For Peer Review

Title: Amyloid-beta Oligomerization in Alzheimer Dementia vs. High Pathology Controls

Running head: Amyloid-beta Oligomerization

Thomas J. Esparza, BS1, Hanzhi Zhao1, John R. Cirrito, PhD1,2,4, Randall J. Bateman, MD1,2,4, David M. Holtzman, MD1,2,3,4 and David L. Brody, MD, PhD1,2*

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1 Department of Neurology, Washington University School of Medicine
2 Hope Center for Neurological Disorders
3 Department of Developmental Biology,
4 Alzheimer’s Disease Research Center, Washington University School of Medicine
* Correspondence to brodyd@neuro.wustl.edu, (314) 362 1381

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ABSTRACT:
Objective: While amyloid-beta (Aβ) peptide deposition into insoluble plaques is a pathological hallmark of Alzheimer’s disease, soluble oligomeric Aβ has been hypothesized to more directly underlie impaired learning and memory in dementia of the Alzheimer type. However, the lack of a sensitive, specific, and quantitative assay for Aβ oligomers has hampered rigorous tests of this hypothesis.

Methods: We developed a plate-based fluorescence immunoassay for oligomeric Aβ sensitive to low pg/ml (<1 pM) concentrations of synthetic Aβ dimers. The assay uses the same Aβ-specific monoclonal antibody to both capture and detect Aβ. Sensitivity was enhanced using single molecule counting technology. The Aβ oligomer assay does not recognize monomeric Aβ, amyloid precursor protein, or other non-Aβ peptide oligomers. We tested frontal cortex lysates and cerebrospinal fluid from Alzheimer’s patients and controls.

Results: Aβ oligomer concentrations in lysates from patients with dementia of the Alzheimer type were tightly correlated with the extent of Aβ plaque deposition (r=0.88), but this relationship was much weaker in non-demented patients with equivalent Aβ plaque pathology (r=0.30). The ratio of Aβ oligomer levels to plaque density fully distinguished demented from non-demented patients, with no overlap between groups. Other Aβ and plaque measures did not distinguish demented from non-demented patients. Aβ oligomers were not detected in cerebrospinal fluid.

Interpretation: The results raise the intriguing hypothesis that the linkage between plaques and oligomers may be a key pathophysiological event underlying dementia of the Alzheimer type. This Aβ oligomer assay may be useful for many tests of the oligomer hypothesis.

Key words: amyloid-beta, oligomer, Alzheimer’s disease,

INTRODUCTION
Amyloid-beta (Aβ) aggregation and deposition is one of the pathological hallmarks of Alzheimer’s disease. However, the presence of fibrillar Aβ plaque pathology in cognitively normal subjects raises the issue of whether one or more additional events are required to cause neurodegeneration and cognitive impairment. A series of recent studies have shown that soluble oligomeric species of Aβ have direct adverse effects, whereas fibrillar or monomeric Aβ seem to be less harmful in vitro and in animal models. Several studies using postmortem tissue from Alzheimer’s patients have demonstrated the presence of soluble oligomeric Aβ species in diseased brains. Thus, oligomerization of Aβ has been proposed to be a key event in the pathogenesis of dementia of the Alzheimer type.

Several groups have previously developed assays to detect amyloid-β oligomers. Strategies include Western blotting, nano-particle based detection, conformation specific antibodies, and immunoassays utilizing the same monoclonal antibody to capture and detect Aβ. However, all of these assays have had limitations in terms of sensitivity, specificity and quantitative reproducibility. One technical challenge for quantitative studies has been the choice of an oligomer standard which is stable and stoichometrically well-characterized; many synthetic oligomeric preparations contain an unstable mixture of monomers, dimers, trimers and higher order oligomers. Xia and colleagues have addressed this problem with the creation of a stable Aβ1-40 Ser26Cys covalently linked disulfide homo-dimer. Under appropriate redox conditions, essentially pure dimer standards can be produced, which allows for a calculation of oligomer levels in units of “dimer equivalents”.

Our objective in developing a sensitive Aβ oligomer assay was to allow assessment of biological fluids and lysates without the need for immunoprecipitation or other methods of concentrating the samples. Aβ may oligomerize at moderate to high concentrations, which could occur artifactually during such procedures. Furthermore, some biological fluids are available only in very limited quantities. Therefore we optimized a 384-well plate format immunoassay utilizing the fluorescent single-molecule counting Erenna platform. Here, we demonstrate the design, sensitivity, specificity of this assay for analysis of Aβ oligomer concentrations. Using
this assay we have uncovered a previously unrecognized quantitative relationship between Aβ oligomer concentrations and plaque deposition in post-mortem human cortical samples.

METHODS:

Preparation of synthetic Aβ dimer standard

Synthetic Aβ1-40Ser26Cys dimer (#64130-1, AnaSpec, Fremont, CA) was initially dissolved in DMSO followed by dilution in phosphate buffered saline (PBS), pH 7.4 containing 0.01% Tween-20. Dimer peptide was isolated by size exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare, Milwaukee, Wisconsin) eluted with 50 mM ammonium acetate, pH 8.5, at a flow rate of 0.5 ml/min. Fractions (0.25 ml) were collected and the presence of peptide was determined by an indirect enzyme-linked immunosorbent assay (ELISA) and Western blot. Amyloid-β positive fractions found to contain only dimer were pooled and lyophilized for use as the standard. Peptide concentration was determined using a combination of direct ELISA against reduced dimer with a known concentration of Aβ1-40 monomer and a modified bicinchoninic acid (BCA) protein assay.

Preparation of HJ3.4-Alexa647 Detection Antibody

For development of the Aβ oligomer ELISA, we selected the Aβ-specific N-terminal mouse monoclonal IgG1 HJ3.4. Alexa Fluor®647 was conjugated to HJ3.4 via a succinimidyl ester moiety using a commercially available labeling kit (#A20173, Invitrogen, Carlsbad, CA). Unreacted dye was removed using the provided gel filtration column and aliquots were prepared and stored at -20°C for use. The extent of labeling was determined to be four moles of Alexa Fluor® 647 dye per mole of antibody using the absorbance at 280 nm and 650 nm as calculated by the provided manufacturer’s equation.

Aβ Oligomer Assay

Unlabeled mouse monoclonal HJ3.4 was used to coat 384-well Nunc MaxiSorp plates (#464718, Nalge Nunc, Rochester, NY) at 20 µg/mL in a carbonate buffer (35mM sodium bicarbonate, 16 mM sodium carbonate, 3mM sodium azide, pH 9.6) using 20 µl/well overnight at 4°C. Plates were washed 5x between steps
with PBS containing 0.005% Tween-20 using a BioTek EXL405 plate washer (BioTek, Winooski, VT). Sample plates were blocked using 0.2 μm filtered 4% BSA (#7030, Sigma-Aldrich, St. Louis, MO) in PBS for 1 hour at room temperature. Samples or dimer standard were analyzed neat or diluted in standard diluent (0.2 μm filtered 0.25% BSA, 0.005% Tween-20, 3 mM sodium azide, 2 μg/mL aprotinin (EMD Chemicals, Gibbstown, NJ), 1 μg/mL leupeptin (EMD Chemicals), in PBS) to 20 μL final volume and loaded. A 9-point standard curve was generated using 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0 pg/ml of Aβ1-40Ser26Cys dimer and loaded in triplicate in all experiments. All samples and standard were kept on ice during processing. Plates were centrifuged at 1000xg briefly after loading samples and standards to ensure that fluid was properly seated at the bottom of each well and to remove any air bubbles. Samples and standards equilibrated with the coating antibody during overnight 4°C incubation. After another wash step to remove unbound solutes, Aβ oligomers were detected with HJ3.4-Alexa647 at 100 ng/mL in PBS containing 0.2 μm filtered 0.1 mg/mL non-fat dry milk plus 0.005% Tween-20. Binding to exposed Aβ oligomers was allowed to occur for 1 hour at room temperature, protected from light. Following a final wash, bound detection antibody was eluted with 0.1 M glycine, pH 2.7, containing 0.01% Triton X-100 for 10 minutes at room temperature. The elution reaction was terminated by neutralization with 4 μL/well of 1M Tris-HCl, pH 8.0. The eluted Alexa Fluor®647 conjugate was then measured on the Erenna® Immunoassay System (Singulex, Alameda, CA). This system uses a spot illumination and single photon counting approach to detect individual Alexa Fluor 647 fluorescence emission events; thus the results are expressed as “detected events.” The standard curve was then used to calculate the concentration of amyloid-β oligomer in units of “dimer equivalents.” The standard curve was fit using the Richards equation, a 5 parameter logistic regression which accounts for asymmetry \(^{36}\), as implemented in Prism 5.0 (GraphPad Software, La Jolla, CA).

Validation of the standard curve

To determine the accuracy and reproducibility of the standard curve, six independent assays were performed on different plates on different days. Each standard curve was run in triplicate to measure % coefficient of variation as a measure of intra-assay variability. Variability between the 6 assays in the calculated
dimer equivalent values following curve fitting was used to measure inter-assay variability. Precision was measured using the % relative error: curve fitting-based calculated dimer equivalents vs. loaded dimer concentration. Using previously recommended criteria for ligand-binding assays, only standard curve values with a sum of the % coefficient of variation and the absolute value of the % relative error less than 30% were accepted as valid for the standard curve. The lower limit of quantitation (LLOQ) was defined as the lowest concentration on the standard curve which met this acceptance criterion.

**Specificity Controls**

Due to the excess of monomeric amyloid-β compared to oligomer in most relevant biological fluids, it is important to determine the ability of the assay to discriminate oligomeric from monomeric amyloid-β species. Monomeric Aβ_{1-38}, Aβ_{1-40}, and Aβ_{1-42} were prepared by incubating lyophilized stock peptide in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (#52512, Sigma-Aldrich) for 30 minutes followed by sonication and drying under vacuum to create monomer films. Monomeric amyloid-β peptides were diluted into standard diluent and measured over a range (156 pg/mL to 10 ng/mL) of concentrations to determine their effect on signal levels. Amyloid-β_{1-40} monomer was also titrated (3.9 pg/mL to 2 ng/mL) into standard diluent containing a fixed concentration (25 pg/mL) of amyloid-β dimer to determine if a monomer saturation effect occurs in the presence of oligomer.

To verify the specificity of HJ3.4 for Aβ over amyloid precursor protein, an immunodepletion assay was performed on brain homogenate from a 9 month old 3xTg-AD mouse containing high levels of human amyloid precursor protein. Whole brain was removed after transcardial perfusion with PBS containing 0.3% heparin and immediately dounce homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, 0.10% SDS, 0.5% deoxycholic acid, 2.5 mM EDTA, pH 8.0) at a 10:1 ratio (RIPA volume/tissue weight) using 25 strokes followed by brief sonication. The resulting homogenate was centrifuged for 20 minutes at 17,000xg at 4°C to remove insoluble protein. Total protein was determined using a standard BCA protein assay. Individual aliquots containing 100 µg of homogenate were immunodepleted using 10 µg of each antibody (HJ3.4, 82E1, 6E10). After overnight incubation, complexes were captured using 150 µg Protein-G.
Dynabeads® (#100.03D, Invitrogen). The resulting immunodepleted supernatants were assayed by Western blot, as described below, to determine affinity in solution for APP.

The specificity of the assay was further validated by determining its response to N-terminally truncated pyroglutamate Aβ (AβpE3)(#29907-01, Anaspec) species, amyloid-BRI (#62-0-92A, American Peptide, Sunnyvale, CA), and amyloid-DAN (#62-0-04A, American Peptide) oligomers. These other peptide oligomers were prepared by incubation of each species at 2.5 µM in PBS containing 0.025% SDS for one hour prior to addition of glutaraldehyde (#G6257, Sigma-Aldrich, St. Louis, MO) (0.0625% final concentration) for 2 minutes to cross-link associated peptides. The reaction was quenched by the addition of 1:20 volume 1M Tris-HCl, pH 8.0. The formation of SDS-stable, fixed oligomeric species was confirmed by Western blot. These other oligomer preparations were then measured in the assay to determine if any non-specific signal occurred.

**Selection and preparation of human frontal cortical tissue**

Human frontal cortical tissue samples were obtained from the Charles F. and Joanne Knight Alzheimer’s Disease Research Center (KADRC) at Washington University School of Medicine in Saint Louis, Missouri. Cognitive status was determined with a validated retrospective postmortem interview with an informant to establish the Clinical Dementia Rating (CDR). For the validation of this assay, we used tissue from cognitively normal subjects with minimal AD pathology (CDR 0, mean age=91.0 ± 7.9 yrs) (n=10), cognitively normal with Alzheimer’s pathology (CDR 0, mean age=90.2 ± 6.8 yrs) (n=14), and mildly demented Alzheimer’s patients (CDR 1, mean age=87.7 ± 7.5 yrs) (n=9). One CDR 1 patient was excluded from the study due to the lack of plaque pathology. Brains from prospectively assessed individuals were obtained at autopsy with a mean postmortem interval of 11.7 ± 5.7 hours. The right hemispheres from each case were coronally sliced and frozen between pre-cooled Teflon coated plates, placed in a freezer rack, and lowered into liquid nitrogen vapor in a cryo-vessel. Following the initial freezing, tissues were stored at -80°C. Tissue was partially thawed and subdivided into blocks of approximately 0.5-1 cm³ and then refrozen for measurement of amyloid-β oligomer or fixed for histological assessment of amyloid-β plaque deposition.

**Preparation and measurement of amyloid-β oligomers in human frontal cortical tissue homogenates**
Frozen cortical samples including both gray and white matter were weighed and immediately dounced and homogenized in ice-cold PBS containing protease inhibitor (137 mM sodium chloride, 7.76 mM sodium phosphate dibasic, 2.17 mM monopotassium phosphate, 2.7 mM potassium chloride, 2 µg/mL aprotinin, 1 µg/mL leupeptin) at a 10:1 PBS volume : tissue weight ratio using a constant 25 manual strokes. The resulting homogenate was centrifuged in a Beckman Optima TLX ultracentrifuge with a TLA-55 rotor at 100,000xg to remove insoluble fibril material. Aliquots of the cleared homogenates were prepared and frozen at -80°C. The concentration of each sample was determined using a standard BCA protein assay. The concentration of PBS soluble amyloid-β oligomers in each sample was measured in triplicate at empirically determined dilutions and expressed as picograms dimer equivalents/milligrams total soluble protein. The accuracy and reproducibility of the method was determined by the ability to recover a known amount of dimer standard, spiked at three concentrations (12.5, 25, 50 pg/mL), in five brain homogenates from cognitively normal subjects without Aβ pathology, measured in six independent assays.

Control for Artifactual Oligomerization

As a control for artificial oligomerization, HFIP-treated monomeric Aβ142 at a final concentration of 1 ng/mL was spiked into the homogenization buffer of five cognitively subjects without Aβ pathology just prior to tissue homogenization. As an additional control for recovery, 12.5 pg/mL amyloid-β dimer was included in separate matched samples prior to homogenization.

Histological Assessment of Aβ Plaque Pathology

Tissues were fixed by overnight immersion in buffered 4% paraformaldehyde, followed by equilibration in 30% sucrose. Sections were cut at 50 µm on a frozen platform cryostat and maintained in a cryoprotectant buffer (2.7 M ethylene glycol, 0.44 M sucrose, 30 mM phosphate buffer, pH 7.4) prior to immunohistochemistry. Floating tissue sections were washed 3× in Tris-buffered saline (TBS) for 5 minutes each and then incubated with 0.3% H2O2 in TBS for 10 minutes at room temperature to block endogenous peroxidase. Following the incubation, sections were rinsed in TBS 3× for 5 minutes each, and then blocked with 5% normal goat serum (NGS) in TBS-X for 30 minutes at room temperature. Sections were then incubated with
polyclonal rabbit anti-PAN-A\(\beta\) (#44136, Invitrogen) in 5% NGS in TBS-X at a 1:700 dilution overnight at 4 °C. The following day, sections were washed 3× in TBS for 5 minutes each time and incubated with a biotinylated secondary goat anti-rabbit antibody in a 1:1000 dilution in TBS-X for 1 hour at room temperature (Vector Laboratories, Burlingame, CA). Following the incubation of the secondary antibody, the sections were washed 3× in TBS for 5 minutes each, incubated with ABC Elite (Vector Laboratories, Burlingame, CA) at a 1:400 dilution in TBS for 1 hour at room temperature, then washed with TBS 3× for 5 minutes and developed with 3,3′-Diaminobenzidine (#D5905, Sigma-Aldrich). Sections were mounted and dehydrated using a standard ethanol-xylene series followed by coverslapping.

**Quantification of plaque pathology**

Histological samples from each patient were scanned using an Olympus Nanozoomer HT System (Hamamatsu, Bridgewater, NJ). The percent of gray matter containing plaque pathology was determined for each sample using the Image J program (NIH). The gray matter boundary was determined using a Nissl stain (cresyl violet) on an adjacent section and redrawn three times to ensure repeatability. During quantitation, the samples were coded so the user was blinded to oligomer assay results. A manual thresholding approach was used. Data from 8 slices per brain sample, spaced every 1 mm were averaged.

**Western Blot**

Samples for Western blot analysis were combined with standard Laemmli buffer and heated to 85°C to denature for 5 minutes. Homogenates containing amyloid-\(\beta\) were denatured in the absence of SDS to prevent artificial oligomerization. Fractions containing A\(\beta_{1-40}\)Ser26Cys dimer were denatured in the absence of 2-mercaptoethanol to prevent reduction of the disulfide-bridge. Protein samples were size separated on NuPAGE® 12% Bis-Tris gels (Invitrogen) in 2-(N-morpholino)ethanesulfonic acid (MES) SDS running buffer at 150 Volts. SeeBlue® Plus-2 prestained standard (Invitrogen) was used to visualize and estimate the progression and size of the sample migration. Gels were then transferred to 0.2 µm nitrocellulose using Towbin buffer (25 mM Tris, 192 mM glycine, pH 8.6) containing 20% methanol at 150 mA for 1 hour. For A\(\beta\) western blotting, membranes were incubated at 95°C for 1 minute in PBS to allow for improved antigen binding and
then cooled in room temperature PBS prior to blocking. Membranes were blocked in 2% non-fat dry milk (NFDM) PBS for 1 hour. Between all remaining steps, membranes were washed 3x for 10 minutes each with PBS-T (0.05% Tween 20). For detection of amyloid-β, the N-terminal mouse monoclonal 82E1 (IBL-America, Minneapolis, MN) was used at 0.1 µg/mL in 2% NFDM PBS overnight at 4°C. For detection of amyloid precursor protein (APP), the mouse monoclonal 6E10 (Covance, Princeton, NJ) was used at 1 µg/mL in 2% NFDM PBS overnight at 4°C. Bound primary antibodies were detected using a sheep anti-mouse-HRP (#NA931V, GE Healthcare) at 50 ng/mL in 2% NFDM PBS and then developed with ECL Advance Reagent (GE Healthcare) followed by exposure to film emulsion.

**Measurement of Overall Amyloid-beta Levels in Human Frontal Cortical Tissue Homogenates**

PBS soluble homogenates prepared as described above were used to assess the overall levels of Aβ by ELISA. The insoluble cortical tissue pellet remaining after PBS homogenization was dounce homogenized in ice-cold 5M guanidine-hydrochloride, pH 8.0, containing protease inhibitor (2 µg/mL aprotinin, 1 µg/mL leupeptin) at a 10:1 guanidine : tissue weight ratio using a constant 25 manual strokes. The lysates were then incubated overnight at 4°C. The resulting guanidine soluble homogenate was centrifuged in a Beckman Optima TLX ultracentrifuge with a TLA-55 rotor at 100,000xg to remove any insoluble material. Aliquots of the cleared homogenates were prepared and frozen at -80°C. The concentration of each sample was determined using a standard BCA protein assay. Overall Aβ levels were assessed using sandwich ELISAs as described previously. These ELISAs do not distinguish between monomeric and aggregated Aβ. Briefly, a mouse anti-Aβ40 antibody (mHJ2) or mouse anti-Aβ42 antibody (mHJ7.4) was used to capture and a biotinylated central domain antibody (mHJ5.1) was used to detect, followed by streptavidin-poly-HRP-40 (Fitzgerald Industries, Action, MA). All ELISA assays were developed using Super Slow ELISA TMB (Sigma) and absorbance read on a BioTek Synergy 2 plate reader at 650 nm. For the insoluble fraction samples, 5M guanidine was included in the standard curve at an equivalent sample ratio to account for any effect caused by guanidine to the curve dynamics. The resulting values have been expressed as pg of Aβ / mg of soluble protein.

**Assessment of X-34 Positive Fibrillar Plaque Pathology**
X-34 dye (generous gift from Dr. Robert Mach, Washington University) was used to stain 50-µm frontal cortex sections adjacent to those used for immunohistochemical assessment of amyloid-β plaque pathology as previously described. X-34 stained sections were scanned using the FITC channel on an Olympus Nanozoomer HT System (Hamamatsu). For quantitative analyses of X-34 staining, scanned images were exported with NDP viewer software (Hamamatsu) and measured using Image J program (NIH). A manual thresholding approach was used and objects identified were inspected to confirm or reject from analysis. During quantitation, the samples were coded so the user was blinded to patient identifiers. Data from 4 sections per brain sample, spaced every 1 mm were averaged.

**Cerebrospinal Fluid Assessments**

Cerebrospinal fluid samples were obtained by lumbar puncture as described previously. Briefly, 20-30 mL of CSF was collected at 8 AM after overnight fasting. Samples were gently inverted to avoid possible gradient effects, briefly centrifuged at low speed, and aliquoted into polypropylene tubes containing ethylenediaminetetraacetic acid (EDTA) prior to freezing at −84°C. Previously frozen, never thawed aliquots of CSF were assessed for amyloid-beta oligomers in a blinded fashion. Total tau, phosphorylated-tau at residue 181 (ptau181), and Aβ₁₋₄₂ values were measured by ELISA (Innotest, Innogenetics) and have been previously reported. CSF ELISA data was generously provided by Dr. Anne Fagan. These subjects were not the same as those used for post-mortem analysis of frontal cortex tissue.

**Statistical methods**

All data were analyzed using Statistica 6.0 (StatSoft, Tulsa, OK) or Prism 5.0 (GraphPad Software, La Jolla, CA). The Shapiro-Wilk normality test was used to determine whether Aβ oligomer levels or Aβ plaque loads were normally distributed. When non-normal distributions in at least one patient group were observed, Mann-Whitney U tests were used to compare the levels between groups. When levels were normally distributed, t-tests or one-way ANOVA with Bonferroni’s multiple comparison tests were used. Pearson product moment correlations and linear regression analyses were performed to assess the relationships between Aβ oligomer levels and Aβ plaque loads in the two groups of patients (CDR 0 with pathology, CDR1). Although
the distribution of Aβ oligomer levels was not normally distributed, there were approximately linear correlations between Aβ oligomer levels and Aβ plaque loads and the distribution of residuals was normally distributed. The 95% confidence intervals of the slopes of the linear regressions were calculated and the slopes of the regressions were compared between groups using an F-test.

RESULTS

We performed several tests of the sensitivity and specificity of the HJ3.4-based, 384 well plate single photon counting immunoassay. The number of single photon detected events rose monotonically with increasing concentrations of synthetic Aβ dimers (Fig. 1A). Levels as low as 1.56 pg/ml were detectible above background (Fig. 1A inset). Using strict criteria for quantitative reliability, the lower limit of quantitation was determined to be 6.25 pg/ml (Table 1), or 0.72 pM (calculated molecular weight of the synthetic dimers 8690.8 Da). The assay did not detect monomeric Aβ at concentrations as high as 10,000 pg/ml (Fig. 1B). Aβ was maintained in monomeric form using the 0.1% HFIP to disrupt hydrogen bonding (Fig. 1C left lane), as otherwise many species of Aβ will aggregate spontaneously in physiological solutions. HFIP at this concentration did not interfere with the sensitivity of the assay (Fig. 1D). Likewise, the assay was similarly sensitive to another synthetic Aβ heterogeneous oligomer preparation, produced by incubating monomeric Aβ under conditions favoring aggregation, and then cross linking the aggregates with glutaraldehyde (Fig. 1D).

The HJ3.4 antibody used in this assay did not recognize amyloid precursor protein. To verify this specificity, an immunodepletion assay was performed on brain homogenate from a 9 month old 3xTg-AD mouse containing high levels of human amyloid precursor protein. Incubation with 6E10, a monoclonal antibody known to recognize both Aβ and amyloid precursor protein, depleted the lysates of immunoreactivity at the expected molecular weight of amyloid precursor protein (Fig. 1E), whereas incubation with 82E1, a monoclonal antibody with known specificity for Aβ over amyloid precursor protein did not. Incubation with HJ3.4, like incubation with 82E1, did not deplete the lysates of amyloid precursor protein immunoreactivity (Fig. 1E). This indicated that HJ3.4 had very low binding to amyloid precursor protein in solution.
The assay was specific for oligomeric Aβ and did not recognize oligomeric forms of other peptides, unlike assays based on conformation specific antibodies. Oligomeric forms of A-Dan, implicated in familial Danish dementia and A-Bri, implicated in familial British dementia, were not detected at concentrations as high as 200 pg/ml (Fig. 1F). Likewise, oligomeric forms of N-terminally truncated and pyro-glutamate modified Aβ were not detected by the assay (Fig. 1G).

In principle, because the HJ3.4 antibody used to coat the plate binds to both monomeric and oligomeric Aβ, high concentrations of monomers could saturate the available binding sites on the plate and reduce the sensitivity of the assay. We found no evidence of such saturation effects at monomeric Aβ concentrations up to 2000 pg/ml (Fig. 1H). Furthermore, when synthetic Aβ oligomers were added to frontal cortex homogenates, the levels detected were greater than 90% of the expected levels (Table 2). Thus, the immunoassay was demonstrated to be both highly sensitive to and specific for oligomeric Aβ.

To demonstrate the utility of the assay, we assessed frontal cortex samples obtained at autopsy from normal elderly controls without significant AD pathology, non-demented (CDR 0) elderly patients with known Aβ plaque pathology, and patients with mild dementia of the Alzheimer’s type (CDR 1) and Aβ plaque pathology. The 3 groups of patients did not differ in age (p=0.83, Kruskal-Wallis Test) or post-mortem interval (p=0.46). Most were female (Table 3). As expected, Aβ immunohistochemistry revealed no plaque pathology in the normal elderly controls (Fig. 2A-C). There were variable degrees of plaque coverage in both the CDR 0 non-demented elderly patients with known Aβ plaque pathology (Fig. 2D-F) and the patients with CDR1 mild dementia of the Alzheimer’s type (Fig. 2G-I). The plaque coverage expressed as % of gray matter area was quantified in a blinded fashion on 8 slices per brain sample. The extent of Aβ plaque coverage did not differ between non-demented elderly patients with plaque pathology and patients with mild dementia of the Alzheimer’s type in this study (p=0.11, Fig. 2J). This is consistent with previous reports on “preclinical” AD.

Adjacent frontal cortex samples from the same patients were homogenized and found to contain variable levels of Aβ oligomers (Fig. 2K). Similar results in demented patients have been reported previously using an
immunoprecipitation and Western blotting-based approach. Aβ oligomer levels for the normal controls were essentially at or only slightly above the lower limit of quantitation. Aβ oligomer in both the CDR 0 with plaque pathology group and the CDR 1 group were significantly elevated (p=0.0003, Mann Whitney U Tests). The Aβ oligomer levels in the CDR 0 plus pathology group were statistically lower than those in the CDR 1 group (p=0.0023). However, there was considerable overlap between groups (Fig. 2K).

Aβ oligomerization did not appear to be an artifact of the homogenization and assay procedures. The addition of 1000 pg/ml of Aβ1-42 added into the homogenization buffer along with brain tissue from normal control subjects before homogenization did not result in any detectible oligomer signal (Fig. 2L). The presence of brain homogenate did not obscure or block oligomer detection, as addition of Aβ dimer to homogenates resulted in the expected elevation in signal.

Interestingly, the quantitative correlation between Aβ oligomer levels and Aβ plaque coverage was very strong in the CDR 1 group (r²=0.88), but less tight in the CDR 0 plus plaque pathology group (r²=0.33, Fig. 2M). The slopes of the regression lines were statistically significantly different (F(1,19)= 52.8, p=<0.0001). Unlike plaque area or oligomer measurements in isolation, the ratio of Aβ oligomer levels to Aβ plaque coverage completely distinguished the two groups, with no overlap in these populations (Fig. 2N).

We were not able to find other measures that completely distinguished CDR 1 from CDR 0 plus plaque pathology samples. Specifically, we assessed measurements of PBS-soluble Aβ1-40, PBS-soluble Aβ1-42, guanidine-soluble Aβ1-40, guanidine-soluble Aβ1-42, X-34 labeled fibrillar plaque areas, and ratios of these parameters to Aβ plaque area (Figs. 3-4). Expected differences from normal controls and correlations between parameters were observed, but there was substantial overlap in every measure between the CDR 1 and CDR 0 plus plaque pathology groups.

Despite the high sensitivity of the assay, oligomeric Aβ was not detected in cerebrospinal fluid samples from separate patients with mild dementia of the Alzheimer’s type (Table 4). Many of these samples had low Aβ1-42, high tau and high phospho-tau, consistent with previously reported cerebrospinal fluid biomarkers of Alzheimer’s disease pathology.
DISCUSSION

In summary, we have developed a sensitive, specific and quantitative assay for Aβ oligomers. Using this assay, we have demonstrated that Aβ oligomer levels are tightly linked to plaque deposition in frontal cortex samples from patients with clinical dementia of the Alzheimer’s type, but much less tightly linked in cognitively normal elderly patients with comparable levels of Aβ plaque deposition. This relationship has not been previously recognized to our knowledge, and this finding exemplifies the utility of the Aβ oligomer assay described here. An important finding is that cognitively normal patients with Aβ plaque pathology can have substantial levels of Aβ oligomers, levels as high as some CDR 1 demented patients. This suggests that oligomer levels per se in brain lysates may not be a direct correlate of dementia, but that the events leading to a tight linkage between plaques and oligomers may play a pathophysiological role in the progression of clinical disease status.

A recent study has demonstrated that soluble oligomers isolated from Alzheimer’s cortex cause cytoskeletal abnormalities at 100-fold less concentration than synthetic dimers in primary hippocampal neuron cultures \(^{52}\). This finding, along with our observation of oligomers in preclinical AD subjects, raises the intriguing possibility that there could be a shift in neurotoxicity in oligomeric species during disease progression. The combination of sensitive toxicity assessments and a quantitatively rigorous Aβ oligomer assay would be greatly beneficial in addressing this question and directing therapeutic development.

Aβ oligomerization has been hypothesized to underlie cognitive deterioration in dementia of the Alzheimer’s type. However, detection of Aβ oligomers has not been reported in the living human brain. In principle, tissue homogenization and oligomer assay procedures could result in artifactual Aβ oligomerization, but such artifactual oligomerization does not appear to be occurring to any appreciable extent using the methods described here. This adds confidence to the conclusion that the Aβ oligomers detected arose \textit{in vivo}, although it does not settle the question of whether they formed pre- or post-mortem. We were unable to detect oligomers in cerebrospinal fluid from patients with mild dementia of the Alzheimer’s type. Importantly, it is not known whether oligomers detected in PBS-soluble brain lysates are freely diffusible \textit{in vivo} in the extracellular space of...
the human brain. Instead, they could in principle be loosely associated with plaques or cells where they could exert locally toxic effects.\textsuperscript{53}

Several limitations of this study should be noted. First, the sample size was relatively small, and consisted exclusively of elderly subjects with late onset dementia. A larger population allowing stratification by ApoE genotype as well as younger subjects and subjects with familial Alzheimer’s disease should be assessed. Second, only frontal cortex was assessed. The temporal lobe, posterior cingulate/precuneus, and hippocampal regions known to be especially vulnerable to Alzheimer’s pathology have not been examined. Third, the size forms of the oligomers assessed have not been resolved. This assay uses ‘dimer equivalents’ as the unit of measure, but does not distinguish between size forms; a trimer or higher order oligomer may bind more than one detecting antibody and therefore be counted as more than one oligomer. The use of this assay on fractions obtained from size exclusion chromatography will be an important area for future investigation. Fourth, the assay does not distinguish between oligomers of Aβ peptides of various lengths including the longer C99 peptide resulting from β-secretase but not γ-secretase cleaved APP. However, in human AD brain extracts, most oligomers appear to be primarily composed of Aβ\textsubscript{1-42}.\textsuperscript{13} Fifth, the timing of when the switch from a loose correlation between Aβ plaque coverage and Aβ oligomer levels to a tight correlation has not been determined. Very mild (CDR 0.5) dementia subjects should be assessed in the future. Sixth, samples such as blood, urine, and brain interstitial fluid have not been systematically assessed and thus the utility of this method for ante-mortem detection of Aβ oligomers has not been established. If Aβ oligomers can be detected ante-mortem, the ratio of oligomer levels to PET PIB binding could be a close analog of the oligomer / plaque ratio shown here to distinguish demented and non-demented subjects. Finally, more extensive analyses of the plaque pathology may be revealing. It is not known whether neuritic dystrophy, microgliosis, post-translational modifications of Aβ, or tau pathologies are related to these findings.

Despite these limitations, these findings raise several interesting questions. Why are some Aβ plaques tightly associated with oligomers and some are not? Could early plaques serve as oligomer binding or sequestration sites which later become saturated and leave Aβ oligomers free to diffuse through the extracellular space of the
brain? What are the effects of ApoE genotype, tau localization, and microglial signaling on the coupling between plaques and oligomers? What is the relationship between plaques and oligomers in transgenic mouse models of Alzheimer’s disease? The use of a sensitive, specific and quantitatively useful assay may assist in addressing these questions and many others. The very high sensitivity may be especially important for applications such as assessments of microdialysis samples, laser capture microdissection analyses, and size fractionation experiments. The quantitative reliability may be useful for the assessment of dose-response relationships in mechanistic, electrophysiological, and behavioral experiments. Finally, the 384-well plate format could lend itself to adaptation to high throughput assays of compounds designed to inhibit Aβ oligomerization.

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**Author contributions:** TJE, JRC, RJB and DLB designed research. TJE and HTZ performed research and primary data analysis. TJE and DLB performed statistical analyses, DMH contributed antibodies. TJE and DLB prepared figures and wrote the paper. The corresponding author DLB has access to all of the primary data and vouches for its integrity.
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TABLE 1: Amyloid-β Oligomer Assay Characteristics

<table>
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<tr>
<th>Amyloid-β Dimer Standards (pg/mL)</th>
<th>200</th>
<th>100</th>
<th>50</th>
<th>25</th>
<th>12.5</th>
<th>6.25</th>
<th>3.125</th>
<th>1.56</th>
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<td></td>
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<td></td>
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<td></td>
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<tr>
<td>%CV mean</td>
<td>2.14</td>
<td>2.86</td>
<td>1.73</td>
<td>3.80</td>
<td>5.63</td>
<td>7.58</td>
<td>5.93</td>
<td>23.67</td>
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<tr>
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<td>10.7</td>
<td>2.27</td>
<td>5.78</td>
<td>6.51</td>
<td>9.25</td>
<td>9.18</td>
<td>30.0</td>
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<tr>
<td>%RE mean</td>
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<td>0.40</td>
<td>-1.97</td>
<td>4.98</td>
<td>-5.30</td>
<td>3.45</td>
<td>-19.67</td>
<td>21.95</td>
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<tr>
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<td>1.05</td>
<td>3.31</td>
<td>7.22</td>
<td>11.45</td>
<td>15.72</td>
<td>29.97</td>
<td>36.89</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>‘sometimes’</td>
<td>‘rarely’</td>
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<td><strong>Inter-assay</strong></td>
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<td></td>
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<tr>
<td>Mean (pg/mL)</td>
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<td>99.60</td>
<td>50.98</td>
<td>23.76</td>
<td>13.16</td>
<td>6.03</td>
<td>3.74</td>
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<td>SD</td>
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<td>0.32</td>
<td>0.43</td>
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<td>0.39</td>
<td>0.51</td>
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<td>0.23</td>
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<tr>
<td>%CV</td>
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<td>19.1</td>
</tr>
<tr>
<td>Samples (N)</td>
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<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
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<tr>
<td>%CV: % coefficient of variation.</td>
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<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

%RE: % relative error.

*Quantitation criteria: Sum of %CV and absolute %RE ≤ 30%

SD: Standard deviation

TABLE 2: Amyloid-β Oligomer Recovery in the Presence of Brain Tissue Homogenate

<table>
<thead>
<tr>
<th>Sample</th>
<th>Low  (12.5 pg/mL)</th>
<th>Medium (25 pg/mL)</th>
<th>High  (50 pg/mL)</th>
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<tr>
<td>Frontal cortex 1</td>
<td>98.6±4.3</td>
<td>88.6±1.5</td>
<td>95.5±2.0</td>
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<tr>
<td>Frontal cortex 2</td>
<td>98.5±7.4</td>
<td>90.9±2.9</td>
<td>95.5±2.4</td>
</tr>
<tr>
<td>Frontal cortex 3</td>
<td>100.3±8.2</td>
<td>93.0±3.0</td>
<td>96.2±1.5</td>
</tr>
<tr>
<td>Frontal cortex 4</td>
<td>96.3±7.8</td>
<td>91.4±5.6</td>
<td>95.6±2.2</td>
</tr>
<tr>
<td>Frontal cortex 5</td>
<td>100.0±4.5</td>
<td>90.8±2.4</td>
<td>95.3±2.1</td>
</tr>
</tbody>
</table>

Mean Percent Recovery (+/- SD) calculated as Aβ oligomers measured / Aβ oligomers added to normal control frontal cortex homogenates * 100. Each sample was measured in triplicate.
TABLE 3: Characteristics of Human Brain Frontal Cortex Samples

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Status</th>
<th>Age (yrs.)</th>
<th>PMI (hrs.)</th>
<th>Gender</th>
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</thead>
<tbody>
<tr>
<td>Patient – 1</td>
<td>CDR 0</td>
<td>85</td>
<td>20.5</td>
<td>female</td>
</tr>
<tr>
<td>Patient - 2</td>
<td>CDR 0</td>
<td>86.3</td>
<td>6.3</td>
<td>female</td>
</tr>
<tr>
<td>Patient - 3</td>
<td>CDR 0</td>
<td>90</td>
<td>9.5</td>
<td>female</td>
</tr>
<tr>
<td>Patient - 4</td>
<td>CDR 0</td>
<td>78.5</td>
<td>15.75</td>
<td>male</td>
</tr>
<tr>
<td>Patient - 5</td>
<td>CDR 0</td>
<td>91.5</td>
<td>16</td>
<td>male</td>
</tr>
<tr>
<td>Patient - 6</td>
<td>CDR 0</td>
<td>95.6</td>
<td>9.1</td>
<td>female</td>
</tr>
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<td>Patient - 7</td>
<td>CDR 0</td>
<td>107.8</td>
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</tr>
<tr>
<td>Patient - 8</td>
<td>CDR 0</td>
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<td>12</td>
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<td>Patient - 9</td>
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<td>12</td>
<td>male</td>
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<tr>
<td>Patient -10</td>
<td>CDR 0</td>
<td>92.1</td>
<td>6</td>
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<tr>
<td>Patient -11</td>
<td>CDR 0 +path</td>
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<td>CDR 0 +path</td>
<td>91.6</td>
<td>16</td>
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<td>Patient -13</td>
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<td>CDR 0 +path</td>
<td>85.8</td>
<td>7</td>
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</tr>
<tr>
<td>Patient -23</td>
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<td>CDR 0 +path</td>
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<td>13</td>
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<tr>
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<td>5.25</td>
<td>female</td>
</tr>
<tr>
<td>Patient -27</td>
<td>CDR 1</td>
<td>80.2</td>
<td>6.3</td>
<td>female</td>
</tr>
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<td>Patient -28</td>
<td>CDR 1</td>
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<td>13</td>
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<tr>
<td>Patient -29</td>
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<td>Patient -33</td>
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<td>94</td>
<td>11.6</td>
<td>female</td>
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</tbody>
</table>

PMI: Post-Mortem Interval; CDR: Clinical Dementia Rating
Table 4: Characteristics of Human Cerebrospinal Fluid Samples

<table>
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<tr>
<th>Subject ID</th>
<th>CDR Status</th>
<th>Age at L.P. (yrs.)</th>
<th>Tau (pg/ml)</th>
<th>Phospho-tau (pg/ml)</th>
<th>Overall Aβ$_{1-42}$ (pg/ml)</th>
<th>Oligomeric Aβ (dimer equivalents pg/ml)</th>
</tr>
</thead>
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<tr>
<td>34</td>
<td>0</td>
<td>68.2</td>
<td>246</td>
<td>164</td>
<td>1080</td>
<td>&lt;6.25</td>
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<tr>
<td>35</td>
<td>0</td>
<td>66.6</td>
<td>216</td>
<td>52</td>
<td>1024</td>
<td>&lt;6.25</td>
</tr>
<tr>
<td>36</td>
<td>0</td>
<td>72.5</td>
<td>198</td>
<td>49</td>
<td>976</td>
<td>&lt;6.25</td>
</tr>
<tr>
<td>37</td>
<td>0</td>
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<td>73</td>
<td>844</td>
<td>&lt;6.25</td>
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<td>38</td>
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<td>81</td>
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</tr>
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<td>39</td>
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<td>74.2</td>
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<td>95</td>
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<td>&lt;6.25</td>
</tr>
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<td>86</td>
<td>924</td>
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<td>383</td>
<td>70</td>
<td>360</td>
<td>&lt;6.25</td>
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CDR: Clinical Dementia Rating, L.P.: Lumbar puncture.
Tau, phospho-tau and overall Aβ$_{1-42}$ were measured by standard ELISA.
**FIGURE LEGENDS**

**Figure 1:** Sensitivity and specificity characteristics of the Aβ oligomer assay. 

- **A.** Standard curve demonstrating a monotonic relationship between detected events and the concentration of synthetic Aβ1-40 Ser26Cys dimer. 
  *Inset:* concentrations of Aβ dimer as low as 3.125 pg/ml were detectible above background. 
- **B.** Specificity for Aβ dimers over Aβ monomers. (Note the log scale of the x-axis) 
- **C.** Western blot demonstrating that the Aβ monomer preparation did not contain dimer and the Aβ1-40 Ser26Cys dimer preparation did not contain Aβ monomer. 
- **D.** Sensitivity of the assay to another type of synthetic Aβ oligomers, prepared using aggregated wild-type sequence Aβ cross-linked with glutaradehyde. 
- **Right:** Western blot demonstrating a mixture of monomers, dimers, trimers and tetramers in this preparation. 
- **E.** Immunodepletion experiment demonstrating that HJ3.4, the antibody used in the Aβ oligomer assay, does not bind APP in solution. Controls: 82E1 is known to require a free amino terminus of Aβ to bind, and therefore does not recognize APP, whereas 6E10 is known to bind both APP and Aβ (Reproduced from Tran et al. 2011). 
- **F.** Specificity for Aβ dimers over aggregated A-Dan and A-Bri, two non-Aβ peptides that readily oligomerize. (Note the log scale of the x-axis) 
- **G.** Specificity for full length Aβ dimers over N-terminally truncated, pyroglutamate modified Aβ oligomers. 
- **H.** Sensitivity to Aβ dimers is unchanged in the presence of high concentrations of Aβ monomers.

**Figure 2:** Oligomerization of Aβ is tightly linked to plaque density in dementia of the Alzheimer type but not in high pathology elderly controls.  

- **A-C.** Aβ immunohistochemistry using a polyclonal rabbit anti-pan-Aβ on frontal cortex sections from normal elderly controls demonstrates the absence of plaque pathology. Scale bar: 1 mm applies to panels A-I. 
- **D-F.** Aβ plaque pathology in frontal cortex sections from non-demented elderly subjects (CDR 0). 
- **G-I.** Aβ plaque pathology in frontal cortex sections from elderly subjects with mild dementia of the Alzheimer’s type (CDR 1). 
- **J.** Gray matter coverage by Aβ plaque pathology was not different in the non-demented elderly subjects with plaques (CDR 0 + plaques) vs. subjects with mild dementia of the Alzheimer’s type (CDR 1). 
- **K.** Aβ oligomer levels in PBS-soluble frontal cortical homogenates. (** p=0.0023, *** p=0.0003, Mann Whitney U tests). Oligomer levels expressed as pg dimer equivalents per mg total protein.
in homogenates. L. Control for artifactual oligomerization of monomeric Aβ during homogenization and analysis. The addition of 1000 pg/ml of monomeric Aβ1-42 spiked into the homogenization buffer along with brain tissue from normal control subjects did not result in any detectible oligomer signal. The presence of brain homogenate did not obscure or block oligomer detection, as spiking in Aβ dimer resulted in the expected elevation in signal (**p=0.0001, 1-way ANOVA). M. Correlations between Aβ oligomer levels (y-axis) and gray matter Aβ plaque pathology coverage (x-axis). r^2 values represent Pearson product moment correlations. N. Ratio of Aβ oligomer levels to Aβ plaque pathology coverage was higher in subjects with mild dementia of the Alzheimer’s type (CDR 1) vs non-demented elderly subjects with plaques (CDR 0 + plaques). There was no overlap between groups (**p=0.0001, Mann Whitney U test).

**Figure 3:** Assessments based on overall Aβ levels did not distinguish tissue from patients with mild dementia of the Alzheimer type (CDR 1) vs. non-demented elderly patients with plaque pathology (CDR 0 + plaque). A. No difference between groups in overall PBS-soluble Aβ1-40 levels, as measured using a standard sandwich ELISA. Data expressed as pg of Aβ per mg total protein. B. Overall PBS-soluble Aβ1-42 levels were not different in the CDR 0 + plaque vs. CDR 1 group, though levels in both groups were higher than in the normal control group (*p < 0.05, *** p < 0.001, Kruskall Wallis ANOVA with Dunn’s post-hoc test). C. Ratio of overall PBS-soluble Aβ1-42 levels to overall PBS-soluble Aβ1-40 levels did not distinguish CDR 0 + plaque vs. CDR 1 groups. The CDR 0 + plaque group had higher ratios than the control group (**p < 0.01). D-E. Overall Guanidine-soluble Aβ1-40 levels and Aβ1-42 levels were not different in the CDR 0 + plaque vs. CDR 1 group. Levels in both groups were higher than in the normal control group for both measures. F. Ratio of overall Guanidine-soluble Aβ1-42 levels to overall PBS-soluble Aβ1-40 levels did not distinguish CDR 0 + plaque vs. CDR 1 groups. The CDR 0 + plaque group had higher ratios than the control group (**p < 0.001). G-H. Correlations between overall PBS-soluble Aβ1-40 and Aβ1-42 levels vs. plaque-positive gray matter. The correlation was only significant (p = 0.0023) for PBS-soluble Aβ1-42 vs. plaque area in the CDR 0 + plaque group. I-J. Ratios of overall PBS-soluble Aβ1-40 and Aβ1-42 levels to plaque-positive gray matter area coverage.
did not distinguish CDR 0 + plaque vs. CDR 1 groups (n. s. not significant, Mann-Whitney U Tests). **K-L.**

Significant correlations between overall Guan-soluble Aβ\textsubscript{1-40} and Aβ\textsubscript{1-42} levels vs. plaque-positive gray matter. (p = 0.01 for Guan-soluble Aβ\textsubscript{1-40} vs. plaque area in the CDR 1 group, p = 0.02 for Guan-soluble Aβ\textsubscript{1-42} vs. plaque area in the CDR 0 + plaque group, p = 0.04 for Guan-soluble Aβ\textsubscript{1-42} vs. plaque area in the CDR 1 group).

**M-N.** Ratios of overall Guan-soluble Aβ\textsubscript{1-40} and Aβ\textsubscript{1-42} levels to plaque-positive gray matter area coverage did not distinguish CDR 0 + plaque vs. CDR 1 groups (n. s. not significant, Mann-Whitney U Tests).

**Figure 4:** Assessments based on X-34 staining of fibrillar plaque pathology did not distinguish between tissue from patients with mild dementia of the Alzheimer type (CDR 1) vs. non-demented elderly patients with plaque pathology (CDR 0 + plaque). **A-I:** Exemplar images of X-34 staining in frontal cortex sections. Scale bar = 1 mm. **J.** Gray matter coverage by X-34 in CDR 0 + plaques group vs. CDR 1 group (n.s., Mann Whitney U test). **K.** Correlations between overall Aβ plaque coverage vs. X-34 positive fibrillar plaque coverage. (Spearman r = 0.65, p = 0.06 for the CDR 1 group). **L.** Correlations between Aβ oligomer levels (dimer equivalents) and gray matter X-34 positive fibrillar plaque pathology coverage. **M.** Ratio of Aβ oligomer levels (dimer equivalents) to X-34 positive fibrillar plaque pathology coverage was higher in the CDR 0 + plaque group (p = 0.02, Mann Whitney U test). However, there was substantial overlap between groups.
Figure 1: Sensitivity and specificity characteristics of the Aβ oligomer assay
204x199mm (300 x 300 DPI)
Figure 2: Oligomerization of Aβ is tightly linked to plaque density in dementia of the Alzheimer type but not in high pathology elderly controls.

200x200mm (300 x 300 DPI)
Figure 3: Assessments based on overall Aβ levels did not distinguish tissue from patients with mild dementia of the Alzheimer type (CDR 1) vs. non-demented elderly patients with plaque pathology (CDR 0 + plaque).

189x274mm (300 x 300 DPI)
Figure 4: Assessments based on X-34 staining of fibrillar plaque pathology did not distinguish between tissue from patients with mild dementia of the Alzheimer type (CDR 1) vs. non-demented elderly patients with plaque pathology (CDR 0 + plaque)