ELSEVIER

Neurobiology of Aging 31 (2010) 889–900

NEUROBIOLOGY OF AGING

www.elsevier.com/locate/neuaging

Early diagnostic accuracy and pathophysiologic relevance of an autopsy-confirmed Alzheimer's disease peripheral biomarker

Tapan Kumar Khan[a], Daniel L. Alkon[a,b,*]

[a] Blanchette Rockefeller Neurosciences Institute, West Virginia University,Morgantown, WV 26506-9301, USA

[b] Department of Neurology, West Virginia University, School of Medicine, Robert C. Byrd Health Science Center, PO Box 9100, Morgantown, WV 26506, USA

Received 25 July 2007; received in revised form 10 July 2008; accepted 11 July 2008

Available online 28 August 2008

Abstract

Treatment of Alzheimer's disease (AD) and the discovery of promising drug candidates depend on early diagnosis. Few currently available diagnostic tests have significantly improved this early uncertainty, while the "gold standard" diagnosis continues to require clinical dementia in life and the presence of pathologic brain lesions of amyloid plaques and neurofibrillary tangles in the brain at autopsy. Here, the inflammatory agonist bradykinin, a small nano-peptide, that induces PKC-mediated phosphorylation of Erk1 and Erk2 in fibroblasts, was applied to punch-biopsy-obtained human skin fibroblasts. Quantitative imaging of the phosphorylated Erk1 and Erk2 bands was then used in a ratio that is mathematically configured into an AD-Biomarker Index (AD-Index). In the population described here ($N = 264$), there were 64 autopsy examinations. Demented individuals were clinically diagnosed as AD with an overall accuracy of 78%. Among the 42 autopsy-confirmed cases for which there were also AD-Biomarker measurements, the overall accuracy of the AD-Biomarker was 98%. Among both the autopsy-confirmed and the clinically diagnosed patients, the AD-Index values were inversely correlated with the duration of disease, i.e., the time from the onset of dementia symptoms. Among the autopsy-confirmed cases, the AD-Biomarker diagnosis showed remarkably high sensitivity (97%) and specificity (100%) compared to clinical diagnosis (sensitivity: 78% and specificity: 20%). Using autopsy validation, the clinical diagnosis was only accurate at 52% level vs. the AD-Biomarker accuracy of 100% for cases with dementia not larger than 4 years of duration. Finally, application of soluble $A\beta_{1-42}$ to the fibroblasts of normal controls induced the abnormal AD-Biomarker phenotype, suggesting the pathophysiologic relevance of this AD-Biomarker measurement. In summary, the AD-Biomarker, as confirmed by autopsy validation, showed significantly higher sensitivity and specificity than did clinical diagnosis, particularly at early stages of disease, and pathophysiological relevance was demonstrated for the mechanistic basis of the AD-Biomarker measurements.

© 2008 Elsevier Inc. All rights reserved.

Keywords: Alzheimer's disease; Peripheral biomarker; Erk1/2 phosphorylation; PKC; Bradykinin; Aβ

1. Introduction

PKC signaling deficits have been implicated in Alzheimer's disease (AD) by pathologic changes in the brains and peripheral tissues of patients with dementia and the characteristic plaques and tangles (Masliah et al., 1991; Etcheberrigaray et al., 1993, 1994; Govoni et al., 1993). Such PKC signaling deficits have been further implicated by AD-specific changes of PKC regulated K^+ channels, AD-specific reduction of PKC isozymes by Aβ (Favit et al., 1998), enhanced phosphorylation of Erk1/2 induced by bradykinin-stimulated PKC (Zhao et al., 2002), and AD-specific reductions of PP2A (Zhao et al., 2003). Furthermore, the potent PKC activator, bryostatin 1, was shown to ameliorate increased levels of Aβ, and decreased survival of double transgenic mice for the *Swedish and London* mutations in the amyloid precursor protein (APP) gene (Etcheberrigaray et al., 2004). Finally, PKC signaling deficits can account for the characteristic loss of recent memory in AD, given the demonstration of memory-specific activation of endogenous PKC in a variety of brain regions specific to

* Corresponding author at: Blanchette Rockefeller Neurosciences Institute, 9601 Medical Center Drive, Rockville, MD 20850, USA.
Tel.: +1 301 294 7188; fax: +1 301 294 7007.
E-mail address: dalkon@brni-jhu.org (D.L. Alkon).

0197-4580/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.neurobiolaging.2008.07.010

diverse animal models of associative memory (Alkon et al., 2007).

Based on this accumulated evidence for the involvement of PKC signaling deficits in AD, we analyzed here the relationship of a PKC-based biomarker (the AD-Biomarker) to autopsy-confirmed and clinically confirmed cases of AD as well as the potential role of Aβ in generating diagnostic changes of AD in peripheral tissues such as skin fibroblasts.

Memory loss as well as cognitive impairment revealed by standardized neuropsychological tests such as the "Mini-mental" questionnaire, in the absence of other neurologic and/or psychiatric signs and symptoms help make an initial diagnosis of AD. This clinical diagnosis of AD becomes more certain with years of disease progression and further compromise of cognitive functions, particularly those involving memory. However, it is universally agreed that the current "gold standard" for making a diagnosis of AD consists of dementia in life and the presence of pathologic brain lesions of amyloid plaques and neurofibrillary tangles in the brain at autopsy.

The recent development of biomarkers for AD can be categorized into three classes: (i) biochemical measures of total tau, hyperphosphorylated tau (p-tau) and Aβ as cerebrospinal fluid (CSF) biomarkers, (ii) biophysical imaging of brain (positron emission tomography (PET)), magnetic resonance imaging (MRI), functional MRI, etc., and (iii) peripheral biomarkers using blood, skin samples and amyloid deposition in peripheral tissues. The diagnostic potential of levels of total tau, p-tau and $A\beta_{1-42}$ as biomarkers for AD has been studied extensively in CSF (Zetterberg et al., 2003; Wahlund and Blennow, 2003; Sunderland et al., 2003). *In vivo* brain amyloid plaque imaging by Pittsburgh compound B (PIB) with PET was found to be a promising biomarker for AD (Klunk et al., 2004). PIB enters into the brain rapidly (within ∼2 min) after intravenous injection and binds amyloid plaques at very low concentration (∼nM), and clears rapidly from brain.

Combination of biomarkers should provide better performance. An inverse relation between *in vivo* amyloid load imaging by the Pittsburgh compound B-positron emission tomography (PiB-PET) and CSF $A\beta_{1-42}$ was obtained (Fagan et al., 2006). The latter has been found to serve as antecedent biomarker of AD. Using a combined test based on evidence that high p-tau and low $A\beta_{1-42}$ levels in the CSF correlated with PET imaging studies improved (95% sensitivity and 83% specificity) the diagnosis of AD (Hansson et al., 2006). It has been shown that longitudinal CSF and MRI biomarkers improve the diagnosis of MCI (mildly cognitive impairment) patients (de Leon et al., 2006).

Generally AD is considered a central nervous system (CNS) disease, however, numerous biological tissues other than CNS, have been reported to be affected. These peripheral abnormalities found in blood cells, skin fibroblasts, peripheral vessels, and the lenses of eyes. Peripheral inflammatory cytokines in blood (Guerreiro et al., 2007; Ray et al., 2007) and p-tau and Aβ from blood plasma (Irizarry, 2004) have also shown some initial promise as biomarkers for AD. Abnormal cytosolic Aβ deposition in eye lenses in AD has been reported (Goldstein et al., 2003). C-reactive protein and homocysteine levels with low $A\beta_{1-40}$ and $A\beta_{1-42}$ were found to be interesting for peripheral AD-Biomarker (Davis et al., 2007; Locascio et al., 2008).

Several peripheral biomarkers have been tested from our laboratories, particularly in skin fibroblasts. These have involved human fibroblast K^+ channels (Etcheberrigaray et al., 1993, 1994), PKC isozymes (Govoni et al., 1993; Favit et al., 1998), Ca^{2+} signaling (Ito et al., 1994), and MAP kinase Erk1/2 phosphorylation (Zhao et al., 2002). In the K^+ channel test, a particular 113pS channel appeared less frequently in AD patients and $A\beta_{1-40}$ impaired this same channel (Etcheberrigaray et al., 1994). Similarly, it was also shown that $A\beta_{1-42}$ secretion was elevated from fibroblasts of familial AD (FAD) patients vs. controls (Scheuner et al., 1996). More recently, a new internally controlled MAP kinase AD-Biomarker revealed AD-specific differences of an Erk1/2 phosphorylation ratio in response to the natural inflammatory agonist and PKC activator, bradykinin (Khan and Alkon, 2006).

The PKC isozymes α, ε, and δ have been implicated in activating α-secretase, increasing the non-toxic soluble amyloid precursor protein (s-APPα), and, indirectly reducing the toxic Aβ, the degradation product of β-secretase-mediated cleavage of APP. MAP kinase Erk1/2 has also been reported to be an endogenous negative regulator of γ-secretase activity (Kim et al., 2006).

Values of Erk1 and Erk2 were found to be abnormal in previous observations of AD brains. A study by Veeranna et al. (2004) found that calpains are activated in AD brains in association with phosphorylated Erk1 and Erk2. After measuring two bands separately they found a relative increase of intensities of the phosphorylated Erk1 band as compared to the same phosphorylated Erk2 for AD brains but not in age-matched control brains. Furthermore, we previously found bradykinin (BK)-induced abnormalities of Erk1 and Erk2 phosphorylation in cultured skin fibroblasts from AD patients in comparison to control cases (Zhao et al., 2002).

The report examines the AD-Biomarker in an expanded population in relation to clinical diagnosis as well as in relation to autopsy diagnosis. Autopsy validation confirmed high clinical and AD-Biomarker diagnostic accuracy for pure AD cases for patients with more than 4 years of disease symptoms, while for early stage patients (i.e., ≤4 years disease duration), and for patients with comorbidity, only the AD-Biomarker showed high diagnostic accuracy.

In addition, for familial AD patients, skin fibroblasts showed enhanced secretion of $A\beta_{1-42}$ (Scheuner et al., 1996), while AD-specific reduction of specific K^+ channels were induced by $A\beta_{1-40}$ in normal human fibroblasts (Etcheberrigaray et al., 1994). For these reasons, we applied $A\beta_{1-42}$ to the normal fibroblasts to assess its impact on Erk1/2 phosphorylation. The pathophysiologic relevance of this

peripheral AD-Biomarker was tested by following $A\beta_{1-42}$-induced abnormalities of the AD-Biomarker.

2. Experiments

2.1. Materials

Bradykinin (molecular weight, 1060.2) was obtained from Calbiochem (San Diego, CA). Erk1/2 (MAPK) and phospho-p44/p42 Erk1/2 antibodies from rabbit were purchased from Upstate Cell Signaling solution (Lake Placid, NY) and Cell Signaling Technology (Danvers, MA), respectively. Tau AT8 monoclonal antibody was purchased from Pierce Biotechnology, Inc. (Rockford, IL). Secondary anti-rabbit with peroxy-conjugation was obtained from The Jackson Laboratories (Bar Harbor, ME). Biotinylated anti-mouse and anti-rabbit secondary antibodies and HRP-conjugated avidin were obtained from Ventana (Ventana Medical Systems, Tucson, AZ). Amyloid beta ($A\beta_{1-42}$) was obtained from American Peptide Company (Sunnyvale, CA).

2.2. Background for clinical diagnosis

Patients were recruited from the general pool of patients of speciality memory/AD clinics at Johns Hopkins University and its affiliated centers. Clinical diagnoses were made according to criteria developed by the National Institute of Neurologic and Communicative Disorders and Stroke and the AD and related Disorders Association (NINCDS-ADRDA). For the clinical diagnosis of AD, only "Probable AD" cases were considered as AD. Patients who met criteria for a clinical diagnosis of "Possible AD" were excluded from this study. In a double-blind framework (i.e., clinicians were blind to the scientists who made the AD-Biomarker measurements and vice-versa), patients reported for evaluation with a complaint of "memory loss" or related dementia symptoms such as "confusion" or "inappropriate judgment". There were no pre-determined selection criteria for enrollment other than the occurrence of some symptom(s) of dementia.

2.2.1. Autopsy diagnosis

An autopsy registry was created and neuropathological examinations were conducted upon death. The pathological diagnosis of AD was conducted according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al., 1991). These criteria have been the most widely used for the pathological diagnosis of AD in the U.S. and abroad, and are based on the semi-quantitative assessment of the density of neuritic plaques on silver-stained sections from various neocortical regions.

For the purpose of clinical–pathological correlation, we used the consensus recommendations for the post-morterm diagnosis of AD from the National Institute on Aging and the Reagan Working Group on diagnostic criteria for the neuropathological assessment of AD (Hyman and Trojanowski, 1997). Those recommendations were taken in concert with the CERAD neuritic plaque score and the neurofibrillary Braak score to express a likelihood (low, intermediate, or high) that the Alzheimer's lesions account for the dementia in the patient.

Further details of the autopsy protocol were as follows: during the post-mortem examination the brain was removed and fixed in 10% buffered formalin. A sample of the frontal pole was frozen and kept at −80 °C. Following two weeks of fixation, the brain was cut into 1-cm coronal slabs. Tissue samples for histology were taken from the cerebral cortex in each lobe, entorhinal cortex, hippocampus, amygdala, basal ganglia, thalamus, brain stem and cerebellum. These tissues were processed, embedded in paraffin, and cut at 10 μm thickness. Sections were stained with hematoxylin–eosin and Hirano silver method, a modification of the Bielchowsky method (Yamamoto and Hirano, 1986). Selected sections were immunostained for α-synuclein for the assessment of Lewy bodies and neurites, and for phosphorylated tau for the evaluation of neurofibrillary changes.

The assessment of neuritic plaques and neurofibrillary tangles was conducted primarily on silver stains (Braak and Braak, 1991). We used immunostains for tau, however, to complement the observations on silver stains and to solve occasional questions. Dementia with Lewy bodies were distinguished by examination of the substantia nigra and locus coeruleus with H&E stains plus α-synuclein immunostains. Tau immunostains were conducted with Tau AT monoclonal antibody with a 1:50 dilution using biotinylated secondary and HRP-conjugated avidin–biotin complex as tertiary. If AD pathology was identified in the autopsy brain, a diagnosis of AD was considered to have been made with or without the presence of co-existing morbidity due to other neuropathological diseases such as that of Parkinson's disease/Dementia with Lewy bodies, etc.

2.2.2. Patient populations

Sample sources, number of cases and their diagnoses are all illustrated in Tables 1A and 1B. Tissue-banked fibroblast samples were first obtained from non-demented controls, clinically diagnosed AD patients, and clinically diagnosed

Table 1A
Patient population and diagnoses (sample source: Johns Hopkins University and its affiliated centers; number of patients = 264)

Category of patients	Number of patients
Clinically confirmed AD patients	159
Clinically confirmed non-AD dementia patients	91
Healthy control cases (without heart disease, arthritis, cancer and no family history of AD)	14
Control cases with family history of AD (these cases were not within the total 264 pool)	16
Autopsy-confirmed patients (these patients were within the total 264 pool)	
Autopsy-confirmed AD patients	64
Autopsy-confirmed AD patients mixed with other dementia	12
Autopsy-confirmed non-AD dementia patients	4

Table 1B
Patient population and diagnoses (sample source: Coriell Cell Repository (Camden, NJ); number of patients = 31)

Category of patients	Number of patients
Clinically confirmed AD patients	10
Clinically confirmed non-AD dementia patients	11
Age-matched control cases	10
Autopsy-confirmed patients (these patients belong to total 31 pool)	
Autopsy-confirmed AD patients	2
Autopsy-confirmed non-AD dementia patients	1

non-AD patients from the Coriell Cell Repository (Camden, NJ). Coriell Cell Repository ($N = 31$) patients included AD, AC (age-matched controls) and non-AD dementia. The AD group included 5 familial AD cases, and 2 autopsy-confirmed cases and the remaining cases were sporadic, i.e., late onset, in nature. The Erk1/2 phosphorylation ratio was first tested on these Coriell Cell Repository samples before testing the samples that were freshly obtained from the clinic.

Clinically obtained samples were originally collected under contract from multiple hospital clinics in a study of an earlier BRNI (Blanchette Rockefeller Neurosciences Institute) biomarker (Zhao et al., 2002). The electronic images of the gels after Western blot from cell lysates after bradykinin stimulation/non-stimulation that were obtained from the earlier study were then made available to BRNI for the present study (sample sources: The Johns Hopkins University and affiliated centers). The images were available for a total of 159 patients who received the clinical diagnosis of AD, a total of 91 patients who received the clinical diagnosis of non-AD dementia (non-ADD, some form of dementia other than AD; such as Parkinson's disease, dementia with Lewy bodies, vascular dementia, frontotemporal dementia, etc.), and 14 age-matched healthy control patients (non-demented patients with no cancer, heart disease, arthritis, stroke and no family history of AD, and a mini-mental score of more than 27). Punch-biopsy samples were obtained from the upper arm, lower back, periumbilical area, or inner aspect of the thigh of patients. Of these 264 clinically obtained samples, a total of 64 also received an autopsy-validated diagnosis based on CERAD pathologic criteria. Not all data were available for every patient. Unavailability of one value (e.g., disease duration, AD-Biomarker value, etc.) occurred at random and for different reasons, such as lack of record keeping, loss of samples, etc. However, all data that were available for all patients were included, i.e., without selection, for all comparisons.

2.3. Biopsies and cell culture

Skin fibroblasts were taken from two different sources: (A) fresh-taken skin fibroblasts within the registry described above, and (B) banked skin fibroblasts from the Coriell Institute for Medical Research (Camden, NJ). The collection and culture of fibroblasts from freshly obtained skin tissue were performed as follows: punch-biopsy skin tissue samples from AD, non-AD dementia patients, and age-matched controls were obtained by qualified personnel at the Copper Ridge Institute (Sykesville, MD) and Johns Hopkins Hospital (Baltimore, MD). Clinical evaluation and skin biopsies were performed at the same time. All patients (or their representatives) signed informed consent forms. The Institutional Review Board at Johns Hopkins University approved the procedure as part of an ongoing research protocol. The outer keratinous layer of the skin tissue (biopsy sample) was removed after thorough rinsing with cold saline solution. The remaining part of the tissue was minced into small pieces (~1 mm). The pieces were kept in T-25 (25 cm^2) cell culture flasks. A few hours were allowed for the cells to adhere to the surface of the culture flasks. Three millilitres of DMEM culture solution containing 45% fetal bovine serum (FBS) and penicillin/streptomycin were carefully added into the flask and placed in a 5% CO_2 and 37 °C incubator for 3 days. After 3 days, 5 mL of additional culture media were added. All flasks were regularly examined and after 7–10 days they became confluent. Cells were trypsinized and expanded according to their number. The total number of cell passages was not allowed to exceed 16.

Banked fibroblasts from AD patients and age match controls were maintained and cultured in T25/T75 culture flasks with DMEM culture medium containing 10% FBS. The total number of cell passages was not allowed to exceed 16.

2.4. Western blot analysis

Confluent (90–100%) skin fibroblasts cells in T25 culture flasks were used for the actual bradykinin treatment. The regular culture medium was first removed and washed three times with DMEM medium without serum. The cells were kept serum-deprived for 16 h. A 10 nM BK challenge was conducted for 10 min (BK^+). For each patient, three pairs of BK and vehicle (DMSO, without BK, BK^-) samples were measured. After the treatment, the medium was removed and washed five times with cold 1× PBS (4 °C) on the ice. The flasks were then placed on dry ice/ethanol for 30 min. Homogenization buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 1% Triton X-100, 1% protease inhibitor mixture, 1% phosphatase inhibitor cocktails) was then added. The flasks were placed on an end-to-end rocker at 4 °C. After 30 min, cell extracts were collected with a cell scraper and sonicated three times on the ice and put on ice for 30 min. Cell lysates were centrifuged at 14,000 rpm (Eppendorf Centrifuge 5417R; Brinkman Instruments, Westbury, NY) for 20 min. The supernatant was collected as a cell lysate. Total protein assay was conducted using a standard BSA protein assay protocol. Cell lysates were mixed with equal volumes of 2× SDS sample buffer and boiled for 10 min. The resulting cocktails were loaded on 4–20% minigradient gels to conduct electrophoresis and this was followed by transfer of the protein onto a nitrocellulose membrane. After blocking the non-specific binding with nonfat milk for 1 h at room temperature, the blots were incubated with antibodies for total

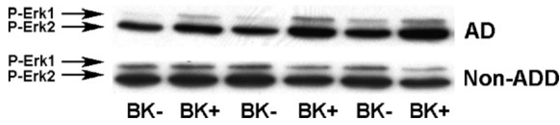

Fig. 1. Representative gels for Western blot analysis of autopsy-confirmed Alzheimer's disease (AD) and non-AD dementia (non-ADD) patients: skin fibroblasts cells were taken by biopsy and grown to 90–100% confluency. Serum starved cells were treated with bradykinin (BK^+) and vehicle (without bradykinin, BK^-) for 10 min and P-Erk was measured by phospho-specific Erk antibody.

phospho-Erk1/2 measurements at 4 °C for 16 h. Blots were washed and the phospho-Erk signal was detected by ECL using anti-rabbit secondary antibodies from Jackson Laboratories and recorded on film. Blots were then stripped with stripping buffer and probed with the anti-Erk antibody and signals were recorded as above. Signal intensity was recorded with a BioRad densitometer. For bradykinin-treated AD cells (BK^+), both the P-Erk1 and P-Erk2 were elevated compared to untreated cells, but not for non-AD dementia patients (Fig. 1). The AD-Biomarker Index (AD-Index, see Eq. (A)) was determined from such densitometric measurements of P-Erk1 and P-Erk2 (with and without BK stimulation) for all available skin fibroblasts from both CAVEAT and Coriell Cell Repository patients.

2.5. Data analysis

Clinical studies, autopsies and AD-Biomarker measurements were all performed at different locations and were unknown to the workers at each location (double blind). Strip densitometric analysis was conducted with software developed by BRNI (Dr. Tom Nelson, Blanchette Rockefeller Neurosciences Institute) to measure the intensities of specific bands from the images. Total intensity of Erk and P-Erk1 and P-Erk2 was measured separately. The ratios of P-Erk1 to P-Erk2 in the presence of BK (BK^+) and in the absence of BK (BK^-) were calculated separately. Values for P-Erk1 and P-Erk2 for each patient were measured in the same lane of the same gel, using the same antibody. In this way, sources of variation were internally controlled. From these measurements an Alzheimer's disease Index (AD-Index) was calculated as follows:

$$\text{AD-Index} = \left[\frac{\text{p-Erk1}}{\text{p-Erk2}}\right]^{BK^+} - \left[\frac{\text{p-Erk1}}{\text{p-Erk2}}\right]^{BK^-} \quad (A)$$

If the AD-Index became positive, the patient was considered as having an AD diagnosis, and if it was 0 or negative the patient was considered to have a non-AD dementia. The sensitivity, specificity, positive predictive value and negative predictive value were determined by a decision matrix test both for AD-Biomarker and clinical diagnosis patients with autopsy confirmation.

Table 2A
AD-Biomarker and clinical diagnosis of autopsy-validated pure AD patients

ID#	Age/sex	Disease duration (years)	AD-Index	AD-Biomarker diagnosis	Clinical diagnosis	Autopsy data
24	81M	2	0.12	AD	Non-ADD	AD
27	80F	–	0.03	AD	AD	AD
42	80F	9	0.01	AD	Non-ADD	AD
45	82F	–	0.05	AD	AD	AD
68	83M	6	0.11	AD	AD	AD
89	87M	11	0.05	AD	AD	AD
94	92F	2	0.04	AD	Non-ADD	AD
99	93F	11	0.24	AD	AD	AD
104	63F	6	0.04	AD	AD	AD
110	81F	0	0.13	AD	AD	AD
111	91F	7	0.02	AD	AD	AD
115	76M	7	0.07	AD	AD	AD
121	89F	4	0.07	AD	AD	AD
150	78F	20	0.10	AD	AD	AD
165	71F	6	0.005	AD	AD	AD
239	80F	6	0.05	AD	AD	AD
247	57F	5	0.01	AD	AD	AD
305	93M	10	0.05	AD	AD	AD
321	83F	8	0.06	AD	AD	AD
496	87M	10	0.05	AD	AD	AD
543	81M	4	0.06	AD	AD	AD
798	86F	6	0.09	AD	AD	AD
797	86M	5	−0.07	Non-AD	AD	AD
800	73F	7	0.07	AD	AD	AD
824	84F	7	0.12	AD	AD	AD

Correctly diagnosed (%): AD-Biomarker diagnosis, 24/25 → 96%; clinical diagnosis, 22/25 → 88%.

Table 2B
AD-Biomarker and clinical diagnosis of autopsy-validated mixed-AD patients

ID#	Age	Disease duration (years)	AD-Index	AD-Biomarker diagnosis	Clinical diagnosis (MMSE[a])	Autopsy data primary diagnosis/secondary diagnosis
18	88F	–	0.07	AD	AD	AD/LBD
83	84F	7	0.01	AD	AD (9)	AD/VD
114	79F	14	0.03	AD	AD	AD/PD
139	77F	6	0.04	AD	AD (6)	AD/VD
142	76F	4	0.02	AD	Non-ADD (15)	PD/AD
154	74M	6	0.11	AD	Non-ADD (16)	AD/LBD
237	89F	4	0.04	AD	AD	AD/LBD
281	69M	3	0.02	AD	PD (20)	PD/AD
283	81M	3	0.02	AD	Non-ADD (27)	PD/AD
461	78M	4	0.21	AD	AD (2)	AD/LBD
510	60M	Months	0.035	AD	AD	AD/VD
513	83M	3	0.02	AD	Non-ADD (11)	AD/PD

Correctly diagnosed (%): AD-Biomarker diagnosis, 12/12 → 100%; clinical diagnosis, 8/12 → 67%.
[a] MMSE: mini-mental score.

2.6. $A\beta_{1-42}$ treatment

Fibroblast cell lines from six control patients were treated with 1.0 μM $A\beta_{1-42}$ (in DMEM culture medium with 10% FBS), for 16 h in 5% CO_2 and 37 °C incubator after reaching 90–100% confluence. After the 16 h of incubation with 1.0 μM $A\beta_{1-42}$, the medium was removed and washed three times with regular culture medium without serum and kept for 16 h. After that bradykinin stimulation/non-stimulation (BK^+/BK^-) experiment was conducted followed by AD-Index measurement as described above.

3. Results

3.1. Performance of AD-Biomarker and clinical diagnoses validated with autopsy data

For autopsy-confirmed pure AD (Table 2A) cases the performance of the AD-Biomarker was remarkably high (96% accuracy for AD-Biomarker, and 88% accuracy for clinical diagnosis, respectively). However, the accuracy of clinical diagnosis (67%) was quite low compared to the AD-Biomarker (100%) for patients who had AD mixed with other dementias (Table 2B). When clinical diagnosis alone, i.e., in the absence of autopsy validation, was used to validate the AD-Biomarker diagnosis of AD, there was an overall agreement of 82%. The agreement was slightly greater (85%) for AD after 4 years vs. before 4 years (80%) from the time of symptom's onset. Clinical diagnosis often agreed with the AD-Biomarker positive diagnosis of AD, but this was frequently not true with non-AD dementia. Among the five autopsy-confirmed non-AD dementia patients, clinical diagnosis was able to only detect one accurately, whereas the AD-Biomarker detected all five cases (Table 2C). From our registry we had 64 patients that had clinical as well as autopsy data. There were 42 patients that had all three types of information, e.g., AD-Biomarker diagnosis, clinical diagnosis and autopsy data. The performance of the clinical diagnosis on 64 autopsy-confirmed patients was moderate (78%) and was quite comparable with other clinical studies. The patients of Fig. 2 were based on Table 2, although the numbers of patients were not equal, because the disease duration, and/or symptom duration were not available for all patients.

3.2. Performance of AD-Biomarker for the healthy non-demented control sub-group validated with clinical diagnosis

The performance of the AD-Biomarker was also quite high for the subgroup of healthy non-demented control (with no cancer, heart disease, arthritis, stroke and no family history of AD, and a mini-mental score of more than 27) cases (Table 3A). To test the potential predictive capability of the AD-Biomarker, biomarker measurements were made on 16 clinically confirmed (not autopsy) non-demented control

Table 2C
AD-Biomarker and clinical diagnosis of autopsy-validated non-AD dementia patients

ID#	Age	Disease duration (years)	AD-Index	AD-Biomarker diagnosis	Clinical diagnosis (MMSE[a])	Autopsy data
331	81F	6	−0.07	Non-ADD	AD (15)	Other[b]
549	74M	3	−0.02	Non-ADD	AD	LBD
569	85M	8	−0.02	Non-ADD	AD (11)	Other[b]
822	79M	8	−0.12	Non-ADD	AD	Other[b]
AG08395	85F	2	−0.02	Non-ADD	PD	PD

Correctly diagnosed (%): AD-Biomarker diagnosis, 5/5 → 100%; clinical diagnosis, 1/5 → 20%.
[a] MMSE: mini-mental score.
[b] Other: taupathy, frontotemporal dementia, vascular dementia, motor neuron disease-inclusion dementia (MNDID).

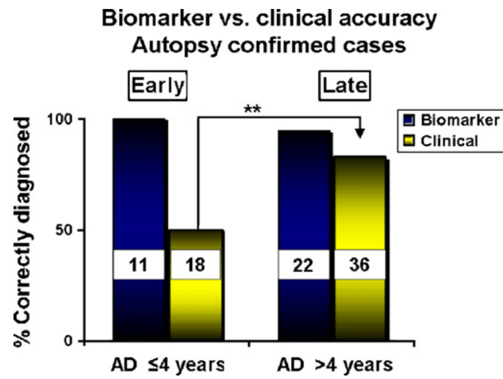

Fig. 2. AD-Biomarker vs. clinical accuracy of autopsy-confirmed patients: the patients were categorized into two groups: (i) early (≤4 years of disease duration) and (ii) late (>4 years of disease duration). Disease duration of the patients was estimated from the date of biopsy and the day that the first symptoms of dementia were observed. In the case of early onset patients (≤4 years of disease duration), AD-Biomarker outperformed clinical diagnosis. For the category >4 years of disease duration, clinical diagnostic performance was improved, but remained somewhat lower than that of AD-Biomarker performances. The number of patients is indicated in the bar for each group.

cases with a family history of AD (Table 3B). Almost half of the tests were positive for the diagnosis of AD. In contrast, only 14% of non-demented control cases (without autopsy and without family history of AD, and no record of cancer, heart disease and arthritis), showed a positive AD diagnosis.

3.3. Early vs. late onset of dementia

The AD-Biomarker accurately distinguished AD from non-AD dementia for cases diagnosed (and skin samples taken) within the first 4 years of symptom onset (Fig. 2). Clinical diagnoses, however, when validated by subsequent autopsy diagnosis were not accurate within the first 4 years of the onset of symptoms of dementia (Fig. 2). When subjected to autopsy validation, both the AD-Biomarker and clinical diagnosis did show high accuracy for patients that were seen (and samples taken) after the first 4 years of the onset of dementia symptoms. Clinical diagnosis of mixed AD patients was not always correct particularly for patients with disease duration of ≤4 years.

Table 3A
Clinically validated AD-Biomarker diagnosis of age-matched healthy control patients (without family history of AD, cancer, heart disease, stroke, and arthritis)

ID#	Age/sex	AD-Index	AD-Biomarker	Clinical (MMSE)[a]
87	79M	−0.02	Non-AD	Non-AD (28)
133	44F	−0.05	Non-AD	Non-AD (30)
174	60F	−0.04	Non AD	Non AD (30)
296	76F	−0.05	Non-AD	Non-AD (29)
297	83M	0.04	AD	Non-AD (29)
357	74F	−0.01	Non-AD	Non-AD (30)
366	72F	−0.18	Non-AD	Non-AD (30)
387	88M	−0.07	Non-AD	Non-AD (28)
411	80F	0.03	AD	Non-AD (28)
464	77F	−0.03	Non-AD	Non-AD (30)
476	83F	−0.03	Non-AD	Non-AD (28)
478	87M	−0.26	Non-AD	Non-AD (27)
479	68F	0.0	Non-AD	Non-AD (28)
556	81F	0.0	Non-AD	Non-AD (30)

[a] MMSE: mini-mental score.

Table 3B
AD-Biomarker performance of clinically confirmed non-demented control cases with family history of AD

ID#	Age (years)	AD-Index	AD-Biomarker diagnosis
73	84	−0.01	Non-AD
102	60	−0.11	Non-AD
133	44	−0.05	Non-AD
137	57	0.12	AD
169	60	−0.01	Non-AD
170	61	0.02	AD
173	62	0.03	AD
178	60	0.11	AD
216	70	−0.06	Non-AD
306	80	0.15	AD
329	77	0.06	AD
377	76	−0.05	Non-AD
487	83	0.07	AD
515	82	−0.01	Non-AD
556	81	0	Non-AD
590	85	0.03	AD

Diagnosed as non-AD by AD-Biomarker (%) = 8/16 = 50%.

3.4. AD-Biomarker identifies AD in mixed dementia (co-morbidity) cases

Fig. 3 presents the performance of the present AD-Biomarker when AD was mixed with other dementia (co-morbidity) in comparison to clinical diagnosis for autopsy-confirmed cases. We had categorized all autopsy-confirmed patients into three groups, namely: (A) pure AD, (B) AD mixed with other dementia (co-morbidity), and (C) non-ADD. The accuracy of the AD-Biomarker was further confirmed by cases of co-morbidity when autopsy validation was available. As illustrated in Fig. 3, the AD-Biomarker accurately diagnosed the presence of AD in all 12

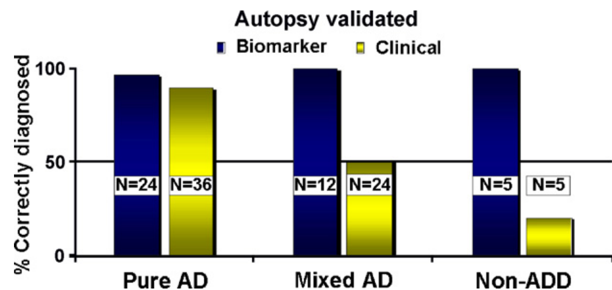

Fig. 3. AD-Biomarker identifies AD in mixed dementia (co-morbidity) cases: comparison of diagnostic performance of AD-Biomarker with clinical diagnosis for demented patients: autopsy-confirmed patients were categorized into three groups: (i) AD (Alzheimer's disease), (ii) mixed (AD diagnosis with other non-AD dementias, such as Parkinson's disease/dementia with Lewy bodies, vascular dementia etc.) and (iii) non-ADD (non-Alzheimer's dementia, such as dementia due to Parkinson's disease/dementia with Lewy bodies, vascular dementia, etc.). N = number of patients depicted in each column for each individual category.

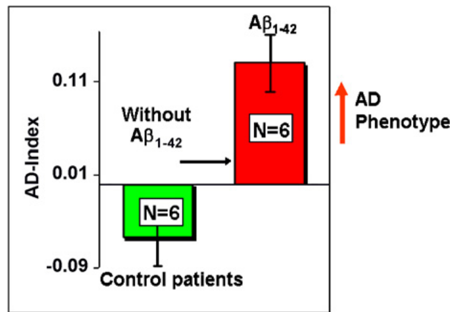

Fig. 4. Soluble Aβ induces AD-Biomarker on human fibroblasts: AD-Biomarker for each 6 normal control skin fibroblast cell lines (Coriell Cell Repository) was determined and the average AD-Biomarker was found to be negative. After over night treatment with 1.0 μM $A\beta_{1\text{–}42}$ of the same control skin fibroblast cell lines, the AD-Biomarker was measured for each cell line. The data show that $A\beta_{1\text{–}42}$ application converts the normal (negative) AD-Biomarker values to the abnormal (positive) values seen for AD patients.

cases when other causes of dementia (e.g., Parkinson's disease/dementia with Lewy bodies, etc.) co-existed with AD. The AD-Biomarker accurately identified 5/5 cases that were "pure" non-ADD without AD pathology (Table 2C). It should also be noted that clinical diagnosis, when validated by neuropathological diagnosis, did not accurately identify AD in these mixed cases and did not accurately distinguish AD from cases of non-AD dementia (Fig. 3).

3.5. Soluble Aβ induces Alzheimer's phenotype of human fibroblasts

Accumulating evidence suggests that soluble $A\beta_{1\text{–}42}$ can induce some of the neuropathology both with *in vitro* (brain slice) and *in vivo* administration. The AD-Biomarker was first measured for fibroblasts from age-matched control patients (Coriell cell repository) and was found to have the expected negative (normal) values of the AD-Biomarker (Fig. 4). After soluble $A\beta_{1\text{–}42}$ treatment (1 μM, 16 h), the fibroblasts were then found to have the AD-specific positive AD-Biomarker (Fig. 4). The first term of the AD-Index from Eq. (A) $[\text{p-Erk1/p-Erk2}]^{BK^+}$ became greater than $[\text{p-Erk1/p-Erk2}]^{BK^-}$ after $A\beta_{1\text{–}42}$ treatment for control fibroblasts. As a result the AD-Index values became positive after $A\beta_{1\text{–}42}$ treatment.

4. Discussion

4.1. Overall accuracy of AD-Biomarker and clinical diagnoses validated with autopsy data

The overall accuracy of the AD-Biomarker on autopsy-validated patients was quite high compared to clinical diagnosis (Table 2), particularly at the early stages of AD (≤4 years from symptom onset). All control patients were not autopsy-confirmed. It is well established that in a general population, ∼10% of people of age 75 years or older have AD and one out of three individual over the age of 85 years and more have AD. Among the control cases some of these may have already experienced the onset of AD to which the present AD-Biomarker may be responsive. It has also been previously established that the non-demented control cases with a family history of AD have an increased possibility to develop AD at older age (Desai and Grossberg, 1999; Pope et al., 2003).

The results of autopsy validation demonstrate the considerable inaccuracy of clinical diagnosis, particularly within the first 4 years of symptoms onset (Fig. 2). The overall accuracy of clinical diagnosis during this early period is slightly greater than 50%. This inaccuracy is particularly marked for non-AD dementia and cases of mixed dementia. In the latter group, the presence of other dementias such as those of Parkinson's disease/dementia with Lewy bodies or multiple strokes apparently masks the co-existing presence of AD. Because therapeutic decisions are likely to be most effective soon after symptoms of dementia begin, another diagnostic tool such as the AD-Biomarker is particularly needed.

The AD-Biomarker involves a ratio of Erk1 and Erk2 phosphorylation in response to the natural PKC agonist, bradykinin. Bradykinin, a nano-peptide, is a potent inflammatory mediator, produced by brain and peripheral cells under pathophysiological conditions such as, trauma, stroke, pain ischemia and asthma. It acts on the B2 bradykinin receptor (BK2bR), a G-protein coupled receptor. Bradykinin activates the PLC/phospholipids-Ca^{2+}/PKC cascade, which interacts with the Ras/Raf/MEK/MAPK signaling pathway that activates Erk1/2 (Yang et al., 2005). The autopsy validation of the AD-Biomarker appears quite promising in that it showed high accuracy for exactly all of the diagnostic challenges not met by clinical diagnosis. The AD-Biomarker correctly identified all of the non-AD dementia cases as well as all of the mixed AD dementia cases (Tables 2B and 2C). Overall accuracy of the AD-Biomarker was high even during the first 4 years within symptom onset (Fig. 2).

Most previously published studies show higher clinical accuracy because either (i) there was no autopsy validation, or (ii) if autopsy validation was included, a preponderance of cases of long-standing duration were studied. One particular previous study (Hogervorst et al., 2003), however, included autopsy validation as well as clinical diagnoses made at two successive times, one early in disease progression, a second much later. In this study, the overall clinical accuracy reported for the first clinical diagnosis (based on subsequent autopsy validation) was approximately 60%, quite close to our observations as discussed above.

4.2. Sensitivity and specificity of diagnosis based on autopsy validation

Among 42 autopsy-validated patients, 37 were AD and 5 were non-ADD. The AD-Biomarker successfully detected 36 AD diagnoses out of 37 autopsy-confirmed AD patients

Table 4A
Decision matrix test results for autopsy-confirmed cases (number of patients = 42)

	Present	Absent	Total
AD-Biomarker diagnosis[a]			
Positive	*a* (true positive), 36	*c* (false positive), 0	$a+c$, 36
Negative	*b* (false negative), 1	*d* (true negative), 5	$b+d$, 6
Total	$a+b$, 37	$c+d$, 5	42
Clinical diagnosis (same patients)[b]			
Positive	*a* (true positive), 29	*c* (false positive), 4	$a+c$, 33
Negative	*b* (false negative), 8	*d* (true negative), 1	$b+d$, 9
Total	$a+b$, 37	$c+d$, 5	42

[a] Sensitivity = $(a/a+b) \times 100 = 97\%$, specificity = $(d/c+d) \times 100 = 100\%$, positive predictive value = $(a/a+c) \times 100 = 100\%$, and negative predictive value = $(d/b+d) \times 100 = 83\%$.
[b] Sensitivity = $(a/a+b) \times 100 = 78\%$, specificity = $(d/c+d) \times 100 = 20\%$, positive predictive value = $(a/a+c) \times 100 = 88\%$, and negative predictive value = $(d/b+d) \times 100 = 11\%$.

and all 5 non-ADD. Decision matrix analysis showed that the sensitivity was quite high (97%) with a very high specificity (100%) for the AD-Biomarker, whereas clinical diagnosis showed moderate sensitivity and very low specificity (Table 4A). The positive and negative predictive values were always higher for biomarker diagnosis compared to clinical diagnosis (Table 4A). The results of decision matrix analysis of all 64 autopsy validated clinically confirmed patients also showed a similar trend (Table 4B).

4.3. Correlation of AD-Biomarker with symptoms duration

This accuracy of the AD-Biomarker to make the correct diagnosis early in the cause of the disease received further support from the significant correlation obtained between disease duration and the magnitude of the AD-Biomarker values as illustrated in Fig. 5A and B. This observed inverse correlation suggests that the AD-Biomarker may very well be abnormal before the onset of symptoms. No correlation was found between age and AD-Index. The correlation between AD-Index and cell passages was not studied here. A negative correlation for all cases showed that at an early stage the AD-Biomarker value was higher and more efficient in detecting the disease.

Table 4B
Decision matrix test results for all autopsy-confirmed cases (number of patients = 64, 42 autopsy-confirmed patients are included from Table 3A)

	Present	Absent	Total
Clinical diagnosis			
Positive	*a* (true positive), 47	*c* (false positive), 4	$a+c$, 51
Negative	*b* (false negative), 11	*d* (true negative), 2	$b+d$, 13
Total	$a+b$, 58	$c+d$, 6	64

Sensitivity = $(a/a+b) \times 100 = 81\%$, specificity = $(d/c+d) \times 100 = 33\%$, positive predictive value = $(a/a+c) \times 100 = 92\%$, and negative predictive value = $(d/b+d) \times 100 = 15\%$.

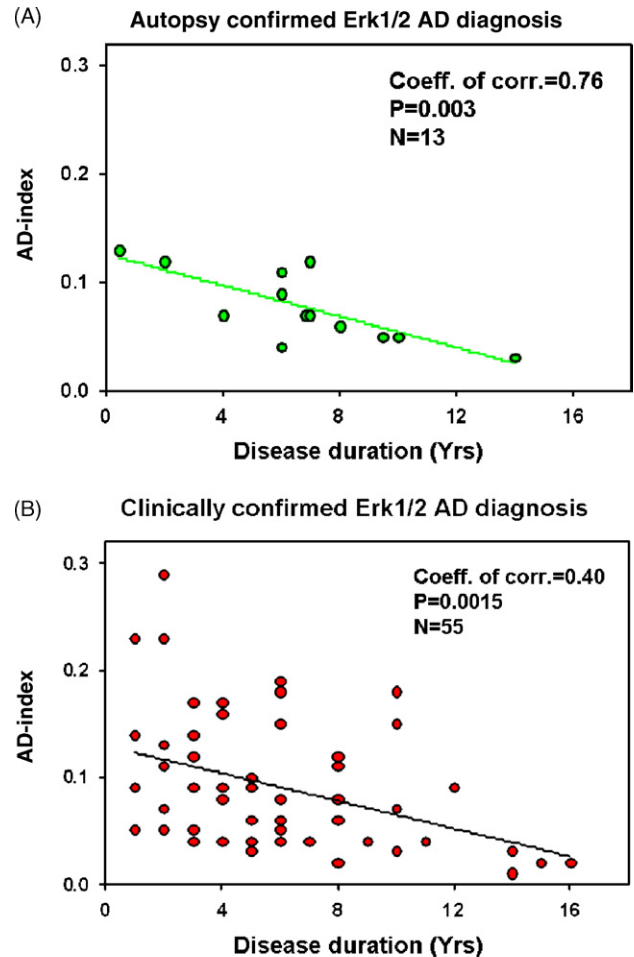

Fig. 5. Correlation of disease duration with AD-Biomarker: (A) Correlation of AD-Biomarker with disease duration for autopsy-confirmed patients (coefficient of correlation = 0.76, $P = 0.003$, $N = 13$). (B) Correlation of AD-Biomarker with disease duration for clinically confirmed patients (coefficient of correlation = 0.40, $P = 0.0015$, $N = 55$). Disease duration of the patients was estimated from the date of biopsy and the day that the first symptoms of dementia were observed. The criteria for selection of data from scanned gels for this study were: (i) signals that were at least 15% higher than that of background and (ii) gels with P-Erk1 > 5% of total P-Erk. N = number of patients for each category.

4.4. Possible underlying mechanisms for a propensity of selective Erk1 activation

AD might particularly involve early inflammation signaling triggered by natural mediators such as bradykinin, TNF-α, and cytokines such as interleukins, etc., that activate PKC-mediated phosphorylation of Erk1 and Erk2. Positive AD-Biomarker value for diagnosis of AD corresponds to higher intensity of P-Erk1 after BK treatment (Eq. (A)) with respect to placebo (without BK). The relative higher activation of Erk1 compared to Erk2 was found to be AD-specific from our study. From a time dependent study of activation of Erk1 and Erk2 by bradykinin in skin fibroblasts, we found that the peak time of activation was 10 min (Zhao et al., 2002). Subsequently, during dephosphorylation, impairment

of phosphatase 2A (PP2A) can contribute to prolongation of Erk1 and Erk2 phosphorylation in AD fibroblasts. It is possible, therefore, that dephosphorylation process of Erk1 was slower for AD cells (Zhao et al., 2003). A similar study by Veeranna et al. (2004) found a relative increase of intensities of phosphorylated Erk1 bands for AD brains as compared to the same phosphorylated Erk2 in comparison to age match control brains.

4.5. Pathophyiological relevance of the AD-Biomarker

Accumulation of Aβ is believed to be one of the first events to occur in AD pathology. It has been reported that the primary human skin fibroblasts cells of symptomatic as well as presymptomatic patients carrying the Swedish familial AD mutation produced excess Aβ protein (Citron et al., 1994; Johnston et al., 1994). $A\beta_{1-42}$ is more prone to aggregate *in vitro* than $A\beta_{1-40}$ and its neurotoxicity is considered to be the main cause of neuronal damage. Prefibrillar aggregates, but not mature fibrils form of Aβ, can impair cell viability when added to cell culture media (Cecchi et al., 2005). 1.0 μM does not represent physiological levels. In a recent report (Cecchi et al., 2007), 1.0 μM prefibrillary $A\beta_{1-42}$ was used to study the susceptibility to amyloid toxicity in familial AD fibroblasts. The authors also conducted cytotoxicity with different concentration of $A\beta_{1-42}$ (0.001, 0.01, 0.1, 1.0 and 10 μM) and found that cell cytotoxicity was higher at 1.0 and 10 μM $A\beta_{1-42}$.

To assess the physiologic relevance of the AD-Biomarker diagnosis, we examined the $A\beta_{1-42}$ induction of AD phenotype. Among the amyloid peptides, $A\beta_{1-42}$ has been found to be more neurotoxic. Furthermore, the Erk1/2 signaling cascade was found to be affected by amyloid peptide incubation (Mok et al., 2006). With $A\beta_{1-42}$ application, the present AD-Biomarker successfully demonstrates AD-Index values that were positive for the presence of AD (Fig. 4). To the best of our knowledge, the elevation of Aβ in fibroblasts from sporadic AD patients has not been tested. However, it has been documented that addition of Aβ to human skin fibroblasts (both sporadic and familial AD) differentially degraded PKC isozyme (particularly PKC α), compare to skin fibroblasts from age-matched control cases (Favit et al., 1998). Another study showed that addition of Aβ to cultured cortical astrocytes from rat brain promoted the phosphorylation of Erk1 and Erk2, particularly Erk1 (Abe et al., 2003). Moreover, the present study is based on abnormal phosphorylation of Erk1 and Erk2 through PKC activation by bradykinin stimulation.

4.6. Goals for an Alzheimer's disease biomarker

The search for a biological marker that can predict or confirm AD is an important area of research. An ideal antemortem AD-Biomarker should satisfy the following criteria: to discriminate from other dementias, such as Parkinson's disease, and dementia with Lewy bodies disease, etc., to detect early stages and the progression of AD, and to monitor therapeutic efficacy.

Among biomarker technologies that are currently under development there are *in vivo* brain imaging, including imaging of amyloid plaques using the PET-Pittsburgh compound (Klunk et al., 2004), molecular measures in cerebrospinal fluid (CSF) (Hansson et al., 2006), and a variety of biochemical measures in peripheral tissues (Mayeux et al., 2003; van Oijen et al., 2006). Of these, none except that reported here has had autopsy-validated efficacy for the *early* identification of AD, i.e., soon after the onset of dementia. Direct measures of amyloid plaques, tau protein, $A\beta_{1-40}$ and/or $A\beta_{1-42}$ in the CSF in more advanced cases may not be useful at earlier times when the initial AD-specific abnormalities may be more closely related to levels of soluble $A\beta_{1-42}$ within the intracellular and extracellular compartments and its effects on synaptic function. While a number of CSF measures are being investigated that are of potential value (e.g., phosphorylated tau, soluble $A\beta_{1-42}$, amyloid-β-derived diffusible ligands, monoamine metabolites, somatostatin, cytokines, lipid metabolites and indicators of oxidative stress), they may not lend themselves to diagnostic screening of large numbers of patients given the invasiveness of the required lumbar puncture procedure, which is a significantly more invasive procedure than the punch biopsy or taking a blood sample. Furthermore, some of the results reported have not shown consistency between laboratories (Irizarry, 2004; Bateman et al., 2007). The lumber puncture procedure is undoubtedly invasive, but is in fact, a standard feature of dementia investigation in many European countries. Nevertheless, a peripheral biomarker would be more convenient for clinicians and for the patients.

Among the autopsy-confirmed cases, the Erk1/2 AD-Biomarker diagnosis showed remarkably high sensitivity (97%) and specificity (100%) compared to clinical diagnosis (sensitivity: 78% and specificity: 20%). The performance of the peripheral biomarker was also comparable with or higher than the accuracy of CSF biomarker combined with PiB-PET imaging (95% sensitivity and 83% specificity) diagnosis of AD (Hansson et al., 2006).

The rationale for the AD-Biomarker describe here is based on the potential systemic impact of AD in addition to AD pathology in the human brain. As previously reviewed (Gasparini et al., 1998), peripheral tissues may reflect some of the systemic abnormalities of AD without causing symptomatic effects.

For useful screening of an elderly population, the biomarker should be non-invasive as possible and relatively less expensive. CSF biomarker sample collection is invasive and expensive compared to any peripheral biomarker. Nonetheless, CSF cannot be routinely collected in evaluation of AD, and lumber puncture is not a wide spread procedure to be followed by primary care. Generally, correctly performed lumber puncture is safe, with the only known side effect of headache due to low pressure generated during the procedure. However, one study found development of neurological prob-

lems after lumber puncture (Hart et al., 1988). In contrast, skin biopsy is a relatively easy procedure and less painful (local anesthesia usually makes this a painless procedure; (Zuber, 2002)), and is frequently used diagnostic tests for skin disorders. The skin biopsy technique involves the use of an inexpensive commercially available circular blade that rotates through epidermis and yields a ∼3 mm wound and that can be taken care by a single suture. For AD-Biomarker studies, the superiority of skin fibroblasts over blood samples has been discussed in a gene expression study (Nagasaka et al., 2005). This study found that blood cells were more susceptible to variation introduced by external stimuli such as fever, infections, and drug effects.

Among peripheral tissues, skin fibroblasts have been successfully used to elucidate the biochemical processes that underlie many inborn errors and metabolic processes that cause neurological diseases, such as Refsum's disease (Steinberg, 1983) and Lesch–Nyhan disease (Stanbury et al., 1983). Development of another biomarker using optical measures of AD-specific Aβ deposition in the human lens is also being pursued (Goldstein et al., 2003). However, measurable Aβ deposition in the lens may only occur at advanced stages of the disease, when significant deposition of plaques and tangles have occurred in the brain. It seems more likely, therefore, that *early* detection of AD may require biomarkers directly involved in the early molecular signaling deficits of AD such as those responsible for initial increases of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$. These might result, for example, from decreased PKC- and Erk1/2-mediated α-secretase activation. Such a deficit could explain reduced levels of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ as well as the autopsy validated, AD-specific differences described above.

Disclosure statement

Both the authors disclose that the work performed was funded by internal institutional resources and there is no conflict and no financial interest. When applicable, appropriate approval and procedures were used concerning human subjects.

Acknowledgements

We wish to acknowledge the important contribution to this work of Dr. Juan C. Troncoso, M.D. of The Johns Hopkins University Department of Pathology. Prof. Troncoso performed all of the brain autopsies and determined their neuropathologic diagnoses. We also thank Dr. Thomas J. Nelson (Blanchette Rockefeller Neurosciences Institute) for design and development of the software used to differentiate P-Erk1 and P-Erk2 band intensities on the Western Blots.

References

Abe, K., Hisatomi, R., Misawa, M., 2003. Amyloid beta peptide specifically promotes phosphorylation and nuclear translocation of the extracellular signal-regulated kinase in cultured rat cortical astrocytes. J. Pharmacol. Sci. 93, 272–278.

Alkon, D.L., Sun, M.K., Nelson, T.J., 2007. PKC signaling deficits: a mechanistic hypothesis for the origins of Alzheimer's disease. Trends Pharmacol. Sci. 28 (2), 51–60.

Bateman, R.J., Wen, G., Morris, J.C., Holtzman, D.M., 2007. Fluctuations of CSF amyloid-beta levels: implications for a diagnostic and therapeutic biomarker. Neurology 68, 666–669.

Braak, H., Braak, E., 1991. Neuropathological staging of Alzheimer-related changes. Acta Neuropathol. 82, 239–259.

Cecchi, C., Baglioni, S., Fiorillo, C., Pensalfini, A., Liguri, G., Nosi, D., Rigacci, S., Bucciantini, M., Stefani, M., 2005. Insights into the molecular basis of the differing susceptibility of varying cell types to the toxicity of amyloid aggregates. J. Cell Sci. 118, 3459–3470.

Cecchi, C., Fiorillo, C., Baglioni, S., Pensalfini, A., Bagnoli, S., Nacmias, B., Sorbi, S., Nosi, D., Relini, A., Liguri, G., 2007. Increased susceptibility to amyloid toxicity in familial Alzheimer's fibroblasts. Neurobiol. Aging 28, 863–876.

Citron, M., Vigo-Pelfrey, C., Teplow, D.B., Miller, C., Schenk, D., Johnston, J., Winblad, B., Venizelos, N., Lannfelt, L., Selkoe, D.J., 1994. Excessive production of amyloid beta-protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. Proc. Natl. Acad. Sci. U.S.A. 91, 11993–11997.

de Leon, M.J., DeSanti, S., Zinkowski, R., Mehta, P.D., Pratico, D., Segal, S., Rusinek, H., Li, J., Tsui, W., Saint Louis, L.A., Clark, C.M., Tarshish, C., Li, Y., Lair, L., Javier, E., Rich, K., Lesbre, P., Mosconi, L., Reisberg, B., Sadowski, M., DeBernadis, J.F., Kerkman, D.J., Hampel, H., Wahlund, L.O., Davies, P., 2006. Longitudinal CSF and MRI biomarkers improve the diagnosis of mild cognitive impairment. Neurobiol. Aging 27, 394–401.

Davis, G.K., Baboolal, N.S., Seales, D., Ramchandani, J., McKell, S., McRae, A., 2007. Potential biomarkers for dementia in Trinidad and Tobago. Neurosci. Lett. 424, 27–30.

Desai, A., Grossberg, G., 1999. Risk factors and protective factors for Alzheimer's disease. Clin. Geriatr. 7, 1070–1389.

Etcheberrigaray, R., Ito, E., Oka, K., Tofel-Grehl, B., Gibson, G.E., Alkon, D.L., 1993. Potassium channel dysfunction in fibroblasts identifies patients with Alzheimer disease. Proc. Natl. Acad. Sci. U.S.A. 90, 8209–8213.

Etcheberrigaray, R., Ito, E., Kim, C.S., Alkon, D.L., 1994. Soluble beta-amyloid induction of Alzheimer's phenotype for human fibroblast K^+ channels. Science 264, 276–279.

Etcheberrigaray, R., Tan, M., Dewachter, I., Kuipéri, C., Van der, A.I., Wera, S., Qiao, L., Bank, B., Nelson, T.J., Kozikowski, A.P., Van Leuven, F., Alkon, D.L., 2004. Therapeutic effects of PKC activators in Alzheimer's disease transgenic mice. Proc. Natl. Acad. Sci. U.S.A. 101, 11141–11146.

Fagan, A.M., Mintun, M.A., Mach, R.H., Lee, S.Y., Dence, C.S., Shah, A.R., LaRossa, G.N., Spinner, M.L., Klunk, W.E., Mathis, C.A., DeKosky, S.T., Morris, J.C., Holtzman, D.M., 2006. Inverse relation between in vivo amyloid imaging load and cerebrospinal fluid $A\beta_{42}$ in humans. Ann. Neurol. 59, 512–519.

Favit, A., Grimaldi, M., Nelson, T.J., Alkon, D.L., 1998. Alzheimer's-specific effects of soluble beta-amyloid on protein kinase C-alpha and -gamma degradation in human fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 95, 5562–5567.

Gasparini, L., Racchi, M., Binetti, G., Trabucchi, M., Solerte, S.B., Alkon, D.L., Etcheberrigaray, R., Gibson, G., Blass, J., Paoletti, R., Govoni, S., 1998. Peripheral markers in testing pathophysiological hypotheses and diagnosing Alzheimer's disease. FASEB J. 12, 17–34.

Goldstein, L.E., Muffat, J.A., Cherny, R.A., Moir, R.D., Ericsson, M.H., Huang, X., Mavros, C., Coccia, J.A., Faget, K.Y., Fitch, K.A., Masters, C.L., Tanzi, R.E., Chylack Jr., L.T., Bush, A.I.,

2003. Cytosolic beta-amyloid deposition and supranuclear cataracts in lenses from people with Alzheimer's disease. Lancet 361, 1258–1265.

Govoni, S., Bergamaschi, S., Racchi, M., Battaini, F., Binetti, G., Bianchetti, A., Trabucchi, M., 1993. Cytosol protein kinase C downregulation in fibroblasts from Alzheimer's disease patients. Neurology 43, 2581–2586.

Guerreiro, R.J., Santana, I., Brás, J.M., Santiago, B., Paiva, A., Oliveira, C., 2007. Peripheral inflammatory cytokines as biomarkers in Alzheimer's disease and mild cognitive impairment. Neurodegener. Dis. 4, 406–412.

Hansson, O., Zetterberg, H., Buchhave, P., Londos, E., Blennow, K., Minthon, L., 2006. Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. Lancet Neurol. 3, 228–234.

Hart, I.K., Bone, I., Hadley, D.M., 1988. Development of neurological problems after lumber puncture. Br. Med. J. 296, 51–52.

Hogervorst, E., Bandelow, S., Combrinck, M., Irani, S.R., Smith, A.D., 2003. The validity and reliability of 6 sets of clinical criteria to classify Alzheimer's disease and vascular dementia in cases confirmed post-mortem: added value of a decision tree approach. Dement. Geriatr. Cogn. Disord. 16, 170–180.

Hyman, B.T., Trojanowski, J.Q., 1997. Consensus recommendations for the postmortem diagnosis of Alzheimer disease from the National Institute on Aging and the Reagan Institute Working Group on diagnostic criteria for the neuropathological assessment of Alzheimer disease. J. Neuropathol. Exp. Neurol. 56, 1095–1097.

Irizarry, M.C., 2004. Biomarker of Alzheimer's disease in plasma. NeuroRx 1, 226–234.

Ito, E., Oka, K., Etcheberrigaray, R., Nelson, T.J., McPhie, D.L., Tofel-Grehl, B., Gibson, G.E., Alkon, D.L., 1994. Internal Ca^{2+} mobilization is altered in fibroblasts from patients with Alzheimer disease. Proc. Natl. Acad. Sci. U.S.A. 91, 534–538.

Johnston, J.A., Cowburn, R.F., Norgren, S., Wiehager, B., Venizelos, N., Winblad, B., Vigo-Pelfrey, C., Schenk, D., Lannfelt, L., O'Neill, C., 1994. Increased beta-amyloid release and levels of amyloid precursor protein (APP) in fibroblast cell lines from family members with the Swedish Alzheimer's disease APP670/671 mutation. FEBS Lett. 354, 274–2748.

Khan, T.K., Alkon, D.L., 2006. An internally controlled peripheral biomarker for Alzheimer's disease: Erk1 and Erk2 responses to the inflammatory signal bradykinin. Proc. Natl. Acad. Sci. U.S.A. 103, 13203–13207.

Kim, S.-K., Park, H.-J., Hing, H.S., Baik, E.J., Jung, M.W., Mook-Jung, I., 2006. Erk1/2 is an endogeneous negative regulator of γ-secretase activity. FASEB J. 2, 157–159.

Klunk, W.E., Engler, H., Nordberg, A., Wang, Y., Blomqvist, G., Holt, D.P., Bergström, M., Savitcheva, I., Huang, G.F., Estrada, S., Ausén, B., Debnath, M.L., Barletta, J., Price, J.C., Sandell, J., Lopresti, B.J., Wall, A., Koivisto, P., Antoni, G., Mathis, C.A., Långström, B., 2004. Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. Ann. Neurol. 55, 306–319.

Locascio, J.J., Fukumoto, H., Yap, L., Bottiglieri, T., Growdon, J.H., Hyman, B.T., Irizarry, M.C., 2008. Plasma amyloid beta-protein and C-reactive protein in relation to the rate of progression of Alzheimer disease. Arch. Neurol. 65, 776–785.

Masliah, E., Cole, G.M., Hansen, L.A., Mallory, M., Albright, T., Terry, R.D., Saitoh, T., 1991. Protein kinase C alteration is an early biochemical marker in Alzheimer's disease. J. Neurosci. 11, 2759–2767.

Mayeux, R., Honig, L.S., Tang, M.X., Manly, J., Stern, Y., Schupf, N., Mehta, P.D., 2003. Plasma A[β]40 and A[β]42 and Alzheimer's disease: relation to age, mortality, and risk. Neurology 61, 1185–1190.

Mirra, S.S., Heyman, A., McKeel, D., Sumi, S.M., Crain, B.J., Brownlee, L.M., Vogel, F.S., Hughes, J.P., van Belle, G., Berg, L., 1991. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 41, 479–486.

Mok, S.S., Losic, D., Barrow, C.J., Turner, B.J., Masters, C.L., Martin, L.L., Small, D.H., 2006. The beta-amyloid peptide of Alzheimer's disease decreases adhesion of vascular smooth muscle cells to the basement membrane. J. Neurochem. 96, 53–64.

Nagasaka, Y., Dillner, K., Ebise, H., Teramoto, R., Nakagawa, H., Lilius, L., Axelman, K., Forsell, C., Ito, A., Winblad, B., Kimura, T., Graff, C., 2005. A unique gene expression signature discriminates familial Alzheimer's disease mutation carriers from their wild-type siblings. Proc. Natl. Acad. Sci. U.S.A. 102, 14854–148549.

Pope, S.K., Shue, V.M., Beck, C., 2003. Will a healthy lifestyle help prevent Alzheimer's disease? Annu. Rev. Public Health 24, 111–132.

Ray, S., Britschgi, M., Herbert, C., Takeda-Uchimura, Y., Boxer, A., Blennow, K., Friedman, L.F., Galasko, D.R., Jutel, M., Karydas, A., Kaye, J.A., Leszek, J., Miller, B.L., Minthon, L., Quinn, J.F., Rabinovici, G.D., Robinson, W.H., Sabbagh, M.N., So, Y.T., Sparks, D.L., Tabaton, M., Tinklenberg, J., Yesavage, J.A., Tibshirani, R., Wyss-Coray, T., 2007. Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. Nat. Med. 13, 1359–1362.

Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., Younkin, S., 1996. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nat. Med. 2, 864–870.

Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., Brown, M.S., 1983. The Metabolic Basis of Inherited Disease. McGraw Hill, New York.

Steinberg, D., 1983. Phytanic acid storage disease (Refsum's disease). In: Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., Brown, M.S. (Eds.), The Metabolic Basis of Inherited Disease, 5th ed. McGraw Hill, New York, pp. 731–747.

Sunderland, T., Linker, G., Mirza, N., Putnam, K.T., Friedman, D.L., Kimmel, L.H., Bergeson, J., Manetti, G.J., Zimmermann, M., Tang, B., Bartko, J.J., Cohen, R.M., 2003. Decreased β-amyloid-42 and increased tau levels in cerebrospinal fluid of patients with Alzheimer's disease. JAMA 289, 2094–2103.

van Oijen, M., Hofman, A., Soares, H.D., Koudstaal, P.J., Breteler, M.M., 2006. Plasma Abeta(1-40) and Abeta(1-42) and the risk of dementia: a prospective case-cohort study. Lancet Neurol. 8, 655–660.

Veeranna, Kaji, T., Boland, B., Odrljin, T., Mohan, P., Basavarajappa, B.S., Peterhoff, C., Cataldo, A., Rudnicki, A., Amin, N., Li, B.S., Pant, H.C., Hungund, B.L., Arancio, O., Nixon, R.A., 2004. Calpain mediates calcium-induced activation of the erk1 2 MAPK pathway and cytoskeletal phosphorylation in neurons: relevance to Alzheimer's disease. Am. J. Pathol. 165, 795–805.

Wahlund, L.-O., Blennow, K., 2003. Cerebrospinal fluid biomarkers for disease stage and intensity in cognitively impaired patients. Neurosci. Lett. 339, 99–102.

Yamamoto, T., Hirano, A., 1986. A comparative study of modified Bielschowsky Bodian and thioflavin S stains on Alzheimer's neurofibrillary tangles. Neuropathol. Appl. Neurobiol. 12, 3–9.

Yang, C.M., Lin, M.I., Hsieh, H.L., Sun, C.C., Ma, Y.H., Hsiao, L.D., 2005. Bradykinin-induced p42/p44 MAPK phosphorylation and cell proliferation via Src, EGF receptors, and PI3-K/Akt in vascular smooth muscle cells. J. Cell Physiol. 203, 538–546.

Zetterberg, H., Wahlund, L.O., Blennow, K., 2003. Cerebrospinal fluid markers for prediction of Alzheimer's disease. Neurosci. Lett. 352, 67–69.

Zhao, W.Q., Ravindranath, L., Mohamed, A.S., Zohar, O., Chen, G.H., Lyketsos, C.G., Etcheberrigaray, R., Alkon, D.L., 2002. MAP kinase signaling cascade dysfunction specific to Alzheimer's disease in fibroblasts. Neurobiol. Dis. 11, 166–183.

Zhao, W.Q., Feng, C., Alkon, D.L., 2003. Impairment of phosphatase 2A contributes to the prolonged MAP kinase phosphorylation in Alzheimer's disease fibroblasts. Neurobiol. Dis. 14, 458–469.

Zuber, T.J., 2002. Punch biopsy of the skin. Am. Fam. Physician 65, 1155–1158.