



# Salivary Biomarkers for Alzheimer's Disease and Related Disorders

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Received: July 16, 2019 /  
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## ABSTRACT

The search for accessible and cost-effective biomarkers to complement current cerebrospinal fluid (CSF) and imaging biomarkers in the accurate detection of Alzheimer disease (AD) and other common neurodegenerative disorders remains a challenging task. The advances in ultra-sensitive detection methods

has highlighted blood biomarkers (e.g. amyloid- $\beta$  and neurofilament light) as a valuable and realistic tool in a diagnostic or screening process. Saliva, however, is also a rich source of potential biomarkers for disease detection and offers several practical advantages over biofluids that are currently examined for neurodegenerative disorders. However, while this may be true for the general population, challenges in collecting saliva from an elderly population should be seriously considered. In this review, we begin by discussing how saliva is produced and how age-related conditions can modify saliva production and composition. We then focus on the

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data available which support the concept of salivary amyloid- $\beta$ , tau species and novel biomarkers in detecting AD and alpha-synuclein ( $\alpha$ -syn) in Parkinson's disease (PD).

**Keywords:** Alzheimer's disease; Amyloid- $\beta$ ; Biomarkers; Parkinson's disease; Saliva; Tau

### Key Summary Points

Peripheral biomarkers for Alzheimer's disease and related disorders have taken centre stage with ultra-sensitive assays developed for amyloid- $\beta$ , tau species and neurofilament light.

Saliva is an alternative peripheral source for accessible and non-invasive disease biomarkers.

Total tau, phosphorylated tau, amyloid- $\beta$  and alpha-synuclein proteins are all detectable in saliva and preliminary investigations have shown potential diagnostic utility. Novel candidates (e.g. lactoferrin) could be used for early disease detection.

Standardisation of saliva collection and storage methods are greatly needed to advance this field further.

## INTRODUCTION

The diagnosis of probable Alzheimer's disease (AD) and other common neurodegenerative disorders remains primarily reliant on a clinical assessment outside the specialist clinic. However, an AD diagnosis can now be supported by positron emission tomography (PET) and cerebrospinal fluid (CSF) biomarkers that detect the hallmark-underlying pathologies of amyloid- $\beta$  (A $\beta$ ) [1] and tau [2]. One of the many challenges that the dementia community face is the detection of the pre-symptomatic phase of the AD using non-invasive, widely accessible and disease relevant biomarkers. In recent times, blood biomarkers have taken

centre stage, with measurements of A $\beta$  species [3–6], the axonal injury marker neurofilament light (NfL) [7, 8] and phosphorylated tau on threonine 181 (P-tau181) [9] showing much promise. There are now international efforts underway to progress these biomarkers to be applicable for clinical use [10]. Without question, a blood biomarker is far more attainable for population screening than PET or CSF; however, it still faces certain logistical limitations. Saliva has been proposed as a potential easily collectable source of biomarkers for the diagnosis and risk assessment for a range of pathological conditions occurring not only in the mouth but also systemically [11]. Disorders that have been targeted include periodontal and oral mucosal diseases, oral, pancreatic, lung and breast cancer, together with diabetes and hepatitis C infection [12]. The major salivary glands secrete saliva in response to cholinergic innervation from cranial nerves VII and IX, which are monitored by the autonomic nervous system (ANS) [13]. This relation to the nervous system suggests that these gland secretions may represent various physiologies of the nervous system. Indeed, central nervous system (CNS) proteins are secreted into the saliva in an age-dependent manner [14, 15]. Furthermore, via passive diffusion, active transport or microfiltration proteins can pass from the blood into the saliva [13, 16]. For these reasons, saliva may contain novel biomarkers for CNS injury or be an alternative and more accessible source in sampling AD-related biomarkers that are currently being eagerly pursued in blood. In this review, we summarise the current evidence for salivary biomarkers in detecting AD and related disorders, while considering important factors related to saliva production, composition and collection in older adults. This article is based on previously conducted studies and does not contain any studies with human participants or animals performed by any of the authors.

## PRODUCTION OF SALIVA AND IMPACTS OF AGING, LOCAL AND SYSTEMIC PATHOLOGY

Saliva collection generally represents a pooled sample of the products from three pairs of major salivary glands (submandibular, sublingual and

parotid), supplemented by numerous minor salivary glands. In addition, this material includes microorganisms, their by-products, host cells from epithelial surfaces, and other components released from the gingival crevices around teeth (gingival crevicular fluid). Therefore, it is important to understand the processes of production and regulation of saliva, and how this may differ in populations, especially older adults, since any variations may impact on the relative validity of proposed biomarkers.

Saliva production varies between different glands, not only in production volume but also in composition [17]. The exocrine glands contain secreting epithelial cells located in structures called acini as the terminal element of the ductal tree within the gland. Acinar cells will produce either dilute saliva with low levels of mucins or mucin-rich secretion. Whilst the parotid glands largely contain non-mucinous acinar cells, submandibular glands are mixed, whereas the sublingual glands and the minor glands located throughout the mouth are largely mucin forming. The ducts and acini are surrounded by myoepithelial cells, a rich blood supply and dense innervation by parasympathetic and sympathetic nerves. Consequently, the steady unstimulated saliva flow occurring throughout the day is made up primarily of glands producing mucinous saliva; 68% from submandibular and sublingual, and around 4% from numerous minor glands. However, when salivary output is stimulated (e.g. during mastication via mucosal and periodontal mechanoreceptors, activated taste bud receptors, smells or thermal changes [18–21]), the percentage contribution from parotid saliva rises from around 1/4 to over 1/2 [22] even though other glands also increase their production. Autonomic innervation is supplied to many (not just acinar) salivary gland cells, although there is some variation between glands in the extent of sympathetic input [23, 24]. In animal models sympathetic stimulation produces a different protein-rich saliva, and mucin production from mucous glands seems to be initiated by parasympathetic stimulation [25]. As a result, whole salivary composition is believed to be influenced by stimulation and the rate of secretion, although

this may be mainly related to dilution effects of molecules from non-glandular sources. Many components of freshly secreted saliva are actively secreted by acinar and ductal cells and so these will not be altered, with the exception of sodium, chloride and bicarbonate salts, which are more concentrated in stimulated saliva following autonomic stimulation and changes in flow rates [26]. Transport mechanisms include exocytosis of protein storage granules, ion pumps, transport proteins and vesicular proteins [17]. Whole saliva will additionally contain some blood components (including immunoglobulins such as IgG) which have entered as a transudate within the gland system as well as the supplemental materials listed above.

Saliva flow is generally considered to be frequently reduced in older individuals. However, there has been some disagreement on the role of direct age-related changes compared to indirect effects such as the influence of medications that are commonly experienced by older adults. Medications which are known to increase the risk of hyposalivation include anticholinergics, proton pump inhibitors, antidepressants, phenothiazines, benzodiazepines, antihistamines, diuretics and various antihypertensives [27, 28]. Statins have also been reported as being responsible for oral dryness in a small interventional study [29]. Hence salivary volume and composition could further be influenced as an outcome, complication or side effect from systemic disease and treatment (including local radiotherapy and systemic medications). These effects may be direct or indirect, mediated by other pathways such as dehydration, which may itself be related to impaired fluid intake, emesis, diarrhoea or polyuria.

The current opinion based on primary research and systematic reviews [30, 31] is that there is an age-related decrease in salivary gland function and thus in xerostomia, and that medication merely enhances this further. However, the primary research is somewhat contradictory for age-related changes in both unstimulated [32–35] and in stimulated [35–38] salivary flow rate, and large inter-individual differences between participants within study groups have been reported. Nagler and

Hershkovich [39] have shown how there is not only a change in volume but also composition (including ions, immunoglobulins and other proteins) with aging, although their participants were taking a range of medications to maintain health. However, this was confirmed in a large cross-sectional study by Dodds et al. [40]. Consequently, it would seem that saliva flow is impaired in older populations, and that aging can also modify the composition of saliva produced. It would seem necessary to accommodate this into study design and data analysis if possible when determining the value of biomarkers in populations of differing or mixed ages.

Finally, psychogenic causes, such as depression, anxiety or stress, can also result in xerostomia mediated by sympathetic stimulation. In cases of AD, stroke or other neurological pathology, patients may complain of dry mouth in the presence of reduced or normal salivary secretion due to altered production [41]. Hence self-reported dryness in some patients may not by itself be an exclusion factor for attempted salivary sampling—it would seem wise to confirm this with intraoral examination even if ultimately collection is unsatisfactory. The impact of these challenges is illustrated by the observations recently reported by Galloway et al. [42] that approximately 1/3 of participants in the recent UK Biobank study were unable to produce an adequate saliva sample, and that this was noticeably increased amongst those with a range of systemic diseases. Indeed only 57.7% of participants with a diagnosis including dementia, AD or Parkinson's diseases (PD) were able to produce an adequate sample for archiving (Table 1). Therefore, it appears that results from salivary analysis, whilst offering excellent potential as a research and management tool, may have to be considered carefully allowing for age, concurrent medication and other risk factors. Each of these factors may independently have an impact on data and these variables should at least be recorded in clinical studies. Likewise, there may be issues with obtaining adequate samples for some participants, especially the elderly and/or those with significant systemic disease.

## CURRENT EVIDENCE OF SALIVARY BIOMARKERS FOR ALZHEIMER'S DISEASE AND RELATED DISORDERS

### Amyloid- $\beta$

The accumulation of A $\beta$  plaques, the foremost hallmark of AD, begins 15–20 years prior to the clinical onset of cognitive and functional decline [43]. Visually assessed and quantitative positron emission tomography (PET) studies using  $^{11}\text{C}$  and  $^{18}\text{F}$  A $\beta$  tracers have consistently verified increased retention in AD dementia and preclinical AD when compared with elderly controls, with the binding typically elevated in the frontal, cingulate, precuneus, striatum, parietal and lateral temporal cortices [1]. This regional distribution visualised by A $\beta$  PET closely follows the categorisation of A $\beta$  burden in post-mortem studies [44]. More recently, it has been confirmed that CSF A $\beta$ 42 is also a reliable marker of amyloid plaque pathology in the brain (as determined at autopsy or through A $\beta$  PET studies [45]), especially when measured in a ratio with CSF A $\beta$ 40. The assessment of A $\beta$  by PET or CSF immunoassays is now included in research criteria for AD [46] as well as in the biological definition of the disease [47]. A $\beta$  peptides can be readily measured in plasma utilising ultra-sensitive immunoassays [5, 6] or targeted mass spectrometry (MS) [3] and emerging evidence suggests that a decreased A $\beta$  peptide ratio can identify cerebral A $\beta$ -positive individuals with high sensitivity and specificity (Table 2).

The widespread peripheral expression of the amyloid precursor protein (APP) would make it seemingly likely that A $\beta$  peptides are present in saliva via interaction with blood or degradation of buccal cells [48]. Indeed, a collection of studies have shown that salivary A $\beta$ 42 is detectable and increased in AD [49–53], with A $\beta$ 40 remaining unchanged. Lee et al. [49] have so far demonstrated the most encouraging findings of salivary A $\beta$ 42. In this study, thioflavin S was added to the salivary sample at collection to inhibit the aggregation of A $\beta$  and a twofold increase in salivary A $\beta$ 42 concentration was observed in AD compared to controls and

**Table 1** Number of UK Biobank participants with selected health conditions (non-cancerous) who attempted to produce a saliva sample at baseline assessment (selected data, adapted from Galloway et al. [42])

Participant groups (selected)	Samples attempted	Samples adequate (%)
All participants in UK BioBanks	120,175	84,721 (70.5)
Diabetes mellitus	743	4787 (64.4)
Cerebrovascular disease	2723	1725 (63.3)
Ischemic heart disease	6839	4474 (65.4)
Alzheimer's and Parkinson's disease	284	164 (57.7)
Clinical depression	7632	5006 (65.6)
Chronic obstructive pulmonary disease	1245	739 (59.4)
Asthma	14,442	10,040 (69.5)
Inflammatory bowel disease	3524	2354 (66.8)
Rheumatoid arthritis	1447	919 (63.5)
Osteoporosis	2652	1721 (64.9)
Acute renal failure/Chronic kidney disease	679	415 (61.1)

patients with PD. An earlier study has also demonstrated an increase of salivary A $\beta$ 42 in AD compared to PD but these significant findings were confined to mild AD and not observed in severe AD [50]. These small pilot studies point towards salivary A $\beta$ 42 being a potentially specific and early biomarker for AD. However, it must be noted that A $\beta$ 42 has also been demonstrated to be unchanged or not detectable in studies using similar antibody-based methodologies [54, 55]. These inconsistent results are similar to the early reports of A $\beta$ 42 in plasma which also utilised standard sandwich enzyme-linked immunosorbent assays [56]. The application of ultra-sensitive immunoassays (e.g. Simoa or SMx-Pro) or targeted MS for salivary A $\beta$  may render more consistent results. Furthermore, no study has yet to compare salivary A $\beta$ 42 to A $\beta$ -positive confirmed AD or tested the salivary A $\beta$ 42/A $\beta$ 40 ratio which may improve the diagnostic accuracy.

### Tau

An aggregated and phosphorylated form of tau protein is the major constituent of neurofibrillary tangles (NFT) in AD. First-generation tau

PET ligands show elevated retention in patients with AD, as compared to controls, with uptake patterns matching histopathology staging schemes for tau [2]. The second-generation tau ligands (e.g. [ $^{18}$ F]MK-6240, [ $^{18}$ F]RO-948, and [ $^{18}$ F]GTP-1) appear to be superior and this is due to their reduced off-target binding to iron, neuromelanin and monoamine oxidase B [2]. The core CSF biomarkers for AD include total tau (T-tau) and tau phosphorylated at threonine 181 (P-tau). Consistently, clinical studies have shown an increase of these biomarkers in AD and in patients with mild cognitive impairment (MCI) [56]. However, the concentrations of these markers are within the normal range in the majority of other primary tauopathies. In blood, increases in plasma T-tau reach statistical significance in large AD cohorts [57, 58]. However, the substantial overlaps between control groups and the poor correlations with CSF levels certainly limit plasma T-tau as being diagnostically useful [57]. The reason for this may be explained by the rapid degradation of tau in blood with a reduced half-life compared to CSF [59]. Preliminary data demonstrating increases of P-tau [9] and



**Table 2** Summary of salivary biomarkers investigated in Alzheimer's disease

Biomarker	Proteomic platform	Observations in AD	References
AChE	Ellman colorimetric method	Decreased (versus healthy controls, $p < 0.005$ )	Sayer et al. [80]
		No change	Bakhtiari et al. [81]
		No change	Boston et al. [82]
A $\beta$ 42	Sandwich ELISA	Increased (versus healthy controls, $p < 0.05$ )	Sabbagh et al. [52]
		Increased (versus healthy controls, $p < 0.001$ )	McGeer et al. [53]
		Increased (versus healthy controls, $p < 0.001$ )	Lee et al. [49]
		Increased (versus healthy controls, $p < 0.05$ )	Bermejo-Pareja et al. [50]
	Luminex ELISA	Not detectable	Lau et al. [54]
Nanobead ELISA	Increased (versus healthy controls, $p < 0.001$ )	Kim et al. [51]	
A $\beta$ 40	Sandwich ELISA	No change	Bermejo-Pareja et al. [50]
	Nanobead ELISA		Kim et al. [51]
Lactoferrin	Sandwich ELISA	Decreased (versus healthy controls, $p < 0.001$ )	Carro et al. [79]
T-tau	Simoa	No change	Ashton et al. [61]
	Sandwich ELISA	No change	Lau et al. [54]
	Luminex ELISA	No change	Shi et al. [55]
P-tau	Luminex ELISA	Increased p181/T-tau (versus healthy controls, $p < 0.05$ )	Shi et al. [55]
	Western blot	Increased s396/T-tau (versus healthy controls, $p < 0.05$ )	Pekeles et al. [62]

N-terminal tau [60] in the blood of patients with AD offer an exciting avenue for blood biomarkers.

We have recently reported the largest AD salivary biomarker study which consisted of 160 age-matched controls, 68 participants with MCI, and 53 participants with AD [61]. Furthermore, this was the first study to utilise an ultrasensitive single molecule array (Simoa) assay to measure T-tau concentration in saliva. However, we found no statistically significant differences across these diagnostic groups. Interestingly, T-tau was detectable in more than 95% of all participants and the concentration was approximately fourfold higher than that in

plasma (cohort mean, 11.85 ng/L). In addition, we demonstrated that there was no association of salivary T-tau with cognitive scores or atrophy measures. This study confirms the preliminary findings by two earlier studies [54, 55]. Shi and colleagues confirmed the presence of tau in saliva by identifying five unique peptide sequences by MS. A subsequent Luminex assay (based on mid-domain tau antibodies) demonstrated no change of salivary T-tau. However, a 1.3-fold increase of the salivary P-tau181/T-tau was observed in AD [55]. In a similar manner, Lau et al. demonstrated no change in salivary T-tau but increases in P-tau [54]. A study using semi-quantitative proteomics investigated a

number phosphorylation sites in saliva [62]. In a ratio with T-tau, phosphospecific antibodies for serine 396 demonstrated increases in AD, while serine 404 was increased in frontotemporal dementia (FTD). However, no association could be drawn with brain atrophy or CSF measures of tau. Furthermore, this study reported no change of P-tau181 in patients with AD. While data on salivary T-tau is consistently negative, phosphorylated tau species in saliva may have greater utility. However, as with P-tau in blood, ultra-sensitive assays are still being refined for reliable detection of P-tau in peripheral fluids.

The presence of tau in saliva may have a number of sources. In a similar manner to A $\beta$ , interactions with the bloodstream or release from buccal cells is likely given that elevated tau isoform levels in isolated buccal cells have been shown in AD [63]. Furthermore, salivary tau may originate from acinar cells that express tau mRNA [64] or nerves that innervate the salivary glands.

### Alpha-synuclein

The presynaptic neuronal protein alpha-synuclein ( $\alpha$ -syn) is the most abundant protein of the intracellular aggregates found in Lewy bodies in PD, dementia with Lewy bodies (DLB) and in glial cytoplasmic inclusions of multiple system atrophy [65]. However, a truncated form of  $\alpha$ -syn (termed non-amyloid component or NAC) is also a major component in the A $\beta$  plaques in patients with AD [66]. CSF biomarkers for  $\alpha$ -syn pathology have been a challenging task but a general trend has emerged of decreased total  $\alpha$ -syn and an increase in phosphorylated and oligomeric  $\alpha$ -syn in PD [67, 68], while level of total  $\alpha$ -syn is markedly increased in AD and correlates with T-tau [69], which may suggest that total  $\alpha$ -syn largely reflects neurodegeneration or synaptic damage [70].

Post-mortem submandibular gland biopsies are positive for Lewy-type  $\alpha$ -syn in patients with PD but not in healthy subjects [71], and this finding has made salivary  $\alpha$ -syn one of the most investigated salivary biomarkers in neurodegeneration. Yet, both increases and decreases in

salivary total  $\alpha$ -syn have been reported [72–74]. On the other hand, increases in oligomeric  $\alpha$ -syn [75] and oligomeric  $\alpha$ -syn/total  $\alpha$ -syn ratio have been demonstrated to be specific for PD [74, 76]. To date, there has been no investigation of salivary  $\alpha$ -syn in AD. In addition to  $\alpha$ -syn, a mutation in the protein DJ-1 has been associated with rare early onset familial autosomal recessive PD. Preliminary data appear to show increases in PD [72, 77, 78] and correlate with motor disability [78].

### Lactoferrin

Lactoferrin is an antimicrobial peptide with A $\beta$ -binding properties which targets bacteria and is abundantly present in human saliva. Carro et al. [79], using an initial MS discovery and subsequent ELISA validation, have shown a substantially decreased concentration of lactoferrin in patients with MCI and patients compared to aged-matched controls, while patients with PD exhibited higher concentrations than controls. In patients with AD, unstimulated lactoferrin was also positively correlated with Mini-Mental State Examination (MMSE) score and CSF A $\beta$ 42 while being negatively correlated to T-tau. In a follow-up study, a concentration cut-off (7.43  $\mu$ g/mL) successfully discriminated people with AD from controls participants. Finally, lactoferrin may have potential in early disease detection. In a longitudinal study, 78% of control participants that presented with “AD-like” concentrations of lactoferrin (< 7.43  $\mu$ g/mL) converted to an MCI or AD diagnosis within 5 years of the baseline collection. None of the control participants with “normal” lactoferrin (> 7.43  $\mu$ g/mL) changed clinical status within the same time frame.

### Acetylcholinesterase

In AD, acetylcholinesterase inhibitors (AChE-I) are the primary drug prescribed for symptom management which mechanistically encourage an increase in acetylcholine (AChE) neurotransmitter release into the synapse cleft.

The activity of AChE in saliva can be measured by the Ellman colorimetric method,

where decreases are seen with age but also further reduced in people with AD compared to aged-matched controls [80–82], albeit not all studies reaching statistical significance. In addition to these findings, within the AD population, AChE is significantly decreased further in AChE-I non-responders when compared to AChE-I responders [80]. In PD, a single report demonstrates an increase in AChE activity compared with age-matched controls, which was an indicator of disease progression as indexed by the Hoehn and Yahr scale [83].

## CONCLUSIONS

In the last decade, there has been considerable advancement in the detection of AD-related biomarkers in non-invasive peripheral materials, such as blood. This remarkable and rapid success has posed a new challenge—can other peripheral sources be utilised for AD-related biomarkers, namely saliva? The data currently available shows that A $\beta$ 42, A $\beta$ 40 and multiple tau species are all detectable in saliva using conventional immunoassays. While salivary A $\beta$ 40 and T-tau are not related to clinical AD disease, increases in A $\beta$ 42, phosphorylated tau species and decreases in lactoferrin may have potential. Yet, how salivary A $\beta$ 42 relates to brain amyloid metabolism, to CSF or plasma levels of A $\beta$ 42, or to brain amyloidosis evaluated by A $\beta$  PET status is not known. On the other hand, the antimicrobial peptide lactoferrin has been shown to correlate well with AD CSF biomarkers and to detect preclinical AD. In PD, multiple forms of  $\alpha$ -syn have been investigated, with oligomeric  $\alpha$ -syn being the most promising for disease diagnosis. Although the non-invasive nature of saliva is an attractive attribute there remain important considerations. Firstly, standardisation in collection (e.g. stimulated versus unstimulated), pre-processing (e.g. centrifugation speeds) and storage (e.g. addition of sodium azide) is currently lacking. While the majority of studies included in this review analysed unstimulated whole saliva, there are very clear distinctions in proteome content between unstimulated and stimulated collections from different glands. It is currently not

known how the concentrations of A $\beta$ , tau and other biomarkers discussed in this review differ between sublingual, parotid, whole unstimulated and stimulated saliva or if their presence in saliva is simply from blood. Secondly, while the successful collection of blood and CSF from a consenting participant is driven by a physician, the access to unstimulated saliva is solely dependent on the participant. This is likely to create large variabilities in the material collected and therefore stimulated saliva could be a preferred method due to the simplicity of the collection method. As previously mentioned, there is substantial evidence available to demonstrate that elderly patients, particularly suffering with dementia, find it difficult to produce an adequate saliva sample. For these reasons, a saliva biomarker would be more suitable for the preclinical phase of disease and not for individuals with significant cognitive impairment. At this moment in time, the limited data in large well-characterised and, in some cases, conflicting reports means we are unable to conclude this with any real certainty. Nonetheless, the current research reviewed in this article cautiously indicates the potential of saliva as a non-invasive biomarker source at the preclinical phase of the disease which should be further investigated to determine the reliability of such biomarkers in detecting disease pathology or monitoring disease progression.

## ACKNOWLEDGEMENTS

**Funding.** No funding or sponsorship was received for this study or publication of this article.

**Authorship.** All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

**Disclosures.** Nicholas J. Ashton is funded by the Wallenberg Centre for Molecular and Translational. Henrik Zetterberg is a Wallenberg



Academy Fellow supported by grants from the Swedish Research Council (#2018-02532), the European Research Council (#681712), Swedish State Support for Clinical Research (#ALFGBG-720931) and the UK Dementia Research Institute at UCL. Henrik Zetterberg has served at scientific advisory boards for Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. Kaj Blennow is supported by the Torsten Söderberg Foundation, Stockholm, Sweden. Kaj Blennow has served as a consultant or at advisory boards for Alector, Alzheon, CogRx, Biogen, Lilly, Novartis and Roche Diagnostics, and is also a co-founder of Brain Biomarker Solutions in Gothenburg AB. Mark Ide has nothing to disclose.

**Compliance with Ethics Guidelines.** This article is based on previously conducted studies and does not contain any studies with human participants or animals performed by any of the authors.

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