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Evaluation and validation of blood-based biomarkers for the early diagnosis of Alzheimer's Disease: from research to clinical application

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1. Introduction

Alzheimer's Disease (AD) is the most common cause of dementia, accounting for approximately 60–80% of all cases worldwide [1]. It is a progressive neurodegenerative disorder characterized by cognitive decline, memory loss, and functional impairment that severely impact quality of life. With the global aging population, the prevalence of AD is projected to rise dramatically, posing an immense public health, social, and economic burden [2]. Despite decades of research, early and accurate diagnosis of AD remains a major challenge, as definitive confirmation still relies on post-mortem neuropathological examination or costly, invasive methods such as cerebrospinal fluid (CSF) analysis and positron emission tomography (PET) imaging [3].

The pathophysiological changes underlying AD, including amyloid-β (Aβ) deposition and tau pathology, begin decades before the onset of clinical symptoms [4]. This preclinical window offers a critical opportunity for intervention, underscoring the urgent need for reliable, accessible, and non-invasive biomarkers to detect disease at its earliest stages. In recent years, blood-based biomarkers have emerged as a promising alternative to traditional diagnostic tools. Advances in ultrasensitive assay technologies have enabled the quantification of low-abundance proteins in plasma, including Aβ42/40 ratios and phosphorylated tau isoforms (p-tau) [5]. These biomarkers have shown strong correlations with established CSF and imaging markers, suggesting their potential for large-scale clinical implementation.

However, translating blood-based biomarkers from research settings to routine clinical practice requires rigorous analytical validation and clinical evaluation. Challenges persist regarding assay reproducibility, biological variability, and the influence of comorbidities and demographic factors. Furthermore, establishing clinically meaningful cut-offs and integrating these biomarkers into

diagnostic algorithms alongside cognitive assessments and imaging tools remains a key step toward their practical adoption.

This research project aims to evaluate and validate a panel of blood-based biomarkers for the early diagnosis of AD, bridging the gap between experimental discovery and clinical application. By addressing both analytical and clinical aspects, this project seeks to develop robust, accessible diagnostic tools that facilitate early detection, patient stratification, and timely therapeutic intervention in Alzheimer's Disease.

2. Alzheimer's Disease

2.1 The global burden

AD represents one of the most pressing public health challenges of the 21st century. It is the leading cause of dementia globally, accounting for the majority of the estimated 55 million people currently living with dementia worldwide. According to the World Health Organization (WHO), this number is projected to reach 78 million by 2030 and 139 million by 2050, driven primarily by population aging and increased life expectancy [6]. The incidence of AD doubles approximately every five years after the age of 65, making it a major contributor to morbidity, disability, and dependency among older adults.

The long duration of illness before death contributes significantly to the overall public health impact of AD, as much of this period is spent in a state of severe cognitive and functional impairment. To quantify this impact, standardized measures to assess the burden of diseases not only in terms of mortality but also disability have been developed. One such metric, the disability-adjusted life year (DALY), combines the years of life lost (YLLs) due to premature mortality with the years lived with disability (YLDs), providing a comprehensive estimate of disease burden across populations. Based on these measures, Alzheimer's disease ranks among the most burdensome conditions globally, not only for affected individuals but also for their families, informal caregivers, and communities.

In the United States, the burden of AD has increased more dramatically than that of many other diseases in recent decades. According to the Global Burden of Disease (GBD) classification, AD rose from the 12th most burdensome disease or injury in 1990 to the sixth in 2016 in terms of DALYs. In that same year, it was ranked fourth for YLLs and 19th for YLDs [7,8]. These figures underscore the extensive human and societal toll of AD. However, it is important to interpret these estimates with caution. Variability in data sources, differences in how disability is defined and measured, and inconsistencies in reporting across states and years may limit the comparability and precision of these estimates. Moreover, DALYs and related measures may not fully capture the social context in which

disability occurs, such as differences in social support, attitudes, and economic resources, which can vary widely both across and within countries.

Globally, the economic and societal implications of Alzheimer's disease are profound. The global economic burden of dementia was estimated at over US$1.3 trillion in 2019, which is expected to more than double by 2030 if current trends continue [9]. Most of these costs arise from long-term care, informal caregiving, and loss of productivity, disproportionately affecting low- and middle-income countries where healthcare resources and social support systems are limited. Beyond financial strain, AD exerts immense emotional and psychological pressure on patients and caregivers. The progressive loss of memory, autonomy, and identity not only complicates clinical management but also challenges societal perceptions of aging and mental health [10]. Furthermore, disparities in access to diagnostic services, delayed detection, and underrepresentation of diverse populations in research exacerbate global inequities in AD care and outcomes.

Given the escalating prevalence, economic costs, and human toll associated with Alzheimer's disease, early detection and intervention have become central priorities in global health policy. Initiatives such as the WHO Global Action Plan on the Public Health Response to Dementia (2017–2025) and the Lancet Commission on Dementia Prevention, Intervention, and Care underscore the urgent need for scalable, equitable, and cost-effective diagnostic strategies [11-13]. Within this context, the development and validation of blood-based biomarkers hold transformative potential for improving early diagnosis, enabling timely intervention, and ultimately reducing the global burden of Alzheimer's Disease.

2.2 Pathophysiological hallmarks

AD is characterized by a complex interplay of molecular, cellular, and systemic mechanisms that culminate in synaptic dysfunction, neuronal loss, and cognitive decline. Historically, the disease has been defined by two neuropathological hallmarks: extracellular Aβ plaques composed of the Aβ peptide and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein (Figure 1). However, contemporary research has expanded this framework to encompass a broader spectrum of pathophysiological processes, including neuroinflammation, synaptic and mitochondrial dysfunction, vascular alterations, and impaired proteostasis. These interconnected mechanisms form a dynamic cascade that begins decades before clinical symptoms, shaping the continuum from preclinical to symptomatic stages of AD.

The amyloid cascade hypothesis, first proposed in the early 1990s, remains a central conceptual model for AD pathogenesis [14]. It postulates that the overproduction or impaired clearance of Aβ42 initiates a cascade of downstream neurodegenerative events. Aβ peptides are generated through sequential proteolytic cleavage of amyloid precursor protein (APP) by β-secretase (BACE1) and γ-secretase. Imbalance in this process results in the accumulation of aggregation-prone Aβ species that oligomerize into soluble protofibrils and ultimately deposit as extracellular amyloid plaques, predominantly in the neocortex and hippocampus.

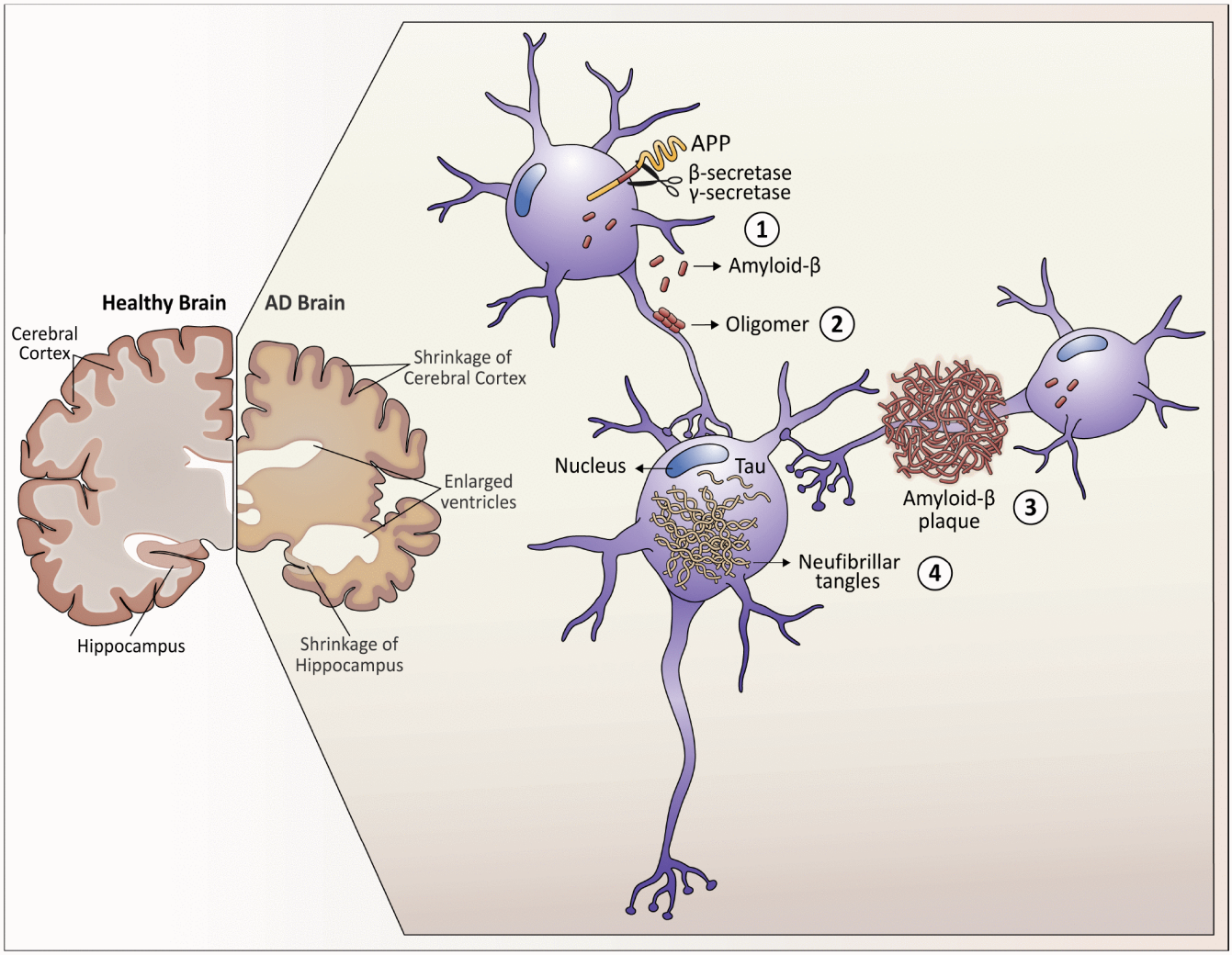

Figure 1. Neuropathological hallmarks underpinning Alzheimer's disease.

Recent evidence emphasizes the neurotoxic role of soluble Aβ oligomers rather than insoluble fibrillar plaques. These oligomers disrupt synaptic plasticity, impair long-term potentiation, alter calcium homeostasis, and promote oxidative stress and neuroinflammation. Moreover, Aβ pathology exhibits a characteristic spatiotemporal pattern, beginning in the neocortex and spreading to other brain regions. Impaired clearance mechanisms, including reduced enzymatic degradation (via neprilysin and insulin-degrading enzyme), compromised glymphatic function, and inefficient transport across the blood–brain barrier, further exacerbate Aβ accumulation. Genetic studies reinforce the amyloid hypothesis, as mutations in APP, PSEN1, and PSEN2 (which encode components of γ-secretase)

cause early-onset familial AD by increasing the production or aggregation propensity of Aβ42. However, emerging data also suggest that Aβ deposition alone is insufficient to produce full clinical dementia, indicating a need to integrate other pathological pathways.

The second major neuropathological hallmark of AD involves the intracellular aggregation of abnormally phosphorylated tau, a microtubule-associated protein predominantly expressed in neurons. Under physiological conditions, tau stabilizes microtubules and supports axonal transport.

In the adult human brain, six tau isoforms are generated by alternative splicing of the MAPT gene, differing by the presence of three (3R) or four (4R) microtubule-binding repeats and zero, one, or two N-terminal inserts. Both 3R and 4R tau isoforms are present in Alzheimer's disease, and their co-aggregation is a defining feature of AD pathology, distinguishing it from other tauopathies that may show predominance of a single isoform [15-17].

The balance and ratio of these isoforms are tightly regulated under physiological conditions, contributing to microtubule stabilization and axonal transport. In Alzheimer's disease, dysregulation of tau isoform expression and post-translational modifications, especially hyperphosphorylation, leads to the formation of neurofibrillary tangles and paired helical filaments, which disrupt neuronal function and drive neurodegeneration [18-20]. Isoform-specific differences in aggregation kinetics and vulnerability to pathological modifications have been shown; for example, 4R tau aggregates more rapidly than 3R tau, and certain isoforms, such as 1N4R, confer increased susceptibility to amyloid-beta-induced toxicity in human neurons [21].

Altered tau isoform expression and aggregation are associated with synaptic loss, cognitive decline, and disease progression in AD, and the specific composition of tau isoforms within neurofibrillary tangles may influence the severity and regional distribution of pathology [22].

In AD, tau undergoes hyperphosphorylation at multiple serine and threonine residues, leading to its detachment from microtubules, misfolding, and aggregation into paired helical filaments (PHFs) that assemble into NFTs.

The tau propagation hypothesis posits that misfolded tau spreads trans-synaptically in a “prion-like” manner, following a predictable anatomical progression described by Braak and Braak staging, from the entorhinal cortex and hippocampus to associative neocortical regions [23-25]. The burden and distribution of tau pathology correlate more closely with the degree of cognitive impairment than amyloid load, suggesting that tau aggregation is a key mediator of neuronal dysfunction and cell death. Molecular imaging using tau PET tracers and fluid biomarkers, such as p-tau isoforms (p-tau181, p-tau217, and p-tau231), has advanced understanding of tau dynamics, demonstrating that changes in specific p-tau species reflect early and region-specific tau pathology, often preceding overt atrophy and cognitive decline.

Neuroinflammation has emerged as a third core component of AD pathophysiology. Activation of microglia and astrocytes in response to Aβ and tau aggregates plays a dual role, initially protective through phagocytic clearance, but chronically detrimental through the release of pro-inflammatory cytokines, reactive oxygen species, and complement factors [26,27]. Genome-wide association studies (GWAS) have identified numerous AD risk genes related to immune function, including TREM2, underscoring the role of the innate immune system in disease susceptibility and progression.

Activated microglia exhibit distinct phenotypes along a spectrum from homeostatic to neurodegenerative states [28]. Persistent activation leads to impaired phagocytosis, altered lipid metabolism, and synaptic pruning. Astrocytes, in turn, undergo reactive astrogliosis, contributing to excitotoxicity, disruption of the blood–brain barrier, and metabolic dysregulation. Reactive astrogliosis is a hallmark response to Aβ and tau pathology, characterized by hypertrophy, upregulation of glial fibrillary acid protein (GFAP), and altered gene expression [29-31]. Peripheral immune cells may also infiltrate the brain, amplifying the inflammatory milieu [32]. This chronic

neuroinflammatory state is now recognized not merely as a secondary response but as an integral driver of neurodegeneration and a potential therapeutic target.

Synaptic loss is the most robust correlate of cognitive decline in AD, as it directly impairs neuronal communication and network integrity [33]. Soluble amyloid beta oligomers disrupt synaptic function by aberrantly activating NMDA receptors, leading to excessive calcium influx, oxidative stress, and downstream activation of calcineurin and cofilin, which destabilize dendritic spine structure and promote spine loss [34,35]. These oligomers also induce endocytosis and altered trafficking of NMDA and AMPA receptors, reducing synaptic strength and impairing long-term potentiation (LTP), while favouring long-term depression [36].

Hyperphosphorylated tau accumulates in dendrites and synapses, disrupting microtubule stability and impairing dendritic spine morphology. Tau pathology also interferes with synaptic vesicle release and receptor trafficking, further reducing synaptic efficacy [33]. Both Aβ and tau promote glial activation, which can lead to excessive synaptic pruning.

Mitochondrial dysfunction is a central driver of energy deficits and increased oxidative stress in AD. Aβ and tau interact with mitochondrial fission proteins (e.g., Drp1), leading to excessive mitochondrial fragmentation and an imbalance in fission–fusion, which reduces ATP production and disrupts calcium buffering at synapses [35,36]. Defective mitophagy leads to the accumulation of damaged mitochondria, further exacerbating oxidative stress and triggering apoptosis via cytochrome c release [39]. These mitochondrial abnormalities impair synaptic vesicle release and dendritic spine maintenance, contributing to synaptic degeneration and cognitive decline. Thus, altered NMDA and AMPA receptor trafficking, impaired dendritic spine structure, reduced synaptic vesicle release, energy deficits, increased oxidative stress, apoptosis, and abnormal mitochondrial dynamics including impaired fission–fusion balance and defective mitophagy are mechanistically linked to cognitive decline in Alzheimer's disease through the combined actions of synaptic loss, soluble amyloid beta oligomers, hyperphosphorylated tau, and mitochondrial dysfunction.

Vascular pathology is increasingly recognized as a significant contributor to AD pathogenesis [40]. Cerebral amyloid angiopathy (CAA), characterized by the deposition of Aβ in cerebral vessel walls, impairs vascular integrity and clearance mechanisms [41]. Reduced cerebral blood flow, endothelial dysfunction, and disruption of the neurovascular unit compromise nutrient delivery and waste removal. These vascular alterations synergize with amyloid and tau pathology to accelerate neurodegeneration. The concept of "vascular–amyloid–tau interaction" highlights the bidirectional relationships between cerebrovascular dysfunction and protein aggregation, suggesting that AD lies on a continuum with vascular cognitive impairment [42].

Beyond the canonical pathways, AD involves widespread proteostatic imbalance. Dysregulation of autophagy and the ubiquitin–proteasome system impairs the clearance of misfolded proteins, contributing to their intracellular and extracellular accumulation [43]. Endosomal-lysosomal dysfunction, observed early in disease, may represent a unifying link between amyloid, tau, and inflammatory pathways [44]. Moreover, alterations in lipid metabolism, RNA processing, and epigenetic regulation are increasingly recognized as modulators of AD risk and progression. The APOE ε4 allele, the strongest genetic risk factor for late-onset AD, influences multiple processes, including lipid transport, Aβ aggregation, and glial reactivity, underscoring the multifactorial nature of disease mechanisms [45].

Modern conceptual models now describe AD as a multifactorial network disorder, in which amyloid, tau, neuroinflammation, and vascular dysfunction interact dynamically over time. The AT(N) framework, introduced by the National Institute on Aging–Alzheimer's Association (NIA-AA), categorizes biomarkers into three primary pathological domains: A (amyloid pathology), T (tau pathology), and N (neurodegeneration) [46]. This model provides a biological definition of AD that transcends clinical symptoms, enabling early and precise diagnosis through multimodal biomarkers. Recent extensions to this framework include inflammatory (I) and vascular (V) dimensions, reflecting

the growing understanding that AD is not a singular proteinopathy but a systems-level disorder influenced by metabolic, immune, and environmental factors [47].

In summary, Alzheimer's Disease is underpinned by a complex interplay of amyloid-β accumulation, tau hyperphosphorylation, chronic neuroinflammation, synaptic failure, mitochondrial and vascular dysfunction, and impaired protein clearance mechanisms. These pathophysiological processes evolve gradually, beginning long before clinical symptoms manifest, and converge to produce widespread neurodegeneration. A modern understanding of AD thus requires an integrative approach that considers the crosstalk among molecular pathways and emphasizes early detection through sensitive, specific blood-based biomarkers that reflect these pathological changes non-invasively.

2.3 The CADRO system

Building on this integrative view of AD pathophysiology, the Common Alzheimer's Disease Research Ontology (CADRO) system was developed as a comprehensive framework to organize and harmonize the diverse, rapidly expanding landscape of AD research. The CADRO system, jointly established by the National Institute on Aging (NIA) and the Alzheimer's Association, provides a standardized taxonomy for categorizing research activities, biological processes, and therapeutic targets across the continuum of AD [48] (Figure 2). Designed to enhance interdisciplinary collaboration and data interoperability, CADRO enables consistent study classification, promotes alignment among funding agencies, and supports strategic prioritization of research investments [49].

At its core, CADRO delineates seven major research categories encompassing the full translational spectrum of AD investigation: (1) molecular pathogenesis and physiology, which includes mechanisms underlying amyloid, tau, and related proteinopathies; (2) diagnosis, assessment, and disease monitoring, focusing on biomarkers, imaging modalities, and digital measures; (3) translational research and clinical interventions, spanning pharmacologic and non-pharmacologic

treatments; (4) epidemiology, addressing risk factors, population trends, and prevention strategies; (5) care, support, and health economics, integrating clinical and societal aspects of dementia care; (6) research resources and data sharing, facilitating open science and reproducibility; and (7) other cross-cutting topics, including neuroinflammation, vascular contributions, and resilience mechanisms.

Importantly, CADRO refines the conceptualization of AD as a multidimensional network disorder rather than a purely amyloid- or tau-centric disease. By explicitly linking mechanistic domains, such as protein aggregation, mitochondrial dysfunction, immune activation, and vascular pathology, CADRO aligns with the systems-biology perspective reflected in the expanded AT(N)IV biomarker framework. This alignment fosters integration between basic discovery science and clinical applications, supporting a precision medicine approach that targets disease mechanisms at individual and population levels. Furthermore, the ontology facilitates interoperability with large-scale databases (e.g., AMP-AD, AD knowledge Portal) and enables meta-analyses that connect molecular signatures with phenotypic and clinical outcomes.

In this context, CADRO serves not only as an organizational tool but also as a conceptual bridge, linking mechanistic understanding, biomarker discovery, and therapeutic development within a unified, data-driven ecosystem. Its structured, modular design ensures adaptability as new discoveries emerge, allowing the framework to evolve alongside advances in genomics, multi-omics integration, and artificial intelligence–based modelling of disease trajectories. By providing a shared language across disciplines, the CADRO system accelerates progress toward translating molecular insights into effective prevention and treatment strategies for Alzheimer’s disease.

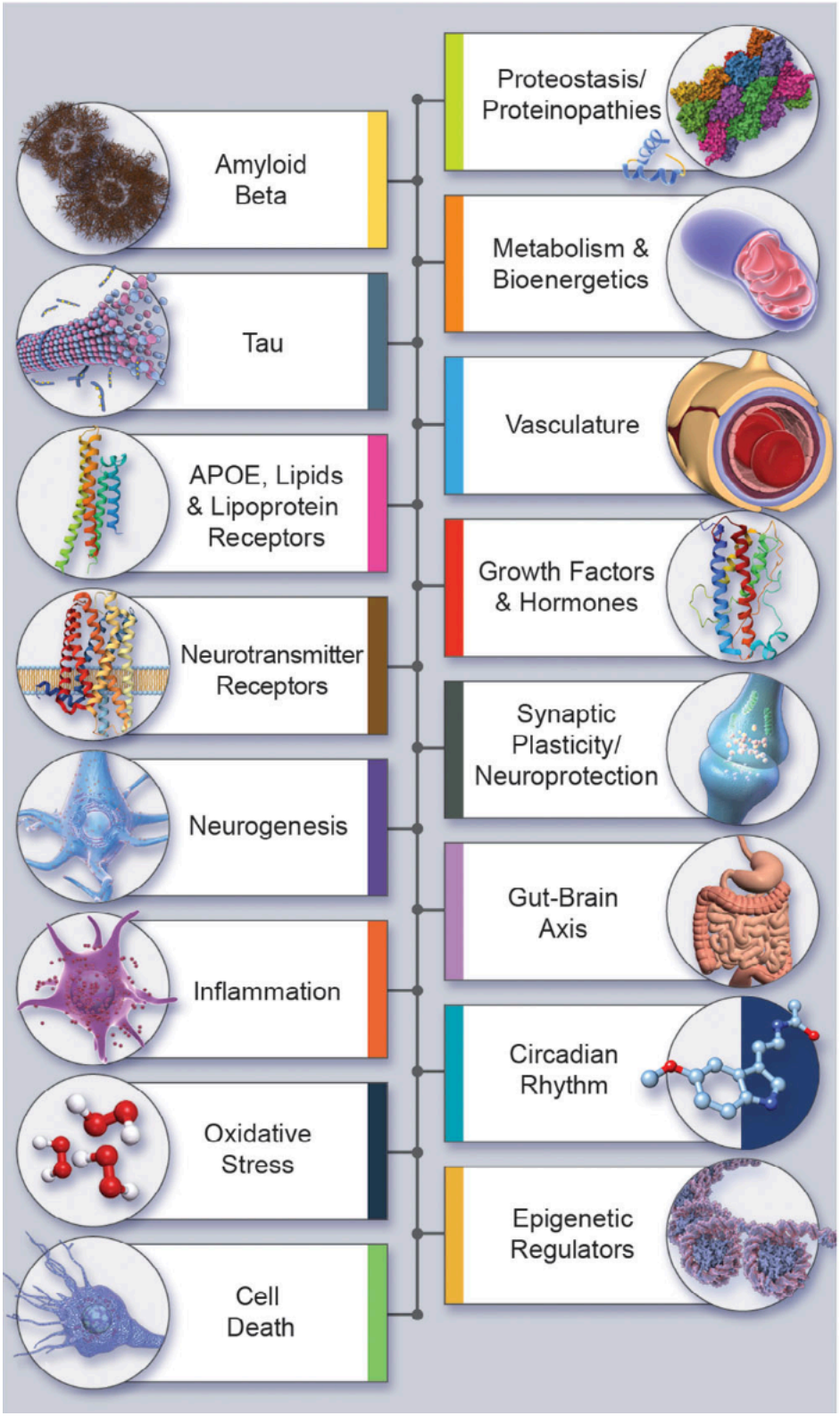

Figure 2. The Common Alzheimer's and Related Dementias Research Ontology (CADRO) categorization system [49].

2.4 The clinical spectrum

The clinical spectrum of AD represents a continuum of progressive neurocognitive decline that evolves over decades, transitioning from asymptomatic brain changes to overt dementia. This continuum reflects the gradual accumulation of pathological processes, Aβ deposition, tau hyperphosphorylation, neuroinflammation, and synaptic loss that precede and accompany clinical manifestations. Modern diagnostic frameworks conceptualize AD as a biological construct rather than a purely clinical syndrome, allowing disease staging based on biomarkers even before cognitive impairment becomes apparent.

The clinical spectrum can be broadly divided into three overlapping stages (Figure 3): i) Preclinical AD; ii) Mild Cognitive Impairment (MCI) due to AD; iii) AD dementia. The earliest phase is the preclinical stage, in which individuals are cognitively normal but have biomarker evidence of amyloid-β accumulation, followed by tau pathology and neurodegeneration. This stage can last years to decades and is detectable only with biomarkers such as amyloid PET, CSF Aβ42/40, or plasma p-tau, as described by [50,51].

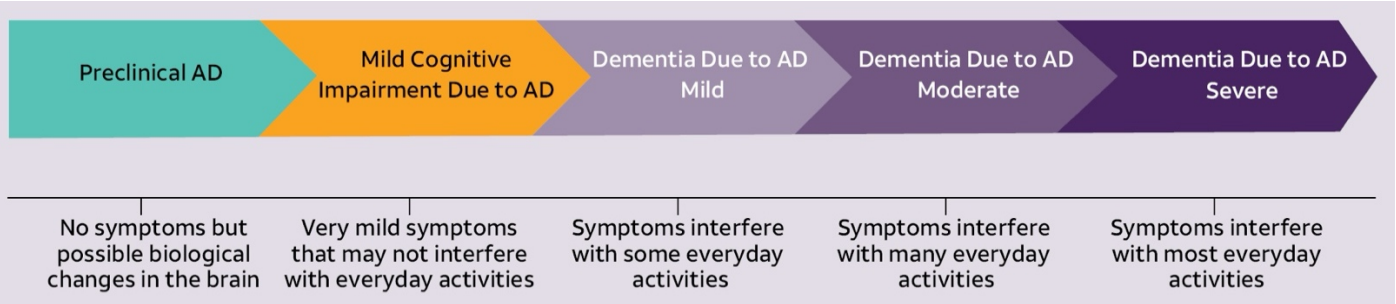

Figure 3. Alzheimer's Disease continuum [2].

As the disease progresses, some individuals develop subjective cognitive decline (SCD), characterized by self-perceived worsening of cognition without objective deficits on testing. SCD is associated with increased risk of progression to objective impairment [3]. The next stage is MCI due

to AD, defined by objective evidence of decline in one or more cognitive domains (often memory), with preserved independence in daily activities. MCI due to AD is associated with a higher risk of progression to dementia compared to normal aging.

The final stage is AD dementia, marked by progressive impairment in multiple cognitive domains and loss of independence in daily functioning. Dementia severity is typically classified as mild, moderate, or severe, based on the degree of cognitive and functional impairment, often using the Clinical Dementia Rating (CDR) scale [52,53]. Neuropsychiatric symptoms such as apathy, depression, agitation, and psychosis may occur at any stage, increasing in prevalence and severity as dementia advances.

In mild dementia, individuals experience prominent episodic memory impairment, often manifesting as rapid forgetting of recent events or conversations. Subtle word-finding difficulties, reduced verbal fluency, and mild spatial disorientation become apparent. Despite these deficits, basic daily activities are largely preserved, although complex instrumental tasks, such as financial management, organizing schedules, or navigating unfamiliar environments, begin to pose challenges.

As the disease advances to moderate dementia, cognitive impairment broadens across multiple domains, including language, visuospatial processing, attention, and executive functioning. Dependence on caregivers increases as patients struggle with routine activities of daily living. Neuropsychiatric and behavioural symptoms, such as apathy, irritability, agitation, or depression, become more frequent and burdensome. Disorientation to time and place intensifies, and patients often lose insight into their deficits, marking a pivotal decline in autonomy and self-awareness.

In the advanced stage (severe dementia), there is global cognitive failure with near-complete loss of verbal communication, severe memory impairment, and profound functional dependence. Recognition of loved ones diminishes, and motor abnormalities, such as rigidity, myoclonus, and dysphagia, may emerge. This stage is often complicated by infections, malnutrition, and immobility-related disorders, which contribute to mortality typically within 8–12 years of symptom onset.

This continuum model aligns with the AT(N) biomarker framework established by the NIA–AA, which categorizes AD based on three biological domains, amyloid (A), tau (T), and neurodegeneration (N), rather than clinical symptoms alone. The integration of these biomarkers into disease staging enables early identification, precise classification, and targeted intervention, long before irreversible neuronal loss occurs.

Thus, the concept of the Alzheimer's clinical spectrum underscores the shift from viewing AD as an end-stage dementia to understanding it as a progressive, multifactorial, and biologically continuous disorder, in which early detection and intervention are key to altering its trajectory.

Although amnestic Alzheimer's disease is the classical presentation, several atypical variants highlight the heterogeneity of cortical involvement and symptom expression, including Posterior Cortical Atrophy (PCA), characterized by predominant visuospatial and perceptual impairments, reflecting occipitoparietal involvement; Logopenic Variant Primary Progressive Aphasia (lvPPA), defined by word-finding pauses and sentence repetition deficits, arising from left temporoparietal degeneration; and Frontal (Behavioral/Executive) variant, marked by disinhibition, apathy, and impaired judgment, resembling frontotemporal dementia [54,55].

While clinically distinct, these phenotypes share the core molecular pathology of Alzheimer's disease, including amyloid-β and tau aggregation, but differ in their regional vulnerability and cognitive manifestations.

In many older individuals, AD pathology coexists with other neurodegenerative and vascular lesions, producing mixed dementia [56,57]. Common comorbid pathologies include cerebral small vessel disease, Lewy body pathology, and TDP-43 inclusions, which can modulate both the clinical phenotype and the rate of progression [58]. This overlap underscores the necessity of multimodal biomarker assessment, integrating neuroimaging, fluid biomarkers, and genetic profiling, to achieve diagnostic precision and guide individualized management strategies.

In summary, Alzheimer's dementia encompasses a clinically diverse and pathologically multifaceted spectrum, where the interplay between disease stage, regional brain involvement, and comorbid pathologies shapes the tempo and expression of cognitive decline.

2.5 The evolution of diagnosis from autopsy to biomarkers

In just over a century, the diagnostic approach to AD has undergone a significant evolution, shifting from reliance on postmortem histopathology to a multimodal biomarker-based approach that enables in vivo detection and characterization of underlying pathologies [59]. Historically, Alzheimer's disease could only be definitively diagnosed after death, through neuropathological examination revealing its pathological hallmarks, Aβ plaques and NFTs. Over the past century, advances in clinical characterization, neuroimaging, and biomarker discovery have transformed AD from a purely pathological entity into a biologically defined, continuum-based disease model that can be identified and monitored during life [60].

When Alois Alzheimer first described the disease in 1906, the diagnosis rested entirely on clinical observation and autopsy findings. The presence of Aβ plaques and tau tangles, confirmed histologically, was the only means of establishing a definitive diagnosis. Throughout much of the 20th century, clinicians relied on clinical criteria and exclusionary diagnoses, such as the Diagnostic and Statistical Manual of Mental Disorders or the NINCDS-ADRDA criteria (1984) [61]. These frameworks defined "probable AD" based on a progressive amnestic syndrome without alternative explanations but lacked biological specificity. Consequently, diagnostic accuracy was limited, and many cases could only be confirmed postmortem, often revealing mixed pathologies.

The late 20th and early 21st centuries marked a paradigm shift toward biological objectivity. Structural magnetic resonance imaging (MRI) enabled visualization of medial temporal lobe atrophy, correlating with hippocampal neurodegeneration. Functional imaging using FDG-PET revealed

regional hypometabolism, particularly in temporoparietal cortices, offering an early window into synaptic dysfunction [62].

Simultaneously, CSF analysis introduced the first biochemical biomarkers of AD pathology. Decreased Aβ42 levels reflected amyloid deposition, while elevated total tau (t-tau) and p-tau indicated neuronal injury and tangle pathology. These biomarkers collectively improved diagnostic confidence and enabled differentiation from other dementias, bridging the gap between clinical and pathological definitions.

A transformative milestone came with the development of PET tracers capable of binding Aβ and tau aggregates in the living brain.

Amyloid PET imaging (e.g., with [^{11}C]PiB or [^{18}F]-labelled tracers) directly visualizes cortical Aβ burden, demonstrating that amyloid accumulation precedes cognitive symptoms by many years. Subsequently, tau PET imaging enabled mapping of neurofibrillary tangle distribution, revealing a close correlation between regional tau burden, neuronal loss, and cognitive decline.

These molecular imaging tools revolutionized diagnosis, allowing *in vivo* confirmation of neuropathological hallmarks once reserved for autopsy and providing quantitative insights into disease staging and progression.

The NIA–AA introduced the AT(N) framework in 2018, marking a decisive transition from syndromic to biological definitions of AD [46]. This model categorizes biomarkers into three domains:

- A (Amyloid pathology): detected by amyloid PET or low CSF/plasma Aβ42 or Aβ42/40 ratio.
- T (Tau pathology): detected by tau PET or elevated CSF/plasma p-tau isoforms (e.g., p-tau181, p-tau217, p-tau231).
- N (Neurodegeneration): assessed via MRI atrophy, FDG-PET hypometabolism, or neurofilament light chain (NfL) levels.

Under this framework, AD is defined by the presence of both amyloid (A+) and tau (T+) pathology, independent of clinical symptoms. This biological staging system acknowledges that pathological processes begin long before dementia emerges and enables identification of preclinical and prodromal AD, fundamentally shifting the diagnostic paradigm toward early detection and prevention.

The most recent evolution in AD diagnostics lies in the emergence of blood-based biomarkers, which promise scalable, cost-effective, and minimally invasive detection. Plasma assays for Aβ42/40 ratios, p-tau181, p-tau217, p-tau231, and NfL have shown strong concordance with CSF and PET measures, allowing widespread screening and longitudinal monitoring. Advances in mass spectrometry and immunoassay technologies have made these biomarkers reliable for clinical and research use, paving the way for population-level risk stratification and early therapeutic intervention.

In just over a century, the diagnosis of Alzheimer's disease has evolved from retrospective confirmation at autopsy to prospective, biomarker-driven identification of living disease processes (Figure 4). This transformation, from clinical observation to molecular quantification, has redefined AD as a biologically measurable, chronically progressive continuum rather than an irreversible endpoint. Modern diagnostic tools now enable detection during the preclinical phase, when therapeutic intervention may hold the greatest potential to alter the disease course.

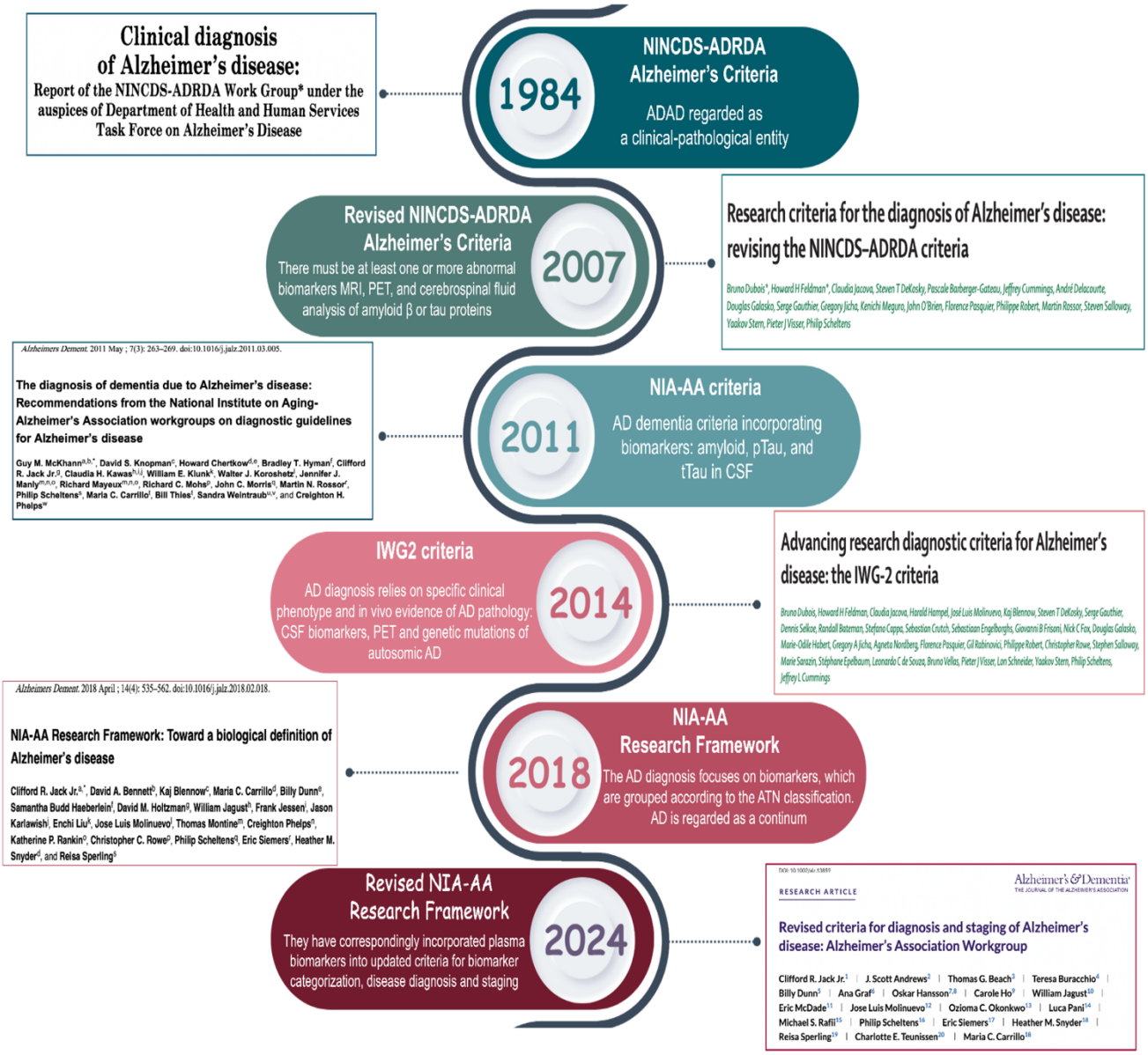

Figure 4. The evolution of Alzheimer's Disease diagnosis [59].

2.6 Current diagnostic tools: cerebrospinal fluid and positron emission tomography biomarkers

Currently, the diagnosis of AD relies on biomarkers that reflect the disease's core pathological processes *in vivo*. Among these, CSF biomarkers and PET imaging represent the gold standards for detecting and characterizing Aβ and tau pathology, as well as neurodegeneration. These tools provide biological confirmation of AD, enable early and differential diagnosis, and support disease staging within the AT(N) biomarker framework.

CSF biomarkers offer a direct biochemical window into the brain's pathological milieu. Because CSF is in close contact with the extracellular space of the brain, it accurately reflects the molecular changes associated with amyloid deposition, tau pathology, and neurodegeneration. CSF analysis, although invasive, has become a cornerstone in both clinical and research-based AD diagnostics. Specifically, three core biomarkers are routinely assessed: Aβ42, tTau, and pTau.

Aβ42 is the primary peptide species that aggregates into amyloid plaques. In AD, CSF Aβ42 concentrations are reduced, reflecting sequestration of the peptide into insoluble cortical deposits. Low Aβ42 or a low Aβ42/Aβ40 ratio indicates amyloid positivity (A+). The Aβ42/Aβ40 ratio is considered more accurate than Aβ42 alone for biomarker assessment in the diagnosis of Alzheimer's disease and related mixed dementias because it corrects for inter-individual and pre-analytical variability in total amyloid production and sampling, thereby improving diagnostic specificity and sensitivity for cerebral amyloidosis. Aβ42 alone can be influenced by factors such as differences in amyloid precursor protein metabolism, CSF dynamics, and technical variability, which may lead to false positives or negatives, especially in the presence of coexisting pathologies or in mixed dementia populations [63-65].

By normalizing Aβ42 to the more abundant and relatively stable Aβ40 isoform, the ratio accounts for individual differences in total Aβ production and mitigates the impact of non-AD-related changes in Aβ42 levels. This is particularly important in older adults, where multiple pathologies (e.g., vascular disease, Lewy body pathology, TDP-43 inclusions) can affect biomarker profiles and confound interpretation [66]. The Aβ42/Aβ40 ratio shows greater concordance with amyloid PET imaging and better discriminates AD from non-AD dementias than Aβ42 alone, as shown by improved sensitivity, specificity, and area under the ROC curve in heterogeneous clinical cohorts [67,68]. Current FDA-approved CSF and plasma biomarker assays for AD diagnosis use the Aβ42/Aβ40 ratio rather than Aβ42 alone, reflecting its superior performance in identifying brain amyloidosis and supporting its use in clinical and research settings.

T-tau reflects axonal injury and neuronal degeneration, and levels are typically elevated in AD.

CSF p-tau, particularly at threonine 181 (p-tau181), threonine 217 (p-tau217), and threonine 231 (p-tau231), is consistently elevated in AD compared to controls and other dementias, and its increase is highly specific for AD-related tauopathy rather than general neurodegeneration [69].

CSF p-tau rises early in the AD continuum, often in parallel with amyloid-β pathology, and can distinguish AD from other neurodegenerative diseases, including frontotemporal dementia and Lewy body dementia, with high diagnostic accuracy [70]. The CSF p-tau levels correlate with both amyloid and tau PET imaging and are associated with cognitive decline and disease progression [71].

The CSF biomarker profile with low Aβ42 (or low Aβ42/Aβ40 ratio) and high p-tau and t-tau is considered the biochemical signature of Alzheimer's disease, with sensitivity and specificity for distinguishing AD from non-AD dementias typically exceeding 85–90%.

Overall, CSF provides early detection, has high diagnostic accuracy, and is relatively low-cost compared to PET. However, the invasive lumbar puncture procedure, potential inter-laboratory variability, and need for assay standardization represent important limitations. PET imaging provides a non-invasive visualization of molecular pathology in the living brain. Using radiolabelled tracers that selectively bind to Aβ or tau aggregates, PET enables regional mapping of protein deposition, quantification of disease burden, and assessment of disease progression. A negative amyloid PET scan makes Alzheimer's disease an unlikely primary cause of cognitive symptoms. Tau PET binds to paired helical filaments of hyperphosphorylated tau within neurons. It reveals the spatiotemporal progression of tau pathology, consistent with Braak staging (from medial temporal to neocortical regions), and correlates strongly with cognitive impairment and disease severity, often more closely than amyloid PET. Additionally, it enables differentiation of AD from primary tauopathies (e.g., PSP, CBD) through distinct binding patterns. Although not specific to AD, FDG-PET measures cerebral glucose metabolism and serves as a functional indicator of neurodegeneration. In AD, FDG-PET shows hypometabolism in temporoparietal and posterior cingulate cortices, distinguishing it from

frontotemporal or subcortical dementias. Overall, PET allows direct visualization of pathology, quantifiable regional information, and high diagnostic accuracy. However, PET imaging for amyloid and tau has limited sensitivity in the earliest (preclinical and prodromal) stages of AD, as amyloid PET may be positive decades before symptoms, and tau PET may not detect subtle pathology in mild cognitive impairment or in cognitively normal individuals, potentially leading to false negatives or delayed diagnosis [72-74]. Second, PET tracers can show off-target binding, particularly in subcortical regions, which may confound interpretation and reduce specificity for Alzheimer's pathology. Third, visual and quantitative interpretation of PET scans is subject to reader variability, technical artifacts, and lack of harmonization across centres, which can affect diagnostic accuracy and reproducibility. Additionally, PET imaging cannot reliably distinguish Alzheimer's disease from mixed or comorbid pathologies, such as Lewy body disease or TDP-43 inclusions, which are common in older adults and may alter clinical presentation [75]. There is also no consensus on optimal cutoffs for tau PET positivity, and different tracers may have variable specificity and affinity, limiting generalizability across studies and clinical settings. Finally, PET is expensive, not universally available, and involves exposure to ionizing radiation, which restricts its use for routine screening or longitudinal monitoring in large populations.

CSF and PET biomarkers have revolutionized the diagnosis of Alzheimer’s disease, transforming it from a syndrome defined by clinical symptoms to a biologically characterized disorder identifiable decades before dementia onset. CSF biomarkers provide molecular quantification of amyloid and tau dynamics, while PET imaging offers spatial visualization of these pathologies in the living brain. However, their limitations hampered their widespread use in clinical practice, creating an urgent need for accessible, scalable, and minimally invasive diagnostic tools to detect the same underlying pathology with comparable accuracy. In this context, blood-based biomarkers have emerged as a groundbreaking advancement with the potential to overcome the constraints of CSF and PET.

2.7 The genetic architecture

The genetic landscape of AD reflects a complex interplay between rare, highly penetrant mutations that cause early-onset familial forms of the disease, and common, low-penetrance variants that modulate susceptibility to the more prevalent late-onset form.

High-penetrance variants are rare autosomal dominant mutations that almost invariably lead to Alzheimer's disease, typically before the age of 65 and sometimes as early as the 30s or 40s. These mutations account for less than 1% of all AD cases, but their study has been pivotal in establishing the amyloid cascade hypothesis. They include pathogenic variants in genes encoding APP, PSEN1, and PSEN2. In contrast to early-onset forms, late-onset Alzheimer's disease (LOAD), which accounts for over 95% of cases, is polygenic and multifactorial, influenced by common genetic variants with modest individual effects and strong environmental and lifestyle interactions. To date, more than 70 genetic loci have been identifies as contributing to LOAD risk, most with low individual penetrance but that cumulatively influence disease susceptibility. Among these, APOE is the strongest genetic risk factor for LOAD, with the ε4 allele significantly increasing risk and lowering the age of onset, while the ε2 allele is protective compared to the common ε3 allele [76,77]. APOE modulates AD pathogenesis through several mechanisms. The ε4 isoform promotes earlier and more abundant Aβ aggregation and impairs Aβ clearance, accelerating amyloid plaque deposition in the brain [78,79]. APOE also influences tau pathology, with ε4 carriers showing increased tau-mediated neurodegeneration and more severe neurofibrillary tangle burden [80].

Beyond amyloid and tau, APOE affects lipid transport, synaptic integrity, glucose metabolism, mitochondrial function, and cerebrovascular health. The ε4 isoform is less efficient at lipid binding and transport, leading to dysregulation of cholesterol and sphingolipids, impaired myelin maintenance, and increased vulnerability to neurodegeneration [81]. APOε4 also exacerbates neuroinflammation by modulating microglial and astrocyte activation, and disrupts blood-brain barrier integrity, further contributing to disease progression.

3. The era of blood-based biomarkers

The blood-based AD biomarker field has seen significant progress driven by technological advances, particularly improvements in assay and measurement platform analytical sensitivity and precision [82]. Several blood-based biomarkers have shown high potential for accurately detecting AD pathophysiology. As a result, there has been considerable interest in applying these biomarkers for diagnosis and prognosis, as surrogate metrics to investigate the impact of various covariates on AD pathophysiology and to accelerate AD therapeutic trials and monitor treatment effects.

3.1 Core biomarkers in blood: amyloid beta 42/40 ratio and pTau

Two core plasma biomarkers have shown good diagnostic and prognostic utility: the Aβ42/40 ratio and p-tau, especially pTau181 and pTau217. Together, they capture the essential pathological signatures of AD, amyloid deposition and tau hyperphosphorylation, and provide a biological basis for early detection and disease staging.

The Aβ42/40 ratio mirrors brain amyloid burden and correlates with CSF Aβ42 and amyloid PET findings. A lower plasma Aβ42/Aβ40 ratio is consistently associated with amyloid PET positivity, poorer cognitive performance, and a higher risk of progression from MCI or SCD to dementia.

Plasma p-tau181 is elevated in AD, even in early stages, and increases with disease progression. It reliably distinguishes AD from other neurodegenerative disorders, such as frontotemporal dementia, and correlates strongly with amyloid PET and tau PET findings. Studies from large cohorts, including the AIBL study, show that p-tau181 effectively predicts brain amyloid status. Ratios like p-tau181/Aβ42 show particularly strong associations with amyloid burden, hippocampal atrophy, and cognitive impairment.

Notably, these biomarkers have high negative predictive value (NPV), meaning that a negative result excludes AD with high accuracy. On the other hand, the positive predictive value is moderate. Thus,

an altered biomarker value cannot diagnose the disease, and further investigations may be required to confirm the suspicion of AD.

3.2 Glial fibrillary acid protein

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein primarily expressed by astrocytes, the major glial cells that maintain CNS homeostasis. In healthy brains, GFAP contributes to astrocytes structural stability and supports their roles in metabolic regulation, neurotransmitter recycling, and blood–brain barrier maintenance. In the context of neurodegeneration, including AD, elevated GFAP reflects astrocyte activation (astrogliosis), a hallmark of neuroinflammation and an early response to amyloid pathology. Astrocytes become reactive in response to Aβ plaque deposition and neuronal injury, undergoing morphological and molecular changes characterized by increased GFAP expression. This reactive astrogliosis plays a dual role: protective in early stages by facilitating Aβ clearance and supporting neuronal metabolism, and detrimental in chronic stages by contributing to neuroinflammation, excitotoxicity, and propagation of neurodegenerative processes.

GFAP is regarded as a cell- but not disease- specific biomarker. Indeed, it is specific to astrocytes, but its levels increase in different pathological conditions, including inflammation, neurodegeneration, traumatic brain injury, and brain metastasis [83]. GFAP is primarily an intracellular protein, but various mechanisms can lead to its release into the extracellular space and bloodstream, particularly under neurodegenerative conditions. The exact pathways of GFAP leakage remain unclear, but astrocyte damage, neuroinflammation, and BBB disruption are key contributors. Astrocyte endfeet, which interact with brain capillaries, may facilitate GFAP release when the BBB is compromised, a process commonly observed in neurodegenerative diseases. Reactive astrocytes may also release GFAP via exocytosis, further contributing to its presence in biofluids [84]. These

mechanisms collectively highlight GFAP's potential as a biomarker for neurodegenerative diseases. With technological advancements over the past decade, GFAP can be easily detected in serum.

3.3 Soluble Triggering Receptor Expressed on Myeloid cells 2

Microglia play a crucial role in maintaining brain homeostasis by clearing debris, supporting synaptic function, and responding to injury [85]. In AD, they become activated around amyloid plaques, exhibiting either protective or harmful phenotypes depending on the disease stage and environment [86]. A key regulator of microglial activity is Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), a receptor that, through its signalling partner DAP12, promotes microglial survival, proliferation, and phagocytosis [87]. Importantly, mutations in the TREM2 gene, such as R47H, significantly increase the risk of developing late-onset AD by impairing its protective functions [82]. This evidence further supports the role of TREM2 in AD pathology.

A soluble form of this receptor, namely soluble TREM2 (sTREM2), generated by proteolytic cleavage of the extracellular domain of membrane-bound TREM2, is released into the CSF. Interestingly, CSF sTREM2 levels fluctuate throughout the course of AD, typically rising during early symptomatic stages and declining as the disease progresses [88].

Elevated CSF sTREM2 has been reported in AD patients as early as 12-14 years before the onset of clinical symptoms [89]. These early increases are thought to reflect microglial activation in response to amyloid deposition and neuronal injury, underscoring the role of neuroinflammation in the initial phases of AD. In cognitively normal individuals with evidence of amyloid pathology, sTREM2 levels are often reduced. However, as the disease progresses to the MCI and early dementia stages, sTREM2 levels increase, suggesting a compensatory microglial response to increasing neurodegeneration. Thus, sTREM2 may serve as a predictor of disease progression.

Longitudinal studies in autosomal-dominant AD further support this evidence. Indeed, elevated CSF sTREM2 levels are associated with slower cortical atrophy and reduced cognitive decline, suggesting a protective microglial response during disease progression. Beyond its predictive value, sTREM2 may thus also provide prognostic information [90].

Taken together, these findings suggest a biphasic pattern in which sTREM2 reflects distinct phases of microglial activation across AD stages. Mechanistically, sTREM2 supports microglial survival and proliferation, stimulates cytokine production, and promotes clustering around amyloid plaques, facilitating their clearance. In animal models, increased sTREM2 levels mitigate amyloid pathology and improve cognitive outcomes, whereas genetic variants that impair sTREM2 function increase AD risk and compromise microglial responses [91].

Clinically, higher CSF sTREM2 levels correlate with slower cortical atrophy and less cognitive decline, consistent with a protective microglial activation phenotype. Conversely, low sTREM2 levels at the MCI stage are associated with a higher likelihood of progression to AD. Moreover, sTREM2 has been linked to imaging biomarkers, including tau-PET and amyloid-PET, particularly in individuals with combined amyloid and tau pathology (A+/T+) [92]. These associations further support its role as a surrogate biomarker of disease activity.

Overall, sTREM2 captures neuroinflammatory activity and microglial engagement in AD pathogenesis. It complements established biomarkers, offering additional value for diagnosis, prognosis, and therapeutic monitoring. While robust evidence supports the role of sTREM2 in CSF as a biomarker of AD, only a few Authors explored the value of blood sTREM2 [24, 25]. Nonetheless, the non-invasive nature of blood-based testing makes blood sTREM2 an attractive candidate for screening and longitudinal monitoring, provided that future studies can standardize measurements and clarify its relationship with CNS pathology.

4. Research project

4.1. Aim

The primary aim is to validate and clinically translate a panel of blood-based biomarkers for AD to support diagnosis, prognosis, and treatment monitoring. The panel includes seven biomarkers indicative of different pathological hallmarks of AD: Aβ42, Aβ40, and the Aβ42/Aβ40 ratio for amyloid pathology; pTau181 for tau pathology; Glial Fibrillary Acidic Protein for astroglial activation; sTREM2 for microglial activation; and serum APOE as a surrogate for APOE genotyping. Biomarkers were measured in both blood and CSF, with saliva explored in a subgroup. Secondary aims included: (i) investigating biological determinants of biomarker levels (age, sex), (ii) assessing correlations across different matrices (CSF, blood, and saliva), (iii) establishing age- and sex-specific reference intervals (Ris) and diagnostic thresholds, and (iv) evaluating diagnostic performance in AD and other neurological diseases. The project leverages a collaboration between the University of Palermo and University College London Hospitals, integrating complementary clinical and laboratory expertise.

4.2 Materials and Methods

4.2.1 Study population

This retrospective observational study was conducted at the University Hospital "P. Giaccone" in Palermo, Italy. The study population consisted of healthy individuals and patients with neurodegenerative diseases, including AD, multiple sclerosis (MS), and transthyretin amyloidosis (ATTR) polyneuropathy. Healthy individuals were i) blood donors aged 18–65 years from the Unit of Transfusion Medicine of the Policlinico Paolo Giaccone of Palermo and ii) outpatients aged >65 years from the project "Discovery of molecular and genetic/epigenetic signatures underlying resistance to age-related diseases and comorbidities (DESIGN, 20157ATSLF)", funded by the Italian Ministry of education, University and Research. Healthy people were selected based on their age, the

absence of organ or system deterioration (including deafness and visual problems), and having no more than one invalidating condition.

Patients were recruited at the Unit of Neurology of A.O.U.P. “Paolo Giaccone”. The diagnosis of AD was made according to the current criteria [93,94]. All patients underwent a complete medical and neurological evaluation, routine blood tests, neuropsychological evaluation, brain MRI, FDG-PET, and CSF withdrawal as routine diagnostic procedures.

AD patients showed brain atrophy in MRI scans, brain hypometabolism at FDG-PET, and AD core CSF biomarker abnormalities, being classified as A +T +N+ [46].

The MS diagnosis was made according to the revised McDonald criteria [95].

The diagnosis of ATTR with polyneuropathy was made according to current recommendations [96,97]. Briefly, it should be suspected in unexplained progressive and disabling neuropathy, especially if associated with systemic symptoms or family history. The diagnosis is confirmed by genetic testing to detect TTR gene amyloidogenic variants, classical biopsy, and bone scintigraphy with diphosphono-1,2-propanodicarboxylic acid, hydroxymethylene diphosphonate, or pyrophosphate to identify amyloid deposits.

All patients signed informed consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Policlinico P. Giaccone, Palermo (n.7/2020).

4.2.2 Sample collection

CSF, plasma, and serum samples were collected from all participants, while saliva samples were collected from a subgroup of AD patients.

Collection of all biological matrices was performed between 8:00 a.m. and 10:00 a.m. in a fasted state. Additionally, all patients were asked to refrain from eating, drinking, smoking, or using oral hygiene products before collection (at least for 8 h). We documented the consumption of alcohol, caffeine, nicotine, and medication in the previous 12 h.

CSF was obtained by a lumbar puncture at the L3/4 or L4/5 interspace using a 21-gauge needle. It was collected in polypropylene tubes, centrifuged at 500g for 20 min, aliquoted into polypropylene tubes, and stored at -80 °C until analysis, according to international consensus protocols [98].

Whole blood was collected by venipuncture immediately before saliva sampling into K_3-EDTA tubes, then centrifuged at 2.500g for 10 min. The obtained plasma was collected, aliquoted into polypropylene tubes, and stored at -80 °C until analysis.

After checking the oral cavity to exclude wounds, lacerations, or inflammatory processes (periodontitis), patients were asked to rinse their mouths with water, then spit unstimulated saliva into a 50 mL polypropylene Falcon tube (approximately 3 mL of saliva was collected). The collected samples were immediately placed on ice and centrifuged at 1.500g for 5 min. After centrifugation, samples were divided into two aliquots in polypropylene tubes; (i) untreated: the aliquot was immediately stored at − 80 °C and; (ii) treated: the aliquot was added with thioflavin S (0.5 mg, Sigma, St. Louis, MO, USA) to prevent Aβ42 aggregation, and sodium azide (0.5 mg, Fischer Scientific, Suwanee, GA, USA) to prevent bacterial growth, before storing at − 80 °C. Before analysis, after thawing, saliva samples were centrifuged at 1.500g for 5 min. and the supernatant was analyzed.

Pre-analytical procedures, including standardized collection, centrifugation, aliquoting, and storage at - 80 °C, were rigorously applied.

4.2.3 Biochemical and genetic analyses

Each biomarker in all biological matricesm (CSF, saliva, and plasma) was measured by CLEIA using the fully automated Lumipulse G1200 analyzer, Fujirebio Inc. Europe, Gent, Belgium), according to the manufacturer's instructions.

CSF Aβ42, Aβ40, and p-tau181 levels were analyzed as part of the clinical routine using the following kits: Lumipulse G β-Amyloid 1–40 CSF, Lumipulse G β-Amyloid 1–42 CSF, and Lumipulse G pTau 181 CSF, respectively. The limit of detection (LoD) was 6.7 pg/mL for Aβ40, 2.78 pg/mL for Aβ42, and 0.282 pg/mL for pTau. The total precision of the assays (%Coefficient Variation [CV]) was 2–3.9 for Aβ40, 2.6–4.5 for Aβ42, and 2.2–8.3 for pTau.

Plasma and saliva Aβ42, Aβ40, and p-Tau181 levels were analyzed using the following kits: Lumipulse G β-Amyloid 1–40 Plasma, Lumipulse G β-Amyloid 1–42 Plasma, and Lumipulse G pTau 181 Plasma, respectively. The LoD was ≤ 0.44 pg/mL for Aβ40, ≤ 0.37 pg/mL for Aβ42, and 0.052 pg/mL for pTau. The total precision of the assays (%CV) was 2.6–4.6 for Aβ40, 4–5.6 for Aβ42, and 2.3–3.9 for pTau in plasma, and 61–9 for Aβ40, 97–14 for Aβ42, and 62–18 for pTau in saliva.

Serum GFAP levels were measured by the Lumipulse G GFAP assay, which has a detection range of 4.0 to 5000.0 pg/mL. The LoD was 1.8 pg/mL, the LoQ at 10 % CV, and the precision was less than 5 % coefficient of variation (CV). As a reference standard, the assay uses full-length recombinant human GFAP protein (LS Bio, Shirley, MA, USA).

Serum, plasma, and CSF sTREM2 levels were measured using the Lumipulse G sTREM2 assay. The LoD is 2.7 pg/mL, the LoQ is 10 % CV, and the precision is less than 5 %CV. The intra-assay CV is <5%. Controls and calibrators were run in duplicate, and the mean of duplicates was used as the final readout. No dilution of the sample was required. For CSF samples, the reported concentration needs to be multiplied by 1.33 to obtain the neat sTREM2 concentration in CSF.

Finally, plasma levels of ApoE isoforms were measured using Lumipulse G ApoE4 and Lumipulse G Pan-ApoE assays. The Lumipulse G Pan-ApoE assay measures all isoforms of the apolipoprotein E, including ApoE2, ApoE3, and ApoE4. The ApoE4/Pan-ApoE ratio is calculated to estimate the presence of the ApoE4 allele and should be interpreted as follows: <5%, absence of ApoE4; 5% - 75%, heterozygous (presence of ApoE4 in combination with ApoE2 or ApoE3); and ≥75%, homozygous (presence of ApoE4 in the absence of ApoE2 or ApoE3). Each patient was genotyped for APOE using real-time multiplex allele-specific PCR (DiaPlexQtm) on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.).

The APOE gene is polymorphic at two single SNPs (rs7412 and rs429358), defining the three different alleles (ε3, ε2, and ε4). The APOE genotyping assay comprises a multiplex Real-Time PCR smart mix and target gene-specific primer. DNA samples were arrayed in a 96-well plate layout and genotyped using the Bio-Rad system. The following thermocycling conditions were used: 50 ◦C for 3 min (1 cycle), 60 ◦C for 1 min (1 cycle), 95 °C for 15 min (1 cycle); 40 cycles of 95 °C for 15 s and 60 °C for 1 min; followed by a final extension step at 66 °C for 1 min.

APO ε4 carriers were defined by the presence of at least one ε4 allele (ε4/ε4, ε4/ε3, ε4/ε2), while APOE ε4 non-carriers were not (ε2/ε2, ε2/ε3, ε3/ε3). Genotyping was determined by the presence or absence of mutations at amino acid positions 112 (rs429358) and 158 (rs7412). The variability of amino acids 112 and 158 is based on SNPs at nucleotide positions 334 and 472, respectively, of the APOE gene. The presence of 334 T/C determines a Cys or Arg in amino acid residue 112 of mature apolipoprotein E, and 472C/T determines Arg or Cys at residue 158.

4.2.4 Statistical analyses

The relationship between measurements in biological matrices was evaluated using Passing–Bablok regression, a nonparametric method robust to outliers and measurement errors in both variables.

Regression parameters and 95% confidence intervals were obtained by bootstrap resampling (quantile method), and the monotonic association between variables was assessed using Spearman's rank correlation coefficient (ρ). Residual analysis of the regression fit was performed to examine deviation patterns and potential heteroscedasticity. Agreement between matrices was further assessed using the Bland–Altman method, calculating mean differences and limits of agreement (mean ± 1.96 SD) on both absolute and logarithmic scales to account for proportional differences. Data normality was assessed using q–q plots and the Shapiro–Wilk and Kolmogorov–Smirnov tests; since most variables were non-normally distributed, nonparametric statistics were used. Group differences were tested using the Kruskal–Wallis and Mann–Whitney U tests, with Holm-adjusted pairwise comparisons where appropriate. Outliers were identified and removed using Tukey's interquartile fences on Cox-transformed data following CLSI recommendations for Ris calculation. Age- and sex-matched analyses were performed with the MatchIt package using 1:1 or 3:1 matching ratios depending on group sizes. Reference intervals (2.5th and 97.5th percentiles) for GFAP were calculated overall and by sex and age using nonparametric or robust methods with 90% bootstrap confidence intervals, truncating negative lower limits to zero. All analyses and visualizations were performed in R (versions 4.0.3–4.5.1; R Foundation for Statistical Computing, Vienna, Austria) using the mcr, ggstatsplot, ggplot2, tidyverse, reference intervals, and MatchIt packages, with statistical significance set at $p < 0.05$.

4.3 Results

The study population consisted of 340 cognitively healthy controls (251 blood donors and 89 outpatients), 100 AD, 50 hereditary transthyretin amyloidosis with polyneuropathy (ATTRv-PN), and 76 MS.

4.3.1 Core biomarkers across different biological matrices

We could not measure biomarkers in treated saliva because the samples were too dense.

Figure 5 shows the distribution of Aβ42, Aβ40, Aβ42/40 ratio, pTau, and pTau/Aβ42 ratio levels across the three biological matrices, i.e. CSF, plasma, and saliva. Significant differences in median levels of all biomarkers across the three matrices were observed, indicating a large effect size ($p < 0.001$). The relationship between the biomarkers across various biofluids was assessed using Spearman's correlation (Figure 6). A positive correlation between pTau levels in CSF and plasma (rho = 0.54 [0.28–0.73]), and Aβ42/40 ratio levels in CSF and plasma (rho = 0.33 [0.02–0.58]) was found. The analysis also indicated significant correlations, both positive and negative, between different analytes in the same matrix (e.g., pTau in CSF vs Aβ42/40 ratio in CSF) or between different analytes in different matrices (e.g., pTau in plasma vs Aβ42/40 ratio in CSF), which are represented in Figure 6. The analysis also highlights non-significant correlations, indicating areas where the relationship between biomarkers does not reach statistical significance (crossed, in the figure). Notably, no significant correlations were found between saliva and CSF or between saliva and plasma for any of the analytes considered.

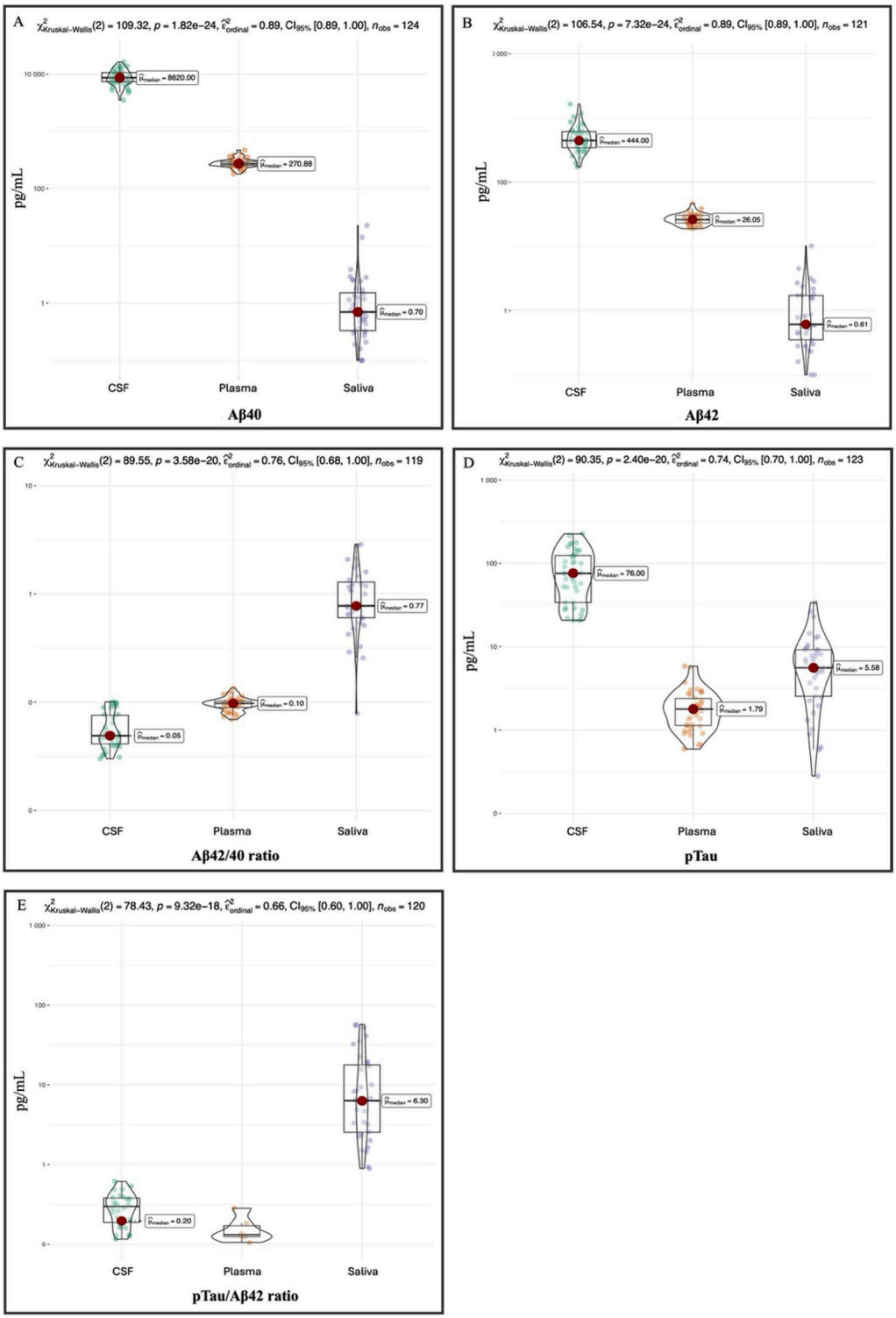

Figure 5. Distribution of Aβ42 (A), Aβ40 (B), Aβ42/40 (C) ratio, pTau (D), and pTau/Aβ42 ratio (E) levels across different biological matrices, i.e., CSF, plasma, and saliva. Significant differences in median levels of all biomarkers were observed across the three matrices. P-value of the Kruskal-Wallis test is reported above. The effect size was large (epsilon squared, reported above). Holm-adjusted p-values of pairwise comparison among all pairs of methods were significant for all pairs.

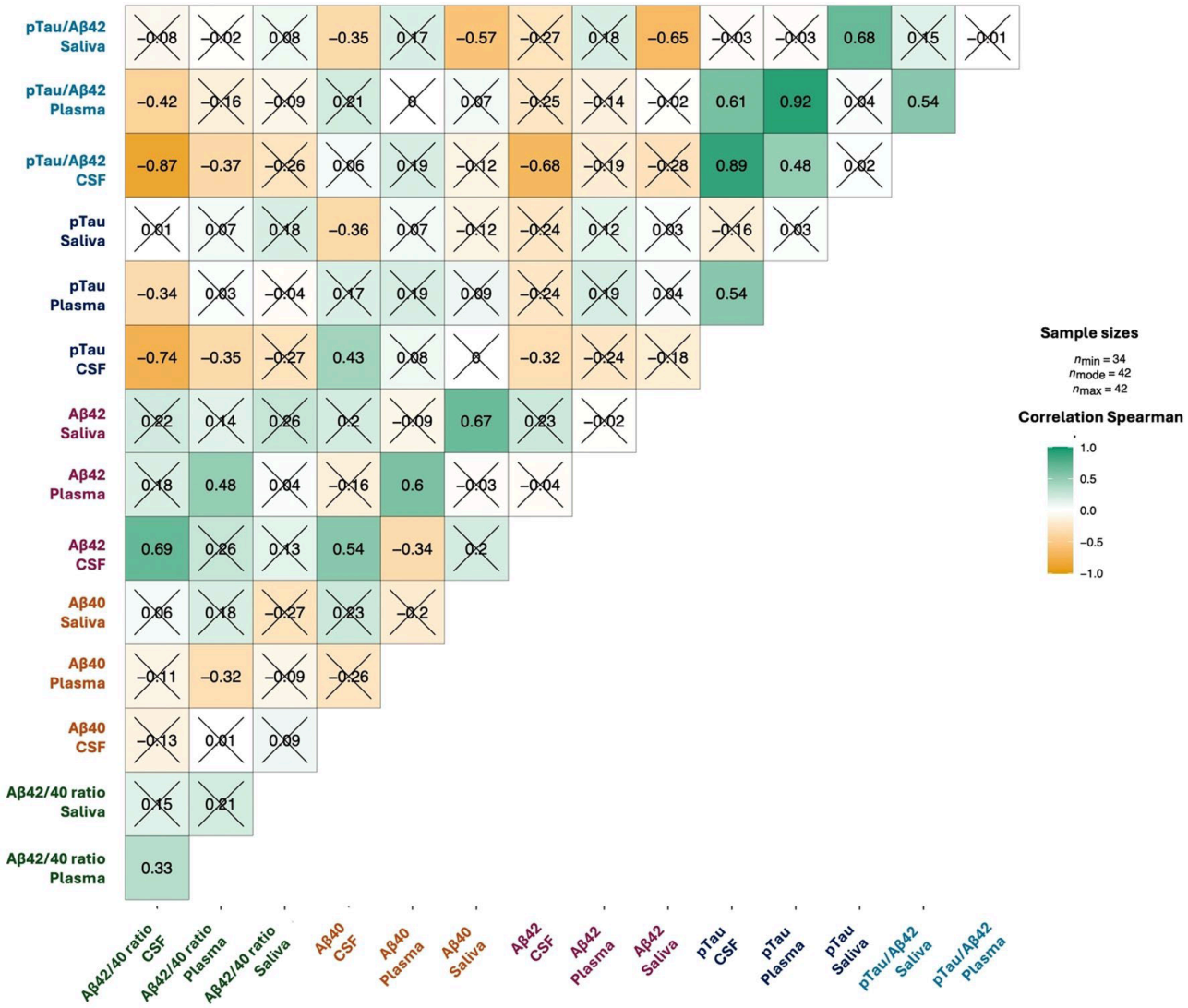

Figure 6. Correlation between biomarkers in the different biological matrices. Non-significant correlations with p-value > 0.05 are crossed. CSF, cerebrospinal fluid; pTau, phosphorylated tau.

The correlation analysis only assesses the strength of association between variables. To assess the (linear) nature of the relationships, a regression analysis (Passing-Bablok) was performed for the significant correlations: Aβ42/40 ratio in CSF vs plasma, pTau in CSF vs plasma, and pTau/Aβ42 ratio in CSF vs plasma. The regression results are shown in Figure 7.

In the case of Aβ42/40 ratio, the regression equation derived is Aβ42/40 ratio plasma = 0.06 + 0.65 * Aβ42/40 ratio CSF, indicating a significant positive constant bias of 0.06 [0.04–0.07], while the slope is not significant, with a value of 0.65 [− 0.4 to 1.05], encompassing 1 in its large confidence interval. The residual plot does not indicate any major violations of the assumptions necessary for a linear model to be appropriate. However, there seems to be a slight concentration of residuals below the zero line as the mean of the estimated values increases, suggesting a slight negative bias in the model, which may warrant further exploration with a larger dataset.

In the case of pTau, the regression equation is pTau plasma = 0.90 + 0.01 * pTau. There is a significant positive constant bias of 0.90 [0.61–1.33] and a slope of 0.01 [0.005–0.014], indicating an increase of plasma pTau of 0.01 per unit of pTau liquor. Such a small slope reflects the large concentration difference between CSF and plasma, with CSF levels being approximately 100-fold higher than plasma levels.

Similarly, the regression equation for the pTau/Aβ42 ratio is pTau/Aβ42 ratio plasma = 0.03 + 0.15 * pTau/Aβ42 ratio CSF. This equation indicates a significant positive constant bias of 0.03 [0.02–0.04] and a slope of 0.15 [0.10–0.26], suggesting that the plasma pTau/Aβ42 ratio increases by 0.15 units for each unit increase in the CSF pTau/Aβ42 ratio. The significant difference in concentration between CSF and plasma for these biomarkers is again reflected in the small slope. The residual plot does not indicate any significant violations of the assumptions.

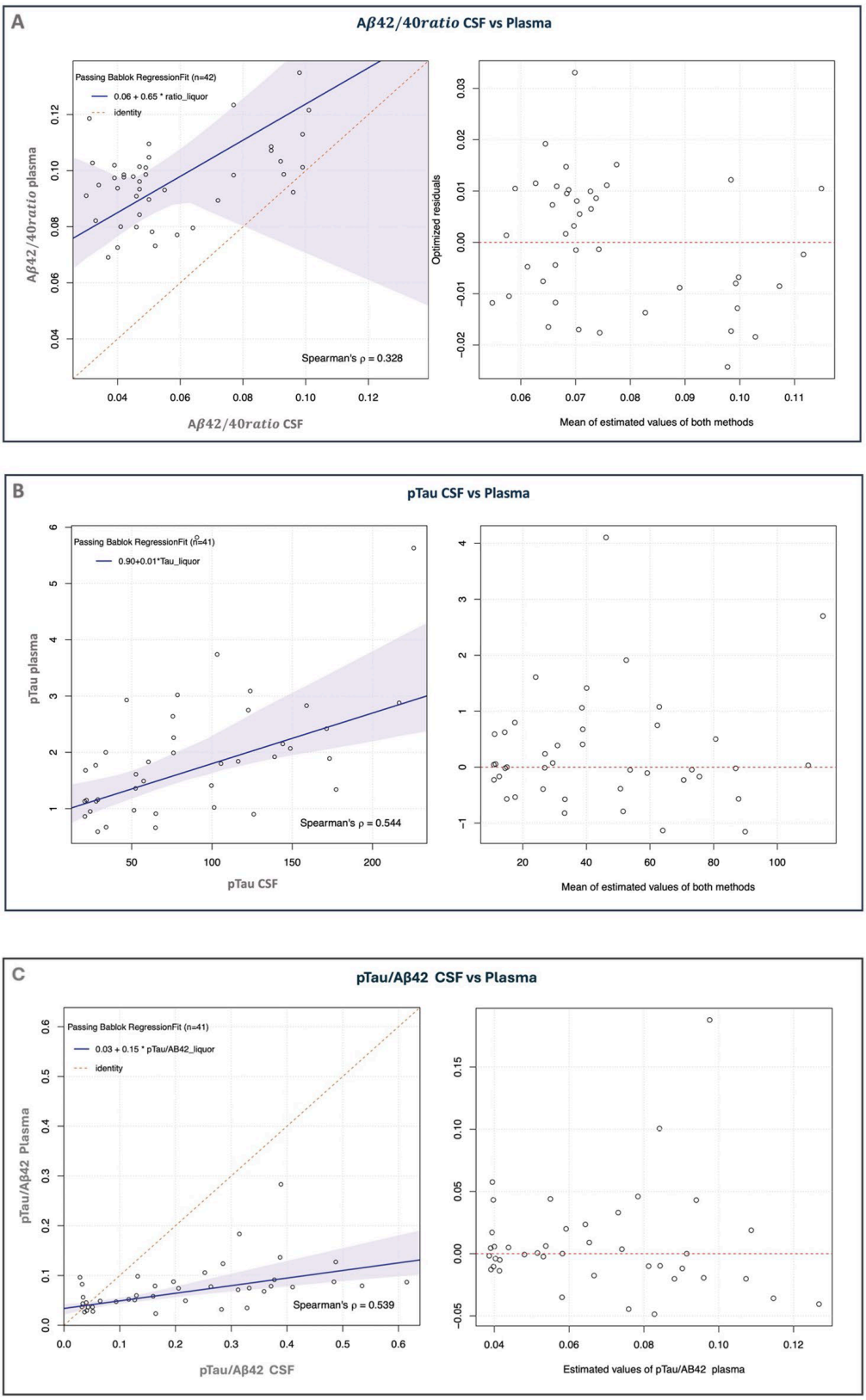

Figure 7. Regression analysis to compare Aβ42/40 ratio and pTau levels in CSF and plasma. In shaded blue are the confidence intervals of the regression line. In dashed red is the identity line. CSF, cerebrospinal fluid; pTau, phosphorylated Tau.

4.3.2 GFAP

4.3.2.1 Establishing reference intervals in healthy controls

Two patients with missing values and four outliers were removed, leaving 334 individuals. Table 1 summarizes the RIs of GFAP levels. When patients were stratified by sex, males had significantly lower levels than females (Figure 8).

Population	RIs of serum GFAP, pg/mL	Lower 90 % CI	Upper 90 % CI
Whole	10.4–92.0	9.8–11.4	69.1–104.0
Male	10.2–60.8	9.2–11.3	46.5–71.2
Female	10.9–107.0	9.8–13.5	97.0–153.3

Table 1. Serum GFAP levels in the whole study population.

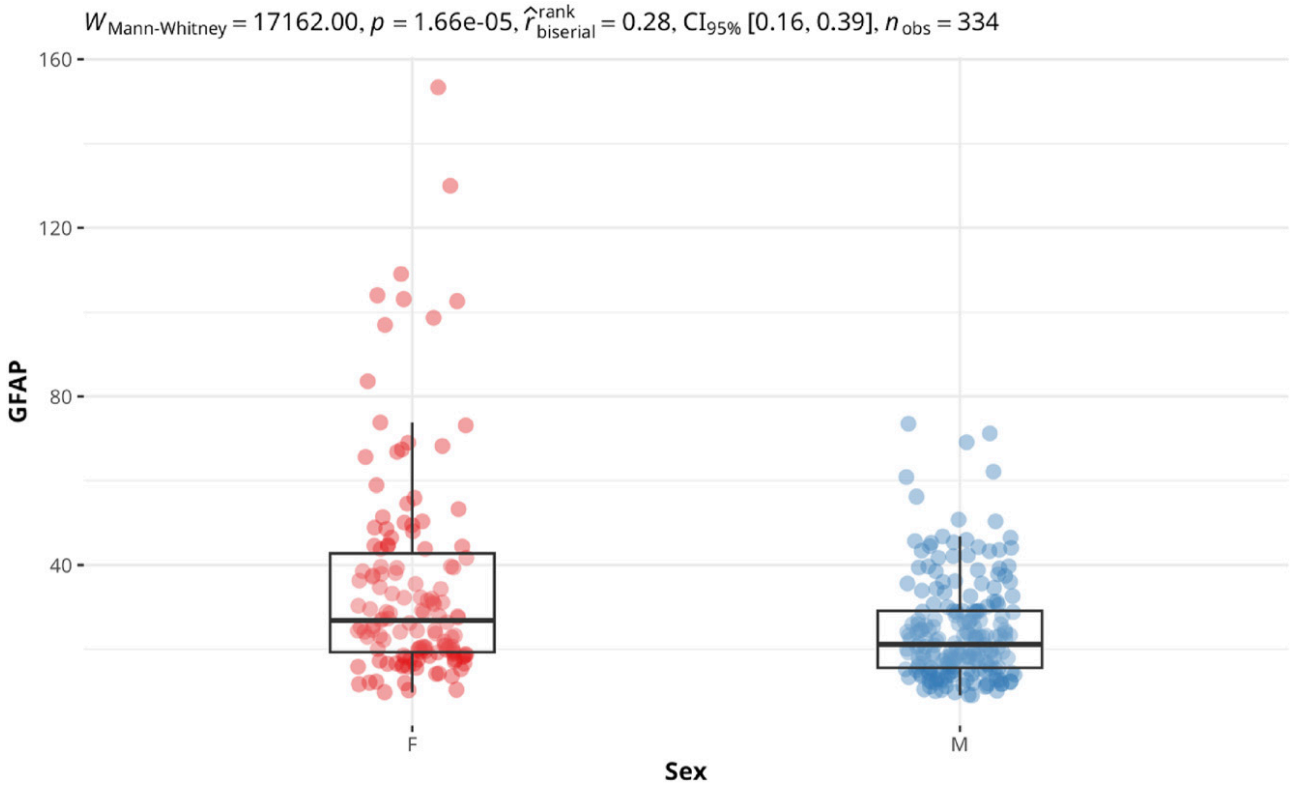

Figure 8. GFAP levels in males and females.

When analysing the relationship between age and GFAP, a moderate positive correlation was observed (Spearman's rho=0.61, $p<0.001$).

To further explore the relationship between age and serum GFAP levels, we grouped the cohort into six age groups. The analysis revealed that the biomarker was stable until age 60, while individuals aged>60 years demonstrated significantly higher GFAP levels than younger age groups. However, the individuals aged 50–60 already show a significant increase with respect to the 18-30- and 30-40-year groups, although to a lesser degree. This pattern varies slightly when considered separately for males and females.

In females, we observed a significant elevation of GFAP from the age of 50 years, whereas in males from 60 years. Indeed, in the 50–60 age group, we observed gender-related differences, with females having increased levels than males (Figure 9).

Knowing this, we tried to establish age- and sex-stratified RIs, grouping the population by sex and age, separating females into 18–50 and >50 age groups, and separating males into 18–60 and >60 age groups (Table 2).

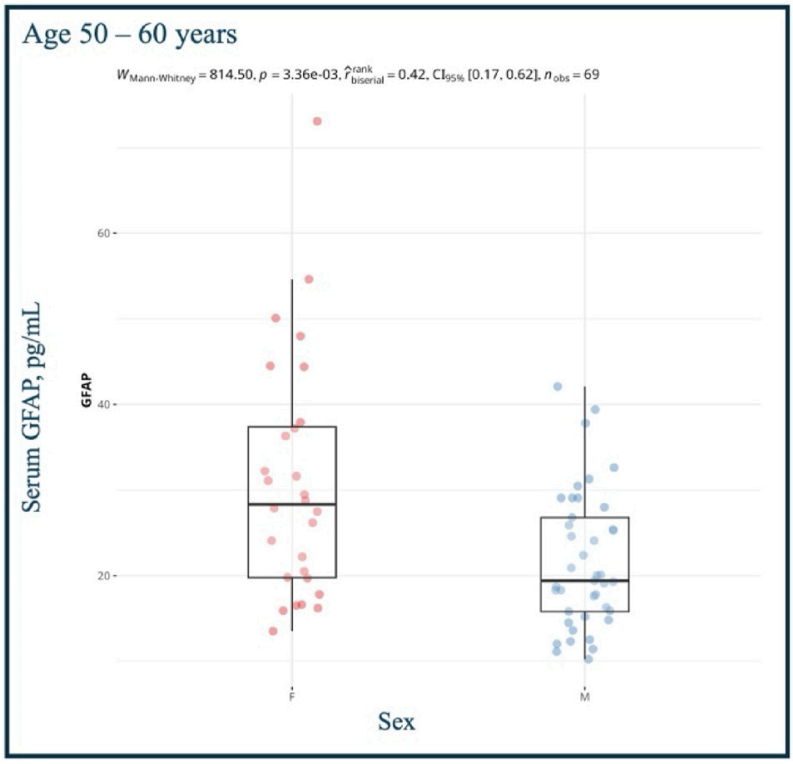

Figure 9. Serum GFAP levels across age and sex.

	Serum GFAP RIs, pg/mL	Lower 90 % CI	Upper 90 % CI
Males, 18–60 years	2.4–34.7	0[t] –5.3	31.5–37.9
Males, >60 years	5.7–62.3	0.1–11.3	56.3–68.1
Females, 18–50 years	5.1–33.9	1.7–8.1	30.7–37.5
Females, >50 years	0[t]–95.5	0[t]	81.5–108.9

[t]indicates the values were truncated at 0.

Table 2. Reference intervals for serum GFAP stratified by sex and age.

4.3.2.2 Diagnostic performance in AD vs other neurodegenerative diseases

Serum GFAP levels were compared between disease groups and their respective age-matched controls (Figure 10). We found that GFAP levels were significantly elevated in MS patients compared with controls, with a median of 24.60 pg/mL in the MS group versus 20.30 pg/mL in the control group ($p = 0.03$). Similarly, individuals with ATTR showed higher GFAP levels than controls (median: 50.20 pg/mL vs. 35.6 pg/mL, $p = 0.02$). In AD, GFAP levels were markedly higher than in controls, with a median of 79.40 pg/mL compared to 39.45 pg/mL, ($p = 2.55 \times 10^{-12}$), suggesting a strong association between GFAP elevation and AD pathology. Overall, GFAP levels were significantly higher in all disease groups than in controls, with the most pronounced elevation observed in AD.

ROC curve analysis was conducted to assess the diagnostic performance of GFAP in distinguishing disease groups from their respective age-matched controls (Figure 11).

GFAP demonstrated a strong discriminatory ability for AD, with an area under the curve (AUC) of 0.86 (0.79–0.93). The optimal threshold determined by the Youden index was 52 pg/mL.

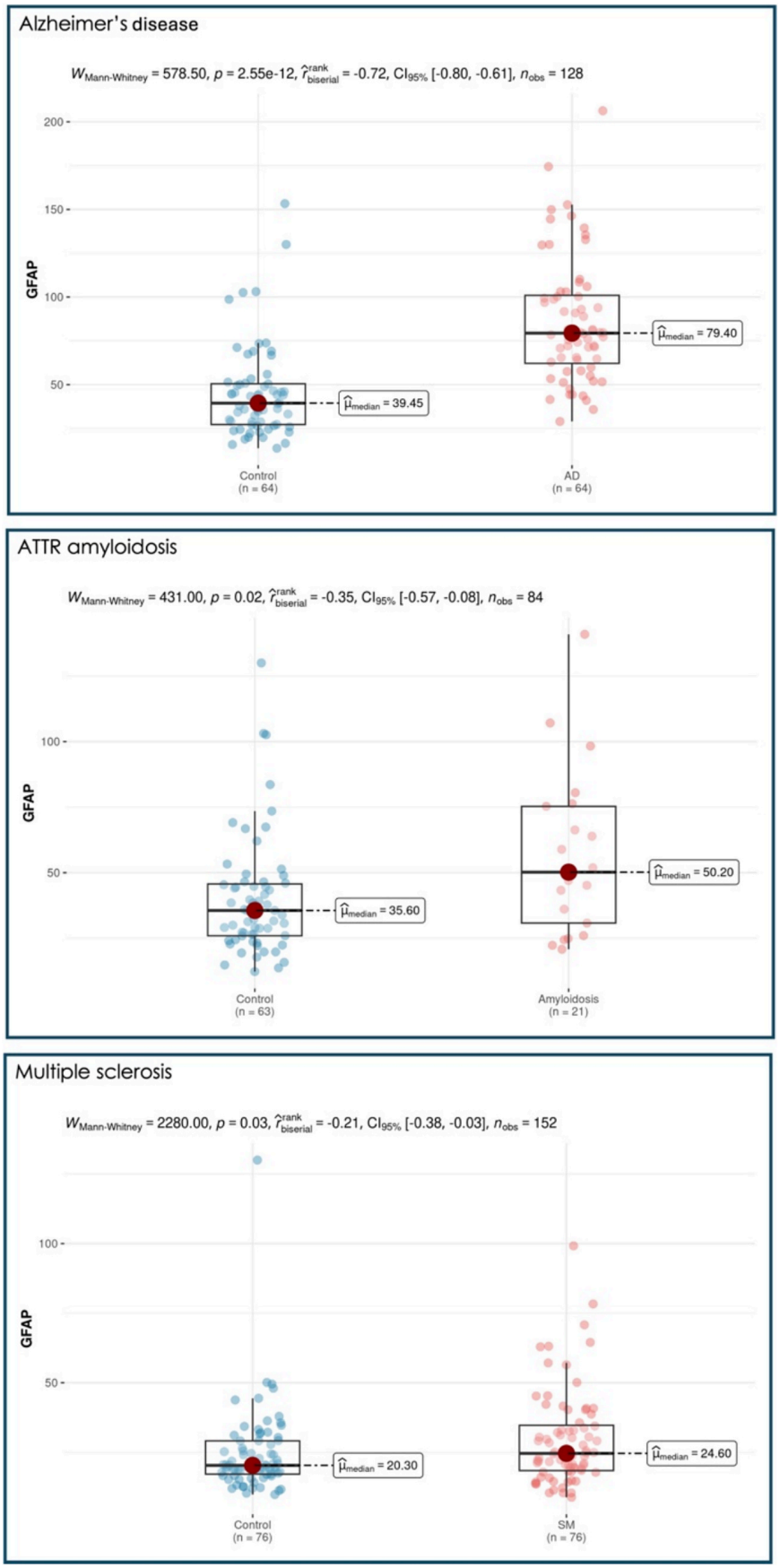

Figure 10. Distribution of serum GFAP levels in the study population.

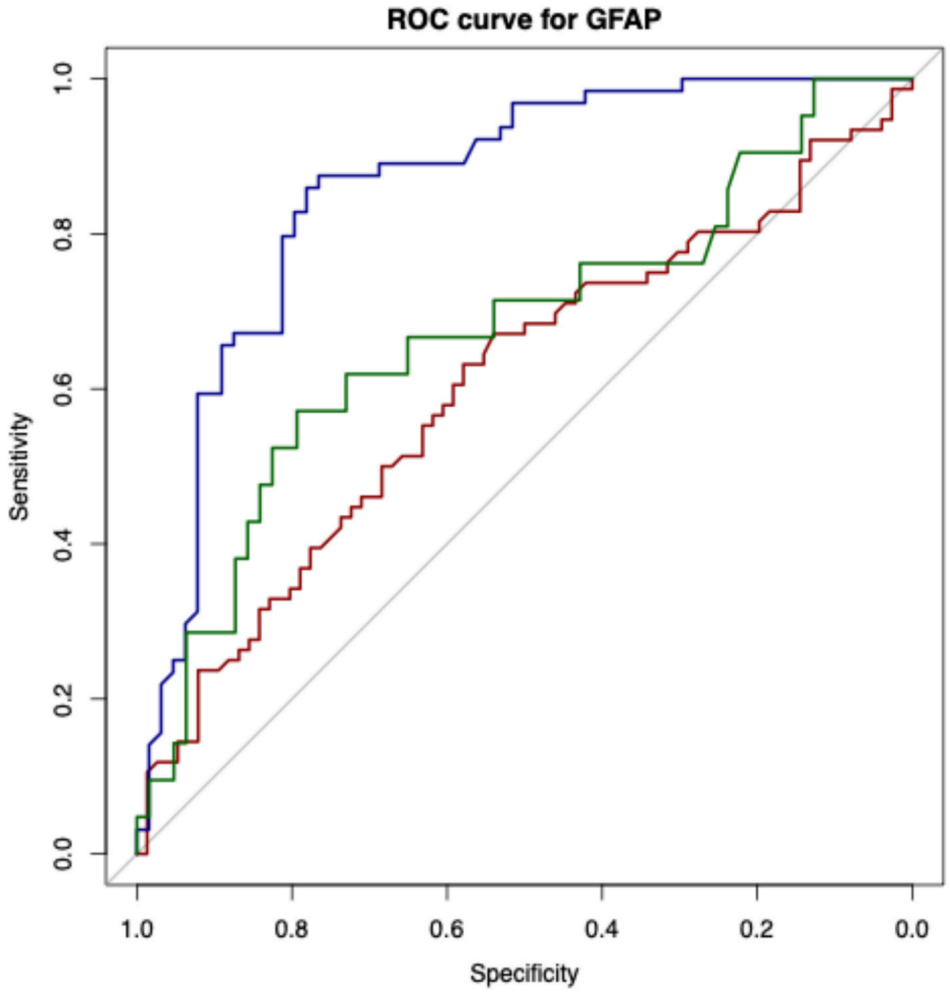

Figure 11. ROC curve analysis of serum GFAP for diagnosing Alzheimer's disease (blue), ATTR amyloidosis (red), and Multiple Sclerosis (green).

GFAP exhibited a more modest diagnostic performance in ATTR, with an AUC of 0.67 (0.53–0.82). The optimal threshold was 47 pg/mL. While GFAP showed some discriminatory power, the relatively lower sensitivity suggests that it may have limited standalone diagnostic utility for ATTR.

For MS, the AUC was even lower, 0.61 (0.51–0.70). The optimal threshold of 21 pg/mL provided low sensitivity and specificity. Overall, GFAP showed the highest diagnostic performance in distinguishing AD from controls, followed by moderate to low performance in distinguishing ATTR and MS from controls.

In AD patients, we explored the possible correlation between GFAP and various neurodegenerative biomarkers. The evaluated biomarkers included serum and CSF biomarkers associated with amyloid pathology, tauopathy, neurodegeneration, and synaptic integrity. GFAP showed significant positive correlations with serum neurofilament light chain (NfL) ($r = 0.42$, $p = 0.01$), CSF NfL ($r = 0.25$, $p = 0.04$), and serum phosphorylated Tau at 181 (pTau) ($r = 0.66$, $p < 0.001$). However, no significant correlation was observed between GFAP and amyloid-related markers (serum or CSF Aβ40, Aβ42, or Aβ ratio) or other neurodegeneration-associated biomarkers. These findings suggest that GFAP is more closely associated with tau pathology and neurodegeneration (as indicated by pTau and NfL levels) rather than amyloid burden.

4.3.3 sTREM2 across different biological matrices

The median concentration of sTREM2 was 2247 pg/mL in serum, 2107.8 pg/mL in plasma, and 4061.5 pg/mL in CSF, demonstrating that CSF has the highest levels, as expected.

Passing–Bablok regression between CSF and serum measurements (n = 102) yielded the following equation:

$$\text{Serum sTREM2} = 1255.70 + 0.21 \times \text{CSF sTREM2}$$

with a Spearman correlation coefficient of $\rho = 0.316$ ($p = 0.0013$). Residuals ranged from –2000 to +4000, with no evidence of marked heteroscedasticity. Although moderately correlated, the regression fit shows substantial scatter around the regression line, indicating that the model does not fully capture the relationship between serum and CSF values (Figure 12).

Bland–Altman analysis showed a mean difference of –2406.06, with limits of agreement between -8301.48 and +3489.37. Analysis of the ratio indicated a mean ratio of 0.55, with limits of agreement from 0.18 to 1.71 (Figure 13). Overall, the analyses indicate wide variability between CSF and serum

values, with proportional differences observed. These findings suggest limited agreement between the two biological matrices.

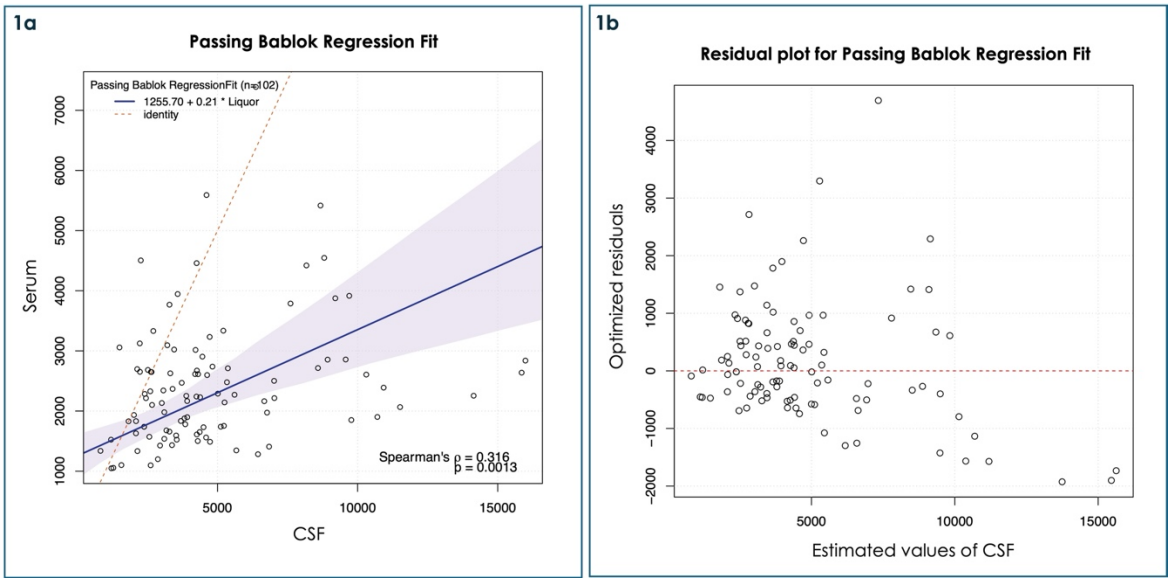

Figure 12. (a) Passing–Bablok regression of serum versus liquor concentrations. The blue solid line represents the fitted regression line, and the shaded area indicates the 95% confidence interval. The red dashed line corresponds to the line of identity (y = x). (b) Residual plot of the Passing–Bablok regression. Optimized residuals (y-axis) are plotted against estimated liquor values (x-axis). The red dotted line marks the zero-residual reference.

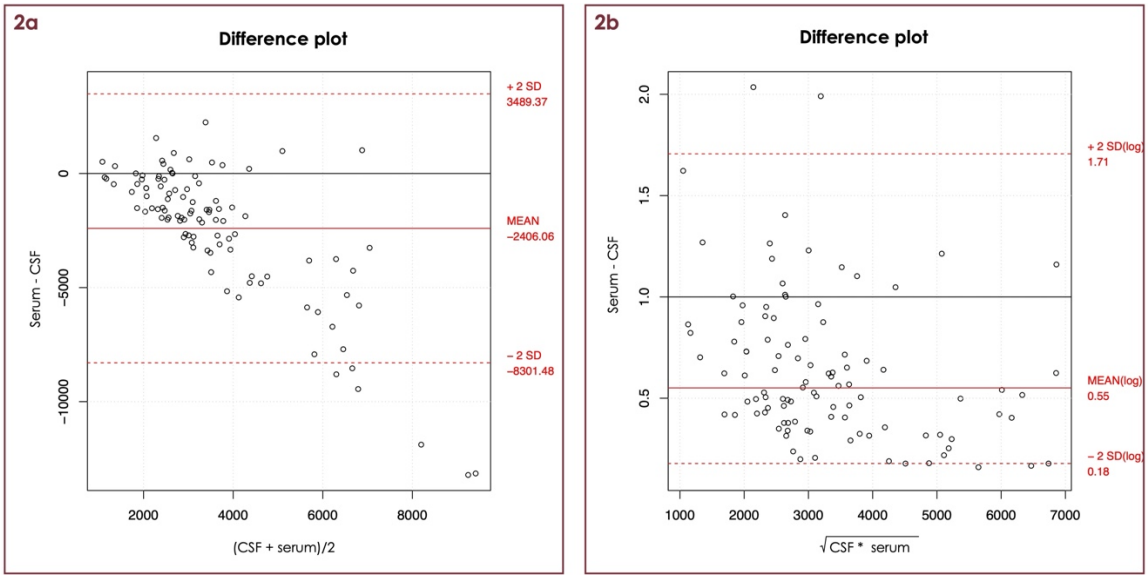

Figure 13. (a) Bland–Altman plot comparing serum and liquor concentrations. Each point represents the difference between serum and liquor values plotted against their mean. The red solid line indicates the mean difference, while the red dotted lines represent the upper and lower limits of agreement (mean ± 2 SD). (b) Ratio Bland–Altman plot. The red solid line marks the mean log ratio of serum to liquor, with red dotted lines indicating the upper and lower limits of agreement (mean ± 2 SD).

In a subset of patients (n = 30), we explored the relationship between serum and plasma. A strong correlation was observed between sTREM2 levels in serum and plasma ($\rho = 0.7419$; $p < 0.001$) (Figure 14). Nonetheless, serum values tended to be slightly higher than plasma values, suggesting that these two biological matrices, while related, should not be used interchangeably.

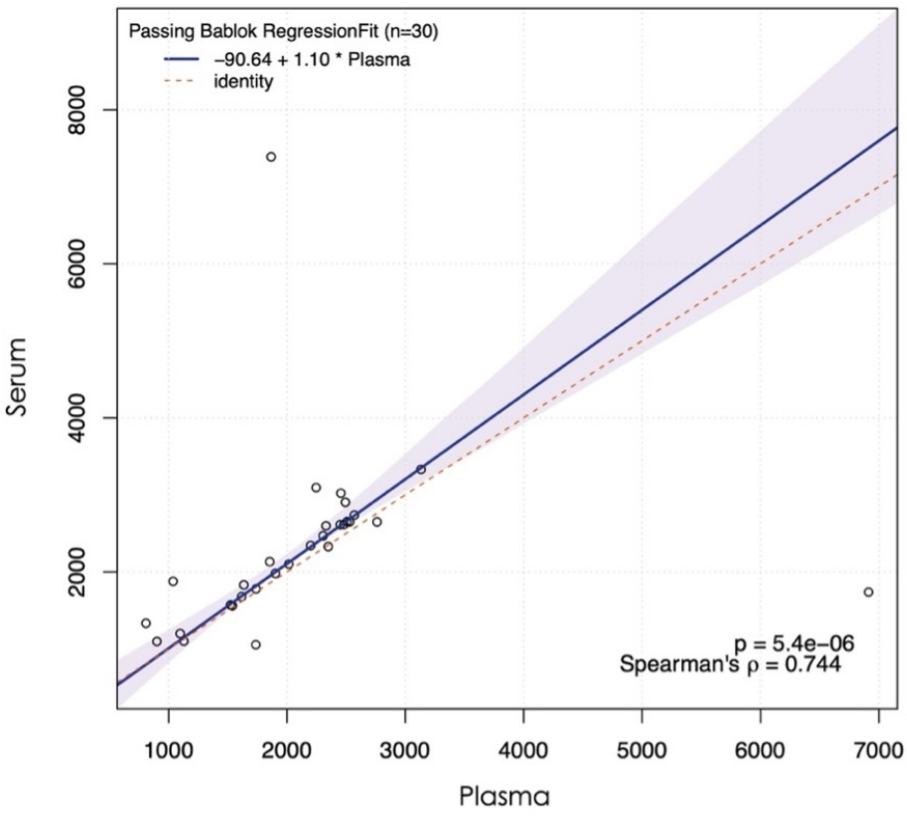

Figure 14. Passing–Bablok regression of serum versus plasma concentrations. The blue solid line represents the fitted regression line, and the shaded area indicates the 95% confidence interval. The red dashed line corresponds to the line of identity (y = x).

4.3.4 APOE genotype and blood levels

Among AD patients, 55% did not carry the Apoε4 allele, either homozygous or heterozygous; 40% were Apoε4 heterozygotes; and 5% were Apoε4 homozygotes. The ratio was significantly higher in homozygous than in heterozygous (Figure 15). Patients with the Apoε4 allele, neither in homo nor heterozygosis, have a median ratio of 0.4%. The Apoε4/Pan-Apoε ratio detects with high accuracy the presence of the Apoε4 allele (0.96, 95%CI 0.92-0.99; $p<0.0001$), with a sensitivity of 97% and a specificity of 98%. The kappa coefficient (Kohen) = 0.94 indicates a high concordance between the Apoε4/Pan-Apoε ratio and genotype.

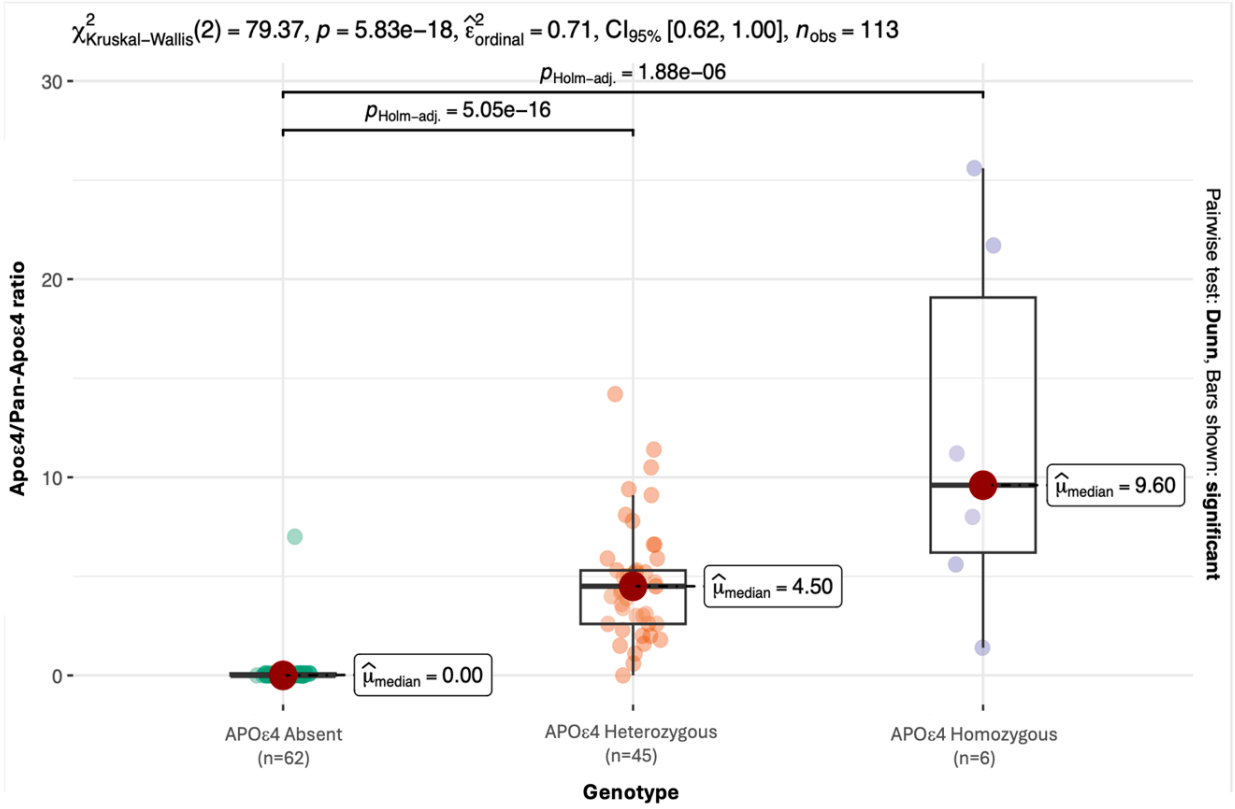

Figure 15. Distribution of patients by Apoε4 genotype.

4.4 Discussion

Alzheimer's disease is increasingly recognized as a global public health priority, with rising prevalence, substantial human and economic costs, and growing demand for timely, biologically grounded diagnosis. In parallel, the therapeutic landscape is shifting from purely symptomatic management toward disease-modifying strategies targeting amyloid and tau. In this context, there is a pressing need for diagnostic tools that are not only biologically accurate but also scalable, minimally invasive, and compatible with routine clinical workflows across diverse healthcare settings. The present study addresses this gap by evaluating a panel of blood-based biomarkers (Aβ42, Aβ40, Aβ42/40 ratio, pTau181, GFAP, sTREM2, and ApoE) measured on an automated chemiluminescent platform, already available in many clinical laboratories.

Our results confirm and extend current evidence that plasma pTau181 and Aβ42/40 ratio capture key aspects of AD-related pathology in a biologically meaningful manner. Plasma pTau181 showed a good correlation with its CSF counterpart, while the Aβ42/40 ratio displayed a weaker but still significant association. Passing–Bablok regression analyses demonstrated approximately linear relationships between matrices, albeit with clear constant biases and concentration-dependent effects, reflecting the expected order-of-magnitude differences between CSF and plasma concentrations. These findings are consistent with previous findings indicating that plasma pTau181 and Aβ42/40 are robust indicators of amyloid and tau pathology and support their use as first-line tests to estimate the likelihood of underlying AD [99-101].

In contrast, salivary measurements of Aβ42, Aβ40, and pTau181 did not show meaningful correlations with CSF or plasma values. Analytical performance was compromised by high viscosity, pre-analytical instability, and limited precision, leading to non-interpretable results in a substantial proportion of samples. Under current technical conditions and with the assays used in this study, saliva cannot be recommended as a reliable biofluid for AD biomarker assessment.

Serum GFAP emerged as a particularly robust biomarker, demonstrating strong discrimination between AD and controls (AUC = 0.86; optimal cut-off ≈ 52 pg/mL). Smaller but significant elevations were also observed in ATTRv-PN and MS, consistent with GFAP's known role as a general biomarker of astroglia activation rather than AD-specific pathology [102]. Two major clinical implications can be derived from these findings. Although GFAP is not disease-specific, in the appropriate clinical context, such as cognitive disorders, elevated GFAP values can strengthen the probability that a positive plasma pTau/Aβ result reflects true AD pathology. Conversely, normal GFAP levels in older adults decrease the likelihood of active neurodegeneration, improving the specificity of the overall BBM panel.

This is the first study to establish GFAP RIs using a fully automated platform. This is a critical step for introducing a biomarker in clinical practice. Specifically, we found that GFAP levels increased with age and were higher in women. Therefore, age- and sex-stratified RIs are essential to minimize false positives, particularly in older or female patients. Implementation without such stratification would likely lead to overdiagnosis in these groups. Correlation analyses showed that serum GFAP was significantly associated with pTau181, but not with amyloid indices. This pattern supports its role as a biomarker of glial reactivity and neurodegeneration, rather than a direct reflection of amyloid pathology. From a practical perspective, GFAP is best positioned as an adjunct to amyloid and tau biomarkers, enriching the biological profile within the AT(N) framework by capturing the "I" (inflammation) dimension.

This is also the first study evaluating blood sTREM2 levels measured by the fully automated platform Lumipulse. As expected, sTREM2 concentrations were highest in CSF. However, the agreement between CSF and serum was limited, with modest correlations and wide Bland–Altman limits, suggesting that the two matrices cannot be used interchangeably. Serum and plasma values, in contrast, showed a strong correlation ($\rho \approx 0.74$), though small systematic biases remained. For clinical

and research standardization, it is therefore advisable to select a single blood matrix for serial monitoring or inter-patient comparisons.

Clinically, CSF sTREM2 remains the gold standard for assessing microglial activation, particularly in complex or research settings. Blood-based sTREM2 demonstrates potential as a screening or monitoring tool, but further assay harmonization and validation against clinical outcomes are required before it can be reliably introduced into clinical workflows. Altogether, these findings emphasize that sTREM2 is a promising index of microglial activation, but that matrix choice and standardization remain critical issues.

The ApoE4/Pan-ApoE protein ratio in serum was highly accurate for identifying APOE ε4 carrier status, achieving an AUC of 0.96 with 97% sensitivity and 98% specificity, respectively ($\kappa = 0.94$ compared with genetic testing). This rapid and non-genetic assay offers several advantages. It can support clinical trial screening and risk stratification without the delays or ethical complexities of genotyping. It serves as a pre-genotype filter, prompting confirmatory DNA testing only when necessary.

Based on the findings of this study and the current literature, a stepwise diagnostic pathway can be proposed for the clinical implementation of blood-based biomarkers, recognizing that their clinical value and optimal use may vary substantially between primary and secondary care, where the pre-test probability of AD differs markedly. In a primary care or general neurology settings, patients typically present with subjective cognitive decline or mild, non-specific symptoms, and the underlying prevalence of biologically defined AD is relatively low [103]. Access to CSF biomarkers and amyloid PET is also limited. In primary setting, blood biomarkers could be used as a triage tool to exclude the disease with high accuracy, if the test is negative, or to identify patients with probable AD who should undergo further evaluation in secondary care to confirm the disease if the test is positive (Figure 16). Therefore, an altered biomarker result, where the clinical suspicion may still be low or non-specific, is not sufficient on its own to establish a diagnosis of AD. Such an approach can

reduce unnecessary referrals to specialized memory centres, minimize the use of invasive testing, and alleviate patient anxiety when AD is not biologically suspected. Conversely, the same result, obtained in a secondary care setting where AD suspicion is high, may support a biological diagnosis, particularly if the biomarker result is clearly positive and consistent with the clinical picture. In this scenario, only patients with an intermediate result should undergo further investigation by CSF analysis or PET to confirm the disease (Figure 16). For example, an amnestic MCI patient with a clearly abnormal pTau181/Aβ42 pattern and elevated GFAP has a high likelihood of AD pathology, allowing for evaluating the eligibility for disease-modifying therapies. Contrariwise, a non-Alzheimerian BBM profile in a patient with atypical clinical features should prompt consideration of alternative diagnoses, such as frontotemporal lobar degeneration, vascular cognitive impairment, or psychiatric conditions. In patients with established dementia, BBMs may also assist differential diagnosis when CSF or PET is unavailable or declined. Although they cannot yet fully substitute for CSF/PET in detecting mixed pathologies, they provide biologically grounded information that complements clinical phenotype, neuropsychological testing, and structural imaging. GFAP appears to reflect astroglial activation and neurodegenerative activity, making it potentially useful for identifying disease activity or for recognizing patients with minimal evidence of ongoing neurodegeneration.

Notably, the NPV of plasma biomarkers is high across both settings, making a negative result helpful to exclude underlying AD pathology, especially at early diagnostic stages [104].

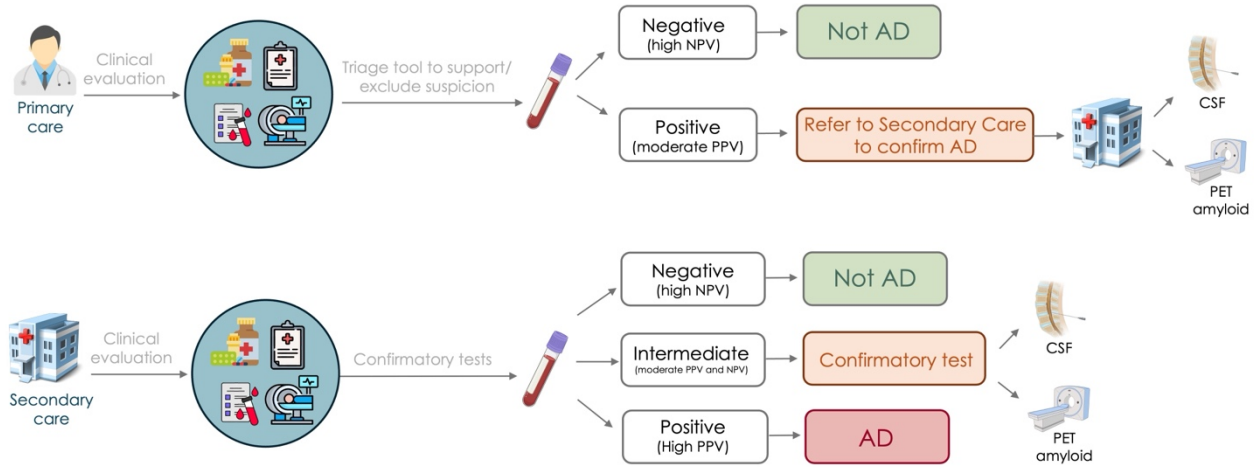

Figure 16. Blood-based biomarkers in primary and secondary care settings.

For the appropriate implementation of BBMs, two principles remain essential regardless of care setting. First, biomarker testing should be performed only after a comprehensive clinical assessment, including detailed medical, family, and pharmacological history; standardized cognitive and functional evaluations; initial laboratory investigations; and structural neuroimaging. These steps are necessary to identify or exclude reversible or alternative causes of cognitive impairment. Second, given the consistently high NPV of BBMs, a negative test result reliably excludes the presence of underlying Alzheimer's pathology.

Finally, while blood biomarkers are most discussed in the context of diagnosis, their use in monitoring may also be valuable. Repeated measurements over time could enhance early detection in individuals at increased risk, particularly in primary care, where most asymptomatic or mildly symptomatic individuals regularly interact with their general practitioners. Such an approach could allow earlier identification of pathological changes and timelier referral to secondary care, ultimately facilitating earlier diagnosis and intervention [104].

The inclusion of MS and ATTRv-PN cohorts in this study also has practical implications. The observation that GFAP is elevated across these conditions underscores the need for clinicians to interpret GFAP levels in the context of known comorbid neurological diseases. An isolated GFAP

increase in a patient with MS and cognitive symptoms, in the context of normal pTau181 and Aβ42/40 ratios, is unlikely to indicate coexistent AD and more likely reflects underlying inflammatory or demyelinating activity. Similarly, non-specific changes in sTREM2 must be interpreted cautiously until matrix-specific reference values and disease-specific patterns are better defined.

The ApoE4/Pan-ApoE ratio adds another clinically relevant dimension. In memory clinics and therapeutic decision-making, rapid identification of APOE ε4 carriers can inform risk counselling and may influence monitoring strategies, particularly in the context of amyloid-targeting therapies. Indeed, we are now in the era of disease-modifying therapies for Alzheimer's disease, and the Lecanemab, a monoclonal antibody targeting amyloid pathology, has already been approved in more than 50 countries worldwide. As these treatments transition into clinical use, careful patient selection has become a critical component of therapeutic decision-making.

A central element in identifying eligible candidates is APOE genotyping. The presence of the APOE ε4 allele, particularly in the homozygous state (ε4/ε4), is strongly associated with an increased risk of amyloid-related imaging abnormalities (ARIA), the most clinically relevant adverse events linked to anti-amyloid monoclonal antibodies [105]. Consequently, individuals who are APOE ε4 homozygotes are generally considered poor candidates for therapy, although the degree of restriction varies across regulatory jurisdictions. In the United States, the FDA permits APOE ε4 homozygotes to receive treatment, but only after a thorough risk–benefit discussion with the prescribing clinician, ensuring that patients fully understand the significantly elevated likelihood of ARIA. In contrast, the European Medicines Agency (EMA) adopts a more conservative stance, recommending against treatment in APOE ε4 homozygous individuals due to the unfavourable safety profile in this subgroup.

As additional disease-modifying therapies emerge, the integration of genetic risk stratification will likely become an increasingly routine component of therapeutic pathways. Despite its growing clinical relevance, particularly in the context of anti-amyloid disease-modifying therapies, APOE

genotyping faces several practical barriers that limit its routine implementation across healthcare systems. Many hospitals, especially in rural or resource-limited settings, lack in-house molecular genetics facilities. Access may depend on sending samples to specialized reference laboratories, which introduces logistical delays and increases costs. This uneven distribution of testing capacity creates disparities in access to personalized therapeutic decision-making. The cost of APOE testing varies widely between countries and even within regions. In some healthcare systems, APOE genotyping is not reimbursed, leaving patients to pay out of pocket. Delayed or incomplete reimbursement frameworks can discourage clinicians from ordering the test. These economic barriers can slow down or prevent appropriate patient stratification for disease-modifying therapies. An emerging strategy to overcome some of the practical limitations associated with APOE genotyping is the use of serum-based APOE protein assays. Although these tests cannot replace genetic analysis, they offer a pragmatic and accessible alternative for preliminary risk stratification, particularly in settings where molecular testing is not readily available.

Serum APOE measurement has shown high accuracy in identifying individuals homozygous for the APOE ε4 genotype, the subgroup at greatest risk for ARIA when treated with anti-amyloid monoclonal antibodies. This is clinically relevant because APOE ε4 homozygosity represents a key contraindication or strong caution criterion for several disease-modifying therapies. Implementing serum APOE assays could therefore support treatment eligibility pathways by flagging patients who are likely to be at substantially increased risk and who may require either confirmatory genotyping or alternative therapeutic strategies.

However, results must be interpreted with caution. Serum protein levels reflect the phenotypic expression of APOE isoforms rather than the exact genotype, and potential confounders, such as metabolic conditions, liver function, or laboratory variability, may influence assay results. For this reason, serum APOE analysis should be viewed as a screening tool rather than a diagnostic substitute

for formal genotyping. When serum APOE suggests ε4 homozygosity or produces ambiguous results, definitive genetic testing remains necessary.

Despite these limitations, the wider availability, lower cost, and faster turnaround of serum-based APOE testing make it a promising adjunct in clinical workflows. Its integration could facilitate broader and more equitable access to disease-modifying therapies by enabling earlier stratification of candidates, particularly in healthcare systems where full genetic testing is difficult to obtain.

This study has several strengths that enhance the robustness and translational relevance of its findings. First, all biomarkers were measured on a fully automated chemiluminescent platform (Lumipulse) that is already implemented in routine clinical laboratories. This minimizes operator-dependent variability and facilitates immediate transfer of the present workflow into practice. Second, the AD cohort was stringently defined by concordant clinical, CSF, and imaging criteria (A+T+N+), reducing diagnostic misclassification. Third, the inclusion of both neurologically healthy individuals and disease controls (MS and ATTRv-PN) allowed us to investigate biomarker behaviour across a spectrum of neurodegenerative and neuroinflammatory conditions. Fourth, the study systematically addressed pre-analytical variables and examined multiple matrices (CSF, serum, plasma, saliva), providing a comprehensive view of matrix-specific performance and limitations. Importantly, age- and sex-specific reference intervals for GFAP were derived in a large control sample, which is directly relevant for clinical implementation.

Nonetheless, several limitations must be considered. The retrospective, single-centre design may introduce referral and spectrum biases and limit the generalizability of the results to other settings and populations. The ATTRv-PN and MS cohorts were relatively small, resulting in wide confidence intervals around the estimates of diagnostic performance for these diseases. Other common dementia aetiologies, such as dementia with Lewy bodies, frontotemporal dementia, and vascular dementia, were not systematically included, preventing a full assessment of differential diagnostic utility across the entire dementia spectrum. The absence of longitudinal data is another important limitation.

Without repeated measurements, it is not possible to determine intra-individual variability, reference change values, or the responsiveness of pTau181, GFAP, and sTREM2 to clinical progression or therapeutic intervention. Furthermore, neuropathological confirmation was unavailable; instead, CSF and PET served as the reference standards, which, although widely accepted, may not fully capture mixed or atypical pathologies, especially in very old individuals.

Finally, the poor performance of salivary assays in this study likely reflects technical and pre-analytical constraints rather than definitive biological irrelevance of saliva as a matrix. However, under current conditions, saliva cannot be recommended for clinical use, and future work in this area will require substantial methodological refinement.

The present findings support a paradigm in which blood-based biomarkers, anchored by plasma pTau181 and Aβ42/40 ratio and contextualized by age- and sex-adjusted GFAP, can be deployed as a first-line triage and enrichment tool for AD in both primary and secondary care. Based on this work and the emerging literature, several priorities for future research and implementation can be identified.

First, prospective multicentre studies with harmonized pre-analytical protocols and external quality assessment schemes are needed to validate the proposed cut-offs and reference intervals across more diverse populations, including individuals of non-European ancestry and those with a high burden of comorbidities. Second, longitudinal cohorts should be established to characterize biomarker trajectories across the AD continuum and to determine how changes in pTau181 and GFAP relate to clinical progression, imaging markers, and treatment response. Such data will be essential for using BBMs not only as diagnostic tools but also as markers of disease activity and therapeutic efficacy.

Third, predictive models that integrate BBMs with clinical, cognitive, imaging, and genetic variables should be developed and tested in real-world cohorts to refine risk stratification and diagnostic probabilities at the individual level. Fourth, health-economic and implementation studies are required to evaluate the cost-effectiveness and feasibility of BBM-based diagnostic pathways in different

healthcare systems, and to ensure that these tools contribute to reducing rather than exacerbating existing inequities in dementia care. Lastly, clear guidance is needed on ethical and communication aspects, particularly for tests that infer APOE genotype or pre-symptomatic risk. Protocols for informed consent, result disclosure, and data governance must be developed in parallel with technical validation to guarantee responsible use.

4.5 Conclusion

This study provides comprehensive clinical validation of a multimodal panel of blood-based biomarkers for Alzheimer's disease, measured on a fully automated chemiluminescent platform suitable for routine diagnostic laboratories. By examining plasma Aβ42, Aβ40, Aβ42/40 ratio, pTau181, serum GFAP, sTREM2, and an ApoE4/Pan-ApoE ratio across multiple biological matrices and disease groups, we demonstrate that blood biomarkers can reliably capture key components of the Alzheimer's pathological cascade and support diagnostic workflows across the clinical spectrum.

Plasma pTau181 and the Aβ42/40 ratio emerged as robust indicators of tau and amyloid pathology, respectively, and showed significant associations with their CSF counterparts. These characteristics position them as effective first-line triage tools, particularly in primary care, where the pre-test probability of Alzheimer's disease is low and access to CSF and PET biomarkers is limited. GFAP provided complementary information reflecting astroglia activation and neurodegeneration, with strong discriminatory power for AD and age- and sex-dependent variation necessitating stratified reference intervals. Although sTREM2 levels were highest in CSF and showed limited agreement with serum levels, blood-based sTREM2 remains a promising biomarker of microglial activation and warrants further standardization. The ApoE4/Pan-ApoE protein ratio demonstrated excellent concordance with APOE genotyping and represents a practical adjunct for rapid risk stratification, particularly in the context of disease-modifying therapies, although it does not fully replace genetic testing.

The integration of these biomarkers into a stepwise diagnostic framework highlights their differential roles across settings. In primary care, negative results can confidently exclude AD, reducing unnecessary specialist referrals and invasive procedures. In secondary care and memory clinics, clearly abnormal profiles can strengthen diagnostic confidence, guide the need for confirmatory CSF or PET studies, and support evaluation for anti-amyloid therapies. In advanced disease, blood biomarkers complement clinical assessment and imaging when CSF or PET are unavailable.

Beyond diagnostic utility, this research project underscores the practical importance of laboratory feasibility, cost, accessibility, and ethical considerations, particularly regarding APOE-related testing, in ensuring equitable implementation of blood biomarkers. Biomarkers measured on automated platforms enable real-world translation and scalability across healthcare systems.

Strengths of this study include rigorous pre-analytical control, use of a clinically implemented analytical platform, comparison across multiple matrices, and evaluation in both Alzheimer's disease and disease controls. Limitations include its single-centre, retrospective design, modest sample size for some conditions, lack of neuropathological confirmation, and absence of longitudinal data.

Overall, the findings support the adoption of blood-based biomarkers as central components of modern Alzheimer's disease diagnostics. They enable earlier, biologically grounded detection; more efficient use of advanced testing; and improved patient stratification for the emerging era of disease-modifying therapies. Future research should focus on multicentre validation, harmonization of assays, longitudinal profiling, integration with multimodal predictive models, and health-economic evaluation to ensure responsible and equitable translation into clinical practice.

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