



## Review

# Clinical and laboratory integration in Alzheimer's disease: From biological biomarkers to diagnostic implementation

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### Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common cause of dementia worldwide. Traditionally diagnosed based on clinical syndromes, AD is now increasingly defined as a biologically identifiable disease, reflecting advances in fluid and imaging biomarkers. Contemporary diagnostic frameworks emphasize the integration of clinical assessment with biological confirmation and staging, primarily through biomarkers of amyloid- $\beta$  deposition, tau pathology, and neurodegeneration. This review provides a structured overview of current clinical staging systems and biomarker-based diagnostic approaches in Alzheimer's disease, with particular emphasis on analytical standardization, interpretive frameworks, and methodological limitations relevant to routine clinical practice. The revised 2024 Alzheimer's Association criteria are discussed, including the numeric clinical staging model and the AT(N) biomarker classification, which together enable biologically grounded diagnosis independent of symptom severity. Core biomarkers are categorized into Core 1 biomarkers, used for biological confirmation of AD pathology, and Core 2 biomarkers, which characterize disease burden, neurodegeneration, and progression. Cerebrospinal fluid (CSF) biomarkers remain the reference standard for *in vivo* biological diagnosis, while blood-based biomarkers—enabled by ultrasensitive analytical platforms—have emerged as scalable and minimally invasive tools for screening, risk stratification, and longitudinal monitoring. Multimodal biomarker profiling further supports the identification of mixed or non-Alzheimer comorbid pathologies, which are common in older populations and critically influence clinical interpretation and therapeutic decision-making. Despite their clinical utility, significant challenges remain. Measurement variability related to pre-analytical handling, assay performance, inter-laboratory differences, and lot-to-lot reagent effects continues to limit universal cut-off definition and broad clinical implementation. International standardization initiatives, reference materials, external quality control programs, and automation-ready assays have substantially improved analytical performance, yet pre-analytical variability—particularly for amyloid- $\beta$ —remains a key unresolved issue. In conclusion, Alzheimer's disease biomarkers provide a powerful framework for biological diagnosis and staging, but their clinical value increasingly depends on rigorous standardization, harmonization, and context-aware interpretation rather than on the discovery of new markers alone.

**Keywords:** Alzheimer's disease, biomarkers, biological diagnosis, diagnostic staging, pre-analytical variability

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**A**lzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive decline and represents the most common cause of dementia in the elderly population. Pathophysiologically, it is defined by amyloid- $\beta$  accumulation, hyperphosphorylation of tau protein, and the accompanying widespread neurodegeneration [1, 2].

For many years, the diagnosis of AD relied predominantly on the definition of a clinical syndrome, with underlying biological

processes regarded as secondary, supportive elements. However, over the past decade—particularly within the last five years—the development and clinical validation of disease-specific biomarkers have led to a paradigm shift, whereby AD is increasingly approached as a biologically definable disorder [3, 4].

In contemporary practice, the diagnostic approach to AD begins with a comprehensive clinical evaluation, followed by biological confirmation and staging using laboratory and

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imaging biomarkers. In 2011, the National Institute on Aging and the Alzheimer's Association (NIA-AA) convened three separate workgroups to develop diagnostic and evaluative recommendations for the preclinical, mild cognitive impairment (MCI), and dementia stages of Alzheimer's disease [5]. In 2018, a single workgroup was convened to update these earlier recommendations, resulting in the publication of a "research framework" intended to be revised over time in response to scientific advances [6]. The most recent revision, coordinated by the Alzheimer's Association and published in 2024, aimed to update the 2018 framework while preserving the core conceptual principles established by earlier NIA-AA workgroups [7]. These foundational principles continue to form the basis of the revised diagnostic criteria. In this chapter, the diagnostic approach to AD is first discussed within the framework of clinical criteria, followed by a detailed presentation of biomarker-based laboratory evaluation.

Accordingly, this review aims to integrate current clinical staging systems with biomarker-based laboratory diagnostics and limitations of the biological markers for AD into routine clinical practice.

## Clinical Staging System in Alzheimer's Disease

The revised 2024 framework maintains the six-stage numeric clinical staging system introduced in 2018, with minor refinements [6,7]. This system applies exclusively to individuals within the AD pathophysiologic continuum, spanning from asymptomatic stages to severe dementia (Table 1):

- **Stage 1:** Biomarker-positive, asymptomatic.
- **Stage 2:** Subtle cognitive or neurobehavioral changes.
- **Stage 3:** Objective cognitive impairment without loss of independence.
- **Stages 4–6:** Progressive loss of independence (mild, moderate, and severe dementia).

This numeric model parallels both the Global Deterioration Scale and FDA guidance for early AD trials, while uniquely anchoring clinical severity to AD-specific biomarker evidence [7]. An important conceptual refinement is the introduction of stage 0, which represents genetically determined AD—such as autosomal dominant AD or Down syndrome-associated AD—in biomarker-negative, clinically asymptomatic individuals. This distinction is essential to avoid conflating genetic determinism with biomarker-defined disease biology in both clinical practice and trial design. In contrast, genetic risk alleles such as APOE  $\epsilon$ 4 are not incorporated into the staging scheme, as they indicate susceptibility rather than established disease. Nonetheless, APOE genotyping has gained clinical importance in the context of anti-amyloid- $\beta$  immunotherapy because of its association with treatment-related risks [7–11]. This staging model emphasizes the severity of cognitive and functional decline, not the phenotypic subtype. It accounts for the heterogeneous and progressive nature of AD, which often involves overlapping cognitive phenotypes and mixed

pathologies. Thus, numeric staging provides a biologically grounded complement to traditional syndromic labels like MCI and dementia, clarifying that biomarker-positive individuals already have AD, not merely "at risk" status [6–8].

## Clinical Diagnostic Criteria in Alzheimer's Disease

The diagnostic process in AD is fundamentally based on clinical evaluation. Clinical diagnosis relies on a detailed assessment of the patient's cognitive complaints, objective confirmation of these complaints through standardized cognitive testing, and evaluation of the impact of cognitive impairment on activities of daily living. Clinical assessment represents a critical step, as it determines in which patient populations and for what purposes biomarkers should be used [7, 12].

In the diagnostic process, the nature of the cognitive complaint is evaluated first. Cognitive concerns reported solely by the individual, without objective confirmation on neuropsychological testing, are defined as *subjective cognitive decline*. This stage may correspond to the preclinical phase of AD. Conditions in which impairment in memory or other cognitive domains is objectively demonstrated, while activities of daily living remain largely preserved, are classified as *mild cognitive impairment* (MCI). Cognitive impairment that affects multiple domains and significantly interferes with daily functioning is considered to be at the dementia level [12].

The typical clinical presentation of AD is characterized by early and predominant involvement of episodic memory. However, atypical clinical phenotypes may also occur, including logopenic primary progressive aphasia, posterior cortical atrophy, or presentations dominated by frontal executive dysfunction. During clinical evaluation, the presence of features such as prominent hallucinations, cognitive fluctuations, early parkinsonism, or rapid disease progression should raise suspicion for non-Alzheimer neurodegenerative disorders in the differential diagnosis [7, 12].

Structural brain imaging is an essential component of the clinical diagnostic workup. Magnetic resonance imaging allows exclusion of vascular pathology and secondary causes of cognitive impairment, while also enabling assessment of Alzheimer-compatible structural changes, such as medial temporal lobe atrophy. Once clinical and imaging findings have been integrated, the diagnostic process proceeds to the stage of biomarker-based biological confirmation [13–15].

## Biomarkers in Alzheimer's Disease

The biologically based diagnosis of AD is intended to complement—not replace—a comprehensive clinical evaluation. Advances in fluid-based biomarkers, particularly blood-based assays, together with the emergence of amyloid- $\beta$ -targeted therapies for early symptomatic AD, have substantially increased the clinical relevance of biomarker-guided diagnosis [14, 15].

**Table 1. Clinical staging of individuals on the Alzheimer's disease continuum**

Stage	Definition	Cognitive status	Activities of daily living (ADLs)
Stage 0	Asymptomatic, deterministic gene carrier	No clinical change; cognitive testing within normal range	Fully independent
Stage 1	Asymptomatic, biomarker evidence only	Objective cognitive tests within expected range; no reported decline	Fully independent
Stage 2	Transitional decline	Cognitive performance within normal range; subtle cognitive or neurobehavioral decline from prior baseline lasting $\geq 6$ months	Fully independent; no or minimal impact on ADLs
Stage 3	Cognitive impairment with early functional impact	Objective cognitive impairment on testing; decline documented by self, informant, or longitudinal testing	Independent, but reduced efficiency in complex ADLs
Stage 4	Dementia with mild functional impairment	Progressive cognitive decline	Dependence in instrumental ADLs; basic ADLs preserved
Stage 5	Dementia with moderate functional impairment	Advanced cognitive decline	Assistance required for basic ADLs
Stage 6	Dementia with severe functional impairment	Severe cognitive and functional decline	Complete dependence for basic ADLs

**Table 2. AT(N) biomarker classification in Alzheimer's disease**

Component	Pathophysiological process	Core biomarkers	Clinical significance
A	Amyloid- $\beta$ deposition	CSF A $\beta$ 42 $\downarrow$ CSF A $\beta$ 42/40 ratio $\downarrow$ Amyloid PET (+)	Presence of Alzheimer's disease biology
T	Tau hyperphosphorylation	CSF p-tau181 p-tau217 Tau PET (+)	Alzheimer-specific pathology
N	Neuronal injury/neurodegeneration	CSF total tau, Neurofilament light chain MRI atrophy FDG-PET	Disease severity and prognosis

At present, biomarker testing is recommended for symptomatic individuals, rather than cognitively unimpaired persons, despite the technical capability to detect disease biology in preclinical stages. While abnormal biomarkers are often sufficient to confirm AD pathology in symptomatic patients, coexisting pathologies must always be considered, as mixed etiologies are common [14, 15].

Higher biological stages, particularly those reflecting tau pathology, increase the likelihood that clinical symptoms are attributable to AD and provide valuable prognostic information. Overall, AD biomarkers constitute essential tools for accurate diagnosis, biological staging, treatment eligibility assessment, and patient counseling. The flexible application of core biomarker categories allows clinicians to tailor diagnostic strategies according to clinical context, biomarker availability, and therapeutic decision-making [16, 17].

Recent advances in ultrasensitive immunoassay technologies have fundamentally transformed the diagnostic landscape by enabling reliable detection of AD-related biomarkers in blood. Traditionally, *in vivo* biological diagnosis relied on cere-

brospinal fluid (CSF) biomarkers and positron emission tomography (PET) to demonstrate amyloid- $\beta$  and tau pathology. Although analytically robust and biologically validated, these approaches are limited by invasiveness, cost, and accessibility. Blood-based biomarkers have therefore emerged as a practical and scalable complement—and in selected contexts, a partial alternative—to CSF- and PET-based diagnostics [8, 13, 18, 19].

## Standardization for The Clinical Use of Biomarkers

The contemporary understanding of AD diagnosis conceptualizes the disorder not merely as a clinical syndrome, but as a biological continuum defined by specific underlying pathophysiological processes. The foundation of this approach is the AT(N) classification system, which comprises amyloid pathology (A), tau pathology (T), and neurodegeneration (N) (Table 2). In the AT(N) classification, the "A" component reflects amyloid- $\beta$  deposition, the "T" component represents tau protein pathology, and the "N" component indicates neuronal injury and neurodegeneration. In clinical practice, the presence

**Table 3. Interpretation of AT(N) biomarker notation**

Notation	Interpretation	Typical findings
A–	No biomarker evidence of amyloid pathology	Normal CSF A $\beta$ 42 or A $\beta$ 42/40 ratio; negative amyloid PET
A+	Amyloid pathology present	Decreased CSF A $\beta$ 42 or A $\beta$ 42/40 ratio; positive amyloid PET
T1–	No evidence of AD-specific tau abnormality	Normal p-tau levels
T1+	Alzheimer-specific tau abnormality present	Elevated p-tau217, p-tau181, or p-tau231
T2–	No evidence of advanced tau deposition	Normal tau PET; normal MTBR-tau243
T2+	Cortical tau deposition present	Positive tau PET; elevated MTBR-tau243, p-tau205
N–	No evidence of active neuronal injury	Normal NfL, MRI, FDG-PET
N+	Neurodegeneration or neuronal injury present	Elevated NfL; cortical atrophy; FDG hypometabolism

of AD biology is primarily defined by positivity for A and T biomarkers, while the N component provides information regarding disease severity and progression [7, 8, 20].

**Amyloid beta biomarkers (A component)** are measured using both fluid-based methods (CSF, plasma) and PET, and they show a high degree of concordance. A $\beta$ 42 levels in fluids may exhibit detectable changes slightly earlier than amyloid PET. In contrast, tau biomarkers display a more complex temporal profile [7, 8, 20, 21].

**The tau (T) component** is no longer considered a single entity but is divided into two biologically and clinically distinct stages, referred to as **T1** and **T2**. **T1 biomarkers** represent early, amyloid-related tau dysregulation and consist of phosphorylated and secreted tau species that are released into CSF and plasma in response to amyloid- $\beta$  pathology. These biomarkers, including p-tau217, p-tau181, and p-tau231, become abnormal very early in the disease course, often years before the onset of clinical symptoms, and demonstrate high specificity for Alzheimer's disease; therefore, they are considered suitable for establishing a biological diagnosis. In contrast, **T2 biomarkers** reflect established cortical tau proteinopathy characterized by the accumulation of neurofibrillary tangles within brain tissue. This stage is typically observed in individuals who already have amyloid pathology and is associated with disease severity, anatomical spread, and clinical progression rather than initial diagnosis. Biomarkers such as MTBR-tau243, p-tau205, and tau PET imaging fall into this category and are primarily used for disease staging and prognostic assessment. Thus, T1 biomarkers answer the question of whether AD biology is present, whereas T2 biomarkers inform how advanced and spatially distributed tau pathology has become [6–8, 20, 21].

Within the AT(N) framework, individuals can be grouped into three broad biomarker categories: Those with normal AD biomarkers; those within the Alzheimer' continuum, encompassing both Alzheimer's pathologic change and biologically defined AD; and c. those with normal amyloid biomarkers but abnormal tau and/or neurodegeneration markers. This latter category reflects evidence of one or more non-Alzheimer neuropathologic processes and has been termed suspected non-Alzheimer's pathophysiology (SNAP) [22]. Importantly,

AT(N) biomarker classification is independent of clinical symptoms, and the term *biomarker profile* is preferred over stage, as it does not imply a fixed temporal sequence or causality [7]. Applying normal/abnormal thresholds to each AT(N) biomarker group yields a set of distinct AT(N) biomarker profiles (e.g., A+T–(N)–, A+T+(N)+), which allow individuals to be classified according to underlying biological patterns rather than solely clinical presentation [8] (Table 3).

### Core Biomarkers in Alzheimer's Disease: Plasma and CSF-Based Approaches

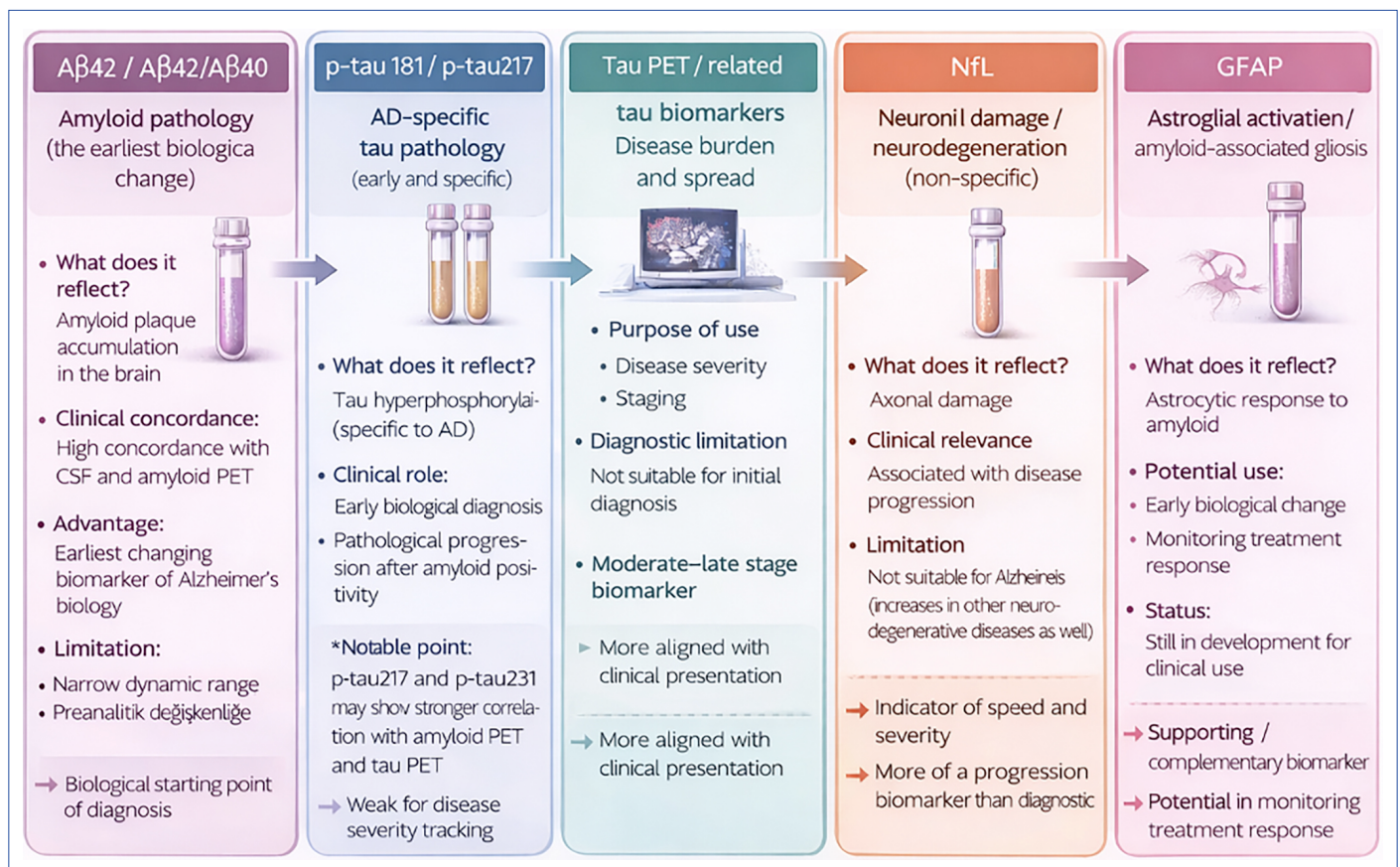
Core biomarkers are a predefined subset of biological markers that directly reflect the defining molecular and cellular pathology of AD and form the basis of a biologically grounded diagnosis. In contemporary diagnostic frameworks, core biomarkers serve two complementary but distinct purposes: Biological confirmation of AD pathology and characterization of disease stage, pathological burden, and progression. Accordingly, core biomarkers are conceptually and operationally divided into Core 1 and Core 2 categories [7, 17, 23].

Core 1 biomarkers are disease-defining markers used to establish the presence or absence of AD biology, independent of clinical stage or symptom severity. In contrast, Core 2 biomarkers are disease-characterizing markers that provide information on the extent of tau pathology, neurodegeneration, and disease dynamics after AD biology has been confirmed [7, 21]. This hierarchical classification ensures a clear separation between biomarkers that answer the fundamental diagnostic question "Is AD biology present?" and those that address "How advanced and active is the disease, and to what extent is neurodegeneration occurring?" Such separation is critical for accurate etiologic interpretation, clinical decision-making, and integration of biomarkers into multimodal diagnostic profiles [7, 21, 24] (Table 4).

**Core 1 Biomarkers: Biological Confirmation of Alzheimer's Disease:** Core 1 biomarkers establish the presence of AD biology and constitute the first-line biological tools once clinical suspicion has been raised (Fig. 1). These biomarkers primarily reflect cerebral amyloid- $\beta$  deposition and early amyloid-driven tau dysregulation and are applicable across the AD continuum, including presymptomatic stages [7] (Table 5).

**Table 4. Summary of biomarkers used in the biological diagnosis, staging, and evaluation of Alzheimer's disease**

Biomarker category	Pathophysiological process	Representative biomarkers	Primary clinical use
Core 1 biomarkers	Amyloid- $\beta$ proteinopathy (A)	CSF A $\beta$ 42 $\downarrow$ CSF A $\beta$ 42/40 $\downarrow$ Amyloid PET (+)	Biological diagnosis
	Phosphorylated and secreted AD tau (T1)	Plasma/CSF p-tau217 p-tau181 p-tau231	Biological diagnosis
	Hybrid amyloid-tau indices	p-tau181/A $\beta$ 42, t-tau/A $\beta$ 42, A $\beta$ 42/40 %p-tau217	Diagnostic accuracy enhancement
Core 2 biomarkers	Established AD tau proteinopathy (T2)	MTBR-tau243, p-tau205 Tau PET	Staging, progression
Neurodegeneration (N)	Neuronal injury and degeneration	NfL (CSF, plasma) MRI atrophy FDG-PET hypometabolism	Severity, prognosis
Inflammation (I)	Astroglial activation	GFAP (CSF, plasma)	Supportive / early change
Vascular copathology (V)	Vascular brain injury	MRI/CT infarcts, white matter hyperintensities	Differential diagnosis
Synucleinopathy (S)	$\alpha$ -synuclein aggregation	$\alpha$ Syn-SAA	Copathology identification

**Figure 1.** Biological biomarkers in Alzheimer's disease.

**Table 5. Comparative overview of biomarkers in Alzheimer's disease**

Biological target	CSF biomarkers	Plasma biomarkers	PET imaging	Clinical role
Amyloid- $\beta$ (A)	A $\beta$ 42 ↓ A $\beta$ 42/40 ↓	A $\beta$ 42/40 ↓	Amyloid PET (+)	Biological diagnosis of AD
Tau pathology – early (T1)	p-tau181 p-tau217 p-tau231	p-tau217 p-tau181	—	AD-specific tau dysregulation
Tau pathology – established (T2)	MTBR-tau243 p-tau205	MTBR-tau243 p-tau205	Tau PET (+)	Disease staging and progression
Neurodegeneration (N)	Total tau NFL	NFL	MRI atrophy FDG-PET hypometabolism	Severity and prognosis
Inflammation (I)	GFAP	GFAP	—	Supportive, early biological change
Copathology – vascular (V)	—	—	MRI/CT infarcts, WMH	Mixed / vascular pathology
Copathology – synuclein (S)	$\alpha$ Syn-SAA	$\alpha$ Syn-SAA	—	Lewy body-related pathology

CSF biomarkers provide high analytical sensitivity and established diagnostic validity. Plasma biomarkers offer scalable, minimally invasive screening and longitudinal monitoring. PET imaging provides spatial localization and in vivo visualization of pathological burden but is limited by cost and accessibility. CSF: Cerebrospinal Fluid; AD: Alzheimer's Disease; MTBR: Microtubule-binding region; NFL: Neurofilament light chain; GFAP: Glial fibrillary acidic protein;  $\alpha$ Syn-SAA: Alpha-synuclein seed amplification assay; FDG-PET: Fluorodeoxyglucose positron emission tomography; MRI: Magnetic resonance imaging; CT: Computed tomography; WMH: White matter hyperintensities.

### a. Plasma Amyloid- $\beta$ Biomarkers -A Biomarkers

Plasma A $\beta$  biomarkers, particularly A $\beta$ 42 concentration and the A $\beta$ 42/A $\beta$ 40 ratio, reflect cerebral amyloid deposition and demonstrate concordance with CSF measures and amyloid PET positivity. The development of highly sensitive analytical platforms—including single molecule array (Simoa), electrochemiluminescence assays, and immunoprecipitation mass spectrometry—has enabled reproducible detection of reduced plasma A $\beta$ 42/A $\beta$ 40 across the AD continuum [25, 26].

Notably, alterations in plasma A $\beta$ 42/A $\beta$ 40 often occur early, in some cases preceding amyloid PET positivity, highlighting their potential utility for early biological screening and risk stratification. However, compared with CSF, plasma A $\beta$  biomarkers exhibit a relatively narrow dynamic range, making them more susceptible to analytical variability and preanalytical confounders. Peripheral production, clearance mechanisms, and pharmacologic influences may further affect plasma A $\beta$  levels. Consequently, plasma A $\beta$ 42/A $\beta$ 40 is best regarded as a biologically informative but analytically fragile Core 1 biomarker, suitable primarily for screening and enrichment strategies rather than standalone diagnostic use in routine clinical practice [7, 26–28].

### b. Plasma Phosphorylated Tau Biomarkers -T1 Biomarkers

Complementing amyloid markers, plasma phosphorylated tau (p-tau) biomarkers represent the most robust blood-based indicators of AD-specific tau pathology and form a central component of Core 1 biomarkers. Among currently available epitopes, p-tau181, p-tau217, and p-tau231 consistently discriminate between amyloid-positive and amyloid-

negative individuals, with elevations observed in both symptomatic and presymptomatic stages. Notably, p-tau217 and p-tau231 demonstrate larger fold-changes and higher disease specificity than p-tau181. From a pathophysiological perspective, plasma p-tau reflects amyloid-driven tau dysregulation and secretion rather than established neurofibrillary tangle deposition. Accordingly, these biomarkers align with the T1 category of the updated AT(N) framework and are particularly valuable for identifying AD biology prior to overt neurodegeneration or dementia [29–31].

**Core 2 Biomarkers: Disease Burden, Neurodegeneration, and Progression:** Once AD biology has been established using Core 1 biomarkers, Core 2 biomarkers assume a central role in disease staging, assessment of pathological extent, and prognostic evaluation. These biomarkers reflect more advanced and established stages of tau pathology and neurodegeneration and are therefore more closely associated with clinical severity and disease progression than Core 1 markers [7, 21] (Table 5).

### a. Advanced Tau and Neurodegeneration Markers -T2 and N Biomarkers 3,11

While plasma p-tau reflects early tau dysregulation, biomarkers of advanced tau aggregation and neurodegeneration are more challenging to assess in blood. The microtubule-binding region (MTBR) of tau—particularly MTBR-tau243—has emerged as a promising plasma marker of established tau proteinopathy, showing closer associations with neurofibrillary tangle burden and tau PET findings. These markers are best classified within the T2 category, reflecting advanced tau pathology [14, 23, 32].

Neurofilament light chain (NFL) is the most widely adopted blood-based marker of neuroaxonal injury and constitutes a

core N biomarker. Plasma NfL levels correlate with CSF concentrations and increase with disease severity and progression but lack disease specificity, as elevations are observed in numerous neurological conditions. Accordingly, NfL reflects neurodegenerative burden rather than AD biology per se [7, 20, 33, 34].

### b. Astrocytic and Inflammatory Biomarkers - I Biomarkers

Glial fibrillary acidic protein (GFAP) has emerged as a clinically relevant blood-based marker of astrocytic activation. Plasma GFAP levels are closely associated with cerebral amyloid pathology and often increase early in the AD continuum, sometimes preceding overt tau abnormalities. Although GFAP is not specific to AD, the magnitude of elevation is typically greater in AD than in many non-AD neurodegenerative diseases. Notably, reductions in plasma GFAP following anti-amyloid therapy suggest potential utility as a treatment-response biomarker, supporting its classification within the I category [7, 20, 35, 36].

### c. Synaptic Injury Biomarkers and Emerging Blood-Based Candidates

Synaptic dysfunction is a central correlate of cognitive decline in AD. While several synaptic proteins, such as neurogranin, perform well in CSF, their translation to blood has been limited by peripheral expression and weak central-peripheral correlations. More recently,  $\beta$ -synuclein and synaptosomal-associated protein 25 (SNAP-25) have shown promise as blood-based indicators of synaptic injury, with associations to cortical atrophy and cognitive impairment. These biomarkers remain investigational but represent an important future direction in refining biological characterization and monitoring disease progression [37, 38].

### d. Cerebrospinal Fluid Core Biomarkers

CSF biomarkers remain the reference standard for biological diagnosis of AD. Within the AT(N) classification, the A category includes CSF  $A\beta_{1-42}$  or the  $A\beta_{1-42}/A\beta_{1-40}$  ratio, the T category comprises CSF phosphorylated tau, and the **N category** reflects neurodegeneration or neuronal injury, as indicated by CSF NfL or total tau (t-tau) [7, 21].

Reduced CSF  $A\beta_{1-42}$  concentrations are strongly associated with cerebral amyloid deposition, whereas elevated CSF p-tau levels are characteristic of AD and correlate with cortical neurofibrillary tangle burden. In contrast, increased CSF t-tau reflects neuronal injury or neurodegeneration more broadly and is not specific to AD, as elevations may also be observed in conditions such as traumatic brain injury, stroke, and Creutzfeldt-Jakob disease [7, 27-30].

## Clinical-to-Biological Diagnostic Workflow in Alzheimer's Disease

This section presents a clinical-to-biological diagnostic workflow for AD from a laboratory and analytical perspective, emphasizing the stepwise use of validated biomarkers and their interpretive integration. The framework illustrates how analytical performance, biomarker categorization, and multimodal

profiling collectively support biological confirmation, disease characterization, and the identification of comorbid pathologic processes across clinical and research settings.

## Clinical-Laboratory Decision Pathway for Alzheimer's Disease Diagnosis

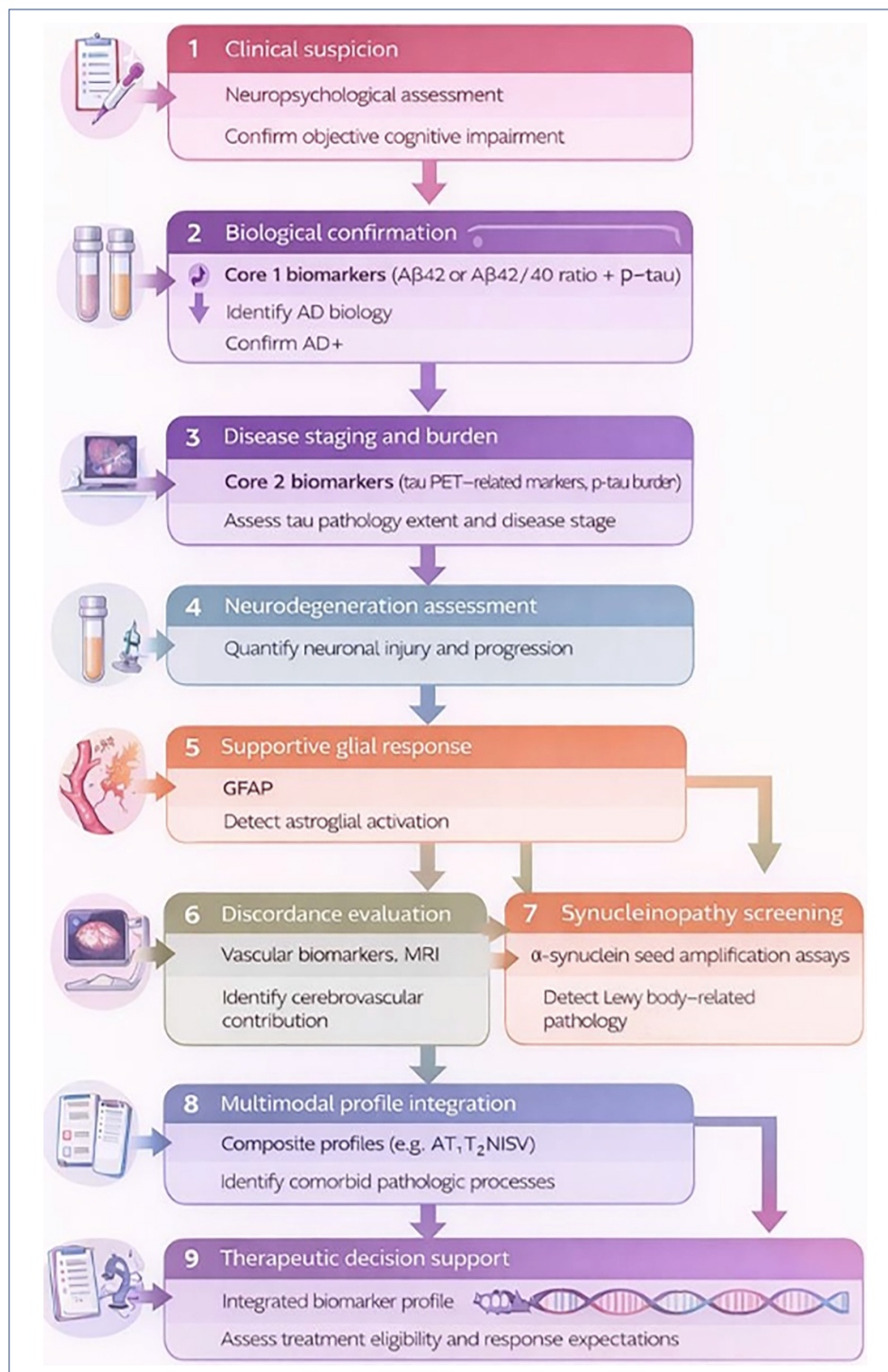
In clinical practice, the diagnostic process begins with the evaluation of cognitive complaints and the objective confirmation of impairment through standardized neuropsychological testing. Once clinical suspicion of AD is established, laboratory-based biological confirmation is pursued using **Core 1 biomarkers** as the first-line analytical approach. Positivity in Core 1 biomarkers provides evidence of underlying AD biology and supports a biologically defined diagnosis. Following biological confirmation, further laboratory and imaging-based assessment of disease stage, pathological extent, and progression is performed using **Core 2 biomarkers** and neuroimaging findings. These markers provide complementary information regarding tau pathology, neurodegenerative burden, and disease dynamics. In cases of discordance between clinical presentation and biomarker results, additional laboratory and imaging markers of **vascular pathology** or **synucleinopathies** should be considered to evaluate the presence of potential copathologies [6, 7].

An expanded representation of this clinical-laboratory diagnostic workflow, detailing the analytical rationale and interpretive integration of biomarkers across diagnostic stages, is provided in the supplementary material (Fig. 2). As illustrated, the workflow incorporates CSF or blood-based amyloid- $\beta$  measures ( $A\beta_{42}$  or  $A\beta_{42}/40$  ratio), phosphorylated tau species, tau PET-related markers, NfL, and supportive biomarkers such as GFAP. Integration of core and non-core biomarkers enables the identification of mixed or alternative pathologies and supports refined etiologic interpretation, prognostic stratification, and informed therapeutic decision-making, including assessment of eligibility for disease-modifying interventions [6, 7, 17, 23-25, 39].

## Multimodal Biomarker Profiles and Identification of Comorbid Pathologic Change

Following biological confirmation and initial disease characterization, further refinement of the diagnostic interpretation often requires evaluation beyond core AD pathology. In this context, multimodal biomarker profiles are conceptually distinct from the biological staging of AD. While biological staging applies exclusively to individuals in whom AD pathology has been established using core biomarkers, multimodal profiles are applicable to all individuals and are designed to characterize the overall neuropathophysiological state, either in conjunction with or independent of AD pathology [5, 7].

Within this extended framework, biomarkers are used not only to define AD biology but also to identify coexisting pathological processes that frequently contribute to cognitive impairment, particularly in older populations. Mul-



**Figure 2.** Clinical-laboratory decision framework for Alzheimer's disease diagnosis.

timodal biomarker profiling therefore extends beyond the traditional AT(N) system by integrating both core and non-core biomarkers into composite profiles (e.g., AT<sub>1</sub>T<sub>2</sub>NISV), with each component expressed either dichotomously or on a quantitative scale [23].

Although comprehensive profiling requires extensive biomarker assessment, partial profiles are often sufficient and clinically informative. This is particularly relevant given

that isolated AD pathology is uncommon with advancing age; cognitive impairment is frequently shaped by multiple coexisting processes, including cerebrovascular disease,  $\alpha$ -synuclein-related disorders, and limbic-predominant age-related TDP-43 encephalopathy (LATE). Multimodal profiles facilitate the identification of both **direct** and **indirect indicators of comorbidity**. Direct indicators include positive  $\alpha$ -synuclein seed amplification assays or neuroimaging evi-

dence of multiple cerebral infarctions. Indirect indicators include biomarker mismatches, such as a **T2-N+** profile, suggesting that neurodegeneration or neuronal injury is driven by a pathology other than AD. For example, an individual with an **A+T2-N+** profile may have biologically defined AD accompanied by an additional neurodegenerative process, such as LATE, contributing independently to neuronal injury and cognitive decline [7, 40, 41].

Clinically, recognition of comorbid pathological change through multimodal biomarker profiling is essential for accurate etiologic interpretation, prognosis estimation, and individualized treatment planning. Such profiles may also influence expectations regarding response to disease-modifying therapies, particularly anti-amyloid- $\beta$  treatments. In research settings, multimodal biomarker profiles support the definition of biologically homogeneous cohorts in early-phase trials and enable stratified or subgroup analyses in later-phase studies, thereby improving the assessment of therapeutic efficacy across heterogeneous patient populations [7, 17, 42–44].

### Laboratory Reporting and Interpretation in Biomarker-Guided Diagnosis

Laboratory reports should present biomarker results not merely as numerical values but within a clearly defined pathophysiological and clinical context. For Core 1 biomarkers, reports should explicitly state whether findings are consistent with or not consistent with AD biology and include relevant methodological details, analytical performance characteristics, and validated cut-off values [7, 17, 18, 20].

Reports for Core 2 biomarkers and NfL should incorporate interpretive comments related to disease stage, neurodegenerative burden, and progression dynamics. GFAP and other inflammatory or glial markers should be presented as supportive findings rather than standalone diagnostic indicators, while the detection of biomarkers suggestive of vascular pathology or  $\alpha$ -synuclein-related disease should prompt consideration of mixed or alternative etiologies [33–38, 45].

### Pre-analytical Variability of Alzheimer's Biomarkers

Despite their central role in the biological diagnosis and staging of AD, currently available biomarkers have important limitations that must be acknowledged in both clinical and research settings.

#### Blood-Based Alzheimer's Biomarkers

Experience gained from CSF biomarkers in AD has clearly demonstrated that not only analytical, but also pre-analytical standardization is a critical determinant of biomarker reliability. Proteins that are prone to aggregation, particularly amyloid- $\beta$ , may adhere to certain plastic materials or undergo *in vitro* aggregation, leading to substantial measurement variability. These observations have prompted systematic

investigations into the effects of pre-analytical variables and the development of standardized protocols for blood, plasma, and serum samples [46, 47].

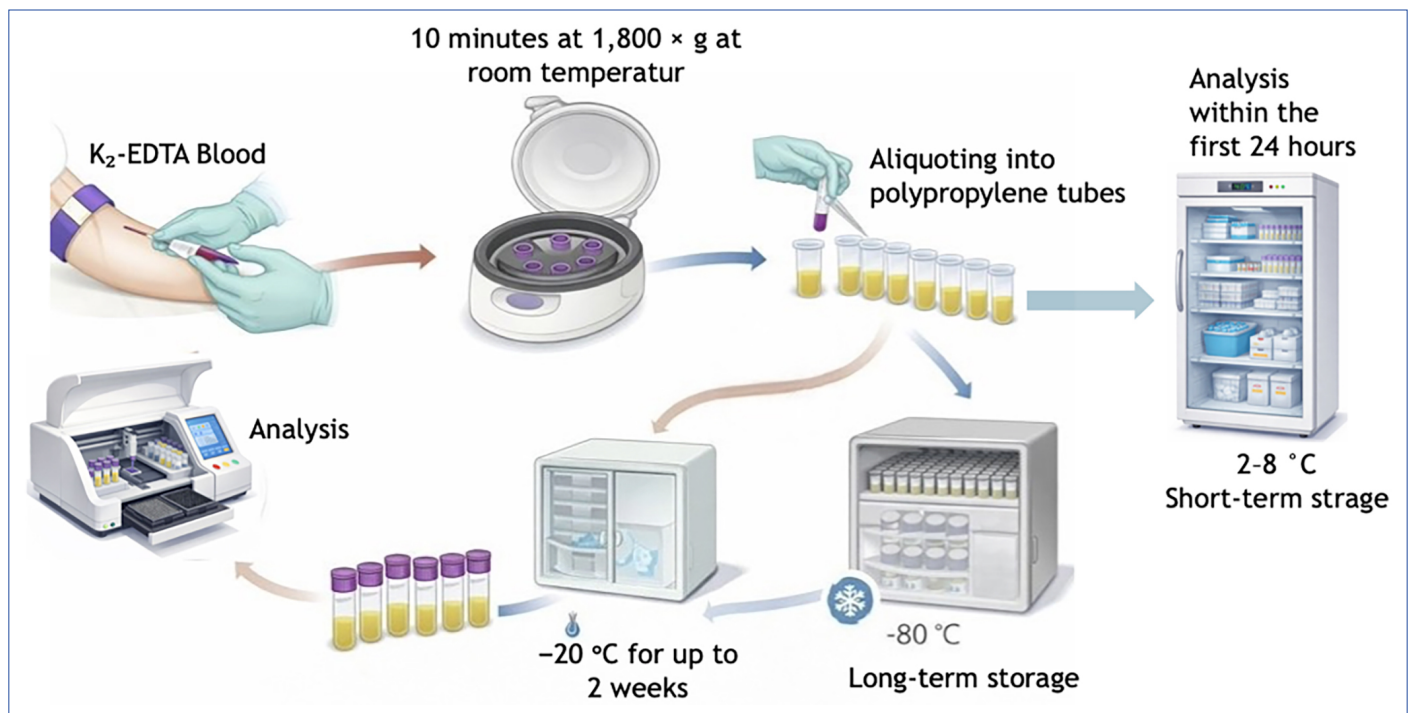
In a large multicenter study, commonly encountered pre-analytical variations across existing cohorts were systematically evaluated, including blood collection tube type, time from blood draw to centrifugation, centrifugation parameters, time from centrifugation to freezing, sample temperature during processing, aliquot volume, and number of freeze–thaw cycles [10]. Based on analyses of A $\beta$ 42/A $\beta$ 40, phosphorylated tau (p-tau181), total tau, GFAP, and NfL, a simple and standardized plasma handling protocol was proposed [46–48].

Systematic inventories of pre-analytical practices across multiple cohorts further revealed pronounced heterogeneity in critical handling steps. While factors such as needle size, blood draw location, tube filling volume, tube inversion, and freeze–thaw cycles showed little variation, substantial differences were observed in delays from blood draw to centrifugation and from centrifugation to freezing, with some studies reporting delays of up to 30 hours and 4 hours, respectively. These variables were therefore ranked as high priority for experimental evaluation [46–49].

Compared with EDTA plasma, values were generally lower in sodium-citrate tubes and higher in lithium-heparin tubes, while serum showed variable effects depending on the assay. Delayed centrifugation or delayed freezing for 24 hours at room temperature resulted in substantial reductions in A $\beta$ 42 and A $\beta$ 40 levels, whereas refrigeration largely mitigated these effects [10]. Prolonged intermittent storage at 4 °C prior to freezing caused pronounced declines in both peptides, while storage at –20 °C did not. In contrast, centrifugation temperature, aliquot volume, and repeated freeze–thaw cycles had minimal effects on A $\beta$ 42 and A $\beta$ 40 [9]. Although the A $\beta$ 42/40 ratio partially attenuated pre-analytical variability, this mitigating effect was assay- and condition-dependent and not universally reliable [48, 49].

Other blood-based biomarkers exhibited distinct pre-analytical sensitivity profiles. GFAP, NfL, p-tau181, and alternative amyloid species were influenced by sample type in a manner similar to A $\beta$  peptides but were generally more stable with respect to delayed centrifugation and short-term storage. GFAP was the only biomarker significantly affected by repeated freeze–thaw cycles, with increased concentrations observed after multiple cycles [50, 51]. In contrast, total tau showed marked instability in whole blood, sensitivity to centrifugation temperature, and vulnerability to prolonged refrigerated storage [52].

Figure 3 illustrates the recommended pre-analytical workflow for blood sample handling to ensure reliable measurement of Alzheimer's disease-related biomarkers. Blood samples are collected by venipuncture using K<sub>2</sub>-EDTA plasma, as alternative sample matrices are known to yield systematically different biomarker concentrations. Following blood collection, samples may be maintained at room temperature or cold con-



**Figure 3.** Recommended workflow for blood sample handling for Alzheimer's disease biomarker analysis.

ditions if processed within <3 hours; however, for processing delays of 3–24 hours, samples must be stored at 2–8 °C to prevent biomarker degradation. Plasma separation is performed by centrifugation for 10 minutes at 1,800 × g at room temperature, followed by aliquoting into polypropylene tubes with volumes ranging from 250 to 1000 μL. Post-centrifugation handling allows for short-term storage at 2–8 °C for up to 24 hours or intermediate storage at -20 °C for up to 2 weeks prior to long-term preservation. Long-term storage is conducted at -80 °C, which represents the recommended condition for maintaining biomarker stability. Samples may undergo up to two freeze–thaw cycles without significant impact on measured biomarker concentrations. This workflow emphasizes strict control of sample type, temperature, and processing time, which are critical determinants of analytical reliability in blood-based AD biomarker studies [48].

These findings underscore that analytical sensitivity alone is insufficient without strict control of pre-analytical conditions in blood-based AD biomarker testing.

### CSF-Based Alzheimer's Biomarkers

Despite their widespread clinical and research use, significant inter-laboratory and batch-to-batch variability has been reported for CSF biomarker measurements. Three major sources of variability must be considered when interpreting CSF biomarker results or defining diagnostic cut-off values: Pre-analytical factors related to CSF collection, handling, and storage; analytical factors related to assay performance, technician skill, and lot-to-lot differences in reagents; and biological or patient-related factors, such as age and comorbidities [28, 48, 53, 54].

After the implementation of certified reference materials for Aβ<sub>1–42</sub>, pre-analytical factors are widely regarded as the final critical source of variability limiting harmonization. Differences in tube type, fill volume, sample transfers, storage conditions, and handling procedures can substantially influence measured biomarker concentrations. Among the core biomarkers, Aβ<sub>1–42</sub> is particularly susceptible to pre-analytical effects, including adsorption to tube surfaces and changes related to sample volume. The persistence of heterogeneous pre-analytical protocols across centers continues to hinder the establishment of universal cut-off values, complicates clinical interpretation, and poses challenges for patient selection in clinical trials [23, 54–57].

To address this gap, a multidisciplinary workgroup convened by the Alzheimer's Association developed a simplified, standardized pre-analytical protocol for routine clinical CSF biomarker testing, with particular emphasis on Aβ<sub>1–42</sub>. This unified protocol systematically addresses key pre-analytical variables, including collection method, blood contamination, tube type and filling volume, sample transfers, mixing, transportation, and short-term stability. Broad adoption of such standardized pre-analytical procedures is essential to minimize variability, enable reliable use of biomarker cut-offs, and strengthen clinician confidence in CSF biomarkers as diagnostic tools for Alzheimer's disease [18, 23, 28].

### Limitations of Biological Biomarkers

Blood and CSF biomarkers play a critical role in the diagnosis and research of Alzheimer's disease (AD). However, measurement variability across studies, laboratories, and analytical

**Table 6. Schematic comparison of Alzheimer's disease cerebrospinal fluid and blood biomarkers**

Dimension	CSF biomarkers	Blood (Plasma) biomarkers
Primary Purpose	Definitive biological diagnosis of AD	Screening, risk stratification, triage
Biological Proximity to Brain	High (direct contact with brain extracellular space)	Low–moderate (blood–brain barrier dilution)
Core Biomarkers	A $\beta$ 42 ↓, A $\beta$ 42/40 ↓ T-tau ↑, P-tau ↑	A $\beta$ 42/40 ↓, APP669-711/A $\beta$ 42 ↑ Tau ↑, NFL ↑
Pathophysiology Reflected	Amyloid deposition, tau pathology, neurodegeneration	Brain amyloidosis (indirect), neurodegeneration
Specificity for AD	High (especially P-tau, A $\beta$ 42/40)	Moderate–low (NFL and tau not AD-specific)
Temporal Sensitivity	Detects pathology very early (preclinical AD)	Early changes possible but later than CSF
Analytical Platforms	ELISA fully automated analyzers, Mass Spectrometry–based Reference Measurement Procedures,	Ultrasensitive immunoassays (Simoa), Immunoprecipitation–Mass Spectrometry
Standardization Status	Advanced (CRMs, reference methods available)	Developing (method-dependent variability)
Inter-laboratory Variability	Reduced with automation and CRMs	Higher; platform- and method-dependent
Pre-analytical Sensitivity	Moderate (tube type, freeze–thaw effects)	High (proteolysis, peripheral interference)
Sample Accessibility	Invasive (lumbar puncture)	Minimally invasive
Clinical Setting	Memory clinics, specialist centers	Primary care, population screening
Regulatory Readiness	High – integrated into NIA-AA biological AD definition	Emerging – not yet standalone diagnostic tools
Key Limitations	Invasiveness, patient acceptance	Lower specificity, strong analytical demands

AD; Alzheimer's disease; CSF: Cerebrospinal fluid; A $\beta$ 42: Amyloid beta 42; A $\beta$ 40: Amyloid beta 40; A $\beta$ 42/40: Ratio of amyloid beta 42 to amyloid beta 40; APP: Amyloid precursor protein; APP669–711/A $\beta$ 42: Ratio of APP fragment (amino acids 669–711) to A $\beta$ 42; T-tau: Total tau; P-tau: Phosphorylated tau; NFL: Neurofilament light chain; ELISA: Enzyme-linked immunosorbent assay; Simoa: Single molecule array; IP-MS: Immunoprecipitation–mass spectrometry; CRM: Certified reference material; NIA-AA: National institute on aging and alzheimer's association; A/T/N: Amyloid/tau/neurodegeneration; NIA-AA: National institute on aging – alzheimer's association.

platforms remains a major limitation for their widespread clinical implementation. Variability may arise at multiple stages, including assay and kit production, sample collection and storage, laboratory procedures, operator- and instrument-related factors, and data processing [28] (Table 6).

To address this challenge, the Alzheimer's Association launched an international external quality control (QC) and proficiency testing program in 2009. Within this program, 85 laboratories across more than 20 countries analyze standardized CSF samples, with centralized data evaluation enabling interlaboratory comparisons, longitudinal monitoring, and identification of outliers. Early rounds demonstrated coefficients of variation of approximately 25% for CSF A $\beta$ 42 and 15–20% for tau biomarkers, exceeding the desired analytical target of 10–15%. Variance component analyses further indicated that batch-to-batch effects represent the dominant source of variability for A $\beta$ 42, whereas between-laboratory differences contribute more substantially to variability in tau measurements [18, 23, 28, 45, 54, 57, 58].

Effective standardization therefore requires close collaboration between academia and industry. Major assay manufacturers are developing analytically validated, automation-ready immunoassays aligned with common reference materials, supported by multisite validation studies, training and certification programs, run-validation controls, and ready-to-use calibrators to minimize analytical variability [28, 46, 55, 57].

In parallel, international reference materials and reference measurement procedures are being established. Under the coordination of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and the European Com-

mission's Joint Research Centre (JRC; formerly IRMM), certified reference materials for CSF A $\beta$ 42, total tau, and phosphorylated tau are being developed together with accuracy-based reference methods, particularly those based on mass spectrometry. Despite this progress, calibration matrix selection and sample preparation remain key technical challenges in achieving full harmonization [28, 57–59].

Large international QC and proficiency testing initiatives further support assay harmonization by continuously monitoring laboratory performance. As a result of these combined standardization efforts, modern automated immunoassays now demonstrate improved precision, reduced lot-to-lot variability, and lower inter-laboratory variation compared with earlier generations of assays [28, 54, 55].

Another important limitation is the intrinsic sensitivity of biomarker modalities. PET, CSF, and blood-based biomarkers are inherently less sensitive than neuropathologic examination for detecting very early or mild AD neuropathologic change. For example, regional tau PET ligand uptake does not directly equate to Braak staging at autopsy. While this may appear as a limitation, it can also be considered a strength, as abnormal core biomarkers generally reflect clinically meaningful AD pathology rather than incidental or sparse neuropathologic findings [6, 7, 57, 60–62].

From a laboratory medicine perspective, the future clinical implementation of Alzheimer's disease biomarkers will depend less on the discovery of new markers and more on rigorous analytical validation, harmonization, and context-appropriate interpretation of existing assays.

## Conclusion

Disease-specific biomarkers are not yet available for all neurodegenerative and age-related brain disorders. As a result, it is not currently possible to determine with certainty which additional pathologies coexist with AD *in vivo* or to quantify the relative contribution of each pathology to the overall clinical phenotype. In conclusion, although current biomarkers provide a powerful framework for identifying AD biology, the frequent presence of comorbid pathologies limits diagnostic certainty, highlighting the continuing need for multimodal evaluation and individualized clinical interpretation. From a laboratory medicine perspective, the clinical value of Alzheimer's disease biomarkers will increasingly depend not on discovery, but on disciplined implementation.

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