

Detection of Misfolded A β Oligomers for Sensitive Biochemical Diagnosis of Alzheimer's Disease

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SUMMARY

Alzheimer's disease (AD) diagnosis is hampered by the lack of early, sensitive, and objective laboratory tests. We describe a sensitive method for biochemical diagnosis of AD based on specific detection of misfolded A β oligomers, which play a central role in AD pathogenesis. The protein misfolding cyclic amplification assay (A β -PMCA), exploits the functional property of A β oligomers to seed the polymerization of monomeric A β . A β -PMCA allowed detection of as little as 3 fmol of A β oligomers. Most importantly, using cerebrospinal fluid, we were able to distinguish AD patients from control individuals affected by a variety of other neurodegenerative disorders or nondegenerative neurological diseases with overall sensitivity of 90% and specificity of 92%. These findings provide the proof-of-principle basis for developing a highly sensitive and specific biochemical test for AD diagnosis.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in the elderly population and one of the leading causes of death in the developed world (Hebert et al., 2003). The disease is typically characterized by a progressive amnesic disorder followed by impairment of other cognitive functions and behavioral abnormalities associated with specific neuropathological changes, in particular accumulation of protein aggregates in the form of amyloid plaques and neurofibrillary tangles (Terry, 1994). Although the etiology of the disease is not yet clear, compelling evidence suggests that misfolding, oligomerization, and accumulation of amyloid aggregates in the brain is the triggering factor of the pathology (Selkoe, 2000; Haass and Selkoe, 2007; Soto, 2003). Amyloid aggregates are composed predominantly of a 42-residue peptide called amyloid- β (A β), which is the

product of the enzymatic processing of a larger amyloid precursor protein (Selkoe, 2000). A β misfolding and fibrillar aggregation follow a seeding-nucleation mechanism that involves the formation of several intermediates, including soluble oligomers and protofibrils (Caughey and Lansbury, 2003; Soto et al., 2006; Jarrett and Lansbury, 1993). Recent findings have shown that A β oligomers, rather than large amyloid fibrils, might be the culprit of neurodegeneration in AD (Walsh and Selkoe, 2007; Haass and Selkoe, 2007; Glabe and Kaye, 2006; Klein et al., 2004).

AD belongs to a large group of diseases associated with misfolding, aggregation and tissue accumulation of proteins (Soto, 2003). These diseases, termed protein misfolding disorders (PMDs), include Parkinson's disease, type 2 diabetes, Huntington's disease, amyotrophic lateral sclerosis, systemic amyloidosis, prion diseases, and many others (Soto, 2003; Luheshi and Dobson, 2009). In all these diseases, misfolded aggregates composed of different proteins are formed by a similar mechanism resulting in the accumulation of toxic structures that induce cellular dysfunction and tissue damage (Caughey and Lansbury, 2003; Soto et al., 2006; Jarrett and Lansbury, 1993).

One of the major problems in AD is the lack of a widely accepted early, sensitive, and objective laboratory diagnosis to support neuropsychological evaluation, monitor disease progression, and identify affected individuals before they display the clinical symptoms (Parnetti and Chiasserini, 2011; Urbanelli et al., 2009). For diseases affecting the brain, a tissue with low regeneration capacity, it is crucial to intervene before irreversible neuropathological changes occur. Therefore, early diagnosis of AD is of utmost importance. Several lines of evidence point that the process of A β misfolding and oligomerization begins years or decades before the onset of clinical symptoms and substantial brain damage (Braak et al., 1999; Buchhave et al., 2012). Recent studies have shown that A β oligomers are naturally secreted by cells and circulate in AD biological fluids (Gao et al., 2010; Head et al., 2010; Walsh et al., 2002; Klyubin et al., 2008; Georganopoulou et al., 2005; Fukumoto et al., 2010). Thus, detection of soluble A β oligomers might represent the best strategy for early and specific biochemical diagnosis of AD. The challenge of this approach is that the quantity of A β

oligomers is likely very small in tissues other than the brain. An additional difficulty for specific detection of A β oligomers is that their sequence and chemical structure is the same as the native A β protein.

Our strategy to detect misfolded oligomers is to use their functional property of being capable of catalyzing the polymerization of the monomeric protein. For this purpose, we invented the protein misfolding cyclic amplification (PMCA) technology in order to achieve the ultrasensitive detection of misfolded aggregates through amplification of the misfolding and aggregation process *in vitro* (Saborio et al., 2001). So far, PMCA has been applied to detect minute quantities of oligomeric misfolded prion protein (PrP^{Sc}) implicated in prion diseases (Morales et al., 2012). Using PMCA, we were able to detect the equivalent of a single particle of misfolded PrP oligomer (Saá et al., 2006b) and strikingly to identify PrP^{Sc} in the blood and urine of infected animals at symptomatic and presymptomatic stages of the disease (Castilla et al., 2005; Saá et al., 2006a; Gonzalez-Romero et al., 2008). The basis for the PMCA technology is the fact that the process of misfolding and aggregation of A β , PrP, and the other proteins implicated in PMDs follow a seeding-nucleation mechanism (Soto et al., 2002, 2006). In a seeded-nucleated polymerization, the limiting step is the formation of stable oligomeric seeds that, depending on the conditions, may take a very long time to form or not occur at all. Once formed, oligomers grow exponentially by recruiting and incorporating protein monomers into the growing polymer. Addition of preformed seeds into a solution containing the monomeric protein accelerates protein misfolding and aggregation (Soto et al., 2006; Jarrett and Lansbury, 1993). Thus, measuring seeding activity could be used to estimate the presence and quantity of oligomers in a given sample. To increase the sensitivity of detection, PMCA combines steps of growing polymers with multiplication of oligomeric seeds to reach an exponential increase of misfolding and aggregation (Soto et al., 2002). In this study, we describe the implementation and optimization of PMCA for highly sensitive detection of misfolded A β oligomers and show its application to detect these structures in the cerebrospinal fluid (CSF) of AD patients.

RESULTS

Cyclic Amplification of Amyloid- β Misfolding

To implement the experimental conditions for A β -PMCA, we performed studies using *in-vitro*-produced oligomeric seeds. Because it is still unknown which of the different species of A β oligomers is most relevant for AD pathology, we decided to work with a mixture of oligomers of different sizes generated during the process of fibril formation. A β oligomers were prepared by incubation of monomeric (seed-free) synthetic A β 1-42 (10 μ M) at 25°C with stirring. After 5 hr of incubation, we observed an abundance of globular oligomers by electron microscopy with only a small amount of protofibrils and fibrils (Figure 1A). These aggregates were positive with the A11 oligomer-specific antibody (Kayad et al., 2003; data not shown). After longer incubation, protofibrillar and fibrillar structures were observed. The size of the aggregates was determined by filtration through filters of defined pore size and western blotting

after SDS-PAGE separation. Oligomers formed by incubation for 5 hr migrated as \sim 170 kDa SDS-resistant aggregates, with a minor band at 17 kDa (Figure 1B).

Low concentrations of seed-free A β 1-42 (2 μ M) were incubated at 22°C with constant shaking (100 rpm) for different times alone or in the presence of distinct concentrations of synthetic A β oligomers, prepared by incubation during 5 hr as described above. A β aggregation was studied by the fluorescence emission of the amyloid-binding dye Thioflavin T (ThT) (LeVine, 1993; Soto et al., 1995). The peptide concentration, temperature, and pH of the buffer are critical to control the extent of the lag phase and reproducibility among experiments. Under these conditions, no spontaneous A β aggregation was detectable during the time in which the experiment was performed (125 hr). However, A β aggregation was observed in the presence of 0.3–8.4 fmol of A β oligomers (Figure 1C). To increase the efficiency of seeding, and thus the limit of detection of A β oligomers, we introduced cycles of amplification, combining phases of polymer growing with multiplication of seeds as in the PMCA assay. For this purpose, we subjected the samples to intermittent shaking, which has been previously shown to dramatically accelerate the seeded conversion of recombinant prion protein (Atarashi et al., 2008). Under these conditions, the kinetic of A β aggregation induced by 8,400, 300, 80, and 3 fmol of A β oligomers was clearly faster and easily distinguishable from that observed in the absence of A β seeds (Figure 1D). This result indicates that, using the A β -PMCA assay, we should be able to detect as little as 3 fmol of A β oligomers in a given sample.

Detection of A β Oligomers in the Cerebrospinal Fluid of AD Patients

To study the usefulness of the A β -PMCA assay to detect seeding-competent A β oligomers in biological fluids, we analyzed aliquots of CSF from 50 AD patients, 39 cognitively normal individuals affected by nondegenerative neurological diseases (NNDs), and 37 patients affected by non-AD neurodegenerative diseases (NANDs) including other forms of dementia. The experiments as well as the initial part of the analysis were done blindly, because the investigator was unaware of which samples were coming from AD or controls. Figure 2A shows the average kinetics of aggregation of five representative samples from the AD, NND, and NAND groups. The result indicates that CSF from AD patients accelerates significantly A β aggregation as compared to control CSF ($p < 0.001$). To determine the effect of individual samples on A β aggregation, we estimated the lag phase (Figure 2B), defined as the time required to get a ThT fluorescence larger than 40 arbitrary units (after subtraction of the blank). This value was selected considering that it corresponds to approximately five times the reading of the buffer alone. We also estimated the P90, which corresponds to the extent of A β aggregation at 90 hr (Figure 2C). By comparing both parameters among the experimental groups, a highly significant difference was observed between AD and control samples from individuals with nondegenerative neurological diseases or with non-AD neurodegenerative diseases. No correlation was detected between the aggregation parameters and the age of the AD patients, which indicates that the levels of the marker are not simply

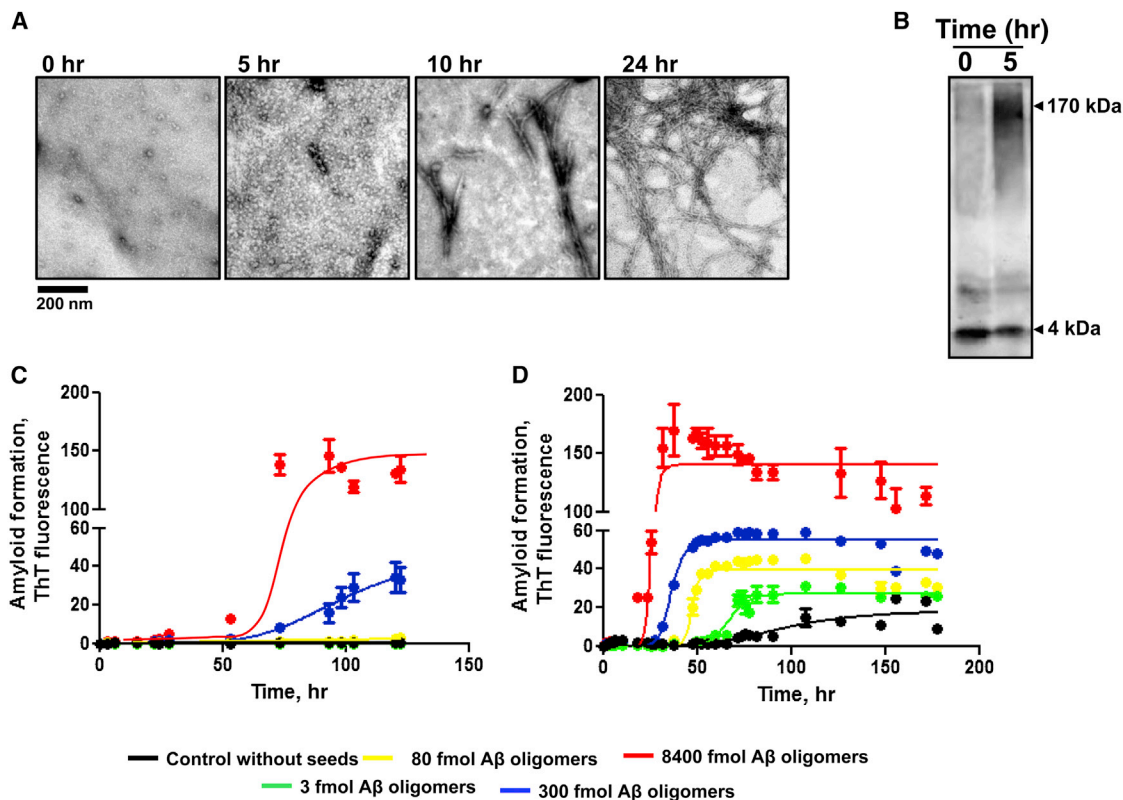


Figure 1. Detection of Synthetic A β Oligomers by A β -PMCA

(A) To prepare A β oligomers, solutions of A β 1-42 at 10 μ M were incubated for different times at 25 $^{\circ}$ C with shaking. At various time points, samples were visualized by electron microscopy after negative staining. At 5 hr of incubation, aggregates consist mainly of globular oligomers, whereas at 10 hr, there are mostly protofibrils, and at 24 hr, we can observe a large amount of long fibrils.

(B) Preparations of oligomers were characterized by SDS-PAGE followed by western blot with 4G8 antibody. The mixture at 5 hr of incubation was also characterized by strong reactivity with A11 antibody and by a native size ranging from 30 to 1,000 kDa, as evaluated by filtration using filters of defined pore size.

(C) Seeding of A β aggregation was studied incubating a solution of 2 μ M seed-free A β 1-42 in 100 mM Tris-HCl (pH 7.4) in the presence of 5 μ M Thioflavin T, with or without different quantities of synthetic A β oligomers (prepared by incubation during 5 hr, as indicated in A) with constant, but low, agitation (100 rpm) at 22 $^{\circ}$ C.

(D) The same samples as in (C) were incubated with cyclic agitation (1 min stirring at 500 rpm followed by 29 min without shaking). Aggregation was measured over time by the Thioflavin T (ThT) binding to amyloid fibrils using a plate spectrofluorometer (excitation: 435; emission: 485 nm). Graphs show the mean and SE of three replicates. The concentration of A β oligomers was estimated assuming an average molecular weight of 170 kDa.

a reflection of age but rather whether or not the patients contain seeding-competent A β aggregates in their CSF. Using the values for lag phase, we calculated the sensitivity, specificity, and predictive value of the A β -PMCA test (Table 1). To determine the appropriate cutoff points and the performance of the test, we carried out a detailed statistical analysis of the receiver operating characteristics (ROC) (Figure S1). In relation to the control group consisting of age-matched individuals with nondegenerative neurological diseases, we estimated a 90.0% sensitivity and 84.2% specificity, whereas for the clinically more relevant differentiation of AD from other neurodegenerative diseases including other forms of dementia, we obtained a staggering 100% sensitivity and 94.6% specificity (Table 1). If confirmed with a larger number of patients, the ability of A β -PMCA to distinguish AD from other forms of neurodegenerative diseases might be very useful in clinic. The overall sensitivity and specificity considering all control individuals was 90% and 92%, respectively (Table 1).

To confirm that A β -PMCA detects a seeding activity associated to A β oligomers present in CSF, we performed immunodepletion experiments. The methodology for efficient immunodepletion of A β oligomers was first optimized by using synthetically prepared A β oligomers. Incubation with Dynabeads conjugated with a mixture of sequence (4G8) and conformational (A11) antibodies led to the complete removal of these structures (Figure 3A). Next, application of immunodepletion to three AD CSF samples showed that the kinetic of A β aggregation in the A β -PMCA reaction was comparable to that observed in control CSF samples, and both were significantly different from the aggregation observed with AD CSF prior to immunodepletion (Figure 3B). A similar result was observed when immunodepletion was done only using the A11 conformational antibody (Figure 3C), which recognizes specifically oligomers (Kayed et al., 2003). These results indicate that the seeding activity observed in AD CSF samples was indeed associated to A β oligomers.

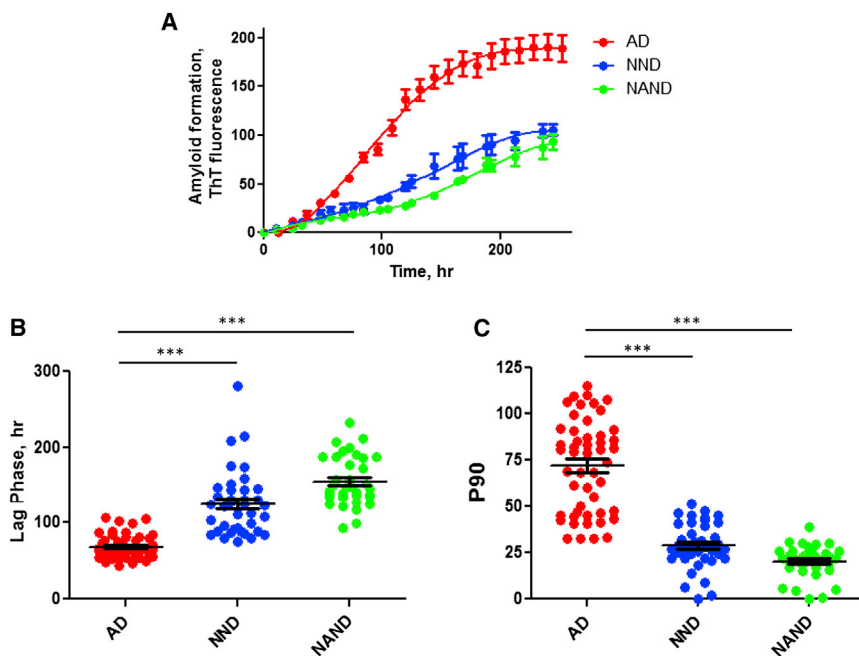


Figure 2. Detection of Seeding Activity in Human CSF Samples from Controls and AD Patients by A β -PMCA

(A) Representative aggregation curves of seed-free A β 1-42 in the presence of CSF samples from AD patients, people affected by non-neurodegenerative neurological diseases (NND), and patients suffering from neurodegenerative diseases other than AD (NAND). The values represent the average and SE of five different patients, representative of the average results in each group. For this purpose, we selected as representative for each group, the patients in which the kinetic parameters (lag phase and P90) were closer to the average obtained in each group. (B) The lag phase of A β aggregation in A β -PMCA was compared for the three groups of patients. Lag phase was defined as the time (in hours) required to reach a ThT signal more than 40 arbitrary units. One sample from the NND group did not reach a ThT signal of 40 during the duration of the aggregation experiment. Thus, the lag phase for this sample is >300 hr. This sample was not included in the graph and was not utilized to calculate sensitivity and specificity in Table 1.

(C) The extent of amyloid formation obtained after 180 A β -PMCA cycles, i.e., 90 hr of incubation

(P90), was measured in each patient. For the graphs in (B) and (C), we show the values obtained in individual samples, which correspond to the average of three independent experiments. Data were analyzed by one-way ANOVA, followed by the Tukey's multiple comparison post hoc test. The differences between AD and samples from the other two groups were highly significant with $p < 0.001$ (***).

DISCUSSION

AD is one of the most important public health problems in the developed world. Among the ten leading causes of death, AD is the only one that lacks effective ways to prevent, cure, or even slow its progression. Part of the difficulties in developing an efficient treatment for this devastating disease is the absence of an early and objective biochemical diagnosis that will enable to recognize patients before substantial brain damage has occurred (Parnetti and Chiasserini, 2011; Blennow et al., 2010; Urbanelli et al., 2009). Indeed, many of the failures in recent clinical trials with compounds aimed to modify the disease progression have been attributed to the lack of early diagnosis that prevents beginning treatment before it is too late (Galimberti and Scarpini, 2012). Currently, diagnosis is achieved by clinical assessment, neuroimaging, and CSF measurements of the

levels of A β 42, tau, and phospho-tau, with definitive confirmation relying on postmortem identification of amyloid plaques and neurofibrillary tangles in the brain (Parnetti and Chiasserini, 2011; Urbanelli et al., 2009). Recent advances in neuroimaging using amyloid tracers offer a promising alternative to increase accuracy of diagnosis (Klunk et al., 2004; Klunk and Mathis, 2008), but it is unclear whether these methods will identify patients before irreversible brain damage has occurred. Identification of biomarkers that could be used for noninvasive, sensitive, and objective detection of the disease process years or even decades before the onset of brain abnormalities and clinical symptoms are of utmost importance (Parnetti and Chiasserini, 2011; Blennow et al., 2010; Urbanelli et al., 2009). Although it is widely accepted that combining the measurements of A β 1-42, total tau, and phosphorylated tau in CSF can provide a relatively high sensitivity and specificity for AD diagnosis (Blennow et al.,

Table 1. Estimation of Sensitivity, Specificity, and Predictive Value for A β -PMCA Using CSF Samples

Groups	Sensitivity (%) ^a	Specificity (%) ^b	Positive Predictive Value (%) ^c	Negative Predictive Value (%) ^d
AD versus NAND	100.0	94.6	96.2	100.0
AD versus NND	90.0	84.2	88.2	86.5
AD versus controls ^e	90.0	92.0	88.2	93.2

For estimation of sensitivity, specificity, and predictive value, we used the results of the lag phase as shown in Figure 2B. Cutoffs were estimated by receiver operating characteristics (ROC) curve analysis using the MedCalc software (see Figure S1).

^aSensitivity was estimated by the formula (true positives/[true positives + false negatives]) \times 100.

^bSpecificity was estimated by the formula (true negatives/[false positives + true negatives]) \times 100.

^cPositive predictive value was estimated by the formula (true positives/[true positives + false positives]) \times 100.

^dNegative predictive value was estimated by the formula (true negatives/[true negatives + false negatives]) \times 100.

^eControls refer to the samples from NND plus NAND.

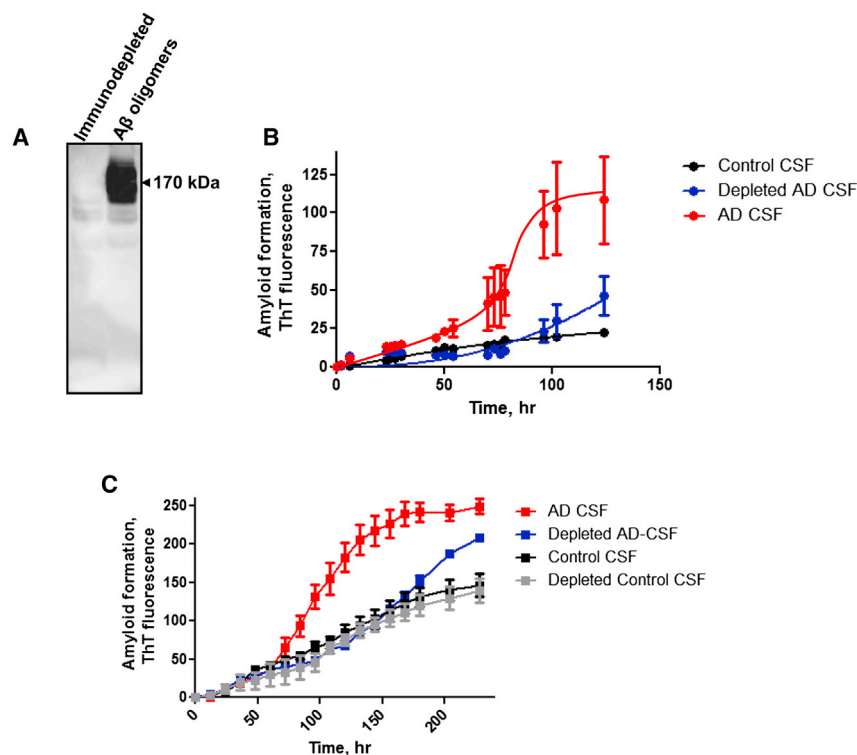


Figure 3. Seeding Activity in AD CSF Is Removed by Aβ Oligomers Immunodepletion

To test whether the seeding activity observed in human AD CSF was dependent on Aβ aggregates, we removed these structures from CSF samples by immunodepletion using Dynabeads coated with a mixture of antibodies recognizing specifically the sequence of Aβ (4G8) and the conformation of Aβ oligomers (A11) (Kayed et al., 2003).

(A) The procedure for immunodepletion was optimized using synthetically prepared Aβ oligomers spiked into human CSF at quantities detectable by western blot. Three consecutive rounds of incubation with antibody-coated Dynabeads were sufficient to efficiently remove the Aβ oligomers observed at around 170 kDa in our western blots.

(B) Samples of AD CSF before or after immunodepletion with 4G8 and A11 antibodies were used to seed Aβ aggregation in the Aβ-PMCA assay.

(C) Samples of AD CSF were also depleted only with the A11 conformational antibody and aggregation monitored by Aβ-PMCA assay. As in Figures 1 and 2, Aβ aggregation was measured by ThT fluorescence emission at different times of incubation. Values represent the average and SE of three different replicates.

2010), other CSF markers are needed that can more specifically differentiate AD from other dementias. It is also necessary to identify markers that correlate with the severity of dementia in patients with AD and, more importantly, can identify the pathological process earlier during the presymptomatic period of the disease.

Recent compelling evidence indicates that the formation of misfolded Aβ oligomers might be a critical event in AD pathogenesis (Walsh and Selkoe, 2007; Haass and Selkoe, 2007; Glabe and Kaye, 2006; Klein et al., 2004). Indeed, both synthetic and natural Aβ oligomers have been shown to induce apoptosis in cell cultures at very low concentrations (Dahlgren et al., 2002; El-Agnaf et al., 2000; Shankar et al., 2007), block long-term potentiation in brain slice cultures (Wang et al., 2002; Walsh et al., 2002), and impair synaptic plasticity and memory in animals (Cleary et al., 2005; Shankar et al., 2007). These findings, in addition to the fact that Aβ oligomers are small and soluble structures, suggest that they could be circulating in biological fluids and offer a specific marker for AD. Considering the well-established observation that the levels of total Aβ1-42 are lower in AD than in controls, it might be surprising that, in our study, we found that Aβ oligomers are elevated in AD patients. However, our assay measures the levels of seeding-competent Aβ oligomers, which are likely a very small proportion of total Aβ in CSF. A rough estimation based on the comparison with the amplification results obtained with synthetic oligomers is that the material we are measuring is below 100 pg/ml (expressed as monomer). Our assay does not distinguish oligomers produced by Aβ40 or Aβ42; thus, in our estimation, oligomers represent a very small fraction (less than 1%) of total Aβ. Therefore, it

is perfectly possible that a decline in total Aβ42 coincides with an overall increase of oligomers in AD patients. Indeed, recent studies have shown that Aβ oligomers might be present in low quantities in CSF and that their levels are elevated in the CSF of people affected by AD (Pitschke et al., 1998; Klyubin et al., 2008; Georganopoulou et al., 2005; Gao et al., 2010; Santos et al., 2012).

In this study, we adapted the PMCA technology to cyclically amplify the process of Aβ misfolding and aggregation, leading to the highly sensitive and specific detection of soluble Aβ oligomers. PMCA was originally developed to replicate the misfolding and aggregation of the prion protein implicated in prion diseases and is now considered a major technological breakthrough that has been instrumental to understand the prion biology and to detect misfolded prions in various biological fluids (Morales et al., 2012). PMCA is a cyclical process that enables exponential increase of the detection signal in a manner analogous to the amplification of DNA by PCR (Soto et al., 2002). PMCA takes advantage of the fact that the process of protein misfolding and aggregation associated with Aβ, PrP, and other proteins implicated in PMDs follows a seeding-nucleation mechanism and is dependent on the formation and number of oligomeric seeds present in the reaction (Soto et al., 2002, 2006). To achieve exponential amplification, PMCA consists of cycles of incubation for the elongation of the polymers, followed by breaking the aggregates to multiply the number of seeds. For amplification of prions, PMCA traditionally uses sonication as a mechanical force to break big polymers into smaller species (Morales et al., 2012). However, as shown by Caughey and colleagues, sonication can be replaced by strong shaking to achieve

similar results using a procedure called shaking-based PMCA or quaking-induced conversion (Atarashi et al., 2008). In adapting PMCA for AD, we found that shaking is more compatible with the fragile nature of A β aggregates.

In our current setting, A β -PMCA enables detection of as little as 3 fmol of A β oligomers. This level of sensitivity can be further increased by additional development of the technology, as was also the case in prion diseases. Indeed, in our original proof-of-concept study using PMCA for prion detection, the increase of sensitivity was only around 60-fold (Saborio et al., 2001), which contrast with the striking 3 billion folds obtained after further development (Saá et al., 2006b). Nevertheless, even with the current status of the A β -PMCA technology, we were able to distinguish with a high degree of sensitivity and specificity CSF samples coming from individuals diagnosed with AD, patients affected by other neurodegenerative disorders, and people suffering from nondegenerative neurological diseases (Table 1). Sensitivity, specificity, and predictive value of the A β -PMCA assay are higher than the currently widely used ELISA tests measuring the levels of A β 42, tau, and phospho-tau in CSF. The sensitivity and specificity of these methods range between 80% and 90%, depending on the study (Mulder et al., 2010; Sunderland et al., 2003; Maddalena et al., 2003). A crucial feature for an assay aimed for routine use in AD diagnosis is its reproducibility and repeatability. Several variables need to be carefully controlled to maintain a good reproducibility, including A β concentration, temperature, buffer composition, pH, shaking rate, etc. It is also possible that, with different batches of A β , the kinetic of aggregation and the parameters most useful to compare different group of samples may be different, but still AD samples induce accelerated A β aggregation compared to controls (Figure S2). Experiments displayed in this article were carried out blindly and independently by two different investigators at distinct times and using samples obtained from three different locations. However, during the course of the study, we found that an entire set of CSF samples coming from a fourth location was not amenable to the assay, because using these samples A β did not aggregate at all, not even after spiking with large concentrations of synthetic oligomers. We suspect that preanalytical factors related to sample collection influenced the assay. We are actively investigating what this interference could be. Nevertheless, more work needs to be done to assess reproducibility in a large number of samples, with distinct reagents, equipment, and samples coming from different locations.

Future studies should include larger populations of AD patients and controls and direct comparisons with the diagnostic accuracy obtained by measuring A β 42, total tau, and phospho-tau in CSF and by positron emission tomography amyloid imaging with specific ligands. Also, it will be important to investigate the presence of A β oligomers in patients affected by mild cognitive impairments, which is considered a precursor for AD (Petersen et al., 2009), as well as in asymptomatic carriers of familial AD to analyze the utility of A β -PMCA for pre-symptomatic detection of AD. Longitudinal studies from individual patients would also be needed to evaluate the relationship between the detection of A β oligomers and the progression of the disease. Finally, adaptation of A β -PMCA

to detect A β oligomers, which may potentially be circulating in the blood of AD patients, may offer a great opportunity for more-routine testing.

EXPERIMENTAL PROCEDURES

Biological Samples

We used CSF samples from 50 patients with the diagnosis of probable AD as defined by the DSM-IV and the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association guidelines (McKhann et al., 1984) and determined using a variety of tests, including routine medical examination; neurological evaluation; neuropsychological assessment; MRI; and measurements of CSF levels of A β 1-42, total tau, and phospho-tau. The mean age of AD patients at the time of sample collection was 71.0 ± 8.1 years (range 49–84). As controls, we used CSF from 39 patients affected by NND, including 12 cases of normal pressure hydrocephalus, seven patients with peripheral neuropathy, seven with diverse forms of brain tumor, two with ICTUS, one with severe cephalgia, three with encephalitis, one with hypertension, and six with unclear diagnosis. The mean age at which CSF samples were taken from this group of patients was 64.6 ± 14.7 years (range 31–83). We also used as controls CSF samples from 37 individuals affected by NAND, including ten cases of fronto-temporal dementia (five behavioral and five language variants); six patients with Parkinson's disease (including four associated with dementia and one with motor neuron disease); six with progressive supranuclear palsy; six with spinocerebellar ataxia (one associated with dementia); four with amyotrophic lateral sclerosis; two with Huntington's disease; one with mitochondria encephalomyopathy, lactic acidosis, and stroke-like episodes; one with Lewy body dementia; and one with vascular dementia. The mean age at sample collection for this group was 63.8 ± 11.1 years (range 41–80). CSF samples were collected in polypropylene tubes following lumbar puncture at the L4/L5 or L3/L4 interspace with atraumatic needles after one night fasting. The samples were centrifuged at 3,000 g for 3 min at 4°C, aliquoted, and stored at -80°C until analysis. CSF cell counts, glucose, and protein concentration were determined. Albumin was measured by rate nephelometry. To evaluate the integrity of the blood brain barrier and the intrathecal immunoglobulin G (IgG) production, the albumin quotient (CSF albumin/serum albumin) $\times 10^3$ and the IgG index (CSF albumin/serum albumin)/(CSF IgG/serum IgG) were calculated (Sellebjerg and Christiansen, 1996). The study was conducted according to the provisions of the Helsinki Declaration and was approved by the Ethics Committee.

Production of Synthetic A β and Preparation of A β Aggregates

A β 1-42 was synthesized using solid-phase N-tert-butylloxycarbonyl chemistry at the W. Keck Facility at Yale University and purified by reverse-phase high-performance liquid chromatography. The final product was lyophilized and characterized by amino acid analysis and mass spectrometry. To prepare stock solutions free of A β aggregated seeds, we used our previously described protocol (Morales et al., 2010), which involves dissolution of aggregates in high pH and filtration through 30 kDa cutoff filters to remove remaining aggregates. To prepare different types of aggregates, solutions of seed-free A β 1-42 (10 μM) were incubated for different times at 25°C in 0.1 M Tris-HCl (pH 7.4) with agitation. This preparation contains a mixture of A β monomers as well as fibrils, protofibrils, and soluble oligomers in distinct proportions depending on the incubation time. The degree of aggregation was characterized by ThT fluorescence emission, electron microscopy after negative staining, dot blot studies with the A11 conformational antibody (Kayed et al., 2003), and western blot after gel electrophoresis using the 4G8 A β antibody.

Cyclic Amplification of A β Misfolding and Aggregation

Solutions of 2 μM aggregate-free A β 1-42 in 0.1 M Tris-HCl (pH 7.4; 200 μl total volume) were placed in opaque 96-well plates and incubated alone or in the presence of synthetic A β aggregates or 40 μl of CSF aliquots. Samples were incubated in the presence of 5 μM ThT and subjected to cyclic agitation (1 min at 500 rpm followed by 29 min without shaking) using an Eppendorf thermomixer, at a constant temperature of 22°C. At various time points, ThT

fluorescence was measured in the plates at 485 nm after excitation at 435 nm using a plate spectrofluorometer.

Determination of Sensitivity, Specificity, and Predictive Value

The differences in the kinetic of aggregation between different samples were evaluated by the estimation of two different kinetic parameters: the lag phase and P90. Lag phase is defined as the time required to reach a ThT fluorescence of 40 arbitrary units. This value was selected because it corresponds to approximately five times the value of the buffer alone. The P90 corresponds to the extent of aggregation (measured as ThT fluorescence) at 90 hr. Sensitivity, specificity, and predictive value were determined using the lag phase data, with cutoff thresholds determined by ROC curve analysis, using the MedCalc software (version 12.2.1.0).

Statistical Analysis

The significance of the differences in A β aggregation kinetics in the presence of human CSF samples was analyzed by one-way ANOVA, followed by the Tukey's multiple comparison post hoc test. The level of significance was set at $p < 0.05$. Statistical tests were performed using the GraphPad Prism 5.0 software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.02.031>.

AUTHOR CONTRIBUTIONS

N.S. designed the experiments, optimized the A β -PMCA technology, carried out many of the experiments with CSF samples, analyzed the results, and prepared the figures. M.S. performed various A β -PMCA assays and analyzed the data. E.S. and F.T. provided some of the CSF samples and characterized them. C.S. is the principal investigator on the project and was responsible for coordinating research activity, analyzing the data, funding, and producing the final version of the article.

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