Review

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Biofluid biomarkers for Alzheimer's disease: past, present, and future

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Abstract: Alzheimer's disease (AD) is a gradually progressive neurodegenerative disease with tremendous social and economic burden. Therefore, early and accurate diagnosis is imperative for effective treatment or prevention of the disease. Cerebrospinal fluid and blood biomarkers emerge as favorable diagnostic tools due to their relative accessibility and potential for widespread clinical use. This review focuses on the AT(N) biomarker system, which includes biomarkers reflecting AD core pathologies, amyloid deposition, and pathological tau, as well as neurodegeneration. Novel biomarkers associated with inflammation/immunity, synaptic dysfunction, vascular pathology, and α-synucleinopathy, which might contribute to either the pathogenesis or the clinical progression of AD, have also been discussed. Other emerging candidates including non-coding RNAs, metabolites, and extracellular vesicle-based markers have also enriched the biofluid biomarker landscape for AD. Moreover, the review discusses the current challenges of biofluid biomarkers in AD diagnosis and offers insights into the prospective future development.

Keywords: Alzheimer's disease; biomarker; cerebrospinal fluid; blood; amyloid

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and the leading cause of dementia and disability in the older population [1]. It is characterized by chronic progressive cognitive impairment including memory and decision-making problems, which eventually lead to functional disability and death from complications [2]. Currently, approximately 50 million people worldwide living with dementia, with AD accounting for 60–80 % of total dementia cases [2]. In China, there are 249.49 million individuals aged 60 years or older, constituting 17.9 % of the total population. The estimated prevalence was 6.0 % for dementia and 3.9 % for AD in this population, indicating that 15.07 million people have dementia, among whom 9.83 million have AD in China [3]. Furthermore, estimates indicate the prevalence of dementia ranges from 2.9 % in individuals aged 60–69 years to as high as 31.9 % in those aged 90 years or older [3], suggesting that the annual prevalence of dementia is expected to climb as life expectancy continues to rise. Moreover, the total annual costs of dementia are expected to reach $507.49 billion in 2030 in China [4]. Without substantial advancements in AD prevention and treatment, the prevalence of AD and the cost of dementia is expected to surge, creating a substantial societal and economic burden [4].

In the past, the diagnosis of AD primarily relied on clinical criteria centered around the presentation of dementia symptoms. Given the insidious onset and gradual progression of AD, this approach can significantly increase the risk of both misdiagnosis and underdiagnosis. Indeed, evidence suggests a misdiagnosis rate of approximately 30 % when compared with neuropathological assessments [5]. The average time from the appearance of symptoms to the diagnosis of AD is around 2.8 years [6], with patients possibly advancing to later disease stages by the time of diagnosis. Furthermore, heterogeneity in the pathobiology of AD may result in distinct clinical presentations and AD often co-occurs with other neurodegenerative and vascular diseases, which complicates the diagnosis of AD. Moreover, disease-modifying treatments seem attainable for AD [7].

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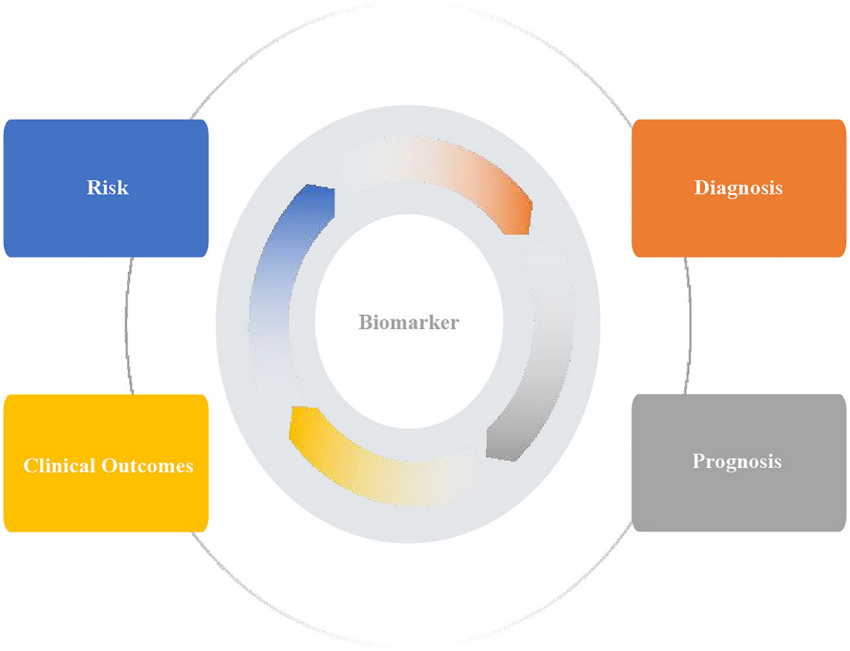

Figure 1: Role of a biomarker in diseases or physiological conditions. A biomarker should indicate at least one of the following: clinical risk, disease diagnosis, disease prognosis, and clinical outcomes for diseases or physiological conditions.

These treatments are believed to be most useful in the earlier stages of the disease and timely diagnosis of AD holds significant importance. Consequently, there is an imminent need for the development of objective early diagnostic and differential diagnostic tools.

A biomarker is a measurable and quantifiable biological parameter or characteristic that can be used to indicate the risk, diagnosis, severity, or progression of a particular disease or physiological condition and the clinical response to treatment (Figure 1). Biomarkers play a pivotal role in the precise and early detection of AD, constituting an essential prerequisite for the efficient management of the disease. Biomarkers of AD include a wide range of substances, such as proteins, genes, metabolites, or other molecules found in tissues, cerebrospinal fluid (CSF), blood, urine, or other biofluids, and specific imaging patterns. In this review, we discuss the progress, challenges, and prospects of research on biomarkers in biofluids, particularly CSF and blood, for AD.

The historical development of AD biomarkers

Figure 2 illustrates the main milestone in the historical development of AD biomarkers. In 1906, Alois Alzheimer made the pioneering discovery of AD, identifying the presence of abnormal protein deposits and tangled fibers in the brain, later known as amyloid plaques and neurofibrillary tangles (NFTs) [8]. The development of detection technology in the 1980s led to significant progress in understanding the neuropathology and clinical concepts of AD [9]. In 1986, Wolozin et al. detected abnormally phosphorylated tau proteins in homogenates of brain tissue using monoclonal antibodies [10], which is a milestone in identifying *in vivo* AD biomarkers. Obtaining pathological information from individuals with AD through biopsy or post-mortem examination is impractical. Consequently, researchers have initiated exploration into alternative methodologies for acquiring pathology-related information.

CSF, being the sole fluid sample obtainable from the central nervous system (CNS), is an ideal source for biomarkers that might reflect alterations in the extracellular space of the brain. In 1995, a series of publications discussing enzyme-linked immunosorbent assays (ELISA) utilizing monoclonal antibodies led to the development of modern AD biomarkers. These assays were designed to measure CSF levels of total tau (T-tau) and phosphorylated tau (P-tau), as well as Aβ42. These publications revealed a significant increase in CSF T-tau and P-tau, accompanied by a significant decrease in Aβ42 in AD [11, 12]. Furthermore, the development of positron emission tomography (PET) with radioactive tracers enabled the visualization of protein deposition in the brains of individuals with AD.

Early biomarkers primarily aimed to distinguish AD from other forms of dementia but could only be detected in advanced cognitive decline. As research evolved, it became apparent that AD pathologies occur before clinical symptoms arise [13–15]. Research efforts have transitioned the

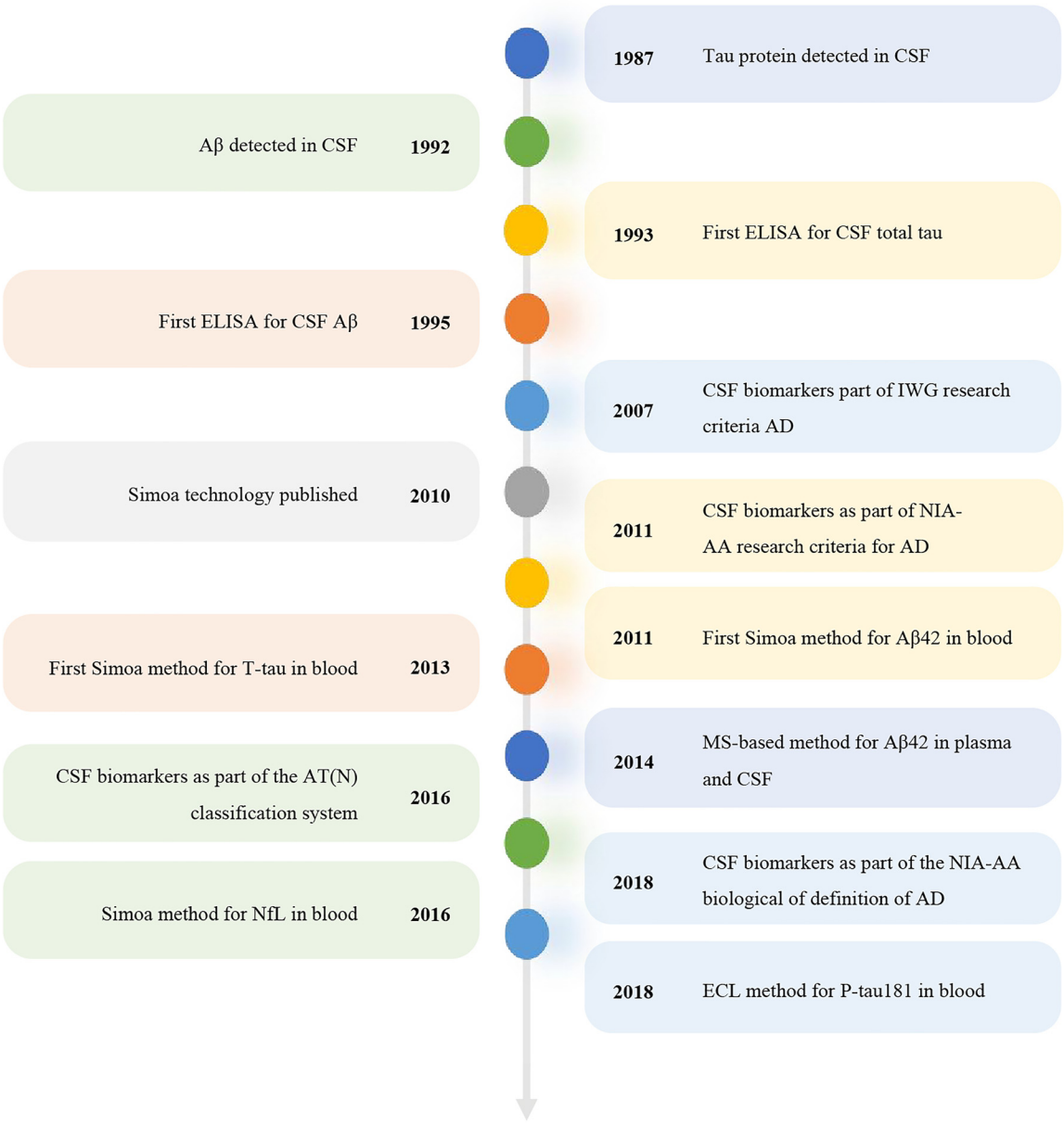

Figure 2: Main milestones in the historical development of AD biofluid biomarkers. AD, Alzheimer's disease; Aβ, amyloid-β; CSF, cerebrospinal fluid; ECL, electrochemiluminescence; ELISA, enzyme-linked immunosorbent assays; IWG, International Working Group; NfL, neurofilament light chains; NIA-AA, National Institute on Aging and the National Institute on Alzheimer's Association; P-tau, phosphorylated tau; Simoa, single-molecule array.

diagnosis of AD from the later dementia stages to the earlier phases, opening up the possibility of pre-symptomatic identification.

In 2007, the International Working Group (IWG) introduced the significance of biomarkers in diagnostic criteria [16]. Subsequently, the National Institute on Aging and the National Institute on Alzheimer's Association (NIA-AA) released AD diagnostic guidelines in 2011, emphasizing the role of biomarkers in diagnosing AD and assessing the risk of disease progression [17–19]. In 2018, the NIA-AA proposed a biological definition of AD as a research framework, which includes amyloid deposition, pathologic tau, and neurodegeneration [AT(N)] biomarker classification system [20]. The AT(N) system subsequently evolved into the ATX(N) system, where the X represents the incorporation of emerging biomarkers [21]. The system is initially designed for imaging and CSF. However, the high cost and unavailability of imaging scans and the perceived invasiveness of CSF sampling have limited their use in community settings and early AD-risk populations.

Blood samples are generally easier and less invasive to obtain than CSF, and thus blood can be a valid source for repeated measurements of biomarkers. Nonetheless, early studies using first-generation blood-based biomarker assays yielded inconsistent findings in the context of AD [22], partly because of lower abundance in plasma, considerable peripheral expression, proteolytic degradation, and matrix effects from plasma proteins [23–25]. Emerging ultrasensitive technologies circumvent the limitations inherent to conventional technologies and provide a 1,000-fold improvement in sensitivity [26, 27]. This has led to renewed enthusiasm for identifying blood-based biomarkers for the diagnosis and prediction of AD in the last decade. Three key developments have contributed to this achievement, including electrochemiluminescence (ECL), single-molecule array (Simoa), and mass spectrometry (MS)-based assays [28] (Figure 3). ECL functions by substitution of the enzyme label in the detection antibody with a molecule that emits light during an electrochemical reaction [29]. Simoa is a refinement of the traditional ELISA technology, which involves compartmentalizing the detection reaction within femtoliter-sized wells. This is achieved by using magnetic beads to capture immunocomplexes, thereby digitizing protein detection [26]. MS-based assays work by calculating the mass-to-charge ratio of the molecules [30]. These technological advancements collectively represent significant progress in biomarker detection and quantification.

Moreover, significant advancements have substantially deepened our comprehension of the pathogenesis and pathophysiology of AD. In addition to the well-established Aβ and tau pathologies, AD is intricately linked to synaptic dysfunction, neuronal loss, neuroinflammation, oxidative stress, and metabolic dysfunction [31]. Consequently, there is a growing emphasis on the thorough investigation of biomarkers involved in these multifaceted processes (Figure 4), which may unravel the intricate molecular signatures that hold promise for improved diagnostics and targeted interventions.

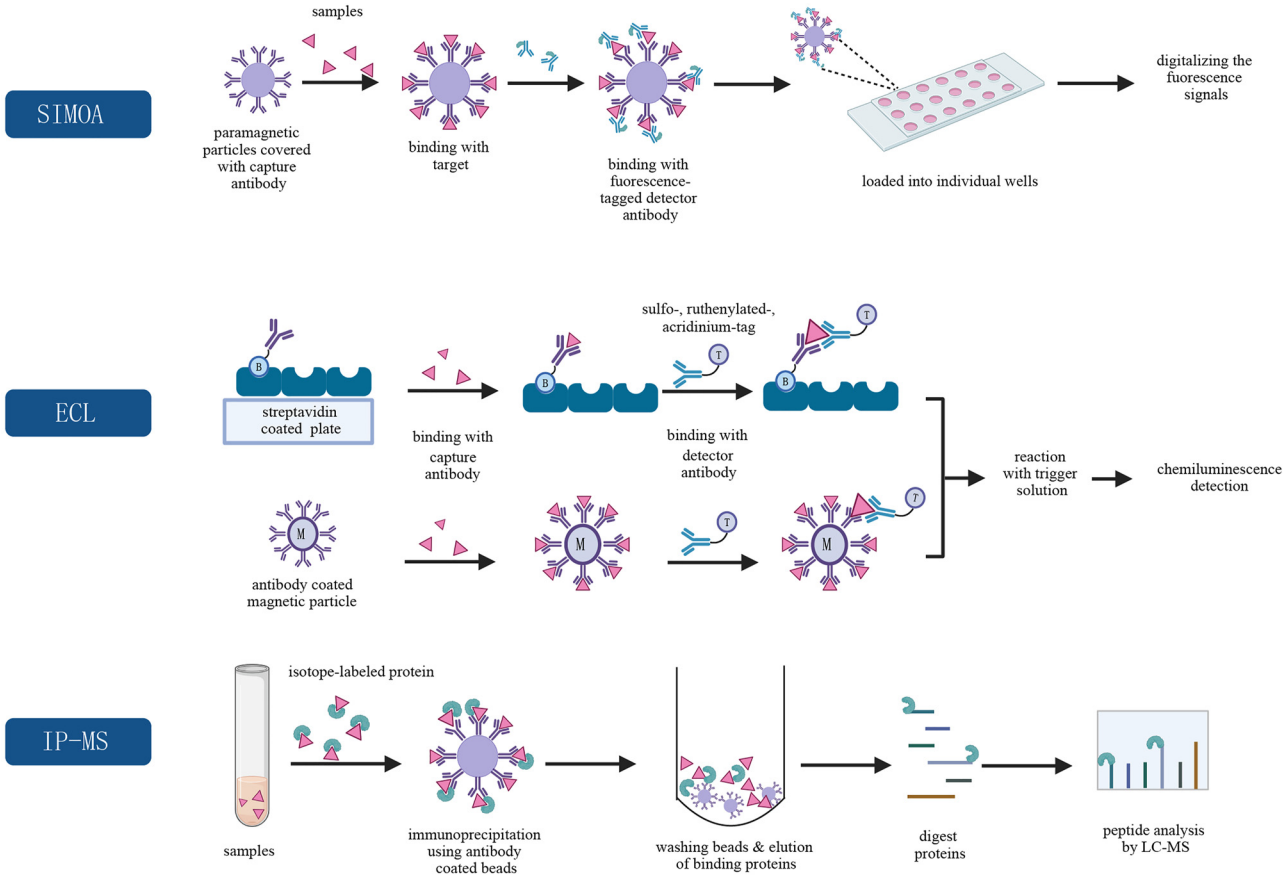

Figure 3: Ultrasensitive techniques for biomarker quantification. B, biotin; ECL, electrochemiluminescence; IP-MS, immunoprecipitation coupled with mass spectrometry; LC-MS, liquid chromatography coupled with mass spectrometry; M, magnetic; SIMOA, single-molecule array; T, tag. Modified with permission from ref [28]. Created with BioRender.com.

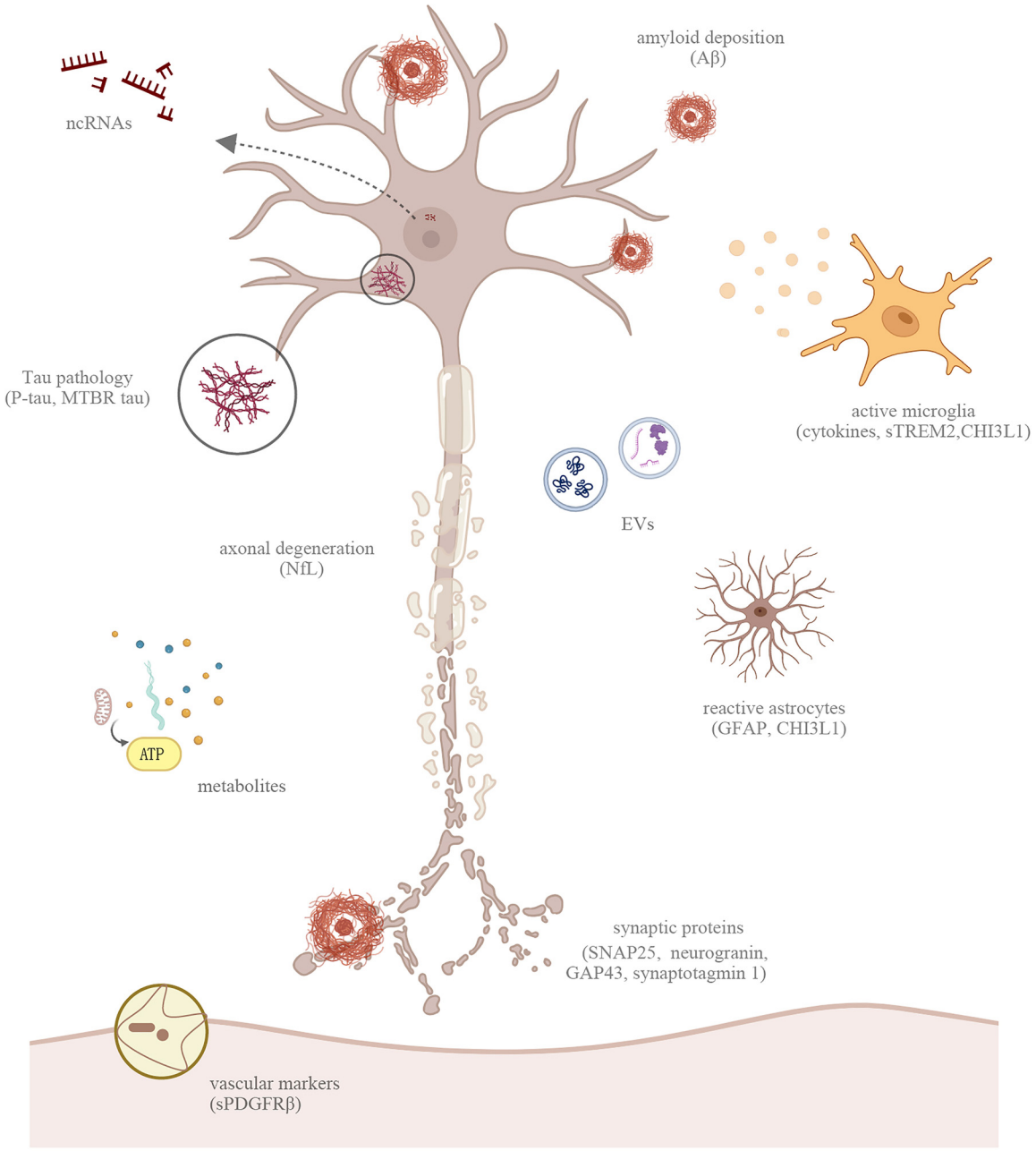

Figure 4: Pathophysiology and related biomarker candidates for Alzheimer's disease. Aβ, amyloid-β; CHI3L1, chitinase-3 like-protein-1; EVs, extracellular vesicles; GAP43, growth-associated protein 43; GFAP, glial fibrillary acidic protein; MTBR tau, non-modified tau species that contain the microtubule-binding region of tau; ncRNAs, non-coding RNAs; P-tau, phosphorylated tau; SNAP25, synaptosome-associated protein 25; sPDGFRβ, soluble platelet-derived growth factor receptor β; sTREM2, soluble triggering receptor expressed on myeloid cells 2. Created with BioRender.com.

ATX(N) system

The AT(N) biomarker framework for AD was initially introduced by the NIA-AA in 2016 [32], with further refinement in 2018 [20]. The framework was creatively proposed by Jack et al. to classify the biomarkers of AD according to pathophysiological characteristics [21] and staged AD at the pathological level [33]. As shown in Figure 5, the initial AT(N) system includes biomarkers for amyloid deposition (A), pathological tau (T), and neurodegeneration or neuronal injury (N), namely, cortical amyloid PET ligand binding, low CSF Aβ42 or Aβ42/Aβ40 for A component; elevated CSF P-tau or cortical tau PET ligand binding for T component; and CSF T-tau, 18F-fluorodeoxyglucose (FDG)

PET hypometabolism, and atrophy observed on structural MRI for N component. Binary classification of the three components yields eight different biomarker profiles, with A−T−(N)− being considered normal, A+T±(N)± being considered Alzheimer's continuum, and A−T+(N)± as well as A−T−(N)+ being considered non-AD pathologic change (Figure 6) [20]. The CSF-based AT(N) classification not only allows monitoring of cognitive decline but also facilitates the prediction of disease prognosis [34]. Imaging biomarkers such as 18F-Florzolotau PET (T) and 18F-FDG PET (N) exhibit limited specificity in diagnosing AD but can effectively assess the severity of cognitive impairment [35]. Despite potential inconsistencies in results between CSF and imaging markers [36], a multicenter study demonstrated the highest concordance (96 %) between CSF biomarkers and amyloid-PET in A+T+N+ cases, followed by A−T−N− cases (89 %) [37]. Furthermore, the combination of imaging markers and CSF biomarkers enhances the diagnostic capability for AD. For instance, microstructure imaging capturing neurodegeneration (N) may increase the clinical sensitivity of CSF A and T biomarkers [38].

As research on the pathogenesis of AD advances, novel potential biomarkers have emerged, including biomarkers related to inflammation/immunity mechanism (I), brain vascular injury (V), and α-synuclein (S). These collectively termed "X" biomarkers have been incorporated into the AT(N) framework to comprehensively represent the entire pathological spectrum of AD and enhance our understanding of its underlying mechanisms. Among them, amyloid deposition and pathological tau-related biomarkers are the core biomarkers of AD, while neurodegeneration and inflammation/immunity are non-specific AD biomarkers that can provide information in staging and prognosis. Brain vascular injury biomarkers and α-synuclein biomarkers, representing common non-AD co-pathologies, play a potential role in differential diagnoses and comprehensive management of AD.

Existing frameworks still have certain limitations, particularly when applied to large populations in the early stages of cognitive decline, prompting the need for alternative, less invasive, and more cost-effective blood biomarkers for widespread population screening [39]. Integrating blood markers into the AT(N) framework not only enhances diagnostic accuracy but also facilitates the repeated monitoring of biological changes, offering potential advantages for the clinical application of biofluid biomarkers [33]. Researchers have undertaken extensive investigations to identify promising representative markers in each category and assess their diagnostic efficacy. Noteworthy blood-based biomarkers identified include the Aβ42/Aβ40, P-tau, and neurofilament light chains (NfL) [40].

Core biomarker

Aβ

Amyloid deposition is one of the hallmark pathologies of AD [41]. The formation of Aβ amyloid plaques involves cleavage of amyloid precursor protein (APP) by α, β, and γ secretases [42]. Specifically, cleavage at sites Asp1 and Glu11

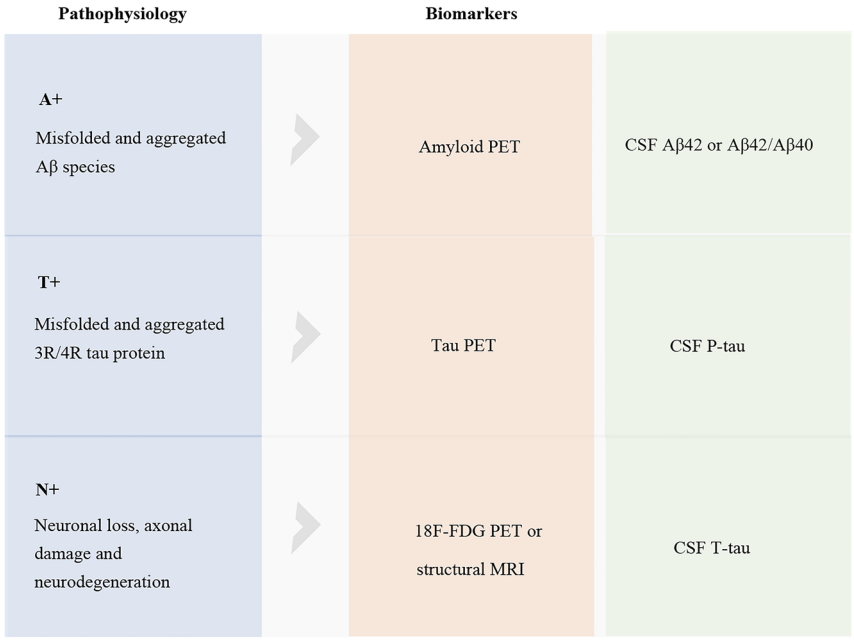

Figure 5: Pathophysiology and corresponding biomarkers according to the AT(N) research framework. Aβ, amyloid-β; CSF, cerebrospinal fluid; FDG, fluorodeoxyglucose; PET, positron emission tomography; P-tau, phosphorylated tau.

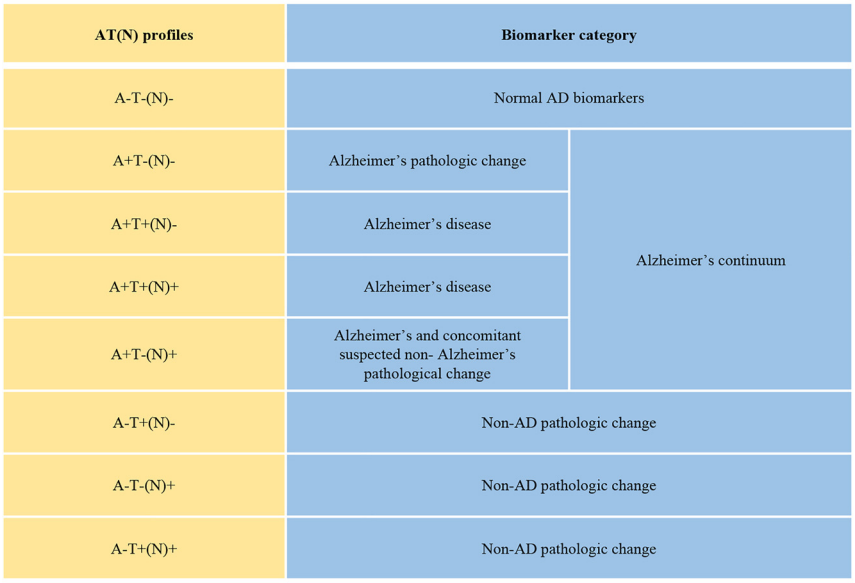

Figure 6: Biomarker profiles and categories according to the AT(N) research framework. Binary classification of the three AT(N) biomarkers results in eight distinct biomarker profiles. Individuals can be classified into one of three biomarker categories based on these profiles: those with normal AD biomarkers, those within the Alzheimer's continuum, or those exhibiting non-AD pathological changes. AD, Alzheimer's disease.

on the β region of APP occurs through the action of the β-site APP cleaving enzyme 1(BACE1), resulting in the production of a C-terminal membrane containing either 89 or 99 amino acid fragments. Subsequent processing by γ-secretase gives rise to various subtypes, including the soluble isoform Aβ40 and the plaque-forming variant Aβ42, which contains additional amino acids (isoleucine and alanine) [43]. The alteration in the free quantity of Aβ accompanying Aβ deposits has prompted extensive research into the utility of Aβ in CSF as a biomarker for AD.

Aβ42 stands out as a specific indicator widely acknowledged for diagnosing AD. As early as 1995, researchers observed a substantial decline in CSF Aβ42 levels in individuals with AD, using an ELISA tailored for Aβ42 (Figure 1) [11]. Diminished Aβ42 concentrations in CSF also emerged as a predictive factor for the progression to AD in individuals with mild cognitive impairment (MCI) [44]. Aβ40 is considered the foundational element for total Aβ production. Studies have demonstrated that using Aβ42/Aβ40, which adjusts for inter-individual differences in Aβ processing and potential preanalytical confounders, enhances consistency with amyloid PET results [45]. Notably, CSF Aβ42/Aβ40 exhibits superiority over CSF Aβ42 alone in distinguishing AD from other dementias [46].

Plasma Aβ42/Aβ40 also demonstrates excellent predictive ability for brain amyloid deposition, which is comparable to CSF Aβ42/Aβ40 and amyloid PET, and its accuracy (area under the curve [AUC]=0.84–0.85) could be further enhanced through incorporating apolipoprotein E (*APOE*) genotypes (AUC=0.88–0.93) [47]. Moreover, plasma Aβ42/Aβ40 predicted the risk of development and progression of AD. In a cohort study involving 62 individuals with amnestic MCI, 52.4 % of those with amnestic MCI and a low baseline Aβ42/Aβ40 progressed to AD within 24 months, compared to 28.8 % of individuals with a high Aβ42/Aβ40 [48]. Additionally, it was found that plasma Aβ42 concentration and Aβ42/Aβ40 ratio were significantly reduced in individuals with AD compared to those with normal cognition, subjective cognitive decline (SCD), or MCI, while the change in plasma Aβ40 was less pronounced [49]. A head-to-head study reveals that MS-based plasma Aβ42/Aβ40 shows the most accurate diagnostic performance [50]. In 2020, Precivity AD™, an MS-based plasma Aβ assay, received approval for the use of AD diagnosis in the United States and Europe, which showed an 86 % agreement with amyloid PET (sensitivity: 92 %, specificity: 76 %) [51].

Plasma Aβ42/Aβ40 holds promise as a diagnostic biomarker for AD. Nevertheless, the change amplitude of plasma Aβ42/Aβ40 is relatively modest (the decrease of plasma Aβ42/Aβ40 is only 10–15 %, while the amplitude of changes in CSF is about 50 % between Aβ-positive and negative individuals) [52], resulting in potentially unstable results. The reason for this variability remains unclear, whether attributed to biological variations or disparities in analytical methods. Therefore, ongoing research is dedicated to exploring oligomeric and misfolding forms of Aβ [53–55] to improve its diagnostic and predictive performance. Aβ oligomer is the most toxic and pathogenic form of Aβ (Figure 7). An elevated CSF level of oligomeric Aβ has been linked to cognitive decline in individuals with AD [56]. In plasma, it effectively distinguished AD from control, showing a correlation with the severity of symptoms [57]. Furthermore, it has been demonstrated that the oligomerization of Aβ is triggered by a structural shift from

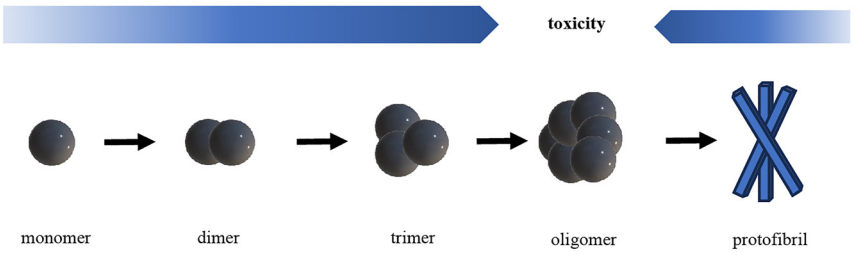

Figure 7: Toxicity of different forms of Aβ. The aggregation of Aβ peptides initiated from Aβ monomers to eventual protofibrils and fibrils. Oligomer is the most toxic form while monomers and fibrils have lower cytotoxicity. Aβ, amyloid-β.

predominantly monomeric α-helical to a β-sheet-enriched secondary structure [58]. Therefore, this misfolding Aβ, detectable through an immuno-infrared sensor, also holds promise for predicting the conversion to MCI and dementia due to AD in cognitively unimpaired (CU) individuals when considered alongside the Aβ42/Aβ40 ratio [54]. Given the numerous biological and analytical challenges encountered by plasma Aβ42/40 alone as a diagnostic biomarker for AD, integrating both structure-based and concentration-based Aβ holds the potential to improve the prognostic performance of this biomarker.

Pathological tau

Tau is a protein found in neurons that interacts with microtubules, containing a domain facilitating the assembly and stabilization of these structures, thereby maintaining cytoskeletal integrity. Hyperphosphorylated tau loses its affinity for microtubules, forming NFTs deposited in the cytosol, compromising cellular structure maintenance. Additionally, this deposition adversely affects normal neuronal functions such as synaptic transmission, axon transport, and signal transduction, leading to gradual neurodegeneration [59].

Elevated levels of P-tau in CSF were observed early in AD [60]. Currently, there are mainly three subtypes of P-tau as biomarkers: P-tau181, P-tau217, and P-tau231. Historically, P-tau181 was considered the predominant tau marker in CSF; however, subsequent research revealed that CSF P-tau217 exhibits greater accuracy compared to CSF P-tau181 [61]. MS studies found that individuals with AD displayed six times higher levels of CSF P-tau217 compared to individuals with other neurological disorders and CU individuals, whereas the increase observed for CSF P-tau181 was only 1.3 times [62]. These P-tau subtypes change as early as two decades before the development of aggregated tau pathology and fail to reflect the tau aggregation and NFTs [63]. Recently, some novel tau protein subtypes have been discovered in CSF, such as non-modified tau species that contain the microtubule-binding region of tau (MTBR tau) and tau species ending at amino acid 368 (tau368). They exist in NFTs and are significantly correlated with tau PET signals [64–67], indicating they might be directly related to tau aggregation and NFTs. However, their corresponding biomarkers have not yet been found in the blood.

In plasma, these P-tau subtypes have also received extensive attention, with P-tau217 recognized for its superior detection efficiency. A multicenter cohort study revealed that plasma P-tau217 demonstrated a remarkable ability to differentiate AD from non-AD, demonstrating a high accuracy (96 %) comparable to the established CSF or tau PET biomarkers [68]. In addition, plasma P-tau217 is elevated in the asymptomatic phase and changes with the progression of AD, thus it might contribute to the early prediction of AD, disease surveillance, and evaluation of drug efficacy. Plasma P-tau231 is also a biomarker indicating Aβ pathology at very early stages [69, 70]. Plasma P-tau231 demonstrated remarkable efficacy in distinguishing individuals with AD with high accuracy (AUC=0.92–0.94) from Aβ-negative CU individuals. It also distinguished individuals with AD from those with other neurodegenerative diseases (AUC=0.93) and those with Aβ-negative MCI (AUC=0.89) [70]. Regarding plasma P-tau181, its elevation was observed in preclinical AD, with a subsequent increase in both MCI and dementia stages. Plasma P-tau181 emerges as an interchangeable marker for evaluating Aβ status in MCI and AD with 18F-Florbetapir PET imaging and 18F-Florzolotau PET imaging [35]. It correlates with CSF P-tau181 and predicts positivity in tau PET (AUC for different brain regions: 0.87–0.91) [71]. Plasma P-tau181 effectively discriminated AD dementia from other neurodegenerative diseases with similar accuracy to tau PET and CSF P-tau181 (AUC=0.94–0.98) [71]. Additionally, plasma P-tau181 showed high diagnostic accuracy in distinguishing between Aβ-positive and Aβ-negative individuals of the Alzheimer's continuum (AUC=0.77) [72].

In summary, plasma P-tau181, P-tau217, and P-tau231 are all potential biomarkers for early diagnosis of AD. The different metabolic characteristics of these biomarkers also determine their unique diagnostic value. Particularly noteworthy are the significant dynamic longitudinal changes observed in plasma P-tau217, correlating with alterations in multiple cognitive domains and cortical

thickness in regions characteristic of AD. In contrast, P-tau231 displays more pronounced cross-sectional changes in response to early Aβ pathology but does not exhibit significant longitudinal changes [73]. In comparison, P-tau217 outperforms P-tau181 in differentiating AD from frontotemporal lobar degeneration (FTLD) and demonstrates a stronger correlation with tau PET signaling [74]. Nevertheless, these blood markers encounter challenges. Confounding factors can alter plasma concentrations of P-tau217 and P-tau181. Specifically, plasma levels of P-tau181 and P-tau217 exhibit an age-related increase between 65 and 70 years, with the most notable elevation observed in Aβ-positive individuals [75]. Furthermore, elevated plasma levels of P-tau181 and P-tau217 are associated with the presence of multiple comorbidities, such as chronic kidney disease, a history of myocardial infarction, or stroke [76]. The research on blood-based tau biomarkers is still in its early stages and needs to be replicated and validated in more studies.

Non-specific biomarker

Non-specific biomarkers are biomarkers that are prevalent in other neurodegenerative diseases or pathological states. Non-specific biomarkers of AD mainly include neurodegeneration-related markers such as NfL, T-tau, and synaptic-related markers, as well as markers related to inflammation and immunity mechanisms. These proteins are not specific to AD; therefore, they cannot be used alone for the diagnosis of AD and are commonly used for disease staging [77].

NfL

NfL is an important component of the axonal cytoskeleton. When axons are damaged, NfL is released, leading to a substantial increase in concentration in CSF and blood [78]. A range of pathological processes, including neurodegeneration, neuroinflammation, ischemia, and trauma, can disrupt the cytoskeletal network, which may result in neurofilament dysfunction, ultimately leading to neuronal and axonal damage [79]. Consequently, the clinical correlation between detecting NfL levels in CSF and blood holds the potential to offer insights into the progression and prognosis of neurological diseases.

CSF NfL is elevated across various neurological diseases, making it a non-specific marker for neuronal and axonal damage without indicating a specific disease pathology. However, it demonstrates the ability to differentiate diseases based on the extent of axonal damage and determine whether cognitive decline is linked to neurodegeneration [80]. Elevated CSF NfL levels are associated with an increased risk of MCI [81]. Moreover, CSF NfL demonstrates greater sensitivity in predicting clinical progression in CU individuals compared to other proposed neurodegeneration markers like neurogranin and T-tau [82]. CSF NfL serves as a valuable indicator for monitoring disease progression and assessing prognosis.

There is a high correlation between blood and CSF NfL concentration, despite blood NfL levels being significantly lower than CSF levels [80]. Studies indicate that plasma NfL, similar to CSF NfL, is elevated in various neurological disorders [79]. In sporadic AD, plasma concentrations of NfL are elevated during the MCI stage and exhibit correlations with cognitive, biochemical, and imaging characteristics of the disease [83]. This elevation holds significance in predicting the progression of AD [49]. In familial AD, blood NfL concentrations begin to increase approximately 10 years before the anticipated onset, and the level of NfL is correlated with the estimated years to symptom onset [84, 85]. The rate of change in serum NfL reached its peak in individuals converting from the pre-symptomatic to the symptomatic stage, and this increase was correlated with cortical thinning [86]. These findings imply the potential utility of blood NfL in aiding disease staging and monitoring progression.

A notable challenge in clinical practice arises due to a significant correlation between NfL concentration and age [87]. Serum NfL is influenced by the aging process, displaying a nonlinear increase in concentration after the sixth decade of life in individuals without neurological diseases [88]. Furthermore, the neurodegenerative process from the peripheral nervous system may also contribute to the elevated blood levels of NfL [89]. Therefore, for older adults potentially experiencing neurodegenerative conditions, it is advisable to incorporate NfL alongside Aβ and P-tau for a comprehensive assessment. While NfL may not be suitable for direct diagnosis purposes in AD, it still holds potential implications for enhancing diagnostic clarity, monitoring diseases, evaluating treatment responses, and predicting long-term prognosis. Moreover, NfL could serve as an indicator of both amyloid-dependent and independent neurodegeneration, which is pertinent when considering the involvement of mixed pathology in AD.

T-tau

There is still controversy over the classification of T-tau. Both CSF and plasma T-tau change in the early stage of familial AD [63] and exhibit a strong correlation with P-tau [90], which makes it desirable to be a T biomarker. However, a substantial increase in CSF levels of T-tau is also observed

in various neurological diseases such as brain trauma [91], stroke [92], and peripheral neuropathies [93], suggesting that T-tau may fall into the N category. In 2018, the NIA-AA research framework defined CSF T-tau as a neurodegeneration-related marker, believing that it cannot represent AD-specific tau pathology [20].

A high level of CSF T-tau is associated with rapid progression from MCI to AD, steerer cognitive decline, and increased mortality in AD. CSF T-tau also differentiates individuals with AD from CU individuals with good performance [94]. Additionally, it is suggested that CSF tau is correlated with postmortem NFT load, implying that neurons affected by NFTs may contribute to the elevated CSF T-tau levels, and thus CSF T-tau might reflect AD-specific neurodegenerative processes.

Although plasma T-tau is associated with an increase in the risk of all-cause or AD [95], it is ineffective in distinguishing individuals with AD from CU individuals [96]. The disparity in the performance of CSF and plasma T-tau may stem from post-translational modification or peripheral metabolism of tau. As depicted in Figure 8, the brain contains the full-length tau, but post-translational modifications result in truncated tau in both CSF and blood. Specifically, tau in CSF comprises N-terminal and mid-region species, while in blood, it predominantly exists in N-terminal forms. These characteristics of tau kinetics might lead to a poor correlation between CSF and plasma T-tau [97] and a significant overlap in plasma T-tau between normal aging and AD [97]. Recently, a novel biomarker designed to specifically detect brain-derived tau (BD-tau) in blood is expected to address this issue [96]. The performance of blood BD-tau is superior to that of traditional blood T-tau. Blood BD-tau is highly correlated with CSF BD-tau and has significant changes in individuals with AD. It can accurately diagnose AD and recognize neurodegeneration with AD specificity (AUC=0.86) [96]. Therefore, BD-tau may have the potential to complement the AT(N) system in the blood.

Synaptic proteins

Synaptic dysfunction is also an important pathological feature of AD, and the associated biomarkers include growth-associated protein 43 (GAP43), neurogranin, synaptosome-associated protein 25 (SNAP25), synaptotagmin 1, and β-synuclein. In CSF, synaptic proteins change earlier than NfL and are highly promising AD biomarkers [98]. Neurogranin, a protein localized in dendrites and associated with protein kinase C, is intricately connected to the compromised synaptic function observed in AD [99] and therefore emerges as a promising biomarker with the potential to identify the disease with high sensitivity in the early stage [100]. Presynaptic proteins also offer valuable potential as biomarkers for AD. GAP43, which is crucial for synapse maintenance and neurite regeneration [101, 102], is diminished in the brain but increased in CSF in individuals with AD. Notably, a positive correlation exists between CSF GAP43 and amyloid deposition as well as tau pathology, underscoring its effectiveness in diagnosing AD (AUC=0.92 for distinguishing AD from the control) [103, 104]. Another presynaptic protein, SNAP25, plays a role in vesicle fusion and exocytosis [105]. CSF SNAP25 levels are elevated in individuals with AD. The longer soluble forms including at least amino acid 32–40 of SNAP25, SNAP25aa40, not only serve as a diagnostic marker (AUC=0.93 for distinguishing AD from the control group) but also aid in differential diagnosis (AUC=0.92 for distinguishing AD from other dementias) [103]. Synaptotagmin 1, a crucial vesicle protein in hippocampal neurons, facilitates rapid neurotransmitter release [106]. Initial studies [107] suggested decreased CSF synaptotagmin 1 in early-onset AD, but later research [108] contradicted this, revealing increased concentrations in AD and MCI, with the highest levels in MCI due to AD. β-Synuclein is a presynaptic protein predominantly expressed in the brain. An increase in CSF β-synuclein in AD was identified in an MS study focusing on synaptic proteins and was corroborated through immunodetection [109, 110]. This specificity to amyloidopathies was reinforced by its unchanged levels in non-amyloid pathologies like FTD [111]. Importantly, the elevation was evident in individuals with MCI due to AD [110], indicating its potential as an early diagnostic and prognostic biomarker.

There is limited research on synaptic protein in the blood, which may be due to lower peripheral expression concentrations. No blood biomarkers have been found that can accurately reflect synaptic pathology until very recently, the assay for blood β-synuclein has been developed. Blood β-synuclein has demonstrated a correlation with cognitive impairment and brain atrophy in AD [112]. Furthermore, blood β-synuclein is associated with amyloid PET positivity in multiple regions and predicts Aβ status in individuals with MCI. Other synaptic proteins remain challenging to measure in blood, even with highly sensitive methods, as demonstrated in recent tests for SNAP-25 [113]. While neurogranin can be identified in blood, its expression extends beyond the brain and its alterations observed in CSF may not be accurately reflected in blood samples [113].

Inflammation and immunity

More and more evidence suggests that inflammation and immunity play an important role in the occurrence and development of AD. Currently, inflammatory/immunity

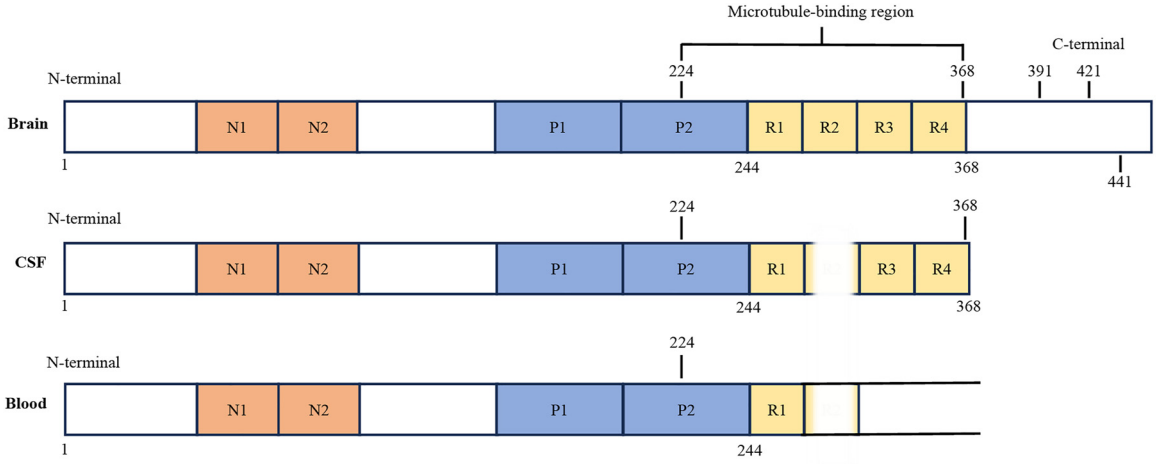

Figure 8: Tau in the brain and biofluids. Tau exists in the full-length form in the brain, while tau in CSF and blood is truncated after post-translational modification. CSF, cerebrospinal fluid.

biomarkers related to AD mainly include glial fibrillary acidic protein (GFAP), triggering receptor expressed on myeloid cells 2 (TREM2), chitinase-3 like-protein-1 (CHI3L1), and systemic inflammatory markers.

GFAP is currently a promising biomarker for AD, which is specifically expressed in astrocytes. A meta-analysis revealed elevated CSF GFAP concentrations in both early-onset and late-onset AD participants compared to CU participants [114]. A longitudinal study demonstrated increased GFAP levels throughout the continuum of AD, spanning preclinical, MCI, and dementia phases, compared to Aβ-negative controls [115]. Plasma GFAP levels also begin to increase during the asymptomatic phase of AD, increasing with the progression of AD disease [116]. The amplitude of changes in plasma GFAP is greater than that in CSF GFAP. Furthermore, plasma GFAP has a stronger correlation with Aβ pathology and has a better ability to differentiate Aβ-positive individuals from Aβ-negative individuals than CSF GFAP (plasma AUC, 0.69–0.86; CSF AUC, 0.59–0.76) [117, 118]. It is currently the only plasma biomarker with higher efficacy than its CSF counterpart.

TREM2 is expressed on the surface of microglial cells. It undergoes ectodomain shedding and produces soluble TREM2 (sTREM2). CSF sTREM2 is increased in individuals with AD [119]. Furthermore, a positive correlation between sTREM2 level and classical CSF biomarkers T-tau and P-tau suggests its potential as a reliable predictor for early-stage AD [120]. Plasma sTREM2 is highly correlated with CSF levels and predicts the risk of AD conversion and cognitive decline [121]. Increased TREM2 mRNA expression in peripheral blood mononuclear cells distinguishes individuals with AD and enables differentiation between disease stages [122, 123] Consistently, Hu et al. revealed a diagnostic accuracy close to 70 % when assessing TREM2 protein in circulating mononuclear cells [124].

CHI3L1, also known as YKL-40, is expressed by astrocytes and microglia in response to proinflammatory cytokines. Increased CHI3L1 concentrations in CSF and plasma are indicative of ongoing inflammatory processes. CHI3L1 is increased in the early stage of AD [125]; however, the increase may not be specific to Aβ pathology, as no significant differences have been found between individuals with AD and those with other neurodegenerative dementias. Instead, the progression of clinical symptoms and brain cortical atrophy appears to be more closely associated with increased CHI3L1 levels [126, 127]. Therefore, it would function more effectively as a staging biomarker rather than a standalone diagnostic biomarker for AD.

Research on inflammatory markers in AD is also controversial. Interleukin (IL) has emerged as a potential biomarker in numerous studies, focusing on alterations in pro-inflammatory cytokines like IL-1α, IL-1β, and IL-6 in individuals with AD [128]. Elevated concentrations of IL-6 and impaired cognitive function have been linked to the progression of AD [129]. Furthermore, systemic levels of IL-6 were associated with cognitive function in carriers of *APOE* ε4 [130]. The soluble form of IL-33 and its decoy receptor sST2 could potentially serve as additional indicators of inflammation in AD, with reduced IL-33 observed in the brain tissue of individuals with MCI and AD [131]. Conversely, plasma IL-33 level was higher in those with MCI and AD compared to CU individuals. However, it is important to note that alterations in these inflammatory markers may not directly correlate with AD pathology, as they could also be influenced

by aging or systemic conditions. Additional clinical evidence is essential to substantiate the feasibility of using systemic inflammatory markers as biomarkers in future research.

Immunity and inflammation are essential processes throughout AD. While they currently fall short of meeting diagnostic specificity requirements, their potential for diagnosis becomes more promising when combined with other core biomarkers such as Aβ and tau.

Co-pathology biomarkers

It has been widely acknowledged that additional pathologies other than amyloid plaques and NFTs often co-occur in AD, including α-synucleinopathy, vascular pathology, and TDP-43 inclusions pathology [132]. The presence of these co-pathologies not only contributes to clinical heterogeneity but also potentially accelerates the progression of AD, resulting in an earlier onset of neurodegeneration and clinical symptoms. Biomarkers of co-pathology of AD reflect concurrent pathological processes and play a crucial role in clinical diagnosis, prognosis, and treatment decisions of AD. For example, an individual experiencing cognitive impairment with co-pathology may not respond as effectively to anti-Aβ immunotherapy compared to someone with biomarker profiles lacking co-pathology indicators. This holds significance not only in clinical trials where a homogeneous cohort with purer AD is preferable but also in the precise management of the disease. Currently, available biofluid assays for co-pathology biomarkers include those that reflect brain vascular injury and α-synuclein pathology.

Brain vascular injury biomarkers

The neurovascular system, comprising neurons, glial cells, and blood vessel cells, is the structural and functional unit of the brain. Its pivotal role involves regulating the permeability of the blood-brain barrier (BBB) and maintaining cerebral blood flow, which ensures normal brain function, neuronal survival, and an optimal microenvironment for information processing [133]. Recently, there has been increasing interest in exploring the impact of vascular biology on the pathophysiology of AD [134]. Heart-type fatty acid-binding protein (H-FABP), extensively studied as a biomarker for myocardial infarction and heart failure [135, 136], has been linked to AD. An increase in CSF H-FABP level has been observed in AD as early as the MCI stage, with higher baseline levels in MCI predicting conversion to AD during follow-up [137]. However, H-FABP levels show a limited ability to differentiate AD from other dementias and a weak association with cognitive impairment [138, 139]. Currently, there is no evidence that H-FABP in the blood is associated with AD. Pericytes are crucial for vascular integrity and BBB regulation in AD [140]. Pericytes release soluble platelet-derived growth factor receptor β (sPDGFRβ) under hypoxic conditions or exposure to Aβ peptides [141]. Multiple studies suggest increased CSF sPDGFRβ is associated with early cognitive decline in AD [142, 143]. Furthermore, this increase predicts cognitive decline in early AD stages irrespective of the Aβ and tau state, suggesting that vascular injury is an early biomarker of cognitive dysfunction independently of Aβ and tau changes [144].

α-Synuclein

The misfolding of α-synuclein plays a pivotal role in the pathogenesis of neuronal synuclein disease, including Parkinson's disease and dementia with Lewy bodies (DLB) [145]. α-Synuclein constitutes the primary component of Lewy bodies and is a common co-pathology in AD, accounting for nearly one-third of cases [146]. Studies indicate that increased level of α-synuclein contributes to Aβ oligonucleation, tau phosphorylation, kinase activation, and tau aggregation in AD [146, 147]. Furthermore, they are associated with amyloid deposition in the asymptomatic phase [147]. There is a positive correlation between CSF α-synuclein and tau, with notably increased CSF α-synuclein concentration in early AD stages. This suggests that CSF α-syn could serve as a diagnostic marker for AD, aiding in differentiation from other neurodegenerative disorders when combined with additional biomarkers [147].

Combination biomarkers of ATX(N)

The continuous refinement of individual biofluid biomarker research has established the groundwork for exploring combinations of biomarkers. Utilizing the strengths and recognizing the limitations of various biofluid biomarkers, researchers have developed diverse models that combine these markers, intending to enhance the diagnostic effectiveness of AD.

The first idea is to combine Aβ and P-tau or T-tau biomarkers. Studies suggest that the combination of Aβ and P-tau or T-tau serves as a reliable and precise marker for diagnosing AD [44]. Moreover, T-tau/Aβ42 and P-tau/Aβ42 ratios demonstrate equivalent diagnostic efficacy to the widely accepted Aβ42/Aβ40 ratio for AD diagnosis (AUC=0.94) [148, 149]. Notably, the P-tau/Aβ42 ratio stands out for its exceptional predictive value for AD pathology and progression [150]. A combination of Aβ42/T-tau or T-tau/Aβ42

in both plasma and CSF shows a higher predictive ability for progression from MCI to AD than individual biomarkers alone [151]. Additionally, combinations like P-tau217 with Aβ42/Aβ40 and P-tau181 with Aβ42/Aβ40 predict brain amyloid PET status, with the latter being superior [71, 152]. Aβ42/Aβ40 combined with percentage of P-tau217 not only has high diagnostic accuracy for identifying AD among individuals with cognitive symptoms in primary and secondary care but also reduce the influence of comorbidities or confounding factors [153, 154]. Therefore, biomarker combination is a promising direction, which deserves extensive research and continuous attention.

Various combinations involving more components of the ATX(N) have also been explored. We previously demonstrated that a combination of plasma Aβ42, P-tau181, and NfL could effectively differentiate AD from CU individuals, achieving an impressive AUC of 0.99. Notably, this combination showed promise in predicting AD 8–10 years before clinical onset, with applicability to both sporadic and familial AD cases [155]. Another noteworthy combination involving P-tau181, NfL, and GFAP in plasma had the potential to distinguish AD from FTD with an AUC of 0.82 [156]. The incorporation of neurophysiological markers into the analysis enhanced the diagnostic significance of P-tau181, NfL, and GFAP in differentiating individuals with AD from CU individuals (AUC=0.99) [156]. Our previous study indicated that the predictive ability of a combination of plasma biomarkers for AD was superior to individual biomarkers [155], which might be attributed to the ability of the combination to decrease potential confounding factors associated with preanalytical procedures to a certain extent [157]. Therefore, despite the simplicity of individual biomarkers, it is recommended to utilize a combination of biomarkers for more accurate detection of AD.

Other biomarkers

In addition to the aforementioned biomarkers, there are also other biofluid biomarkers under exploration, including non-coding RNAs(ncRNA) [158, 159], metabolites [160], and biomarkers using extracellular vesicles (EVs) as carriers [161, 162]. They not only serve as potential biomarkers of AD but also reflect the upstream and downstream mechanisms of AD. Since most disease-modifying therapies such as Aβ-targeting therapies have limited clinical efficacy for AD [155], continuous development of new biomarkers can help discover new therapeutic targets.

NcRNA

NcRNA refers to RNA transcribed from the genome without coding potential [163]. These molecules exert a regulatory function by binding to DNA, RNA, and proteins. Transcriptomic analyses have revealed the abundance of ncRNA in the CNS, exhibiting tissue and cell specificity, and its capacity to dynamically regulate signaling pathways in neurodegeneration [164]. Previous studies have confirmed the involvement of ncRNA in the regulation of BACE activity, tau protein phosphorylation, inflammation, and synaptic plasticity [165].

MicroRNAs (miRNA) represent a class of short ncRNAs typically comprising 20–24 nucleotides. Through binding to the 3′untranslated region (3′UTR) of mRNA, miRNA can modulate mRNA translation by either suppressing its activity or promoting its degradation [166]. Kiko et al. observed reduced levels of miR-34a and miR-146a in plasma, and miR-34a, miR-146a, and miR-125b in CSF of individuals with AD, while levels of miR-29a and miR-29b in CSF were increased [167]. Subsequent studies confirmed these findings and assessed the diagnostic potentials of these differentially expressed miRNAs. Blood miR-125b was found to discriminate AD from CU (specificity: 68.3 %; sensitivity: 80.8 %) [168] and from MCI (AUC: 0.733) [169]. Importantly, miR-125b showed a correlation with cognitive performance in individuals with AD [168]. Plasma miR-34a-5p correlated positively with CSF Aβ42 levels, distinguishing early AD from controls (AUC=0.77) [170]. Serum miR-206 showed significant elevation in individuals with MCI, providing robust diagnostic value (AUC=0.88) [171]. Longitudinal follow-up demonstrated upregulated plasma miR-206 in the pre-dementia stage, predicting cognitive decline at the MCI stage [172]. Inconsistent findings were observed for miR-146a in CSF and serum. A fluorescent miRNA-array showed increased CSF miR-146a in AD [173], while qPCR indicated a reduction, potentially due to blood contamination in CSF [174]. Serum miR-146a increased in MCI but decreased in AD, playing a role in AD progression and correlating with CSF Aβ levels, *APOE* ε4 status, and hippocampal volume [175].

While individual miRNAs show promise as diagnostic biomarkers, their efficacy remains suboptimal due to the intricate pathogenesis of AD. Studies suggest that combinations of miRNAs yield superior diagnostic performance. Kumar et al. identified a seven-miRNA panel with an AUC of 0.953 for AD detection [176]. Kayano et al. demonstrated a combination that accurately distinguishes MCI from controls with 95 % accuracy (AUC=0.962) [169]. We previously identified a set of seven miRNAs accurately estimating P-tau/Aβ42 values in CSF, differentiating AD from controls and

other dementias [158]. Collectively, these findings indicate the potential of miRNAs as effective biomarkers for AD.

While miRNA has received extensive attention due to its important role in gene regulation, there is limited research on biomarkers of circular RNA (circRNA) and long ncRNA (lncRNA). CircRNA, a closed circular non-coding RNA, plays a crucial role in gene regulation by acting as a sponge for miRNAs and regulating RNA binding proteins (RBPs) [177]. Specific circRNAs, including hsa_circRNA_050263, hsa_circRNA_403959, and hsa_circRNA_003022, are found to be upregulated in the plasma of individuals with AD and MCI [178]. Conversely, hsa_circ_0003391 is reduced in the blood of AD participants compared to other forms of dementia and effectively distinguishes AD with an AUC of 0.7283 [179]. Moreover, incorporating additional circRNAs into the panel significantly enhances its ability to identify individuals with AD from those with other dementias, achieving an impressive AUC of 0.968 [159]. This underscores the potential of circRNA biomarkers, along with the need for further exploration in this area.

LncRNA, a subset of ncRNA exceeding 200 nucleotides, regulates gene expression through interactions with DNA, RNA, and RBP [180]. Plasma lncRNA BACE1 demonstrated high specificity (88 %) for AD [181]. This observation was consistent in other studies where BACE1-AS, upregulated in AD plasma, effectively discriminated AD from CU with 68 % sensitivity and 100 % specificity [182]. Additionally, plasma levels of NEAT1 (72 % sensitivity, 84 % specificity) and BC200 (60 % sensitivity, 91 % specificity) successfully differentiated individuals with AD from CU [183]. In our prior study, a combination of seven lncRNAs showed strong performance in AD diagnosis (AUC=0.797), significantly outperforming individual markers [184].

In conclusion, ncRNAs hold promise in diagnosing and differentially diagnosing AD. Notably, miR-125b, miR-206, and miR-146a demonstrate high sensitivity and specificity. The exploration of relationships between peripheral ncRNA markers and other indicators sparks further investigation. However, inconsistencies in ncRNA detection, data processing, and statistical analysis contribute to variability in results. We anticipate more research adhering to stringent standards, and using consistent detection and analysis methods, with a particular emphasis on longitudinal studies.

Metabolites

Metabolites, small molecules typically possessing a molecular weight below 1,500 Da, play a pivotal role in various biological functions, providing valuable insights into disease phenotypes and uncovering pathophysiological mechanisms [185]. Multiple metabolic disorders, such as diabetes and hyperlipidemia, are comorbidities of AD, indicating a potential connection between AD and metabolic dysregulation [186]. As of November 2023, the Human Metabolome Database (available at https://hmdb.ca) contains more than 220,945 entries for metabolites, including both water- and fat-soluble compounds. Metabolomics, an advanced *in vivo* analytical technique facilitating rapid and simultaneous detection of hundreds/thousands of metabolites, has successfully detected metabolic changes and characterized biochemical pathways in AD. This approach aids in identifying biomarkers in the early stages of AD, searching for emerging therapeutic targets, and assessing therapeutic response and disease progression [187, 188].

Several aromatic, branched, and urea cycle amino acids in CSF were associated with CSF Aβ42 and P-tau181 in AD, indicating dysregulated systemic energy metabolism in AD, alongside specific alterations in the tryptophan pathway and creatinine within the CNS [189]. An earlier study identified several novel CSF metabolites associated with indole-3-propionic acid, kynurenine, indole-3-acetic acid, guanosine, and glutathione, which showed high accuracy in distinguishing individuals with AD from controls (AUC=0.96) [190]. Targeted metabolomics on CSF from postmortem-confirmed AD revealed a significant decrease in concentrations of metabolites associated with polyamines and the tryptophan-kynurenine pathway [191]. A diagnostic model, mainly comprising amino acids, nucleotides, and other small molecules, predicting AD occurrence, with an accuracy of 98.7 %, and specificity and sensitivity values exceeding 95 % [192].

Lipid metabolism abnormalities, particularly involving sphingolipids and phospholipids in plasma, are key focuses in AD research. A longitudinal cohort study suggested higher baseline serum ceramide levels (C16:0 and C24:0) are associated with an increased risk of AD [193]. Stage-specific changes in plasma metabolites, such as specific sphingolipids (sphingomyelin with acyl residue sums of C16:0, C18:1, and C16:1, as well as hydroxysphingomyelin with an acyl residue sum of C14:1), are closely associated with AD pathology severity and progression during preclinical and prodromal stages [187]. To identify AD-specific metabolomic alterations, the Alzheimer's Disease Neuroimaging Initiative (ADNI) study revealed correlations between MCI and AD with plasma levels of anthranilic acid, glutamate, taurine, and xanthine in 2,067 participants [194]. Other studies identified metabolites associated with cognitive impairment, dementia risk, and lifestyle factors [195]. Notably, a diagnostic model incorporating 11 serum metabolites demonstrated high accuracy in both diagnosing AD and distinguishing it from other types of dementia [160].

In summary, metabolomics reveals significant alteration in certain neurotransmitters, amino acids, and lipids in CSF and blood of individuals with AD. These changes are involved in pathways closely related to amino acid metabolism, lipid metabolism, and mitochondrial energy metabolism, all of which are intricately linked to the pathophysiology of AD [196]. They provide dynamic biological insights into different clinical stages of AD. Metabolomics holds promise in providing crucial clues for the early diagnosis, treatment strategies, and preventive measures in AD.

EVs

EVs, ranging from 30 to 200 nm, are released by living cells [197]. Nearly all types of cells in the CNS, including neural stem cells, neurons, and glial cells, can release EVs [198]. EVs play a crucial role in intercellular communication, as the proteins, lipids, and nucleic acids they are carrying can reflect and influence various physiological and pathological states of cells [199]. EVs may play a dual role in AD. On one hand, they participate in the formation of pathological proteins such as Aβ and P-tau, propagating them among interconnected neurons and causing cellular damage and neuronal loss [200]. On the other hand, EVs mediate the clearance of these pathological proteins through multiple pathways [201]. Besides, EVs have been implicated in synaptic function [202, 203], neuroinflammation [204], and insulin resistance [205], all of which are essential mechanisms for AD. Of interest, pathological changes in AD precede clinical symptoms over many years, and toxic substances in EVs can be detected early in AD [206]. Thus, EVs may be promising biomarkers for effectively recognizing AD in the asymptomatic phase.

As mentioned earlier, the levels of Aβ42, T-tau, and P-tau in CSF have been recognized as appropriate biomarkers for early identification of AD [19, 207]. In postmortem studies, these pathological proteins were found to colocalize with EVs [208, 209]. Furthermore, their changes in CSF are related to EV-mediated secretion [210]; therefore, assessing these toxic proteins within CSF-derived EVs is expected to improve the diagnostic sensitivity for AD. P-tau in CSF-derived EVs also can reflect the severity of AD, which is supported by the observation of significantly higher levels in advanced AD than in early-stage [210]. In addition, miRNAs in CSF-derived EVs have also been recognized as a potential diagnostic tool for AD, although their expression profiles varied across different studies. Importantly, miR-193b and miR-125b-5p are both changed in EVs derived from CSF, serum, and plasma, despite the variation in expression patterns across different sources of EVs [211].

The small size and cell membrane-like structures of EVs facilitate their relatively easy passage across the BBB [212]. Additionally, the continuous flow of CSF may play a role in mediating the diffusion of EVs from the CNS to the periphery [213]. Consequently, EVs can transport pathological proteins from the brain to the bloodstream, preventing their contents from degradation and contamination by blood components. Specific pathological substances carried by neuronal-derived EVs (NDEs) detected in peripheral blood are anticipated to serve as identifiable biomarkers for AD diagnosis and treatment. Furthermore, detecting NDEs in the blood is proved to be feasible as we previously demonstrated high consistency between Aβ42, T-tau, and P-tau181 in NDEs and those from CSF, suggesting NDEs may reflect AD pathological changes in the brain [214]. This becomes particularly crucial in scenarios where biomarker in free form is affected by the peripheral metabolism or when their concentrations in peripheral blood are subtly changed. Notably, two independent studies identified significantly elevated levels of P-tau396, P-tau181, and Aβ42 in plasma NDEs of individuals with AD. The combination of these biomarkers allowed for accurate detection of preclinical AD up to 10 years before the onset of cognitive impairment [215, 216].

As mentioned in the 2.2.3. Synaptic protein section, while synaptic protein in CSF stands as promising biomarkers for AD, most of the synaptic proteins are challenging to measure in blood possibly due to low peripheral expression; NDEs in blood might solve this issue. We previously found a combination of GAP43, SNAP25, neurogranin, and synaptotagmin 1 in plasma NDEs successfully identify preclinical AD 5–7 years before clinical onset [217]. Consistently, the levels of other synaptic proteins including neuronal pentraxin 2, neurexin 2α, GluA4-containing glutamate, and neuroligin 1 detected in plasma NDEs were also significantly decreased in individuals with AD. This reduction is indicative of the progression of dementia, suggesting they may serve as biomarkers for predicting AD 6–11 years before cognitive decline [218]. These studies collectively highlight the potential of plasma neuronal-derived synaptic protein to reflect the degree of synaptic dysfunction in AD.

MiRNAs are an important component in EVs. Alterations in miRNA from blood EVs have been observed in AD, with specific miRNAs like miR-30b-5p, miR-22-3p, and miR-378a-3p identified by Dong et al. to distinguish AD from CU individuals (AUC: 0.88) [219]. Wei et al. observed a reduction in plasma EV miR-223 in AD and correlations with cognitive status and plasma inflammatory marker levels [220]. Our previous research identified a panel of six miRNAs in blood NDEs capable of detecting preclinical AD 5–7 years before cognitive decline [162]. While various studies support the

potential of miRNAs in plasma NDEs for AD prediction or diagnosis, the specific miRNA types showing elevation or reduction vary. Multicenter studies are crucial for validating these findings.

Undoubtedly, EVs derived from diverse sources demonstrate promising potential in the prediction and diagnosis of AD. However, the intricate process of extracting and characterizing EVs presents challenges, necessitating efficient and high-purity extraction while mitigating interference from other EVs.

Challenges and future directions of biofluid biomarkers for AD

In recent years, a paradigm shift has taken place in the AD field, leading to the development of a new diagnostic framework. Aligned with this framework is a novel disease model that initiates with the evaluation of risk factors for primary prevention, advances to screening for early detection and intervention in the disease, proceeds to diagnosis and staging, and concludes with treatments and the monitoring of their effects [221]. Therefore, the role of the biomarker should be addressed. The goal of biofluid biomarker research in AD is to apply these biomarkers in clinical practice. To achieve this goal, Frisoni et al. [222] proposed a five-phase roadmap for the development of AD biomarkers (Figure 9). In the first phase, preclinical exploratory studies aim to identify potential useful biomarkers. The second phase involves the development and validation of clinical assays for biomarkers, assessing their ability to identify individuals with AD. In the third phase, biomarkers should be evaluated in retrospective and longitudinal cohorts to determine their capacity for early identification or diagnosis of AD and define criteria for screening biomarker positivity. The fourth phase involves prospective studies to ascertain the accuracy of core biomarkers in clinical settings. Finally, the fifth phase focuses on clinical implementation, evaluating the reduction in mortality, morbidity, and disability rates associated with biomarker detection. Among the ATX(N) biomarkers, the first and second stages of Aβ42/40, P-tau, and NfL have been largely completed. The first and second phases of GFAP, as well as the third phase for all biomarkers, are currently ongoing. Limited work on the fourth phase has been undertaken, while research for the fifth phase is pending further refinement of the preceding steps [223].

The establishment of the ATX(N) framework holds significant importance for predicting AD risk, enabling early diagnosis and treatment, and advancing clinical research. However, the current ATX(N) classification system does not achieve the consistency required for use in clinical settings [224]. While the ATX(N) framework was discussed as clinical criteria at the recent Alzheimer's Association International Conference (AAIC), it still primarily serves as a research framework. Further research and refinement are necessary before its integration into clinical practice can be realized. Currently, biofluid biomarkers are mainly used for the identification of individuals with preclinical AD and AD progressors at a lower cost than amyloid PET screening. Furthermore, these biomarkers can help improve target engagement and enrollment in randomized controlled trials (Figure 10). These biomarker tests used in a clinical trial to screen participants for amyloid positivity before amyloid PET does not need to be as accurate as a test intended to replace amyloid PET. A relatively crude marker that minimizes false negatives could substantially decrease the number of individuals requiring PET imaging, streamlining the process of participant selection for clinical trials. Future research not only needs to focus on the efficacy of blood biomarkers in the diagnosis and prediction of AD, but also requires long-term longitudinal research to clarify the relationship between biofluid biomarkers, response to treatment, and clinical symptoms. They will continue to play a crucial role in identifying the disease and creating opportunities for new therapeutic advancements.

While blood biomarkers might serve as a non-invasive, low-cost, and relatively convenient method for early diagnosis and disease monitoring of AD, their reliability faces challenges due to systemic and biological factors. Recent assessments in real-world settings, particularly in populations characterized by significant heterogeneity, reveal that comorbidities, such as chronic kidney disease and myocardial infarction, have a considerable impact on AD biomarker levels [76, 225]. Noteworthy biological factors, including age, body mass index, and circadian rhythm, as well as the influence of concomitant medications and lifestyle variables, contribute substantially to both inter- and intra-individual variation [226, 227]. The susceptibility of blood biomarkers to these diverse factors arises from their metabolic interactions in peripheral tissues, which may lead to potential confusion with biomarkers originating from the periphery. The intricate dynamics of AD biomarkers in the blood involve processes such as dysfunction of BBB, dilution within the circulating blood, adherence to blood proteins or cells, and clearance by peripheral organs [143, 228, 229]. Moreover, the origin of pathological proteins in the blood extends beyond the brain to peripheral organs or tissues, which further amplifies the complexity. NDE is a potential

Figure 9: Five-phase framework to develop biomarkers for Alzheimer's disease.

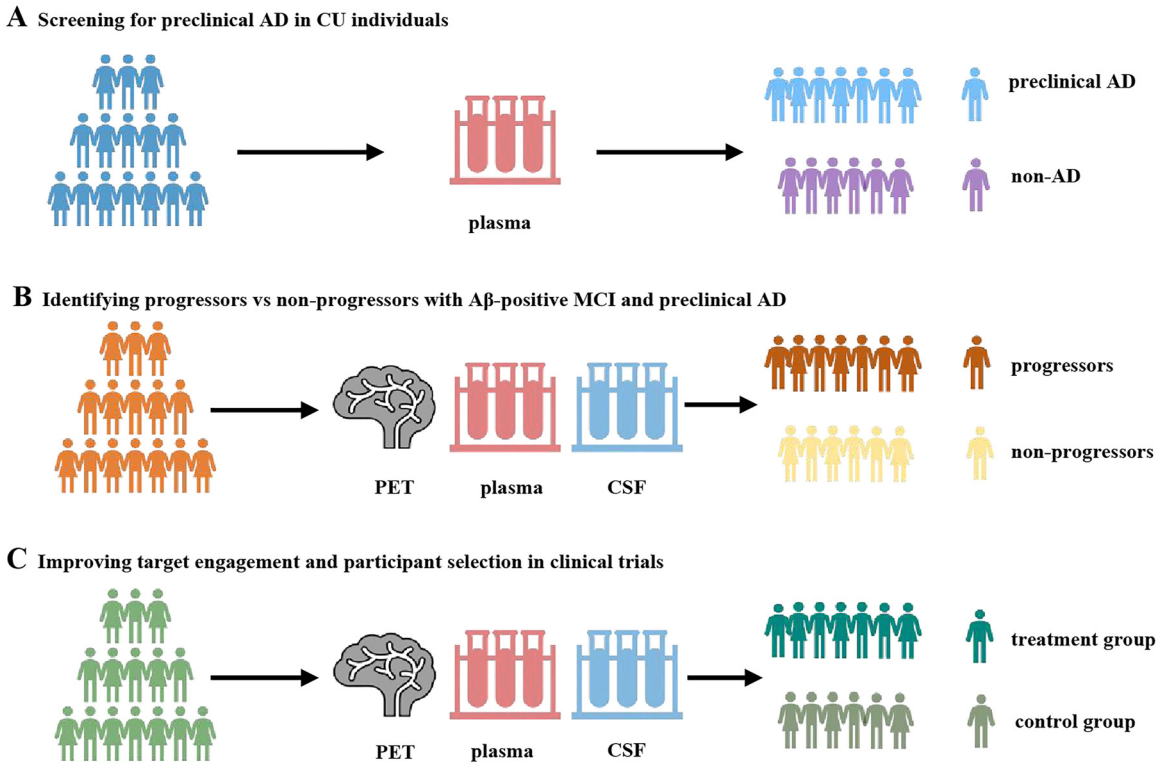

Figure 10: Potential role of AD biomarkers for research purposes. AD biomarkers are used for the identification of individuals with preclinical AD (A) as well as AD progressors (B). They also improve target engagement and enrollment in clinical trials (C). AD, Alzheimer's disease; Aβ, amyloid-β; CSF, cerebrospinal fluid; CU, cognitively unimpaired; MCI, mild cognitive impairment; PET, positron emission tomography.

avenue for regulating the impact of peripheral metabolism; however, the complexity of NDE isolation and characterization poses a practical challenge.

Due to the intricate interaction of biological and systemic factors, it is challenging to define the normal reference ranges and cut-off values for these biomarkers. Real-world studies are imperative for the clinical application of these biomarkers in AD. Various studies, including those in memory clinics and communities [230–232], have explored cutoff values for these biomarkers. While similar studies have been conducted in specialized research cohorts, the performance and cut-off values of these biomarkers in real-world populations, characterized by increased heterogeneity in patient demographics, co-morbidities, and disease presentations, remain to be fully ascertained. Likewise, establishing reference intervals for biomarkers is crucial for interpreting clinical laboratory tests and diagnosing diseases. These intervals represent the range of physiological measurements observed in healthy individuals. Most of the existing biomarker data mainly comes from the European and American populations, and its applicability in the Asian population needs further research and verification. A recent study [233] has preliminarily revealed the reference intervals for plasma Aβ42, Aβ40, T-tau, and P-tau181 measured using Simoa assays in Chinese CU individuals aged 50–89 years. In the future, larger-scale, multicenter research is needed to cover participants of different ages, genders, and races, to better understand the performance of these biomarkers in different populations.

Recently, several new techniques have been developed for the diagnosis of AD. Seed amplification assay has received more attention in the field of neurodegenerative diseases. Using Aβ as a seed, it expands exponentially by a protein-based self-proliferation mechanism. The

amplification power of these assays allows for diagnosis of AD [234]. This method without antibodies may provie a cost-effective choice for AD screening [235]. More studies have shown that Tau and α-synuclein also have potentials to act as seeds [236, 237]. Therefore, seed amplification assays may have potentials for clinical translation.

Even with highly sensitive methods, the results of the AD biomarker are inconsistent across different laboratories. The variability may arise from inconsistency in detection methodologies, data processing, and analytic models, which hinders their use as reliable clinical tools. Therefore, optimization and standardization measures should be addressed to advance the reliability and global implementation of biomarker tests in Alzheimer's and dementia research and diagnosis. The Alzheimer's Association established the Global Biomarker Standardization Consortium to standardize and validate biomarker tests for global clinical practices. Achieving optimization and standardization is a complex and substantial endeavor. Rigorous quality control measures should be implemented to ensure uniformity across diverse laboratories, which involves creating a monitoring tool for assessing biomarker performance between clinical and large research laboratories, utilizing existing testing platforms like MS and immunoassays. This facilitates inter-laboratory comparisons to identify and address variations in measurement techniques and results. The development of universal materials for biofluid biomarkers ensures standardized measures and calibration. Standardizing pre-analytical factors in CSF and blood handling and processing aims to minimize systemic differences in biomarker measurements. Moreover, creating a standardized reporting format for biomarker results, including specifying units of measurement, reference ranges, and other relevant information, supports clinicians in interpreting results accurately. These comprehensive efforts collectively contribute to enhancing the reliability and consistency of biomarker tests in Alzheimer's and dementia research and diagnosis [238, 239].

In summary, biofluid biomarkers have great potential in early diagnosis and disease monitoring of AD, but there are still some challenges and limitations that need to be overcome. Future research will further expand our understanding of these biomarkers, providing more possibilities for early diagnosis and treatment of AD.

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Informed consent: Not applicable.

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