REVIEW ARTICLE

Alzheimer's disease biomarkers and their current use in clinical research and practice

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While blood-based tests are readily available for various conditions, including cardiovascular diseases, type 2 diabetes, and common cancers, Alzheimer's disease (AD) and other neurodegenerative diseases lack an early blood-based screening test that can be used in primary care. Major efforts have been made towards the investigation of approaches that may lead to minimally invasive, cost-effective, and reliable tests capable of measuring brain pathological status. Here, we review past and current technologies developed to investigate biomarkers of AD, including novel blood-based approaches and the more established cerebrospinal fluid and neuroimaging biomarkers of disease. The utility of blood as a source of AD-related biomarkers in both clinical practice and interventional trials is discussed, supported by a comprehensive list of clinical trials for AD drugs and interventions that list biomarkers as primary or secondary endpoints. We highlight that identifying individuals in early preclinical AD using blood-based biomarkers will improve clinical trials and the optimization of therapeutic treatments as they become available. Lastly, we discuss challenges that remain in the field and address new approaches being developed, such as the examination of cargo packaged within extracellular vesicles of neuronal origin isolated from peripheral blood.

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INTRODUCTION

Alzheimer's disease (AD) is a multifactorial neurodegenerative disease that is the leading cause of dementia worldwide [1]. The two neuropathological hallmarks of AD—extracellular amyloid beta (Aβ) deposition and intracellular tau-containing neurofibrillary tangles (NFTs)—begin to change years before symptoms begin, highlighting the need for sensitive and reliable diagnostic tests [2].

The diagnosis of AD has shifted from a syndromal to a biological basis in recent years. As per the clinical guidelines published by the National Institute on Aging and the Alzheimer's Association (NIA-AA) workgroup in 2011, mild cognitive impairment (MCI) and dementia due to AD may be diagnosed based solely on clinical presentation [3, 4]. Biomarkers of Aβ and neurodegeneration, detected via positron emission tomography (PET), structural magnetic resonance imaging (MRI), or in the cerebrospinal fluid (CSF), were only recommended to diagnose preclinical AD or improve the certainty of the MCI or dementia diagnosis [2, 3]. In 2018, the NIA-AA published guidelines for research that shifted AD diagnosis toward a biological paradigm [5]. This document recognizes three biomarker categories for AD—biomarkers of amyloid deposition (A), pathological tau (T), and neurodegeneration (N) (i.e., AT(N))—that can be detected by neuroimaging or in the CSF, but not in plasma [5]. Furthermore, the severity of the disease may be biologically staged with this ATN profile (i.e., from A-T-(N)- to A+T+(N)+) and clinically staged based on the level of cognitive impairment (i.e., from stage 0 (asymptomatic) to stage 6 (severe dementia)) [5].

In 2024, the AA workgroup released updated research guidelines for the diagnosis and staging of AD [6]. Most notably, the guidelines suggest that AD may be diagnosed if any Core 1 biomarker (i.e., amyloid PET, approved CSF biomarkers, and accurate plasma biomarkers) is abnormal [6]. The inclusion of blood-based biomarkers (BBMs) in disease diagnosis and staging is new to the 2024 guidelines, with previous guidelines based solely on clinical presentation, CSF biomarkers, and neuroimaging. Nonetheless, standardized cut-offs and association with clinical prognosis have not been formally established for BBMs [6].

Here, we review recent developments in neuroimaging and fluid biomarkers and their utility in detecting preclinical and clinical AD and monitoring the effects of drug candidates in interventional trials. We also provide a comprehensive list of AD clinical trials that include neuroimaging or fluid biomarkers as endpoints. Since the detection of AD biomarkers in peripheral blood is a quickly developing field, we provide a list of currently available technologies capable of detecting BBMs. Lastly, we discuss the isolation of neuronal-derived extracellular vesicles (NEVs) in the blood and the analysis of their AD-related biomarker content as an avenue toward detecting brain-specific changes in peripheral samples.

NEUROIMAGING BIOMARKERS – MRI

In AD, the earliest site of atrophy is seen in the medial temporal lobe, moving to the parietal, frontal, and cingulate cortices with

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advancing disease [7, 8]. In vivo measurements of brain mass and ventricular volume began in the 1970s with computed tomography, which was used to support a syndromal diagnosis of AD [9]. MRI was subsequently established as a measure of neurodegeneration in regions known to be vulnerable to AD neuropathology [10]. Brain atrophy detected by MRI is related to symptom severity in a specific topographic manner [11]. MRI measures of the entorhinal cortex, superior temporal sulcus, and anterior cingulate can, with moderate accuracy, distinguish between patients with AD, MCI to AD converters, MCI non-converters, and healthy controls [12]. Hippocampal texture and morphology on MRI brain scans have also shown some success in predicting the conversion of MCI to AD [13, 14]. However, AD progresses non-linearly with atrophy rates. The rate of hippocampal atrophy accelerates at a greater rate than cortical atrophy and a deceleration of cortical atrophy has been observed in later AD stages [15, 16]. These variations must be considered when assessing the efficacy of disease-modifying interventions or when providing prognostic information. Further, MRI neuroimaging is mainly based on atrophy and other non-specific features. Non-AD neurodegenerative diseases such as Lewy body dementia and vascular dementia also show increased atrophy rates compared to healthy controls [17]. For these reasons, MRI is useful in a patient's clinical workup but not sufficient to diagnose MCI or dementia due to AD. Instead, MRI may be more useful as a measure of neurodegeneration for biomarker profiling or monitoring treatment effects in clinical trials. Indeed, in a survey of interventional trials of AD drugs (with US Food and Drug Administration (FDA) phase, 3, phase 4, or approved status) listing biomarkers as primary or secondary endpoints, we found that structural or functional MRI was used in 61.8% (Tables 1 and 2; Supplementary Table 1). The use of MRI as endpoint in clinical trials has increased in recent years due to the need to monitor amyloid-related imaging abnormalities (ARIA), a common adverse event associated with amyloid-targeting immunotherapies (Fig. 1) [18].

NEUROIMAGING BIOMARKERS – PET

The development of amyloid PET tracers provided the AD field with a specific biomarker to detect and quantify brain beta-amyloidosis. One of the first amyloid-imaging PET tracers developed was ^{11}C-labeled Pittsburgh Compound-B (PIB) [19]. Post-mortem studies demonstrated that, compared to controls, AD patients show elevated retention of PIB in cortical areas consistent with Aβ deposition patterns [19, 20]. Subsequent longitudinal studies have shown conflicting results, with some reporting a positive association between PIB retention and progression to AD in MCI patients, and others finding no differences in PIB retention between controls, MCI, and AD patients [21–23]. Three second-generation ^{18}F-labeled amyloid tracers with longer half-lives have been FDA- and European Medicines Agency (EMA)-approved for clinical use: ^{18}F-florbetapir, ^{18}F-florbetaben, and ^{18}F-flutemetamol [24]. These tracers correlate with brain amyloid burden and successfully predict AD progression in patients with MCI [25–29].

Much progress has been made in the development and application of tau PET tracers. Tau imaging may be a stronger predictor of cognitive dysfunction than Aβ imaging, especially in normal cognition and amyloid-positive MCI [30–32]. In 2020, the FDA approved the first PET tracer, ^{18}F-Flortaucipir, for imaging tau in cognitively impaired adults [33]. ^{18}F-Flortaucipir-PET may predict longitudinal changes in cognitive impairment more strongly than MRI or amyloid PET [32]. While this tracer overcomes some limitations of other first-generation tau tracers, such as nonspecific white matter binding and high bone uptake, it still faces problems with off-target binding and detecting preclinical AD [33]. Second-generation tau PET tracers (e.g., PI-2620, MK-6240, and RO-948) are superior in terms of off-target binding but are still in the process of achieving clinical validity and utility [34, 35].

Despite advances in PET tracers, PET remains very expensive and is not part of the routine clinical assessment of AD in most of the world. Additionally, amyloid PET cannot differentiate AD from other amyloid-positive diseases such as Lewy body dementia [36]. The half-lives of PET tracers are relatively short (e.g., ~20 min for ^{11}C and 110 min for ^{18}F) so they must be produced and used in the same facility [24]. Nonetheless, PET is commonly used as an endpoint in 36.8% of AD interventional trials that list fluid or neuroimaging biomarkers as primary or secondary outcomes (Tables 1 and 2; Supplementary Table 1). PET is also the most frequently used endpoint in trials of amyloid immunotherapies (Fig. 2).

FLUID-BASED BIOMARKERS

The core AD biomarkers that can be detected in biofluids include those in the Aβ (e.g., Aβ42/Aβ40) and tau (e.g., phosphorylated tau (p-tau)) categories. Non-core biomarkers include those belonging to the categories of neurodegeneration (e.g., neurofilament light chain (NfL)) and inflammation (e.g., glial fibrillary acidic protein (GFAP)). The core fluid biomarkers can be used to diagnose, stage, and monitor AD, while non-core biomarkers can complement disease staging and monitoring or identify co-pathologies [6]. Given the unspecific nature and broader applicability of biomarkers such as NfL and GFAP [37, 38], our focus here will be on fluid Aβ- and tau-related biomarkers.

Of the two main fluid sources of biomarkers for AD, CSF and blood, CSF offers the advantage of having direct contact with the brain and thus being enriched in central nervous system (CNS)-specific proteins. Standard immunochemical assays are sensitive enough and commonly used to quantify Aβ42, total tau (t-tau), and p-tau in the CSF. To date, the Elecsys® (Roche) CSF tests for Aβ42, p-tau181, and t-tau and the Lumipulse® (Fujirebio) CSF test for Aβ42/Aβ40 have been approved by the FDA for diagnostic use. Biomarker ratios measured by both tests show similarly high concordance with amyloid PET status and clinical diagnosis [39, 40]. Moreover, CSF biomarkers are used in 32.4% of AD interventional trials that list fluid or neuroimaging biomarkers as endpoints (Tables 1 and 2; Supplementary Table 1).

Detecting CNS-derived biomarkers in the blood is far more challenging. Due to the blood-brain barrier (BBB) and the high blood to CSF volume ratio, their concentration in the periphery is usually a small fraction of what can be measured in the CSF. Advancements in ultrasensitive immunoassay technologies have only recently allowed for the reliable detection of CNS-derived proteins in the blood. One such technology is the Single Molecule Array (SiMoA®), an automated bead-based enzyme-linked immunosorbent assay (ELISA) with an innovative detection step. Using femtoliter wells to confine individual beads, it can detect signals produced by a single captured molecule of the analyte, increasing sensitivity dramatically in well-optimized assays. SiMoA can detect Aβ42, Aβ40, p-tau, and t-tau in the blood at sub-femtomolar concentrations [41, 42]. Numerous assays developed on the SiMoA platform, such as the ALZpath, Eli Lilly, and Janssen SiMoA kits for plasma p-tau217 and the ADx Neuroscience SiMoA kit for plasma p-tau181 have shown excellent performance detecting amyloid positivity [43, 44]. Several other types of immunoassays currently in use also rely on ultrasensitive detection steps, including those based on chemiluminescence (Fujirebio Lumipulse®; Siemens ADVIA®) or electrochemiluminescence (ECLIA; Roche Elecsys®; Meso Scale Discovery). In parallel, mass spectrometry (MS)-based techniques have also achieved detection of BBMs with high diagnostic accuracy. MS typically requires much more complex sample processing, but it does not rely on an antibody or enzymatic reaction for its detection step. For certain analytes,

Table 1. Interventional trials in AD listing biomarkers as primary or secondary endpoints.

Drug/intervention	Type[a]	ID(s) at clinicaltrials.gov	Total Sample Size[b]	Biomarkers used as primary or secondary endpoints
Donepezil	Cholinesterase inhibitor	NCT00408525; NCT02087865; NCT00403520; NCT00267163	435	fMRI (brain activation patterns; BOLD); MRI (hippocampal volume); PET (brain metabolism)
Donepezil and Memantine	Cholinesterase inhibitor and NMDA antagonist	NCT00768261	39	MRI (hippocampal volume)
Galantamine	Cholinesterase inhibitor	NCT00236574	974	MRI (brain atrophy)
Galantamine and Memantine	Cholinesterase inhibitor and NMDA antagonist	NCT01921972	232	vMRI (whole brain and hippocampal atrophy)
Memantine	NMDA antagonist	NCT00800709; NCT00334906; NCT00255086; NCT00862940	395	MRI (brain atrophy and hippocampal volume); CSF Biomarkers; PET (^{18}F-FDG)
Suvorexant	Small molecule	NCT04629547	200	Amyloid-PET; Tau-PET; CSF Aβ, p-tau, and t-tau; Plasma Aβ, p-tau, and t-tau
Carvedilol	Small molecule	NCT01354444	29	CSF Aβ
NE3107	Small molecule	NCT04669028; NCT05227820	339	fMRI (Glutathione on fMRI MRS, Neurite Orientation Dispersion Density Imaging [DTI-NODDI], Arterial Spin Labeling, functional connectivity by BOLD, neurovascular coupling)
Nilotinib	Small molecule	NCT05143528	1275	Amyloid-PET; Tau-PET; Blood and CSF Aβ42, Aβ40, t-tau, and p-tau
Resveratrol	Small molecule	NCT01504854	119	vMRI (whole brain volume); CSF Aβ40
Simufilam	Small molecule	NCT04388254; NCT04994483; NCT04079803; NCT05026177; NCT03748706	2110	MRI (whole brain, ventricular and hippocampal volume); Amyloid-PET; Tau-PET; CSF p-tau, p-tau181, p-tau217, t-tau, Aβ42, NfL, neurogranin, YKL-40, soluble TREM2, IL-6, albumin, IgG, HMGB1 and other potential CSF biomarkers; Plasma p-tau181, p-tau217, and NfL
TRx0237	Tau-related small molecule	NCT01689246; NCT01689233; NCT03446001	2289	MRI (whole brain)
ALZ-801	Amyloid-related small molecule	NCT04693520; NCT04770220 NCT06304883	669	vMRI (hippocampal volume, ventricular volume and cortical thickness); CSF p-tau181, p-tau217, Aβ40, Aβ42, NfL, t-tau, sTREM2, YKL-40 and neurogranin; Plasma p-tau181, Aβ40, Aβ42, p-tau217, GFAP, and NfL
ALZT-OP1 (cromolyn and ibuprofen)	Amyloid-related small molecule and NSAID	NCT04570644	56	CSF and plasma Aβ42, Aβ40, Aβ38 t-tau, NfL, GFAP, p-tau231, TNF-α, TGF-β1, CD33, TREM2, and neurogranin
AR1001	Phosphodiesterase inhibitor	NCT05531526	800	CSF and plasma biomarkers
Escitalopram	SSRI	NCT00702780; NCT05004987	164	MRI (whole brain and hippocampal volume); CSF Aβ40 and Aβ42
GENUS device	Device	NCT05776641; NCT04055376; NCT05655195	265	sMRI (whole brain and ventricular volume, cortical thickness); fMRI (brain functional connectivity and structure measured by changes in white matter/BOLD; Amyloid-PET (PiB); Tau-PET (MK-6240); CSF amyloid and tau; Blood Aβ42/Aβ40 ratio, Aβ42, Aβ40, p-tau, and NfL
GammaSense Stimulation System	Device	NCT03556280; NCT05206305; NCT03661034	100	fMRI (cerebral network function); Amyloid-PET and CT
Sensory Stimulation System (GS120)	Device	NCT05637801	530	MRI (whole brain and hippocampal volume)

Table 1. continued

Drug/intervention	Type[a]	ID(s) at clinicaltrials.gov	Total Sample Size[b]	Biomarkers used as primary or secondary endpoints
Continuous positive airway pressure (CPAP)	Device	NCT01962779; NCT02474251; NCT04905238; NCT05094271 NCT05988385	863	MRI (white matter integrity and hippocampal volume); Amyloid-PET, Tau-PET; Plasma Aβ40, Aβ42/Aβ40, p-tau181, p-tau217, and NfL; CSF Aβ42, Aβ42/Aβ40, p-tau, and t-tau
Docosahexaenoic acid (DHA)	Supplement	NCT03613844	368	MRI (functional and structural connectivity)
Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)	Supplement	NCT04972643	163	MRI (total brain volume)
Lipoic Acid plus Omega-3 fatty acids	Supplement	NCT01780974	42	MRI (white matter volume)
Mediterranean diet and Omega-3	Supplement	NCT03841539	209	MRI (whole brain and hippocampal volume)
Axona	Supplement	NCT01122329	17	PET (regional cerebral blood flow)
Vitamin E, Vitamin C, Alpha-lipoic Acid, and Coenzyme Q	Supplement	NCT00117403	75	CSF and plasma Aβ42 and Aβ40

Drugs/interventions targeting AD and currently in phase 3, phase 4, or FDA approved were identified from the alzforum.com database. Clinicaltrials.gov was searched for each drug/intervention (and alternative names). The search was limited to Condition: "Alzheimer's Disease" and Study type: "Interventional." From these results, inclusion criteria were interventional trials with fluid and/or neuroimaging biomarkers listed as an endpoint. Terminated and withdrawn trials and trials with incomplete information were excluded. Search was completed in May 2024.

[a]See Table 2 for immunotherapy trials listing biomarkers as endpoints.

[b]Total sample size reported is the sum of participants for all trials conducted or ongoing for each drug/intervention. Participants and biomarkers used are distributed among the listed trials. For a more detailed resource see Supplementary Table 1.

Table 2. Biomarkers used in AD immunotherapy trials.

Drug	Type	ID(s) at clinicaltrials.gov	Total Sample Size[a]	Biomarkers used as primary or secondary endpoints
Aducanumab	Amyloid-related immunotherapy	NCT02434718; NCT04241068; NCT05310071	3229	MRI (ARIA-E and ARIA-H); Amyloid-PET; Tau-PET
Solanezumab	Amyloid-related immunotherapy	NCT00904683; NCT00905372; NCT01148498; NCT02008357; NCT04623242	3439	vMRI (ventricular volume); sMRI (whole brain, hippocampal and cortical volume, cortical thickness); Amyloid-PET (PiB and Florbetapir); Tau-PET (Flortaucipir); PET (brain metabolism by FDG); CSF Aβ40, Aβ42, p-tau181, tau and NfL; Plasma NfL, Aβ40, Aβ42, Aβ fragment-2, and modified Aβ species
Lecanemab	Amyloid-related immunotherapy	NCT01767311; NCT03887455; NCT04468659	4162	vMRI (whole brain, ventricular and hippocampal volume); Amyloid-PET; Tau-PET; CSF Aβ42, t-tau, and p-tau
Lecanemab and E2814	Amyloid-related immunotherapy and Tau-related immunotherapy	NCT05269394	168	Amyloid-PET; Tau-PET; CSF p-tau, t-tau and NfL
Donanemab	Amyloid-related immunotherapy	NCT02624778; NCT04437511; NCT05108922; NCT04640077; NCT03367403; NCT05738486; NCT05508789	4723	vMRI (whole brain volume, ARIA-E, ARIA-H); Amyloid-PET (Florbetapir); Tau-PET (Flortaucipir)
Remternetug	Amyloid-related immunotherapy	NCT05463731	600	Amyloid-PET

See Table 1 for the complete search information. For a more detailed resource see Supplementary Table 1.

[a]Total sample size reported is the sum of participants for all trials conducted or ongoing for each drug/intervention. Participants and biomarkers used are distributed among the listed trials.

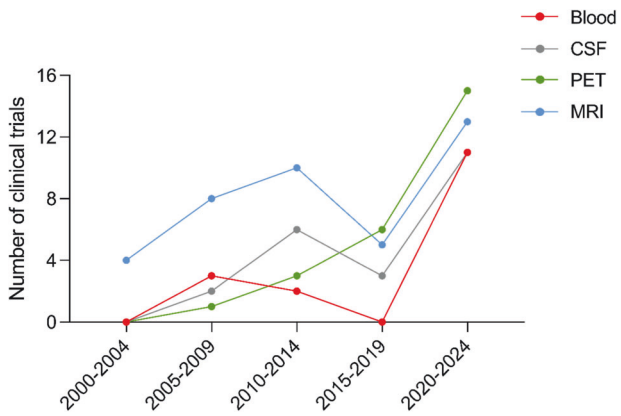

Fig. 1 The number of AD-clinical trials listing biomarkers as primary or secondary endpoints from 2000 to 2024. Data obtained from information compiled in Supplementary Table 1. Figure created in GraphPad Prism version 10.2.3.

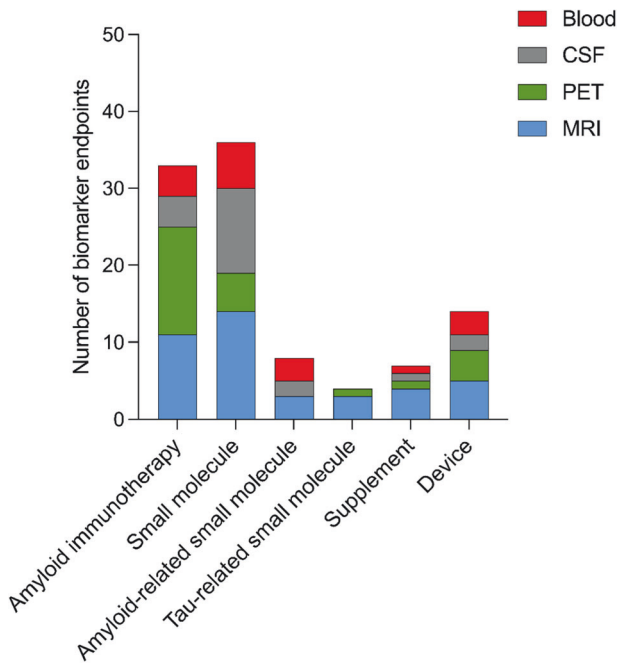

Fig. 2 The number of primary or secondary biomarker endpoints in interventional trials across phase 3, phase 4, or FDA-approved AD therapeutics. Data obtained from information compiled in Supplementary Table 1. Figure created in GraphPad Prism version 10.2.3.

particularly plasma Aβ42/Aβ40, immunoprecipitation coupled to MS (IP-MS) has shown better performance than immunoassays for detecting brain Aβ burden [45]. To facilitate inter-assay and inter-laboratory consistency, the Standardization of Alzheimer's Blood Biomarkers group has developed a standard operating procedure for pre-analytical sample handling for some of the most common BBMs across several platforms [46].

A selection of assay kits currently capable of detecting AD-related biomarkers in peripheral blood, including SiMoA, IP-MS, ECLIA, Microfluidic ELISA, Single Molecule Counting®, and Chemiluminescence assays are summarized in Table 3. Some of the listed kits are already available on the diagnostics market as laboratory developed tests (LDTs). Of note, all were introduced before the US FDA's final rule on LDTs (which significantly increases the agency's oversight and quality system requirements for these tests) was published. In the following sections, we review the recent advancements that have been achieved in fluid biomarker detection with these and other technologies.

CSF BIOMARKERS – Aβ

Aβ was first shown to be secreted into the CSF in 1992 [47]. Around the same time, the aggregation-prone, 42 amino acid form of Aβ (Aβ42) was determined to be the species of Aβ earliest deposited in plaques [48]. The CSF level of Aβ42 is thus reduced upon plaque formation and is a biomarker of a pathologic state associated with amyloid deposition. It has been suggested that the CSF concentration of Aβ42 begins to decline up to 25 years prior to the onset of AD [49]. Moreover, around 90% of patients with MCI and CSF Aβ42 positivity develop AD within 9–10 years, indicating CSF Aβ42 can predict disease progression [50]. A large meta-analysis by Olsson and colleagues—comprising almost 30,000 AD patients and controls—found a strong association between CSF Aβ42 and AD, reporting a CSF Aβ42 AD to control ratio of 0.56 [51].

The ratio of Aβ42 to Aβ40 (a less amyloidogenic, soluble isoform) is often used. CSF Aβ42/Aβ40 levels measured by immunoassays and IP-MS show concordance with amyloid deposition as determined by amyloid PET [52, 53]. In the Swedish BioFINDER cohort, the Aβ42/Aβ40 ratio better correlated with amyloid-PET and differentiated AD from other neurodegenerative disorders compared to Aβ42 alone [54]. Conversely, CSF Aβ42 alone may have superior prognostic ability than the Aβ42/Aβ40 ratio. In the China Cognition and Aging Study, a difference in CSF Aβ42 levels between AD patients and healthy controls appeared 18 years prior to diagnosis, compared to 14 years for CSF Aβ42/Aβ40 [55]. CSF p-tau/Aβ42, measured by the Elecsys® or Lumipulse® immunoassays, also shows high concordance with amyloid PET [56, 57].

Finally, while post-mortem neuropathology confirmation is rare in fluid biomarkers studies, it has been established for CSF Aβ42. In one such study, Strozyk and colleagues showed an association between lower CSF Aβ42 and increased plaque deposition in the neocortex and hippocampus, examining 155 autopsy samples [58].

CSF BIOMARKERS – TAU

Total tau levels in the CSF can be used to estimate the extent of neurodegeneration in AD. The first ELISA protocol for quantifying CSF t-tau was published in 1993 and reported significantly higher CSF t-tau levels in AD patients [59]. In fact, the ratio of CSF t-tau between AD and controls was 2.54 in the meta-analysis by Olsson and colleagues [51]. However, increases in CSF t-tau also occur in acute conditions such as stroke, brain injury, and in disorders without amyloid or tau pathology, such as Creutzfeldt-Jakob disease [60–62]. As more of a 'state marker' of neuronal injury, t-tau is less specific for AD than Aβ42 or p-tau, and thus is often evaluated within a ratio to other biomarkers. For instance, t-tau/Aβ42 in the CSF strongly correlates with amyloid PET [63, 64].

Since NFTs primarily consist of p-tau, CSF p-tau is consistently increased in AD [51]. Unlike t-tau, p-tau is specific to AD and other tauopathies and remains low in most non-AD neurodegenerative disorders [51, 62]. Many studies have shown CSF p-tau correlates strongly with the severity of NFTs, hippocampal volume, and cortical amyloid deposition [65–67].

The three main isoforms of p-tau measured in the context of AD are p-tau181, p-tau217, and p-tau231. Although p-tau181 is the most thoroughly examined and routinely used isoform, recent evidence suggests that CSF p-tau217 can differentiate AD from other neurodegenerative diseases and controls with higher sensitivity and specificity [68]. Further, CSF p-tau217 correlates more strongly with tau PET and longitudinal changes in p-tau217 are greater than p-tau181 [68]. While CSF p-tau217 has the largest

Table 3. Select technologies and kits capable of measuring relevant AD biomarkers in peripheral blood.

Technology	Test name	Analyte	Manufacturer/Developer	Availability
Single molecule array	SiMoA® pTau181 V2.1/Lucent AD	pTau181	Quanterix	LDT and RUO commercial kit
	SiMoA® NfL V2	Neurofilament light chain		LDT and RUO commercial kit
	ALZPath® Plasma pTau217	pTau217	Quanterix/ALZPath	LDT and RUO commercial kit
	Plasma pTau217+	pTau217 and pTau212	Quanterix/Janssen	LDT
	Plasma pTau217	pTau217	Quanterix/Eli Lilly	Not commercially available
IP-MS	Precivity AD®	Amyloid-β1-40, 1-42, and ApoE4	C2N Diagnostics	LDT
	Precivity AD® 2	Amyloid-β1-40, 1-42, and pTau217/Tau217		LDT
ECLIA	Elecsys® Amyloid Plasma Panel	pTau181 and ApoE4	Roche	Not commercially available
	S-PLEX® Human Tau (pT217) Kit	pTau217	Meso Scale Discovery	RUO commercial kit
	S-PLEX® Human Neurofilament L Kit	Neurofilament light chain		RUO commercial kit
Microfluidic ELISA	Ella® Simple Plex Human NF-L	Neurofilament light chain	Bio-Techne	RUO commercial kit
Single molecule counting	SMC® Human NF-L High Sensitivity Immunoassay Kit	Neurofilament light chain	Merck	RUO commercial kit
Chemiluminescence	Lumipulse® G pTau181 plasma	pTau181	Fujirebio	RUO commercial kit
	Lumipulse® G NfL blood	Neurofilament light chain		RUO commercial kit
	Neurofilament Light Assay	Neurofilament light chain	Siemens Healthineers	LDT
Proximity ligation assay	NULISA pTau217	pTau217	Alamar Biosciences	RUO commercial kit

Compiled using information publicly available to the authors as of May 2024.

fold-change between AD and non-AD, it has been suggested that p-tau231 attains abnormal levels earliest in the AD continuum [69]. Nonetheless, the performance of any analyte heavily depends on the quality of the antibody pair used in the immunoassay. In at least some cases, differences in diagnostic performance seen among analytes may be due to antibody quality rather than reflecting pathophysiological processes.

Assessing these CSF biomarkers in combination would further aid preclinical and differential diagnosis. For instance, the CSF p-tau/Aβ42 demonstrated a sensitivity of 88% and specificity of 100% in the differentiation of AD from other dementias, confirmed in post-mortem studies [70]. In the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort, p-tau/Aβ and t-tau/Aβ most accurately predicted clinical decline in MCI patients over 24 months [64].

CSF BIOMARKERS – NOVEL/ATYPICAL TARGETS AND LIMITATIONS

CSF tau assays that measure p-tau205 and the microtubule binding region (MTBR) of tau have shown promising results in tracking tau pathology. The ratio of p-tau205/tau205 in the CSF, determined by IP-MS, more strongly correlates with tau PET than CSF p-tau181 [71]. CSF MTBR containing the residue 243 (MTBR-243), identified by IP-MS, strongly correlates with tau PET and longitudinal increases in insoluble tau [72]. Since these tau species appear later in the disease process (i.e., after p-tau181, 217, and 231), the 2024 AA guidelines categorize these biomarkers as Core 2, which may be useful for biological staging and inform the rate of disease progression [6].

In parallel to the core AD biomarkers, atypical targets and their relation to AD have long been investigated in the CSF. For instance, synaptic proteins are altered in the CSF of preclinical AD patients, preceding symptoms or CSF elevations in t-tau or p-tau [73]. Several groups have described altered levels of neurotransmitters, such as noradrenaline and dopamine, in the CSF of AD patients [74, 75]. Our group has previously demonstrated that the CSF level of irisin—an exercise-induced hormone—positively correlates with CSF Aβ42 and cognitive performance [76, 77]. Markers of glial activation and neuroinflammation in the CSF are also increased in AD and associate with CSF tau, cognitive dysfunction, and cortical thinning [78]. Investigation of biomarkers outside of the established AT(N) framework is crucial for the discovery of novel neuropathological processes and may be relevant in future multi-analyte algorithms to diagnose AD.

An important limitation to the use of CSF biomarkers in clinical practice is that it requires an invasive lumbar puncture, which may cause reluctance in physicians and patients [79, 80]. Common complications include back pain and headache, while rare complications include infections, cerebral hematoma, and cerebral venous thrombosis [81]. Furthermore, CSF acquisition is not a standard clinical procedure; it is restricted to specialized clinics and personnel, and obtaining ethical approval to collect CSF for research or clinical trials can be difficult. It is also important to note that CSF biomarkers reflect the rate of biomarker production and clearance at one-time point [5]. An altered CSF biomarker suggests a pathological state associated with amyloid deposition or NFT formation, unlike neuroimaging biomarkers which can directly measure amyloid or tau load [6].

BLOOD-BASED BIOMARKERS – Aβ

Plasma Aβ42/Aβ40 levels decrease with advancing clinical stage and can be used to identify Aβ pathology early in the AD continuum [82]. As mentioned previously, IP-MS methods have so far performed better than immunoassays in predicting amyloid PET status [45, 83]. IP-MS measures of Aβ40/Aβ42 correlate with CSF Aβ42 and clinical staging and successfully predict amyloid PET

status [84, 85]. Further, the risk of converting to amyloid PET positivity was 15-fold greater for individuals with abnormal Aβ42/Aβ40 levels [86]. The IP-MS-based PrecivityAD (C2N) test for Aβ42/Aβ40 was the first LDT for plasma AD biomarkers to reach the market in 2020. In 2022, the current version of this test, which is aided by age and ApoE proteotype determination (Table 3), predicted amyloid-PET status in cohort samples totaling 686 participants, with an AUC of 0.88 [87]. The inclusion of age and ApoE proteotype—two established risk factors for brain amyloidosis—improved the accuracy of plasma Aβ42/Aβ40 for identifying amyloid-PET status [88].

One major issue encountered with plasma Aβ42/Aβ40 is the small difference seen between Aβ-positive and Aβ-negative individuals. The Aβ42/Aβ40 ratio is reduced by only 8–15% in plasma compared to 40–60% in CSF due to peripheral expression of Aβ [89]. Although less reliant on antibody performance, IP-MS is also subject to the pre-analytical variables that affect immunoassays in general and, as highlighted by data from the Alzheimer's Association quality control program, the inter-laboratory variability for AD biomarker assays can be quite high (20–30%, published CSF data), especially for Aβ [90]. The small effect size that must be measured in plasma Aβ assays, together with pre-analytical factors known to disproportionally impact Aβ (such as analyte stability and surface adsorption) make the performance of plasma Aβ42/Aβ40 more so impacted by intra- and inter-assay variation than other plasma biomarkers [91]. Plasma Aβ42/Aβ40 may thus have additional hurdles to overcome before widespread adoption. The search for CNS-specific forms of Aβ in the plasma, such as by isolating neuronal-derived vesicles (discussed below), may help overcome the biological factors that currently limit the robustness of Aβ as a BBM for AD.

BLOOD-BASED BIOMARKERS – TAU

Plasma p-tau has emerged as a promising biomarker for AD that may reflect both Aβ and tau pathology [92]. Due to limitations with detecting Aβ mentioned above, many non-MS-based tests have focused on p-tau, particularly p-tau181, p-tau217, and p-tau231. Available p-tau immunoassays use antibodies against phosphorylated sites on the N-terminal or mid-domain of tau, as these fragments are more soluble and more frequently secreted compared to aggregation-prone C-terminal tau [93].

Plasma p-tau181 repeatedly correlates with tau and amyloid PET and CSF p-tau181 [94–97]. This biomarker is also increased in preclinical AD stages and further increases with advancing clinical stage [94–97]. Plasma p-tau181 can differentiate AD from cognitively unimpaired older adults, MCI, and non-AD neurodegenerative diseases [94–96, 98, 99]. Higher baseline concentrations of plasma p-tau181 are associated with future development of AD pathology in individuals with normal cognition and MCI [94, 96, 98, 100]. Notably, plasma p-tau181 can predict conversion to AD up to eight years prior to death and, when combined with Aβ42 and NfL, can predict AD eight years prior to clinical onset [98, 101]. In a large clinical-based cohort, higher baseline levels of blood p-tau181, quantified using SiMoA, were associated with an accelerated time to AD onset [100]. Blood p-tau181 was also a better predictor of 5-year AD risk than blood Aβ42/Aβ40, t-tau, and NfL [100]. Furthermore, longitudinal measurements have shown low intra-individual variability in plasma p-tau181, indicating that it may be useful in measuring treatment responses [102].

Plasma p-tau217 can also track CNS changes across the AD continuum and may outperform plasma p-tau181 in several analyses. In a head-to-head comparison of 10 plasma p-tau assays, IP-MS for p-tau217 performed better than all other plasma p-tau assays for predicting Aβ status and progression from MCI to AD [103]. The immunoassays for p-tau217 (Janssen and Eli Lilly) and p-tau181 (ADx Neuroscience, Washington University) also performed well for both outcomes [103], and a novel ECL-based plasma p-tau217 assay developed by Meso Scale Discovery (MSD) outperformed p-tau181 for differentiating AD and controls [104]. Notably, the fold change in plasma p-tau217 between AD and controls is consistently higher than in plasma p-tau181 [104–106]. Plasma p-tau217 also correlates with amyloid and tau PET, CSF and plasma p-tau181, CSF p-tau 217, and with amyloid plaques and tau-containing NFTs in studies of autopsy-confirmed AD [105, 107–109]. Amyloid PET positive, tau PET negative individuals can be differentiated from controls with plasma p-tau217 levels, suggesting that plasma p-tau217 may change before tau aggregation is detectable by PET [110]. In fact, plasma %p-tau217 (p-tau217/non-phosphorylated tau) analyzed by MS was equivalent to FDA-approved CSF tests in determining amyloid PET and superior in determining tau PET [111]. Moreover, individuals with MCI who progressed to AD dementia up to six years later had higher baseline levels of plasma p-tau217 than non-converters [112]. Plasma p-tau217 not only performs well in clinical staging, but it may outperform plasma p-tau 181 in differentiating AD from normal cognition and other neurodegenerative disorders [106, 107, 112].

Plasma p-tau231 has gained traction as a biomarker for AD pathology, showing a correlation with CSF p-tau231, tau PET, and amyloid PET [113]. Plasma p-tau231 measured by Quanterix's SiMoA can distinguish AD from non-AD neurodegenerative disorders, amyloid PET negative controls, and amyloid PET negative MCI [113]. However, plasma p-tau231 detected by ADx Neuroscience's SiMoA kit could not identify AD or asymptomatic amyloid PET positivity [114]. Furthermore, plasma p-tau231 did not outperform p-tau181 in differentiating AD from non-AD neurodegenerative disorders and performs inferiorly to CSF p-tau 231 in detecting amyloid PET positivity [113, 115]. While plasma p-tau231 and p-tau217 are similarly associated with longitudinal changes in amyloid PET, concentrations of plasma p-tau231 may increase earlier than amyloid PET thresholds are reached or increases in plasma p-tau181 are detected [113, 116]. Despite these promising results, longitudinal measures of plasma p-tau231 failed to predict cognitive decline in preclinical AD [117]. While plasma p-tau231 may reach a significant threshold early in AD pathology, succeeding cognitive decline may be better indicated by other p-tau isoforms. Plasma p-tau231 may detect AD pathology before amyloid or tau PET, but larger-scale longitudinal studies and assay standardization are warranted.

Since the majority of plasma total-tau originates from peripheral sources, higher CNS t-tau in AD is unlikely to cause significant increases in plasma t-tau [105]. Recently, an antibody that selectively binds brain-derived tau (TauJ.5H3) was developed [118]. Initial studies report that blood-based brain-derived tau has similar diagnostic performance to CSF t-tau in distinguishing AD from controls and other neurodegenerative disorders, and shows weaker correlations with age, comorbidities, and race/ethnicity than other BBMs [118, 119]. Another promising avenue for detecting CNS-specific t-tau in the periphery is through the isolation of neuronal-derived vesicles from the blood (discussed below).

USE OF BBMS IN CLINICAL PRACTICE AND INTERVENTIONAL TRIALS

BBMs have potential utility for early and accurate AD diagnosis, monitoring of disease progression and treatment effects, and screening for clinical trial eligibility. Compared to neuroimaging and CSF biomarkers, BBMs are minimally invasive, cost-effective, highly scalable, and available outside specialized centers and in low-resource settings [120, 121]. In clinical practice, BBMs have the potential to address the high rates of misdiagnosis and underdiagnosis in AD caused by current testing limitations, which in turn will allow for the optimization of therapeutic intervention [120] In remote settings without access to blood-collection

facilities, AD biomarkers may even be quantified by SiMoA in dried blood spots [122].

BBMs can also improve the design of clinical trials investigating AD drugs. PET and CSF biomarkers are commonly used to screen and recruit large numbers of individuals for trials, in a costly, time-consuming process, where patients with or without AD-specific pathology are selected. Karikari et al. estimate cost-savings of 58% in the recruitment phase of AD clinical trials by pre-screening participants with a blood p-tau test prior to PET scans compared to screening all participants with a PET scan alone [93].

In the past 5 years, the use of BBMs as endpoints in AD interventional trials has increased substantially (Fig. 1). BBMs are included in 26.5% of trials that list biomarkers as primary or secondary endpoints (Tables 1 and 2; Supplementary Table 1) and most frequently in trials of small molecule therapeutics (e.g., Simufilam, Suvorexant, AR1001) (Fig. 2).

Detecting amyloidosis with BBMs will be critical for the success of anti-Aβ immunotherapies (Table 2), as amyloid-positive individuals, who stand to benefit from anti-Aβ treatment, must be selected and monitored [6]. Furthermore, it is important to recruit pre-amyloid individuals (e.g., with plasma p-tau231) so disease-modifying therapies can be initiated before irreversible downstream pathology occurs [113]. Given the importance of tracking Aβ in response to Aβ immunotherapy and the usefulness of BBMs for this purpose, it was notable to find that, among trials for anti-Aβ immunotherapies, only those for Solanezumab included BBMs as primary or secondary endpoints (Table 2). While virtually all large-scale AD immunotherapy trials in recent years have measured and published data on BBMs, few have listed them as endpoints. PET and CSF biomarkers are often prioritized, despite their higher cost and risks, with BBMs being used only for exploratory analyses. This is an interesting reflection of each sponsor's confidence in BBMs at the time of study design and is likely to shift as the biomarker field progresses.

INTERPRETATION OF BBMS

Numerous factors, such as age, genotype, and sex are known to have an influence on AD pathology and biomarker levels and should be considered when interpreting BBM data [88, 123]. As BBMs are incorporated in clinical settings, diagnostic workups and cutoff values can be expected to be refined based on demographic factors and genotype (e.g., C2N's plasma Aβ42/Aβ40 tests incorporate age and ApoE proteotype). Additionally, certain medical conditions such as previous stroke, diabetes, high body mass index (BMI), dyslipidemia, and chronic kidney disease (CKD) have already reported to influence BBM levels [124–127]. In community-based samples, CKD was associated with higher levels of plasma p-tau181, p-tau217, Aβ40, Aβ42, NfL, and total tau [124–127]. While this particular effect may be caused by altered clearance of plasma proteins, others may be a result of direct influence on AD pathology, which is not yet completely understood. The exact extent to which comorbidities can impact the reliability of reference values and the interpretation of BBM data still needs to be determined. In a recent report, Mielke and colleagues found that excluding individuals with either CKD, myocardial infarction or stroke significantly impacted the determination of a normal range for plasma p-tau in community-based samples [124]. In contrast, using cohort samples, other authors found that creatinine (as a proxy for kidney function) and BMI were associated with BBM levels, but did not alter their interpretation in a meaningful way [128].

The impact of population diversity, socioeconomic disparities, and lifestyle on biomarker levels and their interpretation have also only begun to be investigated. Multi-ethnic American studies show conflicting results on the impacts of race/ethnicity on BBM levels [108, 125, 126, 129]. While Mohs et al. found different BBM levels among non-Hispanic Blacks, non-Hispanic Whites, and Hispanics, race/ethnicity did not impact the ability of BBMs to predict amyloid PET positivity [129]. In Chinese cohorts, large studies have confirmed the excellent diagnostic performance of BBMs seen in Western populations, despite differences in APOE4 prevalence, diet, and lifestyle [101, 130]. Contrarily, little data is available on AD biomarkers in developing countries where large longitudinal cohorts are less common. In one notable study including 746 participants of Caribbean-Hispanic ethnicity, 91% of them from the Dominican Republic, authors distinguished AD from cognitively normal controls using plasma p-tau181 with a performance similar to that shown for US- and Europe-based cohorts [131]. While work is still ongoing to locally validate BBMs and account for possible confounding factors, the conservative use of cutoff values advised by AA guidelines, with the inclusion of an indeterminate zone, may be crucial [6].

NEURONAL DERIVED EVS AS A SOURCE OF BBMS

Extracellular vesicles (EVs) are cell-derived membranous structures that vary in their biogenesis, release, composition, and interaction with cells. Numerous physiological roles have been proposed for EVs, particularly as a mode of intercellular communication. Regardless of their physiological relevance, EVs hold promise as a source of biomarkers. EVs carry a sample of the proteins, lipids, and nucleic acids found in their cell of origin [132]. Neuronal-derived EVs (NEVs) may carry membrane proteins specific to neurons, allowing for their identification and immunoprecipitation, as well as AD-specific biomarkers.

Evidence suggests that NEVs can cross the BBB and accumulate in the blood and that their concentration and cargo reflect the pathophysiological changes in AD [133, 134]. Varied levels of Aβ, tau, synaptic proteins, insulin resistance-associated proteins, and RNAs have been detected in NEVs isolated from the plasma of AD patients compared to MCI or healthy controls [135–138]. Furthermore, there are myriad targets that show potential as AD biomarkers in the CSF (e.g. irisin) but are not neuron-specific and thus not relevant when measured in whole plasma. However, the isolation of NEVs from the blood may allow for the detection of CNS-specific changes of non-CNS specific biomarkers.

ISOLATING NEVS

Several methods for isolating EVs exist, differing in specificity, yield, and ease of use. Ultracentrifugation (UC) was the first to be described and has been considered the gold standard method of EV isolation, but several alternative methods have attracted interest and investment in recent years [134]. UC results in high purity, especially when combined with a density gradient (dgUC), but is time-consuming, requires expensive equipment and large volumes of starting material, and has low recovery [134, 139]. Ultrafiltration (UF) and size-exclusion chromatography (SEC) separate samples according to their size with high ease of use but also low recovery [134, 139]. Polymer-based precipitation can isolate EVs using water-excluding polymers, such as polyethylene glycol (PEG), which precipitate the less soluble EVs [134, 139]. Precipitation is an easy, high-yield method with commercially available kits such as ExoQuick ULTRA or miRCURY [134, 139]. The main disadvantages are the co-precipitation of blood proteins, which reduces purity, and the interference from polymers in downstream assays [139]. Generally, a combination of isolation methods is used to improve EV purity and yield.

Importantly, EV isolation based on physical properties alone cannot differentiate NEVs from peripherally derived EVs. Combining these methods with immunoaffinity isolation targeting EV-specific surface proteins is the only technique devised thus far to obtain NEVs. A common protocol to isolate NEVS involves an antibody targeting the L1 cell adhesion molecule (L1-CAM), a neuronal protein that is sorted to EVs [140]. This method has been

used to isolate NEVs containing Aβ42, t-tau, p-tau181, NfL, and synaptic proteins [141]. Although L1-CAM is expressed by neurons, it is also expressed by peripheral tissues, raising important concerns about the specificity of NEVs obtained through L1-CAM IP. Notably, in a 2021 report, authors were unable to find L1-CAM immunoreactivity in EV-enriched SEC fractions isolated from human plasma, despite using an ultrasensitive SiMoA assay as readout [142].

In a continued effort to find targets for isolating NEVs, the ATPase Na + /K+ transporting subunit alpha 3 (ATP1A3) was recently tested, showing promising results [143]. ATP1A3 is a neuron-enriched protein found in EVs isolated from human brain tissue and plasma. Although ATP1A3 expression patterns (as reported by proteinatlas.org) suggest that IP using ATP1A3 might show much higher neuronal specificity than L1-CAM, ATP1A3 expression is also not exclusive to neurons, allowing for the possibility of contamination from peripheral EVs.

Further, low recovery is a common problem with EV IP techniques. In addition to partial or inconsistent elution of EVs from beads, the low concentration of NEVs in human plasma—evidenced by the very low starting concentration of neuron-specific proteins in whole blood—makes isolating useful amounts of pure NEVs challenging [139]. Likewise, detecting protein targets in pure NEV samples can be challenging even for current ultrasensitive methods. Many authors—such as You et al. when describing the novel ATP1A3 method—have resorted to single-event techniques to detect biomarkers in plasma NEVs (e.g., fluorescent nanoparticle tracking analysis, nanoparticle flow cytometry, single-EV visualization by dSTORM) [143]. These, however, are often semi-quantitative in nature and are much further from diagnostic implementation than traditional immunoassays or IP-MS.

More recently, affinity-based proteomic techniques (e.g. Olink, NULISA), which are based on oligonucleotide-conjugated antibodies quantified via polymerase chain reaction or next-generation sequencing, have allowed for a combination of attomolar sensitivity and very high multiplexing (Table 3) [144, 145]. Although the field has so far focused primarily on high-sensitivity, single-target assays, these novel technologies may open to door to multi-analyte biomarker discovery, particularly in samples such as NEVs.

AD-RELATED BIOMARKERS IN NEVS

Several studies have used ExoQuick followed by L1-CAM IP to enrich NEVs from plasma and subsequently quantify their AD-related biomarker content [146–149]. Levels of Aβ42, t-tau, and p-tau181 in plasma NEVs were shown to increase with advancing clinical stage and to correlate with CSF biomarkers [146–149]. Fiandaca and colleagues found that NEV Aβ42, t-tau, and p-tau181 could predict the development of AD up to 10 years prior to clinical diagnosis [146]. Similarly, Winston and colleagues report that NEV Aβ42 and p-tau181 could predict patient conversion from MCI to AD in the following three years [147].

As mentioned above, the recently described ATP1A3 NEVs also show potential as sources of AD biomarkers. By detecting pan-Aβ immunoreactivity in immobilized NEVs, authors could discern AD patients from MCI and controls in a total of 30 cohort samples [143]. These results indicate that core AD biomarkers found in blood-derived NEVs may be of diagnostic and prognostic value. Nonetheless, these studies are limited in size and standardized methods to isolate NEVs have not been established.

NEVs may also carry non-core AD biomarkers. Markers of insulin signaling (e.g., p-Ser IRS-1, p-Tyr IRS-1) are altered in NEVs of AD patients compared to controls [150]. These differences are identifiable up to 10 years before the clinical onset of AD [150]. Additionally, NEV levels of synaptic proteins, such as synaptotagmin, synaptophysin, and neurogranin, may be reduced years before AD onset [136]. Differential expression of micro-RNAs in NEVs has distinguished patients with AD from those with stable MCI, MCI that progresses to AD, and other neurodegenerative disorders [138, 151]. Furthermore, a transcriptomic analysis of EVs from post-mortem brain tissue identified an enrichment of inflammation-associated mRNAs and depletion of synaptic signaling mRNAs in AD compared to healthy controls [152]. It is to be expected that novel NEV biomarkers will be discovered in the coming years as the field continues to develop.

While NEVs are an exciting avenue in the field of BBMs of AD, they are in their infancy compared to the clinically validated core AD biomarkers. The current lack of consensus and standardized protocols regarding NEV isolation methods prevents NEVs from living up to their potential as a source of AD-related biomarkers. Establishing reliable and reproducible NEV isolation and detection methods will encourage the discovery of novel targets and improve our ability to measure CNS-specific changes in the blood.

CONCLUSION

The field of AD biomarkers has developed rapidly in the last decade, closely following technological advancements. The emergence and optimization of ultrasensitive immunoassays and MS techniques are now very close to establishing an equivalence between CSF and blood biomarkers. In turn, core AD biomarkers quantified in these biofluids have reached diagnostic and prognostic accuracies similar to those of PET. Widespread adoption of blood as a source of core AD biomarkers will benefit both clinical practice and interventional trials, due to the technical and financial ease of sampling blood over CSF. Most recently, NEVs isolated from the blood have shown promising results for investigating core and atypical AD biomarkers. Not unlike what happened for BBMs over the past decade, an effort for standardizing NEV protocols is warranted before NEVs can be seen as a robust and reliable source of AD biomarkers.

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AUTHOR CONTRIBUTIONS

FGDF and TRH conceptualized the article. TRH wrote the original draft. TRH and LES created the figures and tables. TRH, LES, FTM, and FGDF reviewed and edited the manuscript. All authors approve the submission of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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