CEREBROSPINAL FLUID BIOMARKERS FOR ALZHEIMER’S DISEASE: EMERGENCE OF THE SOLUTION TO AN IMPORTANT UNMET NEED

CE Teunissen, EAJ Willemse
Neurochemistry Laboratory and Biobank, Dept. of Clinical Chemistry, Neuroscience Campus Amsterdam, VU University Medical Center Amsterdam

Corresponding Author:
Charlotte Teunissen
Neurochemistry Laboratory and Biobank
Dept of Clinical Chemistry
VU University Medical Center Amsterdam
PO Box 7057 - 1007 MB Amsterdam
Phone: +31 20 444 3680
Fax: +31 20 444 3895
e-mail: c.teunissen@vumc.nl

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ABSTRACT
Alzheimer’s Disease (AD) represents an increasing problem as the overall population ages. The identification of reliable biomarkers of AD has, therefore, become increasingly important. This is not only for risk prediction and diagnosis in order to provide appropriate care, but also to identify those patients at high risk of AD development who may be eligible for inclusion in clinical trials of novel therapies. Treatment in the early stages of the disease are urgently needed, as these are expected to yield the greatest benefits.

The cerebrospinal fluid biomarkers amyloid beta (1–42), hyperphosphorylated Tau and total Tau have been most extensively evaluated. Their combination has been shown to be valuable in identifying AD patients, including those who will progress to AD among a wider group of subjects with only subjective memory complaints or in very early disease stages. While commercially available diagnostic tests for these biomarkers are available, implementation in clinical practice is associated with a number of problems, such as absorption of amyloid beta (1–42) to laboratory tubes, a high degree of batch-to-batch variation with the current test, and the lack of certified reference material. Therefore, there is a need for increased automation and implementation on routine diagnostic platforms in order to support the cost-effective and reliable introduction of the tests on a wider scale. As the use of these biomarkers in research and in clinical practice continues to expand, extensive standardisation efforts are being put in place to address the challenges associated with the use of these biomarkers. Together with the development of additional biomarkers for early and differential diagnosis, this casts good foresight to serve the needs of an increasing patient population.

INTRODUCTION
Alzheimer’s disease (AD) is the most common form of dementia and, as such, forms an important threat for the aging population. AD has a strong age-dependent incidence and prevalence, increasing by 100% every 5 years after the age of 65 (Figure 1). Thus, as we live longer, the population at risk is increasing tremendously. Associated with this, the costs related to AD are exploding,
with increasing demands both on diagnostic activities and on costly institutionalized care in particular [1]. A major challenge is that AD has a long preclinical phase (10–20 years), in which the neurodegenerative events progress. This calls for diagnosis and treatment in the earliest possible stage, especially as it is impossible to replace damaged neurons [2, 3].

Historically, AD was a disease for which no antemortem diagnosis was possible and for which there was no hope of a cure. This situation changed following the discovery of amyloid as an important and unique protein in the characteristic plaques seen in brains of patients with AD. This led to the subsequent development of the amyloid cascade hypothesis that postulates that amyloid formation is central in the evolution of AD pathology [5]. In this hypothesis, which is explained in more detail below, extracellular deposition of amyloid, especially the amyloid beta (Abeta) (1–42) isoform (Abeta(1–42), and intracellular deposition of hyperphosphorylated Tau (pTau) are the major pathological hallmarks of AD. Another important advance in our understanding of AD, which has now been incorporated into treatment approaches, was the discovery that the combination of a decrease in cerebrospinal fluid (CSF) concentrations of Abeta(1–42) and an increase in the total Tau (tTau) and pTau proteins can discriminate AD patients from controls with a sensitivity and specificity of over 85% (reviewed in [6]). Ever since the first data suggesting a role for evaluating Abeta and Tau proteins in CSF were published almost 20 years ago [7, 8], the potential utility of these three biomarkers for AD diagnosis (including diagnosis at an early point in the disease) and in monitoring therapy response has been sequentially and very intensively studied. Due to these efforts, the evaluation of Abeta and Tau levels in CSF is being increasingly incorporated into diagnostic criteria and into the inclusion criteria for clinical trials. At the same time, the assays measuring these biomarkers continue to improve and several large pharmaceutical and diagnostic companies are generating diagnostic tests specifically to serve this market. This paper discusses some of the challenges and opportunities associated with biomarker testing in AD.

CLINICAL FEATURES OF AD

AD typically presents as a progressive decline in memory and learning capacity (short-term memory is particularly affected: what did someone tell you 5 minutes ago?), executive function (how do you prepare your sandwich?), language (naming of words like ‘chair’ or ‘table’) and praxis (make a new jigsaw puzzle). These changes reflect the pathology of the disease, as the characteristic plaques and tangles are observed at a high density in the hippocampus and temporal cortex, areas that have a major function in learning and memory formation. The earliest clinical presentation of AD is termed mild cognitive impairment (MCI), and about 50% of patients with MCI and 10% of patients with subjective memory complaints eventually convert to AD [6, 9]. AD is the most common form of dementia, accounting for 60–80% of demented patients [10]. The other forms of dementia are Lewy body dementia (LBD), Vascular dementia (VaD) and Frontotemporal dementia (FTD). The latter is the most frequent form of dementia in patients below the age of 65, and LBD is the most frequent form in patients over 65.

CAUSES AND MECHANISMS OF AD

The cause of Abeta deposition in sporadic AD patients is largely unknown. The major hypothesis is the amyloid cascade hypothesis, with a key initiating role of oligomerisation and aggregation of Abeta, with downstream alterations in Tau protein (Figure 2) [5]. There are different isoforms of Abeta, varying in length between 37 and 43 amino acids. These Abeta isoforms are produced by endosomal processing of the amyloid precursor protein (APP). The Abeta(1–40) isoform is seen at the highest...
concentration in typical AD plaques, whereas Abeta(1–42) is more fibrillogenic and isoforms such as Abeta(3–42) (or pyroglutamate Abeta) are likely to be more toxic [11–13]. The amyloid cascade hypothesis was based on observations of patients with familial forms of AD (which account for only 5–10% of patients), and observations from animal models overexpressing human APP. These familial AD forms are caused by autosomal dominant mutations in the APP and presenilin (PSEN1 and PSEN2) genes among others [14]. These findings suggest that excessive APP/Abeta production maybe the culprit in familial AD. Strikingly, in these familial AD patients, decreased CSF Abeta levels occur more than 20 years before the clinical symptoms appear, while atrophy (shrinking of the brain) occurs 5–10 years before symptom onset [15]. Thus, there is a long preclinical phase of biological changes that can be identified in CSF even before a patient is aware of any cognitive dysfunction.

By contrast, relatively recent genome-wide association studies indicate that sporadic AD, which occurs in 90% of cases, may be a disease of impaired Abeta degradation rather than APP overproduction. This is because genes involved in Abeta degradation, such as clusterin, complement receptor and PICALM have been associated with the sporadic form of the disease [16, 17]. These findings suggest that immune system function, especially the complement system, may have an important role in the development of sporadic AD. Other possibly related mechanisms are defects in the proper folding of the Abeta protein. For example, a misfolded form of ubiquitin (UBB+) is co-expressed with Abeta plaques, indicating that dysfunction of the proteasome which is responsible for degrading damaged or redundant proteins may be closely involved in AD pathology [18].

**USE OF CSF BIOMARKERS IN AD DIAGNOSIS**

The ability of the biomarkers Abeta(1–42), tTau and pTau to discriminate patients with AD from controls has been clearly demonstrated in a large number of studies (reviewed by [6, 19]). The combination of these three biomarkers is especially sensitive and specific for identifying AD patients among those with subjective memory complaints (defined as persons that come to the clinic worried about their memory function, but in whom no objective signs of memory decline can be observed). The predictive value of these biomarkers in identifying patients who will progress to AD among a group of patients in the MCI stage is also quite good, with a sensitivity ranging from 81–95% and a specificity ranging from 72–95% now shown in several studies [20, 21], including multicenter studies [22]. However, the predictive value becomes lower as patients get older [23]. An interesting feature is that the biomarkers can have predictive value not only in patients with subjective memory complaints, but even in cognitively normal individuals – a topic of increasing interest and investigation [9]. A recent study in cognitively normal individuals showed that altered baseline levels of Abeta(1–42) and pTau preceded the onset of clinical symptoms of MCI or dementia [24]. Even more powerful predictors were the rates of change in the ratios of tTau/Abeta(1–42) and pTau/Abeta(1–42), which had a stronger correlation with the risk of progression than the baseline measurements [24]. On the whole, changed CSF biomarker levels seem to be a reflection of the long preclinical period of AD, during which preclinical pathological changes occur in the brains of patients with AD (Figure 3) [25]. In particular, Abeta(1–42) levels plateau long before the clinical symptoms, and appear unchangeable after reaching this plateau.
The use of CSF Abeta(1–42), tTau and pTau in discriminating AD patients from those with other dementias is less good and is most efficient when ratios of biomarkers (e.g. Abeta/pTau) are used instead of single biomarker measurements [26, 27]. Whereas decreased Abeta(1–42) and increased pTau levels are quite specifically observed in AD [26], increases in tTau are frequently observed in other diseases, for instance after cardiac arrest [28], in ischaemic [29] and traumatic brain injury [30], and in several neurodegenerative disorders, probably reflecting general axonal damage (Table 1) [31]. Postmortem examinations show that the pathology of other dementias may include the same proteins as in AD, i.e. amyloid plaques in LBD and Tau pathology in FTD. The CSF levels of Abeta(1–42) that are considered pathologic consequently overlap between AD and LBD, and Tau levels are increased in FTD as well as AD patients [26]. Therefore, novel biomarkers are needed for differential diagnoses in order to better define patient management and to provide disease-specific patient management.

USE OF CSF BIOMARKERS IN THE DEVELOPMENT OF TREATMENTS FOR AD

The use of Abeta(1–42), tTau and pTau as biomarkers during the development of AD treatments has also been explored, but the results have been inconclusive so far. The biomarkers have been advocated as good treatment outcome measures since repeated measurement of consecutive samples in the same patients have shown stability [33]. By contrast, this stability could be considered to illustrate their inappropriateness as outcome measures, since biomarkers should be dynamic and should correlate with clinical outcome measures to indicate a treatment effect. Indeed, the stability of the biomarkers over 2 years may simply prove that a plateau has been reached in clinically diagnosed AD patients, which cannot be changed upon any treatment. Support for this idea comes from a study showing a change in clinical outcome measures which was not related to a

Table 1

Levels of typical Alzheimer’s disease biomarkers in different types of dementia.

| Reference range | SMC | AD | FTLD | LBD | VaD |
|-----------------|-----|----|------|-----|-----|
| Abeta (1–42) (pg/mL) | > 550 | 863 (691–1,045) | 447 (365–535) | ↓↓ | 741 (500–959) | ↓ | 638 (467–790) | ↓ | 627 (432–862) | ↓ |
| tTau (pg/mL) | < 375 | 245 (179–318) | 604 (419–860) | ↑↑ | 350 (250–496) | ↑ | 305 (222–510) | ↑ | 238 (166–430) | - |
| pTau (pg/mL) | < 52 | 45 (36–57) | 83 (63–112) | ↑↑ | 47 (36–63) | - | 52 (40–69) | ↑ | 35 (27–56) | - |

Arrows indicate changes in biomarker levels compared to SMC. Values are expressed as medians (interquartile range). Abbreviations: SMC = subjective memory complaints; AD = Alzheimer’s disease; FTLD = frontotemporal lobar degeneration; LBD = Lewy Body Dementia; VaD = Vascular Dementia. Reference ranges adapted from [32], other values taken from [26].
corresponding change in biomarker levels [34]. Even if the disease became more severe, typical AD biomarkers did not change, while markers indicating neurodegeneration, such as Tau, in general did. It is important to note that most trials to date have included AD patients with clinical symptoms, in whom we know that the pathology is relatively advanced. It is therefore possible that the CSF biomarkers may have greater utility in monitoring the effects of treatment if given much earlier in the course of the disease before the plateau has been reached. Another complicating factor is the fact that most trials targeting amyloid pathology have so far been unsuccessful or have been impeded by side effects [35].

Other amyloid molecules, such as oligomers, or n-terminally or c-terminally truncated or extended isoforms, have been evaluated as alternative biomarkers. Treatments in clinical trials for AD have not only been developed to target amyloid molecules directly; some target cleaving enzymes in the amyloid cascade such as gamma-secretase and beta amyloid cleaving enzyme (BACE), which are responsible for generating Abeta(1–42) by cleaving APP to form amyloid beta peptides (Figure 4). The use of alternative amyloid isoforms as outcome measurements during treatment with these other therapeutic approaches has shown promising results. For instance, the Abeta(1–14), (1–15) and (1–16) isoforms appeared to respond to gamma-secretase inhibitor treatment, so the next step is to establish their predictive value over the long-term [36, 37]. Also, isoforms Abeta(1–34) and (1–50) react very sensitively to BACE1 inhibition therapy and thus could be used as pharmacodynamic biomarkers, which may also be applicable to other therapies [13, 38]. However, robust assays to analyse these forms in high or intermediate throughput approaches would need to be developed in order to fully exploit the use of these isoforms and no such assays are currently available. The level of soluble APP-beta (sAPPβ), a protein released more upstream in the amyloid cascade, could also be used as an outcome measure since it correlates with BACE1 and levels are decreased following BACE1 inhibitor treatment [39]. In recent trials, Abeta(1–42) is seen as a reflection of tissue Abeta pathology with the aim of treatment being to remove Abeta plaques. These treatments are thus based on the assumption that the plaques are the cause of AD and that removal of plaques should lead to a resolution of the disease. However, this remains to be seen, as plaques could also be the end product, and a way to isolate the toxic intermediate products. A recent approach is to target the unfolded protein response, a mechanism to repair misfolded proteins, which not only include Abeta, but also prion proteins which cause Creutzfeld–Jacobs disease, for instance. The presence of cellular prion proteins was found to be crucial for the development of memory deficits in vivo in Alzheimer mice [40] and suppression of this machinery in mice shows enhancement of cognitive performance [41], which suggests potential for translating these approaches into human trials. Another potential use of CSF biomarkers is for patient selection for inclusion in clinical trials, especially for inclusion for early treatment. Since a decrease of Abeta(1–42) has been proven to be a specific marker of AD pathology, even in the preclinical phase, decreased levels of this protein can be used as an objective inclusion marker [42].

Figure 4
Cleavage of the APP protein leading to accumulation of Abeta oligomers. Levels of sAPPβ can be used as a direct readout parameter for the efficiency of BACE1. Abeta, amyloid beta; APP, amyloid precursor protein; BACE1, beta amyloid cleaving enzymes; sAPPβ, soluble APP-beta.
Assays for CSF biomarkers for AD

Historically, Abeta(1–42), tTau and pTau have been measured primarily using enzyme-linked immunosorbent assays (ELISA) and the majority of studies so far have used the Innotest assays. Since all three biomarkers are generally measured simultaneously, assays have also been developed on multiparameter platforms, such as a Lumineux and bead-based platform (MesoScale electrochemoluminescence technology). The Innotest assays have served the field well, and have been incorporated into clinical diagnosis in research and in clinical settings. However, there are some drawbacks associated with these assays, some of which are partly due to the fact that the amyloid proteins are very prone to aggregation and therefore very difficult proteins to work with. Drawbacks include the lack of certified reference material with which to calibrate assays, the absence of internal controls and the presence of significant batch-to-batch variation. Another drawback has been the use of different protocols for the assays used for the three biomarkers, with limited automation involved.

Extensive standardization of every aspect of biomarker analysis is now being addressed through the initiation of an international quality-control program which includes CSF reference materials [43] and in the European Joint Programming project ‘Biomarkers for Alzheimer’s Disease and Parkinson’s Disease’ (BIOMARKAPD). To give an example, the BIOMARKAPD project standardizes procedures for CSF collection, biobanking, thorough assay validation (in agreement with ISO15198 guidelines), assay implementation, clinical interpretation and patient communication. In addition, the project is developing certified reference standards and internal controls for calibration. The effects of variation in all aspects of the procedure are also being extensively tested in this project. For example, different types of polypropylene tubes were shown to absorb Abeta differently. Although they may look similar, transferral of CSF into different tubes resulted in changes in biomarker levels of up to 60% [44]. The project has adopted one collection of tubes with the lowest known absorption, and a further activity of the project is now to compare absorption in biobanking tubes.

Now that implementation of CSF biomarker testing in clinical practice is becoming increasingly common (for example, to support patient diagnosis in unclear cases, and to determine eligibility for trial inclusion), there is a clear stimulus for improving current tests and developing novel tests. Current tests have substantial batch-to-batch variation. For example, batch-to-batch variation accounted for ~22% of the total variation observed in Abeta levels, and around 2 and 10% for tTau and pTau, respectively, in a multicenter study involving 61 laboratories using the Innotest assays [45]. In another study, we showed reduction in variation in Abeta outcomes of internal controls from 13% to 8% as a result of changing from use of multiple lots (n=30 runs) to a single lot (n=18 runs of one lot) [46]. Improvements are needed to lower turnaround time and to reduce batch variation. The expiry of patents on current assays is increasing the degree of interest in developing assays from other companies. Some clinics, such as the Alzheimer Center Amsterdam, offer patients a one-day diagnostic screening, in which patients go through all procedures and tests for diagnosis; this is followed by discussion of the results in a multidisciplinary team with discussion with the patient themselves within a week. In this set-up, a cost-effective and robust approach to the screening of smaller subsets of CSF on a weekly basis would be a great advantage. For an ELISA test to be cost-effective, one must analyse a minimum of 30 patient samples, which can usually only be achieved by infrequent analysis. There is thus a need for automation and implementation of assays on routine platforms, in view of the increase in numbers of samples to be analysed and to minimize assay variation. Nevertheless, centralized analysis by expert laboratories is likely to still be relevant in the future, even if more cost-efficient platforms are developed, in order to achieve sufficiently large analysis loads to perform quality control [13].

Future perspectives

The CSF biomarkers Abeta, tTau and pTau have proven value for diagnosis of AD, and in addition have good predictive value for progression to AD in subjects with MCI. Current biomarker studies are therefore focusing on the development of biomarkers for differential diagnosis between different subtypes of dementia (e.g. AD, FTD, LBD etc), and on the development of blood-based biomarkers for screening. Nevertheless, the current AD CSF biomarkers are already finding their way into clinical trials, to aid the determination of eligibility of patients for inclusion in trials of early disease stages. Current efforts for standardization and optimization of analysis and interpretation of these biomarkers will enhance broad worldwide implementation.

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