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The Past and the Future of Alzheimer's Disease Fluid Biomarkers

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Review

The Past and the Future of Alzheimer's Disease Fluid Biomarkers

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Abstract. Following the development of the first methods to measure the core Alzheimer's disease (AD) cerebrospinal fluid (CSF) biomarkers total-tau (T-tau), phosphorylated tau (P-tau) and the 42 amino acid form of amyloid-β ($A\beta_{42}$), there has been an enormous expansion of this scientific research area. Today, it is generally acknowledged that these biochemical tests reflect several central pathophysiological features of AD and contribute diagnostically relevant information, also for prodromal AD. In this article in the 20th anniversary issue of the *Journal of Alzheimer's Disease*, we review the AD biomarkers, from early assay development to their entrance into diagnostic criteria. We also summarize the long journey of standardization and the development of assays on fully automated instruments, where we now have high precision and stable assays that will serve as the basis for common cut-off levels and a more general introduction of these diagnostic tests in clinical routine practice. We also discuss the latest expansion of the AD CSF biomarker toolbox that now also contains synaptic proteins such as neurogranin, which seemingly is specific for AD and predicts rate of future cognitive deterioration. Last, we are at the brink of having blood biomarkers that may be implemented as screening tools in the early clinical management of patients with cognitive problems and suspected AD. Whether this will become true, and whether it will be plasma $A\beta_{42}$, the $A\beta_{42/40}$ ratio, or neurofilament light, or a combination of these, remains to be established in future clinical neurochemical studies.

Keywords: Alzheimer's disease, amyloid, biomarkers, cerebrospinal fluid, neurogranin, plasma, tau

This paper in the 20th anniversary issue of the *Journal of Alzheimer's Disease* is a review on the development of cerebrospinal fluid (CSF) biomarkers, from early assay development to the current status with fully automated assays and the highest level of standardization, with focus on the most important, but also most troublesome, Alzheimer's disease (AD) biomarker; $A\beta_{42}$. We also review the path from early clinical biomarker studies to the very extensive and consistent clinical validation of the diagnostic performance of the core AD CSF biomarkers we have today. Last, we give an update on recent developments, including biomarkers for synaptic proteins in CSF and the promise of blood biomarkers with potential application as screening tools.

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THE NEED FOR ALZHEIMER'S DISEASE BIOMARKERS

Twenty years ago, there was not much discussion on if, or why, there is a need of biomarkers for AD. At that time, "probable AD" was diagnosed using the exclusion criteria published in 1984 by the Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [1]. Further, the diagnosis could not be set until the patient had reached the relatively advanced stage of clinically overt dementia. At that time, no biomarkers (e.g.,

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amyloid PET scans or CSF tests amyloid-β (Aβ) or tau) for positive identification of AD pathology were available, so this was the only possible way to make the diagnosis.

The identification of Aβ and phosphorylated tau aggregates as the main components of plaques and tangles, respectively [2, 3], opened the possibility to find AD biomarkers by developing assays for proteins related to the core pathology of the disease (plaques, tangles, and neuronal degeneration), and evaluate their performance as diagnostic tests in CSF samples from AD patients and controls. To compare with diagnostic tools in other areas, laboratory medicine tests influence up to 70% of clinical decisions and thus have a central position in clinical medicine [4]. For brain disorders such as AD, the advantage of CSF over blood is its proximity to the brain parenchyma, and that proteins are secreted from the brain extracellular space to the CSF. CSF can be collected by lumbar puncture, a diagnostic procedure in which a needle introduced into the subarachnoid space in the lumbar region (L3/L4 or L4/L5), i.e., at a level that is safely below the end of the spinal cord. CSF collection is a routine procedure in the clinical practice setting in the diagnostic work-up of brain disorders such as infectious CNS diseases, multiple sclerosis, and Guillain-Barré syndrome [5]. However, still today, in the routine diagnostic evaluation of patients with cognitive symptoms, the use of CSF biomarkers varies between countries and medical specialties, being higher in some European countries than in the US and Japan.

It is well known that not only the clinical symptoms in AD often are diffuse and overlap with other disorders, but also that the clinical progression is slow and variable. Further, it is known that the severity of neuropathological changes varies considerably between AD patients and overlaps with pathology found in cognitively unimpaired elderly [6–10], and that the majority of patients with clinical Alzheimer-type dementia have mixed (multiple) pathologies; in addition to plaques and tangles, varying degrees of Lewy body, TDP-43, and other pathologies are often seen [11]. Thus, it was not surprising that an increasing number of papers showed that these purely clinical criteria for AD have poor diagnostic accuracy, also in expert academic centers, with sensitivity and specificity figures of around 70% [12]. The pathological heterogeneity of late-onset AD-type dementia also highlights a need for biomarkers reflecting different types of pathophysiology, such as α-synuclein deposition, as reviewed under the "Biomarker candidates for other aspects of AD pathophysiology" section below.

In general, AD biomarkers have a potential to be used to support a clinical diagnosis, especially in the early stages of the disease, to predict disease progression, to monitor effects of novel drug candidates in clinical trials, and last also in clinical research to deepen our understanding on the pathogenesis of the disease [13].

TWENTY YEARS OF CLINICAL VALIDATION OF AD CSF BIOMARKERS

Early assay developments and clinical neurochemical studies

It is now around two decades since the most commonly used ELISA, the so-called INNOTEST assays, to measure CSF levels of total tau (T-tau), phosphorylated tau (P-tau), and the 42 amino acid isoform of amyloid-β ($A\beta_{42}$) were published [14–16]. These articles showed a marked increase in both CSF T-tau and P-tau in AD, together with a marked decrease in $A\beta_{42}$, a CSF biomarker change that today is known as the "Alzheimer profile". The core AD CSF biomarkers also aid in the differentiation of AD from many differential diagnoses such as depression and Parkinson's disease, with P-tau levels giving substantial aid also in the differentiation from other dementias, such as frontotemporal dementia and Lewy body dementia [15, 17]. These findings were subsequently validated in numerous papers, with a recent meta-analysis showing very consistent changes of these biomarkers in AD patients, with a mean fold change to elderly control groups of 2.54 for T-tau, 1.88 for P-tau, and 0.56 for $A\beta_{42}$ [18]. Studies on the core AD CSF biomarkers (T-tau, P-tau, and $A\beta_{42}$) are also, together with other top candidate CSF and blood AD biomarkers such as VLP-1 and sTREM2, continuously (last update April 2017) curated and presented both individually and in the meta-analysis format at the online Alzbiomarker database, see http://www.alzforum.org/alzbiomarker.

Amyloid PET set the pace in the clinical validation of CSF biomarkers

A problem with studies evaluating the diagnostic accuracy for the CSF biomarkers in clinically diagnosed AD patients as compared with cognitively

normal elderly was that a percentage of normal elderly, as well as patients with other dementias, had biomarker concentrations similar to those found in AD patients. This was often interpreted as being due to suboptimal performance of the AD biomarkers.

Although high diagnostic accuracy of the core AD CSF biomarkers also was validated in studies with diagnosis confirmed by autopsy, with even better performance than studies based on pure clinical diagnoses [19, 20], these studies were largely based on cases with near end stage disease.

The availability of amyloid PET made it possible to identify amyloid pathology *in vivo*, and thereby also offered the possibility to compare the AD CSF biomarkers, especially $A\beta_{42}$, with amyloid load directly in patients and cognitively unimpaired elderly. Importantly, amyloid PET marked a major change in AD biomarker research, since it became clear that 20–30% of apparently healthy elderly showed positive on scans [21]. This knowledge rather quickly changed the view on how to interpret low CSF $A\beta_{42}$ levels in cognitively intact elderly, from poor assay quality or suboptimal biomarker performance, to an indicator of cerebral amyloidosis, and thus possibly of preclinical AD.

After the first paper showing that individuals (regardless of whether they had clinical AD symptoms or were cognitively unimpaired) with low CSF $A\beta_{42}$ had positive amyloid PET scans, and vice versa [22], a large number of papers have consistently found a very high concordance between CSF $A\beta_{42}$ and amyloid PET outcomes, with almost identical diagnostic accuracy to identify AD [23]. Importantly, high concordance between CSF $A\beta_{42}$ and amyloid PET has also been validated in a large prospective and longitudinal clinical study enrolling consecutive patients with early cognitive disturbances at a memory clinic [24]. Further, a large clinical study showed that regional PET measures did not outperform assessment of global cortical amyloid deposition as both measures were highly concordant with CSF $A\beta_{42}$ [25].

Results from some studies suggest that cases showing discordance between CSF $A\beta_{42}$ and amyloid PET most often have low CSF $A\beta_{42}$ but a negative amyloid PET scan [23]. This type of discordancy is much more common in cognitively normal elderly and early mild cognitive impairment (MCI) cases than in late MCI or in AD dementia cases [26], i.e., it is preferentially found in the earlier disease stages. A recent study also found that non-demented elderly who are CSF $A\beta_{42}$ positive but amyloid PET negative continue to build up brain amyloid deposits to the same degree as those who are positive for both CSF $A\beta_{42}$ and amyloid PET [25]. These results suggest that CSF $A\beta_{42}$ may be an earlier biomarker for cortical amyloid deposition than amyloid PET. If this can be verified in independent cohorts, it may govern the clinical decision whether to perform CSF analysis or amyloid PET in patients with mild memory complaints and suspected early AD pathology, e.g., the initiate treatment with anti-amyloid disease modifying drugs if such will be available. Additional factors of relevance for this may include availability, costs, and risks (radiation exposure versus post CSF tap headache), as well as both physician and patient preferences.

Interestingly, discordancy (low CSF $A\beta_{42}$ but negative amyloid PET) is preferentially found in cognitively unimpaired elderly and early AD, while it is rare in AD dementia cases [26]. One study examined a large cohort with non-demented ADNI subjects and found that those with low CSF $A\beta_{42}$, but negative amyloid PET, at baseline accumulated brain amyloid deposits at a rate similar to cases positive for both biomarkers, and at a rate three times higher than those with both biomarkers being normal [27]. These findings suggest that CSF $A\beta_{42}$ may be the earliest AD biomarker, becoming positive before amyloid PET and neurodegeneration.

Evaluating CSF biomarkers for early diagnosis

It may be logical to assume that novel anti-Aβ and anti-tau disease-modifying drug candidates likely will be more effective if treatment can be initiated early in the course of disease, before neurodegeneration is too severe [28], in the MCI stage of AD, or even pre-clinically. Given the diagnostic challenges to accurately diagnose AD in the MCI stage, there was a need to evaluate the CSF biomarkers for early diagnosis, and also for studies in which the core AD CSF biomarkers show a change earliest during the course of the disease.

In 1999, the first paper evaluating the AD CSF biomarkers in MCI patients showed that those who progressed to AD dementia (previously called "converted") during clinical follow-up had the typical AD CSF profile, with biomarker levels being equally abnormal as in the dementia stage of the disease [29]. In this first study, no cognitively stable MCI group was included (to ascertain absence of progressive neurodegenerative disease, follow-up over

several years is needed). In 2006, the first study with an extended clinical follow-up period (4–7 years) was published, showing a very high sensitivity (95%) for the core AD CSF biomarkers to identify prodromal AD, and also a high specificity to differentiate prodromal AD from stable MCI cases and those developing other dementias [30]. A high diagnostic accuracy of the CSF biomarkers for prodromal AD was soon thereafter verified in several large multi-center studies [20, 31, 32].

The $A\beta_{42/40}$ ratio compensates for factors complicating $A\beta_{42}$ measurements

In addition to $A\beta_{42}$, CSF contains several other Aβ species, the most abundant one being $A\beta_{40}$ [33]. Even if CSF $A\beta_{40}$ shows no clear change in AD [18], the CSF $A\beta_{42/40}$ ratio has been suggested to have stronger diagnostic accuracy for AD compared to CSF $A\beta_{42}$ alone [34–36]. Recent studies also suggest that the $A\beta_{42/40}$ ratio has better concordance with amyloid PET than $A\beta_{42}$ [37–39], and that the CSF $A\beta_{42/40}$ ratio is also valuable in the clinical setting [40].

The explanation has been hypothesized to be due to the ratio normalizing the "total" Aβ production level between individuals, so that lowering of CSF $A\beta_{42}$ in high Aβ producers can more accurately be identified, and vice versa [41]. Alternative explanations may involve the ratio normalizing for differences in CSF dynamics (affecting both $A\beta_{42}$ and $A\beta_{40}$ similarly), such as variable CSF production or clearance rate, or in the proportion of CSF diffusing along the spinal cord to the lumbar sac as compared with proportion flowing over the cortex to the venous sinuses. Such differences between individuals may affect $A\beta_{42}$ levels, but may be compensated for by the $A\beta_{42/40}$ ratio. Last, the CSF $A\beta_{42/40}$ ratio may compensate for pre-analytical confounders that affects both $A\beta_{42}$ and $A\beta_{40}$ in the same way [42]. As an alternative to the $A\beta_{42/40}$ ratio, the ratios between T-tau/$A\beta_{42}$ and P-tau/$A\beta_{42}$ have also been evaluated in some studies. Indeed, all three ratios show very high concordance figures [receiver operating characteristic (ROC) area under curves (AUCs) around 0.95] with amyloid PET [24, 37], and another study also found very high concordance figures for all of CSF $A\beta_{42}$ (ROC AUC 0.94) and the T-tau/$A\beta_{42}$ and P-tau/$A\beta_{42}$ ratios (ROC AUCs of 0.98 and 0.97) [43]. Further studies are warranted to evaluate, for example, whether the $A\beta_{42/40}$ ratio, that is based only on amyloid markers, may signal earlier during the course of AD than the T-tau/$A\beta_{42}$ and P-tau/$A\beta_{42}$ ratios, which combine amyloid with neurodegeneration and tau pathology biomarkers.

The core AD CSF biomarkers enter novel diagnostic criteria

In 2007, the International Working Group (IWG) published the first research criteria for the diagnosis of prodromal AD [44], which provided a new conceptual framework based on that AD could be diagnosed before the dementia stage, by the combination of a clinical phenotype of episodic memory disturbances and one or more abnormal AD biomarker (CSF biomarkers, volumetric MRI, and amyloid PET). Similar, but not identical, criteria for MCI due to AD [45] and dementia due to AD [46] have been published by the National Institute on Aging-Alzheimer's Association (NIA-AA) working groups on diagnostic guidelines for AD. Recently, in the updated IWG-2 criteria [47], the CSF biomarkers got a more significant role, together with amyloid PET, while the downstream topographical biomarkers (volumetric MRI and FDG-PET), were assigned as tools to monitoring disease course in AD.

EFFORTS TO MAKE STABLE AND PRECISE ASSAYS

As mentioned above, the most commonly used methods for measurement of the core AD CSF biomarkers $A\beta_{42}$, T-tau, and P-tau in clinical studies and routine diagnostics are ELISAs [9–11], while the multiplex method used in the ADNI study [20] is based on the Luminex technology [48]. Although an increasing number of clinical studies showed very promising results for these biomarkers, it was easy to see a marked difference in the reported absolute levels between the studies, i.e., between clinical cohorts and laboratories [49]. It was also evident that this between-laboratory variability was more pronounced for $A\beta_{42}$ than for T-tau or P-tau [50]. This type of differences in absolute levels may stem from differences in pre-analytical procedures between clinical centers [51], but also from inconsistencies in analytical procedures between laboratories, and not the least from variability in manufacturing procedures for the assays that results in batch-to-batch variations. To monitor the latter, the Alzheimer's Association quality control (QC) program for CSF biomarkers was started in 2009 [50].

Monitoring assay performance in the quality control program

Although strict quality control procedures within a clinical laboratory can assure accurate measurements over time [24] and facilitate implementation in clinical routine diagnostics [52], the between-laboratory differences in absolute levels precludes the introduction of uniform global cut-off levels and thus a more widespread implementation of CSF biomarkers in clinical routine. This is not unique for the AD CSF biomarkers, but a problem for any novel, either fluid or imaging (PET or MRI), biomarker, which simply calls for standardization efforts to solve the problems.

From the start, the QC program had several goals. The main goal was to establish a working program to monitor the performance of the CSF biomarker measurements between laboratories and between batches of reagents, just like any proficiency program that is running for common routine assays such as blood cholesterol, glucose, and tumor markers. Important goals were also to stimulate spin-off projects focused on standardization, as well as Biotech company efforts on development of novel high-quality assay versions, or building CSF biomarker assays on fully automated laboratory instruments.

Disappointingly, between-laboratory CVs have been around 15–25% for the ELISA and Luminex methods since the beginning, without any trend for improvement, despite training efforts and attempts to introduce common standard operating procedures for the methods [53], indicating the need of more automated analytical techniques.

Standardization efforts

In clinical chemistry, the highest level of standardization is through a Certified Reference Material (CRM). For the AD CSF biomarkers, this means a "Gold Standard" large CSF pool with known exact biomarker levels, from which aliquots can be distributed to kit vendors and large laboratories for harmonization of levels between assay formats, and to secure long-term (batch-to-batch) stability of assays. From the Alzheimer's Association QC program, it was known that the largest problem with between-assay and between-batch variability was for CSF $A\beta_{42}$ [50]. Thus, the International Federation of Clinical Chemistry and Laboratory Medicine Working Group for CSF proteins (IFCC WG-CSF, http://www.ifcc.org/ifcc-scientific-division/sd-working-groups/csf-proteins-wg-csf/), was initiated to start working on standardization of this biomarker [54], in close conjunction with the Alzheimer's Association Global Biomarker Standardization Consortium [55].

To set the absolute concentration of $A\beta_{42}$ in the CRM, methods capable of absolute quantification without matrix effects is needed. With the aim to develop such a "Gold Standard" method, called a Reference Measurement Procedure (RMP), a first paper on a selected reaction monitoring mass spectrometry method for CSF $A\beta_{42}$, where isotope-labeled $A\beta_{42}$ was added to the CSF sample prior to sample workup for use as an internal standard, was published [56]. The fully validated CSF $A\beta_{42}$ candidate RMP was published in 2014 [57], and was approved and listed as an RMP (No. C11RMP9) by the Joint Committee for Traceability in Laboratory Medicine [58], which is the regulatory body for reference methods, in 2015.

In the development process of a CRM, the type of matrix for this material needs to be evaluated in so called commutability studies, i.e., studies examining whether the measured values in different candidate CRMs align with individual CSF samples when measured using different assay formats. For $A\beta_{42}$, such a commutability study showed that only native human CSF works as a CRM, and that so-called artificial CSF, or $A\beta_{42}$ spiked into buffer, did not commute, meaning that such materials did not behave like the native peptide present in human CSF when quantified using different assays [59]. For this reason, together with the Joint Research Centre European Commission Science Hub (see https://ec.europa.eu/jrc/en/reference-materials), the IFCC-WG CSF decided to develop three CRMs, with low, medium, and high $A\beta_{42}$ levels, with exact concentrations assigned using the RMP and equivalent methods that have been tested in Round Robin studies for concordance [39]. These three CRMs (or aliquot sets) have also been tested for homogeneity, long-term stability, and other quality measures, and will serve at the top of the calibration hierarchy, for calibration of commercial assays to these reference aliquots, which will make the different assays directly comparable to each other. We see the above as important steps in the standardization of $A\beta_{42}$, the trickiest AD CSF biomarker.

Fully automated instruments for the AD CSF biomarkers

In clinical chemistry, for the majority of protein biomarkers, e.g., PSA for prostate cancer and

troponin-T for myocardial infarction, analyses are performed on fully automated laboratory instruments. These instruments are very exact, and there are no manual steps, meaning that the assays have superior performance as compared with, for example, ELISA methods. In 2016, a paper was published on the full validation and analytical performance a novel β-amyloid (1–42) assay on such a fully automated instrument [60]. Results showed excellent performance (repeatability CVs for human CSF pools of 1.0%–1.6% and intermediate CVs of 1.9%–4.0%) and very low between-batch variability (correlation coefficients for 100 individual samples analyzed using three different batches of reagents of 0.996–0.998). This assay has also been running in the QC program since 2014, with coefficients of variations (CVs) dropping to a mean of 4% (for in total 22 individual QC samples analyzed from 2014–2017) as compared with a mean of 15-16% for the ELISA methods during the same time period. Assays for T-tau and P-tau on fully automated instruments have also been developed and have done one round in the QC program, showing excellent CVs of 3.7% (T-tau) and 1.9% (P-tau). A number of companies are building assays on fully automated laboratory, meaning that clinical laboratories can choose between different platforms for their clinical routine measurements.

These new types of assays will serve as the basis for highly stable and precise results for CSF biomarkers, which, together with certified reference materials, will allow for the establishment of uniform worldwide cut-off levels. We believe that this will lead to a more general use of CSF biomarkers in the routine diagnostic evaluation of patients with suspected AD, and also in clinical trials in novel disease-modifying drugs. Last, highly exact CSF biomarker levels with stable results between batches will allow for both longitudinal studies and merging data from clinical research centers worldwide, in clinical studies on disease pathogenesis.

As mentioned above, the proportion of cognitively unimpaired elderly harboring AD pathology increases with age, particularly after age 65 [7–9, 61, 62], and that the amounts of plaques and tangles varies between late onset AD patients, and overlaps with those found in cognitively unimpaired elderly [8, 61, 63]. Therefore, it will probably not be possible to identify any AD biomarker reflecting pathology that will show a complete discrimination; instead, studies show a clear overlap in both CSF $A\beta_{42}$ and SUVr values between AD patients and controls [64]. It is our belief that reporting absolute biomarker values (instead of reporting back results as being "positive" or "negative") will allow clinicians to manage patients with clearly abnormal biomarker values and those with values close to the cut-off (sometimes called "grey zone" values) differently. The improved performance of the novel automated assays may help in this respect.

EXPANDING THE AD BIOMARKER TOOLBOX TO INCLUDE SYNAPTIC PROTEINS

Despite that the core CSF AD biomarkers reflect central pathogenic mechanisms of the disease, novel biomarkers to monitor additional important molecular mechanisms in AD are constantly sought. One important component of AD pathologic change and pathophysiology is synaptic dysfunction and degeneration. Synapses are the central communication units in the neuronal networks the brain. Synapses consist of a pre-synaptic domain, where synaptic vesicles that contain the neurotransmitters that are released upon activation are located. Neurotransmitter release is a process regulated by a delicate machinery of specific pre-synaptic proteins [65]. After release to the synaptic cleft, neurotransmitters bind to post-synaptic receptors at the dendritic spines and activate a cascade of molecular events to advance the neuronal signal [66]. Synaptic dysfunction and degeneration are likely the direct cause of the cognitive deterioration in AD.

A large body of literature supports a marked degeneration and loss of synapses in grey matter regions in AD, also in the early disease stages [67, 68]. Importantly, severity of synaptic loss is more tightly correlated with degree of cognitive impairment than either plaque or tangle counts [69–71], and synaptic degeneration has been suggested as the best anatomical correlate of cognitive deficits in AD [69, 72]. Further, experimental animal studies suggest that both Aβ fibrils [73] and diffusible Aβ oligomers [74] may disturb dendritic spines by distinct mechanisms. In addition, tau hyperphosphorylation and microglia activation may also contribute to spine loss [75, 76]. Thus, synaptic biomarkers in CSF may serve as tools to explore this important aspect of AD pathophysiology in man, and to examine the link between effects on AD molecular pathology and cognitive symptoms by novel drug candidates with disease-modifying potential. Synapses are plastic structures in the brain and, potentially, synaptic

markers would change rapidly in response to successful treatment.

Early search for synaptic proteins in CSF

Based on semi-preparative scale chromatographic and gel electrophoretic protein separation combined with western blotting and mass spectrometric identification, in the late 1990s we were able to identify synaptic proteins in CSF from the key synaptic compartments, including the presynaptic vesicle proteins synaptotagmin and rab3a, the presynaptic membrane protein SNAP-25, and the dendritic protein neurogranin [77, 78]. These discoveries served as the motivation to initiate a project on production on novel antibodies and detailed mass spectrometric characterization of synaptic proteins in human CSF aiming at developing quantitative immunoassays for reliable quantification in individual samples. A first pilot study in 2010, based on semi-quantitative immunoprecipitation combined with western blotting showed promising results with a marked increase in CSF neurogranin in AD [79].

Dendritic proteins: Neurogranin

Dendritic spines are specialized protrusions on the dendrites, the point where neurons receive and integrate information. Neurogranin is a dendritic protein, expressed in the cortex and hippocampus by excitatory neurons [80, 81], and is known to play an important role in long-term potentiation [82, 83]. Neurogranin expression is highest in associative cortical areas, but levels are markedly reduced in the hippocampus and the frontal cortex in AD, indicating loss of post-synaptic elements [84, 85]. Thus, measurement of neurogranin in CSF may serve as a biomarker for dendritic instability and synaptic degeneration.

After developing novel monoclonal antibodies to measure neurogranin by ELISA, high CSF levels were found to predict prodromal AD in MCI [86]. High CSF neurogranin in AD dementia and prodromal AD has been confirmed in several subsequent papers [87, 88], including in the ADNI study [89]. High CSF neurogranin also correlates with future rate of hippocampal trophy measured by MRI and rate of metabolic reductions on FDG-PET [89]. Interestingly, a recent study suggests that high CSF neurogranin may be specific for AD, and not found in other neurodegenerative disorders such as frontotemporal dementia, Lewy body dementia, Parkinson's disease, progressive supranuclear palsy, or multiple system atrophy [90]. A recent large study confirms that increased CSF neurogranin levels is found in AD dementia and prodromal AD, but not in other neurodegenerative disorders such as frontotemporal dementia, Lewy body dementia, Parkinson's disease, progressive supranuclear palsy, corticobasal degeneration, or amyotrophic lateral sclerosis (Portelius et al., unpublished).

Mass spectrometry characterization of neurogranin in CSF suggests that it is present in CSF as a series of C-terminal peptides [86], while other studies using a sandwich immunoassay combining N- and C-terminal antibodies, which thus measures full-length neurogranin [91], as well as an assay specific for neurogranin peptides ending at position 75 [92], also found high CSF levels in AD and MCI as compared with controls Thus, we need further studies on how neurogranin is processed and released from neurons into the CSF, including studies comparing the diagnostic potential of full-length versus C-terminal neurogranin peptides.

Presynaptic biomarkers

In the presynaptic terminal, the SNARE complex proteins, including synaptosomal-associated protein 25 (SNAP-25), syntaxin-1, and vesicle-associated membrane protein (VAMP)/synaptobrevin, are key components of the molecular machinery that drives fusion of membranes in neurotransmitter exocytosis [93]. While SNAP-25 is located at the synaptic vesicles, synaptotagmin-1 (SYT1) is found in the presynaptic plasma membrane, and is essential for synaptic vesicle exocytosis, and thus neurotransmitter release [94].

The levels of both SNAP-25 and SYT1 are reduced in cortical areas in the AD brain [84, 95], reflecting the synaptic degeneration and loss in AD. Interestingly, using immunoprecipitation mass spectrometry methods, a marked increase in the CSF levels of both SNAP-25 and SYT1 was found in AD dementia and prodromal AD cases [95, 96]. These promising results need validation in future studies, but suggest that a set of synaptic proteins covering different components of the synaptic unit (dendrites – neurogranin, presynaptic plasma membrane – SNAP-25, synaptic vesicles – SYT1) may be valuable tools in clinical studies on the relevance of synaptic dysfunction and degeneration in AD pathogenesis, and maybe also in the clinical evaluation of patients.

BIOMARKER CANDIDATES FOR OTHER ASPECTS OF AD PATHOPHYSIOLOGY

Some candidate biomarkers for other pathologies have been developed, for example measurement of α-synuclein in CSF, that in theory may reflect degree of Lewy bodies and Lewy neurite pathology. Several [97–99], but not all [100–102], studies have found a slight decrease in CSF α-synuclein in disorders with Lewy body pathology, e.g., in Parkinson's disease and Lewy body dementia. However, other studies have shown a marked increase in CSF α-synuclein in AD, which is even more pronounced in Creutzfeldt-Jakob disease [103, 104]. Further, CSF α-synuclein levels correlate with the neuronal injury biomarker tau [101, 104]. Taken together, these findings suggest that CSF α-synuclein is biomarker for neurodegeneration or synapse loss [101, 102, 104]. Novel assays with antibodies specific for other variants of α-synuclein, such as phosphorylated or oligomeric forms [105, 106], may improve performance detect Lewy body pathology.

The studies reporting a genetic association between the triggering receptor expressed on myeloid cells 2 (*TREM2*) gene and AD [107, 108], and the finding that the ectodomain of TREM2 (sTREM2) is secreted into CSF, and that increased CSF levels are found in patients with relapsing-remitting and primary progressive multiple sclerosis [109], initiated an interest for biomarker tools to monitor microglial activation in AD pathogenesis. Several studies have found an increase in CSF sTREM2 in AD that also correlates with CSF T-tau and P-tau levels [110–112]. However, as reviewed in a recent meta-analysis [18], the degree of increase modest, which does not support the use of this biomarker for diagnostic purposes, but it may serve to study microglial activation in clinical studies on AD pathogenesis.

MOVING FROM CSF TO BLOOD: THE PROMISE OF SCREENING TOOLS

Since blood is more accessible than CSF, there is little doubt that blood sampling would be preferable to CSF when it comes to taking fluid samples to measure AD biomarkers, both for clinical diagnosis or screening and for repeated sampling in clinical trials. However, developing blood biomarkers for AD has proven difficult; while the CSF is continuous with the brain extracellular fluid, with a free exchange of molecules from the brain to the CSF, only a fraction of brain proteins enters the bloodstream. Further, blood is a more challenging matrix than CSF for brain biomarkers, for several reasons. First, the minute amounts of brain proteins entering the blood have to be measured in a matrix containing very high levels of plasma proteins, such as albumin and IgG, introducing a high risk of interference in analytical methods [113]. Second, in addition to dilution, brain proteins released into blood may be degraded by proteases, metabolized in the liver or cleared by the kidneys, which will introduce a variance that is unrelated to brain changes and difficult to control for. This limits the potential of finding blood biomarkers for AD [114]. Nevertheless, technical developments in the field of ultrasensitive immunoassays and mass spectrometry have given new hopes [115].

Aβ in plasma

While numerous papers on CSF $A\beta_{42}$ consistently have found a high concordance with amyloid PET measures of plaque burden [23], and a marked decrease in AD, studies on plasma $A\beta_{42}$ as a biomarker reflecting brain amyloid pathology (and thus AD) have been disappointing, with contradictory results, with no or minor changes and large overlaps in both $A\beta_{42}$ and $A\beta_{40}$ levels between patients and controls [18]. This lack of association with disease pathology may be due to the contribution from peripheral tissues to plasma Aβ, as also evidenced by the lack of correlation between plasma and CSF Aβ concentrations [116]. The poor disease association might also be related to analytical shortcomings using ELISA methods or other standard immunoassays, e.g., epitope masking by hydrophobic Aβ peptides binding to plasma proteins [117], or other interferences that might be mitigated by analytical improvements.

In 2011, we published a novel method based on the Single Molecule Array (Simoa) technique for measurement of $A\beta_{42}$ in plasma [118]. This technique is based on immunocapture of the protein biomarker on magnetic beads, which are trapped in femto-liter volume wells, followed by addition of enzyme-labelled detection antibody and digital quantification that allows for exact quantification of $A\beta_{42}$ down to sub-picogram per mL levels (limit of quantification of 0.04 pg/mL). The high analytical sensitivity allows for pre-dilution of samples that may reduce matrix interferences. When evaluating this assay in the large Swedish BioFINDER study cohort, weak but significant correlations were found

between both plasma $A\beta_{42}$ and the $A\beta_{42/40}$ ratio and the corresponding CSF measures, as well as to cortical $^{[18F]}$flutemetamol PET retention [119]. Significantly lower plasma $A\beta_{42/40}$ ratio ($p < 0.002$) was found in both MCI and AD cases as compared with controls.

In an attempt to evaluate if mass spectrometric analysis may give a more accurate quantification of Aβ peptides in plasma, we developed an immunoprecipitation (IP) mass spectrometry (MS) selected reaction monitoring method for quantification of $A\beta_{42}$ and $A\beta_{40}$, where stable isotope-labeled Aβ peptides are added to the sample before analysis (and thus processed and analyzed simultaneously with endogenous Aβ peptides) and using the detergent octyl-glucopyranoside to disrupt complexes between Aβ and plasma proteins such as albumin [120]. In a small pilot clinical study based on clinically diagnosed cases, we were not able to find any significant change, even if there was an apparent trend for a reduction on both plasma $A\beta_{42}$ and the $A\beta_{42/40}$ ratio in AD [120]. Interestingly, using a similar IP-MS method, also involving LysN proteolytic digestion of Aβ peptides before analysis, significantly lower $A\beta_{42}$ concentration and $A\beta_{42/40}$ ratio was found in amyloid PET-positive compared with negative cases [121]. The $A\beta_{42/40}$ ratio was 14% lower in the amyloid PET positive group, which gave an impressive ROC value of 0.89 [121]. These very promising results call for further studies to evaluate plasma Aβ as a screening tool for brain amyloidosis and AD, also including larger clinical cohorts and comparisons of different analytical platforms for measurement.

Tau protein in plasma

Ultrasensitive immunoassay techniques also allow for measurement of tau protein in blood samples [115], with increased tau levels in plasma in AD found using both the immuno-magnetic reduction [122] and Simoa [123], methods. A large study on both the ADNI and BIOFINDER cohorts could confirm an increase in plasma tau concentrations in AD dementia, although with a substantial overlap in levels with controls [124]. Interestingly, longitudinal data showed significant correlations between plasma tau levels and future cognitive decline, as well as increases in atrophy measured by MRI and in hypometabolism measured by FDG PET during follow-up [124]. Thus, current data suggest a minor increase in plasma tau in AD, although with too large overlap with controls to be diagnostically useful. Tau protein in CSF has been found to be present as truncated fragments [125], and it is possible that development of assays based on antibodies for specific tau fragments will improve performance. Alternatively, measurement of T-tau or P-tau in neuron-enriched exosome preparations may improve performance for tau as a blood biomarker [126], but further studies are needed to validate this finding.

Neurofilament light in plasma

We have also developed a highly sensitive Simoa method for the axonal protein neurofilament light (NFL) protein [127]. This assay has many-fold higher analytical sensitivity than assays using the same anti-NFL antibodies based on the electrochemiluminescence (ECL) Meso Scale Diagnostics (MSD) technique or standard ELISA [128], meaning that NFL can be measured also in blood samples from normal individuals who have plasma NFL concentrations that are below the level for accurate quantification when using ECL-MSD or ELISA. In contrast to tau protein, the correlation between plasma and CSF levels of NFL protein is tight [127].

A recent study on the ADNI cohort showed a marked increase in plasma NFL in AD cases (149% of control levels), with a ROC AUC value of 0.87, which is comparable to the core AD CSF biomarkers [129]. While the change in the MCI group was less pronounced, plasma NFL was highest MCI cases with positive amyloid PET scans, and predicted faster cognitive deterioration, higher rate of future both brain atrophy (measured by MRI) and hypometabolism as measured by FDG-PET [129]. Importantly, in a study on 48 familial AD mutation carriers and non-carriers, blood NFL was increased in symptomatic familial FAD cases, but also in pre-symptomatic mutation carriers, with levels correlating with expected estimated year of symptom onset as well as both cognitive and MRI measures of disease stage [130]. These results indicate that blood NFL detects neurodegeneration also in the preclinical stage of AD.

In this context, an important piece of knowledge is that high plasma (or CSF) NFL is not a feature that is specific for AD. Instead, increased levels are found in many neurodegenerative disorders, such as frontotemporal dementia, progressive supranuclear palsy and corticobasal syndrome [131, 132]. Thus, a possible future application for plasma NFL is as a screening test at the first clinical evaluation of patients with cognitive disturbances, e.g., at the primary care unit. Here, plasma NFL might serve as simple, non-

invasive, and cheap screening tool, primarily to rule out neurodegeneration.

CONCLUDING REMARKS

The last 20 years have seen an enormous expansion in research on fluid biomarkers for AD. The core AD CSF biomarkers T-tau, P-tau, and $A\beta_{42}$ (and $A\beta_{42/40}$ ratio) have been evaluated in hundreds of clinical neurochemical studies with extraordinary consistent results, showing high diagnostic accuracy both for AD dementia, but importantly also for prodromal AD. These biomarkers have undergone a phase of standardization and new assay versions on fully automated instruments show excellent analytical performance. The core AD biomarkers are today part of research diagnostic criteria, and we foresee an increased use of these diagnostic tests in clinical routine practice. The AD CSF biomarker toolbox has been expanded with novel biomarker reflecting additional aspects of AD pathology, such as synaptic dysfunction.

We envision further validated assays reflecting other pathologies common in age-related neurodegenerative disorders, e.g., Lewy body and TDP-43 pathology, reaching the stage of clinical applications in the coming years, so that CSF biomarkers can be part in a personalized medicine approach to the clinical evaluation of patients with cognitive disturbances. Last, we hope that blood biomarkers may be implemented as screening tools in the first-in-line clinical evaluation of this group of patients.

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