

Review

Current state and future directions of neurochemical biomarkers for Alzheimer's disease

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Abstract

In this comprehensive review, we summarize the current state-of-the-art of neurochemical biomarkers for Alzheimer's disease. Predominantly, these biomarkers comprise cerebrospinal fluid biomarkers directly related to the pathophysiology of this disorder (such as amyloid β protein, tau protein). We particularly pay attention to the innovations in this area that have been made in technological aspects during the past 5 years (e.g., multiplex analysis of biomarkers, proteomics), to the discovery of novel, potential biomarkers (e.g., amyloid β oligomers, isoprostanes), and to the extension of this research towards identification of biomarkers in plasma.

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the accumulation of extracellular senile plaques and intracellular neurofibrillary tangles (NFTs) in cortical and limbic brain regions (1, 2). Until now, a definitive diagnosis of Alzheimer's

disease can only be made after postmortem examination of the patient. During life only a probable AD diagnosis is possible, based on clinical features and results of neurological and neuropsychological testing, added by the exclusion of other dementias, in particular frontotemporal lobe degeneration (FTLD), dementia with Lewy bodies (DLB) and vascular dementia (VaD). An accurate and early diagnosis is essential for appropriate support and treatment of dementia patients, as drugs for the symptomatic treatment of AD are currently available and drugs that may slow or halt the progression of the disease are being developed.

The clinical diagnostic criteria currently used for dementia disorders [NINCDS-ADRDA criteria for AD, Lund and Manchester criteria for FTLD, Consensus guidelines for DLB and NINDS-AIREN criteria for VaD (3–6)] were initially developed for research purposes. The accuracy of these criteria was then examined in several clinicopathological studies. It was established that a clinical diagnosis of probable AD could be achieved with a sensitivity of 41%–93% and a specificity varying between 23% and 100% (7–9). Similar studies yielded 85% sensitivity and 97% specificity for FTLD (9), 57%–78% sensitivity and 64%–100% specificity for probable DLB (9–11), and 20% sensitivity and 93% specificity for probable VaD (12). Clearly, to improve upon these less than perfect characteristics, the introduction of additional discriminating diagnostic markers (biomarkers) is needed to improve our diagnostic accuracy. Analysis of various brain-specific proteins in cerebrospinal fluid (CSF), i.e., biomarkers closest to the brain's chemical composition, has already proven to be very sensitive and specific in preliminary validation studies (13–16).

In this review, we summarize the studies reporting on CSF biomarkers in AD and other dementias. Furthermore, we particularly pay attention to the technological innovations in this area during the past 5 years, to the discovery of novel, potential biomarkers, and to the extension of this research towards identification of biomarkers in plasma.

Cerebrospinal fluid amyloid β protein₄₂, total tau protein and hyperphosphorylated tau protein

In this paragraph, we summarize the abundant literature that has been published over the past 10 years

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on this topic. For a more extensive review of this topic, we refer to previously published reviews (17–20).

Amyloid β protein₄₂, total tau protein and hyperphosphorylated tau protein in the nosological classification of dementia disorders

The characteristic neuropathological findings in AD brains are senile plaques, NFTs, and degeneration of neurons and their synapses. The major component of senile plaques is amyloid β protein ($A\beta$), while tau protein, particularly hyperphosphorylated tau, is the primary constituent of NFTs. Therefore, levels of both proteins in CSF of dementia patients have been thoroughly investigated as potential diagnostic biomarkers.

Significant decreased concentrations of $A\beta_{42}$ in CSF were found in AD patients compared to controls (15, 21–27), patients with VaD (25), and patients with FTLN (27) (Table 1). Mean CSF $A\beta_{42}$ levels were decreased in patients with DLB compared to normal subjects, but no differences were found between the AD and the DLB group (26). However, it should be noted that in none of these studies a clear-cut discrimination between the various forms of dementia could be achieved.

The level of CSF total tau protein (t-tau), which includes both normal and hyperphosphorylated tau (p-tau), seems to correlate with the number of NFTs in AD postmortem brains (28). Mean CSF t-tau levels were found to be significantly elevated in AD patients as compared to control subjects (15, 23–27, 29–35), DLB patients (25, 26, 32), FTLN patients (27, 30), and patients with VaD (25, 30, 33) (Table 1). However, in FTLN patients a large variation in CSF t-tau levels has been described in different studies, varying from non-detectable to significantly elevated (27, 30, 34, 36–41). This is most likely due to the fact that FTLN comprises at least three neuropathologically different subtypes, i.e., with tau deposition (Pick's disease and familial FTLN with tau pathology, both approximately 20% of FTLN patients) or without tau pathology (60% of FTLN patients) (42, 43). Some of these tau-negative FTLN patients were recently linked to mutations in the progranulin gene (44, 45). Furthermore, the surprisingly low CSF tau levels found in one study (40) can also be explained by methodological issues regarding appropriate sample handling (see also Effect of processing and storage conditions, and reference values) (41).

p-Tau, also measurable in CSF, appeared as another promising biomarker in view of the neuropathological findings in AD. Tau can be phosphorylated at various sites, correlating with three stages of neurofibrillary tangle formation: 1) pre-neurofibrillary tangles (tau phosphorylated at threonine 231; p-tau₂₃₁); 2) intra-neuronal neurofibrillary tangles (tau phosphorylated at threonine 181; p-tau₁₈₁); and 3) extra-neuronal neurofibrillary tangles (tau phosphorylated at serine 199; p-tau₁₉₉) (46). Measurement of p-tau₂₃₁ in CSF improved discrimination of AD from FTLN (47) (Table 1) and measurement of p-tau₁₈₁ levels enhanced the discrimination of AD from DLB (48–50).

Subsequently, many studies followed that investigated all the above-mentioned CSF markers to find the most optimal combination of markers for the discrimination between different dementias. The combination of CSF t-tau and $A\beta_{42}$ concentrations yielded a sensitivity varying between 81% and 94% and a specificity varying between 79% and 95% when differentiating between AD patients and normal controls (15, 24, 25, 27, 32), mostly established with the (imperfect) clinical diagnoses as the golden standard. However, the clinical challenge to distinguish AD from specific other dementia disorders still remained. The specificities for the distinction between AD and DLB, VaD and FTLN were 67%, 48%, and 85%, respectively (25, 27). The $A\beta_{42}$ /p-tau₁₈₁ ratio was found to be a good discriminator between AD and VaD (sensitivity, specificity, negative and positive predictive values all $\geq 85\%$) (51). AD and DLB could be differentiated by the t-tau/p-tau₁₈₁ ratio, but values still overlapped markedly (52). To facilitate simultaneous measurement of these three biomarkers, a new technique has been developed (see Multiplex analysis of $A\beta_{42}$, t-tau and p-tau₁₈₁).

CSF $A\beta_{42}$, t-tau and p-tau analysis can also be useful in the discrimination between sporadic Creutzfeldt-Jakob disease (CJD) and other dementias. CJD is characterized by progressive dementia, pyramidal dysfunction, myoclonus, extrapyramidal signs, visual symptoms and ataxia, but the differentiation from other dementias, especially AD and FTLN, can be difficult very early in the course of CJD. In CSF of CJD patients, very high levels of t-tau were found. Using a cutoff value of 1300 pg/mL, a diagnostic sensitivity of 94% with 90% specificity was achieved, with a positive predictive value of 92% (53). In another study, a CSF t-tau cutoff value of 2131 pg/mL resulted in a sensitivity of 93% and specificity of 100% for the discrim-

Table 1 Overview of cerebrospinal fluid (CSF) findings for $A\beta_{42}$, t-tau and p-tau₁₈₁ in the most common dementia disorders.

Groups	$A\beta_{42}$	t-tau	p-tau ₁₈₁
Normal aging	N	N	N
Alzheimer's disease	↓ ↓	↑ ↑	↑ ↑
Dementia with Lewy bodies	↓	N/(↑)	N
Frontotemporal lobar degeneration	N/(↓)	Variable	N/↑
Vascular dementia	N/(↓)	N/↑	N
Creutzfeldt-Jakob disease	↓	↑ ↑ ↑	N

CSF concentration: N, normal; ↑, increased; ↓, decreased. $A\beta_{42}$, amyloid β_{42} protein; t-tau, total tau; p-tau₁₈₁, tau phosphorylated at threonine 181.

ination between CJD and AD (54). In accordance with the absence of hyperphosphorylation of tau proteins and NFTs in brains of CJD patients, relatively low CSF p-tau concentrations (<85 ng/L) were found (55). Furthermore, the CSF p-tau/t-tau ratio was also found to be a good discriminator; CJD patients could be differentiated from patients with AD and FTLT (55, 56). CSF $A\beta_{42}$ levels were decreased in CJD patients, but to a similar degree as in AD patients (54, 57). The immunodetection of 14-3-3 proteins in CSF has also shown high sensitivity and specificity for CJD (53, 58, 59). However, further research revealed false negative results in some typical, autopsy proven CJD cases (59, 60), and false positive results in patients with other dementias, including AD, DLB, FTD and VaD (61, 62). The concentrations of neuron-specific enolase (NSE) and S-100 protein were also elevated in CSF of CJD patients (63–66). Thus, CJD can be discriminated from other dementias by the combined analysis of $A\beta_{42}$, t-tau, p-tau₁₈₁, 14-3-3 protein, NSE and S-100 protein.

Postmortem verification studies

Although the neuropathological diagnosis of different dementias is considered the gold standard, there are only a few studies that used autopsy-confirmed diagnoses as such. Two studies included only patients with autopsy-confirmed dementias. The first investigated the correlation of 106 antemortem CSF t-tau and $A\beta_{42}$ levels with postmortem dementia diagnoses, and confirmed the earlier observations that elevated t-tau levels and reduced $A\beta_{42}$ levels in CSF are associated with AD pathology, and can help discriminate AD from other dementias (37). Interestingly, there were also a few patients in this study who met the clinical and pathological criteria for AD but had CSF t-tau levels below a pre-defined cutoff value. In the second and recently published study, levels of $A\beta_{42}$, t-tau and p-tau₁₈₁ were determined in CSF samples from 100 autopsy-confirmed dementia and 100 control subjects. AD was optimally discriminated from non-AD dementias using p-tau₁₈₁ and $A\beta_{42}$ (sensitivity 80%, specificity 93%) (67).

Other studies included a few autopsy-confirmed patients within their larger group of dementia patients. Based on CSF levels of t-tau and $A\beta_{42}$, 12 out of 13 patients with postmortem diagnoses could be correctly classified as having AD or not in one study (15). In two other studies, a small number of autopsy-confirmed dementia patients were included, but unfortunately mean CSF tau and $A\beta_{42}$ levels of these subgroups of AD and FTLT patients were not described (17, 40).

Thus, information on CSF biomarkers in autopsy-confirmed AD patients is relatively limited, but generally confirms the observation in the more numerous studies based on clinical diagnosis of dementia syndromes. More studies will likely follow on this issue, also including more patients with autopsy-confirmed dementia diagnoses other than AD. However, it is to be expected that the generally accepted view of CSF $A\beta_{42}$, t-tau and p-tau₁₈₁ will not dramatically change

with these additional studies. Ideally, we still need the conformation of the validation of CSF biomarkers against a randomly selected postmortem sample, in which referral and selection bias, possibly still important in the studies that have been performed up to now has been excluded.

Effect of processing and storage conditions, and reference values

Several pre-analytical factors will have to be considered for correct and reliable analysis of $A\beta_{42}$, t-tau and p-tau₁₈₁. It was reported that sampling of CSF in tubes made of glass or polystyrene leads to up to 30% reduced concentrations of $A\beta_{42}$ relative to collection in polypropylene tubes (68). This was confirmed in one subsequent study (69) that showed that various $A\beta$ peptides were up to 30% decreased in polystyrene tubes, the t-tau concentration decreased by 15%, while the p-tau₁₈₁ concentration remained unaffected. Sampling in polycarbonate or modified polystyrene (polystyrene/acetonitrile mixture) did not affect results (69).

Prolonged storage of CSF at -80°C does not affect $A\beta_{42}$ or t-tau concentrations (70, 71). CSF tau protein is even stable at up to 18°C for more than 2 weeks. However, $A\beta_{42}$ concentrations may decrease by 20% during the first 2 days when stored at 4°C or 18°C , while thereafter they remain stable (71). In contrast, in another study a small increase in $A\beta_{42}$ concentration was demonstrated after storage for 24 h at room temperature (72). We did not observe any effects on p-tau₁₈₁ concentrations (unpublished data) when CSF was stored for 24 h at -20°C , 4°C or 20°C . Neither did a single freeze/thaw cycle affect $A\beta_{42}$ or t-tau concentrations. However, $A\beta_{42}$ concentrations did decrease by 20% after multiple freeze/thaw cycles, whereas t-tau concentrations remained stable (71). We did not observe any effects on p-tau₁₈₁ concentrations after a single freeze/thaw cycle (unpublished data). Furthermore, blood contamination of CSF samples leads to reduced concentrations of t-tau, p-tau₁₈₁ and $A\beta_{42}$ (unpublished observations). Finally, we did not find any indication for a lumbar-ventricular gradient in the concentrations of t-tau, p-tau₁₈₁ and $A\beta_{42}$. However, it was recently reported that the concentrations of $A\beta$ may follow diurnal fluctuations (73), suggesting the need for standardized sampling procedures.

In summary, the following procedures are advised when analyzing t-tau, p-tau₁₈₁ and $A\beta_{1-42}$ in CSF: 1) CSF should be collected and stored in polypropylene tubes; 2) for optimal analysis, CSF samples should be centrifuged, aliquoted and stored at -80°C as soon as possible after withdrawal, although the effects of short-term storage at higher temperatures on the analytical results are minimal; 3) samples contaminated by blood or hemolytic CSF should not be used for analysis; 4) repeated freeze/thawing of CSF should be avoided; 5) aliquots from any of the CSF fractions that are collected can be used (there is no need for a standardization of the volume to be analyzed); and 6) because of possible diurnal fluctuations, CSF withdrawal should be performed at a standard-

Table 2 Reference values for A β_{42} , t-tau and p-tau₁₈₁ in cerebrospinal fluid (CSF).

CSF biomarker	Age, years	Sjogren et al. (77), ng/L	Our laboratory, ng/L
A β_{42}	< 15		> 400
	21–93	> 500	> 500
t-tau	21–50	< 300	< 300
	51–70	< 450	< 350
	71–93	< 500	< 350
p-tau ₁₈₁	> 15		< 85

A β_{42} , amyloid β_{42} protein; t-tau, total tau; p-tau₁₈₁, tau phosphorylated at threonine 181.

ized time point of the day, but the initial observations on this matter await confirmation.

Studies aimed at analyzing and comparing the performance of different laboratories using the same tests for analysis of A β_{42} , t-tau and p-tau₁₈₁ have been rarely reported. In a study with 13 laboratories testing three CSF samples, coefficients of variation (CV) varied from 8.5% to 10.8% (p-tau₁₈₁), 8.5% to 30% (t-tau) and 20% to 60% (A β_{42}) for users of the Innogenetics assays (Innogenetics NV, Ghent, Belgium) (74). In a study with 14 participating laboratories testing a single CSF sample, CV varied from 26% to 29% for these three assays (75). This implies that, as long as a uniform standardization of (pre-)analytical procedures has not been defined, inter-laboratory comparisons of test results are possible to a limited extent only. However, as intra-assay CVs are low [$< 8.3\%$ for all three assays as determined in our laboratory (76)], stable and reproducible results can be produced by one and the same laboratory over time. Finally, given the high inter-laboratory CVs, each laboratory should establish their own reference ranges.

The reference values for CSF t-tau and A β_{42} levels using the sandwich ELISAs by Innogenetics were established in 231 healthy individuals, aged 21–93 years, with a Mini-Mental State Examination score of 28 or above (77). Given that a positive correlation was found between age and CSF t-tau, separate reference values for different age groups were established: < 300 ng/L (21–50 years), < 450 ng/L (51–70 years) and < 500 ng/L (71–93 years). The reference value for CSF-A β_{42} was set to > 500 ng/L (Table 2). In addition, there was no correlation found between blood-CSF barrier (dys)function and levels of t-tau or A β_{42} in CSF. In our own laboratory, we established reference ranges with the Innogenetics assays that are largely in line with these data [A β_{42} : > 400 ng/L (< 15 years), > 500 ng/L (> 15 years); t-tau: < 300 ng/L (< 50 years), < 350 ng/L (> 50 years); p-tau₁₈₁: < 85 ng/L (> 15 years)] (Table 2). For reasons mentioned above, these reference ranges may not be universally applicable.

Multiplex analysis of A β_{42} , t-tau and p-tau₁₈₁

Robust analysis of A β_{42} , t-tau and p-tau₁₈₁ should enhance their usefulness in AD diagnosis. As noted above, analysis of these three biomarkers by ELISA may lead to relatively high inter-laboratory variation.

The recent development of assays based on the xMAP technology may possibly solve this issue. The Luminex xMAP technology (Luminex Corporation, Austin, TX, USA) has several advantages over conventional ELISA. It involves coupling of capture antibodies to microspheres (with a unique color code) and fluorescent dye-labeled detecting antibodies. By using a flow cytometry-based detector, multiple analytes can be detected simultaneously. Compared with ELISA, xMAP technology requires less total assay time, fewer procedural steps and a smaller sample volume. It has a higher reproducibility than ELISA does, because the result of each analysis is the mean of multiple (typical 50–100) readings. xMAP assays quantifying A β_{42} , t-tau and p-tau₁₈₁ in CSF, together with test characteristics for calibration, precision and specificity have been recently described (78). Inter-assay CVs for the three analytes are all $< 8.4\%$ – 10% (76, 78). In contrast to what has been suggested by others (78), we observed that a single correction factor cannot be applied to recalculate xMAP results into ELISA results (or vice versa), suggesting that the introduction of xMAP assays should be accompanied by a complete method validation, including a re-establishment of reference ranges specific for this assay which are different from those used for the ELISAs (76). However, both ELISA and xMAP assays had a comparable analytical performance to differentiate AD patients from either controls or VaD patients (76). In another recent, multicenter study, it was demonstrated that the xMAP assays for A β_{42} , t-tau and p-tau₁₈₁ may identify patients with mild cognitive impairment (MCI) that convert to AD among a population of MCI patients, just as has been described for the ELISA assays (79).

Relation with disease severity

In many studies, the correlation between disease severity and CSF levels of A β_{42} , t-tau and p-tau was investigated. The results were not unanimous. Some studies did find a correlation between one or more CSF biomarkers and disease severity (13, 15, 80–82), while many others did not (14, 29, 30, 83–86). Also, remarkably stable CSF A β_{42} , t-tau and p-tau concentrations over a 6-month period were found in individual AD patients (87). In agreement with these latter findings, decreased CSF A β_{42} levels and increased t-tau levels were already found very early in progression of AD, and thus trading the correlation with disease progression for better diagnostic sensitivity. In line with this, these biomarkers were able to predict progression to AD in MCI patients (88–92). Similarly, marked increases in CSF p-tau levels were found in MCI cases, which at follow-up progressed to AD, compared to stable MCI cases (92, 93). Similar results were achieved using the xMAP technology (94). The t-tau/A β_{42} ratio or the p-tau₁₈₁/A β_{42} ratio in CSF can also be used to predict conversion from cognitively normal to AD (95).

Because it is thought that Alzheimer pathology starts at least 20–30 years before the clinical onset of the disease (96), it could be speculated that altered CSF levels of the above-mentioned biomarkers are

present even before cognitive dysfunction begins. Thus, when drugs with potential effects on the progression of AD reach the clinical phase, CSF diagnostics would be helpful in identifying people at risk of developing AD.

Discovery of novel biomarkers by proteomics

Technological advances over the past few years, and the recognition that it is indeed possible to define biomarkers in CSF for AD, have sparked the interest in proteomics techniques to identify such new biomarkers. Several approaches have been followed, either based on separate technologies, e.g., hypothesis-free two-dimensional gel electrophoresis (2D-GE) combined with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), mass spectrometry (MS) or surface-enhanced laser desorption/ionization (SELDI-TOF) MS, or application of these techniques to unbiased proteomics approaches (protein profiling without any type of pre-selection for biomarkers), or procedures with enrichment for specific protein species (e.g., SELDI-TOF with antibody capture).

Unbiased approaches, 2D-GE and MALDI-TOF analysis

Because of the extensive improvement in the techniques to perform 2D-GE and the increased possibilities to identify proteins by MS, the number of studies aimed at the identification of biomarkers in CSF by using this technique has grown steadily. In 2D-GE, proteins are separated in the first dimension on the basis of their pI by iso-electric focusing (IEF) and in the second dimension by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight. Specific protein spots can be picked out from the gel, trypsin-digested and the resulting peptides are analyzed by (MALDI-TOF) MS or nano-liquid chromatography (LC)-MS. Numerous technical papers have been published in which technical advances and methods have been described to pre-treat or enrich CSF samples for low-abundant proteins. Several pre-treatment strategies (protein precipitation, column filtration, removal of albumin and immunoglobulins) may improve 2D-GE patterns (97), leading to large databases of CSF proteins (98). The use of liquid IEF (instead of solid-phase IEF) followed by SDS-PAGE and MS analysis allows for identification of proteins in the various fractions, but mainly high-abundant proteins are identified and large volumes of CSF are needed (99). An extra IEF step may resolve less abundant proteins (100). Irrespective of the exact technology that has been applied, all the variants on the concept of 2D-GE have led to the definition of combinations of multiple proteins – ranging from 5 to 25 in number – that may discriminate AD from non-AD CSF by 2D-GE [see a recent review for a comprehensive overview (101)] (102–104). Most studies described alterations in abundant, mostly blood-derived proteins that are often without any relation to the presumed pathophysiology

of AD, and that are usually not confirmed by alternative techniques (e.g., ELISA) or in independent studies (105, 106). However, one recent study, in which 2D-GE analysis of plasma proteins combined with LC-MS/MS characterization, demonstrated increased concentrations of complement factor H and α_2 -macroglobulin in AD (107). However, although the findings from the 2D-GE studies indicated a large quantitative difference in the levels of these proteins, after confirmation by ELISA these differences appeared much smaller resulting in sensitivity, specificity and negative/positive predictive values all <68%. Furthermore, the use of such combinations of protein markers relative to the analysis of $A\beta_{42}$, t-tau and p-tau₁₈₁ has not yet been studied. Given the advances that have been achieved so far, it is unlikely that a laborious and relatively insensitive experimental approach such as 2D-GE combined with MS analysis will ever reach clinical application or lead to discovery of novel biomarkers for AD.

SELDI-TOF MS

SELDI-TOF is a technique that probably has more potential than 2D-GE/MS to identify novel biomarkers, as it allows not only for an unbiased approach in the search for biomarkers, but also for more specific identification of proteins, e.g., by applying pre-selection with antibodies.

In a small study using CSF of nine AD patients and ten controls, four proteins (cystatin C, two β_2 -microglobulin isoforms, one unidentified protein) were overexpressed and one protein (VGF polypeptide) was underexpressed in AD CSF (108). In a somewhat larger study (16 FTLD and 12 controls) (109), five proteins were increased in FTLD and five others were decreased. Among the increased proteins were transthyretin and S-cysteinylation of transthyretin, and among the decreased proteins were VGF, truncated cystatin C (amino acids 1–8 deleted) and a chromogranin B fragment. Finally, in a recent comparison with 65 AD and 44 control subjects, a model with five proteins (decreased levels in AD: cystatin C and an unknown 4.0 kDa protein; increased levels in AD: truncated cystatin C, C3a anaphylatoxin des-Arg, $A\beta_{1-40}$) together with t-tau and $A\beta_{1-42}$ analysis optimally discriminated these groups (110). Also in this study, decreased concentrations of VGF were found. In general, relatively high-abundant proteins are selected in the SELDI procedures that, in combination with t-tau and $A\beta_{1-42}$, may provide a useful panel of markers to discriminate either AD or FTLD from controls. Somewhat worryingly, several of the proteins identified in these studies and suggested as biomarkers for AD or FTLD [e.g., (truncated) cystatin C, VGF, transthyretin] have also been nominated as biomarkers for amyotrophic lateral sclerosis (111, 112), schizophrenia (113) or multiple sclerosis (114), suggesting that several of the proteins (or combinations thereof) identified as biomarkers for a specific disease by SELDI-TOF are more likely to be reflections of ongoing generalized neurodegeneration rather than related to specific pathophysiological disease processes. Alternatively, they may

be selectively identified under the applied technical conditions. Obviously, larger validation studies with more clinical subgroups, and validation of the data by independent techniques, need to be performed to assess the clinical validity of these findings.

A β peptidomics identified by SELDI-TOF or gel electrophoresis

Most studies aimed at the identification of A β in CSF, have focused on A β_{1-42} or A β_{1-40} . However, several other studies suggested the presence of numerous other A β species in CSF, i.e., N-terminally or C-terminally truncated species and A β peptides with an elongated C-terminus. Such peptides have been identified by SELDI-TOF or urea-based gel electrophoresis. Moreover, evidence is accumulating that dimeric or higher assembled forms of A β peptides may circulate in the CSF.

By coating the SELDI chip with an anti-A β capture antibody, it is possible to employ SELDI-TOF to specifically detect these alternative A β species present in CSF. In a study with a small number of AD ($n=10$) and control patients ($n=9$), it was found that A β peptides other than A β_{1-42} or A β_{1-40} are present in CSF at abnormal concentrations in AD compared to controls. These new A β peptides could fit with the following sequences: A β_{3-44} (decreased in AD), A β_{3-47} (increased in AD) and a possible dimer of A β (decreased in AD) (115). A β peptides starting at residue 1 and ending at residues 37–40 were present in AD and control CSF at equal concentrations. In addition to these peptides, the same research group as well as another group published the identification of many different truncated A β peptides in CSF (116, 117), of which A β_{1-38} was reported to be decreased in AD compared to controls in one study (116), but not in the other (117). Recently, we performed a similar study with CSF of 18 AD patients, 20 VaD patients and 17 controls (unpublished observations). We observed decreased concentrations of A β_{1-37} , A β_{1-38} , A β_{1-39} and A β_{1-40} in AD compared to either VaD or controls. Furthermore, we found decreased levels of A β_{1-33} , A β_{1-34} , several oxidized A β peptides and possible dimeric and trimeric forms of A β in AD vs. VaD or controls. We uncovered preliminary evidence that a possible dimeric A β_{1-38} peptide was decreased in both AD and VaD. These findings of altered concentrations of assembled forms of A β in CSF are in line with the observation that the concentrations of so-called A β -derived diffusible ligands (oligomers) are altered in AD CSF compared to controls (118).

Urea-based gel electrophoresis allowed a good separation of A β peptides of various lengths (119). By using the A β_{1-42} /A β_{1-37} ratio, AD patients ($n=23$) could be differentiated from patients with DLB ($n=21$), idiopathic Parkinson's disease ($n=21$) or controls ($n=23$) (120). However, a disadvantage of this method may be that quantification of A β peptides is likely to be subjected to larger analytical variation than, e.g., specific ELISAs. A combination of immunoprecipitation with anti-A β antibodies and subsequent MALDI-TOF yielded a significant increase in

A β_{1-16} in AD CSF and, in combination with A β_{1-42} , A β_{1-33} , and A β_{1-39} , may distinguish AD from control CSF (121).

The results of these various studies suggest that several of the identified A β peptides or aggregated forms of A β merit further study as a potential biomarker for AD, in particular by using specific quantification techniques (e.g., ELISA) and in larger patient cohorts (also including other dementia forms).

Other potential CSF biomarkers

In the past years, numerous potential CSF biomarkers for AD and other dementias have been studied, e.g., amyloid precursor protein (APP), apolipoprotein E, α_1 -antichymotrypsin, C-reactive protein, complement C1q, homocysteine, 3-nitrotyrosine, neuronal thread protein, NSE, glial fibrillary acidic protein, S-100B protein, ubiquitin and growth-associated protein-43 [for details, see other reviews (19, 122–124)]. However, none of these proteins and metabolites appeared useful as biomarkers in clinical practice, because of conflicting results between different studies, or because of an inferior capacity to discriminate between different dementias compared to A β_{42} , t-tau or p-tau₁₈₁. More recently studied potential CSF biomarkers are described below (see also Table 3).

Isoprostanes

Oxidative damage may play a role in the pathogenesis of AD. Isoprostanes are exclusive products of free radical-mediated peroxidation of arachidonic acid. Levels of isoprostanes were found to be markedly elevated in both frontal and temporal cortex of AD brains, but not in the corresponding areas of FTL D brains and controls (146, 147). F2-isoprostanes are the most studied species, and their CSF concentrations can be measured by gas chromatography/negative ion chemical ionization MS. CSF F2-isoprostanes

Table 3 Potential cerebrospinal fluid (CSF) biomarkers for dementia.

Group	Potential CSF biomarker	References
Isoprostanes	F2-isoprostane	(40, 125–127)
Cytokines	IL-1 β	(123, 128)
	IL-6	(123, 128, 129)
	IL-12	(130)
	IL-15	(131)
	TNF- α	(123, 128)
Neurofilaments	NFL	(132–138)
	NFH/M	(132)
	NFH	(138, 139)
A β oligomers		(118, 140–142)
Enzymes	BACE-1	(143)
	ACE	(144, 145)

IL, interleukin; TNF- α , tumor necrosis factor α ; NFL, neurofilament light subunit; NFH/M, neurofilament heavy/medium subunit; NFH, neurofilament heavy subunit; BACE-1, β -site amyloid precursor protein cleaving enzyme 1; ACE, angiotensin-converting enzyme.

were found to be significantly increased in patients with probable AD compared to controls (40, 125, 126) and compared to FTLD patients (40). Also, levels were highly correlated with disease severity (125, 126). Furthermore, in AD patients and patients with non-AD dementias the combined analysis of CSF F2-isoprostane levels, $A\beta_{42}$ and t-tau levels resulted in sensitivity of 84% and specificity of 89% (127). Others performed a discriminant analysis based on CSF levels of t-tau, $A\beta_{42}$ and F2-isoprostane and were able to classify 88.5% of patients in a manner that corresponded to their clinical or autopsy diagnosis (40). F2-isoprostane levels were also increased in CSF of subjects with MCI compared to cognitively normal elderly subjects (148). This implies that brain oxidative damage starts early in the course of AD, and that F2-isoprostanes can be used as CSF markers before the onset of symptomatic dementia. Additional studies, including confirmation by independent research groups, are warranted to establish F2-isoprostane analysis as a biomarker for AD.

Cytokines

In AD brains, the pathological lesions, in particular senile plaques are associated with a localized neuro-inflammatory reaction, characterized by activated microglia and astrocytes, with increased expression of inflammatory proteins such as cytokines (149). Several cytokines can be detected in CSF, but the results of CSF cytokine measurements in dementia patients are still controversial [reviewed in (123)]. In AD patients, interleukin-6 (IL-6) was found to be increased, decreased or not significantly different from controls in different studies (123, 128). CSF concentrations of IL-6 were also significantly elevated in VaD patients (128, 129). Differentiation of AD and VaD by measuring CSF IL-6 levels was possible in one study (129), which could not be confirmed by others (128). The same applies for IL-1 β and tumor necrosis factor- α (TNF- α). Some studies found increased CSF levels in AD patients, while others did not (123, 128). CSF levels of TNF- α were also increased in VaD patients (128). In the CSF of FTLD patients, IL-15 was elevated, and IL-12 levels were reduced (130, 131), but the same phenomenon was found in AD patients (130, 131).

Thus, although CSF levels of cytokines in dementia patients appear different from controls, their quantifications cannot yet be used as biomarkers to differentiate between different dementia disorders. However, in the future, CSF cytokines maintain the potential to provide assistance in monitoring treatment effects of neuroprotective drugs, especially anti-inflammatory drugs.

Neurofilaments

Neurofilament proteins (NFs) are the major constituents of the neuronal cytoskeleton. They are predominantly localized in large neurons and myelinated axons and play an important role in neuronal structure and function. NFs consist of three polypeptides

with different molecular weights, known as the light (NFL), medium (NFM) and heavy (NFH) subunits, respectively (150). Because NFs are released from damaged neurons, increased levels of NF in CSF may reflect the degree of neuronal degeneration in neurological disease.

Since 1996, the analyses of NFs in CSF of patients with different neurodegenerative disorders, including dementia, have challenged various researchers. NFL levels in CSF were found to be increased in AD and FTLD patients compared to control subjects (132–138). More specifically, CSF NFL levels were elevated in relatively young patients with FTLD compared to patients with early-onset AD (136, 138). When combining CSF levels of NFL with p-tau₁₈₁ and $A\beta_{42}$, differentiating between FTLD and early-onset AD was possible with a sensitivity of 86% and specificity of 100% (138).

In contrast to studies on NFL, less is known about the CSF levels of the other two NF subunits in neurodegenerative dementias. Higher CSF levels of phosphorylated NFH/M were found in AD patients compared to VaD patients and controls (132). CSF levels of NFH were increased in patients with AD, VaD, FTLD and DLB compared to control subjects (138, 139). However, discriminating between different dementia disorders using CSF NFH levels was only possible in DLB vs. early-onset AD (138).

Although increased CSF levels of NFL and NFH were found in different dementia disorders, their analysis showed no superior, and only limited additional, diagnostic value compared to the frequently used CSF biomarkers $A\beta_{42}$, t-tau and p-tau₁₈₁ (138).

$A\beta$ oligomers

Results from the above-mentioned studies using the SELDI-TOF technology suggest the occurrence of $A\beta$ aggregates, particularly dimers, trimers and possibly larger species, in CSF of AD patients. However, the quantification and definite identification of such $A\beta$ oligomers cannot be achieved by SELDI-TOF, and assays that are more specific are needed for this goal. Already back in 1998, a study was published in which the presence at increased concentrations of $A\beta$ oligomers in CSF was demonstrated by seeded polymerization of labeled $A\beta$ onto $A\beta$ oligomeric seeds, visualized by fluorescence correlation spectroscopy (140). More recently, identification of $A\beta$ oligomers in CSF was achieved by a combination of protein immunodetection and DNA amplification techniques in the so-called bio-barcode assay (118). $A\beta$ oligomer concentrations were increased in patients with AD compared to controls. Advanced $A\beta$ oligomerization may lead to an underestimation of $A\beta$ levels, as the commonly used assays preferentially detect $A\beta$ monomers (141). Recently, $A\beta$ oligomers were detected in CSF by using flow cytometry and fluorescence resonance energy transfer (142). Although such oligomers appear to be promising new biomarkers for AD, more validation studies with large clinically well-defined subgroups of dementia syndromes are needed to establish their potential use as a biomarker. Further-

more, extensive analysis and characterization of the type of A β aggregates that are detected by the respective assays are needed to assess the robustness of such assays.

Enzymes

Various enzymes in CSF have been studied as potential biomarkers for AD, but only few are promising. The β -site APP cleaving enzyme 1 (BACE-1) is responsible for the first proteolytic event in the cleavage of APP leading to amyloid formation. Inhibition of BACE-1 is thought to be a therapeutic approach to AD. CSF BACE-1 activity was found elevated in AD patients in comparison to a group of patients with other dementias (143). Thus, detection of BACE-1 in CSF may have diagnostic applications and may also be useful for monitoring the effects of drugs. However, further research is needed.

Another potentially useful enzyme is the angiotensin-converting enzyme (ACE). There is increasing evidence that ACE plays a role in the development of AD. It has been suggested that decreased ACE activity may influence the susceptibility to AD by a mechanism involving A β metabolism (151). However, no difference in CSF ACE levels was found between patients with AD, DLB and controls (144). Others found elevated CSF ACE activity in AD-converted MCI patients and mild to moderate AD patients as compared to controls (145). A brain-penetrating ACE-inhibitor was able to significantly inhibit CSF ACE activity in AD patients (145). Thus, a role for ACE as a possible marker for disease and drug effects remains a possibility.

Plasma biomarkers for AD

Because blood is more easily accessible than CSF, the search is on for useful plasma biomarkers, especially A β_{40} and A β_{42} . Although there was no relation between plasma A β_{40} and A β_{42} levels and those in CSF (152), or between plasma A β levels and brain A β levels (153), some groups reported increased plasma A β_{42} levels in AD patients or MCI patients that converted to AD (154–156). Plasma A β_{40} levels were not increased in these patients (154, 157). Similar to biomarkers in CSF, an increased ratio of plasma A β_{42} and A β_{40} levels correlated with a decrease in dementia risk (158) and vice versa (159). In most studies, plasma A β_{42} levels were positively correlated with age (154, 157), but not with cognitive impairment or duration of the disease (152, 157). Thus, when interpreting the results of plasma A β levels, the effect of age should be taken into account. Also, the consequence of concomitant medication should be considered. Increased levels of plasma A β_{42} were found in non-demented subjects who used insulin and biguanides, decreased levels in users of ginkgo biloba and statin users, and a non-significant trend to reduced levels was found in users of non-steroidal anti-inflammatory drugs (NSAIDs) (160). Although another study could not confirm the altered plasma A β_{42} levels in statin

and NSAID users (157), these findings will complicate the interpretation of the results.

In conclusion, measurement of plasma A β_{42} levels or the plasma A β_{42} /A β_{40} ratio is currently not useful for the diagnosis of AD, but it may be interpreted as a biological risk factor. Changes related to specific medication should be taken into account.

What is the future of biomarkers for AD?

Still the most successful neurochemical biomarkers for AD are the analysis in CSF of A β_{42} , t-tau, and p-tau₁₈₁. However, CSF analysis of t-tau, p-tau and A β_{42} also has some limitations. Although the test characteristics are excellent to differentiate AD patients from controls, the real challenge is in the differentiation of AD from other dementia disorders that commonly include prion disease (e.g., CJD), VaD, tauopathies (e.g., FTLT, progressive supranuclear palsy) and α -synucleinopathies (DLB, Parkinson's disease). CJD can be identified by CSF analysis of, particularly, 14-3-3 and t-tau proteins with very high sensitivity and specificity. However, only a very small proportion of dementia patients suffer from CJD (incidence of 1–1.5: 1,000,000 people). Furthermore, we demonstrated that AD can be very well differentiated from VaD by calculating the A β_{42} /p-tau ratio, which accurately separates both disorders (sensitivity, specificity and negative/positive predictive values >85%) (51). However, the differentiation of AD from other dementia syndromes remains a challenge and is not optimal by using these CSF biomarkers. Basically, this is caused by low A β_{42} levels in DLB and highly variable t-tau and p-tau levels in FTLT. Therefore, the application of CSF testing seems to be particularly restricted to the identification of AD patients amongst a population of dementia patients.

Essentially, almost all studies aimed at the estimation of the diagnostic accuracy of CSF analysis in dementia syndromes have been carried out in specific and selected patient cohorts as part of retrospective case-control studies, in expert referral clinics, and are usually not based on predetermined cutoff values (phase I and IIa,b) (161). Phase III studies, in which the test should be used with predetermined cutoff values to discriminate between cases and non-cases have been attempted, but mostly failed because of insufficient blinding of the clinicians for the CSF measurements in the process of defining a diagnosis and in the omission of strictly pre-defined cutoff values. Extension of the studies referred here with a phase III clinical effectiveness study, in which the additional value of CSF analysis will be investigated in a cohort of patients visiting memory clinics, will be an essential step forward in the evaluation of the validity of CSF testing in the diagnostic pathway for AD as it appears in daily practice.

A recent study described how new diagnostic tests can be categorized and tested against existing diagnostic pathways (162). Three different types of new diagnostic tests can be defined: 1) replacement tests.

The introduction of a new test is superior to existing diagnostic tests and entirely replaces the old ones; 2) triage tests. Triage tests may be less accurate than existing ones, but may be performed prior to the existing tests as a first line of rapid screening; and 3) add-on tests. This type of test does not replace existing tests and is performed after these existing tests in a specific subgroup of patients. Typically, CSF analysis of t-tau, p-tau and $A\beta_{42}$ should be regarded as add-on tests in the diagnostic pathways, as in cases of an unequivocal clinical diagnosis of AD or non-AD, additional diagnostic investigations including CSF analysis are not indicated. We have recently started a diagnostic phase III study in a population of patients visiting a memory clinic for evaluation of their dementia syndrome, in which the diagnostic value of CSF analysis of $A\beta_{42}$, t-tau and p-tau₁₈₁ will be evaluated in an add-on design.

In conclusion, CSF analysis of t-tau, p-tau and $A\beta_{42}$ is still the standard in the neurochemical diagnosis of AD. Novel biomarkers for AD are extensively searched for by several research groups. Novel technologies, including MS analysis, may lead to the discovery of new biomarkers. Probably the most promising type of new biomarkers is still to be found in CSF. However, if new techniques that allow for the sensitive detection of very low levels of brain-specific proteins are being developed, it is most feasible that biomarker analysis in CSF will be replaced by the more easily accessible blood. The presence of assembled forms of $A\beta$ (dimers, trimers, oligomers) may serve as potential biomarkers for AD. In addition, N- or C-terminally truncated $A\beta$ peptides may also add to the diagnostic specificity of CSF analysis for AD. However, specific and robust tests to detect this variety of $A\beta$ -(derived) peptides and aggregates need to be developed. Furthermore, the analysis of isoprostanes, and possibly other specific metabolites related to the oxidative process in AD, may serve as a biomarker. Confirming studies by other groups should nevertheless be performed. Moreover, for all the newly described potential biomarkers, extensive phase I and II studies will have to be performed before they can be evaluated in clinical practice.

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