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REVIEW

Consensus Paper of the WFSBP Task Force on Biological Markers of Dementia: The role of CSF and blood analysis in the early and differential diagnosis of dementia

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Abstract

Aging of population, and increasing life expectancy result in an increasing number of patients with dementia. This symptom can be a part of a completely curable disease of the central nervous system (e.g, neuroinflammation), or a disease currently considered irreversible (e.g, Alzheimer's disease, AD). In the latter case, several potentially successful treatment approaches are being tested now, demanding reasonable standards of pre-mortem diagnosis. Cerebrospinal fluid and serum analysis (CSF/serum analysis), whereas routinely performed in neuroinflammatory diseases, still requires standardization to be used as an aid to the clinically based diagnosis of AD. Several AD-related CSF parameters (total tau, phosphorylated forms of tau, Aβ peptides, ApoE genotype, p97, etc.) tested separately or in a combination provide sensitivity and specificity in the range of 85%, the figure commonly expected from a good diagnostic tool. In this review, recently published reports regarding progress in neurochemical pre-mortem diagnosis of dementias are discussed with a focus on an early and differential diagnosis of AD. Novel perspectives offered by recently introduced technologies, e.g, fluorescence correlation spectroscopy (FCS) and surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) are briefly discussed.

Key words: *Dementia, Alzheimer's disease, neuroinflammation, cerebrospinal fluid*

Introduction

Along with the aging of population and increasing life expectancy, dementia will become a serious problem for the health care system. Regarding Alzheimer's disease (AD), however, the increasing number of patients has not resulted in achieving accurate standards of *durante vitam* diagnosis so far. Although sensitivity of clinical diagnosis is relatively high (93%), specificity may be lower, e.g., reported as 55% in a multi-center clinical-autopsy study (Mayeux 1998). Although in expert hands the clinical diagnosis of AD is predictive of ADpathology in 80–90% of cases, very early diagnosis of AD, and

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differential diagnosis of unusual presentations of patients with dementia remains difficult on clinical grounds.

With the introduction of potentially successful treatment against dementia prior considered irreversible, like acetylocholinesterase inhibitors in AD (recently reviewed in Bullock 2002; Knopman 2001), the need for an early and differential diagnosis of dementia becomes even more urgent (1998). Biomarkers such as neuro-imaging studies and CSF analysis have been developed as aids to clinical diagnosis. The aim of this review, therefore, is to summarize the current state-of-the-art in the field of neurochemical diagnosis of dementias with a special focus on biomarkers of AD. Finally, new perspectives to search for dementia biomarkers offered by novel sophisticated techniques, like correlation spectroscopy and mass spectrometry are briefly discussed.

Since cerebrospinal fluid (CSF) is in direct contact with the environment of the central nervous system (CNS), it is obvious that any changes in biochemical composition of brain parenchyma should be predominantly reflected in the CSF. A recent review by Reiber (2001) presents a complete concept of distinguishing diffusion of brain-derived proteins into CSF from diffusion of proteins from blood into CSF, allowing proteins that originate from the brain to be prioritized. Lumbar puncture is an easy procedure, with low incidence of complications. In a large study by Andreasen et al. (2001) only 4.1% of all patients experienced post-lumbar headache, and an even smaller ratio of 2% was reported in the study of Blennow et al. (1993). Thus, it is reasonable to postulate that lumbar puncture is a feasible, moderately invasive procedure, and CSF analysis could possibly improve current clinical and neuroimaging-based approaches to diagnosis.

Dementia as a symptom of potentially reversible diseases

Dementia could be one of the symptoms in several potentially treatable diseases of CNS. Therefore, the American Academy of Neurology advises CSF analysis in differential diagnosis of dementia in suspected cases of: metastatic cancer, CNS infection, reactive serum syphilis serology, hydrocephalus, immunosuppression, CNS vasculitis, and in all patients under the age of 55 (1994). These guidelines basically have not changed, and were further confirmed recently (Knopman et al. 2001). Moreover, routine CSF analysis serves as a basis for advanced CSF protein investigation since analysis of *all* blood- and brain-derived proteins in CSF *must* be related to blood–CSF barrier function, as indicated by albumin CSF/serum concentration quotient (Q_{Alb}) (Reiber and Peter 2001).

Among many others, inflammatory diseases of CNS form a group of reversible disorders often characterized by memory disturbances, and CSF analysis should be performed in all suspected cases.

Neuroborreliosis is a CNS disease caused by the tick-borne spirochete *Borrelia burgdorferi*. Several neuropsychiatric complications have been observed in this disorder, including dementia, as reviewed by Fallon and Nields (1994). Neuroborreliosis should be considered as a possible cause of neuropsychiatric symptoms especially in endemic areas. With a combination of parameters routinely analysed in CSF and serum, neuroborreliosis can be confirmed or rejected with a specificity of 96% and a sensitivity of 80% (Tumani et al. 1995). It is usually characterized by blood–CSF barrier dysfunction (increased Q_{Alb}), mononuclear pleocytosis, dominant or isolated intrathecal IgM synthesis, and increased specific antibody indices (Tumani et al. 1995; Reiber and Peter 2001).

In neurosyphilis, another example of CNS inflammatory disease that can present with dementia, normal or only slight dysfunction of blood–CSF barrier is observed, accompanied by intrathecal IgM and IgG synthesis (Reiber and Peter 2001). Serological analyses (VDRL and TPHA) are usually positive and confirm the diagnosis.

Infection with human immunodeficiency virus (HIV) can affect cognitive functions in several manners. CNS involvement is a frequent complication in AIDS, appearing in 30–60% of patients (as reviewed by Luer et al. 1988). In the German study of Poser et al. (1988), 11% of subjects with AIDS presented dementia, a percentage somewhat lower than in American studies (Navia and Price 1987). Memory impairment, and other cognitive disturbances in HIV-related encephalitis are much more frequent and appear earlier than AIDS-related dementia. Infection of CNS with HIV not only leads to encephalitis with cognitive impairment by itself. Even more importantly, immunodeficiency leads to severe infectious complications known as opportunistic infections. These include neurotoxoplasmosis, cytomegalovirus (CMV) infection, herpes simplex virus (HSV) infection, CNS tuberculosis and many others. Since all these conditions are potentially treatable, their differential diagnosis should always include CSF analysis. Immunological findings (intrathecal synthesis of specific antibodies) combined with genetic analysis (polymerase chain reaction, PCR) may even increase accuracy of the diagnosis.

Among patients with CNS Whipple's disease, up to 71% of cases develop cognitive changes (Louis et al. 1996). The signs of systemic infection include

gastrointestinal problems, weight loss, and arthritis, and the symptoms of CNS infection include also supranuclear gaze palsy, altered level of consciousness, and movement disorders (Louis 2003). Cerebrospinal fluid changes in this chronic inflammatory disorder caused by *Tropheryma whippelii* include mild pleocytosis with typical PAS-positive macrophages, called Sieracki's cells, in approximately 30% of cases. PCR with primers recognizing bacterial DNA can substantially increase the sensitivity of the diagnosis.

Evidence has been reported suggesting neuropsychological deficits in the course of multiple sclerosis (MS), including memory impairment and dementia (Rao 1986; Rovaris and Filippi 2000). Typically, MS patients show normal or only slightly increased cell count and blood–CSF barrier disturbances in routine CSF analysis accompanied by intrathecal immunoglobulin synthesis. Oligoclonal IgG bands, and IgG, IgM, and less frequently IgA local synthesis are observed. Intrathecal synthesis of antibodies specific for neurotropic viruses (measels, rubella, and/or varicella-zoster) is very frequently found, a phenomenon known as MRZ reaction. Clinically, sensitivity of MRZ reaction and oligoclonal IgG bands in MS are 89 and 98%, respectively, i.e., comparable to neuroimaging methods (Reiber et al. 1998); however, specificity of oligoclonal bands is much lower as they are observed in many neuroinflammatory conditions. Currently, isoelectric focusing is considered as a method of choice to detect oligoclonal bands in CSF/serum.

Neurochemical markers of Alzheimer's disease

The major cause of dementia in the elderly is Alzheimer's disease. Moreover, in older subjects, AD is a major overall cause of morbidity and death. Neuropathological markers of AD are extracellular β-amyloid deposits (plaques), and deposits of neurofibrillary tangles (NFT) (reviewed in Wiltfang et al. 2001b). Neuronal loss with brain atrophy, disturbances of neurotransmission as well as local inflammatory reaction of glia are also commonly found.

Recently, requirements have been proposed for a test to become an acceptable diagnostic parameter in AD (1998). Ideally, such a test should be: able to detect a fundamental feature of AD pathology, validated in neuropathologically confirmed cases, precise and reliable, non-invasive, simple to perform and inexpensive. A common consensus is that sensitivity and specificity of such a test should not be lower than approximately 85%, and 75–85%, respectively (1998).

β-Amyloid precursor protein (β-APP): Metabolism and impact on AD diagnosis

β-Amyloid plaques are composed mainly of peptides coming from enzymatic cut of β-amyloid precursor protein (β-APP) (Kang et al. 1987). This transmembrane protein is encoded in man by a gene on chromosome 21, and its alternative splicing results in at least eight forms. The form known as β-APP 695 (i.e., one consisting of 695 amino acid residues) is expressed predominantly in the brain (Panegyres 1997). The physiological role of β-APP is not clear so far; however, an involvement in cell-to-cell and matrix interactions is postulated. β-APP is enzymatically processed by α-, β-, and γ-secretases to release several forms of amyloid β-peptides (Aβ). This process is schematically presented in the Figure 1. Interestingly, the discovery of amyloid β peptides ending at different C-termini leads to a conclusion that different γ-secretase activities may exist. If so, perhaps a specific inhibitor might be synthesized to prevent a generation of Aβ1–42 by blocking the cut at the position 42, thus protecting from formation of β-amyloid plaques.

Several studies, including these from our group, report decreased CSF concentration of Aβ1–42 in the CSF (Wiltfang et al. 2002; Lewczuk et al. 2004c), as do two reviews (Blennow et al. 2001; Wiltfang et al. 2001b), whereas another reports that total level of Aβ peptides remains unchanged (Motter et al. 1995). Even more importantly, CSF Aβ1–42 seems to decrease in the course of the disease before a severe collapse of patients' cognitive functions occurs (Riemenschneider et al. 2000). As an example, decreased CSF Aβ1–42 in AD is clearly shown by the SDS–PAGE/immunoblot method (Figure 2). It must be stressed that CSF concentration of Aβ1–42 strongly depends on pre-analytical samples treatment (Wiltfang et al. 2002). Results obtained with methods involving incubation with detergent or heat (e.g., immunoprecipitation in the presence of detergent or SDS–heat denaturation) are up to three-fold higher compared with results obtained without such pretreatment (reviewed in Wiltfang et al. 2002). This finding indicates the presence of a fraction of Aβ peptides not accessible to antibodies, most probably due to binding to carrier proteins.

Mechanisms leading to decreased concentrations of Aβ1–42 in CSF of patients with AD are not yet clear. Accumulation of the peptide in the plaques is suggested by some investigators. This hypothesis, however, cannot explain our results (Otto et al. 2000) of decreased concentration of the peptide in the CSF of the subgroup of patients with Creutzfeldt-Jakob disease (CJD) who did not develop any

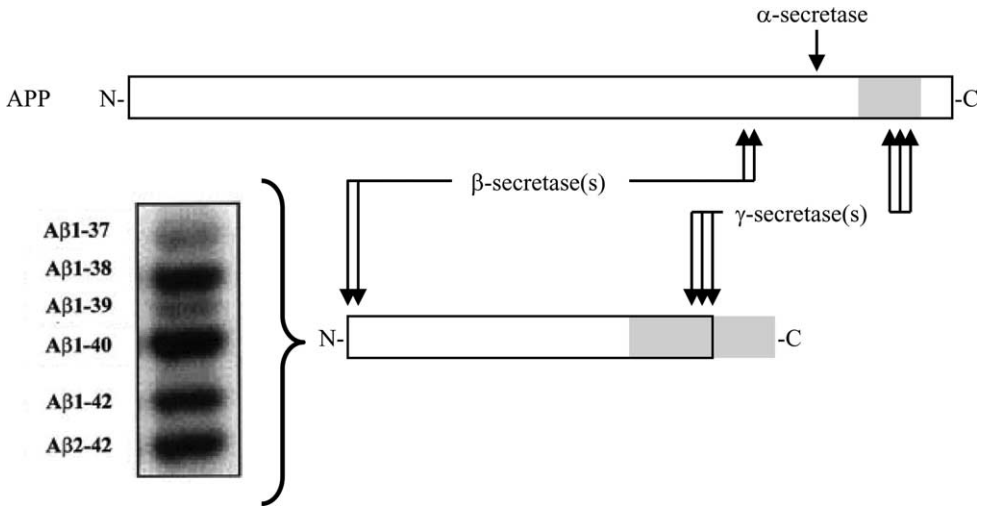

Figure 1. Common cut of amyloid precursor protein (APP) by β- and γ-secretases leads to a formation of at least six different β-amyloid peptides. The 'classical' position of the cut by β-secretase is defined as position-1 of Aβ peptides; however, an alternative cut by β-secretase leads to a release of peptides beginning at position 2 and also, probably, 3. γ-Secretase(s) cut(s) ahead of amino acid isoleucine (position, 40) leads to the generation of Aβ1–40, and the cut ahead of the amino acid threonin (position 42) leads to the generation of Aβ1–42. Recently, peptides ending at C-terminus positions 37, 38, and 39 have also been found (Wiltfang et al. 2002). This may indicate the presence of more than one activity of this enzyme, resulting in the search for a possible specific inhibitor. The gray box indicates the position of the transmembrane domain of APP.

amyloid plaques. Similarly, decreased levels of CSF Aβ1–42 were recorded in bacterial meningitis (Sjögren et al. 2001), a disease which may cause chronic memory deficits but does not present with β-amyloid plaques. In fact, the lowest values found in the large multicenter study of Hulstaert et al. (1999) were observed in two cases of subacute sclerosing panencephalitis and in one case of bacterial meningitis. As an alternative hypothesis, it might be postulated that the formation of Aβ1–42 binding complexes is responsible for decreased CSF concentration of the peptide (Wiltfang et al. 2002). Indeed, evidence for such complexes in the CSF of patients with AD have been recently obtained by fluorescent correlation spectroscopy (Pitschke et al. 1998).

The sensitivity and specificity values of Aβ1–42 alone to distinguish AD from elderly controls were 78 and 81%, respectively, in the study by Hulstaert et al. (1999), and Galasko et al. (1998) reported similar figures of 78 and 83% for sensitivity and specificity, respectively. In our study (Lewczuk et al. 2004c), application of CSF Aβ42 alone resulted in a correct classification of 87% of subjects when non-Alzheimer's dementia and non-demented patients were treated as a control group for AD. Blennow et al. (2001) analysed data from eight studies with total number of 562 AD patients and 273 controls, and reported mean sensitivity and specificity of 85 and 84%, respectively.

We have recently found an additional amino terminal-truncated Aβ peptide, i.e., Aβ2–42 in CSF of patients with AD (Wiltfang et al. 2001a), and this peptide is the second, after Aβ1–42, most abundant Aβ peptide in the frontal lobe of patients with AD. In the CSF, it is present in a subset of 35% of AD cases. Experimental studies with knock-out mice showed that Aβ2–42 was most probably produced by an alternative β-secretase cut, and of particular pathophysiological importance is that this peptide may serve as a first nidus for β-amyloid plaque formation (Wiltfang et al. 2001a).

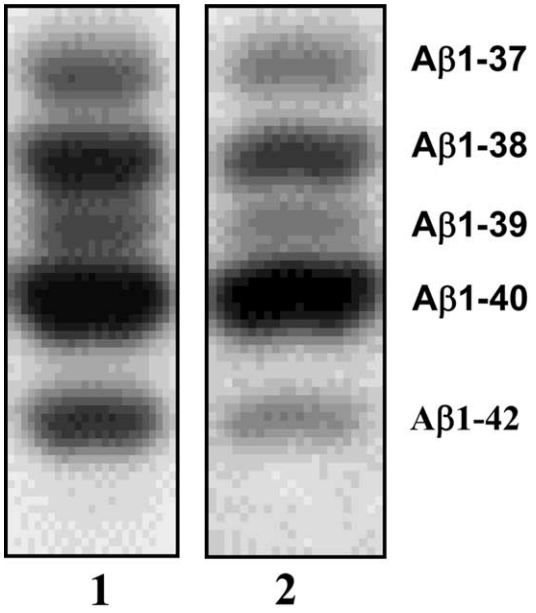

Figure 2. Quantitative Aβ-SDS–PAGE/immunoblot shows decrease in Aβ1–42 in the CSF of a patient with AD (band 2) compared to a control case (band 1). According to Wiltfang et al. 2001a) with modifications.

Application of a urea-based electrophoresis system (Wiltfang et al. 1991) resulted in finding other carboxyl terminal-truncated Aβ peptides, i.e., Aβ1–37/38/39 in human CSF (Wiltfang et al. 2002) as well as in blood (Lewczuk et al. 2004d). This highly conserved pattern was observed in all CSF samples investigated so far, with AD characterized by elevated fraction (%) of Aβ1–40 (Aβ1–40%), reduced Aβ1–42%, and elevated Aβ1–38% in some cases (Wiltfang et al. 2002). Elevation of the latter peptide concentration, observed also in some cases of chronic neuroinflammatory diseases further supports the commonly accepted role of inflammation in developing AD (Eikelenboom et al. 2000; McGeer and McGeer 2001).

Tau protein and its phosphorylated forms

Tau proteins belong to the family of microtubule-associated proteins (MAPs) found in neuronal and non-neuronal cells (for a review, see Buee et al. 2000). The human tau gene is located on the long arm of chromosome 17. Its alternative splicing leads to formation of six forms of the protein ranging from 352 to 441 amino acids. Studies on the role of tau proteins have revealed that their main function is to promote neuronal microtubule stability and assembly. Tau proteins are also involved in promoting microtubule nucleation, growth and bundling, and it is hypothesized that phosphorylation of the tau molecule is an important factor in regulating tau-microtubule interaction (for a review, see Shahani and Brandt 2002). The phosphorylation status of tau is considered to change during development, with a relatively high degree of phosphorylation during the fetal phase followed by a steady decrease with age, most probably as a result of phosphatase activation (Mawal-Dewan et al. 1994; Rosner et al. 1995). An increasing amount of evidence indicates that tau also interacts directly or indirectly with the actin cytoskeleton, playing an important role in regulating the cell's shape, motility and the interactions between microtubule and the plasma membrane (for a review, see (Shahani and Brandt 2002)). Interestingly, as recently reported by the group of E. Mandelkow, tau regulates intracellular traffic of vesicles and inhibits transport of amyloid precursor protein (APP) into neuronal extensions, which leads to accumulation of APP in the cell body (Stamer et al. 2002). Studies with cell culture models indicate that tau is also involved in neurite outgrowth and stabilization (Baas et al. 1991; Knops et al. 1991). Moreover, primary cultures of neurones from genetically modified mice with knocked out tau gene show a significant delay in their axonal and dendritic extensions (Dawson et al. 2001), however, another group reported a completely normal phenotype of mice lacking the tau gene (Harada et al. 1994). These seemingly conflicting results have been recently explained by the finding that other MAPs possibly compensate the missing tau gene (Takei et al. 2000). The role of tau and its phosphorylation by GSK-3 in anterograde transport and in neurite outgrowth has recently been shown by Tatebayashi et al. (2004).

Total tau protein concentration has been extensively studied as an unspecific marker of neuronal destruction in AD. A meta-analysis by Sunderland et al. (2003) was based on the data from 17 reports on Aβ42 and 34 reports on CSF tau in AD, and all studies included in this meta-analysis reported increased CSF total tau in AD. Since an age-related increase of tau concentration has been reported in non-demented controls by some investigators (Buerger et al. 2003), age-dependent reference values of total tau should be considered as has recently been suggested (Sjogren et al. 2001). Increased CSF total tau concentrations are observed in neuropsychiatric disorders other than AD, e.g., Creutzfeldt-Jakob disease (CJD) and stroke (Otto et al. 1997; Hesse et al. 2001). Nevertheless, given the fact that tau most likely can be used to monitor the efficacy of the neuron protective drugs in AD patients, and that CJD and acute stroke are easily distinguishable from AD clinically, should not dampen the value of this marker.

Hyperphosphorylation of tau as a pathological event. It is suggested that some phosphorylation events change the conformational status of the tau molecules, leading to decreased microtubule binding, a reduced ability to promote microtubule assembly, and increased dynamic instability of microtubules. It has been reported that phosphorylation of serine 262 partially abolishes the ability of tau to bind to microtubules (Singh et al. 1996), whereas phosphorylation at threonine-231 and serine-235 markedly influence tau's binding to microtubules (Sengupta et al. 1998). In the brains of patients afflicted with Alzheimer's disease, hyperphosphorylated molecules of tau protein assemble to form intraneuronal filamentous inclusions, neurofibrillary tangles, one of the hallmarks of the disease, and it has been observed that that the number of neurofibrillary tangles correlates closely with the degree of dementia (Alafuzoff et al. 1987; Braak and Braak 1991; Arriagada et al. 1992). Intracellular deposits of hyperphosphorylated tau are observed in several neurodegenerative disorders, known as tauopathies; however, in Alzheimer's disease these inclusions are described only in neurons. Recently it has been suggested that the phosphorylation of tau proteins in

AD happens in a form of evolution with amino acid positions 153, 262, and 231 modified in an early stage of the disease and positions 199, 202, 205, 396, and 404 relatively late (Augustinack et al. 2002). This evolution of phosphorylation sites of the tau molecule corresponds to the morphological evolution of the tangles in AD. Simultaneously, an increased concentration of phosphorylated forms of tau protein is measured in the CSF of AD patients (Vanmechelen et al. 2000; Itoh et al. 2001; Buerger et al. 2002). Several kinases have been shown to phosphorylate tau molecules as recently reviewed (Buee et al. 2000), including stress-activated protein kinases, mitogen-activated protein kinases, glycogen synthase kinase 3, and others.

Phosphorylated tau in CSF as a biomarker of Alzheimer's disease. While the increase in the total tau CSF concentration is considered to reflect unspecific disruption of nerve cells, abnormal hyperphosphorylation of tau is a hallmark of AD (Iqbal et al. 1986), and hyperphosphorylated molecules of tau form neurofibrillary tangles (Grundke-Iqbal et al. 1986). Tau can be phosphorylated at 79 putative positions, serine and threonine being predominant. Recently, independent groups have reported increased CSF concentrations of phosphorylated tau to discriminate AD from other dementia diseases. In studies available so far, mean sensitivity and specificity of tau phosphorylated at different positions varied from 44 to 94 and 80 to 100%, respectively (Blennow et al. 2001). Interestingly, Hu et al. (2002) have shown that pTau396/404 to total tau ratio in CSF could discriminate AD from other dementias and neurological disorders at a sensitivity of 96% and a specificity of 94%. These findings suggest that CSF analysis of tau phosphorylated at serine-396/404 might be more promising than some of the other sites reported to date. It must be also noted that, while processes of hyperphosphorylation of tau dominate in AD, dephosphorylation of these molecules is supposed to happen as well, and the ratio of phosphorylated and dephosphorylated tau molecules must be considered dynamically. Moreover, dephosphorylation of hyperphosphorylated tau seems to be one of the most promising therapeutic targets in AD, as recently reviewed (Iqbal et al. 2002).

In our recent study, we found significantly increased CSF concentrations of pTau181 in the group of AD patients with clinical diagnosis supported neurochemically by decreased A42 in CSF (Lewczuk et al. 2004a). This form seems to be particularly interesting, since pTau181 remains unchanged while total tau is increased after acute stroke (Hesse et al. 2001). This may suggest that pTau181 is not only a marker of simple neuronal loss. Similarly, Vanmechelen et al. (2001) reported significantly increased levels of CSF pTau181 in AD compared to all other groups studied (FTD, LBD, Parkinson's disease, multiple system atrophy, and progressive supranuclear palsy) except for corticobasal degeneration. Parnetti et al. (2001) confirmed that pTau181 was a useful biomarker to distinguish AD from dementia with Lewy bodies. Moreover, in agreement with the results of our study, Vanmechelen et al. (2001) and Nägga et al. (2002) found a strong correlation between total tau and pTau181 independently of the diagnostic group. Similarly, increased CSF concentrations of pTau181 have been reported in subjects with probable AD compared to controls (Nägga et al. 2002), and Papassotiropoulos et al. (2003) found increased CSF concentration of pTau181 associated with a polymorphism of CYP46. The group of C. Hock reported that this gene was associated with an increased risk of late-onset sporadic AD.

Regarding other phosphorylation sites, in an international multicenter study, Itoh et al. (2001) reported a significant overall increase of pTau199 in patients with AD compared to all other non-AD groups. In that study, both sensitivity and specificity of CSF pTau199 for discriminating AD from other studied groups yielded 85% at the cutoff level of 1.05 fmol/ml. Tau phosphorylated at threonine-231 (pTau231) seems to help in the differentiation of AD from relevant diseases, i.e., frontotemporal dementia (FTD), vascular dementia (VD), and Lewy body dementia (LBD) (reviewed in Blennow et al. 2001). A follow-up study revealed increased CSF concentration of pTau231 at the onset of the disease followed by decreasing concentrations of pTau231 but not total tau in a group of untreated AD patients. This may suggest a possible role of this isoform in tracking a natural course of the disease (Hampel et al. 2001). Interestingly, tau protein phosphorylated at both positions threonine-231 and serine-235 turned out to be increased in patients with mild cognitive impairment (MCI) who developed AD during follow-up (Arai et al. 2000). In this study, a simultaneous evaluation of total tau and phosphorylated tau distinguished the group of patients at risk of developing AD from those who complained of having memory impairment but did not have objective memory loss.

In a recently published study, three different forms of phospho-tau forms have been compared regarding their ability to distinguish patients with different forms of dementia as well as non-demented controls. The outcome of this study shows overall equal performance of pTau181 and pTau231, with somehow worse performance of pTau199. Interestingly,

discrimination between AD and dementia with Lewy bodies was maximized using pTau181at a sensitivity of 94% and a specificity of 64%, and pTau231 maximized group separation between AD and frontotemporal dementia with a sensitivity of 88% and a specificity of 92%. Therefore, there seems to be a non significant tendency for phospho-tau proteins to perform differently in the discrimination of particular types of dementias (Hampel et al. 2004)

Apolipoprotein E (ApoE) genotype

ApoE is a protein involved in the transport of cholesterol. Apart of the presence in plasma, it is also produced by astrocytes in CNS to support growth and repair of neurons. The ApoE gene is localized on chromosome 19 and three allele are described, ε2, ε3, and ε4. Recently, a growing volume of evidence has been reported on the association of ApoE ε4 and late-onset familial AD (for review, see Mulder et al. 2000). As many as 40–50% of AD patients possess ε4 allele compared to 15–25% of controls (Strittmatter et al. 1993a). Subjects homozygous for ε4 allele are reported to have 6–8-fold increased risk of developing AD compared to the risk for heterozygotic subjects who have an increased risk of 3–4-fold (reviewed by Mulder et al. 2000). So far, the ε4 allele has been identified as one of the major risk factors studied, independent of gender, age, and ethnic origin of individuals (Farrer et al. 1997). In a large American study with 2188 patients, analysis of ε4 allele showed mild sensitivity and specificity of 65 and 68%, respectively (Mayeux et al. 1998). Thus, it is suggested that genotyping for ApoE allele should be reserved for demented patients, and the presence of one or two ApoE ε4 alleles can improve the specificity of the diagnosis in patients who fulfill the clinical criteria of AD. The mechanisms regulating increased risk of developing AD in cases carrying the ε4 allele are still unclear.

Monoamine-oxidase-B in blood as a biomarker of Alzheimer's disease.

One of the enzymes involved indirectly in oxidative-stress is the amine-metabolizing enzyme, monoamine-oxidase-B (MAO-B, E.C.: 1.4.3.4), a flavin-containing enzyme localized in the outer mitochondrial membrane (Greenawalt and Schnaitman 1970) and responsible for the oxidative deamination of neurotransmitters (noradrenaline, dopamine and serotonin) and exogenous amines (e.g., tyramine) (Weyler et al. 1990). During its catalytic activity it produces hydrogen peroxide. Hydrogen peroxide formed in the reaction is a possible source for oxidative stress, which may cause neuronal cell death in Alzheimer's disease as well as other neurodegenerative diseases (Riederer and Youdim 1993). Several previous studies reported increased activities of MAO-B in the brain and blood platelets of patients suffering from neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Adolfsson et al. 1980; Smith et al. 1982; Gottfries et al. 1983; Oreland and Gottfries 1986; Alexopoulos et al. 1987; Danielczyk et al. 1988; Bonuccelli et al. 1990; Fischer et al. 1994; Zhou et al. 2001). The MAO-B as well as MAO-A activities in the brain of Alzheimer's disease subjects are brain region specific, as shown by the fact that MAO-B activity decreases in the nucleus basalis of Meynert and increases in the temporal pole, whereas the expression pattern in Parkinson's disease as well as Pick's disease are dissimilar (Sparks et al. 1991). The increase in brain MAO-B might be due to transcriptional elevation of MAO-B protein (Nakamura et al. 1990) and predominant in plaque-associated astrocytes in neuropathologically verified Alzheimer brains with astrogliosis, respectively (Jossan et al. 1991; Saura et al. 1994). However, the reason for the increase in platelet MAO-B activity is still unknown, but may be related to transcriptional elevation of MAO-B protein and/or to changes in the cell milieu causing manipulation of the enzyme activity (Song et al. 2000; Damberg et al. 2001; Balciuniene et al. 2002). In accordance with previous studies (Oreland and Gottfries 1986; Parnetti et al. 1994; Götz et al. 1998), we showed, in a longitudinal study of 75-year-old residents in Vienna (Fischer et al. 2002) a highly significant increase in platelet MAO-B activity in Alzheimer type dementia group (Figure 3). A change in the ratio of the number of neurons to the number of supportive tissues in the degenerating brain areas could explain the increased activity of MAO-B in AD brains. However, the augmentation of MAO-B activity in platelets, which only express the B form of the enzyme, points to an additional, as yet unknown, factor which may act on transcriptional regulation (Ekblom et al. 1998) of the amount of enzyme and/or in the kinetic regulation of the molecular activity of MAO-B in platelets (Bongioanni et al. 1997; Song et al. 2000).

Other possible factors

Apart of the 'classic' biomarkers, i.e., tau protein(s) and their phosphorylated forms as well as Aβ peptides, several other candidate biomarkers have been tested, as recently extensively reviewed (Frank et al. 2003).

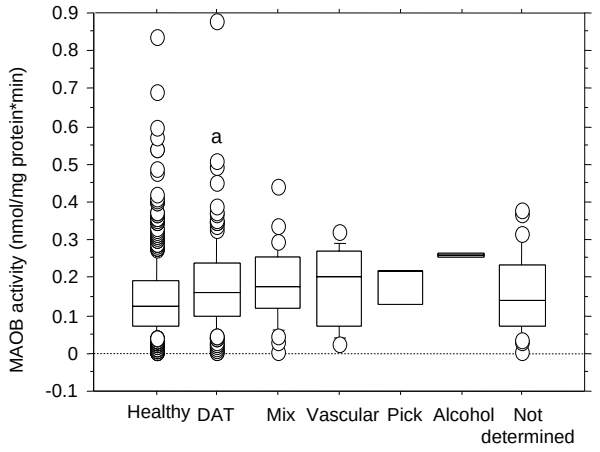

Figure 3. Platelet MAO-B activity level in the VITA and centenarian group. Comparison between different diagnosis groups of dementia to the healthy group. Healthy group ($n=473$, MMSE $=28.1\pm0.06$); dement Alzheimer type (DAT) ($n=129$, MMSE $=13.6\pm0.81$); mix dementia ($n=29$, MMSE $=10.2\pm1.49$); vascular dementia ($n=11$, MMSE $=14.0\pm2.86$); Pick's disease ($n=3$, MMSE $=3.7\pm3.66$); alcohol dementia ($n=2$, MMSE $=12.0\pm1.00$); not determined ($n=32$, MMSE $=20.0\pm2.02$. ANOVA and Fisher-PLSD: $P<0.005$; healthy vs. DAT.

Neuronal thread proteins (NTP) are a family of molecules expressed in CNS. In a post mortem study, brains of AD patients expressed significant increase of NTP immunoreactivity (de la Monte and Wands 1992). Following this finding, CSF examination for NTP revealed increased concentration of NTP which correlated with progression of dementia and neuronal degeneration (de la Monte et al. 1992). Sensitivity and specificity of this protein as a possible marker of AD, however, have not been determined in a large enough number of patients.

The soluble form of the iron-binding protein p97 has been suggested to be increased in AD patients in blood. In one report, all AD patients had elevated serum concentration of this factor, and no overlap with control subjects was observed (Kennard et al. 1996). Recently, this interesting finding has been confirmed by others, although using a different analytical method (Ujiie et al. 2002). Further studies are necessary to confirm p97 as a reliable marker of AD. As another interesting factor investigated in plasma, levels of homocysteine were reported to correlate with the risk of developing AD. A value greater than 14 μmol/l was associated with a two-fold increased risk of developing AD (Seshadri et al. 2002).

Interestingly, recently significantly increased CSF concentrations of transforming growth factor-β1 have been found in Alzheimer's disease by two independent groups of researchers (Tarkowski et al. 2002; Zetterberg et al. 2004).

Since there is growing evidence of the involvement of oxidative and/or nitrative cell damage in the pathogenesis of AD, factors related to lipid peroxidation have been tested as candidate AD biomarkers. Recently, Pratico et al. (2002) reported increased levels of isoprostanes (e.g., 8,12-iso-iPF$_{2\alpha}$-VI) in body fluids of patients with AD. This finding has been further supported by the elevated levels of this factor in the body fluids of transgenic mice expressing AD amyloidosis (Pratico et al. 2001).

Combined analysis of CSF parameters

There are many examples of CNS diseases where a combination of more than one CSF parameter significantly improves the accuracy of the diagnosis. Neuroinflammatory diseases, like neuroborreliosis (Tumani et al. 1995) or multiple sclerosis (Reiber et al. 1998) are representative examples. Similarly, studies have been reported showing increased sensitivity and specificity of combination of CSF parameters in early and differential diagnosis of AD.

In an international multicenter project, combined analysis of Aβ1–42 and tau protein showed 85% diagnostic sensitivity and 58% specificity to distinguish AD from non-Alzheimer types of dementia (Hulstaert et al. 1999). In this study, the mean sensitivity and specificity levels of the individual markers were significantly improved from 74–79 to 86% if both markers were considered simultaneously. In our study (Lewczuk et al. 2004c), we have found slightly better discrimination of patients with AD, non-Alzheimer's dementia and controls when Aβ42 was combined with Aβ40 (i.e., a concentration quotient of Aβ42/Aβ40). This discrimination was further slightly improved by a simultaneous evaluation of CSF total tau concentration, and combination of all these three parameters resulted in a correct separation of 94% of subjects in our study. Andreasen et al. reported sensitivity of 94% in a group of 105 probable AD, and 88% in a group of 58 possible AD when analysis of CSF total tau was accompanied by Aβ1–42 (Andreasen et al. 2001). Specificity in this study was high for differentiating AD from psychiatric disorders and non-demented subjects (100 and 89%, respectively); however, low concentrations of Aβ1–42 found in several cases of LBD resulted in lower specificity to discriminate this disease. The lowest specificity (48%) was found to discriminate AD from vascular dementia, probably because these patients had concomitant pathological features of AD. A study by Kanai et al. (1998) reported similar figures of 71 and 83% for diagnostic sensitivity and specificity, respectively, of simultaneous tau/Aβ1-42 analysis.

By plotting concentrations of Aβ1–42 versus tau, Motter et al. (1995) found a predictive value of 96% for subjects with high tau/low Aβ1–42 to have AD, and a 100% predictive value not to have AD for subjects with low tau/high Aβ1–42. Similar figures, with positive and negative predictive values of 90 and 95%, respectively, at a prevalence of probable AD of 44% were obtained also by Andreasen et al. (2001). To evaluate data of simultaneous analysis of CSF Aβ1–42 and tau, Galasko et al. (1998) used the binary tree-structured classification system obtaining 85.2% of correct diagnosis with sensitivity and specificity of 90 and 80%, respectively. In a recently published study, a combination of low CSF Aβ42 and high CSF pTau181 allowed early-onset AD patients to be distinguished from those with frontotemporal lobar degeneration with a sensitivity of 72% and a specificity of 93% (Schoonenboom et al. 2004).

Somewhat discrepant results have been presented when CSF tau or CSF phosphorylated tau was related to ApoE genotype, while Arai et al. (1995) reported no correlation of total tau to the number of ApoE ε4 allele, and Itoh et al. (2001) reported a similar finding regarding p-tau199. Golombowski et al. (1997) and Blomberg et al. (1996) found that AD patients with ApoE ε4 allele had higher values of CSF tau than these without ApoE ε4. With regard to the combination of ApoE genotype and Aβ1–42 levels, the highest peptide levels in AD patients with no ε4 allele, intermediate in ε4 heterozygous, and lowest in ε4 homozygous have been reported (Galasko et al. 1998; Hulstaert et al. 1999; Riemenschneider et al. 2000). As a possible explanation for this correlation, a high-affinity of binding Aβ1–42 to ApoE is suggested (Strittmatter et al. 1993b). Moreover, sensitivity for the combination of CSF–tau and CSF Aβ1–42 in patients possessing ε4 allele increased from 94 to 99% for probable and from 88 to 100% for possible AD (Andreasen et al. 2001). On the other hand, in a study including more than 400 AD cases, no effect on CSF tau levels was found for the ApoE e4 allele (Andreasen et al. 1999). These observations lead to the conclusion that ApoE genotype should be taken into consideration in interpretation of Aβ1–42 levels, and that combination of ApoE genotyping with other parameters may significantly improve specificity and sensitivity of the diagnosis.

We have recently reported a decreased *fraction* of Aβ1–42 to all Aβ peptides in CSF of patients with AD. In this study, all the AD patients and none of the controls appeared below discrimination line of 8.5%. Plotting the results of relative ratios of Aβ1–42 against Aβ1–38 showed all AD cases and none of the controls in the region with decreased Aβ1–42; however, some AD cases had increased Aβ1–38 similar to subjects with chronic neuroinflammatory diseases (Wiltfang et al. 2002) (Figure 4). This observation points to the presence of chronic (micro)inflammatory reaction in the CNS of subjects with AD. Moreover, this report also presents evidence that severity of dementia correlates negatively with percentage but not absolute value of Aβ peptides shortened at their carboxyl ends.

One of the most demanding aspects of neurochemical analysis of dementia disorders is to find biomarkers capable of predicting development of AD in patients with mild cognitive impairment (MCI). Such an early diagnosis is hoped to allow sufficiently early treatment. According to the epidemiological data of Petersen et al. (1999), approximately 10–15% of MCI subjects develop AD within 1 year. Recently, Andreasen et al. (2003) reported increased positive likelihood ratio for total tau (8.45), phospho-tau (7.49) and Aβ42 (8.2) in the 1-year follow-up of 44 MCI patients progressing to AD. These data suggest that these biomarkers are already altered in an early phase of dementia and that these factors may help to identify MCI subjects who will progress to AD. Similarly, Andreasen et al. (2001) show elevated tau and decreased Aβ42 levels in MCI patients at baseline.

In a recently published study, a combination of three CSF biomarkers, namely tau, pTau181 and Aβ42, could detect incipient AD among patients fulfilling the criteria for MCI with a sensitivity of 68% (95% CI 45–86%) and a specificity of 97% (95% CI 83–100%), therefore suggesting the

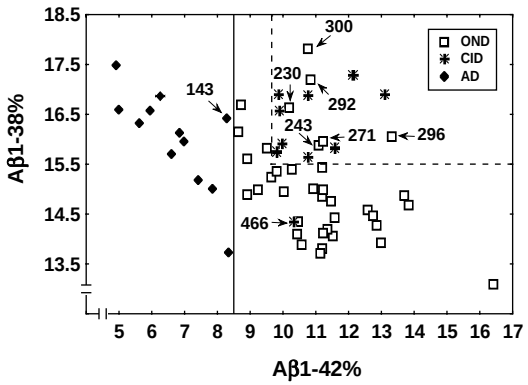

Figure 4. Plot of CSF Aβ1–38 relative concentration ratio (vertical axis, Aβ1–38%) and CSF Aβ1–42 relative concentration ratio (horizontal axis, Aβ1–42%). Cases of Alzheimer's diseases (AD), non-dementive neurological diseases (OND), and chronic neuroinflammatory diseases (CID) have been investigated. With an Aβ1–42% discriminatory value of 8.5%, all AD cases are separated from other subjects resulting in 100% sensitivity and 100% specificity. Increased Aβ1–38% above 15.5% observed in some AD cases corresponds to increased Aβ1–38% in CID and indicates signs of neuroinflammation in these cases of AD. According to Wiltfang et al. 2002) with modifications.

possibility to discriminate the subgroup of patients with MCI who would eventually develop AD from these who would not to offer early treatment for the subjects at risk (Zetterberg et al. 2003).

A limitation in the interpretation of the performance of CSF markers is the lack of standardization of assays. Levels of CSF markers vary both between studies from different centres using the same ELISA method, and between studies using different ELISA methods. Thus, standardization will be a prerequisite for future studies. As part of an EC project, aliquots of CSF pools with different levels of tau and Aβ are now available. This will provide a tool for standardization of methods, and thus an opportunity for direct comparisons of results from different academic centers.

Several pre-analytical and biological confounding factors may influence the analytical outcome for CSF analyses, such as concentration gradients of the protein along the spinal cord, influence of LP hemorrhage, presence of the protein in plasma and passage across the blood–brain and/or blood–CSF barrier, and degradation or loss of the protein in vitro after the CSF tap. For CSF tau and Aβ, the only pre-analytical confounding factor is that all of the proteins have a tendency to stick to the walls of test tubes made of glass and hard plastic, resulting in falsely low levels (Andreasen et al. 1999). Therefore, it is important to tap CSF into non-absorbing test tubes made of polypropylene. Storage of CSF for up to 3 days does not influence levels of these proteins. Thus, CSF samples can be sent to the laboratory at room temperature, after which all CSF samples are frozen before assay.

Another point to consider is the intrinsic characteristics of the antibodies used in the immunoassays for quantification of the proteins of interest. Reported differences in the diagnostic performance of the assay formats might depend on the fact that different antibodies recognize different isoforms, or conformational changes, of the proteins. The characteristics of the monoclonal tau antibodies HT7, BT2 and AT120 have been described in detail (Vanmechelen et al. 2000). Unpublished data from our group show that when combined with HT7, the monoclonal antibody AT120 (a phosphorylation-independent tau antibody) is better than BT2 (recognizing tau, non-phosphorylated at Ser-199), in differentiating AD cases from controls. Further, quantification of β-amyloid may not only depend on the C-terminal antibody, but also on the N-terminal antibody. While the 3D6 antibody requires a free NH2-group at the N-terminus of β-amyloid, other antibodies (e.g., 6E10, 4G8, WO2) also N-terminally truncated β-amyloid species.

Perspectives: Novel techniques to search for AD biomarkers (fluorescence correlation spectroscopy (FCS), mass spectrometry (MS), DIGE™, and multiplexing)

A new approach for analysing the tendency of Aβ peptides to form large aggregates is their detection and quantification in CSF by FCS (Eigen and Rigler 1994). These aggregates are the main component of amyloid plaques that are associated with Alzheimer's disease (Beyreuther et al. 1993). The malignancy of the Aβ peptides originates in their secondary structure rather than in their primary amino acid sequence (Selkoe 1994). Soluble Aβ peptides that consist of a high portion of β-sheet structures form fibrillar, insoluble aggregates by a process described as 'seeded polymerization' (Harper and Lansbury 1997).

FCS detects the fluorescence of labeled molecules that enter a small illuminated confocal volume of less than 1 fl by a random diffusion. Since the diffusion coefficient of molecules depends on their size, it is possible to distinguish between large molecule aggregates and small monomers. Large single aggregates deliver high intensive fluorescent flashes when they pass through the confocal volume, making them easy to detect against the much lower background of monomers. The diagnostic potency of FCS for AD disease was demonstrated 5 years ago for the first time (Pitschke et al. 1998). Synthetic Aβ1–42 peptides that were tagged with a fluorescent marker, were mixed with CSF of AD patients, non-Alzheimer dementia patients or healthy controls. These probes precipitated on amyloid aggregates that preexisted in the CSF in the presence of zinc (Brown et al. 1997). All Alzheimer patients had a significantly higher concentration of large aggregates than the controls or the non-Alzheimer dementia patients. This study demonstrated that the FCS technique is an extremely sensitive tool for use in the future for preclinical and differential diagnosis of Alzheimer's disease. It bears the potential for sensitive and selective detection of malignant amyloid aggregates in plasma to further support diagnosis based on biomarkers evaluated in CSF.

Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) is currently applied in many fields of biological and medical sciences, as extensively reviewed (Merchant and Weinberger 2000). Briefly, crude biological samples (e.g., plasma, cerebrospinal fluid, cell culture supernatants, etc.) are applied on the surface of 'ProteinChips' mimicking chromatographic interactions (e.g., cation/anion exchangers, hydrophobic interactions surfaces, metal affinity surfaces, etc.) and, after washing steps, only those molecules retained on the surface can be further analyzed.

The straightforward opportunity offered by this technology is immuno-affinity chips with specific antibodies covalently bound to the chip surface to capture adequate antigens. These molecules are then desorbed and ionized with a laser shot, and the time of flight of the molecules in the vacuum of the mass spectrometer correlates with the molecular mass of the ions. Applying the mass standards, the molecular mass of the peptides/proteins analysed can be measured with imprecision lower than 0.05%, i.e., about 2–3 Da in the range of the peptides' molecular mass. Polypeptides of interest can be further characterized by gas-phase sequencing, and peptide mapping technology using additional mass spectroscopy technology (e.g., LC–MS/MS). SELDI-TOF-MS has recently been successfully used to analyse amyloid β peptides in supernatants of transfected HEK 293 cells (Frears et al. 1999), and we have been recently the first group to analyse and compare the patterns of Aβ peptides in human CSF and post mortem brain tissue from patients with Alzheimer's disease and non-demented controls (Lewczuk et al. 2003, 2004b).

Another potentially important technique is two-dimensional fluorescence difference gel electrophoresis (DIGE™), which offers advanced opportunity to study protein expression alterations in biological material (Karp et al. 2004). Briefly, on a single gel up to three samples can be co-electrophoresed, which and are then scanned with a three-beam fluorescent laser imager (Typhoon scanner). The crucial step of the procedure is a labeling of proteins with a fluorescent probes (CyDye), which allows to accurately quantify differences in the protein expression patterns (coefficients of variations $<20\%$, up to 3000 individual spots) using a sophisticated software package (DeCyder). As samples from different sources (e.g., from patients and controls) are simultaneously run on a single gel, imprecision of the method afflicts similar proteins in a similar way, i.e., methodological errors are compensated. The detection sensitivity for proteins is in the low nanogram range. In spite of the fluorescent labelling the protein of interest can be further characterized by gas-phase sequencing and peptide mapping. The CSF samples of patients with neurodegenerative disorders and controls will first be depleted from high-abundance proteins by multiple immunoaffinity columns. Following desalting and concentration the samples are studied by DIGE using immobilised pH gradients (IPG, non-linear gradient 3–10) in the first dimension and SDS–PAGE step gradient gels in the second dimension (molecular mass range: 600,000–5000).

Since it is likely that combined analysis of several CSF markers will be necessary to improve diagnostic performance, multiplex methods will be of importance. One such method is the microsphere-based xMap technology (Luminex, Austin, TX, US), which is a multiplex flow cytometric method based on antibodies coupled to spectrally specific fluorescent microspheres (Vignali 2000). Another fluorescent reporter antibody binds the protein captured on the microspheres. Each microsphere is spectrally identified, and quantification is based on the intensity of the reporter signal. In comparison to conventional ELISA methods, this multiplex technology allows simultaneous quantification of up to 100 proteins in a small sample volume, and provides higher reproducibility than multiple ELISA methods. We have used the xMap technology to design multiplex assays for simultaneous quantification of several Aβ and tau isoforms. Preliminary data show equal, or better, diagnostic performance compared with ELISA.

Conclusions

The increasing number of patients with dementia demands improved standards of *intra vitam* diagnosis. The growing body of evidence summarized briefly in this paper may support the use of CSF analysis as part of a diagnostic workup for dementia. When clinical suspicion of an infectious or inflammatory disorder is strong, or in subacute dementia, routine CSF analysis combined with tests for causes of dementia such as infection may yield a specific and often treatable diagnosis. This is further supported by a finding of relatively low incidences of post-puncture complications, especially in the relevant group of patients (Blennow et al. 1993; Andreasen et al. 2001). CSF/serum analysis should be performed according to the generally accepted, theoretically based and practically confirmed concept of CSF-flow rate-related protein diffusion (Reiber and Peter 2001). CSF/serum analysis may provide useful information in patients with conditions currently yet considered untreatable. An early and definitive diagnosis may provide a reasonable time window for starting treatment during a period when the patient has relatively mild impairment and is best able to benefit. As more definitive treatment is tested, very early diagnosis of AD will become imperative. Simultaneous analysis of two or more factors in CSF can significantly improve the accuracy of the diagnosis of AD. Further studies are required to confirm sensitivity and specificity of the markers, including studies in patients who have had post-mortem confirmation of diagnosis. With the introduction of novel, very sensitive techniques, e.g., FCS or mass spectrometry, a further advancement in early diagnosis of Alzheimer's disease is expected.

Statement of interest

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