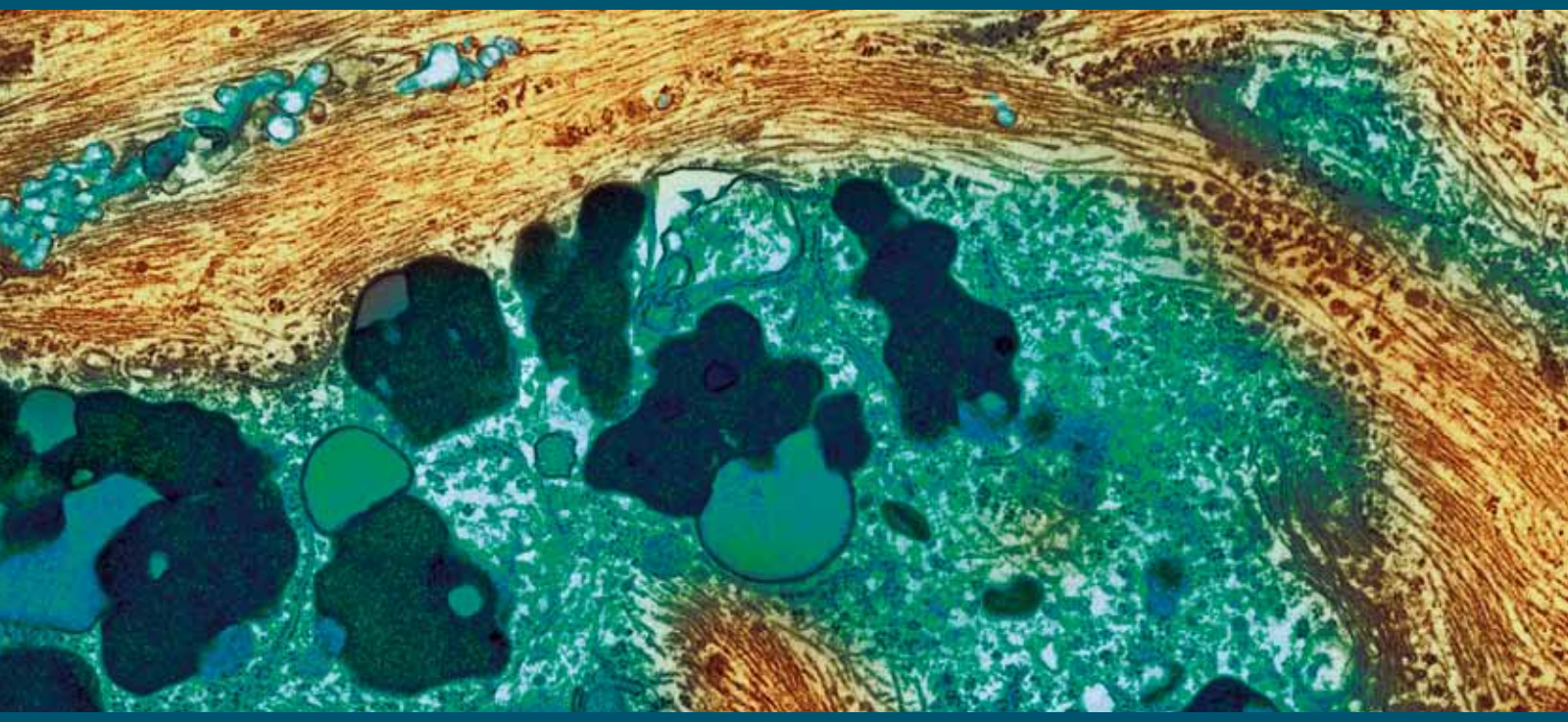
International Journal of Alzheimer's Disease

Should CSF Biomarkers Support a Routine Analysis for Early Diagnosis of Alzheimer's Disease?

Guest Editors: Lucilla Parnetti, Jens Wiltfang, Kaj Blennow, and Tuula Pirttila



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Editorial Board

Contents

Should CSF Biomarkers Support a Routine Analysis for Early Diagnosis of Alzheimer's Disease?, Lucilla Parnetti, Jens Wiltfang, and Kaj Blennow
Volume 2010, Article ID 293587, 2 pages

Cerebrospinal Fluid Analysis Should Be Considered in Patients with Cognitive Problems, Henrik Zetterberg, Niklas Mattsson, and Kaj Blennow
Volume 2010, Article ID 163065, 4 pages

To Know or Not to Know: Ethical Issues Related to Early Diagnosis of Alzheimer's Disease, Niklas Mattsson, David Brax, and Henrik Zetterberg
Volume 2010, Article ID 841941, 4 pages

Are Guidelines Needed for the Diagnosis and Management of Incipient Alzheimer's Disease and Mild Cognitive Impairment?, Katie Palmer, Massimo Musicco, and Carlo Caltagirone
Volume 2010, Article ID 417615, 7 pages

CSF Biomarkers for Alzheimer's Disease Diagnosis, A. Anoop, Pradeep K. Singh, Reeba S. Jacob, and Samir K. Maji
Volume 2010, Article ID 606802, 12 pages

Biological Markers and Alzheimer Disease: A Canadian Perspective, Hyman M. Schipper
Volume 2010, Article ID 978182, 7 pages

Lessons from Multicenter Studies on CSF Biomarkers for Alzheimer's Disease, Niklas Mattsson, Henrik Zetterberg, and kaj Blennow
Volume 2010, Article ID 610613, 5 pages

Standardization of Assay Procedures for Analysis of the CSF Biomarkers Amyloid $\beta_{(1\text{-}42)}$, Tau, and Phosphorylated Tau in Alzheimer's Disease: Report of an International Workshop, Charlotte E. Teunissen, Niek A. Verwey, Maartje I. Kester, Kees van Uffelen, and Marinus A. Blankenstein
Volume 2010, Article ID 635053, 6 pages

Confounding Factors Influencing Amyloid Beta Concentration in Cerebrospinal Fluid, Maria Bjerke, Erik Portelius, Lennart Minthon, Anders Wallin, Henrik Anckarsäter, Rolf Anckarsäter, Niels Andreasen, Henrik Zetterberg, Ulf Andreasson, and Kaj Blennow
Volume 2010, Article ID 986310, 11 pages

cNEUPRO: Novel Biomarkers for Neurodegenerative Diseases, Philipp Spitzer, Hans Wolfgang Klafki, Kaj Blennow, Luc Buée, Hermann Esselmann, Sanna-Kaisa Herruka, Connie Jimenez, Peter Klivenyi, Piotr Lewczuk, Juan Manuel Maler, Katrin Markus, Helmut E. Meyer, Chris Morris, Thorsten Müller, Markus Otto, Lucilla Parnetti, Hilkka Soininen, Susanna Schraen, Charlotte Teunissen, Laszlo Vecsei, Henrik Zetterberg, and Jens Wiltfang
Volume 2010, Article ID 548145, 12 pages

Cerebrospinal Fluid Biomarkers for Dementia with Lewy Bodies, Elizabeta B. Mukaetova-Ladinska, Rachael Monteith, and Elaine K. Perry
Volume 2010, Article ID 536538, 17 pages

Combined Analysis of CSF Tau, Aβ42, Aβ1–42% and Aβ1–40^{ox}% in Alzheimer's Disease, Dementia with Lewy Bodies and Parkinson's Disease Dementia, Mirko Bibl, Hermann Esselmann, Piotr Lewczuk, Claudia Trenkwalder, Markus Otto, Johannes Kornhuber, Jens Wiltfang, and Brit Mollenhauer
Volume 2010, Article ID 761571, 7 pages

CSF Biomarkers Profile in CADASIL—A Model of Pure Vascular Dementia: Usefulness in Differential Diagnosis in the Dementia Disorder, Patrizia Formichi, Lucilla Parnetti, Elena Radi, Gabriele Cevenini, Maria Teresa Dotti, and Antonio Federico
Volume 2010, Article ID 959257, 6 pages

SAGE-Hindawi Access to Research
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Editorial

Should CSF Biomarkers Support a Routine Analysis for Early Diagnosis of Alzheimer's Disease?

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In view of the availability of drugs that may slow or even halt the progression of Alzheimer's disease (AD), the possibility to routinely discriminate individuals carrying incipient AD is utmost important. The CSF biomarkers beta-amyloid and tau are increasingly used in several countries in Europe for their high diagnostic accuracy in detecting early or even prodromal AD. Up till now, thousands of subjects have been studied either in research or routine setting, giving sensitivity and specificity values invariably above 80%. Despite these clinically relevant results, CSF analysis is still considered only a supportive exam in AD diagnostic work-up. We think there is a great need to improve the medical awareness about the importance to perform lumbar puncture as a routine procedure in the diagnostic assessment of cognitive deterioration.

In line with this belief, the first paper of this special issue addresses the usefulness of cerebrospinal fluid analysis for diagnostic definition of cognitive deficits, and the second one further focuses on the ethical issues related to early diagnosis of Alzheimer's disease. As a corollary, the third paper reports some reflections about the actual need in clinical practice of guidelines for diagnosis and management of incipient AD.

The fourth paper illustrates what is known about CSF biomarkers for diagnosing AD, and the fifth paper gives a different perspective according to the Canadian guidelines for dementia diagnosis. An up-dated and comprehensive view of crucial issues emerged from large multicentre studies (which have clearly highlighted the inter-centre variability of these determinations) on CSF biomarkers for AD diagnosis is reported in the sixth paper. Accordingly, the seventh paper nicely describes the efforts done for standardization of assay procedures and the eighth paper further goes into details about the many factors inducing variation of amyloid beta concentration, which heavily hampers, at present, a good inter-centre reliability. Besides the state of art about what is known on classical CSF biomarkers for early diagnosis in routine clinical practice, there also are impressive international research initiatives aimed at identifying new biomarkers for neurodegenerative diseases. This issue is thoroughly addressed by the ninth paper, which gives an overview of the EU-funded consortium cNEUPRO.

The issue of the role of CSF biomarkers in non-AD dementias is also partly addressed in this special issue. An overview on CSF biomarkers in dementia with Lewy bodies (DLB) is given in the tenth paper, and in the eleventh paper the discriminative power of classical and putative CSF biomarkers for differentiating AD, DLB, and Parkinson's disease with dementia (PDD) is accurately described; finally, the usefulness of classical CSF biomarkers in characterizing CADASIL, a genetic model of subcortical vascular dementia, is also reported.

Lucilla Parnetti
Jens Wiltfang
Kaj Blennow

Tuula Pirttilä In Memoriam

Professor Tuula Pirttilä from University of Eastern Finland, Kuopio, Finland, passed away on March 24, 2010. She was fighting against cancer for almost five years. Despite of

her disease, she was constantly working as clinical doctor, scientist, and teacher.

Tuula Pirttilä was born in Kuopio. She studied Medicine at the University of Tampere and got her M.D. in 1983. She completed her residency in neurology in 1990 and got the degree of PhD three years later. After that, she moved to New York, USA and as post doc she carried out doing studies on biomarkers of Alzheimer's disease. She was nominated as docent in neurology at the University of Tampere in 1995. She then returned to Kuopio as associate professor of Neurology in 1997 and was nominated full professor of neurology in 2005.

People who have been working with her, remember her as an excellent clinician, teacher and scientist. Ethical issues were very important for her. She served many years as a chairperson of the Ethical Committee of Kuopio University Hospital. Tuula was also an active performer in patient organisations and in Finnish Alzheimer Research Society. Among many other things she was also chairperson in the working group defining national and European guidelines for diagnosis of Alzheimer disease. Her contribution to the science was mainly focused on biomarkers. She could use excellent material collected in Kuopio Finland including plasma, serum, CSF samples from patients with neurodegenerative diseases, especially Alzheimer's disease. Her group set up a service laboratory at the University of Eastern Finland that is currently serving the whole country. Her contribution to the EU funded cNEUPRO Consortium has been decisive for the success of the initiative, and she gave her enthusiastic contribution until she could.

Tuula showed unbelievable talent and wisdom. She will be remembered as a very warm and ethusiastic person, always available to listen and engage, and her smiles and laughs were contagious.

All of us who had the privilege to work with her are missing a great person, scientist, teacher and clinician.

Hilkka Soininen
Lucilla Parnetti
Jens Wiltfang
Kaj Blennow

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Review Article

Cerebrospinal Fluid Analysis Should Be Considered in Patients with Cognitive Problems

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Hepatologists assay liver enzymes and cardiologists structural heart proteins in serum to diagnose and monitor their patients. This way of thinking has not quite made it into the memory clinics yet, in spite of the availability of validated cerebrospinal fluid biomarkers for key pathological events in the brain in neurodegeneration. Here, we argue that a spinal tap should be considered in all patients who seek medical advice for memory problems and list the highly relevant clinical questions CSF analyses can address.

1. Introduction

Memory problems may be caused by a wide range of neuropsychiatric diseases, including Alzheimer's disease (AD), vascular dementia (VaD), dementia with Lewy bodies, frontotemporal dementia (FTD), to mention a few [1]. Cognitive symptoms may also arise secondary to depression, neuroinflammation and various somatic illnesses. Today, patients with memory problems seek medical advice much earlier than 10 years ago. It is difficult to differentiate benign cognitive deficiencies from AD or other primary neurodegenerative diseases. Memory problems secondary to other diseases may also present a diagnostic challenge.

Patients with memory complaints most often undergo extensive clinical and neuropsychological assessments, and often also one or more brain imaging investigations. We argue that CSF analysis should be considered in the diagnostic work-up of all patients with memory problems to answer a number of highly relevant questions discussed below. Fear of spinal tap-related side-effects should not preclude CSF analyses, since complications are very rare in the elderly, provided that regular precautions well known to any trained physician are taken [2–4].

2. Does the Patient Suffer from Brain Amyloid Pathology?

The robust association of brain amyloid pathology with AD makes this question highly relevant. The easiest and most cost-effective way of giving it a reliable answer is to analyse CSF for the 42 amino acid form of amyloid β (Aβ1-42). Low CSF levels indicate retention of Aβ1-42 in the brain parenchyma [5–8]. This seems to be the earliest biochemical change during the course of AD [9–11]. Low levels of Aβ1-42 may be seen Creutzfeldt-Jakob disease (CJD), also in the absence of significant amounts of brain amyloid pathology [12].

3. Does the Patient Suffer from Neurofibrillary Tangle Pathology?

Tau expression is high in nonmyelinated cortical axons where it serves as a microtubule-stabilizing protein [13]. Hyperphosphorylation of tau causes the protein to detach from the microtubules. This process promotes axonal and synaptic plasticity in the developing brain [14, 15], but is pathological in the adult brain and specifically related

Table 1: CSF biomarkers of pathological findings in relation to differential diagnoses in memory clinic patients.

Diagnosis	Amyloid pathology (Aβ1-42)	Tangle pathology (P-tau)	Cortical axonal damage (T-tau)	Sub-cortical axonal damage (NFL)	Blood-brain barrier dysfunction (CSF/serum albumin ratio)	Inflammation (CSF cell counts, IgG or IgM production)
AD	Yes	Yes	Yes	No	No	No
VaD	No	No	Yes (especially in relation to new brain infarcts)	Yes	Yes	No
FTD	No	Yes (but not in CSF)	Yes (mild)	Yes	No	No
LBD	Yes	No	Yes (mild)	No	No	No
PD	No	No	No	No	No	No
PSP	No	Yes (but not in CSF)	No	Yes	No	No
CJD	No	No	Yes (severe)	Yes (mild to moderate)	No	No
Depression	No	No	No	No	No	No
Lyme disease	No	No	No	Yes (mild)	Yes	Yes (especially IgM)
Multiple sclerosis	No	No	No	Yes	No (mild in 10%)	Yes (especially IgG)
Acute stroke	No	No	Yes	Yes	Yes	No
Normal aging	No	No	No	No	No	No

Abbreviations: CSF = cerebrospinal fluid; Aβ1-42 = the 42 amino acid isoform of amyloid β; P-tau = hyperphosphorylated tau; T-tau = total tau; NFL = neurofilament light; AD = Alzheimer's disease; VaD = vascular dementia; FTD = frontotemporal dementia; LBD = Lewy body dementia; PD = Parkinson's disease; PSP = progressive supranuclear palsy; CJD = Creutzfeldt-Jakob disease.

to a group of disorders referred to as tauopathies, which includes AD and some forms of FTD [16].

Elevated CSF levels of hyperphosphorylated tau (P-tau) protein are the most specific finding suggesting an ongoing AD process in the brain [17]. P-tau levels correlate with cognitive decline in patients with mild cognitive impairment (MCI) [18] and with neocortical neurofibrillary pathology in AD [19]. The reason for the lack of P-tau increase in FTD and other pure tauopathies such as progressive supranuclear palsy and corticobasal degeneration is to date unknown [20, 21].

4. Are there Biochemical Signs of Cortical Axonal Degeneration and How Active Is this Process?

Pathogenic processes that damage axons in the cortex result in increased CSF levels of total tau (T-tau, i.e., all isoforms irrespective of phosphorylation state). T-tau is a dynamic marker of the intensity of the axonal degeneration/damage: the more pronounced increase, the more intense degenerative process, the faster disease progression [17]. Very high CSF T-tau levels are always seen in CJD [22–25], and can be seen in stroke and brain trauma, in which T-tau predicts clinical course and/or outcome [26–28]. The cortical axonal degeneration in AD makes elevated CSF T-tau an obligatory finding. Consequently, a clinical diagnosis of AD should be reconsidered in the absence of tau elevation. Very high levels predict a rapid cognitive decline in AD [29–31] and short survival in DLB [32].

Together, CSF biomarkers of amyloid pathology (Aβ1-42), tangle pathology (P-tau) and cortical axonal degeneration (T-tau) identify AD with dementia and prodromal AD in patients with MCI with 75–95% sensitivity and specificity [33–36].

5. Are there Biochemical Signs of Sub-Cortical Axonal Degeneration and How Active Is this Process?

The best-established CSF biomarker for sub-cortical axonal degeneration/damage is neurofilament light protein (NFL). This type of axonal degeneration is frequently seen in VaD [37–39], FTD [40] and a number of inflammatory conditions, including MS [41] and AIDS dementia [42]. Elevated CSF NFL levels indicate a sub-cortical disease process and help in differentiating pure AD from the conditions listed above. Combined T-tau and NFL increases indicate mixed forms of AD and cerebrovascular disease.

6. Is the Blood-Brain Barrier Damaged?

The best-established biomarker for the integrity of the blood-brain barrier is the ratio of the albumin concentration in CSF to serum (the CSF/serum albumin ratio). Strictly speaking, the CSF/serum albumin ratio is a direct measure of the blood-CSF barrier [43]. However, leaking blood vessels in the brain will eventually result in higher CSF/serum albumin ratio through release of albumin to the brain interstitial fluid which communicates freely with the CSF. Typically, the CSF/serum albumin ratio is normal in patients with pure AD [44], whereas patients with vascular dementia generally present with elevated albumin ratio [45]. The same finding is often present in Lyme disease (neuroborreliosis), where one also may find increased numbers of CSF monocytes and signs of immunoglobulin production within the CNS [46]. Blockage of the spinal canal which, by impairing the flow of CSF distal to the block, allows longer for equilibrium with the circulation and so brings the composition of the CSF nearer to that of plasma (Froin's syndrome), results in elevated CSF/serum albumin ratio in the absence of blood-brain barrier damage.

7. Are there Biochemical Signs of Neuroinflammation?

Basic CSF examinations of inflammatory activity, including white blood cell count and general signs of IgG or IgM production within the CNS are generally negative in AD and other primary neurodegenerative diseases [47]. Distinct positive results speak against pure AD and should motivate further investigation of the patient to exclude neuroborreliosis, multiple sclerosis and other neuroinflammatory conditions that may contribute to the cognitive symptoms.

8. Conclusions

Robust answers to the clinically relevant questions listed above are warranted in the professional evaluation of any patient with memory complaints. Valid CSF biomarkers are available for amyloid pathology (Aβ1-42), neurofibrillary pathology (P-tau), cortical axonal degeneration/damage (T-tau), sub-cortical axonal degeneration/damage (NFL), blood-brain barrier function (CSF/serum albumin ratio) and neuroinflammation (CSF cell counts, IgG and IgM oligoclonal bands and concentrations). The biochemical data should be interpreted with great care together with the whole clinical picture, as well as findings in neuropsychological testing and neuroimaging investigations. Typical changes in relation to different diagnostic entities are summarised in Table 1.

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Review Article

To Know or Not to Know: Ethical Issues Related to Early Diagnosis of Alzheimer's Disease

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In Alzheimer's disease (AD), pathological processes start in the brain long before clinical dementia. Biomarkers reflecting brain alterations may therefore indicate disease at an early stage, enabling early diagnosis. This raises several ethical questions and the potential benefits of early diagnosis must be weighted against possible disadvantages. Currently, there are few strong arguments favouring early diagnosis, due to the lack of disease modifying therapy. Also, available diagnostic methods risk erroneous classifications, with potentially grave consequences. However, a possible benefit of early diagnosis even without disease modifying therapy is that it may enable early decision making when patients still have full decision competence, avoiding problems of hypothetical consents. It may also help identifying patients with cognitive dysfunction secondary to other diseases that may be responsive to treatment already today.

1. Introduction

Much research has been devoted to finding reliable methods for early diagnosis of Alzheimer's disease (AD). The most promising diagnostic modalities are cerebrospinal fluid (CSF) and imaging biomarkers. This research area is largely motivated by the ongoing development of disease-modifying treatment. When available, such treatment will likely be most effective if initiated early in the disease process, before too much irreversible damage has been inflicted on the brain. Early diagnosis in conjunction with disease-modifying treatment will form a preventive strategy. Classically, disease prevention is divided into primary, secondary, and tertiary prevention. Primary prevention is the reduction of risk factors to prevent disease from occurring. Secondary prevention is the detection of presymptomatic disease and early start of treatment to halt disease progression. Tertiary prevention is the prevention of disease progression in patients with symptomatic disease. Each prevention type brings specific medical, ethical, and organizational problems. Diagnosis and treatment of incipient AD in patients with mild cognitive impairment (MCI) would constitute a tertiary prevention, which arguably is the most realistic future scenario. Secondary prevention implies detecting AD pathology and initiating treatment in asymptomatic patients. Such intervention at a stage of little brain deterioration is intuitively tempting, but the low prevalence of incipient AD in the general population makes the strategy extremely problematic [1].

The general outcome of a prevention program depends on the effectiveness of the diagnostic tests and treatments used, wherefore results differ greatly between medical conditions. For example, secondary prevention with screening for cervical cancer in asymptomatic females is quite successful [2], while the effect of secondary prevention of prostate cancer remains debated [3]. With the current lack of disease-modifying AD therapy, few strong arguments favour early AD diagnosis in clinical routine, although this could change within a foreseeable future. Ethical issues of early AD diagnosis should therefore be discussed both to tackle present problems and to prepare health care professionals for a possible future of disease-modifying therapy. Here we

will review ethical considerations of early AD diagnosis. We will mainly discuss consequences for clinical practice, but it should be noted that extended use of early diagnosis might benefit research, since it would likely lead to involvement of more patients in clinical studies. Certain medical conditions present unique ethical considerations, and for dementia these include personal identity and decision-making competence.

2. The Ethics of Uncertainty

For any negative consequence of an erroneous test, one should consider how often misdiagnosis occurs. Several studies show high diagnostic accuracy of CSF biomarkers for incipient AD in MCI, with sensitivity and specificity around 85–90% [4, 5]. This is likely an upper limit when validating tests towards clinical diagnosis of probable AD, since the clinical diagnosis is not always confirmed upon autopsy. However, even a test with a diagnostic accuracy of 90% results in a large number of misdiagnosed persons if the disease prevalence is 50%, which is the typical prevalence of AD in MCI cohorts. Increased specificity may be achieved if testing is restricted to a high-risk group, for example, *APOE* $\varepsilon 4$ carriers. However, since approximately 50% of AD patients lack *APOE* $\varepsilon 4$ [6], the increased specificity would be at the cost of a reduced overall sensitivity. The diagnostic lumbar puncture could be uncomfortable. Severe complications are extremely rare, but postlumbar puncture headache occurs in 2–4% of the patients [7–9].

A test result indicating AD may bring extended followup and stigmatization resulting in feelings of hopelessness, agony, and despair. There might be an increased risk of suicide in dementia, although it is unclear if this is linked to the stigma of diagnosis or caused by mood disorders secondary to the disease itself [10]. From a legal perspective, a test result indicating AD could affect insurance premiums, and the rights to hold a driver's license or own a gun could be questioned. Repeated assessment of competence could be humiliating. This is ethically problematic even in correctly diagnosed AD cases and the ethical consequences in falsely diagnosed cases could be grave. On the other hand, a correct early diagnosis may be clarifying and appreciated by patients even without disease-modifying treatment, and a diagnosis could be valuable since it allows informed planning for the future [11] as we discuss further below. In practice, the attitudes of clinicians vary widely, and some may find it very difficult to "break the bad news" [12, 13].

AD not only concerns the patient but also his or her relatives. An unambiguous diagnosis is likely to benefit the relatives of demented patients [14]. However, it is possible that the worries of first-grade relatives could extend to their own health status and raise demands for further presymptomatic testing [15].

3. Consequences of Early Diagnosis

Studies in MCI patients have not shown significant benefits of early treatment with currently available drugs, but those studies have been conducted on unselected MCI patients. Thus, effects on early AD may be blurred by non-AD MCI patients, and future studies should preferably be conducted on populations enriched for incipient AD. Presently, it is difficult to fully estimate the effects of early treatment, and thus of early diagnosis. However, even without treatment, an early diagnosis might facilitate introduction of tools to help the patient to cope with the progressive decline. In some instances, a diagnosis might also make it easier for the patient to receive assistance from the health care system.

If a false positive diagnosis results in treatment, any harmful side effect is a serious infringe on the basic medical ethics principle of nonmaleficience, summarized in the Latin phrase *primum non nocere* ("first, do not harm"). The risk of serious adverse effects from treatments under development should not be underestimated, considering the meningoencephalitis cases in the AN1792 trial [16]. Although toxicity studies for new drugs are rigorous, rare side effects are only noticed with widespread use. Treatment could also be expensive for patients or society, depending on the local health care funding system. Obviously any investment will be a dead loss in falsely positive cases. Therefore, positive effects of treatment must be weighted against side effects and treatment cost, when determining decision limits for the diagnostic tests.

Early diagnosis might affect the health of an early-stage AD patient, besides treatment effects. As mentioned, detection of incipient AD could question the right to hold a driver's license. It is not known whether mild dementia increases the risk for road accidents. A recent Cochrane systematic review concluded that knowledge is scarce regarding benefits of driver assessment in the elderly for reducing motor vehicle accidents [17]. Implications of early diagnosis could differ between cultures with different roles of the elderly in families and society. Hypothetically, early diagnosis may be more beneficial in individualistic societies, where the cognitive deficient elderly are less supported by their families and have more to gain from early intervention from the health care system. Ultimately, each patient deserves a personal ethical analysis before disclosing an early diagnosis, and it is crucial to involve the patient in this process [18].

A beneficial consequence of investigations aiming at early diagnosis is that they may guide the physician right in the diagnostic algorithms. For example, a test result that does not suggest AD in a patient with mild memory deficits may spur the clinician to look more intensely for other causes of cognitive dysfunction, such as depression, hypothyreosis, and neuroborreliosis. These conditions are all responsive to treatment and must be identified. Test results that do not fit the clinical picture may also identify patients that should be investigated further and/or be referred to a colleague for a second opinion.

4. Personal Identity and Decision-Making Competence in AD

So far, we have raised some ethical issues concerning early AD diagnosis. There are risks associated with the uncertainty of the available diagnostic tools and treatments, and possible detrimental consequences of being diagnosed,

whether correctly or not. We now turn to the philosophically more intricate matter of ethical issues enlightened by the particular nature of AD.

The cognitive and neurological effects of AD hit us where it hurts: at the very core of our personality. Ever since the writings of John Locke (1689) [19], psychological continuity has been widely regarded as essential to personal identity (see Derek Parfit's influential book for a thorough discussion in [20]). It is because of psychological continuity that we remain one and the same person throughout our lives. Loss of memory and of memory function and a general change of cognitive capacities consequentially affect our personality and shake our sense of identity. For many people, such a change presents a nightmarish scenario. Some even state that they would rather die prematurely than live out their life with a severe, personality changing, cognitive disorder. This stated preference is of considerable interest in itself; as mentioned above, the risk of a depressive or even suicidal reaction to a diagnosis should be taken into account. But it also demonstrates a more general problem: There is a potential conflict between my current wishes for my future self, and the actual wishes of my future self. What I want for myself if the disease progresses might not be what I want *as* it progresses.

Normally when we make decisions that affect our future selves, we assume some relevant similarity between our current psychological state and set of preferences and those of our future selves. We decide to quit smoking, for instance, because it is in our predictable future interest not to be addicted to smoking. We invest money so that our future selves will reap the benefits, and we predict that our future self will still be interested in having money to spend. Occasionally, this strategy backfires and we misjudge the needs and interest of our future selves. Nevertheless, the trend seems reliable enough for this strategy to be a reasonable one: the practice of deciding for future selves has a decent track record. If we had less psychological continuity than we in fact do, things would be different and we would probably be wise to live more "in the moment".

Normally, when I make a decision that affects my future self, my future self has some power to veto that decision, and to influence its consequences. I can cancel dinner plans when a current headache makes it in my best interest not to act on former intentions.

In the case under consideration—early diagnosis of AD—we make decisions about a future self that is importantly *not* like us. Depending on the severity of the effects of the disease (and the treatment), the future me might have few interests in common with present me. Too few, in fact, of my present preferences are to be a reliable guide to what future me wants. My present preferences might therefore be held to have less weight, and less authority. Perhaps my current preferences *should not* have authority as to what happens to future me, when the discontinuity is significant. Most of our decisions are covertly *conditional* on this kind of event not happening.

An early diagnosis makes it possible to make arrangements for a decline in cognitive capacities. Early diagnosis of what may result in a profound personality change needs, of course, to be coupled with information of what type of change is to be expected. As noted, one of the benefits of early diagnosis is that the patients can prepare in a suitable manner, by making their lives easier in order to cope with the advancing disease, and also to put off the symptoms and to get the help they need. But one potential drawback here is that the earlier the diagnosis, the greater the potential psychological distance between the agent who makes the decision and the person whom this decision ultimately affects. There are two kinds of ethical problems with this scenario. First, we might misjudge what lies in our future best interest. Second, a radical change in personality might mean that there is a conflict between respecting the current agent's autonomy and that of the future agent.

5. Decision-Making Competence, Substituted Judgments, and Hypothetical Consent

One major benefit of early diagnosis is that it enables us to make rational decisions about what should happen to us once we have lost the ability to decide rationally. In the most dramatic cases, we can be informed about and give consent to treatments that we might later, in a less rational state, not understand the importance of. If we know that we are at high risk of losing certain capacities, we can make our wishes known, draw up a will, and make other preparations. This way we avoid the practical and theoretical quagmire of *hypothetical consent*, when caregivers have to guess what the patient would have wanted. According to the *substituted judgment standard* [21], if an agent loses the ability to make a critical decision, we should aim to substitute the judgment that the agent *would* have made, had he or she been capable. There is however a problem that applies both to the kinds of cases when we have to substitute a judgment and the cases when we listen to the patients' earlier decisions and preferences: what stage of the agent should we listen to? If we try to approximate the decision that the agent *would* have made, if rational, which stage of the agent are we supposed to approximate? Should we listen to an early decision, made by the patient when he/she is clearly competent, or the "latest" decision, made just before the patient is judged unfit/incapable to decide on his/her own? The latter option would be closer to the actual person affected by the decision, but the former is more likely to be the rational, well-informed choice. The principle of autonomy might be in conflict with what is in the patient's best interest here. Even though the first option involves respecting the autonomy of the agent in earlier stages, it is not clear that the agent *should* have authority over later stages of him/herself when there is a substantial personal change involved.

How do we want to be treated as the disease progresses? To give a proper assessment of this issue, the scenario to imagine is not only how we would want to be treated if we get the disease, but also how we would want to be treated if we found out that we *actually, now*, suffer from it, and that we made some decisions earlier about what should happen to us. Would we want those decisions to have authority over our current preferences?

How should we deal with interpersonal conflict of this nature? Is the agent whose best interest we should have in mind only the agent that might, in fact, be affected by the decision? In that case, perhaps we should take earlier stages of the agent into account only if the current stage is temporary, as we do when it comes to temporary insanity or drunkenness.

We offer no solutions to these problems here, but the complications noted mean that we need to be clear about what is involved when faced with an early diagnosis of a predictable change in personality.

6. Conclusions

Several essential questions of ethical implications with early AD diagnosis are unanswered. Perhaps most importantly, knowledge is scarce on how patients actually react to early diagnosis [22]. Further, the ethical implications will change dramatically when disease-modifying drugs become available. Depending on cost, safety, and efficacy, such drugs may transform patients' views on an AD diagnosis from a despair reaction to statements like "I am glad that my doctor found out before I lost too many brain cells". Any disease-modifying treatment will be necessary to evaluate from a cost-benefit perspective. This will be a question for clinicians, politicians, philosophers, and the society as a whole.

Disclosures

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Author Contributions

Niklas Mattsson and David Brax had the idea for this work and drafted the paper. Henrik Zetterberg made important revisions to the manuscript.

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Review Article

Are Guidelines Needed for the Diagnosis and Management of Incipient Alzheimer's Disease and Mild Cognitive Impairment?

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Current research is aiming to push the boundaries of the point at which a diagnosis of Alzheimer Disease (AD) can be made. Clinical syndromes such as Mild Cognitive Impairment (MCI) and various clinical and biological markers of AD may help to identify people in the early stage of AD, before a full dementia syndrome is present. In the first part of this paper, we discuss whether MCI represents incipient AD, and examine some of the methods currently used in research to identify AD patients in the preclinical phase. In the second part, we discuss whether specific guidelines are needed for the diagnosis and management of MCI and incipient AD, and consider the potential impact of this on clinical practice and public health from the perspective of patients, caregivers, and healthcare providers.

1. Introduction: The Concept of MCI and Incipient AD

Currently, dementia of the Alzheimer type is diagnosed clinically according to diagnostic criteria [1]. These criteria include deficits in memory and other cognitive functions, and the symptoms have a gradual onset and progressive deterioration. The H. Braak and E. Braak [2, 3] staging describes an increasing burden of the neuropathological hallmarks of Alzheimer disease (AD), neurofibrillary tangles, and amyloid plaques, with increasing dementia severity, mainly starting in the brain regions associated with memory functioning such as the hippocampus and entorhinal cortex. Evidence now clearly shows that the disease starts before the symptoms of a full dementia syndrome are present. AD related pathology, such as beta amyloid deposition, may already begin occurring even ten to twenty years before a clinical diagnosis of dementia can be made [4]. Furthermore, deficits in cognitive functioning may start to appear three [5], six, or even up to ten years [6] before a clinical diagnosis of AD.

Several syndromes that identify patients at high risk of developing dementia—or "preclinical, early AD" syndromes—have been proposed, including Mild Cognitive Impairment (MCI) and others; for a review see Mariani and colleagues [7]. A decade ago, Petersen et al. [8] proposed diagnostic criteria to describe patients who were neither normal nor demented, but had a mild form of cognitive impairment characterized by memory deficits. The MCI paradigm is based on the assumption that patients with AD develop symptoms, particularly memory impairment, gradually and, therefore, there may be an intermittent stage between normal aging and dementia characterized primarily by episodic memory deficits. These MCI patients have a high risk of progressing to a full dementia syndrome within a few years [8], although, as discussed later, the evolution is heterogeneous.

In the last decade, much focus has been paid to identifying clinically diagnosable syndromes of incipient AD. Criteria for MCI, and other syndromes, have been revised several times [9–11], with interest in identifying specific characteristics of MCI patients who are at the highest risk of progressing to AD. People with MCI progress to dementia at a rate of about 6% to 15% per year [7, 8, 12, 13], although the condition is heterogeneous and some MCI patients do not develop dementia. As discussed in

a recent systematic review of MCI [7], a number of factors have been suggested to further predict conversion to AD in MCI patients, and it is becoming increasingly evident that biomarkers, such as cerebrospinal fluid (CSF) markers including beta-amyloid$_{1-42}$ and total tau protein, may have a good accuracy for identifying impending AD in MCI patients [14] compared to healthy controls. Although the differential diagnostic capacity of using CSF markers between the preliminary states of various dementia disorders has not yet been thoroughly studied, the discoveries regarding AD are pushing the boundaries as to when AD can be identified and diagnosed in individuals.

2. Guidelines for AD and Dementia

Guidelines for the diagnosis and management of AD and other dementias are regularly published, updated, and revised in the USA and elsewhere in the world [15–19]. The latest European recommendations for the diagnosis and management of AD were published by the European Federation of Neurological Societies (EFNS) [17]. These evidence-based guidelines were created through a consensus process to provide guidance for physicians concerning diagnostic evaluation and management of dementia, including pharmacological and nonpharmacological treatment, management of behavioral and psychological symptoms of dementia (BPSD), counseling and support for caregivers, and so forth. The EFNS guidelines state that the recommendations are relevant only to dementia, and do not include MCI. Recently, Dubois et al. [20] published new diagnostic criteria for the early detection of AD. These were proposed as research criteria, rather than for clinical diagnosis and patient management, and incorporate clinical evidence of episodic memory loss (isolated or with other cognitive deficits) with biomarkers (CSF, neuroimaging, genetic) to identify patients in an early, predementia stage of AD. The criteria are novel because rather than focusing on the dementia syndrome they focus on clinical, biological, structural, and biochemical presence of AD in a predementia phase. These, and other, criteria for syndromes of early preclinical phases of AD raise controversial questions in the scientific and medical world. Should physicians diagnose MCI or incipient AD? Should MCI patients be treated? How should physicians and healthcare workers manage the healthcare of patients with MCI? Should dementia guidelines be revised to incorporate MCI? In this paper, we will discuss the disadvantages and advantages of omitting MCI and incipient AD from dementia guidelines, and discuss the potential impact on clinical practice and public health from the perspective of patients, caregivers, and healthcare providers.

3. Does MCI Represent Incipient AD?

The first relevant question to ask in this discussion is whether syndromes such as MCI truly represent an early preclinical phase of AD. Research both from clinical- and population-based samples suggest that MCI patients have a high risk of progressing to a full dementia syndrome; a meta-analyses [12] of longitudinal studies of individuals with MCI reported an annual conversion rate of 8.1% in clinical settings, and 6.8% in community-based settings. Pooled-analysis [13] suggests that about one third of MCI patients will convert to AD, with a higher conversion rate in the first few years following MCI diagnosis. However, it has consistently been shown that not all MCI patients will develop AD [7, 12], and that cognitive impairment in the elderly has heterogeneous risk factors [21] and potentially diverse etiologies and causes [11]. Although neuropathological studies on MCI patients indicate that pathological findings in the brain structures involved in memory represent a transition between normal aging and AD, many concurrent pathological abnormalities are also present in these patients, including vascular lesions and argyrophilic grain disease [22]. Further, neuropathological investigations in patients who progressed from MCI to AD show a heterogeneous pathological outcome [23]. In addition, not all AD patients pass through a clinically identifiable stage of MCI with subjective and objective memory deficits present prior to dementia diagnosis [24]. Thus, the evidence suggests that, although MCI does represent a group of individuals with a high risk of progressing to AD, MCI does not equal incipient AD because not all MCI patients will develop AD.

As mentioned previously, much work has been done to further characterize MCI patients to identify which individuals are most likely to develop AD. Therefore, although patients with a clinical diagnosis of MCI do not necessarily have AD, there are subsets of MCI patients who have a neurodegenerative AD etiology, and are in the early phase of AD. Functional markers, cognitive testing, hippocampal and entorhinal cortex atrophy, neuropsychiatric disorders, genetic factors such as Apolipoprotein E (ApoE) epsilon 4 ($\varepsilon 4$) allele, increased age, low education, and motor dysfunction are all markers that have been shown to identify which MCI patients have the highest risk of developing AD [7]. It is also evident that combining factors (including neuroimaging, clinical, and sociodemographic markers) [25, 26] increases the prediction for identifying which MCI patients will progress to AD. Indeed, Dubois and colleagues' research criteria for the early detection of AD [20] incorporate clinical evidence of cognitive deficits with neuroimaging, CSF, and genetic biomarkers. However, it is not yet clear which markers and cutoff values are the best for identifying incipient AD patients; although Dubois and colleagues make some suggestions for biomarkers, such as hippocampal volume loss, it was emphasized that research should identify and validate other biomarkers and neuroimaging regions of interest in the future. It is worth noting that although combining markers will help to increase the positive predictive value for identifying which MCI patients will develop AD, ultimately this may lead to a drop in the other diagnostic values. Tests and markers with high positive predictive value are likely to have lower sensitivity, especially at the population level. Therefore, very few patients with AD will be able to be identified in the early stage of the disease.

As discussed elsewhere in this special issue, much research effort has been made in the field of biomarkers,

particularly CSF, for identifying patients in the early, predementia, phase of AD. A multicenter study of the CSF markers in MCI patients has shown innovative results concerning the use of beta-amyloid$_{1-42}$, total tau protein, and tau phosphorylated at position threonine 181, which was found to have a good accuracy for identifying impending AD in MCI patients [14]. This was one of the first studies to pool the data from a large range of single center studies. It showed that the positive predictive value of these CSF markers for identifying incipient AD in MCI patients was 62%, with a sensitivity of 83%. However, despite these promising results, the findings should be viewed with caution. An editorial by Petersen and Tojanowski [27] highlighted the clinical variability of the MCI patients as well as the variability in CSF measurements in the different centers in Mattsson and colleagues' work. Further, they acknowledged that although the sensitivity and specificity of the CSF markers were adequate for screening, they did not have high enough accuracy to be used as diagnostic tests, although they may be useful for use in clinical trials [27]. Interestingly, the positive predictive value showed that 62% of MCI patients developed AD when a combination of the three CSF markers was used [14]. However, this positive predictive value needs to be compared with other markers and clinical syndromes. Before taking into consideration other markers, the cumulative conversion rate from MCI to AD across studies is 31.4% [13]. Furthermore, other studies have shown that this positive predictive value can be increased by combining other, less invasive, markers. For example, a prediction model using five factors (functional and olfactory markers, cognitive testing, and MRI measures of hippocampal volume and entorhinal cortex volume) had a positive predictive value of more than 80% for identifying incipient AD in MCI patients [25].

There are important limitations to consider when examining clinical and laboratory markers in MCI patients. First, laboratory samples are mainly taken from clinical patient populations, which may not be representative of the general population. Consequently, the sensitivity of a laboratory measure evaluated in a clinical sample does not reflect the level of sensitivity of the test if used at the population level. Second, a study in the general population showed that half of AD patients have no subjective complaints about their memory performance up to one to three years before AD diagnosis, and one fifth show absolutely no cognitive deficits [24]. This highlights the heterogeneity of patients in the preclinical phase of AD. It is likely that patients who seek medical advice for cognitive problems are at a more advanced stage of cognitive impairment, closer to the diagnosis of AD, which will affect the accuracy of measurements of sensitivity and predictive values in markers such as CSF values. Finally, the multicenter study by Mattsson et al. [14] found that the diagnostic validity of CSF markers had less accuracy in the multicenter analysis than the accuracy reported in single-center settings. This highlights the importance of replicating positive results from single centers, which are often conducted on small study populations, and the need for more multicenter trials and meta-analyses to determine the accuracy of biomarkers for identifying incipient AD.

4. Do We Need Guidelines for the Diagnosis and Management of MCI?

MCI is a frequently occurring syndrome in the elderly. A European longitudinal study [28] reported an incidence rate per 1000 person years of between 11.4 for amnestic-MCI and 33.8 for other types of cognitive impairment, and prevalence data suggests that cognitive impairment syndromes in younger nondemented elderly actually exceed dementia prevalence [29]. Given that MCI is a frequent syndrome in older people, and that individuals with MCI have a high risk of progressing to AD [7, 12], this raises the question of what should be done with such patients. As mentioned earlier, guidelines for the management of dementia often do not include recommendations for patients with MCI. However, many of the issues regarding both diagnosis and management of dementia may also be relevant to the large number of elderly persons suffering from MCI, who may or may not be in the early stage of AD. Clinically, MCI could be viewed, not as a disease or disorder per se, but as a risk factor for AD. Risk factors such as this are commonly addressed in other areas of medicine. Many conditions have been shown to predispose an individual to a higher risk of developing another medical condition, such as hypertension for stroke and myocardial infarction, dyslipidemia for stroke and other vascular disease, diabetes for osteoporosis and so forth. In good clinical practice, clinicians explain the risks associated with such conditions with their patients, and plan appropriate care and management. In this way, MCI should be viewed as a clinical syndrome that has a high risk of progressing to AD. Thus, if clinicians were to consider MCI, not as incipient AD, but as a risk factor for developing AD, certain issues concerning management and treatment that have been raised for dementia, may be relevant also to MCI.

5. Treatment of MCI

First, although dementia disorders such as AD are currently not curable, there are a number of symptomatic pharmaceutical treatments that are recommended in guidelines for dementia [17, 30]. Conversely, there are currently no approved drugs for the treatment of MCI or early preclinical AD. Clinical trials on MCI patients have had limited success; none of the trials, including donepezil, rivastigmine, rofecoxib, galantamine, and vitamin E, have demonstrated convincing effects for delaying progression from MCI to AD [31, 32]. However, other trials are ongoing, and data on subsets of MCI patients have identified potentially promising avenues for future research. For example, although randomized-control trials on cholinesterase inhibitors such as donepezil have shown little or no reduction in the risk of conversion from MCI to AD [33–35], the results of one trial suggested that MCI patients with depressive symptoms treated with donepezil had a lower risk of conversion to AD [36]. Without the availability of effective treatment strategies, and in the absence of approved drugs for the treatment of MCI or incipient AD, one may question the benefits of diagnosing such syndromes. Thus, dementia guidelines, such as those proposed by EFNS, might be right to avoid discussing

disease-specific treatment strategies for MCI, since MCI per se is not a disease, and currently there are no approved drugs for MCI. However, pharmacological treatments are not the only recommendations suggested in current guidelines for dementia, and the other recommendations might be of relevance to MCI patients as well as those with dementia, including suggestions for diagnosis, management of BPSDs, and support for families and caregivers.

6. Nonpharmacological Treatment and Interventions

Although there are no currently approved pharmacological treatments for MCI, nonpharmacological interventions have also been the focus of recent research. Management of risk factors and somatic disorders, lifestyle changes, and cognitive intervention programmes may be beneficial to certain patients, and may be advantageous as they might have less side effects and risks than pharmacological drugs. Cognitive rehabilitation studies in MCI [37–39] have showed improvement in ADL functioning, mood, and memory following training, although there is no evidence for delayed progression from MCI to AD. Further, if MCI patients are told of their high risk of developing AD, there are possibilities for modifying risk factors associated with AD, such as diet, somatic, and lifestyle factors [40]. Further, as it has been shown that MCI has heterogeneous risk factors [21] and that MCI may have diverse somatic, psychiatric, and neurological etiologies [11], the modification of somatic and lifestyle factors may be beneficial to the overall health of MCI patients, even those who are not in a preclinical phase of AD.

7. Disclosure of MCI Diagnosis

There is ongoing debate as to whether a formal diagnosis of MCI should be made and disclosed to patients in the clinical setting. The fact is that increasing numbers of elderly people are approaching physicians complaining of memory problems, and there is a high incidence of both MCI and other syndromes of cognitive impairment in the elderly [28]. There may be some benefits to disclosing a diagnosis of MCI to a patient and discussing the associated risk of developing AD. First, it allows patients to plan for their future before their cognitive impairment becomes severe, including financial planning, and the planning of care and living arrangements for the future. Second, in some cases it may be helpful for individuals to have a formal diagnosis that somewhat explains their symptoms. From the physician's perspective, giving an early diagnosis may be beneficial as the patient will have better cognitive capacities to understand the diagnosis and discuss future treatment and care options; indeed, studies have shown that physicians are often reluctant to disclose a diagnosis of dementia in more severe stages of the disease due to lack of comprehension by the patient [41]. Furthermore, physicians may be concerned that disclosing a diagnosis of dementia may trigger catastrophic events in the patient such as depression or suicide. However, a study by Carpenter et al. [42] examined changes in depression and anxiety both in patients and their relatives before and after the diagnosis of dementia. They found that there were no changes in depression status in either patients or their next-of-kin after a disclosure of a dementia diagnosis, but levels of anxiety decreased substantially after receiving the diagnosis. This supports the idea that receiving a formal diagnosis that explains the symptoms and memory loss can actually be beneficial to a patient.

Interesting lessons can also be learned from studies investigating the effect of disclosing genetic risk of AD to individuals, such as ApoE $\varepsilon 4$, a cardiovascular risk factor which is also associated with an increased risk of developing AD [43]. Both MCI and the ApoE $\varepsilon 4$ genotype are associated with a higher risk of AD but do not represent a definite risk; that is, not all patients with MCI or ApoE $\varepsilon 4$ will develop AD. The REVAL study group [44] investigated the effect of disclosing AD risk (both ApoE genotype and a numeric risk estimate) to family members of AD patients using a randomized-control trial design. They found no differences in short-term psychological symptoms such as anxiety, depression, or distress between people who were told that they had a genetic risk of developing AD (ApoE $\varepsilon 4$ carriers) than those who were not told their genotype. However, when questioned after one year, individuals were more likely to recall their genotype than their numerical lifetime risk estimate of AD [45]. The authors suggested that clinicians, therefore, needed to find an appropriate manner to communicate AD risk and probabilities. Most interestingly, however, was that disclosure of genetic AD risk was associated with a change in behaviors; a large proportion of individuals made changes to their long-term healthcare insurance after learning they were ApoE $\varepsilon 4$ carriers [46] and more than half made changes to their health behavior, such as vitamin intake and exercise [47]. Although specific studies on the disclosure of MCI diagnosis are needed, the results of there studies on disclosing genetic AD risk suggest potentially beneficial effects of informing an at-risk individual that they have a high risk of developing AD.

8. Recommendations for the Management of BPSD

Although there are no approved treatments for MCI, patients with this syndrome might have other related symptoms that can be treated. The EFNS guidelines for dementia stipulate that physicians should be aware of the importance of treating behavioral and psychiatric symptoms for the potential benefit of the patients and carers. This may also be relevant to MCI patients. Studies both from clinical- and population-based samples consistently report a high burden of BPSD in MCI patients, particularly depression, anxiety, and irritability (for a review see Monastero and colleagues [48]). Indeed the pattern of BPSD in MCI are similar to those observed in patients with dementia, and symptoms have been observed in more than one third of patients, with many studies reporting a higher prevalence [48], even up to 85%. Nevertheless, the role of BPSD in MCI remains unclear and,

thus, treatment of specific symptoms or psychiatric disorders in this group of patients is controversial. A population-based study showed that anxiety in MCI patients was associated with an ongoing AD related neurodegeneration [49], and clinical-based studies suggest a similar role for apathy [50–52]. In contrast, depression in MCI is generally not associated with progression to AD [49, 53]. There are currently no clinical trials investigating the effect of treating BPSD and psychiatric disorders in MCI patients, and thus it is not known whether pharmacological treatments are effective either in slowing the progression from MCI to AD or in reducing the severity of neuropsychiatric symptoms in these patients. Indeed, the situation is similar for patients already in the stage of clinical dementia; current guidelines for dementia [17] highlight these controversies.

9. Patient Guidelines and Advice

Guidelines on MCI for healthcare workers as well as patients have already been published in America. In 2010, the American Academy of Physician Assistants [54] published guidelines for physician assistants, stating that they should be able to recognize and treat MCI and dementia. Specific suggestions were made for screening tools, cognitive and somatic evaluation of at-risk patients, as well as tentative recommendations for treatment strategies. A Patient Page, published in the Journal of the American Medical Association [55], aims to provide patients and families with general information about MCI, including possible conditions associated with MCI, a description of diagnostic techniques, and some recommendations for prevention, mainly the management of high blood pressure and other chronic conditions, as well as social activities and dietary changes. A patient and family fact sheet on MCI, published in The Neurologist [56] describes potential causes of cognitive impairment in the elderly, rather than focusing specifically on AD, and describes diagnostic procedures and examination that might be conducted on patient with suspected cognitive problems. It also makes suggestions for life style changes, including social and dietary changes.

10. Conclusions

In conclusion, MCI is not a disease or disorder, but a risk factor for developing dementia and AD. Although studies examining biological markers and other factors in MCI patients have identified markers that further increase the risk of developing AD in these patients, we are still not at a stage where we can accurately identify with 100% accuracy which patients are in the early phase of AD. Consequently, current guidelines for the diagnosis and management of AD and dementia often do not include specific suggestions for MCI. Despite this, many of the recommendations made for patients with dementia, may also be relevant to people with MCI. Therefore, peer-reviewed, consensus guidelines for the diagnosis and management of incipient AD or MCI might be beneficial in the future. However, it is imperative to focus future research on identifying more accurate diagnostic markers to define incipient AD, and to study the effect of informing patients that they have a high risk of developing AD, in terms of psychological reactions and potential benefits for planning appropriate management and prevention strategies in MCI patients.

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Review Article

CSF Biomarkers for Alzheimer's Disease Diagnosis

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Alzheimer's disease (AD) is the most common form of dementia that affects several million people worldwide. The major neuropathological hallmarks of AD are the presence of extracellular amyloid plaques that are composed of Aβ40 and Aβ42 and intracellular neurofibrillary tangles (NFT), which is composed of hyperphosphorylated protein Tau. While the amyloid plaques and NFT could define the disease progression involving neuronal loss and dysfunction, significant cognitive decline occurs before their appearance. Although significant advances in neuroimaging techniques provide the structure and physiology of brain of AD cases, the biomarker studies based on cerebrospinal fluid (CSF) and plasma represent the most direct and convenient means to study the disease progression. Biomarkers are useful in detecting the preclinical as well as symptomatic stages of AD. In this paper, we discuss the recent advancements of various biomarkers with particular emphasis on CSF biomarkers for monitoring the early development of AD before significant cognitive dysfunction.

1. Introduction

Alzheimer's disease (AD) is the most widespread neurodegenerative disease globally [1] and is estimated to afflict more than 27 million people worldwide [2]. AD accounts for at least 60% of all dementia diagnosed clinically. The major pathological hallmarks of AD are the loss of neurons, occurrence of extracellular senile plaques as well as intracellular neurofibrillary tangles (NFT) [3]. Senile plaques are primarily composed of amyloid β-protein (Aβ), which is produced from the amyloid precursor protein (APP) by sequential proteolytic cleavages made by two proteolytic enzymes, β-secretase (β-site APP-cleaving enzyme; BACE) and γ-secretase (Figure 1) [4]. Amyloid plaque is an aggregate of Aβ containing 40–42/43 residues. NFT is primarily composed of hyperphosphorylated form of Tau protein [5]. Tau is synthesized within the neuron and localized in the axon where it promotes stability and assembly of microtubules [6]. During AD progression, tau is hyperphosphorylated and subsequently dissociated from microtubule and polymerized into paired helical filaments (Figure 1) [5, 6]. Although the clinical symptoms of AD are frequently diagnosed in older age, the degenerative process probably starts many years before the clinical onset of the disease [7, 8]. Currently, the diagnosis and treatment of AD is limited. The presymptomatic detection of AD is crucial, as it would facilitate the development of an efficient and rapid treatment of this destructive disorder early on (for recent review see [9–11]).

The biomarkers are the entities whose concentration, presence, and activity are associated with disease. Biomarkers are essential part of disease treatments as they are essential for diagnosis, monitoring the disease progression, detecting early onset of the disease, monitoring the effect of therapeutic intervention, and also avoiding false diagnosis of the disease [19]. An ideal biomarker (1) should be highly specific, (2) should predict the course of illness accurately, and (3) should reflect the degree of response to treatment. The biomarker research for AD has significantly advanced in recent years (Table 1) [9, 10]. The neuroimaging techniques assess the regional structure and function of the brain, as well as assist identifying the biochemical profile of brain dysfunction. The body fluids such as cerebrospinal fluid (CSF), plasma, and urine are considered as important sources for the AD biomarker development (Table 1). CSF is considered a better source for biomarker development as it is in direct contact with the extracellular space of the brain and can reflect biochemical changes that occur inside the brain.

Table 1: Some promising biomarkers in diagnosis of AD.

Category	Markers	Advantages	Limitations	References
Imaging	CT, PET, PIB-PET, MRI	(1) Noninvasive (2) Provides structural and functional details of brain immediately (3) Can reveal disease progression	(1) Expensive (2) Requires experienced personnel (3) The sensitivity and specificity to AD is not satisfactory	[12–14]
Plasma	α_2-Macroglobulin, Complement factor H, Aβ42	(1) Noninvasive (2) Samples are easily accessible	(1) Less correlation to AD (2) Less sensitive and specific for AD diagnosis (due to epitope masking)	[15–17]
CSF	Aβ42, t-tau, p-tau p-tau/Aβ42, t-tau/Aβ42	(1) Can correlate AD directly (2) Highly sensitive and specific (3) Can detect AD progression	(1) Invasive, sample has to be collected by lumbar puncture (2) Irreproducible diagnosis due to sample storage and transportation	[10, 18]

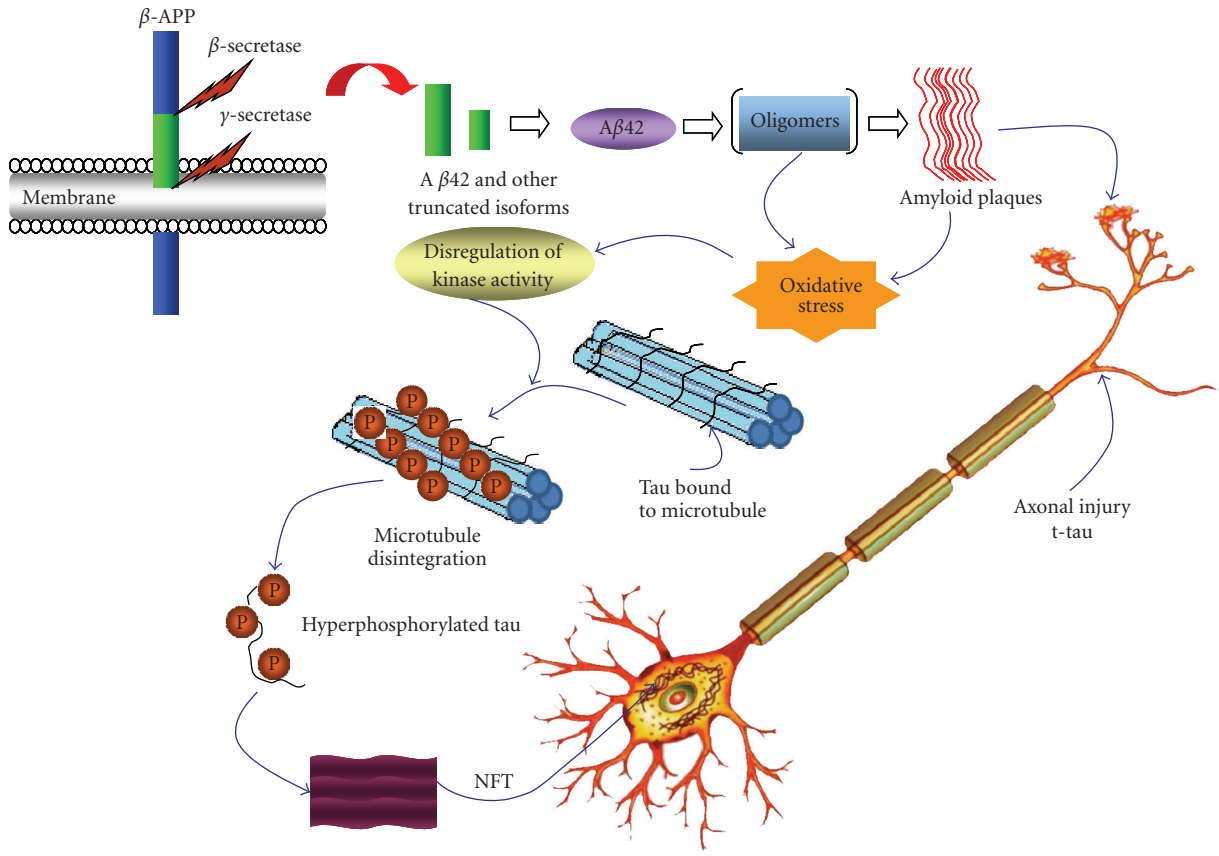

Figure 1: Pathological cascades and potential biomarkers of AD. Proteolytic cleavage of APP first by β-secretase followed by γ-secretase can produce Aβ42 and other shorter Aβ fragments. The subsequent aggregation of Aβ42 results in oligomers and amyloid fibrils. Amyloid fibrils are eventually deposited as senile plaques as shown. The toxicity of oligomers and amyloid fibrils could lead to the cascade of tau-hyperphosphorylation, which is otherwise bound to microtubules, providing microtubule stability. Upon hyperphosphorylation, tau dissociates from microtubules and aggregates into NFT, which could eventually cause increased cytoskeleton flexibility and neuronal death.

Thus far, three CSF biomarkers, Aβ42, total-tau (t-tau), and phosphorylated-tau (p-tau), have been found to have the highest diagnostic potential. Biomarkers of inflammation and oxidative stress and urine-based biomarkers are among the other sources that provide vital information on development and progression of AD. Unfortunately, none of the biomarkers presently available are able to accomplish the disease diagnosis single-handedly. Monitoring more than one biomarker at the same time is suggested to be suitable for detecting the disease progression. The main focus of this paper is to provide insights on the various potential biomarkers with particular emphasis on CSF biomarkers for AD diagnosis. These biomarkers are very promising for early diagnosis of AD.

2. Imaging Biomarkers

Neuroimaging techniques provide structural and functional details of the brain immediately [20, 21]. The imaging techniques are also helpful to predict and monitor the disease progression. Recent progress of functional and molecular neuroimaging [22] could provide insights into brain structure and physiology and also could detect the specific proteins and protein aggregates due to AD in the brain [20].

The loss of brain volume is one of the consequences of AD neurodegeneration [11, 21], and it could be differentiated from normal brain by using computerized tomography (CT) and magnetic resonance imaging (MRI) techniques [23]. These techniques are able to show neuronal loss, atrophy of medial temporal regions, as well as neurofibrillary tangles in the brain of AD patients. Using MRI technique, it is now possible to distinguish atrophy during early stage of AD from the atrophy of normal aging [24]. MRI has also the ability to distinguish AD subjects from normal controls, with a very high sensitivity and specificity [24]. MRI can reveal disease progression from cognitive normalcy to mild cognitive impairment (MCI) and to AD [12]. Discrimination of AD from other forms of dementia, namely, frontotemporal dementia (FTD) and DLB (dementia with Lewy bodies) is also possible based on different atrophy patterns that MRI reveals [25–27]. AD is also associated with metabolic impairment with typical regional pattern in the brain and could be detected by positron emission tomography (PET). If 18F-2-deoxy-2-fluoro-D-glucose (FDG) is chosen for PET, the concentrations of tracer imaged then gives tissue metabolic activity, in terms of regional glucose uptake. PET-FDG has been employed to examine regional cerebral metabolism, which is helpful in distinguishing AD from normal patients [13, 28–30]. Recently, several other radiologically contrast compounds have been developed for PET imaging, which could bind the pathological structures such as amyloid plaques, NFT, activated microglia, and reactive astrocytes, enabling the examination of antemortem pathological changes due to AD. The compounds that have been reported as probes for amyloid plaques in PET imaging include, [18F] FDDNP (2-(1-{6-[(2-[F-18] fluoroethyl) (methyl) amino]-2-naphthyl} ethylidene) malononitrile), 18F-BAY94-9172, 11C-SB-13, 11C-BF-227, and 11C-PIB. The only compound developed that can bind NFT *in vivo* is [18F] FDDNP. The 11C-PIB (PIB, Pittsburgh compound B) has been the most extensively studied and applied in AD research [14, 31]. In individuals with AD, increased retention of PIB shows a very specific pattern that is restricted to brain regions (frontal, parietal, temporal, occipital cortices, and striatum), typically associated with amyloid deposition [32]. A significant number of cognitively normal individuals over the age of 60 show a PIB signal pattern indistinguishable from that of individuals with AD, suggesting that measurement of PIB using PET can detect a preclinical stage of the disease. When PIB-PET was performed along with the Aβ42 concentrations in CSF of AD patients, the PIB-positive group showed low Aβ42 levels in the cerebrospinal fluid [33–35]. This finding is consistent with the "amyloid sink" hypothesis [36, 37], according to which the soluble Aβ42 is retained in the brain once plaques are formed. Besides the radiological studies of amyloid plaques and NFT, the PET imaging agent which images the inflammation due to activated microglia and reactive astrocytes has been developed. For example, increased expression of peripheral benzodiazepine receptor (PBR) has been target for the compound [11C] (R)-PK11195. The study using this compound in conjunction with PIB has suggested that microgliosis occurs concomitantly with amyloid deposition and may have direct role in cognitive dysfunction [38]. Like microglia, the changes in astrocytes on association with plaques could be used as biomarkers. For example, using inhibitors of monoamine oxidase B as radiotracers in AD has targeted the elevation of monoamine oxidase B activity [39, 40]. Although all of these imaging techniques are helpful for diagnosis of AD, the approach faced overlapping symptoms due to other pathological processes and normal aging. The approach also needs expensive instruments and experienced personnel for its application in routine diagnosis of AD.

Fluid Biomarkers. The sampling of CSF and plasma represents the most direct and convenient means to study the biochemical changes occurring in the central nervous system [10, 19, 41]. These fluids are the most attractive resources for ongoing research for discovering AD biomarkers. Most of the research has been performed either with the plasma or CSF, yet CSF represents more suitable source for biomarker discovery.

3. Plasma Biomarkers

Plasma is the liquid portion of blood where red blood cells, white blood cells, and platelets are suspended. Plasma could be easily isolated from whole blood by low speed centrifugation in the presence of an anticoagulant. The easier sampling of blood plasma makes this fluid ideal for biomarker investigation. However, plasma biomarkers as reliable markers for AD have met little success (Table 1). Various blood biomarkers have been proposed, yet changes in the levels of these molecules have proved difficult to verify in independent studies. Multiple studies have identified plasma proteins whose expression levels in AD patients differ from controls. For example, α_2-Macroglobulin (α2M) and complement factor H (CFH) showed an increased expression in AD subjects than in control [15]. Both of these proteins are shown to be present in senile plaques [42, 43]. Similarly, the increased levels of α_1-antitrypsin [44], α1-antichymotrypsin [45, 46], and decreased levels of Apolipoprotein A1 [47] in blood plasma/serum were observed in AD patients compared to healthy controls. Although these proteins may reflect pathological processes observed in AD and could differentiate diseased plasma compared to controls, these differences have yet to achieve sensitivity, specificity, and reproducibility. Irreproducibility might occur due to different analytical methodologies utilized in various laboratories, different choice of anticoagulant and depletion strategy, and storage related problems. The most popular plasma peptide utilized

for biomarker research is Aβ, which is the fundamental element of senile plaques in brain of AD patients [3]. Using ELISA, Aβ can be detected in plasma. The findings from different studies have shown variable results. Some studies have suggested slightly higher Aβ42 or Aβ40 plasma levels in patients with AD than in controls [48]. However, most of the studies have found no change in plasma Aβ concentration between AD and healthy control [48]. It is also suggested that large Aβ42/Aβ40 ratio could indicate the risk factors for AD [49]. These ambiguous results are probably explained by the fact that plasma Aβ is derived from peripheral tissues and does not reflect brain Aβ production. Furthermore, the hydrophobic nature of Aβ makes the peptide bind to plasma proteins, which could result in "epitope masking" [16] and other analytical interferences. Recently, analysis of 18 plasma signaling and inflammatory proteins has accurately identified patients with AD and predicted the onset of AD in individuals with MCI [50]. However, further studies are required to analyze if this set of proteins is the best possible recipe of plasma biomarkers for preclinical AD diagnosis.

4. Urine-Based Biomarkers

Neural thread protein (NTP) levels have been consistently identified as an AD biomarker in urine [51, 52]. With disease severity, the urinary concentration of this protein increases. AD associated NTP (AD7c-NTP) in CSF also showed consistent results [51, 53]. More research needs to be done to study the effects of AD7c-NTP levels upon therapeutic intervention [54–56]. Urinary F2-isoprostanes have been reported to be increased [54–56] or unchanged [57, 58], making them less reliable biomarkers. The utility of urine sample for AD diagnosis has advantage that sample collection is relatively easier and noninvasive compared to CSF and plasma. However, very low protein concentrations and high salt levels make it difficult to use urine sample as a source of biomarker [59].

5. CSF Biomarkers

Cerebrospinal fluid (CSF) is a translucent bodily fluid that occupies the subarachnoid space and the ventricular system around the brain. CSF acts as a "liquid cushion" providing a basic mechanical and immunological protection to the brain inside the skull and it can be obtained via lumbar puncture. Although lumbar puncture is invasive and potentially painful for the patient, CSF is probably the most informative fluid in biomarkers discovery for neurodegenerative disease prognosis [10]. CSF has more physical contact with brain than any other fluids, as it is not separated from the brain by tightly regulated blood brain barrier (BBB). As a result, proteins or peptides that may be directly reflective of brain specific activities as well as disease pathology would most likely diffuse into CSF than into any other bodily fluid. These proteins and metabolites can serve as excellent biomarkers of AD as well as other neurodegenerative diseases. In early course of AD, for an example of MCI, when the correct diagnosis is most difficult, CSF biomarkers would be valuable in particular [10]. Tau and Aβ in CSF represents the earliest and most intensively studied biomarkers [9, 10, 41, 60, 61]. Both proteins are linked to hallmark lesions of AD, amyloid plaques, and neurofibrillary tangles. In the next section, we will discuss the clinical significance of Aβ and tau biomarkers in detail.

5.1. APP, Aβ40/42, and Truncated Aβ in CSF as Biomarkers. One of the major pathological features of AD is the presence of senile plaques primarily composed of Aβ, a proteolytic fragment of the amyloid precursor protein (APP) (Figure 1) [62]. The expression level of APP could serve as diagnostic markers in AD [61]. However, the experimental studies of APP expression level in CSF of AD patients are inconsistent [61]. The inconsistencies between studies ruled out the possibility of CSF-APP being a useful biomarker for AD. APP is expressed in all tissues and could undergo cleavage by either α-secretase or β-secretase to release sAPP-α or sAPP-β, respectively. The processing of APP by α-secretase occurs via nonamyloidogenic pathway, and a reduced CSF level of sAPPα in AD patients has been reported [63]. In contrast, APP processing first by β-secretase and subsequent digestion by γ-secretase leads to formation of Aβ (38–43 residues) peptides. The 42-residue-long Aβ isoform (Aβ42) is highly hydrophobic and forms oligomers and fibrils that accumulate as extracellular plaques (Figure 1) [4]. Because Aβ42 is the dominant component of the plaques seen in AD [64], many groups have investigated the use of Aβ42, as well as the other Aβ species as a diagnostic tool. The amount of total Aβ in CSF is not well correlated with the diagnosis of AD [65]. The majority of studies have demonstrated a decrease of CSF Aβ42 in AD patients [34, 66–70]. However, few reports suggest the increased [71] or unchanged [72] CSF Aβ42 in AD. These differences in observations might be due to the variations in sample assaying protocols and selection of patient groups. Deposition of the peptide in plaques ("amyloid sinks") is considered the underlying basis for the decrease of CSF-Aβ42 levels seen in AD [36, 37]. Although it is not clearly proved, the observation is supported by the strong correlation between low CSF-Aβ42 levels and high plaque burden when measured by PIB imaging [33]. This observation was further supported by the fact that AD mouse model showed low CSF Aβ level with high amount of plaque in the brain [73]. Although it has been shown that CSF Aβ42 levels can identify PIB-positive individuals with highest possible sensitivity and specificity, the decreased CSF levels of Aβ42 have also been reported in other dementia such as FTD [74–76]. Low concentrations of CSF Aβ42 was also found with individuals without PIB-positive plaque [77]. This finding might be explained by the fact that PIB binds fibrillar Aβ not the Aβ oligomers or diffuse plaques [77] that are found in earlier stages of AD process. It is however that CSF Aβ42 has high potential as a biomarker for diagnosis, plaque burden, prognosis and may provide clue of preclinical AD. Aβ40 is unchanged in the CSF of AD patients [21]. However, the decreased Aβ42/Aβ40 ratio is much more pronounced in AD diagnosis than the

reduction of Aβ42 alone. Therefore, Aβ42/Aβ40 ratio might be more useful in AD diagnosis in the early as well as the clinical phases of the disease [78]. Moreover, the presence of several shorter Aβ isoforms in CSF has suggested that Aβ constitutes a large family of peptides with considerable length variations. The carboxy-terminal truncated Aβ peptides for example, Aβ37, Aβ38, and Aβ39 have been found in CSF of AD subjects. In AD patients, an increase in Aβ38 levels, accompanied with a decrease in Aβ42 levels were also observed [79, 80]. Thus, the Aβ42/Aβ38 ratio might prove useful for more precise diagnosis of AD [79, 80]. Immunoprecipitation techniques and mass spectrometry have identified a number of short truncated Aβ isoforms, such as Aβ14, Aβ15, and Aβ16 in the CSF of AD patients. These forms have been reported to be produced through a novel pathway of APP processing involving the β and α secretase actions [81]. In the AD subjects, elevated Aβ16 levels, accompanied with a decrease in Aβ42 levels were reported in CSF [82].

5.2. CSF-Tau as a Biomarker. The protein tau is an intracellular protein, which maintains the stability of microtubules in neurons. In normal individual, only low concentration of tau is present in CSF. The function of tau is tightly regulated by a number of post-translational modifications including phosphorylation at serine and threonine residues. The precise form of tau in CSF and the mechanism for leakage of intracellular tau into CSF is not clearly understood. Despite intense research, the amyloid and tau pathologies remain unclear. Several experimental studies have suggested that hyperphosphorylation and NFT formation is the downstream phenomenon of AD pathologies [83]. However, it is also noteworthy that loss of function of tau due to hyperphosphorylation and subsequent detachment of tau from microtubule could lead to the increased cytoskeleton flexibility and loss of axonal integrity in the brain (Figure 1) [84]. In AD, tau becomes hyperphosphorylated and gets dissociated from microtubule and subsequently polymerized into insoluble paired helical filaments (PHF) [84]. PHF eventually contributes to the formation of neurofibrillary tangles [85, 86]. NFT formation and neuronal degradation is an essential part of AD pathology (Figure 1). Upon significant disruption of neuronal architecture, tau protein could be released into CSF [60]. Therefore, increased levels of tau and hyperphosphorylated tau in CSF can correlate with the onset of neurodegeneration in AD. The total tau (t-tau) concentration in CSF has been investigated by ELISA analysis using monoclonal antibodies against all tau isoforms. Several studies have suggested that t-tau concentration in CSF of AD patients is higher than control [60, 87]. Although the CSF t-tau is very sensitive biomarker for detecting AD, it has limited ability to discriminate AD from other major forms of dementia as t-tau also increased in CSF of others form of dementia including vascular dementia (VAD) and frontotemporal dementia (FTD) [60]. Several studies also used the p-tau in CSF as potential biomarkers since it is the major component of NFT. CSF concentrations of p-tau in AD have been examined using ELISAs based on monoclonal antibodies that can detect its various epitopes of p-tau, namely, (Thr181 + Thr231), (Thr231 + Ser235), Ser199, Thr231, (Ser396 + Ser404), and Thr181 [41, 88, 89]. ELISA study using all antibodies has showed increased CSF concentration of p-tau in AD patients. Moreover, the ability of increased p-tau assays to discriminate AD from normal aging and other dementia is more sensitive and specific than that of CSF concentrations of t-tau and Aβ42 [60, 90, 91]. The experimental evidences of high CSF concentrations of p-tau in only AD patients have suggested that p-tau is not a simple marker of axonal damage and neuronal degeneration, as t-tau, but it is more closely related to AD pathology and the formation of NFT.

5.3. Combined Aβ and Tau in CSF as Biomarkers. It has been suggested that combinations of CSF markers could more successfully discriminate AD from control or other forms of dementia than an individual marker. There are several studies where the diagnostic performance of the combination of CSF t-tau and Aβ42 is analyzed. The evidences have suggested that high CSF concentration of t-tau and low concentrations of Aβ42 could detect AD with high diagnostic sensitivity and specificity [61]. The other combinations of CSF biomarkers have also been evaluated, which suggested that the high CSF p-tau/Aβ42 ratio possesses higher sensitivity and specificity [18] for differentiating AD from normal controls and from subjects with other non-AD dementia than that of the CSF t-tau, p-tau, Aβ42, and ratio of t-tau/Aβ42. It is also suggested that the combination of tau and Aβ42 has more diagnostic potential in terms of sensitivity and specificity in MCI patients to develop future AD [92].

5.4. Oligomers of Aβ in CSF: Promising Biomarkers for Early Diagnosis in AD. Recent studies have suggested that oligomeric Aβs are the most neurotoxic species in AD. Substantial *in vivo* and *in vitro* evidence supports this hypothesis [93–97]. Several *in vitro* neurotoxicity studies have shown that Aβ oligomers are potent neurotoxins [98–104], and the toxicity of some oligomers is higher than that of the corresponding amyloid fibrils [105]. The evidences, which support the fact that Aβ oligomers could be targeted for drug and biomarker discovery include (1) soluble oligomers could inhibit hippocampal long-term potentiation (LTP) [95, 98, 100, 103, 104, 106, 107] and disrupt cognitive function [108] *in vivo*; (2) compounds that bind and disrupt the formation of oligomers have been shown to block the neurotoxicity of Aβ [108, 109]; (3) drugs that reduce the amyloid plaque burden without disruption of oligomers have little effect on recovery of neurological function [110]. Many oligomers such as Aβ-derived diffusible ligand (ADDL)-like Aβ42 oligomers [111], 90 kDa Aβ42 oligomer [112, 113], 56 kDa oligomer of "Aβ*56 [114], and Aβ trimers [115] have shown high *in vivo* toxicity, providing a compelling reason for Aβ oligomers to be used as potential AD biomarkers especially for early diagnosis in AD. In addition, elevated levels of

Aβ oligomers were detected in AD patients and transgenic mice compared to control [116–118]. The elevated level of oligomers could also appear in CSF but with lower concentration. Therefore, highly sensitive techniques are required for oligomer detection in CSF. The study using fluorescence correlation spectroscopy suggested the presence of Aβ oligomers in CSF of AD patients, compared to healthy control [119]. Recently, ultrasensitive, nanoparticle-based, protein detection assay (bio-barcode) showed that the ADDLs concentrations in CSF of AD patients were consistently higher than the nondemented age-matched control [120]. In this study, ADDLs specific antibodies coupled to DNA-tagged nanoparticles were used to capture the oligomers from the CSF of patients with AD. Although the number of AD patients and controls studied was low, the findings were very promising. Although Aβ oligomers are attractive biomarker candidates, several limitations exist to use these species. The concentration of these Aβ oligomers in CSF is very low in comparison with Aβ monomers. Again, the detection of individual Aβ oligomers is difficult since oligomers are metastable and therefore one form of oligomers could transform to another form immediately. Assay sensitivity must reach very high level if one can detect total heterogeneous population of Aβ oligomers in CSF. The monoclonal antibodies specific for only Aβ oligomers could be difficult to develop. Recently, an antibody against Aβ oligomers was developed, which can detect all Aβ oligomers, including oligomers from other amyloidogenic protein [117]. However, using these oligomers specific antibody to diagnose of AD is difficult since it cannot differentiate AD from other neurodegenerative diseases. New analytical methods and novel oligomers-specific antibody must be developed to detect oligomers in CSF of AD patients, which would have ultimate ability to detect early onset of AD.

5.5. Neuronal Biomarkers in CSF. Besides tau and Aβ, neuronal and synaptic proteins could also be used as CSF biomarkers in AD. For example, Visinin-like protein 1 (VLP-1), a calcium sensor protein was shown to be significantly increased in the CSF of AD subjects compared to controls. It is believed to seep out from dented neurons [121]. The sensitivity and specificity of CSF VLP-1 is comparable to CSF t-tau, p-tau, and Aβ42. Combined analysis of Aβ42, p-tau, and VLP-1 has been reported to raise the diagnostic precision of AD. VLP-1 biomarker might also prove useful in indicating the degree of dementia [121]. The neurofilaments, which are structural component of axons, could also be used as biomarkers for discriminating AD patients from other forms of dementia, as their expression levels are high in VAD and FTD [122], while normal levels are found in most AD patients. Another synaptic protein called growth-associated protein (GAP-43) is found in higher levels in CSF of AD than that of controls, and FTD [123]. Furthermore, it has been shown that CSF GAP-43 and t-tau were increased in AD and correlated positively [123], suggesting both biomarkers are reflecting axonal and synaptic degeneration.

5.6. Oxidative Stress Marker in CSF. Besides the formation of amyloid plaque and NFT, AD is also frequently characterized by reactive oxygen species (ROS)-mediated neuronal damage. The oxidative damage in the brain mainly involves lipid peroxidation [124]. Polyunsaturated fatty acids are susceptible to oxidation by reactive oxygen species. Isoprostanes are lipid oxidation products generated due to the reaction between fatty acids and ROS. Therefore, isoprostanes could be used as valuable AD biomarkers. Several studies have suggested that F2-isoprostanes, a group of isoprostanes, are increased in CSF of AD patients compared to healthy control or patients with other dementia [125, 126]. CSF-F2-isoprostanes have also been shown to be increased in patients with MCI and asymptomatic carriers of familial AD mutations. A combined analysis of CSF-Aβ42, tau, and F2-isoprostanes, was able to diagnose AD with a sensitivity of 84% and specificity of 89% [127].

5.7. Inflammatory Biomarkers in CSF. AD pathology involves release of inflammatory mediators. The differential occurrence of several proteins due to inflammatory process in AD might be used as biomarker. These proteins can be detected using ELISA, as well as proteomics approaches. One of the most studied inflammatory biomarkers is α1-antichymotrypsin (A1ACT), which is observed either increased [45, 128] or unchanged [129] in CSF samples of AD patients. However, the contradictory results suggest that more studies must be conducted to raise the possibility of A1ACT to be regarded as an effective biomarker. The study of cytokines, which are produced during inflammation processes in AD, also gave inconsistent results. For example, CSF interleukin-6 (IL-6) levels have been reported to be increased [130–132], decreased [133], or unchanged [134–136] in AD. Studies of IL-6 receptor, Gp130, and tumor necrosis factor (TNF-α) also produced conflicting results [137]. The genetic background, environmental factors, and usage of anti-inflammatory drugs might produce substantial variation in cytokine levels in an individual [138]. This could be the reason for such uncertain results.

6. CSF Biomarkers: A Potential Hope for AD Diagnosis

As discussed in the preceding sections, most biomarker research in AD is based on either brain imaging or is fluid-based. Although imaging techniques are definitive tests for detecting amyloid plaques and atrophy using molecular probe, still antemortem diagnosis of AD and MCI are less successful. More sensitive chemical probes are required to be developed, which would bind oligomers or diffuse plaque. However, imaging techniques being very expensive and requiring more experience for handling the instruments precludes their day-to-day use for AD diagnosis. In fluid biomarker research, CSF has been proved to be a supreme source for biomarkers for several reasons. CSF is in close proximity to the brain, and therefore biochemical changes in the brain affect the composition of biomarkers in CSF. Since

AD pathology is restricted to the brain, CSF is an obvious source of biomarkers for AD. CSF is also a rich source of brain-specific proteins, and changes in these protein levels are observed in CSF with disease progression. CSF biomarkers are also very sensitive to the fine changes in brain that occur in the preclinical stages of the AD. Therefore, CSF is probably the most informative fluid sample available for preclinical as well as symptomatic AD diagnosis. The diagnostic sensitivity and specificity of CSF biomarkers in differentiating AD from healthy controls, and from other forms of dementia is already achieved with satisfactory levels. Moreover, a combination of more than one biomarker in CSF, such as CSF p-tau, t-tau, and Aβ42 is considered to give higher diagnostic accuracy of AD. It can identify AD, prodromal AD, and also can differentiate AD from other dementia with high sensitivity and specificity that is otherwise impossible to achieve.

Although CSF biomarkers have proved to be highly informative, sensitive, and specific for detection of clinical AD and early stage of AD, their regular use in clinic is still limited. One of the major reasons against the vast applicability of CSF in AD diagnosis is lumbar puncture, an invasive method to collect the CSF sample. Other issues including inconsistency of data analysis of CSF sample due to sample collection, transportation, storage, and high expense of the test might limit the use of CSF for routine diagnosis. However, various strategies are available to resolve these issues. For example, the Clinical Neurochemistry Laboratory in Gothenburg, Sweden and Alzheimer's Association, have together started a quality control program, the objective of which is to standardize CSF biomarker measurements between both research and clinical laboratories [10]. This program would obviously enhance the diagnostic precision of CSF markers, thus enabling them to support a routine analysis for diagnosis of AD.

7. Future Direction

According to the current clinical diagnostic criteria, AD diagnosis cannot be made until the patient has dementia, which is defined as cognitive symptoms severe enough to interfere with social or occupational activities [139]. This might hinder the preclinical diagnosis of AD. The disease modifying drugs will be most effective and will have most therapeutic value if these are administered in the earliest stage of AD, before amyloid plaques and NFT become prevalent. Since AD is a multifactorial neurodegenerative disorder both at clinical and neuropathological level, development of biomarkers with 100% efficiency in terms of sensitivity and specificity is difficult to achieve. Also, the effectiveness of the disease modifying drugs could vary from one subgroup to another subgroups, making the utility of biomarkers in clinical trial and drug discovery difficult. Combined analysis of CSF biomarkers represents more suitable diagnostic tool to detect AD patients or detect individuals with MCI. Moreover, sensitive assays should be developed to detect amyloid oligomers in CSF and in the brain. This would raise the possibility for the diagnosis of early onset of AD.

Abbreviations

Aβ: Amyloid β protein
AD: Alzheimer's disease
ADDL: Aβ-Derived diffusible ligand
APP: Amyloid precursor protein
BACE: β-site APP-cleaving enzyme
BBB: Blood brain barrier
CT: Computerized tomography
DLB: Dementia with Lewy bodies
FTD: Frontotemporal dementia
MCI: Mild cognitive impairment
MRI: Magnetic resonance imaging
NFT: Neurofibrillary tangles
PET: Positron emission tomography
PIB: Pittsburgh compound B
p-tau: Phosphorylated-tau
VAD: Vascular dementia.

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Review Article

Biological Markers and Alzheimer Disease: A Canadian Perspective

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Decreased β-amyloid$_{1-42}$ and increased phospho-tau protein levels in the cerebrospinal fluid (CSF) are currently the most accurate chemical neurodiagnostics of sporadic Alzheimer disease (AD). A report (2007) of the Third Canadian Consensus Conference on the Diagnosis and Treatment of Dementia (2006) recommended that biological markers should *not* be currently requisitioned by primary care physicians in the routine investigation of subjects with memory complaints. Consideration for such testing should prompt patient referral to a specialist engaged in dementia evaluations or a Memory Clinic. The specialist should consider having CSF biomarkers (β-amyloid$_{1-42}$ and phospho-tau) measured at a reputable facility in restricted cases presenting with atypical features and diagnostic confusion, but not as a routine procedure in all individuals with typical sporadic AD phenotypes. We submit that developments in the field of AD biomarker discovery since publication of the 3rd CCCDTD consensus data do not warrant revision of the 2007 recommendations.

1. Introduction

The advent of a biological marker that reliably indicates the presence of Alzheimer disease (AD) and distinguishes the latter from other dementing disorders would greatly assist the medical management of this common neurodegenerative condition. The successful integration of such a marker in routine clinical practice would confer the following benefits: (1) the accurate and expeditious diagnosis of sporadic AD, (2) curtailment of ancillary biochemical and imaging studies currently employed to exclude other causes of dementia, (3) the capacity to recognize AD in subjects with major affective disorders, clouded sensorium, depressed levels of consciousness, and other illnesses that often preclude assignment of a dementia diagnosis by conventional means, (4) possible surveillance of AD severity, progression, and impact of therapeutic interventions, (5) prognostication of conversion to incipient AD in individuals with mild cognitive impairment (MCI), and (6) treatment arm assignment and stratification of volunteers enrolled in clinical trials. In this paper, we review criteria for ideal biomarkers of sporadic AD, chemical biomarkers currently in vogue, and a national perspective on the clinical use of AD biomarkers in Canada based on the Third Canadian Consensus Conference on the Diagnosis and Treatment of Dementia [1].

2. Biological Markers and Sporadic AD

A biological marker of disease may be defined as a measurable change in the physical composition of an organism that indicates the presence of the illness. Biomarkers currently under investigation for the early diagnosis of AD include brain volume or activity measurements derived from neuroimaging techniques, such as positron emission tomography (PET) or magnetic resonance imaging (MRI) and chemical indices detected in various body fluids. Neuroimaging modalities are labor-intensive, expensive, and not universally available, prompting intense research efforts towards the development of effective chemical biomarkers and other practical neurodiagnostic tools. Chemical markers of AD fall within three general categories: (i) *genetic markers*, (ii) *genetic modifiers,* and (iii) *biological markers*. Mutant forms of amyloid precursor protein, presenilin-1, and presenilin-2 are proven *genetic markers* of AD. While useful for

predicting disease in rare kindreds with familial AD (<10% of all AD cases), they play little or no role in tracking disease progression or efficacy of therapeutic intervention in these patients. Moreover, these genetic markers have little or no relevance for the management of individuals with the far more common, sporadic form of the disease [1, 2]. Carriers of the apolipoprotein E (*APOE*) $\varepsilon 4$ allele, a *genetic modifier*, are at increased risk for the development of sporadic AD, manifest dementia symptoms earlier than $\varepsilon 4$-negative persons with the disease, and exhibit accelerated conversion rates from MCI to AD [3]. However, testing for the $\varepsilon 4$ allele cannot be used as a diagnostic marker of sporadic AD because its presence does not guarantee that that the disease exists or will occur nor does its absence exclude the condition. True *biological markers* of AD, in contradistinction to genetic markers and modifiers, inform on the presence or absence of AD at the time of measurement ("state" indicators) and may therefore serve as diagnostic modalities of the disease.

3. Criteria for an "Ideal" Biological Marker of Sporadic AD

Principles set forth in a Consensus Report on Molecular and Biochemical Markers of AD sponsored by the Alzheimer's Association (US) and the National Institute on Aging have served as a guiding light for the development of AD biomarkers worldwide [2]. This landmark report recommended that an "ideal" biological marker of AD meet the following criteria [4]:

(i) reflect a fundamental aspect of CNS pathophysiology in AD (plausibility);

(ii) indicate the actual presence of AD and not merely increased risk;

(iii) exhibit high sensitivity and specificity (in the range of 80% or better for each);

(iv) be efficacious in early or preclinical AD (e.g., MCI);

(v) monitor disease severity or rate of progression;

(vi) indicate efficacy of therapeutic intervention;

(vii) be noninvasive, inexpensive, and readily available.

In subsequent reports on this topic, it was also deemed desirable that (viii) the efficacy of the putative biomarker be corroborated by at least one other independent laboratory and that its accuracy (criterion (iii)) be demonstrated in discriminating AD not only from cognitively-healthy controls but from patients with various non-AD dementias [5].

4. Biological Markers of Sporadic AD

In this section, we review the utility of CSF β-amyloid$_{1–42}$ ($A\beta_{1–42}$) and tau/phospho-tau (p-tau) measurements as clinical biomarkers of sporadic AD. Other candidate chemical biomarkers of the disease currently commercially available or under investigation include urine AD7C-neuronal thread protein (marketed by Nymox Pharmaceutical Corp., Montreal), CSF and urinary F_2-isoprostanes, other redox reporter molecules, plasma biospectroscopy, and a host of blood proteins, mRNAs, microRNAs, cholesterol metabolites, and transition metals. The latter will require further validation before they can be recommended for routine clinical use and will not be discussed further. Readers interested in these candidate biomarkers may consult recent literature from the author's laboratory and others on this topic [1, 6–19].

(i) *CSF* $A\beta_{1–42}$: Amyloid fragments are plausible AD biomarkers because they represent a hallmark pathological process in the affected brain (senile plaque formation). Evidence from numerous studies worldwide indicates that concentrations of the amyloid peptide fragment $A\beta_{1–42}$ are abnormally diminished in the CSF of patients with sporadic AD and MCI [19, 20]. A meta-analysis involving 18 studies of CSF $A\beta_{1–42}$ as a diagnostic marker of AD revealed an effect size of 1.56 (95% CI: 1.43–1.69) [21]. In 2003, an analysis of CSF $A\beta_{1–42}$ data derived from 13 studies (~600 AD and 450 control subjects), all utilizing the Innogenetics ELISA for the peptide, indicated an overall sensitivity and specificity of 80% and 90%, respectively, for distinguishing AD from cognitively-healthy controls [22]. However, CSF $A\beta_{1–42}$ may decline in other degenerative CNS conditions including Lewy body dementia (LBD) [23–25], amyotrophic lateral sclerosis (ALS) [26], multisystem atrophy [27], and Creutzfeldt-Jakob disease (CJD) [24, 28]. Thus, in a multicentre study involving 150 AD, 100 normal elderly controls and 79 cases of non-AD dementia, the specificity of CSF $A\beta_{1–42}$ in differentiating AD from normal subjects was 81% whereas it was only 59% relative to non-AD dementias [29]. Important data concerning the use of CSF biomarkers in the management of AD are now emerging from the Alzheimer Disease Neuroimaging Initiative (ADNI), a large, multi-institutional prospective study designed to correlate clinical phenotypes with imaging and chemical biomarkers in >800 rigorously-ascertained subjects with normal cognition, MCI, and AD [30]. A first such report [31] confirmed the stratification of cognitively normal, MCI and AD subjects based on declines in CSF $A\beta_{1–42}$ levels (205.6 ± 55.1, 162.8 ± 56.0, and 143.0 ± 40.8 pg/ml for the 3 groups, respectively; $P < .001$). Moreover, baseline CSF $A\beta_{1–42}$ concentrations successfully predicted the deterioration of neuropsychological measures in the normal and MCI cohorts (but not AD persons) over an ensuing 12-month period. *Plasma amyloid*: augmented plasma β-amyloid$_{1–42}$ ($A\beta_{1–42}$) concentrations have been reported in several kindreds with *familial* AD [32], but these families comprise a very small proportion of the entire AD population. Measurements of CSF or blood total $A\beta$ peptide, $A\beta_{1–40}$ or soluble APPα/β concentrations have thus far not proven useful in the diagnosis of sporadic AD [20, 22, 33–35] although identification of novel amyloid peptide fragments in AD biofluids using mass spectrometry techniques may still yield markers of diagnostic significance [19, 36].

(ii) *CSF total tau:* CSF total (t) tau reflects neurofibrillary tangle formation in the AD brain but is also a fairly non-specific marker for neuronal destruction in a wide range of degenerative and nondegenerative CNS disorders. Elevated levels of total tau protein (t-tau) have been consistently

encountered in AD CSF. An effect size of 1.31 (95% CI: 1.23–1.39) for CSF tau as an AD diagnostic was disclosed in a meta-analysis involving 35 studies [21]. In 2003, a review of CSF t-tau data from 41 studies (over 4000 AD and control subjects) that used either the Innogenetics or Athena ELISA disclosed a sensitivity and specificity for the diagnosis of AD of 80% and 90%, respectively (akin to the meta-analysis of CSF A$\beta_{1–42}$) [22]. In the robust ADNI study [31], CSF t-tau increased progressively from 69.7 ± 30.4 to 101.4 ± 62.2 to 119.1 ± 59.6 pg/ml in normal, MCI, and AD subjects, respectively ($P < .001$). As in the case of CSF A$\beta_{1–42}$, CSF t-tau is less effective in discriminating AD from other dementias, with specificities of 57% for suspected non-AD dementias [29] and 69% for autopsy-confirmed cases [24]. Elevated concentrations of CSF t-tau may also predict progression of cognitive deterioration in MCI, especially in patients without extensive periventricular white matter lesions [37]. High levels of CSF t-tau may also arise in fronto-temporal dementia (FTD) [38], vascular dementia [39], CJD, and (transiently) in acute ischemic stroke [40]. CSF t-tau values in LBD [23] and vascular dementia [41] may be intermediate between those of the cognitively-normal elderly and subjects with AD. Interestingly, 34% of individuals with FTD in one study exhibited significantly *suppressed* levels of CSF tau, a finding not seen in the AD cohort [42].

(iii) *CSF phospho-tau:* Phospho-tau isoforms are tenable AD biomarkers because they reflect a known pathophysiological process in AD brain (neurofibrillary tangle formation). A number of laboratories have documented significant increases in levels of hyperphosphorylated tau in AD CSF relative to cognitively-intact controls using antibodies against various phosphorylated epitopes of tau (p-tau). CSF p-tau is elevated in "incipient AD" [43] and MCI [44, 45] and is therefore a relatively early biomarker of the disease. In the aforecited ADNI report [31], levels of threonine 181 p-tau in the CSF of persons with normal cognition, MCI, and AD were, respectively, 24.9 ± 14.6, 35.5 ± 18.0, and 41.6 ± 19.8 pg/ml ($P < .001$). Use of CSF p-tau to monitor disease progression may be limited by dilutional factors unless combined with MRI measurements of hippocampal atrophy [46]. Of note, p-tau levels in AD CSF are reportedly elevated relative to other dementing and nondementing neurological disorders [22, 47, 48]. As such, and in contradistinction to t-tau, enhanced CSF p-tau levels may differentiate AD from FTD [49, 50], Lewy body dementia [51], vascular dementia [52], PD [53], ALS, acute stroke [54], schizophrenia [55], and major depression [53]. Despite a previous report to the contrary [56], CSF concentrations of threonine 181 p-tau may be augmented in sporadic and variant CJD [57].

(iv) *CSF A$\beta_{1–42}$ and p-tau combined:* CSF A$\beta_{1–42}$ and p-tau, when measured together, exhibit sensitivities and specificities (versus other dementing disorders) in the range of 80%–90% [58]. The positive and negative predictive values of the combined test are 90% and 95%, respectively, assuming a prevalence rate of 45% [20]. This biomarker combination reflects disease pathophysiology (*vide supra*), identifies AD in early stages (e.g., MCI), and is relatively inexpensive. Some posit that CSF A$\beta_{1–42}$ represents the *stage* of AD (with concentrations diminishing progressively as a function of disease duration), while t-tau and p-tau are indicators of disease *intensity* (with higher CSF levels connoting more rapid progression) [59]. It has been suggested that the extent of CSF tau elevation and A$\beta_{1–42}$ suppression may correlate with the *APOE* ε4 allele burden [60] although the extent to which genetic factors impact CSF biomarker levels remains uncertain. In patients with MCI, the biomarker combination may prognosticate for imminent conversion to AD with sensitivities/specificities in the range of 83%–90% [61, 62]. The markers also exhibited efficacy in delineating "nonprogressors" in "mixed" (amnestic and nonamnestic) MCI over a 3-year median follow-up period [63] and may assist in distinguishing MCI from anxiety and depression [64]. A large European-American multi-institutional trial employed a cutoff CSF A$\beta_{1–42}$/p-tau ratio predetermined from an established AD cohort (at 85% sensitivity) to detect AD in 750 MCI individuals followed longitudinally for at least two years or until dementia intervened. The investigators identified incipient AD in the MCI subjects with 83% sensitivity, 72% specificity, 62% positive predictive value, and 88% negative predictive value. The authors concluded that although the test was accurate in identifying incipient AD, intersite assay variability limited its performance relative to previous results from single-centre studies, underscoring the need for standardization of clinical procedures and analytical techniques [65]. In another recent multicentre study, AD-like CSF biomarker ratios were noted to be more frequent among individuals with subjective (but no objective) cognitive impairment (SCI; 52%) than in healthy controls (31%; $P < .01$), suggesting that AD may be the cause of SCI (and not only MCI) in a significant proportion of elderly subjects [66]. To our knowledge, CSF A$\beta_{1–42}$ and tau determinations have not yet proven helpful as indices of therapeutic efficacy in AD.

(v) *CSF biomarkers: further considerations:* (a) In the majority of AD biomarker studies, the validity of the data were limited because receiver operating characteristic curves (plotting the relationship between sensitivities and specificities) were generated on the basis of clinical diagnoses without autopsy corroboration. While *prospective* AD biomarker studies are in principle more valuable than retrospective analyses, the former are less likely to include neuropathological diagnoses [67]. (b) The immunoassay procedures invoked to measure CSF A$\beta_{1–42}$ and tau are not trivial, and interlaboratory variability is commonplace. (c) Athena Neurosciences charges US$905 to MDs and $1,335 to insurance companies for the combined tau and CSF A$\beta_{1–42}$ assays per sample. It was announced this year that the cost of AD biomarkers would be defrayed by the Canadian government pending documentation of need. Regardless, the cost may not be prohibitive if it obviates the need for additional testing (e.g., neuroimaging). (d) In a study of 342 AD, MCI, and cognitively normal individuals subjected to 428 research lumbar punctures, the adverse effect rate was low (e.g., post-LP headaches in 0.93%), and the procedure was generally well tolerated (low pain and anxiety scores in visual analog scales) [68]. Yet, CSF examination by

lumbar puncture is more invasive than venipuncture or urine analysis and currently not suitable for mass screening of elderly persons with AD risk factors or mild memory impairment. The latter could warrant revisiting in the event that effective measures to prevent AD were to become available.

5. The Third Canadian Consensus Conference on the Diagnosis and Treatment of Dementia

Canadian Consensus Conferences on the Diagnosis and Treatment of Dementia were held in 1989, 1998 and, most recently, in March 2006 (Montreal) in attempt to standardize the diagnostic and therapeutic management of AD and related dementias in our country [69]. The structure and organization of the 3rd CCCDTD followed guidelines of the AGREE collaboration [70]. The project was funded by major government health institutes, geriatric and Alzheimer societies, and unrestricted grants from the pharmaceutical industry. Acknowledged leaders representing the disciplines of neurology, geriatric medicine, geriatric psychiatry, and neuropsychology, with liaisons from family practice, participated in the 3rd CCCDTD. PubMed and Embase electronic databases (supplemented by individual investigator files) spanning from January 1996 to December 2005 were surveyed for pertinent literature on nine designated topics. Publications were included for review based on their quality as determined by Jadad criteria [71]. The strength of evidence was graded according to the Canadian Task Force on Preventive Health Care [72]:

(I) Evidence obtained from at least one properly randomized controlled trial. (II-1) Evidence obtained from well-designed controlled trials without randomization, (II-2) Evidence obtained from well-designed cohort or case-control analytic studies preferably from more than one centre or research group, or (II-3) evidence obtained from comparisons between times or places with or without the intervention. Dramatic results in uncontrolled experiments are included in this category. (III) Opinions of respected authorities based on clinical experience, descriptive studies, or reports of expert committees. The valence and strength of recommendations were assigned using the following grading system [73, 74]. (A) There is good evidence to support this maneuver. (B) There is fair evidence to support this maneuver. (C) There is insufficient evidence to recommend for or against this maneuver, but recommendations might be made on other grounds. (D) There is a fair evidence to recommend against this procedure. (E) There is good evidence to recommend against this procedure. Background papers and sets of recommendations for each topic were posted online and voted upon by all conferees. Recommendations receiving at least 80% support were considered to have achieved consensus. The full list of approved recommendations is available on the websites of the 3rd CCCDTD (http://www.cccdtd.ca/) and the Alzheimer Society of Canada (http://www.alzheimer.ca/). Eighteen background articles accruing from this exercise were published in the October 2007 issue of *Alzheimer's & Dementia*.

6. 3rd CCCDTD: Role of Biomarkers

To ascertain the role of biomarkers in AD for the 3rd CCCDTD, the author reviewed a total of 186 papers: 137 generated from surveillance of the electronic literature (see Section 5) using the search terms "Alzheimer disease" AND ("Biological Marker" OR "Biomarker"), and an additional 49 articles from the author's files. The analysis led to the following *conclusions* [1]

(i) AD is a public health concern of epidemic proportions for which current diagnostic (and therapeutic) modalities remain insufficient.

(ii) The advent of a biological marker that differentiates early, sporadic AD from normal aging and other dementing disorders would represent a significant advance in the evaluation and management of this neurodegenerative disorder. An accurate, minimally invasive biological marker of early sporadic AD would serve the public interest by facilitating patient and family counselling, enabling stratification of subgroups for enrolment in clinical drug trials, and improving the interpretation of treatment outcomes. The introduction of a chemical marker that differentiates "malignant" MCI cases at high risk for deterioration to AD from neuropsychologically similar cases destined to manifest "benign" aging-associated memory changes would be particularly useful. Biomarkers may also prove helpful in situations where concomitant medical or psychiatric conditions confound or preclude neuropsychological testing, for example, major depression, delirium, suppressed consciousness, or individuals who are otherwise uncooperative for detailed cognitive testing. (Although conjectural and not listed among the published conclusions of the 3rd CCCDTD, it should prove interesting to determine whether measurement of AD biomarkers in patients with normal pressure hydrocephalus assists in the selection of appropriate candidates for (and improves the success rate of) surgical shunting.)

(iii) Although several candidate biomarkers of sporadic AD have been identified and commercialized, none currently fulfills criteria for an ideal test (see Section 3).

(iv) Decreased $A\beta_{1-42}$ and increased phospho-tau protein concentrations in the CSF are currently the most accurate and reproducible chemical neurodiagnostics of sporadic AD. These biomarkers also show promise as prognosticators in subjects with MCI. However, CSF evaluation by spinal tap remains impractical for mass screening of elderly individuals with symptoms of memory impairment or AD risk factors.

(v) Platelet APP isoform ratios, plasma or urinary F_2-isoprostane levels, blood biospectroscopy, and other modalities under investigation may fulfil several criteria for an "ideal" biological marker of early sporadic AD (Section 3). However, further experimentation and validation will be needed before these candidate biomarkers can be considered for clinical use. Similarly, all AD biomarker candidates arising from mass spectrometry and other proteomic applications [19, 75, 76] will require stringent clinical evaluation for their suitability as bonafide diagnostic tools.

(vi) Given the complexity of AD pathology, it is likely that combinations of individual biomarkers will provide more accurate diagnostic and prognostic data that any single marker assayed in isolation (akin to use of multiple biochemical indices to characterize liver failure, cardiac ischemia, or connective tissue disease).

On the basis of the literature analysis and aforementioned conclusions, the following *recommendations* reached consensus (see Section 5) and were published by the 3rd CCCDTD [1].

6.1. To Primary Care Physicians. (i) "Biological markers for the diagnosis of AD should not, at this juncture, be included in the battery of tests routinely used by primary care physicians to evaluate subjects with memory loss (Grade C, Level 3). Consideration for such specialized testing in an individual case should prompt referral of the patient to a specialist engaged in dementia evaluations or a Memory Clinic."

6.2. To Specialists. (i) "Although highly desirable, there currently exist no blood- or urine-based AD diagnostics that can be unequivocally endorsed for the routine evaluation of memory loss in the elderly (Grade C, Level 3). The non-invasiveness of such tests, if and when they become available, would be suitable for mass screening of subjects with memory loss presenting to specialists in their offices and Memory Clinics.

(ii) Due to their relative invasiveness and availability of other fairly accurate diagnostic modalities (clinical, neuropsychological and neuroimaging), CSF biomarkers should not be routinely performed in all subjects undergoing evaluation for memory loss (Grade D, Level 2).

(iii) CSF biomarkers may be considered in cases where there are atypical features and diagnostic confusion. CSF biomarkers may be useful in differentiating frontal variants of AD from FTD (Grade B, Level 2).

(iv) When a decision to obtain CSF biomarkers is made, combined $A\beta_{1-42}$ and p-tau concentrations should be measured by validated ELISA (Grade A, Level 1). It may be best to convey the CSF samples to a centralized facility (commercial or academic) with a track record in generating high-quality, reproducible data.

(v) CSF biomarker data in isolation are insufficient to diagnose or exclude AD (Grade C, Level 3). They should be interpreted in light of clinical, neuropsychological, other laboratory and neuroimaging data available for the individual under investigation."

It is the opinion of the author and Dr. Howard Chertkow (Chair, 3rd CCCDTD, personal communication) that developments reported in the field of AD biomarker discovery since publication of the 3rd CCCDTD consensus data do not warrant revisal of the 2007 recommendations. However, this remains an area of intensive research worldwide and further insights from large-scale initiatives such as ADNI, or validation of blood- or urine-based markers of the disease, may prompt a sea-change in the way AD biomarkers are exploited in Canadian clinics.

Disclosures

Hyman M. Schipper has served as consultant to Osta Biotechnologies, Molecular Biometrics Inc., TEVA Neurosciences and Caprion Pharmaceuticals. He holds stock options in Osta and equity in Molecular Biometrics.

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Review Article

Lessons from Multicenter Studies on CSF Biomarkers for Alzheimer's Disease

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Several single-center studies have confirmed the usability of cerebrospinal fluid (CSF) biomarkers for the diagnosis of Alzheimer's disease (AD), even in early disease stages. Large scale multicenter studies have principally confirmed this, although such studies have also indicated the presence of significant intercenter and interlaboratory variations in biomarker measurements. Such variations may hamper the development of biomarkers and their introduction into clinical routine practice. Recently a quality control program run by the Alzheimer's Association was started in order to harmonize procedures of laboratories world-wide. This program provides both standardized guide lines and external control CSF samples, and will allow longitudinal evaluation of laboratory performance.

1. Introduction

The ongoing development of disease modifying treatment for Alzheimer's disease (AD) puts pressure on researchers to develop reliable biomarkers for diagnosis, disease progression and monitoring of treatment effect. For these purposes, the most promising biomarkers are imaging and cerebrospinal fluid (CSF) markers [1–3]. The core CSF biomarkers are β-amyoid42 (Aβ42), total-tau (T-tau), and phosphorylated tau (P-tau), where the most frequently used P-tau isoforms are tau phosphorylated at the threonine amino acid residues 181 or 231 [1]. CSF Aβ42, T-tau and P-tau correspond to the principal neuropathological elements of AD: extracellular amyloid plaques, axonal degeneration, and intraneuronal tangles. Indeed, autopsy studies and imaging studies have revealed that CSF biomarker concentrations correspond well to brain alterations [4–7]. The accessibility of CSF for analysis and the low cost of CSF biomarker measurements favor their usage for detection, and monitoring of pathological processes in the brain. Several studies have shown that AD patients have decreased CSF Aβ42 and increased T-tau and P-tau compared with healthy controls [8]. Noteworthy, T-tau and P-tau correlate in AD patients and controls but not in several other neurological diseases. T-tau is increased in several neurodegenerative conditions and is a sensitive but unspecific AD biomarker [9]. P-tau, on the other hand, may add specificity for AD in dementia investigations [10].

2. CSF Biomarkers in Early Diagnosis

Brain alterations are likely to start long before onset of clinical dementia. Thus, biomarkers that detect changes in the brain are likely altered at a clinically early stage. It has been proposed that biomarker alterations occur dynamically during the disease process, with Aβ and Tau markers changed first, followed by distortion of brain structure, decline of memory and ultimately clinical dysfunction [11]. Supporting the use of CSF biomarkers for early diagnosis, decreased Aβ42 and increased T-tau and P-tau are seen in patients with mild cognitive impairment (MCI) later diagnosed with AD. In a well-controlled monocenter study, Hansson and coworkers followed 137 MCI patients for 4–6 years, during which 57 patients were diagnosed with AD. The CSF biomarkers sampled at baseline had

a sensitivity of 95% and a specificity 83% for incipient AD [12]. Since clinical AD diagnosis lacks some accuracy versus autopsy confirmation, it is probably not possible to achieve a significantly higher diagnostic accuracy for biomarkers towards clinical diagnosis. In line with this, consensus reports recommend that AD biomarkers should have at least 85% sensitivity and 75%–85% specificity [13]. As demonstrated by the study by Hansson and coworkers as well as other studies [14–20], this goal is achievable with the CSF biomarkers Aβ42, T-tau, and P-tau even in early stages.

3. Multicenter Studies of Diagnostic Accuracy

The results from the above mentioned studies are encouraging, but it should be noted that diagnostic biomarkers generally perform better in homogenous monocenter studies than in more challenging heterogeneous multicenter studies. So far, only a few large scale multicenter studies of CSF AD biomarkers have been published. In the DESCRIPA study, by Visser and coworkers, CSF samples were taken from 193 MCI and (subjective cognitive impairment) SCI patients and 89 controls at 9 centers in Europe [21]. All samples were analyzed at one laboratory. The results confirmed that a CSF AD profile, as defined by Hulstaert et al. [22], predicted AD in MCI with an odds ratio of 27 (95% CI 1.6–460) in amnestic MCI patients. However, the study was complicated by the fact that all controls were enrolled at one center, and CSF sampling procedures differed between centers. Also, 31% of healthy controls presented a CSF AD pattern, indicating a lack of specificity of the CSF biomarkers. This finding may seem controversial but is consistent with a by now large body of literature, showing that around one third of cognitively normal elderly display AD-like changes in their brains at autopsy [23], in their CSF [24, 25] or on PET scans imaging amyloid [26–28]. Longitudinal studies will tell us whether these individuals do have preclinical AD, or whether silent AD pathology is part of the normal aging process. Recent studies using repeated cognitive assessments [29] or functional magnetic resonance imaging measures of cortical network integrity [30] favor the first of the two scenarios.

Shaw and coworkers have published CSF biomarker results from the Alzheimer's Disease Neuroimaging Initiative (ADNI) [31]. The ADNI is a multicenter, longitudinal study, launched in 2004. In this study, optimal biomarker cut-offs where established in autopsy confirmed ADNI-independent AD patients and normal controls, with diagnostic accuracies ranging from 70% to 87%. The ADNI cohort was recruited at 56 clinical centers, which potentially could introduce large center-dependent variations. To minimize such variations, all participating centers followed the ADNI procedure manual. All samples were analyzed at the ADNI Biomarker Core laboratory at the University of Pennsylvania. The study included 196 MCI patients and 37 of these had been diagnosed with AD at the 12-month follow-up. A majority (87–89%) of these 37 presented a CSF AD profile at baseline. This similar to what was seen in patients with mild AD at baseline (N = 100, 88%–91%). However, as in the DESCRIPA study, a large proportion (34%–38%) of cognitively healthy controls (N = 114) had the same biomarker pattern, again indicating a lack of specificity in relation to clinical diagnosis.

In a third multicenter investigation, published in JAMA in 2009, we enrolled 750 MCI patients, 529 AD patients, and 304 healthy controls from 12 centers in Europe and the United States [32]. Four laboratories were involved, enabling evaluation not only of Intercenter, but also of interlaboratory differences, as discussed below. Cut-offs for the combination of Aβ42, T-tau, and P-tau were constructed in AD patients and controls, with sensitivity 85% in accordance with the above mentioned consensus document. This yielded 88% specificity towards healthy controls. In MCI patients followed for at least 2 years, the sensitivity of the biomarkers was 83% and the specificity 72%. The lower specificity compared to the monocenter study by Hansson et al. could partly be explained by the shorter followup, which makes it hard to verify the benign nature of all stable MCI cases. However, Intercenter variations may also have influenced the results and blurred some effects of the biomarkers. For the remaining part of this review, we will focus on such variations, and possible ways of eliminating them.

4. Intercenter Biomarker Variations

CSF studies on AD patients and controls report different biomarker concentrations, reference ranges, and diagnostic cut-offs [8]. CSF Aβ42 levels in AD patients in some studies even exceed the levels in controls in other studies. Such fluctuations make it hard or impossible to compare CSF biomarkers between centers and studies. These Intercenter variations come as no surprise. Rather, it is more rule than exception that a novel clinical chemical measurement present variations between centers, due to preanalytical or analytical confounding factors. Such variations are traditionally tackled by quality control programs, which until recently have been lacking for CSF dementia markers. For the CSF biomarkers Aβ42, T-tau, and P-tau, possible confounding factors include preanalytical, analytical, and assay factors [33–35]. Preanalytical factors include, for example, usage of different test tubes and differences in sample handling and storage. Analytical factors include differences in pipetting technique and other laboratory procedures. Finally, important assay factors are batch-to-batch variations and differences in standards and coating of antibodies. A growing number of laboratories are performing CSF analyses for dementia diagnostics. To facilitate the use of biomarkers in research settings and to enable their implementation in clinical routine, the Intercenter variations must be dealt with. Basic CSF parameters, including albumin and immunoglobulin levels, typically have interlaboratory coefficients of variation (CV) below 10%. This is a reasonable ultimate aim also for dementia markers. A handful of multicenter studies have investigated interlaboratory variations in CSF dementia markers, and we will now summarize the results of these studies.

5. The First International Quality Control Surveys

In 2006, Lewczuk and coworkers published the first international quality control survey of neurochemical dementia diagnostics [36]. A ventricular CSF sample was collected from a neurosurgical patient. Fourteen laboratories in Germany, Austria, and Switzerland participated and performed CSF biomarker measurements according to local routines. Different commercially available ELISAs were used for Aβ measurement. Three laboratories used a method from The Genetics Company (Zürich, Switzerland) measuring Aβx-40 and Aβx-42, with no specific N-terminal amino acid capture. Thirteen laboratories used the Innotest (Innogenetics, Ghent, Belgium) ELISA for Aβ, which specifically measures Aβ1-42. Fourteen laboratories used Innotest for T-tau, and 11 laboratories used Innotest for P-tau. Interlaboratory coefficients of variation ranged from 21% to 38%, with the highest numbers reported for Aβx-42 (38%) and Aβ1-42 (29%). Most laboratories analyzed samples in duplicates and the intra-assay imprecision was mostly low, exceeding 5% only for T-tau (5.3%) and Aβ1-42 (7.5%).

Verwey and coworkers published another quality survey in 2009 involving 20 laboratories with measurements performed in 2004 and 2008, allowing evaluation of longitudinal stability [37]. For this study, large CSF pools with different biomarker patterns were constructed and samples were distributed to 13 laboratories in 2004 and to 18 laboratories in 2008. A majority of laboratories used the Innotest ELISA for T-tau and P-tau, but in the 2008 run some laboratories had introduced the Luminex method AlzBio3 (Innogenetics, Ghent, Belgium). ELISA methods generally give higher values for CSF biomarkers than Luminex methods, but the methods have equivalent diagnostic accuracy for AD [38, 39]. Aβ42 was measured with the Innotest ELISA, or ELISAs from The Genetics Company, Biosource (Invitrogen, Camarillo, USA) or an in-house assay. Three laboratories used the AlzBio3 method for Aβ42. Interlaboratory CVs were high for Aβ42 ELISAs in both 2004 (31%) and 2008 (37%), and somewhat lower when only including laboratories using the Innotest (30% and 22%, resp.). CVs were smaller for T-tau (2004: 21%, 2008: 16%) and P-tau (2004: 13%, 2008: 15%). The AlzBio3 method had CVs ranging from 14%–22%, but the low number of laboratories performing this assay makes interpretation difficult. Nine laboratories participated in both rounds of the survey and intra-laboratory CVs for these were 25% for Aβ42, 18% for T-tau and 7% for P-tau.

These two studies show large interlaboratory variations for Aβ42, and smaller but significant variations for T-tau and P-tau. Since these studies used centrally distributed control CSF, several preanalytical confounding factors were eliminated. Remaining possible causes of the variations include local differences in analytical routines, machinery differences and batch-to-batch variations in analytical kits. The latter provides a major challenge for kit vendors, and emphasizes the need to have a robust production of antibodies, standard solutions, and analytical plates.

6. The Swedish Brain Power Survey

In the multicenter study mentioned above, published in JAMA in 2009, most centers sent their samples to the Clinical Neurochemistry Laboratory in Mölndal for analysis. However, samples from Amsterdam, Munich, and Kuopio, Finland were analyzed at local laboratories. The study therefore provides information about interlaboratory differences. Subset of samples from Amsterdam, Munich and Kuopio were rerun at the laboratory in Mölndal, and values for Aβ42 and T-tau from all three local laboratories differed more than 2CVs from values measured in Mölndal, using Mölndal CVs for the assays (around 10%). However, even for centers where samples were run at the laboratory in Mölndal, considerable Intercenter variations were seen. Possible sources of these variations include the preanalytical procedures of subject selection, lumbar puncture, sample handling, and storage. Such variations were seen in particular for Aβ42, but to a less extent also for T-tau and P-tau.

7. The Alzheimer's Association Quality Control Program

The studies outlined above present clear evidence for Intercenter and interlaboratory variations in CSF biomarker measurements. This makes it difficult to compare studies, which may hamper development of CSF biomarkers. With the rapid development of disease modifying treatment the AD scientific community must not lose momentum in biomarker development. Standardization of collection and handling of samples is vital for this, such as suggested by, for example, the German Competence Net Dementias [40]. Accordingly, it was decided at the International Conference on Alzheimer's Disease (ICAD) in Vienna in 2009 to start an international quality control (QC) program for CSF AD biomarkers. This program is run by the Alzheimer's Association and administrated from the Clinical Neurochemistry Laboratory in Mölndal. The program is open for public, private, research, clinical, and pharmaceutical laboratories. Participating laboratories receive a chart with recommended guidelines for lumbar puncture and sample handling and storage. In line with the ADNI procedures manual, the intention of this chart is to harmonize local routines to eliminate confounding factors responsible for Intercenter variations. In a second part of the program, participating laboratories receive QC CSF samples, constructed in Mölndal. These are analyzed and results are reported to the QC program coordinator. The participating laboratories then receive feedback on their analysis compared to the other laboratories. The first round of the program has just been completed and data are being analyzed. The QC program will continue with multiple rounds each year, enabling the tracking of longitudinal changes in performance.

8. Conclusions

Multicenter studies have confirmed the high diagnostic accuracy of CSF biomarkers for AD, even at early stage,

before onset of dementia. In particular, the high diagnostic sensitivity of CSF biomarkers achieved in the ADNI trial shows that harmonization of sample collection and handling allows the usage of the biomarkers even in a widespread multicenter setting. This advocates the use of CSF biomarkers in clinical studies, where they may be used to enrich trials with MCI patients with incipient AD. However, to facilitate the development of biomarkers and to enable their introduction in clinical routine, interlaboratory and Intercenter differences should be systematically analyzed. This is achievable within the Alzheimer's Association QC program.

Disclosures

Kaj Blennow has served in a scientific advisory board for Innogenetics. Henrik Zetterberg has served in a scientific advisory board for GlaxoSmithKline.

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Research Article

Standardization of Assay Procedures for Analysis of the CSF Biomarkers Amyloid $\beta_{(1\text{-}42)}$, Tau, and Phosphorylated Tau in Alzheimer's Disease: Report of an International Workshop

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Large variation in assay performance and outcomes of CSF Aβ1-42, total Tau (Tau), and phosphorylated Tau (pTau) (at amino acid 181) levels is observed between laboratories. The aim of this study was to assess the differences in assay procedures between several experienced international laboratories, as potential sources of error. 14 groups performed the Aβ42, Tau, and pTau assays according to the guidelines of the manufacturer. Differences in analytical procedures between the laboratories were monitored. At least 23 items in assay procedures were identified that varied between the laboratories, including procedures for washing, pipetting, incubation, finishing, and sample handing. In general, the inter- and intra-assay variation between the groups was generally below 10% for all three assays. We concluded that 17 international centers that use the same assays for Aβ42, Tau and pTau on a regular basis do not uniformly adhere to the procedures recommended by the manufacturer. For harmonization of intercenter results of these biomarkers standardization of protocols is highly needed.

1. Background

In the aging population the number of Alzheimer Disease (AD) patients is expected to increase [1]. However, the diagnostic accuracy of the clinical criteria is relatively low (sensitivity 80% and specificity of 70%) [2]). With this in mind, biological markers in body fluids are urgently needed to sustain diagnosis, as they are an objective tool and reflect ongoing processes. Biomarkers can aid not only in early diagnosis or in differential diagnosis but also in estimation of prognosis and, ideally, monitoring progression of this disease.

The concentrations of Amyloid-beta$_{(1\text{-}42)}$ (Aβ42), total Tau (Tau), and Tau phosphorylated at position 181 (pTau) in cerebrospinal fluid (CSF) of AD patients can be used as biomarkers [3]. Several laboratories measure these three biomarkers in CSF, and a major challenge is to translate the technology from the lab to clinical practice. To reach this goal, the technique should be robust and laboratories should be adequately experienced [4]. In addition, results obtained in different centres should be comparable to the highest possible degree [5]. The comparability of results between different centres is crucially dependent on the performance of the biomarker tests in the various institutions, and this can be assessed with an external quality assessment scheme. No such scheme was available and that is why we took the initiative in 2004 to send samples to a number of laboratories with previous experience in performing these CSF biomarker assays, with their own ELISA assays. The results revealed large variation in the concentrations of the three biomarkers between the different laboratories and a difference in variation at each evaluated time point [6]. Overall variation for Tau was slightly better in 2008 than in 2004, since the mean interlaboratory CV was 21% in 2004 and 16% in 2008. For pTau the mean between-laboratory

CV increased slightly, that is, from 13% in 2004 to 15% in 2008. The largest overall change was seen for Aβ42, where the between-laboratory variation increased from 31% to 37%. The introduction of other ELISA methods appeared to be responsible for this overall increase in variation. Laboratories that used the Innotest assays improved the between-laboratory variation of Aβ42 from 30% in 2004 to 22% in 2008, suggesting that experience and standardization of assay procedure may contribute significantly to reduce between laboratory variation. With the aim to improve between-laboratory performance we set out to identify the specific differences in procedures between laboratories. For this, we organised a hands-on workshop at the end of 2009.

2. Methods

26 participants from 17 different international centres with previous experience in performing the assays were divided in 14 groups, and every group performed the Aβ42, Tau, and pTau assays. The participants used their own pipettes. Identical samples containing pooled anonymised CSF samples, with concentrations of these biomarkers covering concentrations observed in controls, in AD patients and an intermediate value, were provided to the groups. Standard curves were diluted by each group according to the protocol, starting with dissolving the solid powder. All groups analysed the same samples, and the assays were performed simultaneously in the same laboratory and used the protocol (incubation procedures) as provided by the manufacturer. The standards and samples were analysed in duplicate. One exception was the incubation temperature of the Tau assay, which should be in an incubator at $25 \pm 2^{\circ}$C, and was at room temperature due to practical reasons. During the performance of the assays, the two persons in every group discussed their usual laboratory practice in performing the assays and differences were recorded. The intra- and interassay variation in concentrations of the pooled CSF samples was calculated. The analysis was done blindy for the concentrations.

3. Data Analysis

The standard curves were calculated using a 5-parameter logistic (5PL) curve fitting on the Biorad Microplate Manager Software. The mean and standard deviations of the concentrations in the pools were calculated per group.

4. Results

4.1. Variation in Assay Procedures. Table 1 lists the items that were noticed to vary among the laboratories participating in the workshop. The items involved procedures for pipetting, incubation, washing, finishing, and sample handling.

4.2. Inter-Assay Variation during the Workshop. Several items listed in Table 1 were standardised during the workshop due to its setup. Therefore, we were able to evaluate the inter-assay variation under circumstances that excluded several items that varied between the laboratories (Table 2), providing an indication of the relative contribution of these sources of error. Figure 1 shows the concentrations of the CSF pools for each group. The results of two groups strongly deviated from the mean outcomes. The cause was identified as lack of experience (group 3) and an error in the calculation of the dilution of the standard to make the standard curve (group 11). For Tau, no outliers were identified while, for pTau, the outcomes of the concentration of the highest pool were deviating in group 14. The numbers in Table 1 provide the concentrations and variation coefficients.

The intra-assay variation based on the concentrations of the unknowns was high for pool 1 and 2 of Abeta but on average was below 10% for Tau and pTau.

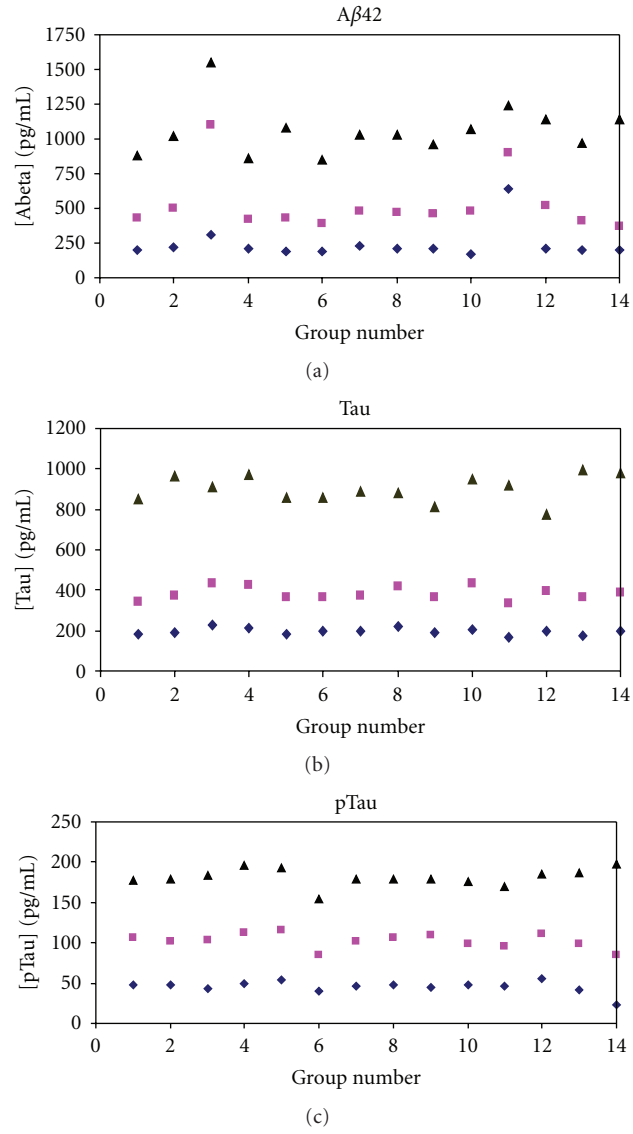

Figure 1: Concentrations of (a) Aβ42, (b) Tau, and (c) pTau in CSF pools containing low, medium, and high concentrations of these biomarkers per group. Aβ42, Tau, and pTau concentrations in samples were analysed blinded for the concentrations. Triangle: pool 3; Square: pool 2; Diamond: pool 1.

Table 1: Procedures that varied between the laboratories.

Pipetting	Standardised due to the design of the workshop
(1) Use of different pipette tip for each standard-sample-dilution tips compared to one tip	
(2) Wiping off the tip with a tissue	
(3) Some labs apply "inverse pipetting"	
(4) Use of a multichannel pipet rather than a single-channel pipet for pipetting the samples of Aβ42 from the dilution plate and pipetting secondary antibodies	
(5) The labs use different brands of pipettes	
Incubation	
(6) Some labs incubate the Aβ42 and Tau ELISA at 25°C, others at room temperature	Yes: at room temperature
(7) Several labs report problems with the air conditioning in summer	Yes
(8) Several labs shake the plate (at 300 rpm) during the whole incubation period while several other labs do this just for one hour and then incubate the plate without further shaking, other labs shake it several minutes, and some do not shake at all during the incubation	Yes
(9) Several labs always incubate the samples in the dark	
Washing	
(10) Several labs use an automatic wash machine, others do it by manually	Yes
(11) Tau: several labs wash 5 times instead of 4 times	Yes
(12) The concentrated wash buffer: several labs heat it up to dissolve the crystals. others do not, they wait untill all crystals are dissolved	
(13) Different labs use different amounts of wash buffer (300 or 400 μL)	Yes
Finishing	
(14) The TMB incubation: several labs perform it in dark, others do not	Yes
(15) Several labs stop the incubation with 50 μL H_2SO_4, others use 100 μL	
(16) Several labs use different brands of microplate readers and different reference values for the reader.	Yes
(17) Different curve fitting procedures are followed (straight line, 5 PL, etc.),	Yes
Sample handing	
(18) Several labs close the standard vial during the procedures	
(19) Between the different assays (Aβ42, Tau, and P-Tau) the samples are stored by several labs at 4°C. Others use new aliquots stored at −20°C. Others leave the samples at room temperature.	Yes
Other	
(20) Several labs do not use the polypropylene plate for the Aβ measurement, which is delivered by the assay manufacturer.	Yes
(21) Several labs use gloves to perform the assays.	
(22) Aβ42: Several labs do not use the 1500 pg/mL standard.	Yes
(23) Several labs use MilliQ water, others use distilled water.	Yes

Table 2: Variation in outcomes of CSF concentrations of the three biomarkers between the 14 groups.

	$A\beta_{1\text{-}42}$				Total Tau			$P\text{-}Tau_{181P}$		
	Concentration (pg/mL)	Inter-assay variation % CV (all groups)	% CV (group 3 and 11 removed)	Intra-assay variation (stdev)	Concentration (pg/mL)	Inter-assay variation % CV	Intra-assay variation (stdev)	Concentration (pg/mL)	Inter-assay variation % CV	Intra-assay variation (stdev)
Pool 1	203	49.3	7.63	24.3 (23.6)	196	8.4	4.3 (2.0)	46	16.5	8.5 (7.1)
Pool 2	448	39.6	10.33	11.4 (19.3)	385	8.7	3.6 (3.6)	102	9.3	3.5 (5.0)
Pool 3	1002	17.0	9.99	4.6 (9.5)	902	7.4	2.9 (2.9)	181	6.2	3.0 (2.1)

5. Discussion

5.1. Procedural Differences. The principle aim of this study was to address the issue of procedural differences as a source of variation between outcomes of CSF biomarker analysis. One of the results of workshop was a list with differences in procedures among the labs, containing 23 items. Variation was observed in all phases of the protocol. Some of the items were prescribed in the protocol but were not adhered to. Examples of these items are the use of polypropylene plate for sample predilution, the inclusion of 1500 pg/mL standard in the curve, the use of 50 μL H_2SO_4in the Aβ42 assay, and the use of 5PL curve fitting and incubation temperature of $25 \pm 2^{\circ}$C for total Tau.

The type of pipette is not prescribed by the manufacturer, neither was the mode of pipetting. We do not expect wiping off the tips, which was historically done with specific types of tissues; inverse pipetting and the pipette brand are factors inducing much variation, as long as one mode of pipetting of the samples is consistently used during the assays. The use of a single pipette versus using a multichannel pipette may influence the time needed to fill an entire plate and can be important when incubation time is short, such as for Aβ42. Use of a single tip can influence the standard curve accuracy. For preparation of standards a new tip for each concentration is required. During pipetting in the plate, multiple tips are recommended. However, the magnitude of this effect, if any, should be tested, to provide a better basis for recommendation.

Incubation temperatures, incubation in the dark, and shaking were other items that varied between the laboratories. Room temperature can vary from 18°C in winter to 30°C in summer time. Whether this variation in incubation conditions is relevant for the current tests is not known but likely. This should be tested, and explicit information regarding influence of shaking and temperature requirements should be provided in assay protocols.

Regarding exclusion of the 1500 pg/mL standard in the curve, this is considered highly relevant. We did a recalculation of the pools for one of the groups on a curve fitting without 1500 pg/mL standard and the outcomes differed by 7.6%, 9.4%, and 7.2% for pool 1, 2 and 3 from the original data. This is therefore seen as an important additional source of variation.

Washing procedures (number of washing steps, volume, purity of washing solutions) are important issues influencing variation and background. This can lead to removing antibody-antigen complexes from the plate when washing too much, as well as insufficient removal of unbound complexes, thereby causing high background. Therefore, our recommendation is to adhere to the protocols from the manufacturers. Furthermore, incomplete dissolution of crystals from the washing buffer may lead to aberrant buffer concentration when only part of the buffer is used.

The difference in volume of the stop-solution H_2SO_4 probably does not influence the outcomes, as long as it is performed for all samples in one test similarly, as the pH of the reaction mixture is only marginally influenced by variation in the volume of the stop solution.

Sample handling (storage at 4°C or repeated freezing) can be very important as well, specifically for instable proteins. For the current proteins, Aβ42, Tau, and pTau, a previous study has shown that repeated freezing and storage at 4°C do not have a significant effect on tau concentrations while Aβ42 concentrations may be reduced due to storage for a few days at 4°C [7]. Results from another study, however, reported no effect of storage temperatures on Aβ42 concentrations (Blennow, personal communication). Closing the vial is important to avoid losing the contents upon accidentally falling, and to avoid contamination.

5.2. Inter- and Intra-Assay Variation in the Current Study. For scientific purposes, ideally, the influence of each of the procedures listed in Table 1 on the intra- and interassay variation is systematically tested. The current study excluded several potential sources of error, indicated in the last column of Table 1, such as differences in incubation temperatures, washing, variation in standardised curve fitting, and lot-to-lot variation. The inter-assay variation in results obtained during the workshop was below the limits for intra-assay variation for all assays, with a few exceptions (Table 2), suggesting that standardisation indeed leads to reduced inter-assay variation. Variation for tau assays was on a whole lower than that for Aβ42. This may be caused by increased experience as Aβ42 assay was started with during the workshop and the participants may have needed some time to get used to the laboratory. Alternatively, the observed variation may be the normal variation for these assays, as these differences were similar to what has been reported before in [6] and what we observe in our own laboratory.

Table 3: The effect of using multiple assays of a single lot.

		>6 lots		1 single lot	
$A\beta_{1\text{-}42}$	Concentration (pg/mL)	465	888	496	1003
	% CV	13.9	12.1	7.6	8.4
	n	37	37	17	17
Tau	Concentration (pg/mL)	622	193	621	181
	% CV	9.5	10.5	7.1	7.1
	n	31	31	19	19
$P\text{-}Tau_{181P}$	Concentration (pg/mL)	123	38	135	41
	% CV	7.7	10.7	5.7	9.9
	n	30	30	18	18

n: number of determinations, which was performed within a total period of four years for the left two columns (multiple lots), and within a period of 1.5 years for the single lots.

The importance of lot-to-lot variation is stressed by the results in Table 3, showing the reduction of inter-assay variation in our laboratory when we started using multiple assays from the same batch, purchased at once.

Analytical variation for the current tests has so far been published sparsely. The variation could be reduced if for instance clear quality control criteria and WHO-approved standards would be available. The issue of quality control is currently addressed in a large multicenter study, and our initiative has been adopted by the Alzheimers Association. That study will among others lead to established reference values.

The issue of preanalytical variation is not addressed in the current study. Standardisation of CSF collection and biobanking procedures would be another strategy to tackle pre-analytical variation. We recently published such guidelines for standardised CSF collection and biobanking protocols, that was based on a broad consensus between multiple centers [8]. Adherence to these guidelines will reduce variation induced by pre-analytical factors as well and increase the quality of studies aimed at discovery and validation of novel biomarkers.

In conclusion, the evaluation of the workshop showed that even under standardised conditions as in this workshop, with the same protocol and laboratory circumstances, inter-assay variation is comparable to intra-assay variation and is acceptable for the large majority of groups. The influence of several items that varied between the labs should be studied and protocols should be adapted accordingly. Provision of information on the influence of specific items in the data sheets might be needed to convince the users of this requirement. Lastly, it is required that protocols are strictly followed. Reduction in variation is most critical for CSF concentrations around the cutoff points especially if these guide decision making in individual patients. Efforts for standardisation and the establishment of international reference laboratories and reference values will ultimately increase the reliability of the assays. This will provide a basis to include these biomarkers assays more prominently in the diagnostic workup of Alzheimer's disease.

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Research Article

Confounding Factors Influencing Amyloid Beta Concentration in Cerebrospinal Fluid

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Background. Patients afflicted with Alzheimer's disease (AD) exhibit a decrease in the cerebrospinal fluid (CSF) concentration of the 42 amino acid form of β-amyloid ($A\beta_{42}$). However, a high discrepancy between different centers in measured $A\beta_{42}$ levels reduces the utility of this biomarker as a diagnostic tool and in monitoring the effect of disease modifying drugs. Preanalytical and analytical confounding factors were examined with respect to their effect on the measured $A\beta_{42}$ level. *Methods*. Aliquots of CSF samples were either treated differently prior to $A\beta_{42}$ measurement or analyzed using different commercially available xMAP or ELISA assays. *Results*. Confounding factors affecting CSF $A\beta_{42}$ levels were storage in different types of test tubes, dilution with detergent-containing buffer, plasma contamination, heat treatment, and the origin of the immunoassays used for quantification. *Conclusion*. In order to conduct multicenter studies, a standardized protocol to minimize preanalytical and analytical confounding factors is warranted.

1. Introduction

By the year of 2000, it was estimated that more than 25 million people suffered from dementia, with Alzheimer's disease (AD) being the most common subtype accounting for around 50 percent of all cases [1, 2]. Histopathological hallmarks of AD include intracellular neurofibrillary tangles composed of tau protein and extracellular deposits of neurotoxic β-amyloid ($A\beta$) visualized as amyloid plaques [3–5]. The cerebrospinal fluid (CSF) concentrations of $A\beta$ peptides in combination with the tau protein and its hyperphosphorylated forms have been found to support the clinical diagnosis of AD [6]. Not only do these biomarkers fulfill the criteria for an ideal diagnostic test according to the guidelines of the Working group on molecular and biochemical markers of Alzheimer's disease [7], but $A\beta$ have also been suggested to be a driving force in the disease process. The amyloid cascade hypothesis proposes that an imbalance in $A\beta$ production and clearance leads to an increase in $A\beta$ load and that this initiates taupathology and neuronal degeneration which ultimately causes dementia [8]. The hypothesis is derived from cases affected by rare familial forms of AD wherein mutations in the amyloid precursor protein (*APP*) gene or in the presenilin-encoding (*PSEN1* and *PSEN2)* genes, which are involved in metabolizing the APP protein, invariably lead to AD pathology. The 42 amino

acid long form of Aβ (Aβ_{42}) has also proven to be the best established CSF biomarker for amyloid pathology in the brain. Aβ has therefore become the primary target of many clinical trials in their search for novel treatment strategies as well as a core biomarker candidate for monitoring disease-modifying effects [9].

Recently a large multicenter study assessed the diagnostic value of the 42 amino acid long form of Aβ (Aβ_{42}), total tau (T-tau), and tau phosphorylated at threonine 181 (P-tau$_{181}$) in identifying subjects with incipient AD among patients with mild cognitive impairment (MCI) and they were found to provide good accuracy [10]. The neuropathologic correlates distinguishing those of the MCI patients thought to await in the precedent stage of clinical overt AD [11], are seemingly reflected by these biomarkers. Although the biomarkers show reasonable accuracy to discriminate controls from AD patients as well as prodromal AD in MCI patients [12–15], it has been shown in population-based studies that healthy elderly people who later develop AD have reductions in CSF Aβ_{42} levels while tau levels are normal [16, 17]. However, there is a high discrepancy in the reported concentrations of these biomarkers [18] leading to different cut-off values between different centers with the highest variability shown for Aβ_{42} [10]. This type of between-center variability in analytical results may be due to differences in preanalytical procedures for CSF collection and sample processing, analytical procedures and techniques, and to batch-to-batch variation for the immunoassay kits. It has been suggested that preanalytical confounding factors such as CSF collection, storage, and adsorption to tube-walls contribute to the highest magnitude of errors [19]. This paper aims at assessing these preanalytical confounding factors together with other factors such as blood contamination, blood-brain barrier dysfunction, sample pretreatment and differences in assay performance regarding the impact on measured Aβ_{42} levels.

2. Material and Methods

2.1. CSF and Plasma Samples. All CSF samples were obtained by lumbar puncture (LP) between the L3/L4 and L4/L5 intervertebral space. Except when otherwise noted, a volume of 10–12 mL CSF was collected in polypropylene tubes followed by centrifugation (2000 × g, 10 min, 4°C) and storage in smaller aliquots at −80°C. Plasma was obtained by the centrifugation (2 500 × g, 10 min, 4°C) of whole blood in EDTA tubes (BD, art. nr. 367864). The plasma was aliquoted into polypropylene tubes and stored at −80°C pending analysis. All samples were thawed at room temperature (RT), if nothing else is declared. The samples used for evaluation of confounding factors were aliquots from samples sent for routine diagnostic purposes. All samples were decoded so that no information could be linked to an individual patient.

2.2. Subjects. Two case control studies assessed the differences in CSF Aβ_{42} levels due to differences in pretreatment, the first study comprised 15 AD and 15 control samples and the second comprised 20 AD and 20 control samples. The patients who received the diagnosis of AD fulfilled the DSM-III-R criteria of dementia [20] and the criteria of probable AD defined by NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders—Stroke/Alzheimer's Disease and Related Disorders Association) [21]. Healthy controls were mainly recruited from senior citizens' organizations, while a few were spouses of study patients. Controls were not included if they had a history or subjective or objective signs of a cognitive disorder.

The study was conducted according to the provisions of the Helsinki Declaration and was approved by the ethics committee of the Universities of Gothenburg and Lund and the Karolinska Institute, Sweden.

2.3. CSF Analysis. Unless otherwise stated, the CSF Aβ_{42} concentrations were obtained using the Innogenetics NV INNO-BIA xMAP technology (INNO-BIA AlzBio3) [22]. For practical reasons some tests were analyzed using the established Innogenetics enzyme-linked immunosorbent assay (ELISA) (INNOTEST β-amyloid$_{1–42}$) [23], using a slightly modified protocol [24], which has previously been shown to correlate well with the Innogenetics AlzBio3 assay [22]. Since preanalytical factors were to be assessed and not absolute Aβ_{42} levels, using different assays should pose no problems. In a case control study, these assays were compared with four other commercially available Aβ_{42} immunoassays from Innogenetics (INNO-BIA plasma Aβ forms) [25], Meso-Scale Discovery (MSD 96-Well MULTI-SPOT, Human/Rodent (4G8) Abeta Triplex Ultra-Sensitive Assay) [26], and The Genetics Company (hAmyloid β42 ELISA) [27, 28], to evaluate their performance in discriminating between AD patients and healthy controls. All analyses were performed according to manufacturers' instructions; however, the Innogenetics ELISA was also performed by replacing the detection antibody with the 4G8 monoclonal antibody. The capture and detection antibodies for each assay are summarized in Table 1. Whenever practically possible the samples for a specific experiment were run on the same plate in order to eliminate errors caused by interassay variability.

2.4. Statistical Analysis. Since several variables were found to be skewed the nonparametric Friedman's or Wilcoxon tests were used for pairwise comparisons while the Mann-Whitney U-test was employed for unpaired comparisons. The data is presented as median and percentiles (5th and 95th). Correlation analyses were performed using the Spearman correlation coefficient (rho). Receiver operating characteristic (ROC) analyses were performed to evaluate the discrimination power of the different assays using the area under the curve (AUC). SPSS 15.0 was employed for all univariate analyses.

2.5. Sample Collection and Assessment of Confounding Factors

2.5.1. Adsorption to Test Tubes and Lumbar Catheter Walls. Freshly collected CSF from ten different subjects was tapped into glass (Schott, art. nr. 2317103), polypropylene (Sarstedt, art. nr. 60.549), and polystyrene (Sarstedt, art. nr. 55.476)

Table 1: Properties of evaluated commercially available Aβ assays. The various capture and detection antibodies employed in the different Aβ_{42} immunoassays. The differences in antibody epitope recognition and the part of CSF diluted in buffer during the incubation render methodological differences both qualitatively and quantitatively. aa, amino acids.

	Innotest Elisa	Innotest Elisa (4G8)	The genetics	INNO-BIAAlzBio3	INNO-BIAAβ forms	MSD Triplex
Capture (epitope)	21F12 (42 C-terminal)	21F12 (42 C-terminal)	W02 (aa 5–8)	4D7A3 (42 C-terminal)	21F12 (42 C-terminal)	Not declared (42 C-terminal)
Detection (epitope)	3D6 (aa 1–5)	4G8 (aa 17–24)	G2-13 (42 C-terminal)	3D6 (aa 1–5)	3D6 (aa 1–5)	4G8 (aa 17–24)
CSF (% v/v)	25	25	50	75	75	50
Aβ	1–42	x-42	x-42	1–42	1–42	x-42

tubes. The tubes were incubated at RT for one hour on a Boule mixer and thereafter stored in a freezer (−80°C) pending analysis.

Six samples of CSF were divided into three aliquots of which one was run through a lumbar catheter (Braun-Perifix, art. nr. 4513150), one was run through a lumbar pressure meter catheter (Mediplast, art. nr. 6061650008), while the third aliquot was analyzed without any pretreatment.

2.5.2. Incubation, Storage, and Collection of CSF. To test whether Aβ_{42} is sensitive to handling at RT, eight freshly collected samples, within 3 hours after withdrawal, were divided into two aliquots. The first aliquot was analyzed immediately, while the other was analyzed after 24 hours of incubation at RT. Six control samples were set on each plate to adjust the levels according to the interassay variability.

In order to test if Aβ_{42} is sensitive to freezing eight samples of CSF were collected freshly and divided into two aliquots. The first aliquot was analyzed immediately while the second was stored at −80°C for at least one week pending analysis. Two control samples, each analyzed in two duplicates, were used to adjust for interassay variability. The samples were analyzed using Innogenetics ELISA.

Data on the long-term storage stability of Aβ_{42} in CSF at −80°C were retrieved from repeated analyses ($N = 214$) of an internal quality control sample (aliquots of a large CSF pool) during 26 months. The control sample was analyzed in duplicates at each occasion using the Innogenetics ELISA.

To test for the impact of incubation at RT in combination with freezing, twelve samples of CSF were collected freshly and divided into seven aliquots of which one was immediately frozen. The remaining six aliquots were stored at either +4°C or RT for four hours, 24 hours or three days and thereafter frozen pending simultaneous analysis using Innogenetics ELISA.

To test for the sensitivity of different processes of freezing, different freezing temperatures, and different thawing conditions freshly collected CSF from ten subjects was divided into eight aliquots and analyzed. Two aliquots were tested for the process of freezing; one was frozen on dry ice/ethanol and thereafter stored at −80°C for three days while the other was immediately stored at −80°C. Three aliquots were frozen at −20°C at stable temperature, −20°C in an auto-defrosting freezer, or −80°C for three days. Three aliquots were stored at −80°C for three days and thereafter tested for thawing in a fridge (+4°C), at RT and in a water bath at +20°C.

To test whether the initial LP conditions influence the biomarker level, freshly collected CSF from nine subjects was either collected at RT or in tubes placed on ice. The samples were left to incubate for three hours and thereafter frozen at −80°C.

2.5.3. Aβ Spinal Chord Gradient. The Aβ_{42} CSF spinal chord gradient was assessed by sequentially withdrawing four fractions of 10 mL CSF from seven patients; fraction 1 (0–10 mL), 2 (11–20 mL), 3 (21–30 mL) and 4 (31–40 mL).

2.5.4. Contamination of Blood and Blood-Brain Barrier Dysfunction. To examine if blood contamination influence CSF biomarkers a blood sample was diluted by water and freeze-thawn in dry ice/ethanol in order to lyse erythrocytes. Serially diluted blood equivalent to an erythrocyte level of 200, 1000 and 5000/μL (0.004–0.1% v/v) was then added to 10 different CSF samples (95% v/v) and compared with the corresponding water-diluted CSF (95% v/v). The samples were analyzed using Innogenetics ELISA.

To test the influence of blood-brain barrier (BBB) dysfunction on CSF biomarkers a plasma sample was serially diluted by water to an albumin level corresponding of approximately 0.25, 0.50, 1.0 and 2.0 g/L (0.625–5% v/v) when added to 8 CSF samples (90% v/v) with an albumin level of approximately 0.20 g/L. The samples were compared with corresponding water-diluted CSF (90% v/v). This series represents different degrees of BBB permeability with a CSF/serum albumin ratio of 5, 11, 18, 30 and 55, respectively (5 equals the CSF baseline level ((0.20 g/L / 40 g/L (serum albumin)) × 1000)). The samples were analyzed using the Innogenetics ELISA.

2.5.5. The Influence of an Overnight Fast on Plasma Aβ_{42} Levels. Blood was withdrawn at three different occasions from nine cognitively healthy subjects to test whether fasting or subsequent food intake influences the plasma baseline Aβ_{42} levels. Plasma was preferred as opposed to CSF due to the inconvenience of repeated fluid sampling by LP on healthy subjects. The baseline blood sample was withdrawn

at nine o'clock in the morning following a nonstandardized breakfast, the follow-up sample after three weeks at the same time in the morning following an overnight fast and the postprandial sample one hour after a standardized breakfast the same day. The samples were analyzed using Innogenetics INNO-BIA plasma Aβ forms.

2.5.6. Diurnal Variability. CSF from 14 psychiatrically and neurologically healthy subjects undergoing knee prothesis surgery was serially collected by LP with an 18-Gauge Portex epidural needle at baseline, after four to six hours (mean 5.3 hours) and after 24 hours, for further details on this procedure see Anckarsäter et al. [29]. The samples were immediately stored at −80°C pending analysis.

2.5.7. Centrifugation and Heat Denaturation. CSF from ten subjects was divided into three aliquots each of which one was analyzed without any pretreatment. The other two aliquots were centrifuged for ten minutes (2000 × g), at RT or at +4°C to evaluate the effect of Aβ precipitation during centrifugation. Furthermore, 18 samples of CSF were divided into two aliquots of which one was boiled at 100°C in a heating block for ten minutes. Both samples were thereafter centrifuged at +4°C for ten minutes (2000 × g).

CSF from 15 AD patients and 15 controls were divided into two aliquots of which one was preincubated for 15 minutes at 100°C in a heating block. Both aliquots were centrifuged (2000 × g) at +4°C for ten minutes and analyzed using the Innogenetics ELISA.

2.5.8. Sample Pretreatment Affecting Assay Analyses. A fresh CSF sample was used to assess the impact of incubation at RT on changes in pH. The baseline pH was measured within 30 minutes from LP.

A CSF pool was serially diluted (50%, 25%, 6.25% and 3.125% v/v) in different buffers as well as in the provided assay buffer. Different buffer concentrations (10, 50 and 100 mM phosphate buffered saline (PBS) (pH 7.4)), and different buffer substances (PBS, Tris and HEPES (pH 7.4)), were tested for the effect on the measured Aβ_{42} concentration. Furthermore, the effect on assay performance at different pH was evaluated in 50 mM Tris (pH 7.4, 8, and 9). The addition of 0.1% v/v Tween 20, 0.05% v/v Triton X100 and 0.5 mg/mL BSA to 50 mM PBS (pH7.4) was used to further assess the improvement of Aβ_{42} detection.

2.5.9. Epitope Masking and/or Assay Specific Variability. Divergences in measured Aβ concentration levels between different commercially available Aβ_{42} assays were evaluated and the assays were compared with respect to how well they perform in discriminating between AD patients and healthy controls. CSF from patients with AD (n = 20) and healthy controls (n = 20) was analyzed using assays from Innogenetics (ELISA, Abeta forms, and AlzBio3), Meso-Scale Discovery (Triplex), and The Genetics (ELISA). The commercial Innogenetics ELISA was also tested by replacing the detection antibody with the monoclonal antibody 4G8. Neat and threefold diluted (0.05% Tween 20 in PBS) CSF samples were analyzed according to the instructions from the manufacturers.

3. Results and Discussion

3.1. Adsorption to Test Tubes and Lumbar Catheter Walls. The Aβ_{42} levels were significantly altered by storage in different test tubes. The Aβ_{42} levels significantly decreased when CSF was stored in polystyrene tubes (208 ng/L (126–467)) compared with polypropylene (271 ng/L (152–478)), as previously shown by others [23, 30, 31], rendering a significantly decreased Aβ_{42} level of as much as 35% (mean decrease 19%; P = .002). The Aβ_{42} level also decreased when CSF was stored in glass tubes compared with polypropylene, however it did not reach statistical significance. Some of the previously reported differences in absolute values of Aβ_{42} might thus be due to adsorption to different test tubes probably caused by the hydrophobic nature of this analyte. In consequence, standardization of collection tubes is necessary in order to be able to compare absolute concentration values among different centers.

Adhesion of Aβ_{42} to the lumbar catheter walls during LP might also render a difference in analyte concentration and would urge for standardization. However, two different catheters were tested of which none significantly altered the concentration of Aβ_{42} as compared with the baseline level.

3.2. Incubation, Storage and Collection of CSF. It is essential for reliable biochemical analysis that the stability of a biomarker is thoroughly investigated in order to implement the appropriate preanalytical handling. Eight CSF samples were analyzed within three hours from withdrawal and after 24 hours of incubation at RT. No significant difference between the paired samples was detected in the Aβ_{42} levels suggesting that this biomarker is stable when left for at least a day at RT. Furthermore, no significant alteration in the level of Aβ_{42}, as previously described [32], was found between fresh CSF and CSF that had undergone one freeze/thaw cycle. Nor were there any significant differences between the baseline Aβ_{42} levels of freshly frozen samples and samples frozen after incubation at RT or at +4°C for four hours, 24 hours or three days. These results are in contrast to one study, which found Aβ_{42} to be decrease by 20% after two days incubation at RT, while no difference was found when comparing fresh CSF to frozen/thawed CSF [33]. However, the fresh CSF had been incubated at RT during two days, which would mean that the level of Aβ_{42} implemented as a baseline value was decreased and thus also the Aβ_{42} level in the frozen CSF. Furthermore, the study had a very limited sample size which may contribute to the divergent results. Another study showed, contradictory to ours, that the Aβ_{42} concentration was increased after 24 hours of incubation at RT [34]. However, the CSF was not centrifuged prior to incubation which seems to affect the outcome, see below.

The storage stability of CSF Aβ_{42} at −80°C was assessed through an internal quality control sample which was analyzed on a weekly basis during a time period of 26 months. The coefficient of variation (CV) on 214 different

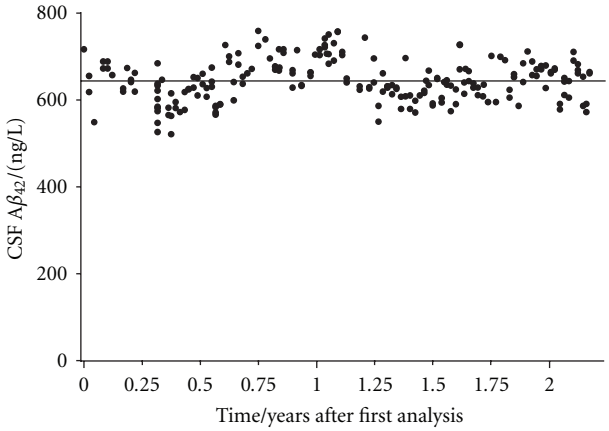

FIGURE 1: The mean $A\beta_{42}$ variation in CSF during 26 months. An internal quality control sample was run on 214 different occasions rendering a mean ± SD value (—) of 643 ± 48 ng/L and a CV of 7.5%.

runs was 7.5%, which is less than the inter assay CV (7.7%) reported by the manufacturer (Innogenetics NV) and consequently signify the storage stability of $A\beta_{42}$ during the accounted time period (Figure 1). The storage stability of $A\beta_{42}$ in CSF during shorter time periods has previously been reported further supporting this finding [35].

No significant changes accounting for larger differences than the intraindividual assay CV (3.8%) was seen for neither of the various routes for freezing, the different storage temperatures, nor the diverse thawing procedures. Moreover, no difference in $A\beta_{42}$ concentration was found when comparing CSF collected and incubated in tubes placed on ice during the LP procedure compared with CSF collected and incubated at RT. This is an indication of the $A\beta_{42}$ stability and supports the use of this peptide as a CSF biomarker. However, other proteins may be sensitive to storage at −20°C. Storage at −20°C causes, for instance, a truncation in Cystatin C revealed by a peptide artifact identified by gel electrophoresis and mass spectrometry [36, 37]. Therefore, it is recommendable to store CSF samples at −80°C as a precaution to possible future analyses.

3.3. Aβ Spinal Chord Gradient. Since CSF proteins originating from brain cells may have a decreasing rostro-caudal concentration gradient, while proteins released from the leptomeninges and blood derived proteins have a lower ventricular than lumbar CSF concentration, withdrawal of different CSF volumes might affect the outcome of biochemical analysis [38]. By withdrawing a small volume of CSF the biochemical composition might only reflect that of the lumbar dural sac and the withdrawal of a too large volume might influence the analysis as to increase the concentration of a brain specific protein. No spinal chord gradient was detected for $A\beta_{42}$ when successively withdrawing four 10 mL portions CSF; that is, the four portions did not significantly differ in their $A\beta_{42}$ level. Although there was no gradient for CSF $A\beta_{42}$ along the spinal chord, it is still recommended to take a standardized volume of CSF at LP since other proteins such as albumin [39] and especially neurotransmitters [40] will be affected.

3.4. Contamination of Blood and Blood-Brain Barrier Dysfunction. It is not uncommon that the CSF gets contaminated by blood during the LP procedure [41]. Since the concentration of proteins in CSF is about 0.5% that of blood [42] only a minor leakage could lead to an altered biomarker profile. Furthermore, blood contamination of CSF could lead to an increase in protein degradation already visible after 6 hours of incubation [43]. Therefore, it is important that contaminated CSF is discarded and that CSF is centrifuged as soon as possible after LP to get rid of contaminants invisible to the eye. Consequently, the addition of 0.1%, 0.02% and 0.004% (corresponding to 5000, 1000 and 200 erythrocytes/μL) of blood to CSF should provide reliable information on the impact of contamination, unnoticeable to the eye, on the $A\beta_{42}$ levels. However, no significant changes accounting for larger differences than the intraindividual assay CV was seen when comparing CSF contaminated with blood to neat CSF (Figure 2(a)).

Neat CSF was compared with CSF with added plasma, corresponding to a CSF/serum albumin ratio of 5, 11, 18, 30 and 55, (i.e., a range from normal to pathological blood-CSF barrier function), and the $A\beta_{42}$ concentration in the diluted CSF was significantly ($P = .008$) decreased by as much as 49% (228 ng/L (165–378)) compared with the neat CSF $A\beta_{42}$ concentration (433 ng/L (291–851)) (Figure 2(b)). One explanation to the decrease might be a high concentration of several proteins that bind $A\beta$ in plasma, such as albumin [44], α2-macroglobulin [45] and low-density receptor related protein-1 [46], and it might explain the fact that numerous studies have found no correlation between CSF and plasma levels of $A\beta$ biomarkers [32, 47], for review see [48]. It may be important to consider the albumin ratio when evaluating the concentration of $A\beta_{42}$ in CSF in disorders with severe impairment of the BBB, such as acute meningitis [49], due to the impact of plasma on the measured CSF $A\beta$ levels.

3.5. The Influence of an Overnight Fast on Plasma $A\beta_{42}$ Levels. Even though the absolute CSF $A\beta_{42}$ values have diverged among different centers the decreased $A\beta_{42}$ levels in AD compared with controls have been consistent. The possible influence of an overnight fast or food intake on $A\beta_{42}$ levels has been brought forward mainly due to inconsistencies in studies concerning the plasma levels of $A\beta$ [48]. However, in this study there was no significant difference between the baseline plasma $A\beta_{42}$ level compared with either fasting or postprandial levels. Furthermore, it would thus seem unlikely that the CSF $A\beta$ levels would be affected when the plasma levels were not.

3.6. Diurnal Variability. Diurnal variability in CSF $A\beta$ levels would give cause for a standardized sampling time for everyday clinical routine. In a previous study, wherein 6 mL of CSF was withdrawn each hour, it was shown that $A\beta$ had a large diurnal variability [50]. During a time period of

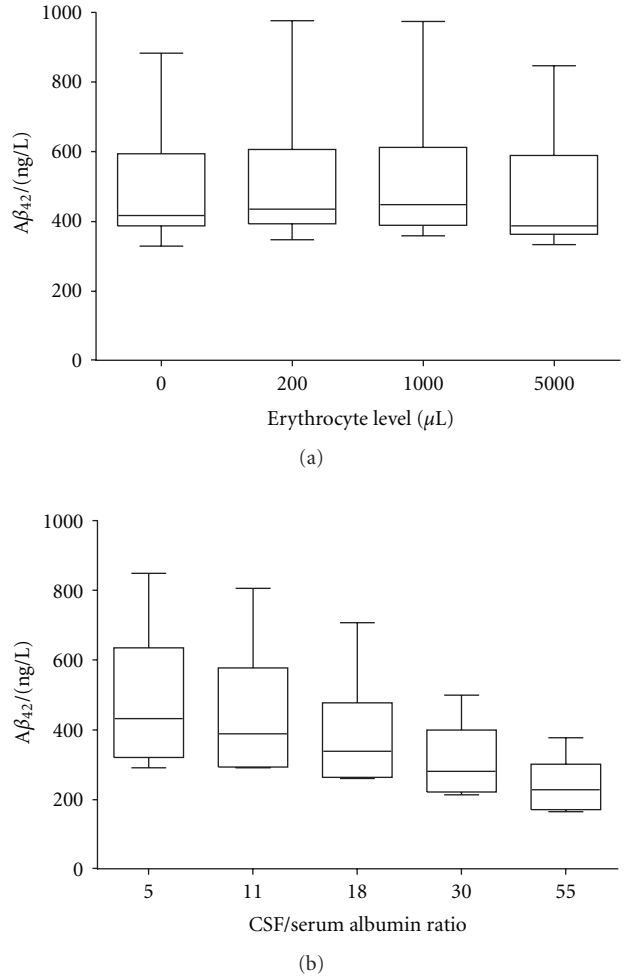

Figure 2: Boxplots with whiskers representing minutes and max values. (a) The effect of blood contamination on CSF Aβ_{42} levels. The addition of blood to CSF representing an erythrocyte level of 200, 1000 and 5000 /μL of blood did not affect the Aβ_{42} level compared with neat CSF. (b) The effect a blood-brain barrier dysfunction on Aβ_{42} levels. Plasma was added to CSF at a concentration representing a CSF/serum albumin ratio of 11, 18, 30 and 55. The Aβ_{42} concentration was significantly decreased ($P < .01$) at all added plasma concentrations.

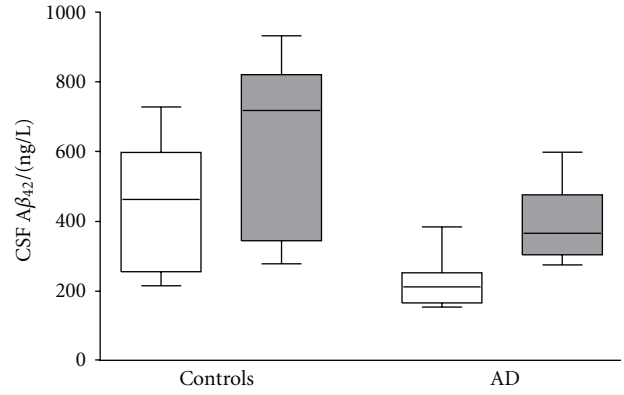

Figure 3: Boxplot (whiskers represent minutes and max values) of measured concentrations of Aβ_{42} in untreated and heat denatured CSF. The white boxes represent untreated CSF and dark boxes represent heat denatured CSF. Each box represents 15 samples (15 AD or 15 controls).

36 hours, the Aβ levels peaked at 12 hours and 23 hours with troughs at baseline and 25 hours with significant fluctuations of more than 50% within 6 hours. However, no complete return to baseline values was seen for Aβ. Our data showed more stable levels with a slight but significant decrease of 9.3% ($P < .001$) in CSF Aβ_{42} after 4–6 hours (mean 5.3 hours), which tended to return to baseline levels after 24 hours (4.4% lower than baseline; $P = .002$).

In this study, our attempt was to reflect the variation in CSF withdrawal time that might be a reality in some clinical settings. One explanation to the difference between our results and the study by Bateman et al. [50] might be that a smaller CSF volume was taken, which may cause less effect on the CSF dynamics. Even though the CSF Aβ_{42} level does not seem to be influenced by circadian rhythms to any greater extent, other analytes might be which would support a standardized time interval during the day for CSF withdrawal.

3.7. Centrifugation and Heat Denaturation. Ten samples were divided into three aliquots in order to test for precipitation during centrifugation, with and without cooling during the process. There was a significant ($P = .002$) decrease in the concentration of Aβ_{42} in both of the centrifuged samples (RT 203 ng/L (138–340); +4°C 203 ng/L (139–341)) when compared with the noncentrifuged samples (228 ng/L (147–354)). This indicates that a portion of Aβ_{42} in CSF might originate from cells that have undergone lysis that would precipitate together with the cells during the process of centrifugation. Furthermore, the Aβ fraction accessible to the antibody was further addressed by exposing CSF to heat denaturation. Herein, 18 CSF samples were divided into one heat exposed aliquot versus one unexposed aliquot and both were submitted to centrifugation prior to analysis. The Aβ_{42} concentration increased significantly ($P < .001$) in the heat treated samples (278 ng/L (170–471)) as compared with the untreated samples (210 ng/L (127–357)). This result was replicated in a case control study were the increase of Aβ_{42} was larger in the AD patient group (71%, $P < .001$) compared with the control group (42%, $P < .001$) (Figure 3). Consequently, the ROC analysis revealed a decreased discriminating power between AD and controls after heat treatment (AUC = 0.796) compared with the untreated samples (AUC = 0.907). The correlation for both test samples was high ($r_s > 0.8$, $P < .001$), when comparing untreated versus treated samples, indicating methodological stability.

3.8. Sample Pretreatment Affecting Assay Analysis. Factors known to affect the solubility and stability of proteins were investigated for its cofounding effects during analysis. Different buffer concentrations (10, 50 and 100 mM PBS, pH 7.4) and different buffer substances (PBS, Tris and

HEPES, pH 7.4) did not affect the CSF Aβ_{42} measurement performance compared with the provided assay buffer. The pH of CSF was investigated and found to increase rapidly in RT from a starting value of pH 7.9 and reaching a plateau at pH 8.7 already after five hours. To test if the Aβ_{42} antibody binding capacity is altered due to differences in pH during analysis, which could be a confounding factor if employing a buffer with a low buffer capacity, pooled CSF was tested in three different pH systems (50 mM Tris, pH 7.4, 8 and 9). Compared with the levels of Aβ_{42} obtained in provided assay buffer system, the different pH tested did not alter the Aβ_{42} levels. Furthermore, BSA (0.5 mg/mL) and two different detergents (0.05% Triton100 and 0.1% Tween20) were added to test if the signal of Aβ_{42} could be improved by possibly decreasing the negative effects of protein interaction with the solid surface of the beads and/or the air-liquid interface. The signal was equally improved for all three additives (data not shown) as compared with the assay buffer system and the detergent effect was further assessed, what follows.

3.9. Epitope Masking and/or Assay Specific Variability. One hypothesis for the decreased level of Aβ_{42} in CSF from AD patients is that plaques in the brain act as sinks for Aβ_{42}, preventing it from reaching the CSF. In CSF Aβ may either exist as a free soluble peptide, as oligomers [51], or bound in complex with carrier proteins such as α-2-macroglobulin [52], apolipoprotein E (ApoE) [53], apolipoprotein J (ApoJ; Clusterin) [54, 55], albumin [44], low-density lipoprotein receptor-related protein-1 (LRP) [56], and transthyretin [57]. The APOE ε4 allele is the strongest known genetic risk factor for AD [58, 59]. Carrier proteins such as ApoE are thought to play a part in the Aβ clearance and an altered clearance effect, in this case, is thought to be allele specific due to a decreased binding efficiency between Aβ and ApoE4 as compared with the other isoforms [60].

CSF samples were treated with either detergent or heat denaturation, which has previously been shown to increase the Aβ_{42} measureable level [61, 62], to assess the fraction of possibly bound/epitope masked Aβ_{42} in proportion to the free Aβ_{42} in untreated samples and whether the total amount of Aβ_{42} could further improve the differentiation between AD and controls. The measured concentration of Aβ_{42} increased after the threefold dilution with the detergent containing buffer. The most striking increases were found for the xMAP assays AlzBio3 and Aβ forms. The Aβ_{42} median concentration for the neat CSF samples varied by more than a factor of eight, between the different assays tested (Figure 4(a)). The variation in the median is still present in the diluted samples but markedly reduced to less than a factor of three. To further investigate the result, the correlations between the assays were calculated (Table 2). Almost all tests, both neat and diluted, resulted in a difference in Aβ_{42} concentrations between AD and controls with $P < .005$. Only the ELISA assay from The Genetics failed to reach significance at this level with $P = .086$ and $P = .017$ for neat and diluted CSF, respectively.

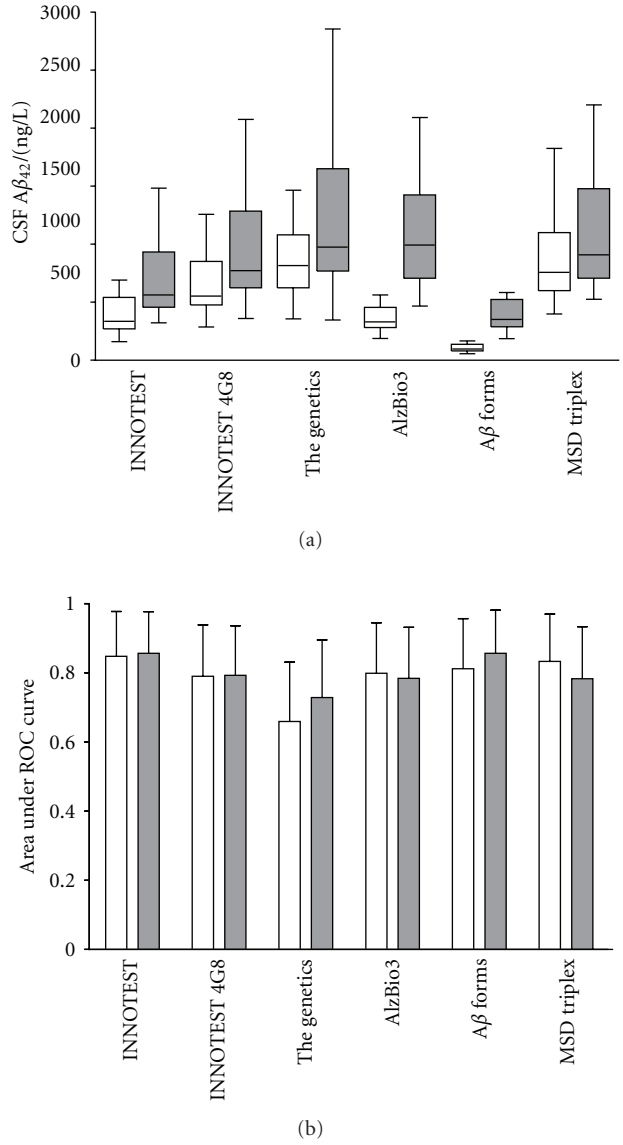

Figure 4: (a) Boxplot (whiskers represent minutes and max values) of CSF Aβ_{42} concentrations using different assays. For each kit the CSF was analyzed twice, neat and threefold diluted in a detergent-containing buffer. Each box represents 40 samples (20 AD + 20 controls). (b) Area under ROC curve for different assays with whiskers representing a 95% confidence interval. Neat, and threefold diluted CSF are represented by white and dark boxes/bars, respectively.

The area under the ROC curve was used as a measure of the discrimination power for the assays (Figure 4(b)). Most of the assays performed equally well in discriminating between AD patients and healthy controls and there were no specific trend in the performance when the CSF was threefold diluted. Even though there are large differences in Aβ_{42} concentration depending on which assay is used most of the correlations between the assays are strong which indicates that the differences in measured concentrations are not due to cross reactivity for other substances than

Table 2: Correlation matrix for the evaluated Aβ assays. The correlation between the levels of Aβ_{42} in different assays. The correlation coefficients for the neat and diluted CSF samples are shown in the upper right and lower left part, respectively.

	Innogenetics ELISA (4G8)	Innogenetics ELISA	The Genetics ELISA	Innogenetics AlzBio3	Innogenetics Aβ forms	MSD Triplex
Innogenetics ELISA (4G8)	1	0.94	0.90	0.87	0.69	0.97
Innogenetics ELISA	0.96	1	0.82	0.93	0.81	0.92
The Genetics ELISA	0.93	0.88	1	0.75	0.53	0.87
Innogenetics AlzBio3	0.92	0.94	0.89	1	0.88	0.84
Innogenetics Aβ forms	0.78	0.86	0.67	0.86	1	0.66
MSD Triplex	0.98	0.96	0.93	0.93	0.77	1

Aβ_{42}. One possible explanation for the variation is that kit manufacturers have different sources for the Aβ_{42} standard that is used for calibration. This result highlights the need for an external Aβ_{42} control program that would allow manufacturers to calibrate their assays towards a common standard. The reason for the increase in measured concentration of Aβ_{42} upon dilution is at present unknown but might involve dissociation of Aβ homo- or heterocomplexes which would liberate more Aβ_{42} that are otherwise masked for detection. If this is true the results from the diluted samples would more truly reflect the total Aβ_{42} concentration, which potentially could be an even better biomarker than the "free" Aβ_{42} measured in the undiluted samples. However, there were no dramatic changes in the discriminating power in the diluted CSF compared with neat samples. Besides, methodological reasons for the increase cannot be excluded since the most dramatic changes are for the two assays based on the *x*MAP technology (Innogenetics' AlzBio3 and Aβ forms). Herein, it is clearly shown that divergences in absolute Aβ_{42} levels between different centers could be explained by the fact that different ELISAs are utilized with different protocols as well as assay methodologies (Table 1). However, when different centers employ the same ELISA from the same manufacturer divergences often still remain [63]. Another factor affecting the Aβ concentration inconsistency might be the result of a lot-to-lot variability [64].

Detergent and heat treatments give rise to a similar increase in the measured level of Aβ_{42} in the AD groups, 75% and 71%, respectively. In contrast, the detergent treated control CSF diverged from the heat denatured by a more pronounced increase (83% versus 42%). Whether the divergences in the increase of Aβ_{42} levels between the two differently treated CSF samples of the controls and the discriminating power between neat/detergent CSF compared with native/denatured samples could be explained by the differences in study sample, methodological reasons or differences in complex stability needs to be addressed by further studies.

4. Conclusion

Due to the high between-center variability (possibly caused by preanalytical and analytical factors) of reported Aβ_{42} levels in CSF, possible confounding factors were assessed in relation to the CSF Aβ_{42} levels. The confounding factors found to influence the preanalytical procedures for CSF collection and sample processing, analytical procedures and techniques ultimately leading to altered Aβ_{42} concentrations are summarized below.

Preanalytical Factors. (i) Increased Aβ_{42} concentration in noncentrifuged CSF samples possibly due to a release of the analyte caused by cell lysis—it is important to centrifuged CSF within a standardized time interval after LP.

(ii) Decreased Aβ_{42} levels due to adsorption of analyte to different types of test tubes—standardization of test tubes used for CSF sampling that is, polypropylene.

(iii) Pretreatment of CSF with detergent-containing buffers or heat denaturation lead to an increase in Aβ_{42} levels—probably due to dissociation of Aβ bound to proteins or release of Aβ from oligomers. For these reasons a standardization of dilution factors, buffer additives and sample processing is necessary prior to analysis.

(iv) The CSF Aβ_{42} concentration decreased at the addition of plasma corresponding to a CSF/serum albumin ratio of 11–55—probably due to the binding of free Aβ to plasma proteins.

Analytical Factors. (i) Different immuno-assays employing various antibodies and possibly dissimilar sources for the calibrator peptides lead to divergences in the absolute Aβ_{42} concentration—between center comparisons cannot be made when employing different assays. This problem cannot be solved until an international Aβ golden standard is available.

Even though the CSF concentration of Aβ_{42} does not seem to be affected by a spinal chord gradient, circadian rhythms, blood contamination or by storage/thawing conditions other proteins may be. It is necessary to use a standardized protocol to allow for between-center comparisons, for a detailed protocol see Blennow et al. [9].

Disclosure

K. Blennow has participated in an advisory board for the Innogenetics. The other authors have nothing to disclose.

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Review Article

cNEUPRO: Novel Biomarkers for Neurodegenerative Diseases

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"clinical NEUroPROteomics of neurodegenerative diseases" (cNEUPRO) is a Specific Targeted Research Project (STREP) within the sixth framework program of the European Commission dedicated to the search for novel biomarker candidates for Alzheimer's disease and other neurodegenerative diseases. The ultimate goal of cNEUPRO is to identify one or more valid biomarker(s) in blood and CSF applicable to support the early and differential diagnosis of dementia disorders. The consortium covers all steps required for the discovery of novel biomarker candidates such as acquisition of high quality CSF and blood samples from relevant patient groups and controls, analysis of body fluids by various methods, and finally assay development and assay validation. Here we report the standardized procedures for diagnosis and preanalytical sample-handling within the project, as well as the status of the ongoing research activities and some first results.

1. Introduction

The diagnosis of Alzheimer's Disease (AD) is currently based primarily on clinical symptoms. Whereas the sensitivity of the clinical diagnosis for possible and probable Alzheimer Dementia according to National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria is over 80%, its specificity is rather low [1]. The term mild cognitive impairment (MCI) was introduced for subjects who complain about verifiable cognitive disturbances but who show a preserved general cognitive functioning and no impairment in the activities of daily living [2]. These patients can be further subdivided into those with an impaired memory function (amnestic MCI) and those whose memory is preserved but who show disturbances of language, executive function, or visual-spatial skills (Nonamnestic MCI) [2]. If only one of the above-mentioned cognitive domains is impaired, patients are called single-domain MCI; if two or more domains are affected, they are referred to as multidomain MCI. Although the term MCI is solely descriptive and allows no conclusion on the aetiology, the classification allows some prediction of the course of the disease. For amnestic MCI patients, the risk to convert to Alzheimer's Dementia is 10–15% per year [3]. Yet, an accurate early diagnosis in MCI patients or even a predictive diagnosis in individuals without cognitive disturbances is still virtually impossible. As there is evidence that pathological biochemical changes start many years before the occurrence of functional symptoms, identification of biological markers in individuals with early-stage dementia is the most promising way to facilitate a predictive diagnosis [4–6].

Improving the early and predictive diagnosis of AD is of paramount importance if, in the future, preventive and disease-modifying therapies become available. In this regard, enormous efforts are under way. Although most therapies failed to show efficacy in Phase III trials, there are still some promising approaches like Aβ lowering compounds, inhibitors of inflammation, inhibitors of tau phosphorylation and aggregation, and compounds interfering with cholesterol metabolism under investigation [7]. Although the brain has some limited regenerative capacity, neurons are still difficult to replace [8, 9]. Therefore, it is clear that maximal benefit for the patients can be expected when the treatment can be initiated as early as possible in the course of the disease. Furthermore, biologically valid and clinically accurate biomarkers may serve in the development of novel therapeutic strategies and may provide important information in clinical trials of therapies [10].

Well-documented biomarkers for AD in cerebrospinal fluid (CSF) include alterations in A$\beta_{1\text{-}42}$, total-tau, and phospho-tau [10]. Importantly, these particular changes are detectable in early dementia stages as well as in individuals with mild cognitive impairment (MCI) who are at high risk of conversion to AD [11]. When analyzed in well-characterized clinical samples, the measurement of A$\beta_{1\text{-}42}$, tau, and phospho-tau in cerebrospinal fluid generally allows the diagnosis of AD and even the prediction of the conversion from MCI to AD with a specificity and sensitivity of about 85% [12]. However, some report a lower sensitivity of below 50% for single biomarkers when these biomarkers are measured as part of a routine diagnostic test in a memory clinic [13]. This drop in sensitivity can be explained by the fact that in clinical practice the reference cohort is not a group of cognitively healthy individuals but consists of patients with other neurodegenerative and neurologic diseases who may also have slightly elevated total-tau, phospho-tau, or A$\beta_{1\text{-}42}$ levels [13]. The application of these markers in the differential diagnostic of neurodegenerative diseases therefore proves to be particularly problematic [14]. Consequently, there is a need for additional and more sensitive CSF biomarkers for the early and differential diagnosis of Alzheimer's Disease.

There is the additional problem of lumbar puncture to obtain CSF, since although the rate of complications during and after lumbar puncture is below 2–4% and restricted to mild to moderate postlumbar puncture headache [15–18], it must still be seen as invasive method for which special precautions must be taken. Consequently, there is a pressing need for new biomarkers in more easily accessible body-fluids such as peripheral blood.

Clinical proteomics is a fast developing field dedicated to the search for new biomarkers applicable to support the clinical diagnosis [19]. At present, a number of potential new biomarker-candidates for AD have been reported from proteomic studies [20, 21]; unfortunately, however, the published data is often contradictory and in many cases, a solid reassessment by other methods and with independent samples is required [19].

Taking this into account, the EU-project Clinical Proteomics for Neurodegenerative Diseases (cNEUPRO) is not only dedicated to the detection of potential new biomarker candidates for neurodegenerative diseases in CSF and blood, but also to the implementation of in-depth reassessments and validation studies. Finally, promising biomarker candidates will be studied for their suitability as routine test analytes by prototype assays.

2. cNEUPRO: The Consortium, Goals, and Workflow

cNEUPRO (http://www.cneupro.eu/) is a Specific Targeted Research Project (STREP) within the sixth framework program of the European Commission. It started in April 2007 and is coordinated by Jens Wiltfang, University of Duisburg-Essen. For the general aims of cNEUPRO, (see Box 1). The consortium consists of 14 academic partners (University of Duisburg-Essen, Centre Hospitalier Universitaire de Montpellier, Sahlgrenska Academy at the University of Gothenburg, VU University Medical Center, University of Ulm, University of Newcastle upon Tyne, University of Aveiro, University of Szeged, University of Perugia, Ruhr-University Bochum, Heinrich Heine University of Duesseldorf, University of Eastern Finland Kuopio, Institut de la Santé et de la Recherche Médicale, University of Erlangen) as

The general AIMS of cNEUPRO are:
(i) Detection of new biomarkers for the early, predictive and differential diagnosis of neurodegenerative diseases in CSF and blood
(ii) Development of new diagnostic assays for the early and predictive diagnosis of neurodegenerative diseases in CSF and blood
(iii) Contribution to the standardization of neurochemical dementia diagnosis
(iv) Establishment of two neurochemical dementia diagnosis reference-centers in Hungary and Portugal

Box 1: General aims of cNEUPRO.

well as four small to medium enterprises. (Matrix Advanced Solutions Germany GmbH, MicroDiscovery GmbH, Protagen, BioGenes GmbH).

cNEUPRO integrates almost all different levels of biomarker research: the primary phase involves the comprehensive clinical characterization of patients and standardized sample-acquisition and handling by specialized geriatric psychiatrists and neurologists. These samples are subsequently used in the search for candidate biomarkers, their biochemical identification by mass spectrometry, and their reassessment in a second, independent set of high quality samples. Finally, the identified biomarkers will be integrated into novel prototype assays (Figure 1).

The research within cNEURPO concentrates on individuals diagnosed with MCI at baseline who subsequently either developed AD, other dementias, or who did not progress to dementia. As the samples had been taken at baseline, clinical information obtained during follow-up allows the identification of predictive biomarker candidates retrospectively. In addition, clinical samples from patients with early AD at baseline or other dementias in the early stages are also included in the analysis.

In the search for new biomarker candidates in CSF or blood, hypothesis-free proteomic approaches such as urea-based gel electrophoresis, Multidimensional liquid chromatography, combined with two-dimensional differential gel electrophoresis (2D-DIGE), several mass spectrometric methods (e.g., SELDI-TOF, MALDI-TOF, nanoLC-MALDI-TOF/TOF, nanoLC-ESI, nanoLCQFTMS), and array-based methods are conducted. Additionally, specific and potentially interesting molecules are studied in detail in the sense of "hypothesis-driven approaches". The most promising biomarker candidates will be selected with the aid of biostatistical tools. Where applicable, published information in terms of the biological function or a possible role of selected candidates in the pathophysiology of AD will also be considered. The selected candidates will be reassessed with a further independent high quality clinical sample of age- and sex-matched patients and controls and with assays allowing for intermediate sample throughput and quantitative comparisons. For those biomarker candidates that can be successfully validated, cNEUPRO will devise novel poly- and monoclonal antibodies. Finally the biomarkers will be integrated into novel ELISA-type assays and, if appropriate, in Multiplex-Assays.

An essential prerequisite for a successful multicenter biomarker-discovery study is the standardization of the clinical diagnostics, the preanalytical sample handling procedures, and the measurements of the known biomarkers total-tau, phospho-tau, and A$\beta_{1\text{-}42}$ in CSF. To this end, two neurochemical dementia diagnosis reference centers in Hungary and Portugal are currently being established, and European standard operating procedures for clinical diagnostics and preanalytical sample handling have been defined.

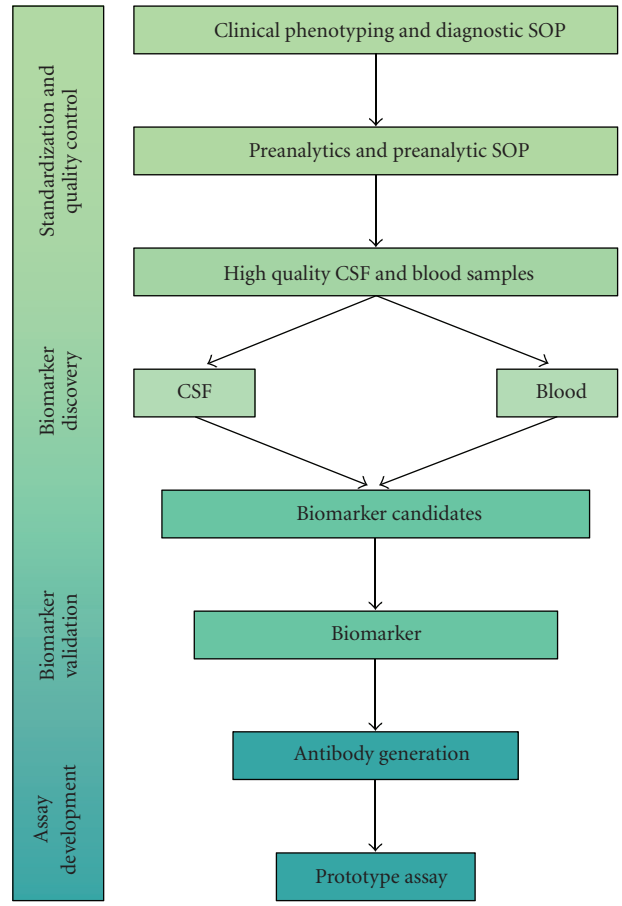

Figure 1: Workflow within the project.

3. Current State and First Results of cNEUPRO

3.1. Neurochemical Dementia Diagnosis-Reference Center in Hungary Launched. In Hungary, 42 Dementia Centers are responsible for the diagnosis and treatment of demented

Diagnostic standard operating procedure
(i) AD is diagnosed according to the NINCDS-ADRDA criteria [27].
(ii) DLB is diagnosed according to the criteria of McKeith [28].
(iii) VaD is diagnosed according to the NINCDS-AIREN criteria [29].
(iv) FTLD is diagnosed according to the consensus criteria of Neary [30].
(v) CJD is diagnosed according to the WHO criteria (Geneva 1998).
(vi) CDR is used for staging of Dementia [31].
(vii) MMSE is used for grading of dementia [32].
(viii) A follow-up of two years must be retrievable.
(ix) Additional neuropsychological testing is desirable but not mandatory.
(x) A CT or MRI scan must be available.
(xi) The ApoE genotype should be determined.
(xii) CSF should be obtained and the concentration of $A\beta_{1\text{-}42}$, tau and phospho-tau should be determined.

Box 2: Diagnostic standard operating procedure. AD = Alzheimer's Disease, DLB = Dementia with Lewy-bodies, VaD = Vascular Dementia, FTLD = frontotemporal lobar degeneration, CJD = Creutzfeldt-Jacob Disease, CDR = Clinical Dementia Rating Scale, MMSE = Mini Mental Status Examination. References in the box: [27–32].

patients. Before 2009, the CSF analysis of $A\beta_{1\text{-}42}$, total-tau, and phospho-tau to support dementia diagnostics was not possible for these centers. As one of the aims of cNEUPRO, the first reference center for neurochemical dementia diagnosis in Hungary was launched in Szeged. With the support of the cNEUPRO consortium, state-of-the-art diagnostic and methodological standards have been implemented, and the center takes part in an ongoing quality control program organized by Kaj Blennow from Sahlgrenska University Hospital, Mölndal, Sweden. During its first twelve months of operation, the neurochemical dementia diagnosis reference center in Szeged has received a total of 54 CSF samples from 14 different Dementia Centers in Hungary. This neurochemical dementia diagnosis center will now try to provide its service to further Dementia Centers in Hungary and to start collecting samples for scientific purposes.

3.2. Diagnostic and Preanalytical Standard Operating Procedures. Due to substantial intercenter variations, the reported accuracy of CSF biomarkers is considerably lower in multicenter studies than in single center surveys [22–24]. To this end, a multicenter study, supported by cNEUPRO, provides guidance on how to establish, validate, and audit CSF tau cutoff values using an unbiased, two-stage multicentre strategy [25]. Furthermore, a hands-on workshop was organized by members of the cNEUPRO consortium (paper submitted to the same issue of IJAD). The aim of the workshop was to assess the differences in assay procedures as potential sources of error. During this workshop, 14 groups simultaneously performed the $A\beta_{1\text{-}42}$, total-tau, and phospho-tau assays according to the guidelines of the manufacturer. At least 23 items in assay procedures were identified that varied between the laboratories, including procedures for washing, pipetting, incubation, finishing, and sample handling. Thus, even if centers use the same assays for $A\beta_{1\text{-}42}$, total-tau, and phospho-tau measurement on a regular basis, they do not uniformly adhere to the procedures recommended by the manufacturer. The results of the workshop stress the importance of standardization of assay protocols. To facilitate biomarker research on a multicenter level, standard operating procedures for the clinical diagnosis and the preanalytical sample handling have been defined by the cNEUPRO consortium (Boxes 2 and 3). The standard operating procedures for sample acquisition, handling, and storage defined by cNEUPRO meet the quality standards required for proteomic studies in CSF [19] and are in agreement with the recently published guidelines for CSF collection and biobanking from the BioMS-eu network [26].

3.3. Investigated CSF Biomarker Candidates for AD Related to Amyloid Precursor Protein (APP) Processing and Tau Pathology. In the last decade, the levels of $A\beta$ peptides and tau proteins in CSF have gained increasing importance in supporting the clinical diagnosis of AD [10, 33]. As no single marker alone allows for a diagnosis with the desired accuracy, several combinations of CSF-biomarkers ($A\beta_{x\text{-}42}$, $A\beta_{x\text{-}40}$, total-tau, phospho-tau) have been proposed [12]. For these markers, a diagnostic accuracy of up to 94% has been achieved in single center studies [12]. Within cNEUPRO, Welge et al. reported a sensitivity and specificity of 88% in the discrimination of AD subjects from other dementias and from elderly depressed individuals with cognitive complaints, by combining the measurement of $A\beta_{1\text{-}40}$, $A\beta_{1\text{-}38}$, and phospho-tau [34]. With the use of MALDI-TOF mass-spectrometry for the study of CSF samples from AD patients, an oxidized form of $A\beta_{1\text{-}40}$ ($A\beta_{1\text{-}40}{}^{ox}$) was identified. Quantification by SDS-PAGE/western immunoblot revealed elevated $A\beta_{1\text{-}40}{}^{ox}$ levels in patients with AD as compared to probable vascular dementia and controls [35]. Taken together, these pilot studies suggest that besides $A\beta_{1\text{-}42}$, additional variants of $A\beta$ peptides may turn out to be specifically altered in AD patients.

Although combinations of these CSF biomarkers were reported to have a high predictive value in single-center studies, their application in multicenter-studies is hampered by relatively high intercenter variations. In an associated multicenter study, including 750 patients with MCI who were

Preanalytical standard operating procedure for CSF:
(i) Cerebrospinal fluid (CSF) is collected by lumbar puncture (LP). Ventricular CSF can also be included but should be clearly labelled as such.
(ii) CSF is collected in polypropylene tubes.
(iii) A standardised volume (10–12 mL) is collected.
(iv) Samples contaminated with more than 500 red blood cells/μL should not be included.
(v) Collected CSF is centrifuged at approximately 1,000–2,500 x g at +4°C or room temperature for 10 minutes within 1 hour after the sampling.
(vi) The supernatant is pipetted off, gently mixed to avoid possible gradient effects and aliquoted in portions in polypropylene tubes.
(vii) The samples are stored at −80°C without having been thawed and re-frozen.

Preanalytical standard operating procedure for serum and plasma:
(i) Serum and plasma are collected by vein puncture.
(ii) Plasma is collected into polypropylene tubes containing EDTA.
(iii) Serum is collected into polypropylene tubes without additives.
(iv) Collected blood samples are centrifuged at approximately 1,000–2,500 x g at +4°C or room temperature for 15 minutes within 2 hour after the sampling.
(v) The supernatant is pipetted off, gently mixed to avoid possible gradient effects and aliquoted in portions in polypropylene tubes.
(vi) The samples are stored at −80°C without having been thawed and re-frozen.

Box 3: Preanalytic standard operating procedures for CSF and blood.

followed for at least two years, the conversion to AD could be predicted with a sensitivity of 83% and a specificity of 72% by the ratio of A$\beta_{1\text{-}42}$/phospho-tau and total-tau. These values are substantially lower than those seen in several single center studies [24]. The highest intercenter variations were reported for A$\beta_{1\text{-}42}$. As this is probably due to its high potential to form aggregates and to stick to test tubes, alternative markers related to APP processing have been investigated within cNEUPRO. In an associated multicenter study, sAPPα and sAPPβ, two proteins secreted in the CSF after the α- or β-secretase cleavage of APP, were assessed in 188 patients with MCI or mild to moderate AD. In previous studies, sAPPα and sAPPβ were found to be unchanged [36, 37] or decreased [38–40] in the CSF of AD patients. Within cNEUPRO, sAPPα and sAPPβ levels in CSF of MCI and AD patients with elevated total-tau and reduced A$\beta_{1\text{-}42}$ CSF concentrations were compared to those from patients without a respective CSF biomarker profile. Both were found to be higher in the CSF from patients with an AD-indicative biomarker profile [41]. Taken together, these results suggest that sAPPα and sAPPβ may be indicators of altered APP expression and/or metabolism. Reports on their value as candidate biomarkers are however so far contradictory.

In a different study which was supported by cNEUPRO, six novel N-terminal APP-fragments with molecular masses of approximately 12 kDa and starting at amino acid 18 of the APP sequence were detected in CSF by mass spectrometry. In a subsequent small pilot study, six of six AD patients and five of five controls could be classified correctly by the combined evaluation of five of the six fragments [42]. Additionally, Immuno-MS analysis of CSF has led to the detection of eleven novel APP fragments, which begin N-terminally to the β-secretase cleavage site, and end one amino acid before the proposed α-secretase cleavage site (APP/Aβ peptides) [43]. Interestingly, seven of the twelve APP/Aβ peptides were significantly upregulated in AD [43].

3.4. CSF-Biomarker Candidates for AD Investigated within cNEUPRO, Which Are Not Related to APP Processing or Tau Pathology. One of several kinases that have been suggested to be involved in the abnormal hyperphosphorylation of tau is the MAP-kinase ERK1/2. In a methodological pilot study, ERK 1/2 and its doubly phosphorylated, activated form have been detected in a small number of CSF samples from patients with AD, MCI, and frontotemporal lobar degeneration (FTLD) [44]. To evaluate the usefulness of ERK 1/2 as a potential novel CSF biomarker, ERK1/2 levels in CSF are currently being studied in a total of 110 CSF samples from partners within the consortium with a chemiluminescent 96 well assay format.

In accordance with a previous report [45], research within cNEUPRO found glial fibrillary acidic protein (GFAP), a marker for astrogliosis, to be increased in CSF of AD and sporadic Creutzfeldt-Jacob Disease (sCJD) patients. CSF samples of 18 AD patients, 22 sCJD cases, and 18 from nondemented controls were analyzed with the use of a commercially available ELISA. In AD, a remarkable elevation in CSF GFAP levels with no overlap to controls was observed. Although a significant increase in GFAP could be observed in CJD as well, this was not as pronounced as in AD [46]. Consequently GFAP might have some additive value as part of a biomarker supported diagnosis, although it lacks specificity for AD.

Chronic inflammation associated with oxidative and nitrosative stress is another aspect which is considered to be important in the pathophysiology of AD [47]. The most common protein markers of oxidative and nitrosative stress are protein-bound carbonyls and 3-nitrotyrosine [48]. An

increased oxidation of certain proteins and an increased concentration of 3-nitrotyrosine have been reported in tissue [49] and CSF [50–52] of AD patients, but there is also contradictory data indicating no difference between AD and controls [53]. In a study conducted by members of the cNEUPRO consortium, where the concentrations of 3-nitrotyrosine and total protein carbonylation were measured, no change was found in CSF of AD patients [48]. Yet, slightly reduced levels of protein carbonyls were detected in ApoE-ε4 carriers as compared to ApoE-ε4 noncarriers [48]. These results suggest that the concentrations of total protein carbonyls and 3-nitrotyrosine are at this stage not suitable to monitor the chronic inflammatory processes related to AD.

3.5. Investigated CSF Biomarkers for Other Neurodegenerative Diseases. In addition to promoting the early and predictive diagnosis of AD, cNEUPRO is also dedicated to search for new biomarkers to support the diagnosis of other neurodegenerative diseases such as sCJD, FTLD, vascular dementia (VaD), Dementia with Lewy bodies (DLB), Parkinson's Disease (PD), and Parkinson's Disease Dementia (PDD).

Two-dimensional differential gel electrophoresis (2D-DIGE) followed by MALDI-TOF mass-spectrometry indicated that CSF from patients with sCJD differed from CSF from patients with other neurological deficits on the basis of several protein spots. Among these, several previously identified surrogate markers of sCJD such as 14-3-3 protein, neuron-specific enolase, and lactate dehydrogenase were identified. Additionally, an unidentified protein of 85 kDa was found to be significantly increased in sCJD patients [54].

In a separate cNEUPRO investigation, SELDI-TOF mass spectrometry was applied in the analysis of CSF from 32 sCJD patients, 32 controls, and 31 patients with other dementias. Ubiquitin, an 8.6 kDa protein involved in protein degradation, was found to be elevated in the CSF of sCJD cases. This could be confirmed by reassessment with western immunoblots. In the study population, the accuracy of a biomarker-based classification of the samples could be significantly improved by including Ubiquitin in addition to tau, and 14-3-3 protein [55]. This finding is in accordance with several previous reports where Ubiquitin was also found to be elevated in the CSF of sCJD patients [56]. As there is also evidence for altered levels of CSF Ubiquitin in AD [57–59] and vascular dementia [60], it seems that this observation is related to neurodegenerative processes in general and not to a specific disease. Yet, in the Steinacker study CSF Ubiquitin levels in sCJD were higher than those in other dementias [55]. Therefore, Ubiquitin may still be a good biomarker for sCJD if, as with tau protein [61], disease-specific cut-off values are applied.

S100B, another astroglial marker, may also be useful to support the diagnosis of sCJD. Within cNEUPRO, S100B was measured in 54 CSF samples from patients with sCJD, AD, and control patients with the use of a commercial ELISA. Supporting previous findings [62, 63], S100B was shown to be highly elevated in sCJD with no overlap to the other groups [46]. Others have found elevated S100B in familial CJD cases [64], but also in CSF [65] and serum [66] of AD patients. These findings suggest that more attention might be paid to the use of astroglial markers in supporting the differential diagnosis of dementias [46].

With respect to FTLD, cNEUPRO found elevated mean levels of the TAR DNA-binding Protein 43 (TDP-43) and reduced A$\beta_{1\text{-}42}$ levels [67, 68]. In line with the reported increased gene expression of TDP-43 in brain tissues [69], elevated 45 kDa TDP-43 levels were found in the CSF of 12 patients with FTLD as compared to 13 nondemented controls by western-immunoblot [67].

In the same sample, the assessment of different Aβ peptide species, sAPPα and sAPPβ, by electrochemiluminescence-based multiplex assays indicated no significant difference for sAPPα and sAPPβ between the groups. However, reduced A$\beta_{1\text{-}42}$ levels were found in FTLD [68]. These findings are supported by several earlier studies which found CSF-levels of A$\beta_{1\text{-}42}$ in FTLD to be lower than in nondemented controls and higher than in AD [70–73]. However, there are also contradictory publications, regarding levels of Aβ species which did not find reduced CSF A$\beta_{1\text{-}42}$ concentrations in FTLD [74, 75]. Although TDP-43 and fragments of APP processing are currently not suitable as biomarkers because of a large overlap between the different diagnostic groups, these findings may still reflect aspects relevant for understanding the pathophysiology of these disorders.

In an associated study focussed on the biomarker supported differential diagnosis of AD, PD, PDD, and DLB, CSF A$\beta_{1\text{-}42}$, total-tau, and phospho-tau were measured in the CSF of a total of 80 patients. Although some significant differences in the average biomarker measurements were found between the groups, only AD patients could be effectively differentiated from patients with other dementias by phospho-tau. For A$\beta_{1\text{-}42}$, total-tau, and phospho-tau, a large overlap between the other neurodegenerative diseases was observed. Interestingly, only in DLB were A$\beta_{1\text{-}42}$ and total-tau found to correlate with the duration and the severity of dementia [76]. Consequently, more and better biological markers are needed to support the differential diagnosis of these dementias [77].

A marker with a potential specificity for synucleinopathies may be the lysosomal hydrolase β-glucocerebrosidase. In addition to a previous report linking a reduced activity of β-glucocerebrosidase to PD [78], a reduced activity of β-glucocerebrosidase was specifically found in DLB within cNEUPRO. In CSF from nondemented controls, patients with AD or FTLD, no differences in β-glucocerebrosidase activity were found. In contrast, the activity of α-mannosidase, another lysosomal hydrolase, was found to be significantly reduced in all investigated neurodegenerative diseases as compared to controls [79]. In order to support the hypothesis that CSF β-glucocerebrosidase activity might be a novel CSF biomarker of synucleinopathies, the data need to be confirmed in larger studies.

3.6. Investigated Blood-Biomarker Candidates Related to APP Processing. Several recent studies aimed at identifying AD biomarkers in blood were specifically targeted at determination of Aβ peptides in blood plasma or serum [20].

Within a cNEUPRO associated substudy of the German Kompetenznetz Demenzen (http://www.kompetenznetz-demenzen.de/), $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ were assessed in blood plasma from 257 individuals with multiplexing technology on the Luminex platform. A statistically significant decrease of the $A\beta_{1\text{-}42/1\text{-}40}$ ratio was found in the plasma of the patients with early AD and MCI of AD type whose clinical diagnoses were backed up by corresponding findings in the CSF [80]. Moreover, the cNEUPRO associated French "Three-City study" found that a reduction of the ratios $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ as well as $A\beta_{x\text{-}42}/A\beta_{x\text{-}40}$ was associated with an increased risk of developing dementia within the next two years [81]. In contrast, several other published studies have not reported significant differences in Aβ peptide concentrations in blood plasma between AD patients and controls [82–84]. In summary, there is no definitive conclusion as to whether plasma Aβ reflects the changing level of central amyloid [20]. Due to the substantial interindividual variations and a large overlap between the diagnostic groups, measuring the individual concentrations of Aβ peptides in plasma is not suitable to support the clinical diagnosis of different dementia disorders. However, there is preliminary evidence that specific forms of Aβ peptides in plasma prove to be helpful in the differential diagnosis of AD and other dementias. In a retrospective pilot study which was supported by cNEUPRO, vascular dementia could be differentiated with a sensitivity and specificity of >80% from other dementias and depressive controls by the ratio of $A\beta_{1\text{-}38}/A\beta_{1\text{-}40}$ [85].

Currently, highly sensitive assays for the detection of Aβ peptides in blood and CSF are available for $A\beta_{x\text{-}38}$, $A\beta_{x\text{-}40}$, and $A\beta_{x\text{-}42}$. For a detailed analysis of additional variants of Aβ peptides in blood plasma, a highly sensitive two-dimensional gel separation method was established within cNEUPRO. Using this method, at least 30 different Aβ peptides were observed [86]. Semiquantitative analysis revealed that the peptides $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ accounted for less than 60% of all Aβ peptides that were detected by the specific antibody that was used in this study. At least 10% of the detected Aβ peptides appear to be N-terminally truncated [86]. One possible source of these N-terminally truncated Aβ peptides detected in human plasma is mononuclear phagocytes. Cultures of human mononuclear phagocytes were shown to secrete complex Aβ peptide patterns characterized by a high proportion of N-terminally truncated variants [87]. Furthermore, the secretion of Aβ peptides from human mononuclear phagocytes was differentially regulated in response to cell culture conditions [87] and was elevated in cell cultures of mononuclear phagocytes from AD patients as compared to controls [88]. Additional work is under way to evaluate several N- and C-terminally truncated Aβ peptides in plasma as potential biomarkers for AD.

3.7. Currently Ongoing Research in cNEUPRO. The identification of valid biomarkers in blood is highly desirable because they have the advantage of being easily accessible. The search for potential biomarker candidates in plasma or serum is complicated by the presence of a number of highly abundant proteins. These proteins which are believed to have only small diagnostic potential make up about 90% of the whole plasma proteome [89]. As a first step towards biomarker discovery in serum, it was shown that the depletion of 12 high abundant serum proteins by immuno affinity chromatography columns resulted in an increased number of detected peaks by subsequent analysis with SELDI-TOF mass spectrometry [90]. In contrast, CSF proteomics for biomarker discovery in neurodegenerative diseases is particularly attractive because of the proximity of CSF to the brain. Again, the removal of highly abundant proteins resulted in an improved detection of low abundant CSF proteins including brain-derived proteins. Additional separation procedures were introduced to account for the large dynamic range of the expression levels and to simplify the analysis of proteolytically generated peptides by mass spectrometry. For a comparative analysis of individual clinical samples and for a relatively in-depth search for potential novel biomarkers, reproducibility is an absolute requirement. Therefore, different multiaffinity depletion methods followed by gel-nanoLC-MS/MS and spectral counting have been evaluated for the in-depth, label-free quantitative analysis of CSF. Depletion in spin-filter format, coupled to gel-LC-MS/MS, provided a robust method that yielded ~800 CSF proteins per analyzed sample, with acceptable reproducibility of protein identification (71%–74% in technical replicates) and quantification (17%–18% CV on spectral counts). To control for reproducibility, the same workflow was implemented in two separate laboratories within cNEUPRO. This proteomics approach was subsequently applied in both laboratories to the independent analysis of two separate cohorts of 20 individual CSF samples each. In both cohorts the patients were clinically diagnosed, and CSF was taken according to the cNEUPRO standard operating procedures. Both discovery sets of samples included CSF samples from five control subjects, from five subjects with mild cognitive impairment without conversion to AD, from five patients with mild cognitive impairment with conversion to AD within the follow-up of 2 years, and five patients with AD. Both datasets contained ~1100 identified proteins with a total of ~1600 unique CSF proteins in the common dataset and an overlap of ~500 between the two laboratories. The biostatistical analysis is currently on-going to select the most promising candidates for a reassessment by targeted mass spectrometry and antibody-based methods in a larger set of samples.

4. Conclusion

Within the first two years, cNEUPRO confirmed sAPP, various Aβ peptide variants, GFAP, S100B, and ubiquitin as biomarker candidates known from previous studies. Additionally, further APP fragments were discovered and TDP-43 as well as β-glucocerebrosidase and ERK 1/2 were proposed as potential novel candidate biomarkers for the early and differential diagnosis of neurodegenerative diseases (Table 1). Because of the high complexity of the blood proteome and probably because of its distance from brain pathology, novel biomarkers in serum or plasma are still elusive. To promote biomarkers in support of the clinical diagnosis of neuropsychiatric disorders in Europe, cNEUPRO devised European standard operating procedures

Table 1: List of candidate biomarkers investigated in the context of cNEUPRO. CON: control patient, AD: Alzheimer's Disease, OD: other dementia, VaD: vascular dementia, MCI: Mild cognitive impairment, sCJD: sporadic Creutzfeldt-Jacob Disease, FTLD: Frontotemporal lobar degeneration, ALS: Amyotrophic Lateral Sclerosis, DLB: Dementia with Lewy bodies.

Biomarker candidate	Context/Function	Method	Patients	n	Result	Ref.
Investigated CSF candidate biomarkers for AD related to APP processing						
$A\beta_{1\text{-}42/1\text{-}38}$ ratio	APP processing	ELISA/MSD	CON	30	Reduced in AD	[34]
			AD	44		
			OD	87		
$A\beta_{1\text{-}40}{}^{ox}$	APP processing	Western blot	CON	30	Elevated in AD	[35]
			AD	30		
			VaD	37		
sAPP	APP processing	Luminex	MCI	81	Elevated sAPPα/β in patients with elevated tau and reduced $A\beta_{1\text{-}42}$	[41]
			AD	69		
			OD	38		
APP/Aβ	APP processing	LC-MS	CON	3	Elevated in AD	[43]
			AD	3		
12 kDa sAPP	APP processing	LC-FTICR-MS	CON	6	Elevated in AD	[42]
			AD	5		
		Western blot	CON	6	Elevated in AD	[42]
			AD	6		
Investigated CSF candidate biomarkers for AD not related to APP processing						
GFAP	Marker for astrogliosis	ELISA	CON	12	Elevated in AD	[46]
			AD	18		
			sCJD	22		
Total protein carbonylation	Neuro-inflammation	ELISA	CON	18	No difference between AD and CON	[48]
			AD	22		
3-nitrotyrosine	Neuro-inflammation	ELISA	CON	18	No difference between AD and CON	[48]
			AD	22		
ERK 1/2	MAP-Kinase	western blot/electrochemi-luminescence	MCI	9	Pilot study, no statistics	[44]
			AD	4		
			FTLD	2		
Investigated CSF candidate biomarkers for other dementias						
S100B	Marker for astrogliosis	ELISA	CON	12	Elevated in sCJD	[46]
			AD	18		
			sCJD	22		
TDP-43	DNA binding protein	Western blot	CON	13	Elevated in FTLD and ALS	[67]
			FTLD	12		
			ALS	15		
			ALS+FTLD	9		
85 kDa protein	Unknown	2D-DIGE/MALDI-TOF	CON	6	Elevated in sCJD	[54]
			AD	24		
			sCJD	36		
			DLB	6		
Ubiquitin	Protein degradation	LC-MS/WB	CON	32	Elevated in sCJD	[55]
			sCJD	32		
			OD	31		
α-Mannosidase	Lysosomal Hydrolase	Enzyme activity assay	CON	23	Reduced in all dementias	[79]
			AD	20		
			FTLD	20		
			DLB	17		

Table 1: Continued.

Biomarker candidate	Context/Function	Method	Patients	n	Result	Ref.
β-Glucocerebrosidase	Lysosomal Hydrolase	Enzyme activity assay	CON	23	Reduced in DLB	[79]
			AD	20		
			FTLD	20		
			DLB	17		

for preanalytical sample handling and established a neurochemical dementia diagnosis reference center in Hungary. cNEUPRO has now started to select the most promising biomarker candidates from two proteomic studies within cNEUPRO and to reassess the most promising biomarker candidates with larger sample size and independent methods to finally integrate them into novel prototype assays.

To increase the accuracy of a biomarker-based diagnosis, biomarkers in body-fluids have been combined with other biological markers such as structural and functional neuroimaging and neuropsychological testing [91]. Whether the new biomarker assays which will be developed within cNEUPRO will be useful in such a multimodal diagnostic workup remains to be elucidated.

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Review Article

Cerebrospinal Fluid Biomarkers for Dementia with Lewy Bodies

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More than 750,000 of the UK population suffer from some form of cognitive impairment and dementia. Of these, 5–20% will have Dementia with Lewy Bodies (DLB). Clinico-pathological studies have shown that it is the low frequency of DLB clinical core features that makes the DLB diagnosis hardly recognisable during life, and easily misdiagnosed for other forms of dementia. This has an impact on the treatment and long-term care of the affected subjects. Having a biochemical test, based on quantification of a specific DLB biomarker within Cerebrospinal Fluid (CSF) could be an effective diagnostic method to improve the differential diagnosis. Although some of the investigated DLB CSF biomarkers are well within the clinical criteria for sensitivity and specificity (>90%), they all seem to be confounded by the contradictory data for each of the major groups of biomarkers (α-synuclein, tau and amyloid proteins). However, a combination of CSF measures appear to emerge, that may well be able to differentiate DLB from other dementias: α-synuclein reduction in early DLB, a correlation between CSF α-synuclein and Aβ42 measures (characteristic for DLB only), and t-tau and p-tau181 profile (differentiating AD from DLB).

1. Introduction

Presently, in the United Kingdom (UK), it is estimated that over 100,000 people have been diagnosed with Dementia with Lewy Bodies (DLB), accounting for 15–20% of the total number of recorded cases of dementia. By 2050, up to 1.8 million UK inhabitants will be affected by dementia [1], thus raising the number of DLB sufferers by 3-fold. These figures inevitably reflect the rapid increase in the ageing populations in UK and Europe. Thus, according to the National Office of Statistics (2009), by 2033, 23% of people in UK will be aged 65 or over, and of them 15–25% will have some form of cognitive impairment.

1.1. DLB Clinical Symptomatology. Clinically, DLB is characterised by fluctuation of cognitive abilities alongside distinctive psychopathological symptoms, including recurrent, regular visual hallucinations and delusions [2]. Neurologically, 25–50% of the DLB patients have extrapyramidal symptomatology, including rigidity and bradykinesia, alongside hypophonic speech, masked facies, stooped posture, and a slow and shuffling gait, whereas the resting tremor is less common [3, 4].

A diagnosis of DLB requires presence of at least two core features (Table 1): fluctuating cognition; definite, regular hallucinations; or spontaneous parkinsonian movement disorders (present in 78% of DLB patients [4, 5]). In addition, suggestive features of the probable DLB diagnosis include Rapid Eye Movement (REM) sleep behaviour disorder, severe neuroleptic sensitivity, and low dopamine transporter uptake in basal ganglia as demonstrated by SPECT and/or PET imaging [5]. In studies using postmortem diagnosis of DLB as the gold standard, an appropriate diagnosis of DLB can be made with a sensitivity of 78–83% and a specificity of 85–95% (using [3] clinical criteria, [6, 7]), with the presence of visual hallucinations early in the course of dementia being a strong predictor of DLB at autopsy [8]. However, these values are achieved when the criteria are used in the specialist clinic, and thus should be considered as a maximal rather than an average value. Indeed the sensitivity and specificity for

Table 1: Clinical diagnostic criteria for DLB.

Core features	Features supportive of diagnosis
A progressive, significant cognitive decline	Repeated falls
Fluctuations in cognition	Syncope
Recurrent, well-formed visual hallucinations	Transient loss of consciousness
Spontaneous motor features of parkinsonism	Neuroleptic sensitivity
	Systemized delusions
	Hallucinations in other modalities

Adapted from Mckeith et al. [5].

DLB diagnosis appear to be somewhat lower when related to subjects coming from hospital environment, with sensitivity and specificity being 60% and 85%, respectively [9].

1.2. Differentiation of DLB from Other Dementias. In the clinical setting DLB is commonly misdiagnosed as Alzheimer's Disease (AD) or Parkinson's Disease (PD) due to their overlapping clinical symptoms [10]. Differential diagnosis is essential, as around 50% of DLB patients are hypersensitive to conventional antipsychotic medication have worsening delusions and exacerbated motor symptoms [11].

The diagnosis of DLB, probable or possible dementia of AD, and vascular dementia (VaD) is largely based on clinical and neuropsychological assessment, using the current diagnostic criteria for different dementias (DSM-IV-TR and specific criteria for each disorder, e.g., NINCDS/ADRDA for AD, NINDS/AIREN for VaD, and International Consensus Criteria for DLB, not included in DSM-IV-TR criteria). The validity and reliability of these existing criteria have relatively good specificity, but low sensitivity for detecting distinct types of the dementing process, making them of limited value for routine clinical practice.

The neuroradiological investigations also have limitations in differentiating distinct types of dementia since many dementia sufferers have a degree of generalised brain atrophy, ventricular dilatation, white matter lesions, and/or ischaemic (sub)cortical changes, though a recent study showed that hippocampal atrophy [12] may differentiate DLB from AD and VaD. HMPAO SPECT occipital lobe hypoperfusion, largely thought to be characteristic for DLB [13, 14], has now been reported to be similarly present to an extent in other forms of dementia (see [15], reviewed in [16]). The latest studies measuring brain amyloid load by [^{11}C]PIB-PET also show that not all individuals (89%) with a probable diagnosis of AD will have an increased amyloid brain load [17]. Similarly, many DLB subjects have widespread PIB binding [18], very similar to that seen in AD subjects. Recent studies have also demonstrated the usefulness of ^{123}I-metaiodobenzylguanidine (MIBG) myocardial scintigraphy for the diagnosis of DLB, with marked reduction of cardiac MIBG uptake being a specific marker of Lewy body diseases [19, 20]. However, this procedure is not well validated as the DaTSCAN-SPECT and remains an unlicensed product for this indication. We have to highlight that a majority of the DLB MIBG myocardial scintigraphy studies (with exception of few, e.g., [21, 22]) have been conducted in Asia, where heart disease rates are low. Since MIBG myocardial scintigraphy can be abnormal in heart failure and, to some extent, severe ischaemic heart disease [23], further studies are needed to explore its clinical utilisation for DLB diagnosis in the Western countries where the prevalence rates of heart diseases are much higher (reviewed in [24]).

1.3. Clinico-Neuropathological Correlates of DLB. The similarities between different dementias may be explained by the degree of overlapping pathology [25] and their attributable risks for cognitive impairment in the elderly [26]. In particular, coexisting AD pathology (tangles and plaques) is known to modify clinical symptoms, disease course, and progression. Thus, DLB individuals with additional neocortical tangles often lack the typical DLB symptom profile (e.g., lack of core symptoms including fluctuation, visual hallucinations, and parkinsonism), showing pronounced memory deficits, severe construction deficit, and a clinical presentation more characteristic for AD [27, 28]. Similarly, approximately 70% of DLB patients have neuropathological changes characteristic of AD, and, clinically, they tend to have more profound cognitive impairment than those with "pure" DLB [29]. Correspondingly, at least 59% of AD patients have LBs, usually restricted to amygdala and sparing the neocortical regions, with their number increasing as the disease progresses [30]. Interestingly, the presence of LBs in the amygdala appears to increase the risk for major depression in AD by nearly 5-fold [31]. Furthermore, in AD subjects with similar severity of cognitive impairment at baseline and comparable Braak stages at autopsy, those with concomitant neocortical LB pathology (referred to as Lewy body variant of AD) generally have faster cognitive decline and accelerated mortality compared to those with "pure" AD [32].

The neuropathological phenotype, as discussed above, influences the clinical presentation in dementia subjects. To date, the detection of the dementia hallmarks, for example, tangles and plaques (for AD), Lewy bodies and Lewy neurites (for DLB), and cerebrovascular changes (for VaD), with the exception of the amyloid deposits, that can be visualised with PIB) is largely confined to neuropathological assessment. The ultimate diagnostic goal, therefore, remains to develop methodology for peripheral detection of the molecular substrates of the characteristic dementia neuropathological hallmarks in the cerebrospinal fluid (CSF), blood and blood derivates and/or urine, to aid the diagnosis, and to monitor the treatment and progression of the disease process. This is particularly important in DLB, in the light of the difficulties of diagnosing this type of dementia in routine clinical setting.

1.4. Diagnostic Tools for DLB. The currently available neuroimaging techniques and associated diagnostic tests for

Table 2: Cost and time implications of neuroimaging and neurophysiological techniques and laboratory tests.

Neuroimaging technique	Cost (£)	Time (minutes)
CT	80–100	10
MRI	200	30
SPECT	250	15–60
DaTSCAN-SPECT	750	180–360
MIBG myocardial scintigraphy	500	180
EEG	250	30–45
Blood	2.71	5
Urine	2.12	5
CSF	10	35–40

Abbreviations: CT: computerised tomography; MRI: magnetic resonance imaging; SPECT: single-photon emission computed tomography; DaTSCAN-SPECT: [123I]ioflupane single-photon emission computed tomography; MIBG: ^{123}I-metaiodobenzylguanidine; EEG: electroencephalogram; CSF: cerebrospinal fluid.
Please note that all costs and times are estimates based on UK NHS data and do not include professional interpretation of results.

dementia (also used to aid the diagnosis of DLB) represent a high financial burden to Health Care Systems (Table 2). These costs will inevitably rise on an annual basis in the light of the rapidly growing population, as reviewed above. In contrast, tests conducted using samples of bodily fluids (blood, urine, and CSF) are considerably less expensive, more readily available, and patient friendly.

Currently, there are no conclusive methods to test for DLB, and recommended CSF or other peripheral biomarkers for routine use in the differential diagnosis of DLB are lacking. Disease biomarkers, defined as "analytes in biological samples, (that provide) any measurement that predicts a person's disease state or response to a drug…" [33] need to fulfil additional criteria, for example, to reflect the central brain pathological process, be reproducible and have over a 90% specificity and sensitivity to changes in phenotype of the condition in order to allow for it to be considered clinically useful for diagnostic purposes [34]. From a clinical perspective, a single biomarker offering such diagnostic and prognostic capability would be the preferable choice.

The CSF contains an abundance of proteins, including neuron- and astrocyte-related and synapse-specific proteins [35]. Among these, the main constituents of Lewy bodies (α-synuclein), neurofibrillary pathology (tau protein), and amyloid deposits (Aβ) are detected. Their peripheral CSF detection provides an opportunity to aid the neurochemical diagnosis of various dementia processes. This is particularly important for DLB in the light of the diagnostic difficulties in routine clinical setting, as discussed above.

2. Materials and Methods

This paper is based on literature searches of several databases, including Medline/Ovid, Scopus, Web of Knowledge, and PubMed. All searches were restricted to literature published in English between the years 1980–2010 (January 1980–June 2010). For the purpose of this paper we concentrated on research studies only on CSF biomarkers and did not include biomarker studies conducted in bloods and/or blood derivates (including plasma/serum), urine, or genomic studies. Review articles were also included if they addressed either individual CSF biomarkers or in the context of CSF proteomic studies conducted in DLB and Lewy body disease. In addition, all articles were reviewed for possible relevant references. The key words used for these searches were Dementia with Lewy bodies (DLB), Lewy body, cerebrospinal fluid (CSF), biomarkers, proteomics, amyloid protein, tau protein, and synuclein.

2.1. CSF Biomarkers for DLB

2.1.1. α-Synuclein as a DLB Biomarker

α-Synuclein Processing and Role in DLB. The main components of LBs are amyloid-like fibrils composed of α-synuclein (also referred to as non-amyloid component of plaques or NACP, synelfin, and SYN1). Although the actual function of α-synuclein remains ambiguous, the protein is involved in both synaptic rearrangements and, as a chaperone in the formation of SNARE (Soluble NSF Attachment protein receptors) complexes, is implicated in the synaptic vesicle trafficking in the human nervous system (reviewed in [36]).

α-Synuclein is a relatively small (112–140 amino acids in length) presynaptic nonsecreting protein and makes up around 1% of the total protein within the brain and less than 0.001% of the CSF proteome [37]. The α-synuclein phosphorylation, occurring on serine 129, is crucial in mediating α-synuclein neurotoxicity and in modulating protein structure, including fibrillar aggregation (see [38], reviewed in [36]). The hippocampal CA2 region is thought to be especially vulnerable to this α-synuclein post-translational modification [39]. Similarly, mutations in the *SNCA*, or Synuclein-Alpha (non-A4 component of amyloid precursor) gene [40] as well as the overproduction of α-synuclein [41] in some rare cases of familial DLB give rise to the characteristic pathology in DLB, and provide further insights into the altered processing of this synaptic protein.

Detection of CSF α-Synuclein in DLB. The monomeric, soluble α-synuclein (molecular weight 14–19 kDa), but not the higher molecular species (e.g., oligomers and polymers, present in blood and brain tissue), is found in the CSF see [42, 43]). However, the presence of higher molecular aggregates (>150 kDa) consisting of α-synuclein, complement C3 precursor, complement C4-B precursor, and Ig gamma-1 chain -C region in the absence of soluble monomeric α-synuclein, has also been described [44].

The limited number of currently available studies has provided rather conflicting results regarding the measures of CSF α-synuclein in DLB subjects: decreased and unchanged to even elevated levels have all been reported (see Table 3, [45]). Furthermore, a recent study found that, although α-synuclein detection in CSF may not be useful for differentiating DLB from AD, the CSF α-synuclein measures

were significantly correlated with duration of the DLB ($P < .05$), but not with the AD [46]. This would suggest that the reduction of the CSF α-synuclein may reflect the extent of Lewy-related pathology characteristic of more advanced stages of DLB [47]. It must be noted, though, that the Noguchi-Shinohara et al. study [46] did not include control subjects, and as an alternative, a group of "matched" DLB patients with similar MMSE score results to the AD patients were included, instead.

α-Synuclein CSF levels appear to be similar in various dementia types, including DLB, AD, frontotemporal dementia (FTD) or VaD, and those of control subjects [48]. However, the findings for AD subjects were not conclusive, and lower levels of CSF α-synuclein, compared to controls, have also been reported ($P < .001$; Table 3; see [49]). In the latter study, the decrease in α-synuclein in the CSF of AD subjects was significantly associated with the disease duration, suggesting that it well reflects the extent of advanced AD neuropathology and profound brain synaptic loss, including α-synuclein as described previously [50].

In contrast to the above studies, Mollenhauer et al. [51] and Kasuga et al. [52] reported differences in CSF α-synuclein levels between AD, PD, DLB, and control subjects. There was a significant decrease in CSF α-synuclein in PD and DLB individuals, in comparison to the AD and control groups ($P = .025$; see [51]) and those with other dementias, including FTD, PSP, VaD, normal pressure hydrocephalus, and unspecified dementias ($P < .01$; see [52]). The α-synuclein gene (SNCA) duplication, which in some instances is associated with somewhat more aggressive clinical presentation of both motor and cognitive symptoms [53], appears not to influence the α-synuclein expression in the CSF, since the affected carriers with the DLB/PDD clinical phenotype have a similar CSF α-synuclein levels as DLB individuals [52].

The α-synuclein levels appear not to be associated with the extent of cognitive impairment (as assessed by MMSE), DLB, or AD disease duration, age, or gender [52]. However, the α-synuclein CSF levels are correlated with those of Aβ42 and restricted to DLB subjects [52], suggesting that there may well be a close relationship between the amyloid and α-synuclein brain processing and deposits in DLB subjects. In a recent study, the significant reduction in the α-synuclein CSF levels was present even in DLB subjects with mild dementia [44], further indicating that lower CSF α-synuclein protein may be an early marker for the disease.

Methodological Limitations of Available α-Synuclein Analytical Tools. The reasons as to why various studies are markedly different must be addressed in order to promote better study designs. Thus, it has been questioned whether the technique recommended by Tokuda et al. [83] (which requires concentration of CSF samples) could lead to inaccuracies. In addition, the assays used in some of the studies introduced incubation of the CSF samples for 48 hours at 4°C. The latter experimental step is associated with oligomerization of the α-synuclein *in vitro*, and thus may be a contributing factor for both the observed decrease and variability in the reported concentration of α-synuclein in CSF.

The choice of immunoprobes (N-terminal end being more consistently present in the CSF than the C-terminal end portion of the protein), as well as differential expression of distinct α-synuclein isoforms (with a modified C-terminus) in ageing and neurodegenerative disorders, may also underlie the reported differences in the detection of α-synuclein in the CSF (reviewed in [36, 45]). Similarly, the findings of decrease in α-synuclein in DLB as a function of the disease duration [46] may reflect the central neuropathological process of the disease and the consequent molecular changes associated with the latter. The duration-dependent decrease in CSF α-synuclein suggests the extent of α-synuclein aggregates in DLB [84] and their inability to pass the brain blood barrier [84, 85]. We have also previously reported a significant depletion of α-synuclein in more advanced stages of AD, preceded by a transient upregulation of the protein in both CSF and brain tissue occurring in the Braak stage 4 [45, 50, 86]. Further correlative clinico-neuropathological studies will need to follow to explore the neuropathological correlates of the α-synuclein CSF changes in DLB and associated dementia syndromes.

2.1.2. Tau Protein as a DLB Biomarker

Tau Protein Detection in DLB. The microtubule-associated Tau Protein represents an integral component of the paired helical filaments (PHFs). The truncation of the protein at Glu391 and/or its phosphorylation (regulated by a number of kinases) are the crucial step in the self-assembly of the protein into PHFs, found within various neurofibrillary structures (e.g., neurofibrillary tangles, neuritic plaques, and dystrophic neurites; see Figure 1) commonly associated with many neurodegenerative disorders, such as AD and DLB [87].

Tau proteins are also present in the CSF and have been extensively investigated in various dementia syndromes. However, this protein (which is also referred to as Beta-2 transferrin or desialyated transferrin) is not routinely found in blood or other body fluids, and its presence has been detected in plasma only transiently, following a stroke [88].

Phosphorylated Tau Protein in DLB CSF. Since hyperphosphorylated tau protein occurs in neurodegenerative disorders associated with neurofibrillary pathology, it is not surprising that phosphorylated tau epitopes, for example, threonine 231 (p-tau231), threonine 181 (p-tau181), and serine 199 (p-tau199), have been recommended by a consensus group as promising biomarkers to differentiate AD from other dementias, provided that they have a sensitivity level of 85% or greater and a specificity level of at least 75% [89]. These posttranslational modifications of the tau protein have been extensively assessed in various types of dementias, and in particular how well they differentiate DLB from AD using CSF samples [57]. These studies have indicated that p-tau231 is the initial post-translational modification of the tau protein found in the CSF, potentially making it a key biomarker in the early detection of tauopathies, for example, AD [90].

Table 3: CSF biomarkers for DLB.

Study	Biomarker(s)	Technique	No. of subjects	No. of controls	Results
Mollenhauer et al. [51]	α-synuclein	Sandwich ELISA	38 DLB; 13 AD; 8 PD; 8 CJD	13 neurological controls	In PD and DLB, α-synuclein levels significantly reduced ($P = .0305$) compared to AD and control subjects.
Mukaetova-Ladinska et al. [45]	α-synuclein γ-synuclein IgG	Dot blot	5 LBD; 9 AD; 3 VaD	8	Postmortem ventricular CSF analysis. Elevation of both α- and γ-synucleins in AD, LBD, and VaD compared to controls. An increase in α- and γ-synucleins seen from Braak stage III onwards. Results not influenced by age at death or postmortem delay.
Noguchi-Shinohara et al. [46]	α-synuclein	ELISA Assay	16 DLB; 21 AD	(A subgroup of 13 DLB patients matched for duration of disease and MMSE score to those of the AD subjects)	α-synuclein levels do not differ between DLB and AD patients. Lower levels of α-synuclein in CSF correspond to DLB duration ($P < .05$).
Ohrfelt et al. [49]	α-synuclein	ELISA	15 DLB; 66 AD; 15 PD	55	Similar levels of α-synuclein in PD, DLB, and control subjects, whereas, in AD, α-synuclein levels significantly lower compared to controls ($P < .001$). AD subjects with MMSE <20 had significantly lower level of α-synuclein than AD subjects with MMSE $\geq$20.
Spies et al. [48]	α-synuclein	ELISA	40 DLB; 131 AD; 39 FTD; VaD 28	Two groups: Group A 57 (aged > 50); Group B 55 healthy volunteers	No significant difference in α-synuclein levels between DLB, AD, FTD, VaD, or control subjects.
Ballard et al. [44]	α-synuclein	Western blot	12 DLB	9	Significant lower levels of α-synuclein in DLB compared to controls ($P < .05$). Mildly cognitively impaired DLB subjects (MMSE > 24) also had lower levels than controls ($P < .007$).
Kasuga et al. [52]	α-synuclein/ t-tau/p-tau181 /Aβ42	ELISA	34 DLB (including 2 with SNCA duplication); 31 AD; 21 other dementias (12 FTD; 2 PSP, 2 normal pressure hydrocephalus; 2 VaD; 3 unclassified)	No control group	α-synuclein significantly lower in DLB than in AD ($P < .05$) or other dementias ($P < .01$). CSF α-synuclein levels correlated with Aβ42 level in DLB only ($r = 0.43$; $P = .01$). CSF t-tau and p-tau181 levels as well as Aβ40/Aβ42 ratio levels significantly lower in DLB in relation to AD ($P < .01$), but similar to other dementias.
Arai et al. [54]	t-tau	Sandwich ELISA	6 DLB; 8 FTD; 6 PSP; 3 CBD	19 (data taken from previous study)	Similar levels of tau in AD and DLB ($P = .78$), but higher than in controls.
Parnett et al. [55]	t-tau/p-tau181 /Aβ42	HT7-AT270 Assay and ROC analysis	43 DLB; 80 AD	40	Strong correlation between t-tau and p-tau independent of diagnostic group ($r = 0.904$). No differences between DLB and AD for Aβ42. The significant increase in p-tau181 in AD ($P = .039$) has 80% sensitivity at differentiating between AD and DLB.

Table 3: Continued.

Study	Biomarker(s)	Technique	No. of subjects	No. of controls	Results
Clark et al. [56]	t-tau/Aβ40 /Aβ42 (includes also postmortem correlation)	ELISA	3 DLB; 74 AD (including 4 genetic AD and 10 LBVAD); 10 FTD; 5 CJD; 3 GSS syndrome; 11 miscellaneous neurologic conditions	73	DLB subjects had 2-fold higher level of t-tau in relation to controls, but two-fold lower levels in relation to AD. No differences in t-tau between LBVAD and AD ($P = .30$). Aβ42 highly depleted in DLB in comparison to both control (8-fold) and AD (4.4-fold) subjects.
Hampel et al. [57]	p-tau181/p-tau231/p-tau199	ELISA	22 DLB; 108 AD; 24 FTD; 7 VaD; 22 OND	23	Decrease in p-tau199, p-tau231, and p-tau181 ($P < .001$) in DLB compared to AD, with similar levels to other studied dementia groups.
Parnetti et al. [58]	t-tau/p-tau181 / Abβ42	ELISA	19 DLB; 23 AD; 20 PD; 8 PDD	20	DLB mean CSF t-tau levels significantly lower than in AD patients ($P = .039$), but significantly higher in PD, PDD, or control subjects. p-tau181 elevated in AD, but similar between DLB, PD, and PDD groups.
Vanderstichel et al. [59]	t-tau/ p-tau181 / Aβ42	ELISA assay	60 DLB; 94 AD	60**	Higher levels of p-tau181 in AD than in DLB and controls. p-tau181 was the most statistically significant single variable of the 3 biomarkers to discriminate between AD and DLB.
Simic et al. [60]	t-tau/ p-tau181/ p-tau199	ELISA assay	2 DLB; 11 AD; 5 FTD; 8 VaD	13	p-tau181 differentiates AD and DLB with a sensitivity of 91% and a specificity of 95%.
Koopman et al. [61] (autopsy confirmed dementias)	t-tau/p-tau181 /Aβ42	ELISA	18 DLB; 95 AD; 10 FTD; 6 CJD; 16 VaD	No control group	DLB group had similar level of t-tau, p-tau181, and Aβ42 as the other dementia groups (FTD, CJD, and VaD); these dementia subjects had significantly lower t-tau ($P = .025$) and p-tau ($P < .0001$) and higher Aβ42 ($P = .001$) in comparison to AD.
Mattson et et al. [62] (autopsy confirmed dementias)	t-tau/p-tau181 /Aβ42	ELISA	750 MCI (420 stable MCI; 14 incipient DLB; 271 incipient AD; 28 incipient VaD; 7 incipient FTD; 10 other dementias); 529 AD	304	MCI subjects who developed DLB had significantly lower levels of t-tau and p-tau181 at baseline compared to AD and incipient AD ($P < .01$), significantly lower Aβ42 CSF levels in relation to control ($P < .001$), stable MCI, and AD subjects ($P < .01$).
Spies et al. [63]	Aβ1-42/Aβ1-40/ t-tau/p-tau181	ELISA	16 DLB; 69 AD; 26 VaD; 27 FTD	47	Significantly lower levels of Aβ40 in DLB and VaD in relation to AD ($P < .01$). AD had similar Aβ40 level to controls ($P = .384$). The Aβ42/Aβ40 ratio significantly lower in AD in comparison to other dementia groups ($P < .001$). Aβ42/Aβ40 ratio improves differentiating AD from VaD, DLB, and FTD than Aβ42 measures alone ($P < .01$). Aβ42/Aβ40 ratio performed equally well as the combination of Aβ42, p-tau181, and t-tau in differentiating AD from FTD and non-AD dementias.

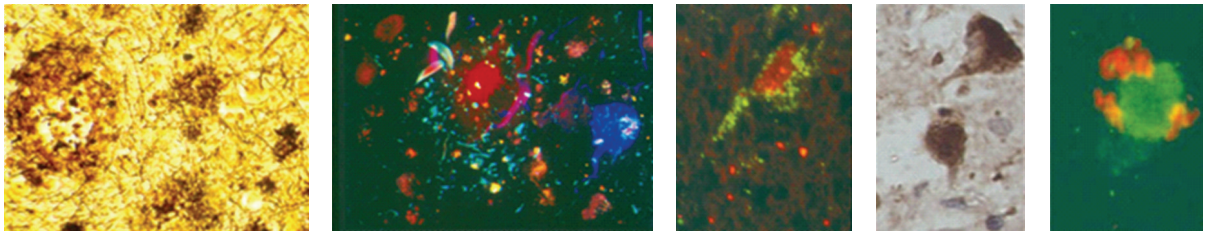
Table 3: Continued.

Study	Biomarker(s)	Technique	No. of subjects	No. of controls	Results
Bibl et al. [64]	Aβ peptides	Aβ-SDS-PAGE/ immunoblot	21 DLB; 23 AD; 21 PDD	23	The significant increase of a novel peptide with an Aβ-like immunoreactivity (Aβ1-40*) in DLB patients relative to PDD has a sensitivity of 81% and a specificity of 71% using a cut of point of 0.954% but failed to be classified as a sole biomarker.
Bibl et al. [65]	Aβ peptides/tau	Aβ-SDS-PAGE/ immunoblot and ELISAs for Aβ1-42 and tau	25 probable DLB; 18 probable AD	14	The ratio of Aβ1-42/Aβ1-38 and Aβ1-42/Aβ1-37 when combined with absolute tau levels produced a diagnostic test with 100% sensitivity and 92% specificity. This ratio discriminated between AD and DLB with a high specificity ($P = 6.6 \times 10^{-6}$).
Wada-Isoe et al. [66, 67]	Aβ42/p-tau 181	ELISA assay	22 DLB; 34 AD	37	No significant difference in p-tau levels in AD and DLB, but a significant increase in the p-tau/Aβ42 ratio in AD in comparison to DLB.
Vanderstichele et al. [59]	Aβ42	ELISA	6 LBD; 39 AD; 10 other dementias, neurological and psychiatric disorder patients*	12	Significant decrease in CSF Aβ42 levels in both AD (P <.000l) and LBD ($P = 0.002$), relative to the control group.
Maetzler et al. [18]	Aβ42	ELISA	9 DLB; 12 PDD; 14 PD no dementia	No control group	Lower levels of Aβ42 in DLB and PDD compared to the nondemented PD subjects ($P = .024$). DLB-PIB-positive subjects had lower levels than the PIB-negative subjects ($P = .044$), but similar Aβ42 levels to the PIB-negative subjects who had dementia ($P = .42$).
Boström et al. [68]	Mg/Ca/Cu	Mass spectrometry	29 DLB	51	Levels of Mg/Ca/Cu increased in CSF in DLB relative to controls, although increases in Cu not significant. The CSF-Mg concentration had a sensitivity of 93% and a specificity of 81% to detect DLB.
Molina et al. [69]	Nitric-oxide metabolites (L-arginine to L-citrulline)	Ionic -exchange chromatography	22 DLB	13	Not statistically significant difference in NO metabolite concentration between DLB and controls.
Molina et al. [70]	Neurotransmitter (NT) amino-acid (AA) concentrations	Ion-exchange chromatography	21 DLB	26**	No significant differences between control and DLB groups in relation to glutamate, aspartate, and GABA levels; however, higher concentrations of asparagine (+25%) and glycine (+21%) in DLB.

Table 3: Continued.

Study	Biomarker(s)	Technique	No. of subjects	No. of controls	Results
Schultz et al. [71]	Cocaine- and Amphetamine-Regulated Transcript (CART)	Radio-immunoassay	12 DLB; 14 AD	12	Significant decrease (30%) in CART in DLB versus controls ($P < .0001$), DLB, and AD ($P < .05$), but concentrations of CART did not indicate DLB progression. CART levels correlated with p-tau protein concentration.
Schultz et al. [72]	Transthyretin (TTR)	Radio-immunoassay	13 DLB; 59 AD	13	No significant differences of TTR concentrations between AD and DLB.

Please note that ELISA assays for t-tau, p-tau, and Aβ42, unless otherwise specified, refer to commercially available sandwich ELISA assays.
Abbreviations: DLB: Dementia with Lewy bodies; LBD: Lewy Body disease; AD: Alzheimer's Disease; LBVAD: Lewy Body variant of Alzheimer's disease; PD: Parkinson's Disease; PDD: Parkinson disease dementia; CJD: Creutzfeldt-Jakob disease; GSS: Gerstmann-Straussler-Scheinker syndrome; FTD: Frontotemporal dementia; VaD: Vascular Dementia; PSP: Progressive supranuclear palsy; OND: other neurologic disorders (e.g., mild psychiatric or neurologic symptoms); CSF: Cerebrospinal fluid; Aβ: Amyloid-beta peptide; t-tau: total tau; p-tau: phosphorylated tau; ELISA: Enzyme-Linked Immunosorbent assay; Aβ-SDS-PAGE: Aβ-sodium dodecylsulphate-polyacrylamide gel electrophoresis; ROC: Receiver Operating Characteristic; MMSE: Mini Mental State Examination; PIB: ^{11}C-labelled amyloid ligand Pittsburgh Compound B.
*Specifically vascular dementia ($n = 3$); hypoxia during cardiac arrest ($n = 1$); cerebrovascular lesion ($n = 1$); unspecified dementia ($n = 2$); depression ($n = 3$).
**Age-matched control.



(a) (b) (c) (d) (e)

Figure 1: Neuropathological hallmarks of dementia. Senile plaques (a) containing amyloid protein and neurofibrillary pathology (b) consisting of altered tau protein are the hallmarks of Alzheimer's disease and ageing. In dementia, loss of synaptic proteins affects both allo- and neocortical areas. In Dementia with Lewy body, intraneuronal aggregates of α-synuclein give rise to Lewy bodies ((d),(e)) that are also found in normal ageing and in other neurodegenerative diseases, including AD and PDD. Light microscopy ((a), (d)); confocal microscopy ((b), (c), (e)). Labelling: Bielschowsky silver (a), tau immunohistochemistry (b), synaptophysin labelling (c), and alpha-synuclein immunohistochemistry ((d), (e)). Magnification: x100 (a) and x400 ((b)–(e)).

CSF p-tau181 in DLB. Although p-tau231 is a relatively consistent biomarker of all dementias (including DLB), increased concentrations of p-tau181 in the CSF appear to be often implicated in DLB (see [91], Table 3). The CSF elevation of p-tau181 is not restricted to DLB, but also found in several other neurodegenerative disorders, thus reflecting the presence of overlapping neurofibrillary pathology. Nevertheless, p-tau181 measures appear to be different in the two dementia types: AD subjects have significantly higher CSF level of p-tau181 compared to control and DLB subjects (see [59]; Table 3), and this differentiates AD from DLB, with a sensitivity of 91% and a specificity of 94% [60]. However, in autopsy-confirmed AD, the diagnostic accuracy of CSF p-tau181 to discriminate AD from DLB showed lower sensitivity and specificity (75% and 61%, respectively, and 73% diagnostic accuracy; see [61]).

The data from the latter study were similar to those of Hampel et al. [57] which found an increase in p-tau181 CSF measures to have a sensitivity of 94% and a specificity of 64% for differentiating AD and DLB. However, the combination of p-tau231 and p-tau199 did not produce promising results in differentiating between these two dementia syndromes: specificities of p-tau231 and p-tau199 were 64% and 50–64%, respectively. The latter may be due to the low sensitivity and specificity of p-tau199 CSF measures (both ranging between 25–30%) in differentiating between AD, other dementia subtypes, and control subjects [60].

CSF Total Tau and Relationship to p-tau Measures in DLB. One of the earliest attempts in evaluating the potential of CSF measures of total tau protein (t-tau) in dementia studies was that by Arai et al. [54], which reported significantly elevated CSF t-tau protein levels in AD subjects in comparison to PD patients (Table 3). In the follow-up study [92], CSF t-tau levels were determined in a number of dementia syndromes including FTD, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and DLB, as well as a control group. Although CSF t-tau was elevated in the

Table 4: CSF studies in Parkinson's disease.

Study	Type(s) of Biomarker	Sample(s) taken	Type(s) dementia
Almonti et al. [73]	Metals	CSF	PD
Asai et al. [74]	Orexin	CSF	PD
Bibl et al. [75]	Amyloid-Beta/ Tau	CSF/Plasma	VaD
Compta et al. [76]	Amyloid-Beta/ Tau	CSF	PD/PDD
Lunardi et al. [77]	DA and metabolites*	CSF	PD
Salehi and Mashayekhi et al. [78]	BDNF	CSF	PD

BDNF: Brain-Derived Neurotrophic Factor; DA: Dopamine; CSF: Cerebrospinal fluid; VaD: Vascular Dementia; PD: Parkinson's Disease; *Homovanillic acid (HVA), Dihydroxyphenylacetic acid (DOPAC).

DLB group, there was no significant difference between the DLB and AD subjects (using the previous data from the AD patients). In contrast, Parnetti et al. [55] reported differences in both CSF t-tau and p-tau levels between AD and DLB patients with a greater difference for the p-tau CSF (Table 3).

These findings of higher t-tau and p-tau CSF measures in AD subjects in comparison to other forms of dementia were confirmed again in a later study by the same group (see [58]; Table 3), with the DLB subjects, although having lower t-tau than AD ($P = .039$), still exhibiting 2-3 fold higher level of CSF t-tau measures than those in PD, PDD, and control subjects. Furthermore, data from this study indicated that, of the 19 patients with DLB, half displayed high levels of t-tau in their CSF, similar to those of the AD subjects. Interestingly, PD subjects with dementia also showed an elevation of t-tau and p-tau compared with PD and control groups, and this was also accompanied by a decrease in amyloid peptides [76], similar to previous dementia studies. Similar findings of highly elevated CSF t-tau and p-tau181 have now been reported for some autopsy-confirmed DLB patients [93].

Concentrations of both t-tau and p-tau do not correlate with the DLB disease duration [58]. However, significant inverse correlation between t-tau levels and MMSE ($r = -0.54$; $P = .02$) along with a Milan Overall Dementia Assessment (MODA) (a standardised assessment for staging dementia used globally but developed within Italian clinics [94]) ($r = -0.66$; $P = .002$) score has been reported, similar to findings of a previous study [55]. One of the explanations for this may be the significantly lower levels of t-tau and p-tau181 already present in incipient DLB [62], suggesting that the cognitive changes may well be influenced by additional factors, for example, neuronal cell loss, vascular insults, and so forth.

CSF Tau Protein Changes in Autopsy-Proven DLB. The presence of LBs may have a damaging effect upon the neuronal cytoskeleton (reviewed in [36]), and thus, may contribute to the altered levels of tau within the CSF in DLB subjects. Indeed, elevated levels of CSF total-tau (considered a marker of axonal neuronal damage) have been confirmed in cases with a definite diagnosis of AD, Lewy Body variant of AD, as well as DLB alone (see [56]; Table 3), thus reflecting the described impairment in axonal transport [95] and axonal loss (reviewed in [96]) underlying the development of both AD and LB pathologies. However, the CSF t-tau findings are not conclusive, and a contrary report of a decrease in t-tau in DLB was also described in a similar study conducted on autopsy-confirmed sample [61]. The latter may well reflect the more advanced stages of the dementia disease process, characterised by both generalised axonal and neuronal loss, as reported previously (see [97], reviewed in [98]).

Recent correlative biochemical and neuropathological studies have also highlighted the relationship between the CSF tau measures (p-tau181 and p-tau231) with the extent of brain neurofibrillary pathology (e.g., neuritic plaques and neocortical neurofibrillary tangles) in AD subjects [99, 100], thus confirming that the CSF tau protein measures reflect closely the brain accumulation of the characteristic AD hallmarks of the disease, the neurofibrillary pathology. However, the findings for CSF p-tau181 are not conclusive, as previous studies have reported lack of association of this CSF tau measurement with neurofibrillary pathology [101, 102]. The differences in the reports may arise from the differences in timing of obtaining the CSF samples in relation to autopsy (ranging from approximately one year [101, 102] to 6 years [99]), suggesting that the CSF p-tau measures close to death do not necessarily reflect the true extent of neurofibrillary pathology in the brain, as detected using immunohistochemical [101] or immunobiochemical [90] methods, since the presence of end stages of neurofibrillary tangles (the so-called "ghost tangles", consisting of the core of the paired helical filaments; see [103]) could not have been addressed.

2.1.3. Amyloid-Beta (Aβ) Peptides as a Biomarker

Aβ Processing. Aβ peptides play an important role not only in the AD pathogenesis [104], but also in DLB. It is suggested by interacting with α-synuclein that the amyloid peptides promote aggregation, enhance the accumulation of α-synuclein pathologies, and accelerate cognitive dysfunction [105]. It is, therefore, reasonable to examine amyloid peptides for their potential diagnostic value in DLB.

CSF Aβ Peptides in DLB. The altered brain processing of APP, leading to accumulation of extracellular amyloid deposits throughout the brain tissue of the affected individuals, is also seen in the periphery, for example, CSF and blood/blood derivates. Thus, the CSF decrease of Aβ peptide 1-42, although characteristic of AD, is also found in DLB and PDD (see [64]; Table 3), and this may reflect the similar extent of Aβ deposits in these diseases (reviewed in [106]). In support of the latter are the recent findings of CSF Aβ42 loss in DLB subjects being accompanied by lower CSF levels of cystatin C [107], a peptide that inhibits fibril formation and oligomerization of the amyloid peptide [108]. Further evidence for the role of the amyloid brain deposits in

downregulating CSF Aβ42 levels comes from a PET imaging study, which demonstrated that the PIB binding in DLB was associated with cognitive impairment ($P = .0006$) and decrease in CSF Aβ42 ($P = .042$; see [18]).

The shorter amyloid peptide (Aβ1-40) also appears to be decreased in the CSF of AD subjects, and significantly more in those with a clinical diagnosis of DLB and vascular dementia [48]. Furthermore, the ratio between the longer and shorter CSF amyloid peptides (Aβ42/Aβ40) appears to be superior in discriminating between AD and other neurodegenerative disorders, including DLB, than the Aβ42 measure alone ($P < .01$), with the former being equally robust as the combination of Aβ42, p-tau181, and t-tau (see [63]; Table 3). In contrast, in AD (but not DLB and PDD) there was a slight increase in CSF Aβ1-37 [64], and this may reflect the slight differences in the Aβ brain deposition between AD and DLB, in terms of the deposition occurring later in the course of DLB [109] or the faster rate of disease progression in DLB [32].

In the Bibl et al. [64] study, a novel Aβ-like peptide (considered to be an oxidised α-helical form of Aβ, designated as Aβ1-40*) was detected in all recruited participants (including the nondemented controls) and was significantly increased in those affected by DLB (in comparison with PDD patients), and to a lesser degree in AD as compared to PDD and control subjects. In fact, the CSF Aβ1-40* measures had 81% sensitivity and 71% specificity at differentiating between DLB and PDD. Furthermore, the ratio of Aβ1-42 to Aβ1-37 significantly differentiated the control subjects from those with DLB, PDD, and AD and differentiated the AD subjects from those with DLB and PDD [64]. These findings suggest that the Aβ CSF patterns vary between AD, DLB, and PDD, and introduction of Aβ ratios improves the diagnostic CSF test accuracy for the dementia differential diagnosis, which is not the case when sole measurements of Aβ1-42 are reported. Similarly, the differential diagnostic value of Aβ peptide patterns in combination with tau protein assays appears to be improved. The ratio of Aβ1-42 to Aβ1-38 and Aβ1-42 to Aβ1-37, when combined with t-tau levels, has 100% sensitivity and 92% specificity in differentiating AD from DLB and control subjects [65].

A study by Parnetti et al. [58] investigated a wider range of subjects, including those with DLB, PD, PDD, and AD. In comparison to PD, PDD, and AD subjects, the DLB group had the lowest CSF level of Aβ42, and the latter was negatively correlated with the dementia duration. In addition, DLB patients had a significantly higher t-tau CSF measures relative to PD, PDD, and controls. However, differences in CSF levels of p-tau, although significantly elevated in AD, failed to discriminate the disease entities within the Lewy body disease (LBD) spectrum, irrespective of presence of dementia (DLB, PDD, or PD). It is important to note that 4 out of 23 AD patients in this study had a CSF analytes composition remarkably similar to that of the DLB group.

The above set of data is similar to that of Vanderstichele et al. [110]. The latter group reported a significant decrease in CSF Aβ42 in both AD ($P < .001$) and LBD ($P = .002$) patients, relative to the control group (data for DLB subjects were not extracted separately in this study). In contrast to their previous findings [64], later CSF studies, further supported by ^{123}I-MIBG cardiac scintigraphy observations, found no significant difference in the CSF Aβ42 measures between AD and DLB subjects [65, 67]. However, the cardiac scintigraphy provided the best discrimination between the two dementia groups. Thus, the DLB subjects had significant elevation of washout rate in comparison to both the AD and control groups [67].

2.2. Miscellaneous Biomarkers

Inflammatory Markers. The biochemical and neuropathological studies in DLB have highlighted a number of novel putative molecular candidates present in the CSF (Table 3). Inflammation is associated with amyloid accumulation in dementia [111], and inflammatory markers have also been detected and investigated in the CSF. However, in DLB, the interleukin CSF levels, specifically, IL-1β and IL-6, did not discriminate DLB from control subjects [112]. Similarly, CSF measures of the precursor peptides for enkephalins and substance P (midregional proenkephalin A and N-terminal protachykinin A, resp.), involved in inflammation and pain, although decreased in dementia disorders, including DLB, appear not to have the power to differentiate various dementia syndromes from acute neuroinflammatory disorders [113], suggesting that the CSF measures of these two neuropeptide precursor fragments could reflect the extent of neuroinflammation and reduction in neuronal activity, common among these diseases.

CART Neuropeptides. The hypothalamic region in DLB shows profound atrophy on MRI brain scans, and this may underlie the characteristic fluctuating clinical symptoms in this dementia syndrome. Thus, any alterations in molecular patterns associated with these characteristic morphological changes can be useful in developing a biomarker for DLB. The neuropeptide Cocaine and Amphetamine-Regulated Transcript (CART) is expressed selectively in neurons in the hypothalamic region. A study by Schultz et al. [71] reported a significant reduction by 30% in the CSF CART levels in DLB compared to both control and AD subjects. The depletion of CART may be a causative factor in the dysfunction of the dopaminergic system seen in DLB. Interestingly, CSF CART levels correlate with p-tau protein levels, but they do not appear to correlate with the DLB disease progression [71]. This suggests that neuroimaging techniques that could detect dysfunction of the dopaminergic system (a consistent finding in various DLB studies [114]) could also be a key in the future diagnosis of DLB.

Brain Neurotransmitters. Brain neurotransmitters play an essential role in various psychological and cognitive functions. The relatively widespread cholinergic [115, 116] and dopaminergic [117, 118] changes in DLB indicate a more generalised neurotransmitter dysfunction that may also be detected in the periphery. To test this hypothesis, Molina et al. [70] measured the concentration of various amino acids (AA) considered to reflect the neurotransmitter

changes within the CSF. Of these, levels of asparagine and glycine, but not glutamate, glutamine, aspartate, and GABA, were raised in both CSF and plasma from DLB patients compared with age-matched controls, with only the plasma levels reaching statistical significance. This suggests that the plasma measures of asparagine and glycine may be useful for the DLB diagnosis. Interestingly, higher levels of glycine are also found in other neurological disorders.

Posttranslational Protein Modifications. In vitro oxidation and nitration of α-synuclein is associated with the aggregation of this protein [119], which leads to the characteristic intraneuronal filamentous inclusions characteristic of DLB pathology. Interestingly, a study conducted by Molina et al. [69] found an increase in Nitric Oxide (NO), associated with protein nitration processes, in CSF in DLB patients in comparison to the control group. Not only does this give an indication of further pathological processes within DLB, but it also highlights the need for further research to determine the clinical relevance of NO as a biomarker for the diagnosis of DLB.

Markers of Cytoskeletal Changes. An increase in neurofilament (NF) in the CSF is also indicative of neuronal degeneration, as seen in neurodegenerative disorders, especially AD. However, De Jong et al. [91] did not find any NF elevation within CSF of DLB patients relative to patients affected by late onset AD, despite an increase in cortical NF containing neurons in DLB. This indicates that peripheral NF concentration does not reflect the cortical NF expression. One of the limitations of this study was the small sample size and the lack of postmortem verification of the diagnosis. Further studies on larger clinical samples are now needed to overcome these limitations.

Metal Homeostasis. Dysfunction in metal homeostasis has been implicated as a causative factor for neurodegeneration. In a study conducted by Bostrom et al. [68] measurements of magnesium (Mg), calcium (Ca), iron (Fe), copper (Cu), zinc (Zn), rubidium (Rb), strontium (Sr), and caesium (Cs) were taken from the CSF samples. Ca and Mg levels were elevated in DLB compared to AD, VaD, and control subjects. In this study, the combined Ca and Mg CSF measurements had a sensitivity of 93% and a specificity of 85% (using cutoff values of $\leq$48.0 mg/L for Ca and $\leq$27.3 mg/L for Mg) to differentiate DLB from AD. Since clinical criteria state that biomarkers must have a specificity and a sensitivity of $\geq$80% (as discussed above [120]), the latter findings may well support the use of Ca and Mg CSF measures as potential biomarkers for DLB.

Thyroid Hormone-Binding Protein Transporter of Thyroxine. Transthyretin (TTR) changes are not only characteristic for familial amyloid polyneuropathy, but also for other neurodegenerative disorders, such as AD and dementia in general [121]. It is thought that TTR has a "neuroprotective" role in AD, via the prevention of formation of Aβ fibrils. Despite TTR proteins having a strong linkage with DLB-type pathology, the study by Schultz et al. [72] did not find any correlation between CSF concentrations of TTR and clinical presentation of DLB.

The clinical designs of the above studies (sample size, inclusion criteria, differences in clinical assessments, number of dementia types analysed, defining control groups, etc.) all differed, and these differences need to be considered when interpreting the results. With the exception of the CSF measures of CART and metal compounds, there are no putative biomarkers that emerge to differentiate DLB from normal ageing and/or other types of dementia.

2.3. Additional CSF Biomarkers in Parkinson's Disease (PD). The overlapping clinical symptoms between DLB and PD make the differentiation of these two clinical entities difficult. Not surprisingly, our searches for DLB CSF biomarkers highlighted a number of studies that included PD and PDD subjects. While these studies may not ultimately highlight a suitable biomarker for DLB, they should be considered, even if only for evidence of further exclusion criteria.

BDNF. Brain-derived neurotrophic factor (BDNF) is important for sustaining existing neurons while promoting the growth of new neurons. Therefore, depletion of BDNF could account for the loss and damage of neurons during various neurodegenerative disorders. In PD, elevated CSF concentrations of BDNF relative to those of control subjects have been reported [78]. Since BDNF is a significant mediator of PD pathology, its role should be assessed further in other neurodegenerative conditions, specifically DLB due to similarities with PD in terms of existing pathology.

Dopamine and Dopamine Metabolites. As discussed above with respect to assessment of the transmitter system, indirect measurement of Dopamine (DA) concentration via its metabolites, Homovanillic acid (HVA), and Dihydroxyphenylacetic acid (DOPAC) may give additional information about factors that contribute to the pathology of neurodegenerative conditions where DA dysfunction occurs. One particular study measured DA, HVA, and DOPAC in different stages of PD [77]. This study also reported an exponential decrease of total DA in the CSF with disease progression, with a rapid drop during the initial phases of the disease onset, whereas an increased HVA/DA ratio (which indicates DA turnover) correlated largely with disease duration.

Hypocretin-1. Another potential biomarker highlighted due to the recognition of sleep-related disorders in neurodegenerative conditions is hypocretin -1. This peptide was initially associated with regulation of the sleep/wake cycle, along with various autonomic dysfunctions. It has since been identified in the CSF. A recent study conducted on PD subjects found a 40% decrease of hypocretin-1 in the prefrontal cortex and a 25% decrease in the ventricular CSF when compared to controls [122]. Using CSF samples from a wider range of neurological disorders, for example, DLB, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD),

along with PD, can help to characterise the pathological substrates of these sleep-related disorders [123]. However, while sleep disorders are symptomatically linked with these dementia syndromes, it seems as though one of the major indicators of sleep dysfunction (the reduction of orexin) is not altered in the CSF [74, 123].

Metals. Metals can contribute to both prooxidant and antioxidant processes within the body. Investigations of the substantia nigra in PD patients have demonstrated an increase of iron (Fe), which, due to the production of free radicals during its catalysis, could be a major contributor to oxidative damage [124]. This makes it the metal of interest when pursuing possible biomarkers for PD, along with Chromium (Cr) and Lead (Pb), which have been linked to PD cases. These three metals have been shown to be significantly reduced in CSF of PD patients relative to those of the controls [73]. However, these observations have to be evaluated in comparison to DLB patients.

3. Conclusions

Despite the neuroradiological advances aiding the clinical diagnosis of distinct subtypes of dementias, their overlapping clinical and neuropathological features make clear differentiation difficult in clinical practice. Similarly, the dementia clinical symptomatology varies from patient to patient, along with disease progression and severity. This has an impact on the pharmacological treatment and long-term care of the affected subjects. It is particularly important for DLB patients who have severe side effects to antipsychotic medication.

Although a number of research studies provide evidence that some of the investigated CSF biomarkers are well within the clinical criteria for sensitivity and specificity (>90%), they all seem to be characterised by the contradictory data for each of the major groups of biomarkers: α-synuclein, tau, and amyloid proteins. Similarly, results from the miscellaneous biomarkers studies have proved disappointing and nonconclusive. Having said that, a combination of CSF measures appear to emerge, which may well be able to differentiate DLB from other dementias: α-synuclein reduction in early DLB, a correlation between CSF α-synuclein and Aβ42 measures (characteristic for DLB only), and t-tau and p-tau181 profile (differentiating AD from DLB). Their usefulness in clinical setting needs to be explored further.

Identifying highly specific and sensitive peripheral analytes that reflect the key hallmarks of dementia that can be used in clinical setting is an imperative. Such analytes have been successfully identified for AD, having high sensitivity and specificity in differentiating this neurodegenerative disorder from other forms of dementia. Further work concentrating on improving the currently available CSF α-synuclein analytical tools may lead to further insights about the peripheral α-synuclein processing, which, either alone, or in a combination with known or novel analytes, may aid the differential diagnosis of DLB. In this respect, CSF proteomic studies (reviewed in [125]) have provided promising results, for example, identifying eight novel proteins which can differentiate between AD, PD, and DLB with a 95% specificity and sensitivity (see [79]; Table 5). Similarly, proteomic studies based on specific pathological features of neurodegenerative conditions have also proved noteworthy. By exploiting the fact that substantia nigra (SN) is the most vulnerable region within the brain to be affected by oxidative stress, Basso et al. [80] collected postmortem tissue samples from this region from subjects with PD and reported that 9 of the 44 proteins had a changed pattern of expression in the PD patients compared with the controls. The most significant of these proteins was the upregulation of peroxiredoxin II, a characteristic indication of oxidative stress (Entrez Gene).

Table 5: Proteomic studies.

Study	Technique(s) used	Sample(s) taken	Type(s) of dementia
Abdi et al. [79]	iTRAQ	CSF	DLB/AD/PD
Basso et al. [80]	MALDI-TOF-MS	SN tissue	PD
Davidsson et al. [81]	2D gel electrophoresis	CSF	AD
Wada-Isoe et al. [66]	SELDI-TOF-MS	Serum	DLB/AD
Yin et al. [82]	LC-MS/MS and 2-DE	CSF	AD/PD

Although promising, further DLB proteomic studies are warranted for better methodological approaches to include a larger number of well- defined samples and be able to address either the central or associated brain disease (dementia) process(es) and how they are reflected in the periphery (CSF, blood/blood derivates, and/or urine). Their further testing in routine clinical settings, alongside with the currently available clinical screening and diagnostic tools, should enhance the early dementia diagnosis and also aid the monitoring and therapeutic outcomes for DLB-affected subjects.

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Research Article

Combined Analysis of CSF Tau, Aβ42, Aβ1–42% and Aβ1–40^{ox}% in Alzheimer's Disease, Dementia with Lewy Bodies and Parkinson's Disease Dementia

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We studied the diagnostic value of CSF Aβ42/tau versus low Aβ1–42% and high Aβ1–40^{ox}% levels for differential diagnosis of Alzheimer's disease (AD) and dementia with Lewy bodies (DLB), respectively. CSF of 45 patients with AD, 15 with DLB, 21 with Parkinson's disease dementia (PDD), and 40 nondemented disease controls (NDC) was analyzed by Aβ-SDS-PAGE/immunoblot and ELISAs (Aβ42 and tau). Aβ42/tau lacked specificity in discriminating AD from DLB and PDD. Best discriminating biomarkers were Aβ1–42% and Aβ1–40^{ox}% for AD and DLB, respectively. AD and DLB could be differentiated by both Aβ1–42% and Aβ1–40^{ox}% with an accuracy of 80% at minimum. Thus, we consider Aβ1–42% and Aβ1–40^{ox}% to be useful biomarkers for AD and DLB, respectively. We propose further studies on the integration of Aβ1–42% and Aβ1–40^{ox}% into conventional assay formats. Moreover, future studies should investigate the combination of Aβ1–40^{ox}% and CSF alpha-synuclein for the diagnosis of DLB.

1. Introduction

Reduced amyloid-β (Aβ) 42 peptide concentrations and elevated tau levels in cerebrospinal fluid (CSF) represent supportive features of Alzheimer's dementia (AD) diagnosis [1]. These biomarkers have shown their major diagnostic value in comparison of AD to controls, but overlapping values have hampered sufficient diagnostic accuracy in differentiating other kinds of dementia, especially vascular dementias and dementia with Lewy bodies [2]. The specificity of Aβ42 in the differential diagnosis of AD and other dementias could be improved by measuring the relative Aβ1–42 concentration in CSF as compared to the sum of the peptides Aβ1–40, Aβ1–38, Aβ1–37, Aβ1–39, and oxidized Aβ1–40 (Aβ1–40^{ox}) as a percentage value (Aβ1–42%) [3]. Moreover, the percentage value of Aβ1–40^{ox} (Aβ1–40^{ox}%) has been proposed as a potentially helpful CSF biomarker in diagnosing DLB [3, 4].

This study investigates the additional diagnostic value of these novel CSF biomarker candidates as compared to the well-acknowledged combined analysis of tau and Aβ42 in differentiating the dementias AD, DLB, and PDD. For this purpose, CSF levels of tau, Aβ42, Aβ1–42%, and Aβ1–40^{ox}% were determined in CSF of 45 patients with probable AD, 15 with probable DLB, 21 with PDD and 40 nondemented disease controls (NDC). Their respective diagnostic accuracies for each relevant differential diagnostic quest were analyzed.

2. Patients and Methods

2.1. Patients. We investigated 121 CSF samples that were referred to our laboratory between 1999 and 2004. CSF concentrations of tau, Aβ42, Aβ1–42%, and Aβ1–40 were measured. Aliquots of these samples had been studied

previously under another objective and focussing a distinct issue of differentially diagnosing dementias [3].

CSF of patients with DLB and PDD, respectively, came from the Paracelsus-Elena Klinik, Kassel, a hospital specialized in the management of movement disorders. CSF samples of AD patients and nondemented disease controls came from the memory clinic and from wards at Goettingen University.

A psychiatrist and a neurologist rendered diagnoses based on thorough clinical examination, neuropsychological assessment, clinical records, and best clinical judgment. The investigators were blinded to the neurochemical outcome measures. Investigations were carried out with the informed consent of patients or their authorized caregiver. The study was conducted under the guidelines of the Declaration of Helsinki [5] and approved by the ethics committee of the University of Goettingen and Hessen.

The nondemented disease controls consisted of two subgroups.

2.1.1. Neurological Diseases without Dementia Syndrome. The 15 patients (6 women and 9 men) underwent lumbar puncture for routine investigation of central nervous affection. The patients were suffering from Parkinson's disease ($n = 6$), polyneuropathy ($n = 2$), genetically reconfirmed Huntington's disease ($n = 2$), spinocerebellar ataxia ($n = 2$), peripheral facial nerve palsy ($n = 1$), autosomal dominant hereditary spastic spinal palsy ($n = 1$) and amyotrophic lateral sclerosis ($n = 1$). The Mini Mental Status Examination (MMSE) score in patients with cognitive complaints ($n = 8$) was 28.0 ± 1.5 (mean ± SD). None of these patients displayed clinical features of dementia syndrome DSM IV or NINCDS-ADRDA criteria [6]. Age of this subgroup was 66.7 ± 6.9 years (mean ± SD).

2.1.2. Depressive Cognitive Complainers. The 25 depressive patients (16 women and 9 men) underwent lumbar puncture for differential diagnosis of cognitive complaints during the course of disease. The diagnosis of depression was made according to the criteria of DSM IV and cognitive impairment was assessed by MMSE at minimum. Patients with persistent cognitive decline for more than six months, MMSE score below 26 or clear focal atrophy in brain imaging (CT or MRI) were excluded. Age of this subgroup was 63.2 ± 10.4 years (mean ± SD).

2.1.3. Patients with Alzheimer's Disease. 45 patients (27 women and 18 men) fulfilled DSM IV criteria and NINCDS-ADRDA criteria for clinical diagnosis of AD [6]. Structural (CT or MRI) or functional (SPECT or PET) brain imaging displayed global cortical atrophy, or temporal, parietotemporal, or frontotemporal focal atrophy, or marked hypometabolism of these regions.

2.1.4. Patients with Dementia with Lewy Bodies (DLB) and Parkinson's Disease Dementia (PDD). Dementia with Lewy bodies (DLB, $n = 15$, 3 women and 12 men) was diagnosed according to the consensus criteria [7]. Patients presented with at least two core features according to the criteria and with parkinsonism less than one year before onset of dementia. Enrolled patients were hospitalized for several days to evaluate fluctuating cognition, extrapyramidal symptoms, and visual hallucinations.

Parkinson's disease dementia (PDD) was diagnosed in 21 patients (6 women and 15 men) according to UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria for idiopathic Parkinson's disease and the consensus criteria [7, 8]. All patients presented parkinsonism at least one year before onset of dementia.

The mean age and MMSE score of patient groups are given in Table 1.

2.2. Test Methods

2.2.1. Preanalytical Treatment of CSF. CSF was drawn by lumbar puncture into polypropylene vials and centrifuged (1000 g, 10 min, 4°C). Aliquots of 200 μL were kept at room temperature for a maximum of 24 hours before storage at −80°C for subsequent Aβ-SDS-PAGE/immunoblot. The samples were not thawed until analysis. The freezers had an automatic temperature control and alarm system, so that relevant temperature changes during the time of storage can be excluded. CSF for total Aβ and tau ELISA analysis was stored at +4°C and analyzed within two days. The protocol of preanalytical CSF handling was harmonized between the two centres of Goettingen and Kassel.

2.2.2. ELISA for Total-Tau and Aβ 1–42. The ELISAs Innotest hTAU Antigen ELISA and Innotest β-Amyloid$_{(1-42)}$ ELISA Innogenetics (Ghent, Belgium) served for quantification of CSF tau and Aβ 1–42, respectively. Both ELISAs were conducted according to published standard methods [9].

2.3. Aβ-SDS-PAGE/Immunoblot. Aβ peptide patterns were analyzed by Aβ-SDS-PAGE/immunoblot. For separation of Aβ peptides and subsequent detection, 10 μl of unconcentrated CSF were boiled in a sample buffer for SDS-PAGE, and Aβ-SDS-PAGE/immunoblot was conducted as published elsewhere [10, 11]. CSF samples of each individual patient were run as triplicates. Bands were quantified from individual blots of each patient relative to a four point dilution series of synthetic Aβ peptides using a charge coupled device camera. The detection sensitivity was 0.6 pg (Aβ1–38, Aβ1–40) and 1 pg (Aβ1–37, Aβ1–39, Aβ1–42), respectively. Signal acquisition was linear within a range of 3.8 magnitudes of order [10]. The inter- and intra-assay coefficients of variation for 20 to 80 pg of synthetic Aβ peptides were below 10% [10, 11].

2.4. Statistical Analysis. Aβ peptide and tau levels were expressed as absolute values (ng/ml). The data on Aβ peptide levels were obtained from individual blots of each patient. Aβ peptide values were determined in absolute (ng/ml) and percentage values relative to the sum of all investigated Aβ peptides (Aβ1–X%). We have characterized patient groups by mean and standard deviation (SD).

Table 1: Age, MMSE, Total tau, Aβ42, Aβ1–42%, and Aβ1–40^{ox}% in the CSF of the diagnostic groups.

Diagnosis	NDC ($n = 40$) mean ± SD	AD ($n = 45$) mean ± SD	DLB ($n = 15$) mean ± SD	PDD ($n = 21$) mean ± SD
Age	64.5 ± 9.3	70.9 ± 9.2	71.4 ± 7.6	73.2 ± 7.2
MMSE	28.6 ± 1.4	19.4 ± 5.8	19.2 ± 3.0	18.1 ± 7.2
Total tau (ELISA)[1]	0.23 ± 0.14	0.62 ± 0.34	0.37 ± 0.29	0.31 ± 0.24
Aβ1–42 (ELISA)[1]	0.79 ± 0.27	0.41 ± 0.14	0.37 ± 0.17	0.51 ± 0.22
Aβ42/Tau (ELISA)[1]	4.74 ± 3.03	0.87 ± 0.58	1.63 ± 1.35	3.16 ± 2.72
Aβ1–42% (Aβ-SDS-PAGE/immunoblot)[2]	11.65 ± 3.53	4.38 ± 0.89	7.13 ± 2.13	7.54 ± 2.07
Aβ1–42% (Aβ-SDS-PAGE/immunoblot)[2]	0.77 ± 0.5	0.88 ± 0.27	1.78 ± 0.70	1.05 ± 0.48

[1] Aβ peptide concentrations as measured by ELISA (ng/ml or ratio)
[2] Aβ peptide values of Aβ1–42 and Aβ1–40^{ox}, respectively, relative to the sum of all measurable Aβ peptides in the Aβ-SDS-PAGE/ immunoblot

The Mann-Whitney U-test was employed for comparisons of diagnostic groups. Multiple comparisons were not performed. Correlations of measured values were estimated by Spearman's Rho. The two-sided level of significance was taken as $P < .05$. The global diagnostic accuracies were assessed by the area under the curve (AUC) of receiver operating characteristic curve (ROC). Cutoff points were determined at the maximum Youden index [12], providing a sensitivity of ≥80%. The statistical software package SPSS, version 12.0, was used for computations.

3. Results

3.1. Test Results. The mean age of NDC was significantly younger than each of the dementia groups ($P < 5 \times 10^{-2}$). The dementia groups did not significantly differ from each other in age. The mean MMSE score did not significantly differ between the dementia groups.

Patients with neurological diseases without dementia syndrome exhibited higher levels of CSF Aβ1–40^{ox}% ($P = 6.1 \times 10^{-4}$) and lower levels of Aβ1–42 ($P = 1.3 \times 10^{-2}$) than depressive cognitive complainers. Nevertheless, for simplification, statistical analysis considered the two groups as one (NDC).

Table 1 summarizes mean age, MMSE, as well as CSF total tau, Aβ42, Aβ1–42%, and Aβ1–40^{ox}% levels of the diagnostic groups.

3.1.1. Neurochemical Phenotype of AD versus NDC. AD was characterized by decreased values of Aβ42 ($P = 1.8 \times 10^{-10}$) and Aβ1–42% ($P = 2.8 \times 10^{-15}$). In contrast, tau ($P = 4.8 \times 10^{-10}$) and Aβ1–40^{ox}% ($P = 1.1 \times 10^{-2}$) were elevated in AD.

3.1.2. Neurochemical Phenotype of DLB versus NDC. DLB patients showed lower levels of Aβ42 ($P = 3.3 \times 10^{-6}$) and Aβ1–42% ($P = 2.3 \times 10^{-5}$), but higher Aβ1–40^{ox}% concentrations than NDC ($P = 9.0 \times 10^{-6}$). Tau levels tended to be increased, but failed the level of significance.

3.1.3. Neurochemical Phenotype of PDD versus NDC. PDD patients showed lower levels of Aβ42 ($P = 1.4 \times 10^{-4}$) and Aβ1–42%($P = 1 \times 10^{-5}$) than NDC. Aβ1–40^{ox}% was elevated in PDD ($P = 1.7 \times 10^{-2}$). Tau was unchanged between PDD and NDC.

3.1.4. Neurochemical Phenotype of AD versus DLB and PDD. AD displayed lower Aβ1–42% levels than DLB ($P = 5.9 \times 10^{-7}$) and PDD ($P = 4.2 \times 10^{-7}$). Aβ42 levels did not significantly differ from DLB and PDD. Aβ1–40^{ox}% was lowered in DLB ($P = 2.6 \times 10^{-6}$), but did not significantly differ from PDD. Tau levels were elevated in AD as compared to DLB ($P = 2.8 \times 10^{-3}$) and PDD ($P = 7.1 \times 10^{-5}$), respectively.

3.1.5. Neurochemical Phenotype of DLB versus PDD. The main differences were elevated levels of Aβ1–40^{ox}% in DLB ($P = 1.3 \times 10^{-3}$). Aβ42 was lower in DLB ($P = 3.0 \times 10^{-2}$), whereas Aβ1–42% and tau were not significantly altered among the two groups.

3.2. Correlations. Analysis of each diagnostic group gave the following significant correlations. In NDC, Aβ42 and Aβ1–42% were positively correlated to each other. Higher values of Aβ1–40^{ox}% were correlated with male sex. Negative correlations were observed between Aβ1–42% and age as well as Aβ1–40^{ox}% and MMSE score. In AD, Aβ42 was positively correlated with Aβ1–42% and male sex, respectively. In PDD, Aβ42 was positively correlated with Aβ1–42%, but negatively with tau levels. No significant correlations were observed in the DLB group.

3.3. Estimates. The results of ROC analysis for each relevant differential diagnostic testing are summarized in Table 2. Figures 1–3 show Receiver operator curves for the most relevant differential diagnostic testings.

4. Discussion

4.1. Biomarker Patterns in the Different Dementia Groups. In agreement with numerous previous studies, we found high levels of tau accompanied by low CSF Aβ42 levels in AD in contrast to nondemented disease controls [2, 13]. In DLB and PDD, these biomarkers displayed a rather unspecific pattern: tau proteins have been found to be in

Table 2: Cutoff points, sensitivities, and specificities.

differential diagnosis	Parameter	cut off	sensitivity	specificity	Youden index	AUC	95%-CI
AD versus DLB	Aβ42/tau	1.163	80%	53%	0.33	0.664	0.483–0.845
	Aβ1–42%	5.093	80%	100%	0.80	0.933	0.872–0.994
	Aβ1–40^{ox}%	1.144	89%	87%	0.76	0.908	0.802–1.014
AD versus PDD	Aβ42/tau	1.450	91%	67%	0.58	0.775	0.630–0.919
	Aβ1–42%	5.730	93%	86%	0.79	0.889	0.773–1.005
	Aβ1–40^{ox}%	1.104	87%	43%	0.30	0.592	0.420–0.763
AD versus DLB and PDD	Aβ42/tau	1.450	91%	56%	0.47	0.728	0.610–0.747
	Aβ1–42%	5.093	80%	92%	0.72	0.907	0.834–0.981
	Aβ1–40^{ox}%	1.104	87%	61%	0.48	0.723	0.600–0.847
DLB versus PDD	Aβ42/tau	3.229	93%	43%	0.36	0.663	0.487–0.840
	Aβ1–42%	8.855	80%	33%	0.13	0.597	0.395–0.799
	Aβ1–40^{ox}%	1.244	80%	71%	0.51	0.810	0.667–0.952
DLB versus AD and PDD	Aβ42/tau	0.546	80%	26%	0.06	0.560	0.396–0.723
	Aβ1–42%	5.198	87%	61%	0.59	0.765	0.658–0.871
	Aβ1–40^{ox}%	1.144	87%	80%	0.67	0.877	0.769–0.985

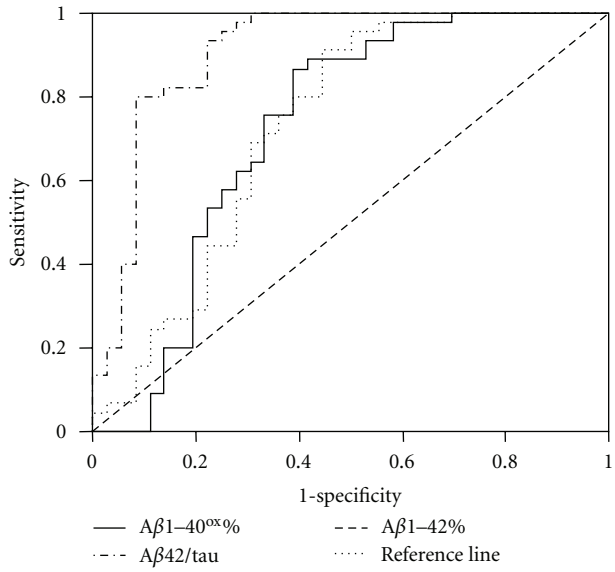

Figure 1: Receiver operator curves for detection of AD among DLB and PDD as a combined group using Aβ42/tau, Aβ1–42% and Aβ1–40^{ox}%, respectively.

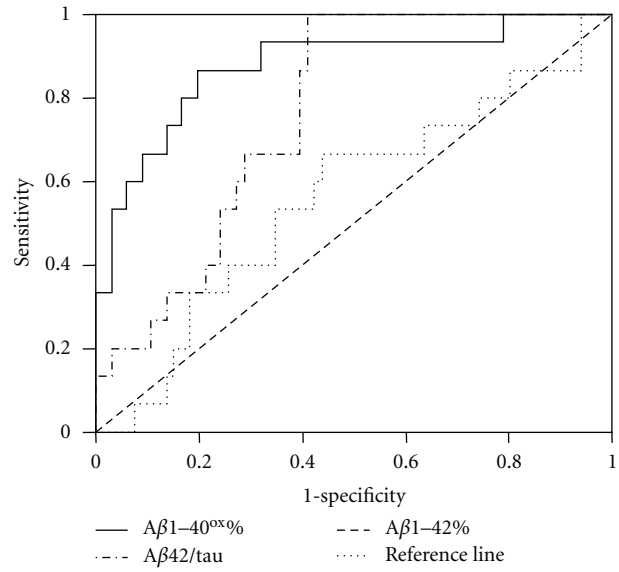

Figure 2: Receiver operator curves for detection of DLB among AD and PDD as a combined group using Aβ42/tau, Aβ1–42% and Aβ1–40^{ox}%, respectively.

a normal range or slightly increased, paralleled by mildly to moderately decreased CSF Aβ1–42 levels [14–19]. Rises of CSF tau levels have also been detected in Creutzfeldt-Jakob Disease (CJD), vascular dementias and after acute stroke [13, 20, 21], indicating tau to be a sensitive biomarker for neurodestruction, but unspecific for the underlying disease process. The range of results for tau levels in DLB and PDD may result from some unexpected variance of values depending on the actual dynamic of neuronal decay at the time of lumbar puncture. Moreover, clinical diagnosis of DLB and PDD may be confounded with AD and vice versa. The selection of control groups varies among the different studies. In the present study, we compare dementia groups to diseased controls that include neurodegenerative disorders, like Parkinson's disease. This may lead to a higher overlap of CSF tau values than in studies in which healthy controls served for comparison. Especially, when taking into account that PDD may be considered as a clinical state of Parkinson's disease.

The decrease of raw CSF Aβ42 concentrations can also be found in dementias other than AD, but then often in the wake of an overall drop of CSF Aβ peptides [3, 4]. In contrast, the selective decrease of the Aβ1–42 concentration as compared to constant Aβ-overall concentrations is more specific for AD [3]. In line with previous results, the diagnostic accuracy between AD and other dementias could be clearly improved

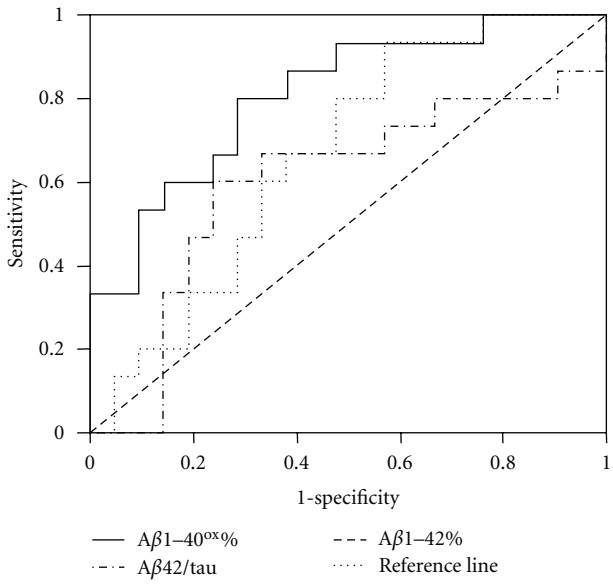

Figure 3: Receiver operator curves for differentiating DLB from PDD using Aβ42/tau, Aβ1–42% and Aβ1–40^{ox}%, respectively.

by scaling Aβ42 as a percentage portion of the sum of all investigated Aβ peptides (Aβ1–42%) [3].

Regarding Aβ1–40^{ox}%, the present study confirms our previous results of its elevated CSF levels in DLB [4]. Remarkably, Aβ1–40^{ox}% was only mildly elevated in AD and PDD as compared to controls, leading to a considerably smaller area of overlapping values in comparison to DLB.

4.2. Diagnostic Accuracies for AD and DLB Using Aβ42/Tau, Aβ1–42% and, Aβ1–40^{ox}%, Respectively. According to the references of The Working Group on "Molecular and Biochemical Markers of Alzheimer's Disease" [22], reasonable diagnostic accuracies of the tau/Aβ1–42 ratio have been reported for detecting AD among nondemented, either healthy or diseased controls [23]. The specificity of this marker combination declined considerably down to 58% when differentiating AD from other neurodegenerative dementias, due to a large overlap of values [9]. We found similar results in the present study. In contrast, disease specific changes of CSF Aβ peptide patterns in AD and DLB enabled higher accuracies for their differential diagnosis, also in discrimination to PDD. With accuracies of 80% at minimum, low CSF levels of Aβ1–42% were the most accurate biomarker for diagnosing AD among PDD alone and in a combined group of DLB and PDD. For the differentiation of AD from DLB, Aβ1–42% and Aβ1–40^{ox}% yielded comparable accuracies of 80% at minimum. The differential diagnosis of DLB and PDD could be made at a sensitivity and specificity of 80% and 71%, respectively, using Aβ1–40^{ox}% as the most accurate biomarker. These accuracies fall within the range of the aforementioned requirements or come close to it [22].

The reason for relative Aβ peptide values being superior to raw Aβ levels include: (i) Aβ1–42, but not Aβ1–40/Aβ1–42 showed a *U*-shaped natural course in normal aging [24]; (ii) in contrast to absolute Aβ peptide values, the relative abundances remained largely stable after different preanalytical procedures [11, 25]; (iii) referencing Aβ1–42 to Aβ1–40 avoids false positive and negative AD diagnosis in patients with constitutionally low and high CSF Aβ42 levels, respectively [26]; and (iv) dementias with low Aβ42 levels in the course of an overall decrease of CSF Aβ peptides will be sorted out from the diagnosis of AD [3]. The whole amount of CSF Aβ peptides measurable in the Aβ-SDS-PAGE/immunoblot is closely correlated to CSF Aβ1–40 levels [26]. This makes it possible to insert the ratio Aβ1–42/Aβ1–40 as a substitute for Aβ1–42%. Thus, the above considerations apply to both Aβ peptide ratios and percentage Aβ peptide values.

4.3. Conclusions. We consider CSF Aβ42/tau to be a sensitive biomarker for detection of AD, but not specific enough for excluding other forms of dementia, like DLB and PDD. Yielding contrasts of 80% or greater, decreased CSF Aβ1–42% and elevated Aβ1–40^{ox}% are promising biomarker candidates for AD and DLB, respectively. However, the pathophysiological meaning of these biomarkers in the development of AD and DLB remains to be clarified.

The further progress of Aβ-peptide patterns as applicable biomarkers requires validation in independent studies on neuropathologically confirmed cases. Under this respect, we recently showed that Aβ1–40^{ox}% does not differ among clinically and neuropathologically defined cases of DLB [27]. The major component of Lewy bodies, α-synuclein, displayed reduced CSF levels in Parkinson's disease and DLB as compared to AD and controls [28, 29]. For future studies on differentially diagnosing DLB, we propose the investigation of combined CSF α-synuclein and Aβ1–40^{ox}% levels. Furthermore, there is a need for translating the measurement of Aβ1–42% and Aβ1–40^{ox}% into more common assay formats, like ELISA [30].

4.4. Limitations of the Study. Our results are limited by the reliance on clinical diagnosis results, because of potential misclassification. Another point of concern is the size of patient groups for DLB and PDD.

Abbreviations

Aβ peptides:	amyloid-beta peptides
Aβ-SDS-PAGE/immunoblot:	amyloid-beta-sodium-dodecyl-sulphate-polyacrylamide-gel electrophoresis with western immunoblot
AD:	Alzheimer's disease
CCD-camera:	charge coupled device camera
CSF:	cerebrospinal fluid
DLB:	dementia with Lewy bodies
ECL:	enhanced chemiluminescence
ELISA:	Enzyme Linked Immunosorbent Assay

MMSE:	Mini-Mental-Status Examination
NINCDS-ADRDA:	National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association
NDC:	nondemented disease controls
PDD:	Parkinson's disease dementia
SDS:	sodium dodecyl sulphate.

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Clinical Study

CSF Biomarkers Profile in CADASIL—A Model of Pure Vascular Dementia: Usefulness in Differential Diagnosis in the Dementia Disorder

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Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is considered a model of pure vascular dementia (VD) because it occurs in young adults unlikely to have concomitant age and Alzheimer's Disease-(AD-) related pathology. CSF levels of β-amyloid 1-42 (Aβ42), total tau protein (t-tau), and phosphorylated tau-protein (p-tau), well accepted biomarkers of AD, were evaluated in 10 CADASIL patients, 22 AD patients, and 17 healthy age-matched subjects. Innotest β-amyloid 1-42, Innotest hTAU-Ag, and Innotest Phospho-tau 181p sandwich enzyme-linked immunoassay were used to determine CSF biomarkers levels. A case-control statistical analysis was carried out. CSF Aβ42 levels were significantly lower in CADASIL patients and considerable overlap with AD whereas t-tau and p-tau levels were normal and significantly different with respect to AD. A significant altered CSF biomarkers profile in a pure VD supports the use of CSF Aβ42, t-tau, and p-tau levels in the differential diagnosis of VD and AD.

1. Introduction

Despite the increased use of widely accepted diagnostic criteria over the last 1-2 decades, the differential diagnosis between Alzheimer disease (AD) and vascular dementia (VD), the most common causes of dementia in the elderly, is not always easy in the clinical practice. Many cognitively impaired patients, with a progressive history of classical cortical dementia, show different degrees of concomitant vascular lesions [1]. Vascular comorbidity may be present in 30%–60% of AD patients [2], and, conversely, AD pathology may be present in 40%–80% of VD patients [3], thus hindering diagnosis accuracy. Further, because the prevalence of both AD and VD increases with age, the coexistence of AD and VD in the elderly patients would also be expected to occur.

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is an inherited microvascular disease associated with a wide range of symptoms including migraine, mood disorders, and recurrent subcortical TIA/strokes [4, 5]. Two thirds of CADASIL patients early develop subcortical dementia, usually in the fourth-fifth decades of life [6]. Due to its monogenic nature and occurrence in young adults, CADASIL is considered a useful model of VD, in whom age- and AD-related pathology unlikely to coexist [7].

Beta-amyloid$_{1\text{-}42}$ (Aβ42), total tau (t-tau), and phospho-tau (p-tau) proteins, indices of amyloid deposition, axonal damage, or tangle formation respectively, have been suggested as biomarkers for the diagnosis in dementia disorders [8]. The CSF profile of these proteins in patients with AD is characterized by decreased Aβ42 and increased t-tau and p-tau levels [9, 10]. On the contrary in VD, studies on these CSF biomarkers showed conflicting results: t-tau levels have been reported to be increased [11–13], normal [14, 15] or intermediate [16, 17], but in any case much lower than

in AD; Aβ42 CSF levels in VD have been reported to be moderately decreased [18] or significantly overlapping with AD [19]. Few works deal with p-tau reporting either normal [20] or increased CSF levels [21] in VD.

Since previous studies showed contradictory results in VD, we evaluated CSF biomarkers in CADASIL, a young-onset monogenic disease which offers a unique opportunity to define CSF biomarkers profile in a pure VD. For this purpose we assessed CSF Aβ42, t-tau, and p-tau levels in ten CADASIL patients, comparing the results with those obtained for twenty-two AD patients and seventeen control subjects.

2. Patients and Methods

After obtaining informed consent, we measured CSF Aβ42, t-tau, and p-tau levels in CADASIL patients, AD patients, and control subjects.

CADASIL group included ten genetically confirmed patients (age range 49–66 years), followed in our department and already described in previous works by our group [22, 23]. Patients have been enrolled in a study on CSF biomarkers for the early diagnosis of dementia approved by local ethical committee. Detailed demographic data and molecular features of CADASIL patients are reported in Table 1.

The AD group consists of twenty-two patients with probable AD (age range 58–74 years) diagnosed in our department according to the NIN-CDS-ADRDA criteria [24]. AD patients with more than one cardiovascular risk factor and patient with even 1-2 white matter lacunes were excluded. None of the AD patients was under treatment for dementia at the time of lumbar puncture.

The control group included seventeen age-matched subjects with polyneuropathy and without signs of cognitive decline or central nervous system lesions.

Overall cognitive performance was evaluated in all three groups by Mini-Mental-State Examination (MMSE). MMSE scores below 26 indicate the presence of cognitive impairment.

CSF was obtained by standard procedures, collected in polypropylene tubes and immediately centrifuged. All blood contamination-free samples were stored at -80°C until assay, CSF levels of Aβ42, t-tau, and p-tau were determined by sensitive sandwich enzyme-linked immunoassay ELISA: Innotest β-amyloid 1-42, Innotest hTAU-Ag, and Innotest Phospho-tau 181p (specific for tau proteins phosphorylated at threonine 181) (Innogenetics, Ghent, Belgium). All determinations were done in duplicate.

3. Statistical Analysis

The nonparametric test of Kruskal-Wallis was used instead of the Analysis of Variance (ANOVA) because Levene's test rejected the hypothesis of equality of sample variances. Pairwise group comparisons were then made using the nonparametric test of Mann-Whitney with Bonferroni correction of type-I error probability.

A statistical significance level of 95% (type-I error probability, P, less than .05) was considered for all tests.

Statistical analysis was executed with the statistical package SPSS 10.

4. Results

Descriptive statistics of clinical features and CSF levels of Aβ42, t-tau, and p-tau, including median and interquartile distance (25th to 75th percentile), for CADASIL, Alzheimer patients, and controls are reported at the bottom of Table 1. Figures 1(a)–1(c) supply a box plot representation of CSF grouped data.

Aβ42. CSF Aβ42 levels were significantly lower both in CADASIL and in Alzheimer patients than in controls (Kruskal-Wallis and Bonferroni-corrected Mann-Whitney tests, $P < .05$). However CSF Aβ42 levels in CADASIL did not significantly differ from those in Alzheimer (Bonferroni-corrected Mann-Whitney test, $P > .05$). Group differences are illustrated in Figure 1(a): grey boxes, which represent group interquartile intervals (25th to 75th percentiles), overlap only for CADASIL and Alzheimer data.

t-tau and p-tau. Statistically significant higher values were found in Alzheimer with respect to both control and CADASIL subjects (Kruskal-Wallis and Bonferroni-corrected Mann-Whitney tests, $P < .05$), while these last two groups are not result statistically different (Bonferroni-corrected Mann-Whitney test, $P > .05$). Figures 1(b) and 1(c) show clear differences.

5. Discussion

Many studies evaluated changes in CSF Aβ42 and tau protein in prodromal stages of AD or in other types of dementia. Tau is an axonal protein that binds to tubulin in microtubules, promoting their assembly and stability [25]. Elevated CSF levels of t-tau and p-tau have been interpreted as indicators of ongoing neuronal and axonal degeneration and/or the presence of neurofibrillary tangles in the brain [26]. β-amyloid (Aβ) peptides are a major component of amyloid plaques deposited in the brain of patients with different neurodegenerative diseases. Decreased CSF Aβ42 levels have been ascribed to accumulation in senile plaques [27]. However alternative mechanisms, such as formation of a CSF chaperon complex with high-affinity binding and epitope masking of Aβ42, have also been reported [28].

Low CSF Aβ42, high t-tau, and p-tau levels are the typical pattern in AD, whereas in VD, until now, results on CSF biomarkers are conflicting [17, 18].

Ethnic and methodological differences may account for this discrepancy, as high assay variability is present amongst laboratories using the same ELISA test [29]. However VD is a heterogeneous entity and different authors may study different subpopulations of patients, and it is difficult to assess the specificity of CSF biomarkers profile. Furthermore, as many patients with VD also show cholinergic lesions of aging and AD, possible overlaps of vascular pathology and AD cannot be ruled out [30–32].

Table 1: Clinical and molecular features of CADASIL patients.

n°	CADASIL patients	age	sex	MMSE	mutation	CSF t-tau (pg/ml)	CSF A$\beta_{1\text{-}42}$ (pg/ml)	CSF p-tau (pg/ml)
1	PV	66	M	24	r207c eter ex4	200	307	40
2	GP	53	F	19	r207c eter ex4	205	193	30
3	MM	50	M	25	r207c eter ex4	174	275	32
4	BAS	52	M	23	r607c eter ex11	295	487	48
5	SS	57	M	15	r607c eter ex11	170	520	32
6	TA	49	M	21	r1076c eter ex20	296	675	45
7	DPM	58	F	29	frame shift aa127–158 stop codon aa159 ex4	438	746	43
8	JF	49	M	24	g528c eter ex11	166	585	29
9	SME	67	M	25	r1076c eter ex20	120	863	27
10	FUS	39	M	23	R332C ex 6	114	244	15
CADASIL patients (10)		54±8,4[a]	2F/8M[a]	23±3,8[a]		187 [155–295][b]	504 [267–693][b]	32 [28–43][b]
Alzheimer patients (22)		66±8,4[a]	15F/7M[a]	18±4,6[a]		1063 [748–1582][b]	340 [225–378][b]	106 [70–151][b]
controls (17)		58±9,9[a]	11F/6M[a]	30±2,2[a]		197 [167–210][b]	875 [793–1024][b]	32 [26–38][b]

MMSE: minimental state examination; [a]values are expressed as mean ± S.D.; [b]values are expressed as median [25th–75th percentile].

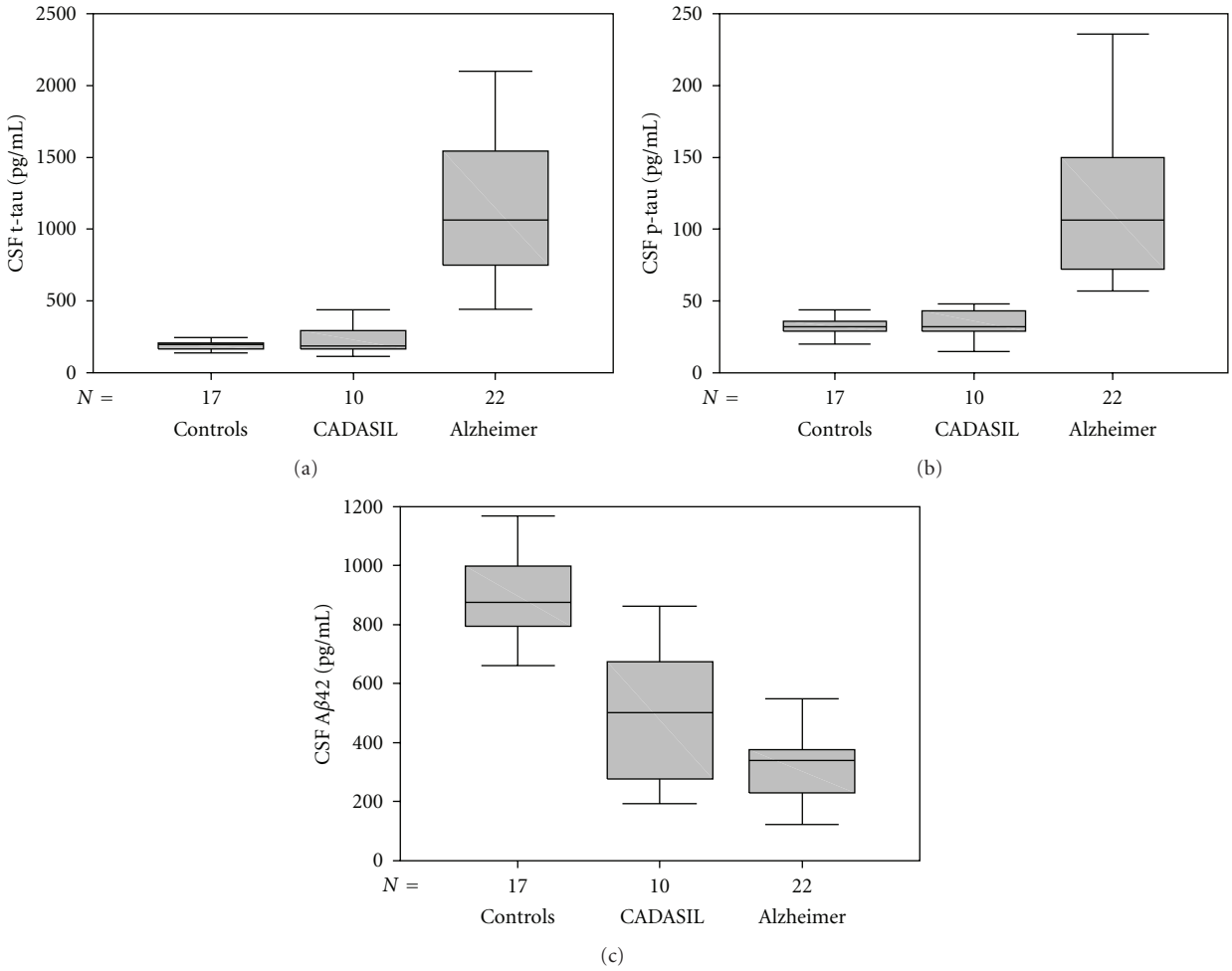

Figure 1: Box plots of CADASIL and Alzheimer patients and control subjects. They include median (horizontal line within box), interquartile interval, that is, 25th to 75th percentile (grey box) and range of variation (whiskers): (a) t-tau data, (b) p-tau data, and (c) A $\beta_{1\text{-}42}$ data.

In a previous study we reported significantly lower CSF Aβ42 levels in CADASIL patients than in controls whereas CSF t-tau and p-tau did not differ between the two groups [33].

In the present study the CSF biomarkers levels of the same CADASIL patients have been compared with those of AD patients showing an altered profile in CADASIL patients: Aβ42 levels were markedly decreased and considerably overlapped with AD, whereas t-tau and p-tau levels were normal and significantly different with respect to AD.

Recent studies on CSF biomarkers profile in sporadic VD showed a clear decrease of Aβ42 levels [19]. The mechanism of decreased CSF Aβ42 levels in cerebrovascular disease is unclear however the presence of these features in sporadic VD and in patients with a young-onset vascular disorder like CADASIL suggests that altered CSF Aβ42 levels may be related to subcortical vascular lesions and independent from an age- and AD-related pathology. Recently, a link between white matter lesions and low CSF Aβ42 has also been reported [34]. Moreover, the significant overlap of CSF Aβ42 levels in a pure VD and AD suggests to rule out any possible correlation between decreased CSF Aβ42 levels and accumulation in senile plaques and strengthens the most recent hypotheses about alternative mechanisms of Aβ42 reduction [28].

Normal t-tau and p-tau CSF levels we found in CADASIL contrast with the majority of the results of other groups on t-tau and p-tau values in sporadic VD. However, in the latter group of patients the possible presence of additional neuropathological changes associated with age and AD cannot be ruled out. Since CADASIL represents a model of pure VD, we can argue that t-tau and p-tau CSF levels in CADASIL patients closely reflect a pathological condition almost exclusively due to cerebrovascular features. We can thus suggest that CSF neurochemical phenotypes, especially t-tau and p-tau levels, sufficiently discriminate between AD and VD.

In conclusion, although our little sample size can reduce the statistical power of our results, significant differences in CSF biomarkers profile between pure VD, AD, and controls were found. These data support the use of CSF Aβ42, t-tau, and p-tau levels in the differential diagnosis of VD and AD.

In any case, this study represents a preliminary investigation whose statistical results should be confirmed by further examinations on larger data samples.

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