then plasma was aliquoted in 1.5 mL polypropylene tubes (Sarstedt, Ref. 72.690.001) and stored at –80 °C until analysis. This approach allowed a direct comparison between the clinical practice protocol (A) and our standardized research protocol (B). CSF samples were collected by lumbar puncture, then centrifuged, aliquoted in 1.5 mL polypropylene tubes (Sarstedt, Ref. 72.690.001) and stored at –80°C until their analysis. The detailed protocol for CSF samples collection used at our facility has been previously documented [39].

Clinical plasma samples (A), pTau₂₁₇ were measured prospectively every week in the fully automated Lumipulse G600II platform with commercially available kits from Fujirebio Europe (Ghent, Belgium) between May and November 2024, using different reagent lots. Plasma samples processed in the research laboratory (B) were analyzed in one batch in November 2024 using a consistent reagent lot. Plasma A β_{1-42} /A β_{1-40} samples were measured only in participants with eGFR <60 mL/min/m² in the Lumipulse fully-automated platform G600II using commercially available kits (Fujirebio Europe, Ghent, Belgium). On the day of testing, plasma samples were brought to room temperature, thoroughly mixed, centrifuged at 2000 g for 5 min, and transferred to specific cuvettes for analysis on the Lumipulse platform.

CSF biomarkers, A β_{1-42} , A β_{1-40} , pTau₁₈₁ and tTau, were measured as part of the validation process for blood biomarkers. These measurements were conducted during routine runs scheduled every two weeks following previously reported methods [40]. Based on CSF biomarker levels, participants were categorized as amyloid positive (A +, CSF A β_{1-42} /A β_{1-40} < 0.062) or negative (A-). The validation of this cutoff value has been described in prior studies [40].

The study was approved by the Sant Pau Ethics Committee (Protocol code: EC/22/202/6880) following the standards for medical research in humans recommended by the Declaration of Helsinki. All participants or their legally authorized representative gave written informed consent to participate in biomarkers research studies.

Stability of samples and batch-to-batch variability (fridge vs freezer; lot-to-lot variability)

To evaluate the stability of the analysis, 15 consecutive blood samples collected in EDTA-K2 tubes were either processed within a 4-h timeframe in the clinical laboratory, as previously described, or stored overnight at 4 °C (range: 2–8 °C) before being processed the following morning, resulting in an 18-h delay. Additionally, since different production lots were used for the pTau₂₁₇ analysis, we recorded lot references to assess potential lot-to-lot variability. Finally, potential differences between the clinical (A) and research (B) laboratory protocols were evaluated by analyzing 30 plasma samples processed simultaneously under both protocols, as described earlier. A descriptive flowchart of the study is shown in Fig. 1.

Statistical analysis

Data normality was evaluated using the Shapiro–Wilk test. For continuous variables following a normal distribution, Student's t-test was employed. For those not normally distributed, Wilcoxon rank-sum tests or log-transformed linear regression were applied. ANCOVA was conducted for group comparisons. Chi-square test was used to assess differences in categorical variables, with Fisher's exact test applied to group comparisons with smaller sample sizes.

Diagnostic accuracy of plasma pTau₂₁₇ was evaluated through receiver operating characteristic (ROC) analysis. We calculated the areas under the curve (AUC) for plasma pTau₂₁₇ and logistic regression models that combined plasma pTau₂₁₇ with other variables. A basic model incorporating Age and Sex served as a reference to assess the added diagnostic value of plasma pTau₂₁₇. We compared the accuracy of plasma pTau₂₁₇ and regression models using DeLong's test adjusted for multiple comparisons via the Bonferroni method. Sensitivity, specificity, and Youden's J index were calculated across a range of cutoffs to differentiate A+ from A- participants within the cohort. Concordance of pTau₂₁₇ with CSF amyloid status was analyzed. Using cutoffs derived from predictive models in retrospective research samples from our prior work [6], we stratified the participants into low, medium, and high risk of CSF amyloid pathology, applying both strict (97.5% sensitivity and specificity) and lenient (95% sensitivity and specificity) cutoffs, and

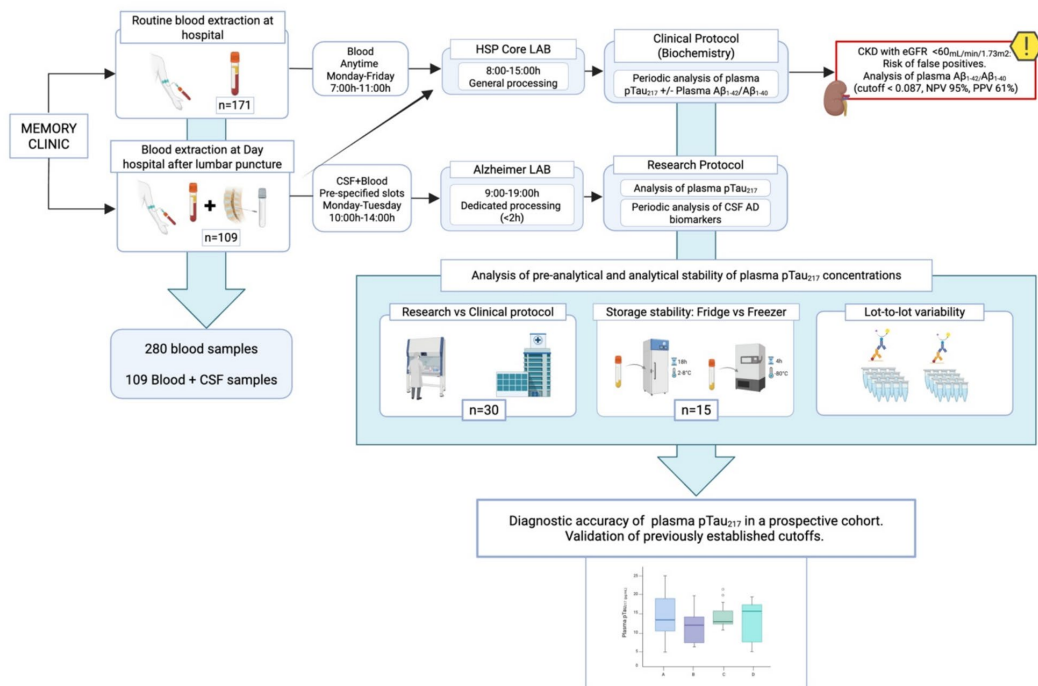


Fig. 1 Flowchart of the implementation of plasma biomarkers in clinical routine. The figure illustrates the workflow for processing plasma and CSF samples in a real-world memory clinic setting. Patients with mild cognitive impairment (MCI) or mild-to-moderate dementia, who were clinically suspected of having Alzheimer's disease (AD) but had contraindications for lumbar puncture (LP), underwent routine blood extraction at the hospital for plasma pTau₂₁₇ measurement. These blood samples followed the standard hospital protocol, being sent to the core laboratory and subsequently to the biochemistry (protein) department for the analysis of AD plasma biomarkers. For patients with no contraindications for LP, who were diagnosed with MCI or mild dementia and were under 85 years of age, both LP and blood extraction were conducted at the Day Hospital. CSF and subsamples of plasma were sent to the Alzheimer Laboratory and processed according to the research protocol. Plasma samples were also sent to the core laboratory and subsequently analyzed for AD plasma biomarkers in the biochemistry department. Additionally, in a subset of participants with an eGFR < 60 mL/min/m², the Aβ₁₋₄₂/Aβ₁₋₄₀ ratio was analyzed to help mitigate the limited but potential impact of pTau₂₁₇ false positives in this population. Plasma pTau₂₁₇ concentrations from both processing protocols (clinical and research) were compared. Variability due to storage conditions and lot-to-lot differences was assessed. Finally, the accuracy of previously established cutoffs for plasma pTau₂₁₇ was validated within this prospective cohort, and newly calculated cutoffs obtained from this prospective cohort were proposed. Created in BioRender. (2025) <https://BioRender.com/x23u713>

compared their performance with newly calculated cutoffs derived from this prospective cohort. We evaluated the variations in cutoff performance (negative predictive value [NPV], positive predictive value [PPV], and global accuracy) across different clinical scenarios based on the expected prevalence of amyloid pathology. All analyses were performed using R statistical software (version 4.2.1), with an alpha level set at 0.05.

Results

Study participants

We included 280 participants with plasma measures. They were further divided in two groups according to

whether CSF was also obtained or not (Table 1). Compared to participants who underwent lumbar puncture, participants without CSF were older (72 vs 74 years, $p=0.008$), had higher plasma pTau₂₁₇ concentrations (0.18 vs 0.25 pg/mL, $p=0.036$), were more likely to be in more advanced stages (42.4% were GDS ≥ 4 vs 19.4%, $p<0.001$) and had higher prevalence of moderate-severe kidney dysfunction defined as eGFR < 60 mL/min/1.73m² (11% vs 22%, $p=0.001$). There were no differences in sex distribution or years of education between both groups, and the distribution of clinical diagnosis was also comparable ($p=0.4$).

Table 1 Characteristics of patients with and without CSF

Characteristic	With CSF, N = 109 ^a	Without CSF, N = 171 ^a	p-value ²
Age (years)	72 (7)	74 (8)	0.008
Sex (female)	62 (57%)	84 (49%)	0.2
Education (years)	11.1 (4.5)	12.1 (4.8)	0.082
Plasma pTau ₂₁₇ (median[IQR])	0.18 [0.10–0.46]	0.25 [0.13–0.54]	0.036
Plasma A β _{1–42} /A β _{1–40} (median[IQR])	0.070 [0.063–0.078]	0.074 [0.068–0.079]	0.4
Amyloid status			> 0.9
A-	57 (52%)	NA	
A +	52 (48%)	NA	
GDS			< 0.001
≤ 3	87 (81%)	95 (58%)	
≥ 4	21 (19%)	70 (42%)	
eGFR (mL/min/1.73m ²)			0.001
> 90	33 (30%)	24 (14%)	
60–90	64 (59%)	110 (64%)	
< 60	12 (11%)	37 (22%)	
Clinical diagnosis			0.4
Alzheimer's Disease	44 (40%)	74 (43%)	
FTLD	14 (13%)	21 (12%)	
LBD	13 (12%)	28 (16%)	
OND	33 (30%)	45 (26%)	
Unknown	5 (4.6%)	2 (1.2%)	

^a Unless otherwise specified, numeric variables are shown as mean(SD). Categorical variables are shown as percentage (%)

² Wilcoxon rank sum test, Pearson's chi-squared test, Wilcoxon rank sum exact test, Fisher's exact test

Abbreviations: pTau₂₁₇ phosphorylated tau 217, A β _{1–42} Amyloid β _{1–42}, A β _{1–40} Amyloid β _{1–40}, GDS Global Deterioration Scale, eGFR estimated glomerular filtration rate, FTLD Frontotemporal Lobar Degeneration-related disorders, LBD Lewy Body Dementia, OND Other Non-neurodegenerative

Within the subset with CSF (Table 2), 57 were A- and 52 were A+. The A+ participants were older (70 vs 75 years, $p < 0.001$) and had higher plasma pTau₂₁₇ concentrations (4.5 folds higher in A+, $p < 0.001$).

Accuracy, PPV and NPV of historical cutoffs and AUC of pTau₂₁₇ in the prospective clinical cohort

We evaluated in the prospective clinical cohort the accuracy of the cutoffs obtained in the retrospective cohort. According to our previously calculated two-threshold strict cutoffs (sensitivity 97.5% = 0.13 pg/mL, specificity 97.5% = 0.55 pg/mL), the proportion of participants classified as low, medium or high risk of amyloid pathology in the whole cohort were 34.9%, 45.9% and 19.3%, respectively. Applying more lenient cutoffs (sensitivity and specificity above 95%), the proportions were 54.1%, 18.3% and 27.5%, respectively.

In the subset with confirmatory CSF, we evaluated the accuracy of historical cutoffs to discriminate between A+ and A- patients prospectively. The prevalence of A+ was 48%. In our prospective evaluation, the global accuracy for historical strict cutoffs in this prospective setting was 96.6%, with NPV 95% and PPV of 100%. The global

accuracy of historical lenient cutoffs was 92.1%, with a NPV of 88% and a PPV of 100%. Applying the strict cutoffs, only two observations were misclassified, both identified as A- by plasma pTau₂₁₇ but as A+ by CSF A β _{1–42}/A β _{1–40}: a male with a final clinical diagnosis of LBD and a female with clinical diagnosis of FTLD. Of note, both patients were CSF pTau₁₈₁ negative. From the seven observations misclassified with lenient cutoffs, six were female, six were CSF pTau negative, and two had etiologic diagnosis of LBD, two of FTLD and three of AD. Using combined cutoffs (sensitivity 97.5% and specificity 95%), global accuracy was 97.03%, leaving 37.6% of the sample in the intermediate zone. The performance of the three historical combinations is shown in Fig. 2A.

Cutoffs calculated in the prospective evaluation

The AUC for detecting A positivity obtained from the prospective cohort was 0.95 (95% CI 0.91–0.98) for plasma pTau₂₁₇ (Supplementary Fig. 1). The models of age and sex or the combination of age, sex and plasma pTau₂₁₇ did not outperform plasma pTau₂₁₇ alone. The single cutoff that maximized the Youden index was 0.185 pg/mL, with a sensitivity of 86.5% and a specificity of

Table 2 Characteristics of patients with CSF sample

Characteristic	A-, N = 57 ^a	A +, N = 52 ^a	p-value ²
Age (years)	70 (7)	75 (5)	< 0.001
Sex (female)	28 (49%)	34 (65%)	0.087
Education (years)	11.3 (4.8)	10.9 (4.3)	0.6
Plasma pTau ₂₁₇ (median[IQR])	0.11 [0.08—0.15]	0.49 [0.24—0.74]	< 0.001
Plasma Aβ ₁₋₄₂ /Aβ ₁₋₄₀ (median[IQR])	0.080 [0.068—0.087]	0.063 [0.061—0.071]	0.006
GDS			0.047
≤ 3	50 (88%)	37 (73%)	
≥ 4	7 (12%)	14 (27%)	
eGFR (mL/min/1.73m2)			0.010
> 90	24 (42%)	9 (17%)	
60–90	26 (46%)	38 (73%)	
< 60	7 (12%)	5 (9.6%)	0.56
Clinical diagnosis			< 0.001
Alzheimer's Disease	1 (1.8%)	43 (83%)	
FTLD	12 (21%)	2 (3.8%)	
LBD	9 (16%)	4 (7.7%)	
OND	30 (53%)	3 (5.8%)	
Unknown	5 (8.8%)	0 (0%)	

^a Unless Otherwise Specified, Numeric Variables Are Shown As Mean(SD). Categorical variables are shown as percentage (%)

² Wilcoxon Rank sum test, Pearson's Chi-squared test, Wilcoxon rank sum exact test, Fisher's exact test

Abbreviations: pTau₂₁₇ phosphorylated tau 217, Aβ₁₋₄₂ Amyloid β₁₋₄₂, Aβ₁₋₄₀ Amyloid β₁₋₄₀, GDS Global Deterioration Scale, eGFR estimated glomerular filtration rate, FTLD Frontotemporal Lobar Degeneration-related disorders, LBD Lewy Body Dementia, OND Other Non-neurodegenerative

91.2%, NPV of 88.1% and PPV of 90%, with an accuracy of 89%.

Using a two-cutoff approach to stratify the risk of AD, we found no significant differences in the biomarker's overall accuracy compared to our retrospective cohort. However, the optimal strict and lenient PPV and NPV cutoffs varied. For strict cutoffs (97.5% sensitivity and specificity), the optimal NPV threshold was 0.12 pg/mL, closely matching the previous 0.13 pg/mL, while the optimal PPV threshold decreased to 0.33 pg/mL from 0.55 pg/mL. For lenient cutoffs (95% sensitivity and specificity), the optimal NPV threshold was 0.14 pg/mL, lower than the previous 0.19 pg/mL, and the PPV threshold decreased to 0.24 pg/mL from 0.39 pg/mL.

Applying the prospective cutoffs, the proportion of cases with medium risk was 37.6% using strict cutoffs, and 23.9% with lenient cutoffs. The global accuracy of the prospective cutoffs to detect CSF amyloid positivity was similar to that of previously reported cutoffs, as the misclassification rates were 2.94% for the strict cutoffs and 7.23% for the lenient combination, which were comparable to 3.39% and 7.87%, respectively, using the previous historical cutoffs. The accuracies of different cutoff combinations in our prospective assessment are shown in Table 3. The performance of the three prospective

combinations is shown in Fig. 2B, and the proportion of participants classified in low, medium or high risk of having AD with the use of different cutoff combinations is shown in Fig. 3. Clinical and demographic details on the individuals in the grey zone are provided in Supplementary Text 1.

Variations in PPV, NPV, and global accuracy across different cutoff combinations in clinical scenarios with varying prevalence of amyloid pathology

In our prospective cohort, the prevalence of amyloid pathology was approximately 50%. We evaluated the impact of varying prevalence rates on accuracy and predictive values. Supplementary Fig. 2 illustrates the NPV increase and PPV decrease in settings with lower A+ prevalence, such as primary care during cognitive screening. Conversely, in settings with higher A+ prevalence, such as memory clinics, the PPV increases while the NPV declines. The global accuracy of plasma pTau₂₁₇ was above 90% in the context of A+ prevalences ranging from 20 to 60%.

Performance of historical cutoffs in the subset without CSF

The risk stratification distribution in the subset without CSF, was largely comparable to that of patients with confirmatory CSF. With strict cutoffs, 24.6% are classified

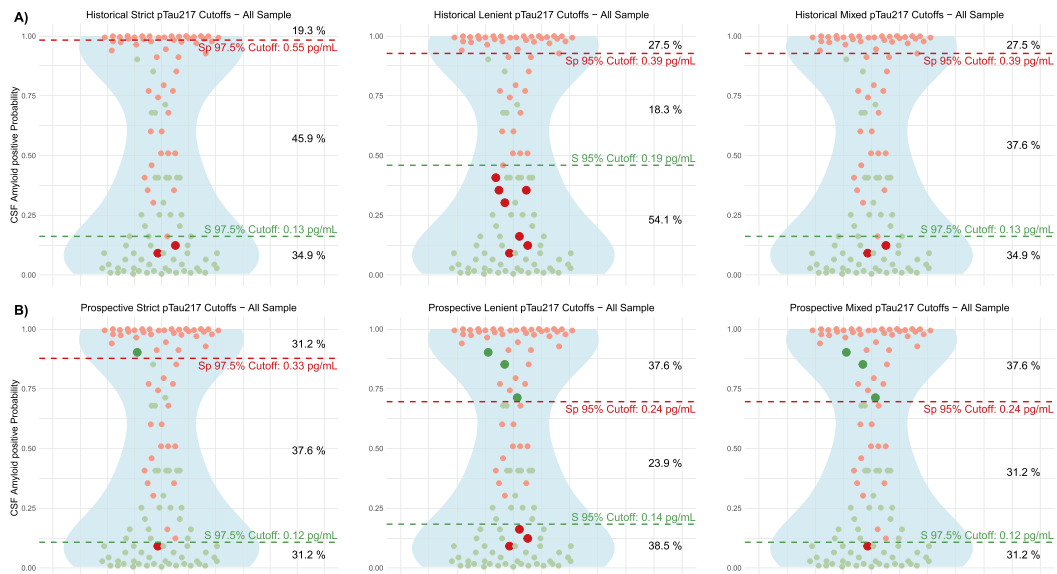


Fig. 2 Application of previously established historical cutoffs (**A**) and newly calculated cutoffs (**B**) to the prospective cohort. The figure illustrates the application of previously established cutoffs to the validation cohort, represented by dashed red and green lines at distinct levels on the Y-axis, each corresponding to specific sensitivity and specificity values. Red dots denote individuals with amyloid positivity in cerebrospinal fluid (CSF), while green dots represent amyloid-negative cases. Participants with pTau₂₁₇ concentrations above the dashed red line are classified as high risk for amyloid CSF positivity, those between the dashed red and green lines are classified as medium risk, and those below the dashed green line are categorized as low risk. Misclassifications into high or low-risk groups are indicated with distinct marker sizes and colors. On the right, the figure displays the percentages of the sample allocated to each risk category. pTau₂₁₇: phosphorylated tau 217. S, sensitivity. Sp, specificity

Table 3 Diagnostic performance of different cutoff combinations of plasma pTau₂₁₇ to detect A+ participants

Cutoffs	Values	Sensitivity	Specificity	NPV	PPV	Accuracy	Prevalence	Gray Zone
Historical strict cutoffs [6]	0.13—0.55	91.3	100	94.7	100	96.6	47.7	45.9
Historical lenient cutoffs [6]	0.19—0.39	81.1	100	88.1	100	92.1	47.7	18.3
Historical mixed cutoffs [6]	0.13—0.39	93.8	100	94.7	100	97.1	47.7	37.6
Prospective strict cutoffs	0.12—0.33	97.1	97.1	97.1	97.1	97.1	47.7	37.6
Prospective lenient cutoffs	0.14—0.24	92.7	92.9	92.7	92.9	92.8	47.7	23.9
Prospective mixed cutoffs	0.12—0.24	97.4	91.7	97.1	92.6	94.7	47.7	31.2

pTau₂₁₇ phosphorylated tau 217, NPV Negative predictive value, PPV Positive predictive value. Threshold units for pTau₂₁₇ are in pg/mL

as pTau₂₁₇ positive, 50.8% fall within the gray zone, and 24.6% are pTau₂₁₇ negative. For lenient cutoffs, the distribution is 36.8% pTau₂₁₇ positive, 22.8% in the gray zone, and 40.4% pTau₂₁₇ negative—both showing a slight increase in the gray zone and an increase in positive cases compared to the CSF group.

Effect of chronic kidney disease on plasma pTau₂₁₇ accuracy

In this cohort, twelve participants with available samples of CSF (five A+ and seven A-) had moderate or severe

kidney dysfunction (eGFR < 60 mL/min/1.73m²). Using historical strict cutoffs (0.13 pg/mL—0.55 pg/mL), four of the five A+ cases were correctly classified as high AD risk according to plasma pTau₂₁₇, and the remaining case was classified as medium risk (pTau₂₁₇ of 0.5 pg/mL). Interestingly, in this case, the Aβ₁₋₄₂/Aβ₁₋₄₀ ratio in plasma (0.067) was below the previously proposed cutoff for individuals with eGFR < 60 mL/min/1.73m² (0.087). This observation remains consistent with our classification algorithm, which suggests that the Aβ₁₋₄₂/Aβ₁₋₄₀ ratio may serve as a valuable biomarker in cases

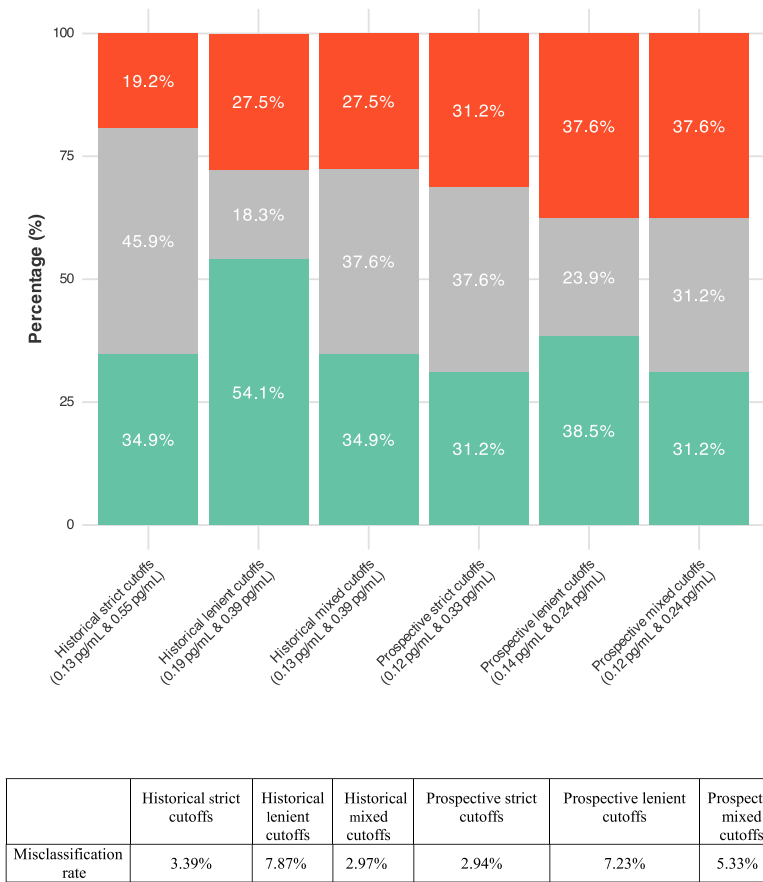


Fig. 3 Risk stratification areas across different cutoff combinations. Stacked area chart of the distribution of the different risk areas across different two-threshold approaches in the subset with CSF. In the table we show the misclassification rate related to each approach. We can observe that lenient cutoffs reduce the grey zone with the tradeoff of higher misclassification rate

of moderate to severe kidney dysfunction. The remaining seven A- cases were classified as either negative (two) or within the gray zone (five). We found differences in the prevalence of participants with eGFR <60 mL/min/m² in the group without CSF. This variable did significantly impact pTau₂₁₇ concentrations after adjustment for age and GDS, which were the three variables identified as the primary contributors to differences in pTau₂₁₇ concentrations between the groups.

Pre-analytical variability

Short-term storage conditions

For the pTau₂₁₇ stability study, 15 plasma samples were either frozen within a 4-h window in accordance with

the clinical laboratory protocol or stored in the refrigerator (4°C) for 18 h before freezing, introducing a storage delay. As shown in Fig. 4A, levels of pTau₂₁₇ were not significantly different between both short-term storage conditions ($p = 0.334$). The mean coefficient of variation (CV) between both conditions was 2.19% and the mean percentual change was 1.09%.

Clinical vs. research protocol

To evaluate potential differences between the clinical and research laboratory protocols, we compared plasma pTau₂₁₇ concentrations of 30 plasma samples that were processed under both protocols as described above. As illustrated in Fig. 4B, pTau₂₁₇ levels in Condition

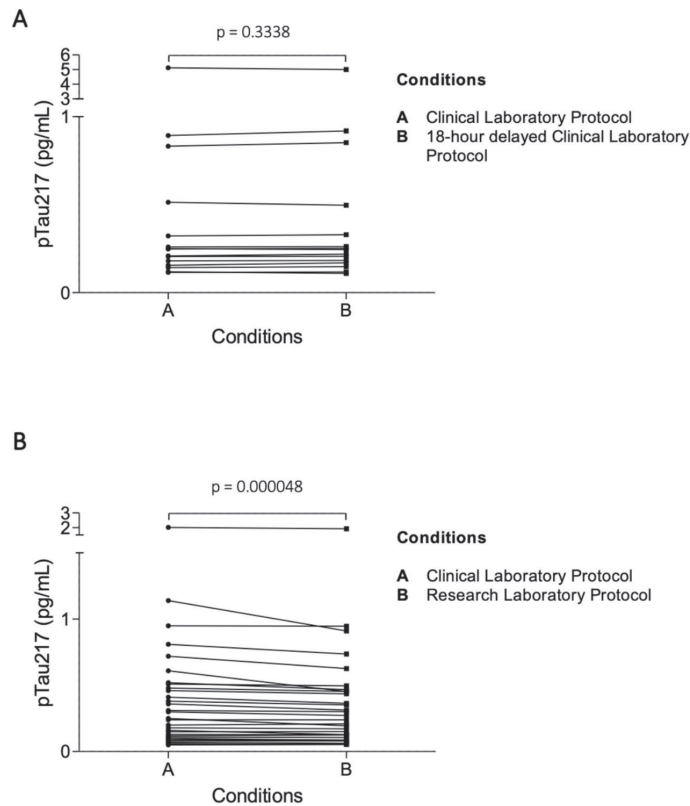


Fig. 4 Variability associated to storage conditions (**A**) and comparison between Clinical and Research protocols (**B**). **4A** displays the results of paired Wilcoxon tests comparing clinical laboratory storage conditions (4 h vs. 18 h), revealing no significant differences between them ($p = 0.3338$). In contrast, **B** presents paired Wilcoxon tests comparing the clinical (**A**) and research (**B**) protocols, showing slightly higher pTau217 concentrations under the clinical protocol ($p = 0.000048$)

A did differ significantly from those in Condition B ($p < 0.001$), finding slightly higher concentrations in clinical protocol. The mean CV between both protocols was 6.3% and the mean percentual change was 7.07%.

Analytical variability: lot-to-lot variability

To assess potential lot-to-lot variability, we calculated the inter-assay CV for plasma pTau₂₁₇ across two control levels. Both control samples were included in 12 assay runs, with either the high or low control included in the remaining 14 runs. The overall CV across 26 different runs and three different lots of the pTau₂₁₇ assay (#4097, #4129 and #5023) was 3.2% for the low control (0.49 pg/mL) and 1.8% for the high control (3.90 pg/mL). Lot-to-lot variability is presented in Supplementary Table 3.

Discussion

In this study, we evaluated the diagnostic performance of plasma pTau₂₁₇ in a real-world memory clinic prospective application. We found that the implementation of this analysis is feasible and that different combinations of cut-offs showed excellent diagnostic accuracy (92–96%), similar to that reported in the primary retrospective cohort. We also found that variations in the pre-analytical process, short-term storage conditions, or the use of different batches of analytical reagents had minimal impact on plasma pTau₂₁₇ concentrations. We observed small but statistically significant differences in plasma pTau₂₁₇ concentrations between clinical and research protocols, with minimal impact on biomarker performance. Importantly, the absence of differences between short-term storage conditions highlights its robustness, making it highly suitable for clinical laboratory use. Overall, the results support the potential of plasma pTau₂₁₇ for integration

into real-world clinical workflows and underscore the importance of evaluating such differences before implementation in routine clinical practice.

Previous studies have extensively evaluated the diagnostic performance of plasma pTau₂₁₇ compared to CSF and PET, demonstrating its high accuracy in detecting amyloid pathology across different clinical stages. Our prior research identified plasma pTau₂₁₇ as the most effective standalone biomarker to detect amyloid pathology in CSF, with an area under the curve (AUC) of 0.94, a performance that remained consistent across different cognitive stages. Our results align with those reported by Feizpour et al. [28], who validated the Lumipulse G pTau₂₁₇ assay against PET imaging, showing strong discrimination between A+ and A− individuals (AUC 0.91–0.94). While their study relied on PET as a reference, our study compared pTau₂₁₇ to CSF biomarkers, which are more widely used in clinical practice. Similarly, Cecchetti et al. [41] reported high diagnostic accuracy (AUC > 0.95) for plasma pTau₂₁₇ in a prospective cohort, consistent with our findings.

Regarding storage conditions, pTau₂₁₇ stability was evaluated by comparing the effect of freezing plasma samples within 4 h compared to keeping them refrigerated for 18 h before freezing. Results indicated no significant differences in pTau₂₁₇ concentrations between both conditions, suggesting that pTau₂₁₇ remains stable for up to 18 h under refrigeration. This finding supports the feasibility of transporting samples to external laboratories for analysis, benefiting centers without in-house testing capabilities, and thus expanding its accessibility. Consistent with our findings, a recent study using Liquid Chromatography–tandem Mass Spectrometry (LC–MS/MS) also demonstrated pTau₂₁₇ stability following delayed centrifugation and storage [42].

Analytical variability is another crucial factor in the practical implementation of a biomarker. It is important to recognize that data fluctuations arising from lot-to-lot differences could have a significant impact on the stability of cutoffs. However, no significant variability was observed in our study indicating consistency in pTau₂₁₇ measurements across different assay lots and ensuring that results were attributable to the presence or absence of AD pathophysiology and not to analytical variability. On the other hand, when assessing potential variations in pTau₂₁₇ concentrations across different laboratory protocols (clinical versus research), we observed slight but significant differences, highlighting the importance of evaluating such differences prior to its implementation in clinical practice. Collectively, these findings provide valuable insights into the sample collection, storage, and analysis of pTau₂₁₇ using an automated platform, representing

essential knowledge to support its implementation in a clinical setting.

We evaluated the generalizability of our findings to participants without a reference standard. In our cohort, we observed biomarker differences between individuals eligible for CSF analysis and those who underwent blood-only testing. Four key factors, age, clinical stage, chronic kidney disease and the inclusion in the clinical protocol, may explain these differences. Patients who did not undergo lumbar puncture were generally older, with more comorbidities and in more advanced clinical stages, with an expected higher prevalence of amyloid pathology. These findings underscore the importance of considering the context of use when interpreting biomarker results [43]. This is particularly relevant for blood-based biomarkers, as patient selection criteria in clinical practice may differ from those in CSF-based studies. The effect of renal dysfunction on plasma pTau₂₁₇ concentrations has been previously reported [44]. The effect of age and clinical syndrome on the accuracy of plasma pTau₂₁₇ has been further investigated in other platforms. Theriault et al. [45] demonstrated that in patients with mild cognitive impairment, the PPV of pTau₂₁₇ increased with age, exceeding 95% in patients aged 90+, while the NPV declined in older individuals due to the higher prevalence of Aβ pathology. In our study we found the same behavior of the biomarker in different prevalence scenarios, with the corresponding implications for the interpretation of its results.

The main strength of our study lies in its reflection of the real-world context of a memory clinic. By incorporating the prospective measurement of plasma pTau₂₁₇ into our workflow alongside the clinical laboratory, we demonstrated its feasibility for integration into our diagnostic clinical routine. On the other hand, the analysis of paired blood samples under varying pre-analytical conditions allowed us to confirm the stability of the biomarker, ensuring its reliability across different handling processes. Additionally, leveraging the prospective design, we examined the variability across multiple assay lots to assess consistency in measurements, a critical factor for its successful implementation in clinical workflows. These methodological strengths enhance the robustness and applicability of our results. But our study has also some limitations. On the one hand, not all participants had CSF samples available as a reference and those who did tended to be slightly younger and at earlier disease stages, introducing a potential bias. On the other hand, as the study was conducted at a single specialized memory clinic, the results reflect this context but should be validated in other settings where the target population may have different characteristics. Another limitation is that the pTau₂₁₇ concentration in the low control used for

lot-to-lot comparisons (0.49 pg/mL) is closer to the average levels seen in A+ individuals than in A- individuals. Lastly, the validation has been performed against CSF and not against PET or neuropathology.

Conclusion

The implementation of plasma biomarkers for identifying AD has important implications in clinical practice, including simplified logistics and resources in the diagnostic process. The stability under different storage conditions, the low lot-to-lot variability of plasma pTau₂₁₇ measurements in an automated platform, and the robustness of its accuracy under different protocol measurements result in high diagnostic performance. However, to maximize its clinical utility, when using the two-threshold approach, it is essential to optimize the trade-off between diagnostic accuracy and the proportion of individuals classified within the gray zone, ensuring a balance between precision and practical applicability. Additionally, results must be interpreted within the specific clinical setting, as factors like disease prevalence can influence biomarker performance. Altogether, our findings support the integration of plasma pTau₂₁₇ into routine clinical practice in memory clinics, providing clinicians with a reliable biomarker for AD diagnosis and facilitating the selection of patients for disease-modifying treatments.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13195-025-01779-7>.

Supplementary Material 1.

Acknowledgements

We thank all the participants in the study, the clinical team members involved in their assessment, and the laboratory technicians responsible for sample handling and analysis. This work has been carried out within the framework of the Doctoral Program in Medicine of the Universitat Autònoma de Barcelona.

Authors' contributions

DA, MT and AL designed the study. JA, RF, NZ, SR-G, IR-B, JEA-I, LM-B, JG-C, JS-G, MC-I, IB, II-G, MS-S, JF, AL, MT, DA acquired data relevant for the study. MC-I, II-G, JF, AL, MT, DA contributed vital reagents/tools/patents. MC-I, II-G, MS-S, JF, AL, DA obtained funding for the study. DA, MT, JA, RF performed statistical analysis. DA, MT, JA, RF contributed in analysis and interpretation of data. DA, MT, AL, JF participated in study supervision or coordination. JA, RF, MT and DA drafted the first version of the manuscript.

Funding

The SPIN cohort received funding from the Fondo de Investigaciones Sanitarias (FIS), Instituto de Salud Carlos III (PI21/00791, PI14/01126, PI17/01019, PI20/01473, PI13/01532, PI16/01825, PI18/00335, PI22/00785, PI19/00882, PI18/00435, PI21/00791, PI22/00611, INT19/00016, INT23/00048, PI17/01896, PI24/00598 and AC19/00103) and the CIBERNED program (Program 1, Alzheimer Disease to AL), jointly funded by Fondo Europeo de Desarrollo Regional, Unión Europea, "Una manera de hacer Europa". The SPIN cohort was also supported by the National Institutes of Health (NIA grants 1R01AG056850-01A1; R21AG056974; and R01AG061566), by Generalitat de Catalunya (2017-SGR-547, SLT006/17/125, SLT006/17/119, SLT002/16/408), "Marató TV3" foundation

grants 20141210, 044412 and 20142610, a grant from the Fundació Bancaria La Caixa to RB (DABNI project), Fundació Catalana Síndrome de Down and Fundació Víctor Grifols i Lucas. Horizon 21 Consortium is partly funded by Jérôme Lejeune Foundation.

MCI acknowledges support from the Alzheimer's Association (AARG-22-973,966) and Global Brain Health Institute (GBHI, ALZ-18-543,740), the Jérôme Lejeune Foundation (#1913 Cycle 2019B), the Societat Catalana de Neurologia (Premi Beca Fundació SCN 2020). IRB was supported by Instituto de Salud Carlos III through the Río Hortega Fellowship "CM22/00052" and co-funded by the European Union. JEA-I was supported by Instituto de Salud Carlos III through the Río Hortega Fellowship "CM22/00219" and co-funded by the European Union. JA was supported by Instituto de Salud Carlos III through the Río Hortega Fellowship "CM21/00243" and co-funded by the European Union. LMB was supported by Instituto de Salud Carlos III through the Río Hortega Fellowship "CM23/00291" and co-funded by the European Union. J.G.C. is supported by a *Río Hortega* grant (CM23/00176) from the Carlos III National Institute of Health of Spain, partly funded by the European Social Fund Plus. This study was also funded by the Instituto de Salud Carlos III (Ministerio de Asuntos Económicos y Transformación Digital, Gobierno de España) through the projects INT21/00073, PI20/01473 and PI23/01786 to J.F.), the Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas CIBERNED Program 1, partly jointly funded by Fondo Europeo de Desarrollo Regional, Unión Europea, Una Manera de Hacer Europa. This work was also supported by the National Institutes of Health grants (R01 AG056850; R21 AG056974, R01 AG061566, R01 AG081394 and R61AG066543 and 1RF1AG080769-01 to J.F.), the Departament de Salut de la Generalitat de Catalunya, Fundació Tatiana Pérez de Guzmán el Bueno (IIBSP-DOW-2020-151 to JF and SG) and Horizon 2020-Research and Innovation Framework Programme from the European Union (H2020-SC1-BHC-2018-2020 to J.F.), Brightfocus, and Life Molecular Imaging (LMI) to J.F.

Data availability

Raw anonymized data and code for statistical analysis are available upon reasonable request. All requests should be sent to the corresponding author detailing the study hypothesis and statistical analysis plan. The steering committee of this study will decide whether data/code sharing is appropriate based on the novelty and scientific rigor of the proposal. All applicants will be asked to sign a data access agreement.

Declarations

Competing interests

DA, participated in advisory boards from Fujirebio-Europe, Roche Diagnostics, Grifols S.A. and Lilly, and received speaker honoraria from Fujirebio-Europe, Roche Diagnostics, Nutricia, Krka Farmacéutica S.L., Zambon S.A.U., Neuraxpharm, Alter Medica, Lilly and Esteve Pharmaceuticals S.A. D.A. declares a filed patent application (WO2019175379 A1 Markers of synaptopathy in neurodegenerative disease). JA reported receiving personal fees for service on educational activities from Lilly, Esteve, Fujirebio-Europe and Roche diagnostics. S.R.-G. received honoraria from Esteve Pharmaceuticals S.A. for educational activities AL declare a filed patent application (WO2019175379 A1 Markers of synaptopathy in neurodegenerative disease). AL has served as a consultant or on advisory boards for Almirall, Fujirebio-Europe, Roche, Biogen, Grifols, Novartis, Eisai, Lilly, and Nutricia, outside the submitted work. JF reported serving on the advisory boards, adjudication committees, or speaker honoraria from AC Immune, Adamed, Alzheon, Biogen, Eisai, Esteve, Fujirebio, Ionis, Laboratorios Carnot, Life Molecular Imaging, Lilly, Lundbeck, Novo Nordisk, Perha, Roche, Zambón, Spanish Neurological Society, T21 Research Society, Lumind foundation, Jérôme-Lejeune Foundation, Alzheimer's Association, National Institutes of Health USA, and Instituto de Salud Carlos III. JF reports holding a patent for markers of synaptopathy in neurodegenerative disease (licensed to ADx, EPI8382175.0). No other competing interests were reported. MCI participated in an advisory board from Roche Diagnostics an dreceived speaker honoraria from Roche Diagnostics, Neuraxpharm, Lilly, Esteve Pharmaceuticals S.A and Adium Pharma.

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Received: 10 March 2025 Accepted: 3 June 2025

Published online: 05 July 2025

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4.3. Publication 3: Plasma p-tau217 as a biomarker of Alzheimer's disease pathology in individuals with Down syndrome

Huber, Hanna. Arranz, Javier. Arslan, Burak. Leuzy, Antoine. Kittel, Oscar. di Molfetta, Guglielmo. Benejam, Bessy. Videla, Laura. Barroeta, Isabel. del Hoyo Soriano, Laura. Maure-Blesa, Lucía. Rodríguez-Baz, Íñigo. Arriola Infante, José Enrique. Illán-Gala, Ignacio. Bejanin, Alexandre. Montoliu-Gaya, Laia. Lleó, Alberto. Carmona-Iragui, María. Alcolea, Daniel. Blennow, Kaj. Zetterberg, Henrik. Fortea, Juan. Ashton, Nicholas J. Plasma pTau217 as a biomarker of Alzheimer's disease pathology in individuals with Down syndrome. Nat Commun 16, 9900 (2025).

<https://doi.org/10.1038/s41467-025-65882-x>. IF 15.7. Q1.



Plasma p-tau217 as a biomarker of Alzheimer's disease pathology in individuals with Down syndrome

Received: 23 July 2025

Accepted: 24 October 2025

Published online: 10 November 2025



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Diagnosing Alzheimer's disease (AD) in adults with Down syndrome (DS), a population with a high genetically determined risk of AD, remains challenging. In this large observational study including $n = 2329$ samples from the Down Alzheimer Barcelona Neuroimaging Initiative (DABNI) and euploid controls from the Sant Pau Initiative on Neurodegeneration (SPIN) with and without symptomatic AD, we investigate if the strong diagnostic performance of plasma p-tau217 observed in sporadic AD extends to the DS population. Plasma p-tau217 discriminated cognitively stable individuals with DS from those with AD dementia with an AUC of 0.96 (95% CI, 0.95-0.97), and from those with prodromal AD with an AUC of 0.90 (95% CI, 0.87-0.92). Amyloid β ($A\beta$) positive and $A\beta$ negative individuals with DS were distinguished with an AUC of 0.95 (95% CI, 0.92-0.99). In this study, we demonstrate that plasma p-tau217 is highly accurate in detecting amyloid β positivity and predicting clinical progression in individuals with DS, outperforming other plasma biomarkers. These findings support its use as a reliable, noninvasive tool for early AD detection and management in individuals with DS.

Down syndrome (DS) is the most frequent cause of intellectual disability of genetic origin affecting 5.8 million people worldwide. Due to improved treatment of cardiac and hematological disease in DS, life expectancy has increased, and the leading cause of death in DS individuals nowadays is dementia¹⁻³. It has been estimated that the lifetime risk of developing Alzheimer-type dementia in adults with DS is >90%^{4,5}. The triplication of the amyloid precursor protein (*APP*) gene, located on chromosome 21, is sufficient to cause early-onset Alzheimer's disease (AD), making DS the most prevalent genetically determined form of the condition⁶. By age 40, nearly all individuals with DS

develop the core pathological hallmarks of AD, amyloid β ($A\beta$) plaques and neurofibrillary tau tangles⁷. Despite minor differences as the timing of initial amyloid accumulation, changes in amyloid positron emission tomography (PET) in DS appear to be comparable to autosomal dominant AD, which strongly supports early amyloid dysregulation in individuals with DS⁸. Diagnosing dementia in adults with DS is challenging, as dementia-associated cognitive decline is often obscured by the variability in intellectual disability⁹. Cerebrospinal fluid (CSF), molecular imaging, and now, blood biomarkers, have shown high diagnostic accuracy for detecting AD in DS while showing

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similar changes as in autosomal dominant AD^{5,10–16}. However, despite these similarities in core AD biomarkers, DS is associated with a high number of comorbidities and complex medical conditions differentially affecting biochemical and hematological parameters compared to euploid individuals¹⁷. The most pressing clinical need in DS is the development of therapies to prevent or delay the onset of AD. Despite being an optimal population for prevention trials—due to their higher prevalence of AD compared to families with autosomal dominant AD and their more uniform pathophysiology than sporadic AD¹—few trials have been conducted in individuals with DS.

Plasma phosphorylated tau217 (p-tau217) has recently emerged as a highly accessible and specific biomarker for the detection of biological AD, demonstrating concordance with CSF biomarkers and utility for monitoring longitudinal changes, including during the preclinical stage¹⁸. In this present observational study, we examine the diagnostic accuracy of p-tau217, and further blood biomarkers including p-tau231, p-tau181, Neurofilament light (NfL) and glial fibrillary acidic protein (GFAP), in detecting AD in DS.

Here, we show that plasma p-tau217 is a superior blood-based biomarker with high accuracy for the detection of A β pathology and the diagnosis of symptomatic AD in individuals with DS. Our findings highlight the potential of plasma p-tau217 for the early detection and monitoring of A β pathology and disease progression in individuals with DS, supporting its use in the recruitment of DS participants for therapeutic trials.

Results

Participant characteristics

Participant characteristics are summarized in Table 1 and Supplementary Table S1. A total of 2329 samples from 1372 participants were included in the study. This included 1332 samples from participants with DS (mean [SD] age at baseline, 42.7 [11.0] years; *n* [%] 614 females [46.1%], Table 1); 837 individuals were classified as asymptomatic (aDS), 145 as prodromal AD (pDS) and 351 as AD dementia

(dDS); and 997 samples from euploid individuals (69.1 [11.1] years; *n* [%] 608 females [60.1%]) classified as either cognitively normal (CN; 350 individuals), mild cognitive impairment due to AD pathology (MCI-AD; 304 individuals) or AD dementia (343 individuals) at the respective visit. Participants attended mean [SD] 3.0 [1.2] visits (range, 2–8 visits) within mean [SD] 3.0 [2.3] years (range, 0–11 years).

Plasma p-tau217 levels by clinical diagnosis and accuracy in discriminating diagnostic groups

In participants with DS, the highest plasma p-tau217 concentration was observed in dDS (mean [SD], pg/mL, 2.87 [1.42]) and pDS (1.79 [0.99]) which could be distinguished from aDS (0.56 [0.48]) with high accuracy (AUC 0.96 [95% CI, 0.95–0.97] and 0.90 [95% CI, 0.87–0.92], respectively, Fig. 1 and Table 2). Plasma p-tau217 levels across age in individuals with DS are presented in Supplementary Fig. S1. In the euploid individuals, we found a similar pattern with the highest p-tau217 levels in AD (mean [SD], pg/mL, 1.25 [0.62]), followed by MCI-AD (0.99 [0.48]) and CN (0.33 [0.16]). The levels were substantially lower compared to DS. The discrimination of cognitively unimpaired and prodromal/demented individuals (CN vs. AD, AUC, 0.97; 95% CI, 0.96–0.98; CN vs. MCI-AD, AUC, 0.96; 95% CI, 0.95–0.97) was highly accurate. The AUCs of prodromal vs. symptomatic groups are presented in Table 2. Next, we derived a binary p-tau217 cut-off for the clinical diagnosis (aDS vs. dDS) in the DS cohort using the Youden index (>1.46 pg/mL; Youden index, 0.79; sensitivity, 84.8%; specificity, 94.1%, NPV, 85.1%, PPV, 84.8%). In the euploid cohort, the cut-off value (CN vs. AD) was >0.61 pg/mL (Youden index, 0.85; sensitivity, 89.2%; specificity, Table 2).

Plasma p-tau217 levels by amyloid status and accuracy in discriminating abnormal A β

For a subset of 1061 samples (*n* = 772 euploid individuals, *n* = 289 DS individuals) paired CSF (*n* = 959) and/or amyloid PET biomarkers

Table 1 | Baseline characteristics

	All	Individuals with Down syndrome				Euploid individuals			
		aDS	pDS	dDS	p-value ^a	CN	MCI-AD	AD	p-value ^a
Age, mean [SD], years	54.0 [16]	38.8 [9.5]	51.3 [5.2]	53.6 [5.4]	1.32e-60	57.0 [12.5]	73.2 [6.1]	72.8 [7.0]	6.48e-99
Sex, count, female / male	1222/1107	353/483	74/71	187/164	0.08	218/132	186/118	204/139	0.28
MMSE score, mean [SD]	24 [7]	NA	NA	NA	NA	29 [3]	25 [4]	20 [8]	7.84e-48
CSF A β 42/40, mean [SD]	0.06 [0.03]	0.08 [0.02]	0.05 [0.01]	0.05 [0.01]	3.58e-25	0.10 [0.02]	0.04 [0.01]	0.05 [0.01]	1.14e-190
Plasma p-tau217, mean [SD], pg/mL	1.12 [1.14]	0.56 [0.48]	1.79 [0.99]	2.89 [1.47]	1.48e-90	0.33 [0.16]	0.99 [0.48]	1.27 [0.72]	4.32e-51
Plasma p-tau231, mean [SD], pg/mL	13.5 [9.4]	10.1 [5.7]	16.8 [7.3]	22.6 [10.7]	4.39e-43	9.3 [8.9]	14.5 [7.4]	17.3 [10.2]	0.06
Plasma p-tau181, mean [SD], pg/mL	18.1 [12.9]	13.0 [10.4]	23.1 [10.6]	31.0 [12.6]	5.15e-48	12.3 [9.9]	21.8 [11.4]	22.6 [9.9]	2.23e-07
Plasma NfL, mean [SD], pg/mL	17.0 [15.8]	11.6 [7.6]	21.7 [10.8]	34.2 [20.3]	8.36e-50	10.4 [8.2]	15.8 [7.6]	23.0 [25.2]	9.75e-14
Plasma GFAP, mean [SD], pg/mL	109.1 [72.1]	109.9 [73.0]	224.7 [128.3]	368.8 [208.1]	4.41e-60	110.8 [54.1]	218.0 [93.0]	268.6 [114.0]	2.55e-27
Centiloids	42.1 [42.6]	23.5 [32.7]	75.4 [37.3] ^a	71.1 [36.9] ^a	1.82e-05	23.0 [51.2]	56.5 [25.1]	73.6 [47.7]	0.45
FU time, median [SD], years	3.0 [1.2]	3.5 [2.3]	3.0 [1.8]	2.0 [1.9]	0.01	4.0 [2.2]	2.0 [0.5]	2.0 [0.5]	0.03
Number of visits, median [SD], years	3.0 [2.3]	3.0 [1.2]	3.0 [1.5]	3.0 [1.4]	3.97	3.0 [1.0]	2.0 [2.0]	2.0 [2.4]	1.69e-03

P-values have been corrected for multiple testing using Bonferroni correction.

In the DS cohort, no MMSE was performed; imaging biomarkers were available for a subgroup (*n* = 102).

A β amyloid beta, AD Alzheimer's disease aDS asymptomatic Down syndrome, CN cognitively normal, CSF cerebrospinal fluid, dDS Down syndrome with dementia, *f* female, FU follow-up, GFAP glial fibrillary acidic protein, MCI mild cognitive impairment, *m* male, MMSE minimal state examination, NfL neurofilament light, pDS presymptomatic Down syndrome, p-tau phosphorylated tau.

^aP-value refers to the comparison of the 3 diagnostic groups, results from 1-way ANOVA.

^bCompared using Pearson's Chi Square test.

^cNo significant difference was found for pDS (*n* = 10) vs. dDS (*n* = 13) group comparison from two-sided t-test (*P* = 0.7891; *t* = 0.2709, *df* = 21).

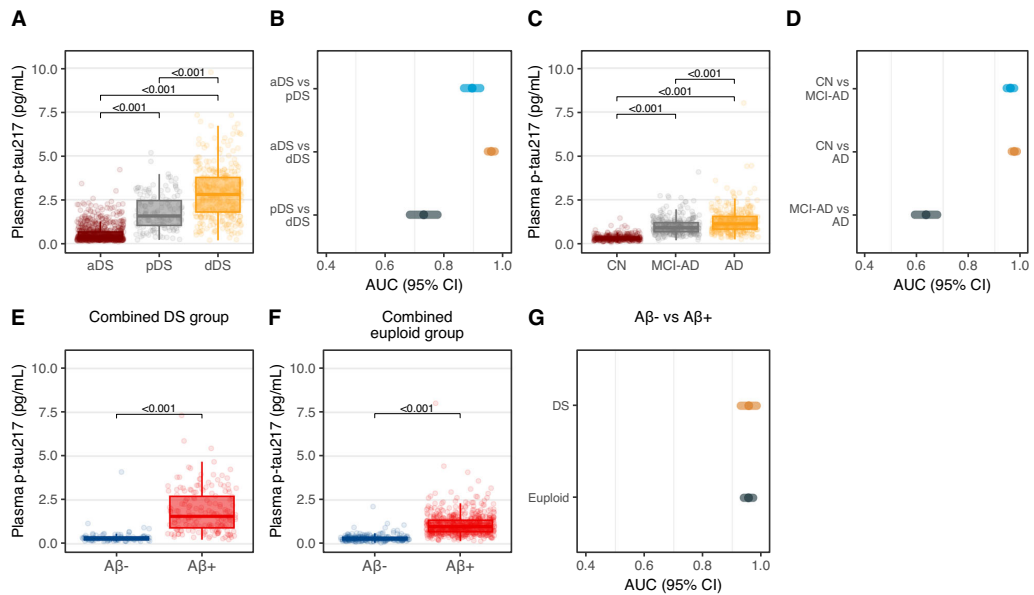


Fig. 1 | Plasma levels and diagnostic accuracy of p-tau217 in individuals with DS and euploid controls with and without AD pathology. Presented are the plasma concentrations (A, C) and the diagnostic performance (B, D) of p-tau217 in the clinical groups in individuals with Down syndrome and euploid controls. Show the plasma concentrations of p-tau217 in Aβ positive and negative individuals with (E) and without DS (F). In G the diagnostic accuracy of plasma p-tau217 in discriminating Aβ positive and negative individuals in both cohorts is presented. In A, C the cognitively stable individuals are presented in burgundy, mildly cognitively impaired individuals in gray and individuals with dementia in yellow. In B, D, G the cognitively stable group is plotted in cyan, the mildly cognitively impaired group in light peach and the dementia group in gray. In E, F the Aβ-group is displayed in blue, the Aβ+ group is displayed in red. aDS vs. dDS, $n = 1154$; aDS vs. pDS, $n = 955$; pDS vs. dDS, $n = 483$; Aβ+ vs. Aβ-, $n = 266$. Exact p-values: A: aDS vs pDS, $p = 2.09E-30$; aDS vs dDS, $p = 5.36E-96$; pDS vs dDS, $p = 2.29E-19$; C: CN vs MCI, $p = 6.18E-73$;

CN vs AD, $p = 2.62E-73$; MCI vs AD, $p = 2.44E-08$; E: $p = 3.06E-34$, F: $p = 2.59E-114$. P-values were derived from two-sided independent t-tests for pairwise group comparisons; p-values were not corrected for multiple testing. Boxplots display the median, IQR (bounds of the box), and whiskers extending to the minimum and maximum values within $1.5 \times \text{IQR}$; individual data points are shown with jittered dots (A, C, E, F). The performance of plasma biomarkers in predicting the diagnostic and Aβ groups was analyzed using AUC ROC analyses; the AUC and respective 95% CI are presented in the forest plot (B, D, G). Source data are provided as a Source data file. Aβ amyloid beta, AD Alzheimer's disease, aDS asymptomatic Down syndrome, AUC area under the curve, CI confidence interval, CN cognitively normal, CSF cerebrospinal fluid, dDS Down syndrome with dementia, IQR interquartile range, MCI mild cognitive impairment, pDS presymptomatic Down syndrome, ROC receiver operating characteristics.

($n = 102$) were available. In DS participants, plasma p-tau217 was 4.7-fold higher in Aβ+ vs. Aβ- individuals (mean [SD], pg/mL, 1.86 [1.23] vs. 0.39 [0.44]) and predicted abnormal CSF Aβ42/40 or amyloid PET with high accuracy (Aβ+ vs. Aβ-, AUC, 0.95; 95% CI, 0.92–0.99). Notably, p-tau217 levels of Aβ- asymptomatic individuals were similar in the DS and euploid groups (mean [SD], pg/mL, 0.33 [0.14] in DS; 0.31 [0.13] in the euploid cohort), while p-tau217 levels in Aβ+ asymptomatic individuals were substantially higher in DS (mean [SD], pg/mL, 0.88 [0.53]) compared to the euploid group (mean [SD], pg/mL, 0.49 [0.29]). Once more, euploid individuals followed the same pattern with plasma p-tau217 levels being 3.4-fold higher in Aβ+ compared to Aβ- individuals (mean [SD], pg/mL, 1.10 [0.62] vs. 0.33 [0.20]) and an AUC of 0.96 (95% CI, 0.94–0.97, Fig. 1 and Table 2). The optimal cut-off for defining Aβ positivity using p-tau217 was >0.59 pg/mL in DS (Youden index 0.79; sensitivity, 87.0%; specificity, 92.1%) and >0.50 pg/mL in the euploid cohort (Youden index 0.81; sensitivity, 89.3%; specificity, 91.8%). In line with current literature^{19,20}, analyses adjusted for age and sex did not substantially differ from unadjusted results; adjusted data as well as predictive value of age alone in the DS group are reported in Supplementary Table S2. Discriminative performance of plasma p-tau217 in individuals with DS was consistently high when using different threshold for amyloid PET positivity (AUC [95% CI]; 18 CL: 0.92 [0.82–1]; 25 CL: 0.96 [0.88–1]; 30 CL: 0.96 [0.89–1]; 35 CL: 0.95 [0.87–1]).

Plasma p-tau217 compared to p-tau181, p-tau231, NfL and GFAP Plasma p-tau181, p-tau231, NfL, and GFAP levels increased progressively based on clinical diagnosis, with the lowest concentrations observed in aDS, and the highest concentrations observed in dDS. This pattern closely mirrored findings in the euploid cohort (Supplementary Figs. S1–S4).

GFAP demonstrated the highest AUC among p-tau181, p-tau231 and NfL in predicting clinical outcomes (aDS vs. dDS; AUC, 0.95; 95% CI, 0.93–0.96) and identifying abnormal Aβ status (AUC, 0.88; 95% CI, 0.83–0.93) within the DS cohort (Supplementary Figs. S1–S4). Among these biomarkers in the DS cohort, p-tau231 presented the weakest accuracy in predicting clinical stage (aDS vs. dDS; AUC, 0.87; 95% CI, 0.85–0.90) and Aβ positivity (AUC, 0.79; 95% CI, 0.74–0.85). Comparable biomarker performance was also observed in the euploid cohort. Compared to p-tau217, GFAP, p-tau181, p-tau231, and NfL showed significantly lower accuracy in predicting disease progression in DS and euploid individuals (aDS vs. dDS; p-tau217 vs. p-tau181, mean difference between areas [95% CI], p-value from DeLong comparison, 0.05 [0.03–0.06], $p < 0.0001$; p-tau217 vs. p-tau231, 0.09 [0.06–0.11], $p < 0.0001$; p-tau217 vs. NfL, 0.03 [0.02–0.05], $p < 0.0001$; p-tau217 vs. GFAP, 0.02 [0.00–0.03], $p = 0.014$; and CN vs. AD; p-tau217 vs. p-tau181, mean difference between areas [95% CI], p-value from DeLong comparison, 0.12 [0.08–0.15], $p < 0.0001$; p-tau217 vs. p-tau231, 0.14 [0.09–0.18], $p < 0.0001$; p-tau217 vs. NfL, 0.11 [0.08–0.15], $p < 0.0001$,

Table 2 | Discriminative performance and cut-off values of p-tau217 in DS and euploid individuals with and without AD pathology

	AUC [95% CI]	Participant samples (N)	Cut-off p-tau217 level (pg/mL)	Youden index	Sensitivity [95% CI] (%)	Specificity [95% CI] (%)	Likelihood ratio
Individuals with Down syndrome							
Clinical diagnosis							
aDS vs. dDS	0.96 [0.95–0.97]	1154	1.460	0.789	84.8 [80.6–88.2]	94.1 [92.3–95.5%]	14.35
aDS v. pDS	0.90 [0.87–0.92]	955	0.695	0.657	89.4 [83.3–93.5]	76.36 [73.2–79.1]	3.77
pDS vs. dDS	0.76 [0.71–0.80]	483	1.775	0.351	76.0 [71.1–80.2]	59.2 [50.9–66.9]	1.86
A β status							
A β + vs. A β -	0.95 [0.92–0.98]	266	0.585	0.791	87.0 [81.3–91.2]	92.1 [84.6–96.1]	11.06
Euploid individuals							
Clinical diagnosis							
CN vs. AD	0.97 [0.96–0.98]	660	0.605	0.846	89.2 [85.4–92.1]	95.4 [92.6–97.2]	19.56
CN v. MCI+AD	0.96 [0.95–0.97]	632	0.495	0.796	91.4 [87.7–94.1]	88.2 [84.21–91.2]	7.71
MCI+AD vs. AD	0.63 [0.59–0.68]	634	1.015	0.219	59.2 [53.8–64.4]	62.7 [57.1–68.0]	1.59
A β status							
A β + vs. A β -	0.96 [0.94–0.97]	821	0.495	0.811	89.3 [84.5–92.8]	91.8 [89.3–93.7]	10.82

AUC from ROC analyses discriminating clinical groups and CSF/PET amyloid positive/negative individuals. AUC and 95% CI are presented. Youden's J statistics were used for cut-off points selection. A β amyloid beta, AD Alzheimer's disease, aDS asymptomatic Down syndrome, AUC area under the curve, CI confidence interval, CN cognitively normal, CSF cerebrospinal fluid, dDS Down syndrome with dementia, MCI mild cognitive impairment, pDS presymptomatic Down syndrome, ROC receiver operating characteristics.

p-tau217 vs. GFAP, 0.08 [0.04–0.11], $p < 0.0001$; Supplementary Figs. S2–S5; Supplementary Tables S3 and S4). Accordingly, p-tau217 outperformed the other plasma biomarkers in predicting A β positivity (DS A β + vs. A β -, p-tau217 vs. p-tau181, mean difference between areas [95% CI], p -value from DeLong comparison, 0.13 [0.08–0.17], $p < 0.0001$; p-tau217 vs. p-tau231, 0.13 [0.08–0.18], $p < 0.0001$; p-tau217 vs. NFL, 0.13 [0.06–0.16], $p < 0.0001$, p-tau217 vs. GFAP, 0.09 [0.04–0.14], $p = 0.0003$; euploid A β + vs. A β - for p-tau217 vs. p-tau181, mean difference between areas [95% CI], p -value from DeLong comparison, 0.12 [0.07–0.17], $p < 0.0001$; p-tau217 vs. p-tau231, 0.13 [0.05–0.18], $p < 0.0001$; p-tau217 vs. NFL, 0.09 [0.05–0.14], $p = 0.0001$, p-tau217 vs. GFAP, 0.07 [0.03–0.11], $p = 0.0012$; Supplementary Figs. S2–S5; Supplementary Tables S3 and S4).

Combining plasma biomarkers (all biomarkers, p-tau217 + NFL + GFAP, p-tau217 + NFL, and p-tau217 + GFAP) did not substantially increase diagnostic accuracy for clinical stage and amyloid positivity (aDS vs. dDS, p-tau217 alone AUC [95% CI], 0.96 [0.95–0.97], p-tau217 + GFAP + NFL + p-tau231 + p-tau181, AUC [95% CI], 0.97 [0.96–0.98]; DS A β + vs. A β -, p-tau217 alone, AUC [95% CI], 0.95 [0.92–0.98], p-tau217 + GFAP + NFL + p-tau231 + p-tau181, AUC [95% CI], 0.95 [0.92–0.98]; Supplementary Table S5).

Discussion

Plasma p-tau217 showed similarly high accuracy in detecting A β pathology and disease progression in individuals with DS compared to those in a euploid population, though the optimal cut-off thresholds were higher in DS. Furthermore, plasma p-tau217 outperformed the other tested plasma biomarkers in predictive accuracy. Our findings highlight the potential of plasma p-tau217 for the early detection and monitoring of A β pathology and symptomatic AD in individuals with DS, supporting its use in the recruitment of DS participants for therapeutic trials.

Recent approvals of anti-amyloid monoclonal antibodies for AD are backed by strong evidence from randomized, placebo-controlled trials demonstrating that reducing A β plaque load can lead to improved outcomes for individuals with early-stage AD^{21,22}. Despite this progress, individuals with DS and AD-type pathology have been excluded from these trials²³. There is now momentum for including the DS population in disease-modifying or prevention trials²³, similar to trials with *PSEN1* or *APP* mutation carriers. Biomarkers, especially

minimally invasive blood biomarkers, will play a critical role in recruitment and may also help assess outcomes¹⁰.

This study emphasizes the strong diagnostic performance and potential usefulness of plasma p-tau217 in individuals with DS, aligning with findings in sporadic AD^{18,24}. We demonstrated that p-tau217 had a similarly good performance in identifying true positive (A β +) and true negative (A β -) DS individuals (NPV/ PPV, 85.1%/ 84.8%, respectively). However, when assessing symptomatic AD diagnosis, NFL¹⁵, GFAP⁴¹ and p-tau212²⁵ also showed high accuracy. In earlier studies with smaller cohorts, plasma p-tau217 was identified as superior biomarker of tau pathological brain changes²⁶ and predicted A β accumulation and progression to dementia in DS²⁷. Consistent with our findings that GFAP had the second highest predictive accuracy for clinical stage and amyloid status among the biomarkers we investigated, plasma GFAP was reported to be associated with A β accumulation in DS²⁷.

Notably, as shown in this present study, the optimal biomarker cutoffs for AD pathology in DS differ; p-tau217 levels were substantially higher in the DS compared to the euploid cohort, which might be due to a higher amyloid and tau burden in DS^{28,29}. Similarly, with a p-tau217 value of 0.59 pg/mL achieving 87.0% sensitivity and 92.1% specificity, a significantly higher threshold to detect A β abnormality was observed in DS compared to the euploid group (0.50 pg/mL; 89.3% sensitivity, 91.8% specificity). This was also true for the clinical diagnosis, possibly due to the presence of preclinical AD in the aDS group, as supported by substantially higher p-tau217 levels in asymptomatic A β + individuals in the DS compared to the euploid group and the aforementioned higher amyloid and tau burden in DS^{28,29}. The accuracy of p-tau217 in discriminating pDS from dDS group was, in line with other biomarker findings²⁵, substantially lower than for other group comparisons—which may result from a slight underrepresentation of pDS individuals in our dataset, likely reflecting the rapid progression from pDS to dDS over only a few years.

In this study, the predictive accuracy of p-tau217 did not differ when adjusted for sex compared to unadjusted results. This is in line with earlier findings, where biological sex did not influence clinical and biomarker profiles of AD in adults with DS^{20,30}. Despite the evidence of sex differences in disease onset/progression and various disease mechanisms during aging in sporadic AD^{31–33}, its influence seems to be lesser in genetic forms of AD.

A limitation of this study is that these cutoff values for the ALZ-path p-tau217 are only applicable for this specific test and cannot be translated to other immunoassays detecting p-tau217, e.g., the Lumipulse G pTau217 Plasma test. Moreover, not for all individuals CSF or, especially for this cohort, amyloid PET imaging biomarker confirmation was available, which is often a challenge in this population. Evaluation of longitudinal biomarker trajectories was not in the scope of this analysis.

To conclude, the results of this present study illustrate that plasma p-tau217 is an accurate and widely accessible blood-based biomarker with significant potential for the early detection and monitoring of A β pathology and disease progression in individuals with DS, supporting its use in the recruitment of DS participants for therapeutic trials.

Methods

Participants

Before starting the study, all protocols, participant information, and consent forms are approved by the Sant Pau Research Ethics Committee (IBSP-NGF-2018-36 and IIBSP-DOW-2014-30). Investigators provide detailed explanations of the study's purpose, methods, and potential, and participants can request additional information or withdraw at any time. Before enrollment, consent and assent are obtained from the participants and their legally authorized representatives for collecting, analyzing, and storing biological samples, with participants informed about possible sharing of anonymized data and samples with other researchers. Confidentiality is maintained according to Spanish law (LOPD 3/2018). All study procedures comply with the principles of the Declaration of Helsinki³⁴.

Participants with DS with or without symptomatic AD were recruited from a population-based healthcare program that involves annual neurological and neuropsychological evaluations. Adults with DS of both sexes over the age of 18 years^{5,34} who expressed interest in research were included in the Down Alzheimer Barcelona Neuroimaging Initiative (DABNI). Euploid controls with or without symptomatic AD were recruited from the Sant Pau Initiative on Neurodegeneration (SPIN)³⁵ cohorts at the Sant Pau Memory Unit, Barcelona, Spain. Both cohorts are followed annually, and all data, including repeated measurements during follow-up visits, have been included in the present analysis to evaluate the performance of blood biomarkers in predicting clinical outcome. Euploid participants were classified as cognitively normal (CN), mild cognitive impairment due to AD pathology (MCI-AD) or AD dementia (AD); participants with DS were classified as asymptomatic (aDS), with prodromal AD (pDS) or with AD dementia (dDS) after an extensive medical history revision, a physical examination, and a neuropsychological assessment as described previously^{5,15,34}. In DS individuals, the two preliminary diagnoses made by two blinded neurologists and neuropsychologists based on clinical and neuropsychological information were reviewed in a consensus clinical meeting and a unified diagnosis was established. Clinical classification and study procedures are performed at each visit. In a subgroup, CSF and imaging AD biomarkers are collected. Sex of participants was determined based on self-report. Participants have not been financially compensated for participating in the study.

Plasma, CSF, and imaging biomarkers

Plasma biomarkers were quantified in singleclates using Single Molecule array (Simoa) technology; p-tau217 concentration was measured with the ALZpath p-tau217 v2 assay¹⁸ (104371; Quanterix, Billerica, MA, US); p-tau231 with an in-house assay developed at the Neurochemistry Laboratory, University Gothenburg, Sweden³⁶; p-tau181 with the p-tau181 v2.1 assay (104111; Quanterix, Billerica, MA, US), and NFL and GFAP with the Neurology-2-Plex-B assay (103520; Quanterix, Billerica, MA, US) by scientists blinded to participant information. Plasma samples were diluted 4-fold for the Neurology-2-Plex B assay and 2-fold

for all p-tau assays. For all biomarkers, the intra-assay CV was $\leq 6.1\%$ and the inter-assay CV was $\leq 7.0\%$.

A β -positivity (A β +) was defined as CSF A β 42/40 ratio <0.072 ($n=1028$)³⁷, CSF A β 42 <550 pg/mL ($n=77$; Fujirebio's Lumipulse G60011) or Centiloid >30 on amyloid PET using [18 F]-florbetapir ($n=102$)³⁸ irrespective of cognitive status. Discriminative performance of plasma p-tau217 in individuals with DS has been tested for four additional thresholds (18 CL, 25 CL, 35 CL).

Statistics and reproducibility

Data are shown as mean and standard deviation unless otherwise described. Group differences were assessed using one-way ANOVA for continuous variables and Pearson's Chi Square test for categorical variables. Pairwise comparisons were adjusted for multiple testing using the Bonferroni correction. The performance of plasma biomarkers in predicting the diagnostic and A β groups was evaluated using area under the curve (AUC) values receiver-operating characteristic (ROC) analyses and DeLong's test to compare AUC values for different biomarkers. All available biomarker measurements were included and treated as independent observation to enhance sensitivity in diagnostic classification by maximizing data usage from repeated assessments. The evaluation of longitudinal trajectories of the blood biomarkers was therefore not in the scope of this analysis. No statistical method was used to predetermine sample size. No data were excluded from analysis. Analyses adjusted for age and sex using binary logistic regression models did not significantly differ from unadjusted results; adjusted data is reported in the Supplementary section. Exact sample sizes for each group comparison are reported in Table 2. Youden's J statistic was used for cut-off point selection. Positive (PPV) and negative predictive value (NPV) for plasma p-tau217 positivity were assessed in relation to clinical diagnosis.

All statistical analyses were performed using GraphPad Prism 9 for Windows, MedCalc version 23.0.9, or R version 4.3.3. The following R packages have been used: ggplot2, dplyr, ggsignif, pROC, and ggsci. Significance levels were set at $P < 0.05$.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Due to consenting issues and IRB requirements, we cannot make the dataset publicly available in a repository, as explicit consent for public sharing was only introduced recently in our cohort and is therefore not in place for many participants. However, the DABNI cohort has supported dozens of international collaborations, and data can be shared following standard data transfer agreements. Requests can be directed to Juan Fortea (jfortea@santpau.cat) or Ana Bueno (abueno@santpau.cat). Access is limited to bona fide researchers for scientific purposes, and requests are typically processed and accepted within 2–3 months. Biomarker data displayed in this study are provided in the Supplementary Information/Source data file. Source data are provided with this paper.

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Acknowledgements

This work was supported by the Instituto de Salud Carlos III (INT21/OO073, PI20/O1473, PI23/O1786 [J.F.]; CP24/O0112 [L.D.H.S.]; CM23/O0291 [L.M.B.]; CM22/O0052 [I.R.B.]; CM22/O0219 [J.E.A.]; CP20/O0038 [A.B.]), the Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas CIBERNED Program 1, partly jointly funded by Fondo Europeo de Desarrollo Regional, Unión Europea, Una Manera de Hacer Europa, the National Institutes of Health (R01 AG056850; R21 AG056974, R01 AG061566, R01 AG081394, R61AG066543, 1RF1AG080769-01 [J.F.]), the Departament de Salut de la Generalitat de Catalunya, Fundación Tatiana Pérez de Guzmán el Bueno (IIBSP-DOW-2020-151 [J.F.]), Horizon 2020—Research and Innovation Framework Programme from the European Union (H2020-SC1-BHC-2018-2020 [J.F.]; Marie Skłodowska-Curie grant agreement No 860197 (MIRIADE) [H.Z.]), Brightfocus, and Life Molecular Imaging (LMI) [J.F.], the European Union [L.D.H.S., L.M.B., I.R.B., J.E.A., A.B., and H.Z.]; the Jérôme Lejeune Foundation (2326-GRT-2024A [L.D.H.S.]), the Alzheimer's Association (AARG-22-923680 [A.B.]; ZEN-21-848495, SG-23-1038904 QC [K.B.]; ADSF-21-831376-C, ADSF-21-831381-C, ADSF-21-831377-C, ADSF-24-1284328-C [H.Z.]), the Ajuntament de Barcelona, in collaboration with Fundació La Caixa (23S06157-001 [A.B.]), the Swedish Research Council (2017-00915, 2022-00732 [K.B.]; 2023-00356, 2022-01018, 2019-02397 [H.Z.]), the Swedish Alzheimer Foundation (AF-930351, AF-939721, AF-968270, AF-994551 [K.B.]), Hjärtfonden Sweden (ALZ2022-0006, FO2024-0048-TK-130, FO2024-0048-HK-24 [K.B.];

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<https://doi.org/10.1038/s41467-025-65882-x>

FO2022-0270 [H.Z.], the Swedish state under the ALF-agreement (ALFGBG-965240, ALFGBG-1006418 [K.B.]; ALFGBG-71320 [H.Z.]), the European Union Joint Program for Neurodegenerative Disorders (JPND2019-466-236 [K.B.], JPND2021-00694 [H.Z.]), La Fondation Recherche Alzheimer [K.B.], the Kirsten and Freddy Johansen Foundation [K.B.], Familjen Rönströms Stiftelse [K.B. and H.Z.], an anonymous philanthropist and donor [K.B. and H.Z.], the Alzheimer Drug Discovery Foundation (201809-2016862 [H.Z.]), the AD Strategic Fund [H.Z.], the European Partnership on Metrology [H.Z.], the European Union's Horizon Europe Research and Innovation Programme and by the Participating States (NEuroBioStand, 22HLT07 [H.Z.]), the Bluefield Project [H.Z.], Cure Alzheimer's Fund [H.Z.], the Olav Thon Foundation [H.Z.], the Erling-Persson Family Foundation [H.Z.], Stiftelsen för Gamla Tjänarinnor [H.Z.], the National Institute for Health and Care Research University College London Hospitals Biomedical Research Centre [H.Z.], and the UK Dementia Research Institute at UCL (UKDRI-1003, [H.Z.]).

Author contributions

N.J.A. and J.F. contributed equally to this work and share senior authorship. J.A., B.B., L.V., I.B., L.H.S., L.M.B., I.R.B., J.E.A.I., I.I.G., A. Lléo, M.C.I., D.A., and J.F. acquired the data. B.A., O.K., G.D.M., and L.M.G. performed the laboratory analysis. A.B., H.Z., K.B., J.F., and N.J.A. conceived, designed or supervised the study and the laboratory and data analysis. H.H. and A. Leuzy performed the statistical analysis. H.H., J.F., and N.J.A. drafted the manuscript, which all authors critically reviewed for important intellectual content. H.H. and A. Leuzy created and edited the figures. All authors have read and approved the final manuscript.

Funding

Open access funding provided by University of Gothenburg.

Competing interests

A Lléo has served as a consultant or on advisory boards for Almirall, Fujirebio-Europe, Roche, Biogen, Grifols, Novartis, Eisai, Lilly, and Nutricia outside the submitted work. M.C.I. has served on an advisory board for Roche and has received honoraria for educational events from Esteve, Lilly, Neuraxpharm, Roche and Adium pharma outside the submitted work. D.A. participated in advisory boards from Fujirebio-Europe, Roche Diagnostics, Grifols S.A. and Lilly, and received speaker honoraria from Fujirebio-Europe, Roche Diagnostics, Nutricia, Krka Farmacéutica S.L., Zambon S.A.U., Neuraxpharm, Alter Medica, Lilly and Esteve Pharmaceuticals SA outside the submitted work. D.A., A. Lléo and J.F. report holding a patent for markers of synaptopathy in neurodegenerative disease (licensed to ADx, EPI8382175.0; WO2019175379 A1) outside of the submitted work. K.B. has served as a consultant and at advisory boards for Abbvie, AC Immune, ALZPath, AriBio, Beckman-Coulter, BioArctic, Biogen, Eisai, Lilly, Moleac Pte. Ltd, Neurimmune, Novartis, Ono Pharma, Prothena, Quanterix, Roche Diagnostics, Sanofi and Siemens Healthineers; has served at data monitoring committees for Julius Clinical and Novartis; has given lectures, produced educational materials and participated in educational programs for AC Immune, Biogen, Celdara Medical, Eisai and Roche Diagnostics; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper. H.Z. has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alektor, Alzinova, ALZpath, Amylyx,

Annexon, Apellis, Artery Therapeutics, AZTherapies, Cognito Therapeutics, CogRx, Denali, Eisai, Enigma, LabCorp, Merry Life, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Quanterix, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures sponsored by Alzecure, BioArctic, Biogen, Cellectricon, Fujirebio, Lilly, Novo Nordisk, Roche, and WebMD, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program outside the submitted work. J.F. reported serving on the advisory boards, adjudication committees, or speaker honoraria from AC Immune, Alnylam, Adamed, Alzheon, Biogen, Eisai, Esteve, Fujirebio, Ionis, Laboratorios Carnot, Life Molecular Imaging, Lilly, Lundbeck, Novo Nordisk, Perha, Roche, Zambón, Spanish Neurological Society, T21 Research Society, Lumind Foundation, Jérôme-Lejeune Foundation, Alzheimer's Association, National Institutes of Health USA, and Instituto de Salud Carlos III outside the submitted work. J.F. reports holding a patent for markers of synaptopathy in neurodegenerative disease (licensed to ADx, EPI8382175.0). N.J.A. has served at scientific advisory boards or has given lectures sponsored by Biogen, Eli-Lilly, Quanterix, VJ Dementia and Roche outside the submitted work. The remaining authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41467-025-65882-x>.

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Peer review information *Nature Communications* thanks the anonymous reviewer(s) for their contribution to the peer review of this work. A peer review file is available.

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<https://doi.org/10.1038/s41467-025-65882-x>

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4.4. Brief clarification on the methodology

The specific materials and methods will be described within each article of the thesis. In this section, we will provide an overview of the cohorts analyzed across the three studies and a summary of the shared methodology.

4.4.1. SPIN cohort(18)

The SPIN cohort study began in 2011 as an umbrella program with the aim of integrating individual observational clinical studies conducted at the Sant Pau Memory Unit. The SPIN cohort currently includes cognitively normal participants, patients with subjective memory complaints, mild cognitive impairment, mild dementia due to Alzheimer's disease, frontotemporal lobar degeneration-related syndromes (FTLD-S), and dementia with Lewy bodies (DLB).

The main goal of the SPIN cohort is the discovery and validation of biomarkers. In all participants, informed consent is required for the collection of clinical (medical and neuropsychological), biofluid (blood and CSF), neuroimaging (MRI and nuclear medicine), and neurophysiological data (polysomnography, electroencephalography, and actigraphy).

Inclusion criteria require the acquisition of biofluid samples, a detailed neurological and neuropsychological assessment, and a structural 3T brain MRI. A subgroup of participants undergo video-polysomnography and/or neuroimaging studies involving radiotracers such as 18F-fluorodeoxyglucose (18FDG), amyloid PET, or Tau PET. Participants are followed annually for a minimum of 4 years and are offered the possibility of repeating imaging studies and/or CSF assessments every two years.

Cognitively unimpaired individuals represent a highly relevant group within the SPIN cohort. These volunteers are often spouses or children of patients who receive information about our studies during outpatient visits to the Sant Pau Memory Unit.

All volunteers receive an initial consultation with a neurologist, in which the SPIN protocol is explained in detail, inclusion/exclusion criteria are reviewed, and informed consent is obtained. As in other observational studies, the SPIN cohort follows a blinded design, and biomarker results are not disclosed to volunteers unless they carry clinical relevance or in cases in which the participant may be eligible for pharmacological prevention trials.

On the first study visit, a neurologist and a neuropsychologist perform a complete review of the medical history, a full physical examination, and a standard neuropsychological evaluation, followed by lumbar puncture and blood extraction. Neuroimaging studies may be scheduled on separate days.

Between 2011 and 2025, the SPIN cohort has included 4859 participants, and gathered 3327 CSF samples, 5278 plasma samples and 1493 MRI studies.

4.4.2. DABNI cohort(96)

The Alzheimer-Down Unit was founded in 2012 as a collaboration between Hospital de Sant Pau and the Catalan Down Syndrome Foundation. Its main objective is the prevention, early detection, and treatment of Alzheimer's disease (AD) in adults with Down syndrome (DS). To achieve this goal, a health plan for adults with DS was developed, which includes comprehensive medical and neuropsychological evaluations performed at least once per year as part of routine screening. In individuals with symptomatic AD, assessments are conducted every 6 months or more frequently if clinically required. The health plan incorporates structured annual or semiannual neurological and neuropsychological evaluations (semiannual in symptomatic AD participants), conducted by experienced clinicians, with visit frequency individually adapted according to each patient's needs.

All adults with DS attending the Alzheimer-Down Unit are first enrolled in the health plan and subsequently invited to voluntarily participate in the DABNI cohort, which involves additional assessments beyond routine healthcare visits under a protocol harmonized with the SPIN cohort.

DABNI was established as a population-based, multimodal biomarker study of AD, with the aim of comprehensively integrating clinical, cognitive, and biomarker data in adults with Down syndrome aged 18 years and older. This initiative is built upon the first population-wide screening program for neurological conditions in individuals with DS and was created by Hospital de Sant Pau and the Catalan Down Syndrome Foundation in Barcelona.

DABNI is a longitudinal cohort with a comprehensive multimodal biomarker program designed to advance interventions and clinical research in Alzheimer's disease associated with Down syndrome (DSAD). In addition, DABNI actively contributes to the development of therapeutic strategies by participating in clinical trials targeting AD in adults with DS.

The DABNI cohort is fully integrated within this health plan and includes individuals who provide informed consent and/or assent to participate in research studies. It includes adults with DS of both sexes aged over 18 years, recruited through the population health program for the prevention, early detection, and treatment of Alzheimer's disease and other co-occurring neurological conditions.

Individuals with all levels of intellectual disability (mild, moderate, severe, and profound), with or without associated medical conditions, are eligible for participation.

This initiative constitutes a multimodal biomarker study of AD aimed at understanding the natural history of the disease in this population. Specifically, DABNI includes clinical data from annual neurological, neuropsychological, and nursing assessments, as well as optional biomarker evaluations including neuroimaging, plasma, cerebrospinal fluid (CSF), neurophysiological, and genetic assessments.

Between 2012 and 2025, 1232 participants with Down syndrome were included in the DABNI cohort and provided 2223 plasma samples, 403 CSF samples and 590 MRI studies.

4.4.3. Processing and storage of samples in SPIN and DABNI cohorts

Lumbar puncture is proposed to participants in both SPIN and DABNI, but it is optional and not required for enrollment in both cohorts. When available, CSF is collected and processed in polypropylene tubes following international recommendations(97,98). The first 2 mL of CSF are transferred to the general laboratory for cell count and analysis of glucose and protein levels. An additional volume of 15–20 mL is transferred to our laboratory, where samples are processed and aliquoted within the first two hours after lumbar puncture. Aliquots are stored at –80 °C until analysis.

The core CSF AD biomarkers (A β 1-42, A β 1-40, total Tau, and phosphorylated Tau) are routinely measured in all participants in the LUMIPULSE automated platform (Fujirebio - Europe). Our clinical cut-offs for these core AD biomarkers were validated in a group of patients who underwent amyloid PET(99). Our laboratory participates in the Alzheimer's Association CSF biomarker quality control program(100,101).

Blood samples are drawn from all SPIN cohort participants at the time of the lumbar puncture, without requiring prior fasting, although the time elapsed since the last meal is recorded. All samples are transferred to our laboratory, where they are centrifuged and aliquoted within two hours after collection, and then stored at –80 °C until analysis.

APOE genotyping is routinely determined in all SPIN participants either through direct sequencing of exon 4 followed by visual inspection of the resulting electropherogram, or through GWAS-based sequencing to identify the two coding polymorphisms that define the three apoE isoforms.

4.4.4. Analytical platforms and assays

Plasma biomarkers were quantified using two high-sensitivity immunoassay platforms: the LUMIPULSE G600II (Fujirebio, Japan) and the Single Molecule Array (Simoa) platform (Quanterix, Billerica, MA, USA). Both technologies are widely used for clinical and research applications and enable accurate measurement of low-abundance biomarkers in blood.

The LUMIPULSE G600II is a fully automated chemiluminescent enzyme immunoassay (CLEIA) platform, designed for high-throughput testing with standardized workflows and minimal operator variability. In the first manuscript of this thesis, plasma concentrations of pTau217, pTau181, A β 1–42, and A β 1–40 were measured using commercially available assays on the LUMIPULSE platform. Analytical reproducibility was assessed using low- and high-concentration quality control samples. The kits/reagents were provided by Fujirebio®.

In the third manuscript, plasma biomarkers were quantified using Simoa technology, which enables ultra-sensitive detection by isolating single immunocomplexes on paramagnetic beads. Measurements were performed in single replicates using validated assays: plasma pTau217 was measured with the ALZpath pTau217 v2 assay (104371; Quanterix), pTau181 with the pTau181 v2.1 assay (104111; Quanterix), pTau231 with an in-house assay developed at the Neurochemistry Laboratory, University of Gothenburg (Sweden), and NfL and GFAP with the Neurology 2-Plex B assay (103520; Quanterix). Plasma samples were diluted four-fold for the Neurology 2-Plex B assay and two-fold for all pTau assays. All analyses were performed by personnel blinded to clinical information. Across all biomarkers, inter-assay CVs were $\leq 7.0\%$, demonstrating high analytical precision.

Overall, the use of fully automated and highly sensitive immunoassay platforms in this thesis ensured robust, reproducible, and clinically applicable measurement of plasma biomarkers across different study settings.

4.4.5. Data integration

All patients and volunteers are evaluated by a cognitive neurologist in the Memory Unit during a first outpatient visit lasting approximately 45 minutes. During this visit, written informed consent is obtained, and a structured medical history, neurological examination, and cognitive assessment are performed. Clinical data are systematically collected using REDCap, with predefined operational definitions for each variable to ensure consistency and data quality. Based on this evaluation, a syndromic diagnosis and an initial

etiological hypothesis are established, and additional diagnostic tests are requested when diagnostic certainty cannot be achieved at the first visit.

Once clinical information and the diagnostic plan are entered into REDCap, an automated report is generated, which serves as the official clinical record of the first visit. When mild cognitive impairment or early dementia is suspected, a comprehensive neuropsychological assessment is also requested following the initial neurological evaluation. When a progressive neurodegenerative disorder is suspected, cerebrospinal fluid biomarkers are requested to assess Alzheimer's disease pathology, and brain magnetic resonance imaging is performed to evaluate patterns of atrophy and the presence of strategic lesions. During the results visit, test findings are reviewed and a final etiological diagnosis is established.

Clinical, laboratory, and imaging data are integrated through multiple platforms, including REDCap for clinical information, Microsoft Access for laboratory data management, and dedicated computing clusters for neuroimaging data. All data sources are subsequently harmonized and consolidated into a centralized MySQL database. Using in-house pipelines developed in R, Python, and related tools, cleaned and integrated datasets are generated on a daily basis, allowing researchers within the unit to access up-to-date, multimodal data for analysis. We have developed visualization tools using ShinyApp® to facilitate personalized data extraction and visualization of neuropsychological test results.

The Down Syndrome Unit has specific organizational characteristics, with neurological assessment and neuropsychological evaluation performed on the same day and longer visit durations; however, data integration follows the same procedures as in the Memory Unit.

OVERALL SUMMARY OF THE RESULTS

5. OVERALL SUMMARY OF THE RESULTS

Overall, this research shows that plasma biomarkers, particularly pTau217, provide a highly accurate, robust, and clinically feasible tool for detecting and biologically characterizing Alzheimer's disease in both sporadic and genetically determined forms. In real-world memory clinic settings, plasma pTau217 demonstrated strong concordance with CSF amyloid status, large effect sizes across clinical stages, and high diagnostic accuracy using two-threshold approaches, enabling reliable confirmation or exclusion of Alzheimer's pathology in most patients. Its consistent performance on fully automated clinical platforms supports both scalability and routine clinical implementation.

Beyond diagnostic utility, prospective analyses confirmed the analytical stability of plasma pTau217 under routine pre-analytical conditions, with minimal variability and consistent performance in real-world clinical practice. This enabled broader biological characterization of patients, including those in advanced stages or unable to undergo lumbar puncture. In individuals with Down syndrome, plasma pTau217 showed similarly high accuracy for detecting amyloid pathology and differentiating clinical stages, outperforming other plasma biomarkers and tracking disease progression across the Alzheimer's disease continuum. Together, these results establish plasma pTau217 as a reliable and accessible biomarker for diagnosis, staging, and clinical implementation, substantially expanding access to biological evidence of Alzheimer's disease.

OVERALL SUMMARY OF DISCUSSION

6. OVERALL SUMMARY OF DISCUSSION

In this thesis, we investigated the diagnostic performance of blood-based biomarkers across several complementary cohorts, forming a unified translational framework that spans analytical validation, clinical performance, and generalizability across biological contexts. We first analysed plasma pTau217, pTau181, and the A β 42 and A β 40 peptides using the fully automated LUMIPULSE platform in a retrospective cohort comprising cognitively unimpaired individuals, patients with prodromal Alzheimer's disease, and with dementia, as well as participants with other degenerative and non-degenerative conditions. This study allowed us to derive optimal cut-offs and their agreement with CSF biomarkers, and to examine the potential influence of biological and clinical modifiers on biomarker concentrations. Using a two-cut-off approach with sensitivity and specificity thresholds of 97.5% or 95%, we observed very high diagnostic performance (94–96%) for discriminating amyloid status as defined by CSF biomarkers. Although renal function was found to affect plasma biomarker levels, its effect size was small compared with the impact of CSF amyloid positivity and was partially mitigated by the use of A β 42/40 ratios. In parallel, we demonstrated that disease prevalence strongly modulates predictive values, with negative predictive value decreasing at high amyloid prevalence and positive predictive value decreasing at low prevalence, highlighting the importance of clinical context when interpreting blood-based biomarkers.

Having established strong performance in the retrospective study, we next assessed whether these results remained stable in prospectively evaluated patients in our memory clinic, thereby testing the translational robustness of the biomarker in real-world conditions. The second article of this thesis focuses on the validation of these cut-offs in a prospective memory-clinic cohort of symptomatic patients. Diagnostic accuracy remained high (92–96%), and grey-zone proportions were consistent with those observed retrospectively. This study also investigated whether specific pre-analytical conditions, such as storage at refrigerator or freezer temperatures during the first 24 hours, differences in

pre-analytical sample handling protocols, or assay-related factors including intra- and inter-lot variability could influence biomarker concentrations. We found that these variables either had no impact on pTau217 levels or only minimal effects that did not reduce its diagnostic performance, supporting the robustness of this biomarker for routine hospital use under heterogeneous laboratory conditions.

Finally, the third article extends this diagnostic framework to Down syndrome, a genetically determined and biologically distinct population, providing an external validation of the pathophysiological relevance of plasma pTau217. Our third article examines the diagnostic performance of pTau217 measured using the ALZpath assay on the SIMOA platform in individuals with Down syndrome (DS). We observed high accuracy for discriminating A β -positive from A β -negative individuals (defined by PET or CSF), with an AUC of 0.95 and sensitivity and specificity close to 90%. pTau217 also showed strong diagnostic precision for classifying asymptomatic (aDS) versus symptomatic individuals (prodromal DSAD, pDS; and dementia-stage DSAD, dDS), with AUC values >0.90, demonstrating that the biomarker retains its discriminative power across disease stages even in a population with chronically elevated amyloid burden and different biological baselines.

Taken together, these three studies provide converging evidence that plasma pTau217 is not only analytically reliable and diagnostically accurate, but also biologically meaningful and clinically scalable across diverse settings and populations.

This thesis demonstrates that blood-based biomarkers constitute robust and clinically meaningful tools for the diagnosis of AD across distinct biological and clinical contexts. Plasma pTau217 consistently emerged as the top-performing marker, achieving diagnostic accuracy comparable to cerebrospinal fluid gold-standard measures, thereby supporting its potential role as a minimally invasive alternative in both research and clinical settings. The implementation of a two-cut-off strategy further enhanced diagnostic performance by balancing sensitivity and specificity, while acknowledging an indeterminate grey zone that reflects the biological continuum of AD and

the inherent uncertainty at early or intermediate stages. Importantly, the replication of these findings in prospective cohorts, together with the strong analytical robustness of plasma pTau217 on fully automated platforms, underscores the reliability and translational readiness of this biomarker. Finally, the demonstration of similarly high diagnostic performance in DSAD highlights the broad applicability of blood biomarkers, while also emphasizing the need for population-specific interpretation and cut-off definition due to systematic differences in biomarker concentrations.

Several aspects deserve further discussion

The first part of this discussion focuses on the appropriate selection and interpretation of cut-offs. It addresses three closely related aspects: the context of use and intended purpose of the biomarkers, the strategy used to define cut-offs, and the balance between diagnostic accuracy and gray zones. These aspects are intrinsically connected, as decisions made in one directly influence the others.

The first aspect to consider is the context of use of blood biomarkers, including diagnosis, staging, prognosis and other applications(46). We will first focus on their primary intended use, diagnosis. The diagnostic accuracy of any biomarker, particularly its predictive values, depends on the pre-test probability of disease (prevalence) and may therefore vary substantially across different clinical settings. In specialized units (Memory Clinics), the most widely used strategy to implement plasma pTau217 is the application of two cut-offs (at different levels of sensitivity and specificity: 90%, 95%, or 97.5%)(60). Patients are classified as positive (above the high/specific cut-off), negative (below the low/sensitive cut-off), or within an intermediate or “grey” zone, the width of which varies depending on the degree of “risk” (misclassification of positives or negatives) deemed acceptable. Using lower sensitivity and specificity values (e.g., 90%) narrows the grey zone at the expense of a higher proportion of false positives/negatives. In contrast, employing 97.5% sensitivity and specificity increases the proportion of patients falling within the grey zone but reduces the risk of false classifications.

It has also been observed that different assays across diverse cohorts report varying percentages of patients classified in the grey zone(102). International recommendations suggest that blood biomarkers should leave fewer than 20% of patients in the grey zone(45). Importantly, clinicians in the Memory Unit have access to additional information relevant for accurate clinical phenotyping (targeted history and physical examination, neuropsychological assessment, functional testing, and neuroimaging), which narrows diagnostic suspicion and improves patient selection and pretest probability. In this context, this approach can be applied to the selection of patients for disease-modifying therapies (DMTs) or for inclusion in clinical trials (Figure 8). Depending on the clinical context, even clear positive or negative biomarker results might require confirmatory tests (CSF or amyloid PET). In the first and second manuscript of this thesis, we explored different cut-offs with the aim to optimize accuracy and gray zones. Although the cut-offs showed good diagnostic performance, the grey zones exceeded the recommended threshold (<20%). The emerging trend is to adopt predefined cut-offs with slightly lower sensitivity and specificity (95% or even 90%)(103), to reduce the size of these grey zones. As we describe in both articles, the current goal is to identify the optimal balance between diagnostic accuracy and the proportion of individuals classified within the grey zone.

In contrast, in primary care, due to the lower disease prevalence and the more limited availability of complementary tests and examinations that enable more accurate patient phenotyping, the strategy must be more conservative. A single cut-off prioritizing sensitivity could be used for screening. Thus, a negative result may help rule out AD pathology, while a positive result would require referral to a specialized memory unit for diagnostic confirmation (Figure 9). This approach would avoid classifying patients within a grey zone, which would introduce additional complexities to the screening process. The recommendations of use of a single cut-off for screening are sensitivity >90% and specificity >75%(104). However, Palmqvist et al. (2025)(103) reported that the diagnostic accuracy of plasma pTau217 in primary care improved by 7% when using a two-cut-off approach with pre-established thresholds. Therefore, both approaches could be valid in this context while meeting accuracy recommendations for screening.

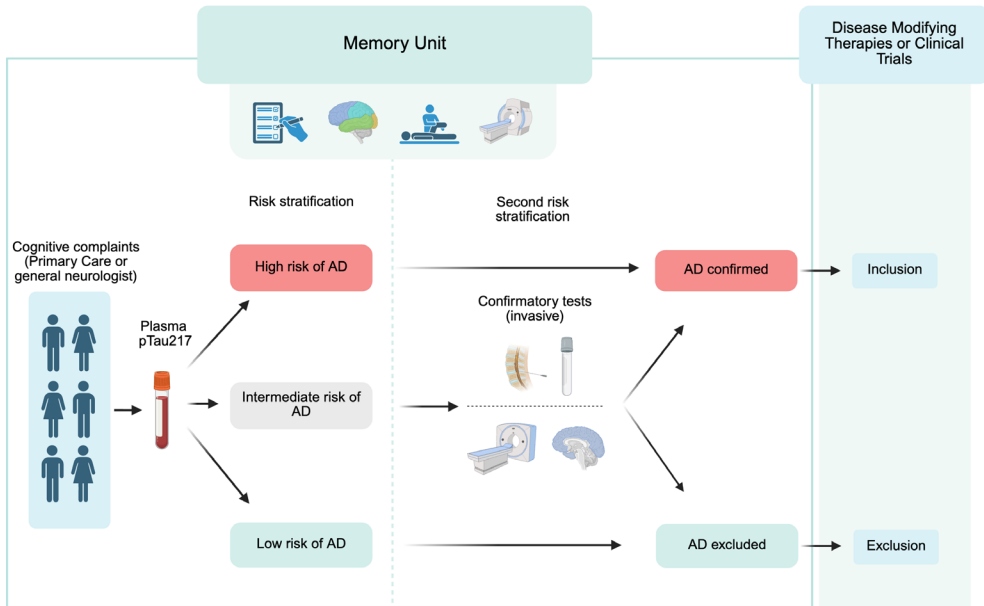


Figure 8. Context of use of plasma biomarkers in a Memory Unit

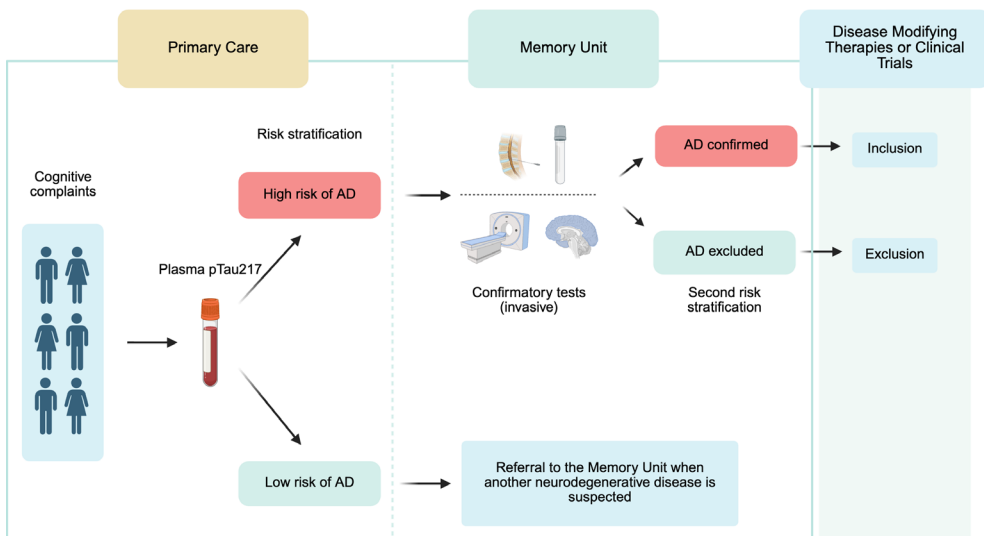


Figure 9. Context of use of plasma biomarkers in Primary Care

It is important to note that when there is high clinical suspicion of a neurodegenerative condition other than Alzheimer's disease, referral to specialized units remains advisable for further evaluation (not everything is AD). To date, population-wide screening in asymptomatic individuals is not recommended, although it may become feasible in the future depending on the outcomes of ongoing secondary prevention clinical trials.

In the context of clinical trials, plasma biomarkers could reduce screening failures by avoiding unnecessary complementary tests within the trial and prioritizing patients who are true candidates for the therapy. Altogether, this would help optimize the development and approval of new drugs by enabling a more efficient identification of eligible participants.

Their utility for disease staging remains to be fully established. There is emerging evidence from sporadic Alzheimer's disease indicates that blood-based tau species, both phosphorylated and non-phosphorylated, follow distinct temporal trajectories. Inspired by CSF staging frameworks(105) several blood-based staging models have been proposed using pTau217 thresholds alone, the pTau217/npTau217 ratio, or combinations with other tau species (e.g., pTau205, eMTBR-tau243, NTA-tau), showing strong concordance with amyloid-PET, tau-PET, cortical atrophy, and clinical progression(65,106). Chronologically, pTau231, pTau217, pTau212, pTau181, and GFAP appear to increase early and correlate with amyloid-PET, while continuing to rise across the disease continuum. In contrast, pTau205 changes later, marking early aggregated tau pathology consistent with initial Braak stages on tau-PET. At more advanced stages, additional tau species, –including MTBR fragments and non-phosphorylated forms such as eMTBR-tau243 and NTA-tau, –also become abnormal and show stronger associations with more advanced tau-PET stages(107–109). However, this temporal sequence has not been consistently replicated across cohorts and requires validation in well-phenotyped, real-world clinical populations. Moreover, it may differ in Down syndrome–associated Alzheimer's disease, where pathology appears more compressed, with earlier and more aggressive A β accumulation and earlier tau-PET positivity following amyloid-PET(110–112).

Another relevant aspect to consider is that plasma, CSF and PET imaging capture fundamentally different biological processes. Fluid biomarkers provide a real-time “snapshot” of protein production and clearance, whereas PET reflects the cumulative aggregation of pathology over many years(106,113). This temporal mismatch explains why plasma–PET correlations are not always one-to-one: plasma tau may increase before aggregates reach PET-detectable levels(114). Indeed, plasma changes often precede imaging, partly due to the limited sensitivity of current tau-PET tracers; post-mortem work indicates that a positive [18F]flortaucipir scan generally corresponds to Braak stage IV or higher(115) and partly because plasma assays measure soluble tau species, whereas PET targets insoluble aggregates(94).

In this context, the current classification in AA diagnostic guidelines of plasma biomarkers into Core 1 and Core 2 categories also warrants consideration. Core 1 biomarkers are state biomarkers that change early in the disease continuum and are considered diagnostic. They include amyloid PET measures, cerebrospinal fluid biomarkers (such as A β 42/40 and phosphorylated tau ratios), and accurate plasma markers, particularly pTau217. Core 1 can also be used for early staging. In contrast, Core 2 biomarkers become abnormal later and mainly reflect disease stage and prognosis, including tau PET and other biofluid measures of aggregated tau. Current evidence suggests that the wide dynamic range of plasma pTau217 makes it useful for early diagnosis, identifying amyloid positivity as defined by CSF A β 42/40 or amyloid PET. However, it is important to note that what we mean by ‘early diagnosis’ is dependent on the cut-off used to define amyloid positivity in the gold standard, which should be sensitive, such as higher CSF A β 42/40 ratios or lower amyloid PET Centiloid thresholds. When more stringent amyloid cut-offs are applied (for example, higher Centiloid thresholds or lower CSF A β 42/40), the time window moves closer to tau PET positivity. Therefore, plasma pTau217 cut-offs derived using more specific amyloid thresholds are also able to identify tau positivity with high accuracy. For this reason, the same plasma biomarker can capture both, Core 1 and Core 2, by applying different cut-offs(65). Importantly, these cut-offs may differ between sporadic Alzheimer’s disease and Down syndrome

Alzheimer's disease, as shown in the third manuscript of this thesis and in recent studies(111).

Regarding clinical staging, it is well established that in sporadic Alzheimer's disease plasma pTau181 increases across clinical stages(20). A similar pattern has been observed in Down syndrome, where plasma pTau181 increases across asymptomatic, prodromal, and dementia stages(116). Plasma pTau217, GFAP and NfL have also shown a stepwise increase across clinical stages in both sporadic Alzheimer's disease and Down syndrome Alzheimer's disease(117) as shown in the first and third manuscripts of this thesis. The implications of these findings in the Down syndrome population are discussed in a dedicated section of this discussion.

Finally, plasma biomarkers have shown promise in prognosis, monitoring treatment response or detecting complications (such as amyloid related imaging abnormalities,ARIA) in clinical. We will expand this topic in the future directions section.

Another important issue to address is the cut-off definition strategy, that is intrinsically linked to the intended context of use. Blood biomarkers are beginning to be applied (as of 2025) in real-world clinical practice, and their use is now gradually expanding from highly specialized units to general neurology services. One of the main challenges of this transition is that the two-cut-off approach has been used to minimize misclassification, ensuring that individuals who fall into the grey zone can undergo more advanced confirmatory testing. Outside specialized memory clinics, this raises a dilemma: whether to apply a two-cut-off strategy without easy access to confirmatory tests (CSF or amyloid PET), or to use a single cut-off that has not been validated in that population, with an increased risk of diagnostic error.

It is true that the sensitivity and specificity of pTau217 using a single cut-off are high, close to 90% for several assays. Current recommendations state that a biomarker should reach at least 90% sensitivity and specificity to be considered a confirmatory diagnostic test(104). pTau217 approaches this performance, but in some cohorts, it does not consistently meet this threshold(118). Altogether,

three assumptions emerge regarding the use of two cut-offs; i) It implicitly acknowledges that pTau217 is not yet diagnostically equivalent to its gold standards (amyloid PET or CSF). ii) It requires either access to confirmatory testing or limiting the size of the grey zone at the cost of increased diagnostic error. iii) At present, the diagnostic error associated with a single cut-off is considered unacceptably high. To date (early 2026), universal cut-offs are not yet recommended due to analytical variability, including platform and assay-related differences, lot-to-lot variability, calibrators, biological matrix effects, and pre-analytical factors. However, methodological standards and minimum performance thresholds are recommended for a test to be considered clinically useful(119). Harmonization of validation criteria and methodologies is advocated, rather than the adoption of a single universal cut-off.

As an example of cut-off generalization, Palmqvist et al.(103) proposed LUMIPULSE pTau217 cut-offs with sensitivity and specificity set at 95%, identifying 0.22 pg/mL as the low/sensitive cut-off and 0.34 pg/mL as the high/specific cut-off, reaching high accuracy and predictive values in five different cohorts. In the first manuscript of this thesis, the 95% sensitivity cut-off was 0.19 pg/mL and the high/specific cut-off was 0.39 pg/mL, leading to 18.3% of individuals falling in the grey zone. These values are very similar but not identical. Overall diagnostic accuracy was similar despite the use of different cut-offs. In Palmqvist et al., accuracy ranged from 92% to 94% across cohorts, in line with the 92% accuracy observed in our first study. However, applying the more sensitive cut-offs to our cohort would have resulted in at least a moderate number of false negatives.

Another relevant aspect is that cut-off values may be influenced by cohort characteristics. Early-onset forms of Alzheimer's disease (EOAD) are likely to show higher individual pTau181 levels than late-onset Alzheimer's disease (LOAD), as they often represent atypical or more aggressive variants of the disease(120). However, at the population level, the prevalence of these early-onset cases is much lower, so cut-off strategy in young patients may shift downwards. In contrast, with increasing age, plasma pTau217 levels in cognitively normal individuals may rise, reducing the separation between

cognitively normal amyloid negative and Alzheimer's disease and potentially requiring higher cut-offs, even if individual pTau217 levels in older patients are lower than in early-onset Alzheimer's disease. A similar consideration applies to clinical stage, as preclinical Alzheimer's disease has a lower burden of amyloid pathology and, consequently, lower pTau217 concentrations than Alzheimer's disease dementia(103,121). This age-related effect in AD pathology was not observed for the ratio pTau217/npTau217. Therefore, cut-off strategies are likely to be adapted. In any case, these considerations are exploratory and there is a clear need for continued studies in well-defined and age-specific populations. One of the key findings of this thesis, reported in manuscript 3, is that individuals with DSAD exhibit higher plasma biomarker concentrations, necessitating distinct biological and clinical cut-offs, likely reflecting the substantially greater amyloid and tau burden observed in this population(122), and a more aggressive biological progression..

A third point for discussion concerns the balance between diagnostic performance and grey-zone classification. In line with the previous sections, it is important to link grey-zone proportions to diagnostic accuracy, as their interplay determines how results should be interpreted. The goal is to classify as positive / negative more than 80% of patients while maintaining high diagnostic accuracy(46). Grey-zone proportions and diagnostic performance are only comparable across studies if the same criteria are used to define cut-offs, for example, setting sensitivity and specificity at 95% or at 97.5% and using the same gold standard. One cannot infer that a particular cut-off is superior solely because it yields a smaller grey zone, nor solely because it yields higher diagnostic accuracy. As discussed, both aspects are cohort-dependent, and even within automated platforms, generalizability may be challenging. Commercial assay manufacturers are currently competing to demonstrate the best balance between maintaining high diagnostic accuracy and reducing the proportion of individuals classified within the grey zone.

The next point of this discussion focuses on pre-analytical and analytical considerations for implementing plasma biomarkers in clinical practice, to which the manuscripts of this thesis have contributed by expanding current knowledge.

Although several pre-analytical factors known to alter biomarker concentrations have been studied, such as renal or hepatic impairment, hemolysis, and vascular risk factors(95,123–125), many additional potential influences remain to be explored. Whereas CSF provides a relatively isolated and stable compartment for biomarker measurement, numerous potential interactions exist in blood, making complete control of all confounders inherently more challenging. These include comorbidities such as sleep disorders (affecting clearance or circadian fluctuations), the presence of a third space (e.g., in patients with severe heart failure), hypercoagulability disorders, medications that may interfere with biomarker catabolic pathways, peripheral synthesis of the biomarker, and conditions associated with increased catabolism such as infections, sepsis, or other external insults that could cause transient and reversible elevations. Previous studies have suggested that biomarker ratios, including $A\beta_{42}/40$, $p\text{Tau}_{217}/A\beta_{42}$, and $p\text{Tau}_{217}/np\text{Tau}_{217}$, are less sensitive to the effects of renal clearance compared with absolute biomarker concentrations(49,72). Consistent with these observations, the first manuscript of this thesis showed that the plasma $A\beta_{42}/40$ ratio was less affected by renal function than absolute biomarker levels; however, its effect size was marginally different from that of plasma $p\text{Tau}_{217}$ alone. Fortunately, the effect size associated with amyloid-positive pathology on plasma $p\text{Tau}_{217}$ generally far exceeds the influence of most of these pre-analytical conditions. Nonetheless, borderline $p\text{Tau}_{217}$ values near the cut-off, or extreme clinical scenarios such as end-stage renal disease on dialysis, may still raise interpretative uncertainty.

In relation to the point discussed above, there is ongoing debate as to whether the $p\text{Tau}_{217}/A\beta_{42}$ ratio truly outperforms $p\text{Tau}_{217}$ alone, in terms of higher diagnostic accuracy or reduction of gray zones. The FDA approved the Fujirebio LUMIPULSE plasma $p\text{Tau}_{217}/A\beta_{42}$ assay; however, in at least one large real-world cohort (Mayo Clinic), false-positive rates approaching 40% were reported when using the recommended cut-off for amyloid pathology (Alzforum). While this was initially attributed to potential lot-to-lot or kit-related issues, concerns have also been raised regarding the inclusion of plasma $A\beta_{42}$ in the ratio. Plasma $A\beta_{42}$ is particularly sensitive

to pre-analytical factors, shows limited dynamic range between amyloid-positive and amyloid-negative individuals, and exhibits substantial biological and day-to-day variability(126,127). Notably, in-house pTau217/A β 42 assays have shown better performance than the FDA-approved version, suggesting that further assay optimization by Fujirebio will be required. Some studies have shown that the use of the ratio did not improve accuracy but decreased gray zones(103). Other studies have suggested that the pTau217/A β 42 ratio does not substantially reduce grey-zone classifications(128) and that the susceptibility of plasma A β 42 to pre-analytical handling variability may further limit the practical implementation of this ratio in routine clinical settings(126). In the first manuscript of this thesis, we evaluated the pTau217/A β 42 ratio and found no significant differences in diagnostic accuracy or gray-zone balance compared with pTau217 alone; therefore, only pTau217 cut-offs were reported. For all the reasons discussed above, the use of the ratio does not appear to provide substantially higher diagnostic performance than pTau217 alone, particularly when considering the increased risk associated with the susceptibility of A β 42 to pre-analytical variability. In this context, Roche and Eli Lilly are seeking regulatory approval in both the United States and Europe for an Elecsys plasma assay measuring pTau217 alone, while Fujirebio may pursue regulatory strategies for both the ratio and the single-analyte approach.

As a final point on pre-analytical factors, in our second study we showed that storage conditions during the first 24 hours did not significantly alter pTau217 concentrations. This provides greater confidence for the implementation of these biomarkers in settings where samples cannot be processed and frozen within a few hours after collection.

In regards of analytical considerations, in this thesis, plasma pTau217 was assessed using two analytical platforms in different study settings, with LUMIPULSE used in the memory clinic cohorts and Simoa (ALZpath) in the Down syndrome study. Although these platforms were not directly compared within the present studies, external head-to-head evaluations(129,130) have reported comparable diagnostic performance and analytical precision

despite platform-specific differences in absolute concentrations and cut-offs. In a multicenter study in which our center participated, the ALZpath plasma assay demonstrated high diagnostic performance in sporadic Alzheimer's disease, with AUCs ≥ 0.92 across three independent cohorts for discriminating A β positivity and > 0.93 for discriminating tau PET positivity(131), yielding performance comparable to that observed in our third manuscript in Down syndrome, and in our first manuscript in sporadic Alzheimer disease measured with LUMIPULSE.

LUMIPULSE offers full automation and a single standardized clinical assay, whereas Simoa provides multiple assays, including ALZpath, allowing greater flexibility but also greater analytical heterogeneity. Across studies, concentrations measured by both platforms are highly correlated, with LUMIPULSE showing slightly larger fold-changes and potentially smaller grey-zone proportions. Both platforms perform better when CSF is used as the reference standard than when amyloid PET is based on visual reads. In both assays pTau217 remains stable after short-term storage at room temperature, refrigeration, or two weeks at -20°C . The robustness of LUMIPULSE under different pre-analytical and analytical conditions was further confirmed in our prospective study, showing low coefficients of variation and reproducible results. In the second and third studies, both LUMIPULSE and Simoa showed low analytical variability, with intra- and inter-assay coefficients of variation below 7% for both platforms; CVs were slightly lower for LUMIPULSE (approximately 2–4%) compared with Simoa (approximately 5–7%), indicating marginally higher analytical precision of the fully automated platform. In the GBSC round-robin study and in a head-to-head analysis by Anastasi et al.(132), the LUMIPULSE plasma pTau217 assay ranked among the best-performing pTau217 platforms. It showed one of the highest fold changes in the round-robin evaluation and was also among the top assays in terms of AUC in Anastasi et al., although with minimal differences compared with other platforms, including MSD, NULISA and ALZpath on the Simoa platform.

It is also important to explain why, despite being the most promising blood biomarker before 2020 and showing high diagnostic performance with

AUC values around 0.90, plasma pTau181 has not matched the diagnostic performance of pTau217. While pTau217 levels in plasma are very low in A β -negative individuals, often close to zero, pTau181 shows higher baseline concentrations in cognitively unimpaired individuals. Indeed, pTau181 levels were higher in cognitively unimpaired A β -negative older adults than in younger A β -negative individuals or patients with frontotemporal dementia(20), suggesting that pTau181 is not as specific for Alzheimer's disease pathology. In addition, pTau217 has a wider dynamic range, allowing better separation between diagnostic groups. Although pTau181 may still be useful from a prognostic perspective, the broader dynamic range of pTau217 provides a clear advantage, as pTau217 increases in close association with amyloid plaque accumulation on PET imaging (70,92,133).

After discussing cut-off considerations and pre-analytical and analytical aspects, we next describe the impact of our studies on clinical practice, how blood biomarkers were progressively implemented, and how this work contributed to expanding current scientific knowledge.

Based on the results of our first study, plasma pTau217 was incorporated into routine clinical practice in our Neurology Department following institutional approval. We then conducted a prospective, real-world validation, which constitutes the second manuscript of this thesis and confirmed the retrospective findings. The introduction of blood-based biomarkers into clinical practice has markedly expanded biological confirmation of Alzheimer's disease, enabling diagnosis or exclusion in a much larger proportion of patients. This is particularly relevant for individuals without a clear indication for lumbar puncture (e.g., advanced GDS stages or low clinical suspicion of AD) or with contraindications to the procedure. As a result, biological evidence for the presence or absence of Alzheimer's disease is now available in over 90% of patients in our cohort, compared with approximately 30% when relying on CSF biomarkers alone. Importantly, this change also affected the composition of patients undergoing CSF assessment across studies. In manuscript 1, when blood biomarkers were not available as a standalone diagnostic option, CSF sampling included a more heterogeneous range of GDS stages. In contrast, in

manuscript 2, CSF was primarily reserved for patients at GDS 3, as individuals at more advanced stages were increasingly evaluated using plasma biomarkers alone. Consequently, the CSF subgroup in manuscript 2 shows a more restricted clinical profile, reflecting changes in clinical workflow rather than differences in the underlying population.

After our prospective study, we periodically evaluate the performance of the cut-offs and consider adjusting them as additional data and evidence are incorporated. For this reason, at the end of 2025 we replaced the retrospective strict cut-offs with the newly derived prospective cut-offs in manuscript 2, as these better fit the biomarker distribution of the prospective cohort. Within Geneva roadmap framework, our first manuscript contributes evidence aligned with phase 3, by evaluating the use of blood biomarkers in retrospective and longitudinal cohorts. Our second manuscript addresses phase 4, through prospective validation in a real-world clinical setting. At present and partly supported by the evidence generated in these studies (although not exclusively by them), we are now in phase 5 of the Geneva roadmap, focused on clinical implementation.

Taken together, the points discussed above highlight the importance of designing care pathways appropriately from the outset, since the aim of implementing blood biomarkers in clinical practice is to facilitate early diagnosis by reducing the costs and waiting times associated with more complex tests. For this reason, up to now the biomarker has typically been requested after an initial visit to the Memory Unit. In the future, however, it is likely that patients will increasingly arrive to their first Memory Unit visit having already undergone blood biomarker testing. This shift raises several ethical considerations. Patients and their families must be aware of, and understand, the implications of undergoing a blood test that can diagnose a neurodegenerative and potentially disabling condition before even being assessed by a specialist, potentially receiving a prodromal-stage diagnosis during their very first visit. It is also essential to ensure that the indication for testing is appropriate, that the concepts of positive and negative predictive values are correctly interpreted in each particular context, and that the patient

has been properly phenotyped both cognitively and functionally. Nonetheless, the potential benefits are clear. When properly implemented, this approach could shorten the diagnostic process, enabling patients to access disease-modifying or symptomatic therapies sooner, participate in clinical trials, begin early cognitive stimulation, organise social support resources, adapt their living environment, and plan for the future.

Our work has contributed to the growing body of evidence and has been considered in studies beyond our center. Our first study was included as supporting evidence in the Alzheimer's Association clinical practice guideline on the use of blood-based biomarkers(104). In this guideline, plasma pTau217 was classified as having a low certainty of evidence, while the plasma A β 42/40 ratio was classified as having a medium certainty of evidence according to the GRADE framework, both for screening and diagnostic purposes. In addition, the same study was eligible for inclusion in the meta-analysis led by J. Therriault(118), which evaluated the discriminative accuracy of all available plasma pTau biomarkers (pTau181, pTau217, and pTau231) against established Alzheimer's disease diagnostic standards, including amyloid PET, tau PET, CSF ratios and neuropathological assessment. Of the 6,429 studies identified through the literature search, 312 were assessed for eligibility and 113 were included in the final analysis. Plasma pTau217 emerged as the highest-performing biomarker for identifying biologically defined Alzheimer's disease, with a pooled sensitivity of 88.1%, a pooled specificity of 88.7%, and AUC of 91.1%, with moderate certainty of evidence. Importantly, this meta-analysis reports a finding that we have discussed and will further develop throughout this thesis; diagnostic performance was consistently higher in cognitively impaired individuals than in asymptomatic populations, reflecting the greater underlying burden of Alzheimer's disease pathology at symptomatic stages. In contrast, the lower biomarker elevations observed in preclinical Alzheimer's disease result in reduced diagnostic accuracy, indicating that different cut-off strategies and acceptable trade-offs between sensitivity and specificity are required depending on the target population and the clinical context.

A final and particularly important aspect addressed in this thesis is how plasma biomarkers for Alzheimer's disease should be interpreted in individuals with Down syndrome. People with Down syndrome develop genetically determined Alzheimer's disease due to trisomy 21 and triplication of the APP gene. They also present additional particularities, such as increased central and peripheral inflammation, a higher prevalence of autoimmune disorders like hypothyroidism and celiac disease, and higher BMI(96,134,135). Although this population offers a unique opportunity to study Alzheimer's disease in a predictable manner providing insights into sporadic AD the pathophysiological mechanisms do not behave identically, and conclusions obtained in one form of Alzheimer's disease cannot always be directly extrapolated to the other.

In manuscript 3 of this thesis, we showed that the diagnostic performance of blood biomarkers, particularly pTau217, is comparable to that observed in the euploid population. However, we demonstrated that baseline plasma pTau217 concentrations are higher in individuals with Down syndrome compared to sporadic AD, being more accentuated during symptomatic stages, which may necessitate different diagnostic cut-offs. This is relevant as diagnostic guidelines of blood biomarkers have not included persons in the Down Syndrome Alzheimer's disease continuum.

A further major challenge in this population is detecting cognitive decline or progression to dementia in the context of pre-existing intellectual disability (clinical staging). Although specific cognitive assessments are used for this purpose, blood biomarkers may offer greater utility than CSF in distinguishing prodromal from dementia-stage disease(134,136,137). In manuscript 3 we showed that blood biomarkers can discriminate symptomatic stages (pDS vs. dDS), with AUCs of 0.76 for plasma pTau217. Although these values are modest, they exceed those observed for other pTau CSF biomarkers. This behavior is particularly relevant in Down syndrome given its distinctive pathophysiological features, with accelerated disease processes and a shorter time window for clinical conversion from prodromal to dementia stages. In this context, CSF tau biomarkers may become abnormal earlier and therefore remain useful for state diagnosis, but they may have limited ability

to capture ongoing biological and clinical progression once symptomatic stages are reached. A similar pattern has been observed in sporadic Alzheimer's disease, where CSF tau markers are elevated in both MCI-AD and AD dementia compared with cognitively normal individuals, but reach comparable levels across these symptomatic stages, consistent with previous reports(106,113,138). Together, these findings suggest a deceleration in the rate of CSF tau increase after symptom onset in both Down syndrome and sporadic Alzheimer's disease. In contrast, plasma tau biomarkers continue to rise across the symptomatic continuum, particularly pTau217(131). In the Down syndrome population, the main value lies in the shorter time window available to identify disease progression, not only for diagnosis or prognosis but also with important implications for clinical trials and for identifying participants at the optimal time for therapeutic intervention, which plasma biomarkers may help to capture ("before A β Bothers Tau")(111,139,140).

As a final consideration, the implementation of easily accessible blood biomarkers raises several ethical challenges, particularly in relation to preclinical Alzheimer's disease, self-testing, and therapeutic coverage within national health systems.

Outside clinical trials or observational research settings, the diagnosis of Alzheimer's disease at preclinical stages is currently not recommended, as no approved treatments are indicated for these early phases. Moreover, individuals may show biological evidence of Alzheimer's disease while remaining a decade away from symptom onset, or potentially never developing clinical symptoms at all, given the prevalence of Alzheimer's disease pathology in the community(141). Nevertheless, continued research in this population is essential, particularly in the context of ongoing prevention trials such as AHEAD 3–45 and TRAILBLAZER-ALZ 3. In line with this, several studies have shown that the diagnostic performance of blood biomarkers is lower in preclinical Alzheimer's disease, as expected due to the lower cerebral amyloid burden(118). In 2025, Salvadó et al. published a study evaluating plasma pTau217 using both immunoprecipitation and immunoassay-based approaches(121). They recommended a two-step strategy combining plasma-

based screening with confirmatory CSF or PET testing, which reduced false positives without compromising sensitivity. In that study, although positive predictive values were similar when applying strict specificity cut-offs (97.5%; PPV ~80% vs ~85%), overall accuracy was higher for immunoprecipitation-based methods compared with immunoassays (88% vs 82%).

In Down syndrome-associated Alzheimer's disease, a population excluded from most major clinical trials and therapeutic guidelines due to the higher risk of ARIAs, there are ongoing clinical trials targeting preclinical stages (139) (ABATE, HERO, ALADDIN). This underscores the need for accurate and readily accessible diagnostic tools for this population, as addressed in our third manuscript. In this context, the implementation of pTau217 on the LUMIPULSE platform using population-specific cut-offs for this genetically determined Alzheimer's disease population is currently ongoing at our site.

The next ethical challenge is the potential availability of blood-based diagnostic tests directly to the public is highly controversial, since testing without appropriate clinical guidance could lead to misinterpretation and psychological harm; results must be contextualized by experienced clinicians, as a positive finding in a healthy individual could have devastating consequences without proper explanation and support.

Finally, clinicians and communicators must clearly convey that increased diagnosis in early symptomatic stages will not automatically translate into access to disease-modifying therapies, as rising healthcare demand and financial and logistical constraints are expected to limit treatment availability, leading, particularly in the early years, to careful selection of those most likely to benefit. For these reasons, the expanding accessibility of plasma biomarkers must be accompanied by responsible communication and realistic management of patient expectations.

6.1. Limitations

It is important to acknowledge the main limitations of this thesis. First, only two analytical platforms were used (Simoa–ALZpath and LUMIPULSE–Fujirebio), both of which are immunoassays dependent on calibration curves and subject to lot-to-lot variation, with slightly lower CV in the LUMIPULSE platform. These platforms were not directly compared within this thesis, as each was applied in different studies, and LUMIPULSE was used only in the sporadic Alzheimer’s disease population, whereas Simoa was applied in both populations. However, despite these differences, the ALZpath pTau217 assay on the Simoa platform in sporadic Alzheimer’s disease has shown diagnostic performance comparable to that observed in our third manuscript in Down syndrome(131). Beyond platform availability, the lack of assay commutability between LUMIPULSE and Simoa represents an additional limitation. Although plasma pTau217 values measured on both platforms are highly correlated, reference materials are not commutable, preventing the use of universal calibrators and cut-offs. While pTau217 shows similarly high fold-changes and AUCs on both platforms, absolute concentrations and dynamic ranges differ. Notably, plasma–CSF correlations remain only moderate overall and are weak in Alzheimer’s disease for most assays, with LUMIPULSE being the only platform showing a detectable correlation with CSF pTau217 in this setting (round robin). Another limitation is that, despite collaboration with external centers for reagent supply or analytical support (e.g., UGOT), both cohorts (SPIN and DABNI) are single-center cohorts. The cut-offs derived in this thesis have not yet been validated in external cohorts. The absence of CSF pTau217 or amyloid PET as gold standards across all three studies further limits the direct anchoring of plasma measurements to central pathology. Finally, we included diverse populations, including individuals with Down syndrome-associated Alzheimer’s disease; however, most participants of the three studies were White/Caucasian.

6.2. Final Summary

The evaluation of the diagnostic performance of blood biomarkers, measured across different analytical platforms, under diverse pre-analytical and analytical conditions, and in distinct forms of Alzheimer's disease (sporadic and genetically determined, as in Down syndrome), is essential to ensure their safe, reliable, and generalizable implementation in real-world clinical practice. Given the emergence of effective therapies, early and accurate identification of Alzheimer's pathology is critical, and blood biomarkers play a key role in expanding accessibility to the widest possible population. Moreover, considering the historical discrimination faced by individuals with intellectual disabilities, such as those with Down syndrome, studying the specific behavior of biomarkers in this population allows their correct interpretation and helps delineate both the similarities and differences relative to sporadic Alzheimer's disease.

In this thesis, we derived plasma pTau217 cut-offs for sporadic Alzheimer's disease in a retrospective cohort, validated them in a prospective real-world memory clinic cohort, assessed the influence of pre-analytical and analytical factors on diagnostic performance and evaluated diagnostic accuracy in genetically determined forms of Alzheimer's disease (DSAD). Moreover, our findings have led to the practical implementation of plasma pTau217 in our center, improving diagnostic pathways by increasing diagnostic certainty earlier in the diagnostic process through a less invasive and more cost-effective approach than CSF, particularly for patients with contraindications to lumbar puncture or who decline the procedure. Altogether, these findings contribute to a better understanding of the disease's pathophysiology across Alzheimer's variants and enable more efficient interpretation of biomarker results within their intended contexts of use.

CONCLUSIONS

7. CONCLUSIONS

- 1) Blood-based biomarkers demonstrate high diagnostic accuracy for discriminating Alzheimer's disease pathology, with plasma pTau217 showing the strongest performance, comparable to CSF gold-standard measures. The application of a two-cut-off strategy further improves diagnostic accuracy at the cost of defining an indeterminate grey zone, whose size depends on the acceptable risk of misclassification.
- 2) In prospective cohorts, plasma pTau217 measured on a fully automated platform maintains high diagnostic performance consistent with retrospective findings, showing stability across different short-term storage conditions, low coefficients of variation across reagent lots and analytical runs, with minor variability related to pre-analytical protocols.
- 3) Blood biomarkers show comparable diagnostic performance in Alzheimer's disease associated with Down syndrome. Plasma pTau217 achieves AUC values >0.90 for detecting Alzheimer's disease pathology in individuals with Down syndrome and discriminates cognitive stages with similarly high accuracy. However, biomarker concentrations, and consequently diagnostic cut-offs, differ from those observed in sporadic Alzheimer's disease.

FUTURE DIRECTIONS

8. FUTURE DIRECTIONS

8.1. Clinical deployment of blood biomarkers across healthcare settings

Looking ahead, the biomarkers most likely to reach routine clinical practice will be those that can be implemented on automated platforms, enabling generalization and broad accessibility beyond specialized memory clinics. Assays that demonstrate strong pre-analytical stability and robust analytical performance across diverse laboratory conditions will be essential, as many hospitals cannot adhere to highly standardized protocols. Because analytical variability is inherent to immunoassays, the separation between cases and controls must be sufficiently large to avoid diagnostic misclassification in real-world settings. Plasma pTau217 measured on automated platforms such as LUMIPULSE currently fulfills many of these requirements, facilitating its deployment in routine neurology and potentially in primary care.

The expansion of blood-based testing will profoundly improve accessibility compared with lumbar puncture. However, it also raises important challenges, particularly regarding the diagnosis of individuals in preclinical or minimally symptomatic stages, for whom disease-modifying therapies may not yet be accessible. The increasing use of plasma biomarkers must therefore be accompanied by careful definition of clinical contexts of use, appropriate referral pathways, and alignment with treatment availability, especially as healthcare systems adapt to the growing demand generated by earlier and broader testing.

8.2. Integration with multimodal and emerging biomarkers. Moving towards precision medicine

There is growing interest in biomarkers that capture the key molecular pathways involved in Alzheimer's disease, including neuroinflammation, synaptic markers such as β -synuclein(142,143), SNAP-25 and VAMP-2(144), and markers of copathology that will help in the differential diagnosis, as

those that measure protein aggregation as extracellular vesicle-derived tau and TDP-43 species(145), and misfolded protein amplification assays such as α -synuclein seed amplification for Lewy body disease(146).

A growing ecosystem of emerging biomarkers is expected to complement plasma pTau217 across neurodegenerative diseases. In addition, new analytical platforms, including biosensors(147,148), retinal imaging(149), and minimally invasive matrices such as saliva(150,151), skin(152), dried blood spots(153,154) and digital biomarkers for cognition (Altoida,Tabcat), speech and sleep (actigraphy) may further expand accessibility and enable decentralized testing(155). Future clinical use of plasma pTau217 will not occur in isolation but as part of an integrated biomarker framework, combining neuroimaging, fluid biomarkers, genetic data and digital biomarkers(114,155,156).

8.3.Expanding contexts of use:Prognosis,monitoring disease progression and treatment response

Emerging evidence also highlights the prognostic value of plasma biomarkers beyond their diagnostic utility. In a large real-world memory-clinic cohort from Fundació ACE, elevated plasma pTau181 was associated with a higher risk of progression from MCI to Alzheimer's disease dementia (HR = 1.84) (155). Consistently, Groot et al.(156) showed that two different pTau217 assays (Janssen on Simoa and Lilly on MSD) predicted conversion from MCI-AD to dementia with AUCs > 0.88, and that the annual increase in biomarker levels was higher in individuals who progressed. Similarly, longitudinal data from the Sant Pau SPIN cohort demonstrated that higher baseline pTau217 levels were associated with faster cognitive decline and increased risk of progression to clinical Alzheimer's disease, independent of clinical stage, and that pTau217 rises progressively from preclinical to symptomatic phases, supporting its use for risk stratification and disease monitoring(157). Building on this prognostic framework, Osenkopppele et al. proposed a two-step recruitment strategy for preclinical AD and MCI-AD trials to enrich for individuals at higher risk of clinical progression, using plasma pTau217 for initial screening followed by tau-PET

in those with elevated pTau217, thereby improving screening efficiency and reducing the number of required tau-PET scans(133). Together, these studies establish plasma tau biomarkers, particularly pTau217, not only as diagnostic tools but also as robust prognostic markers, enabling individualized risk stratification, monitoring of disease trajectories, and more efficient design of clinical trials enriched for participants most likely to progress.

Other plasma biomarkers have also shown prognostic value in Down syndrome Alzheimer's disease. In a longitudinal study, plasma GFAP discriminated symptomatic from asymptomatic individuals with high AUC and was higher in progressors(158), while plasma NfL predicted clinical progression in Cox models and showed distinct longitudinal trajectories in progressors versus non-progressors, with AUCs across clinical groups (aDS, pDS, dDS)(159). Following studies from our site will assess the prognostic value of different pTau species, evaluating how rapidly individuals with positive biomarkers progress compared with those with negative biomarkers, adjusting for age.

A major future application of plasma biomarkers will be in longitudinal monitoring and therapeutic response assessment. Both pTau181 and pTau217 decrease following amyloid plaque removal with anti-amyloid therapies such as Lecanemab (Clarity-AD) and Donanemab (TRAILBLAZER-ALZ-2), closely paralleling reductions in amyloid-PET and clinical benefit. GFAP also declines with treatment, reflecting reduced astroglial activation, supporting the role of plasma biomarkers as pharmacodynamic readouts.

In contrast, plasma biomarkers are not yet reliable for predicting or diagnosing ARIA, and MRI remains essential for safety monitoring(160). Ongoing research into vascular and inflammatory markers such as complement proteins, combined with genetic risk factors such as APOE ε4, may eventually allow individualized ARIA risk stratification. As disease-modifying therapies become more widely used, the development of reliable blood-based markers of progression, treatment response and adverse events will be central to the safe and effective implementation of precision medicine in Alzheimer's disease.

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9. BIBLIOGRAPHY

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