

Brain amyloid- β oligomers in ageing and Alzheimer's disease

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Alzheimer's disease begins about two decades before the onset of symptoms or neuron death, and is believed to be caused by pathogenic amyloid- β aggregates that initiate a cascade of molecular events culminating in widespread neurodegeneration. The microtubule binding protein tau may mediate the effects of amyloid- β in this cascade. Amyloid plaques comprised of insoluble, fibrillar amyloid- β aggregates are the most characteristic feature of Alzheimer's disease. However, the correspondence between the distribution of plaques and the pattern of neurodegeneration is tenuous. This discrepancy has stimulated the investigation of other amyloid- β aggregates, including soluble amyloid- β oligomers. Different soluble amyloid- β oligomers have been studied in several mouse models, but not systematically in humans. Here, we measured three amyloid- β oligomers previously described in mouse models—amyloid- β trimers, A β *56 and amyloid- β dimers—in brain tissue from 75 cognitively intact individuals, ranging from young children to the elderly, and 58 impaired subjects with mild cognitive impairment or probable Alzheimer's disease. As in mouse models, where amyloid- β trimers appear to be the fundamental amyloid- β assembly unit of A β *56 and are present in young mice prior to memory decline, amyloid- β trimers in humans were present in children and adolescents; their levels rose gradually with age and were significantly above baseline in subjects in their 70s. A β *56 levels were negligible in children and young adults, rose significantly above baseline in subjects in their 40s and increased steadily thereafter. Amyloid- β dimers were undetectable until subjects were in their 60s; their levels then increased sharply and correlated with plaque load. Remarkably, in cognitively intact individuals we found strong positive correlations between A β *56 and two pathological forms of soluble tau (tau-CP13 and tau-Alz50), and negative correlations between A β *56 and two postsynaptic proteins (drebrin and fyn kinase), but none between amyloid- β dimers or amyloid- β trimers and tau or synaptic proteins. Comparing impaired with age-matched unimpaired subjects, we found the highest levels of amyloid- β dimers, but the lowest levels of A β *56 and amyloid- β trimers, in subjects with probable Alzheimer's disease. In conclusion, in cognitively normal adults A β *56 increased ahead of amyloid- β dimers or amyloid- β trimers, and pathological tau proteins and postsynaptic proteins correlated with A β *56, but not amyloid- β dimers or amyloid- β trimers. We propose that A β *56 may play a pathogenic role very early in the pathogenesis of Alzheimer's disease.

Keywords: amyloid- β ; Alzheimer; dimer; trimer; A β *56; oligomer

Introduction

In Alzheimer's disease functional, structural and biochemical abnormalities appear one or two decades before the clinical symptoms of dementia develop (Jack *et al.*, 2010; Sperling *et al.*, 2011; Bateman *et al.*, 2012). Longitudinal neuropsychological studies indicate that cognitive impairment begins approximately a decade before individuals report any symptoms (Amieva *et al.*, 2008). These observations suggest that Alzheimer's disease, like many common chronic diseases of ageing, consists of an asymptomatic followed by a symptomatic phase.

While the cause of Alzheimer's disease is unknown, genetic studies suggest it is due to the formation of abnormal amyloid- β aggregates (reviewed in Ashe and Zahs, 2010). Neuropathological, genetic and molecular data indicate that the microtubule binding protein tau may mediate the disease process; the pattern of neurodegeneration correlates with tau neuropathology and tau mediates amyloid- β induced neurotoxicity and cognitive deficits (Gomez-Isla *et al.*, 1997; Rapoport *et al.*, 2002; Roberson *et al.*, 2007). However, Alzheimer's disease has never been linked to tau mutations, arguing against a role for tau in initiating the disease. The amyloid cascade hypothesis consolidates these observations (Hardy and Higgins, 1992), positing that specific aggregates of amyloid- β trigger molecular events leading to widespread neurodegeneration mediated by tau. An important focus of recent studies is to identify the specific amyloid- β aggregates involved in the amyloid cascade.

Although insoluble, fibrillar aggregates of amyloid- β constitute the chief neuropathological hallmark of Alzheimer's disease, they correlate poorly with brain dysfunction in humans (Giannakopoulos *et al.*, 2003; Bennett *et al.*, 2004). Several soluble aggregates (oligomers) of amyloid- β have been studied in animal models (Lesné *et al.*, 2006; Cheng *et al.*, 2007; Shankar *et al.*, 2008; Gandy *et al.*, 2010; Reed *et al.*, 2011). Three specific amyloid- β oligomers isolated directly from the brains of patients with Alzheimer's disease or transgenic mouse models of Alzheimer's disease have been assayed for their effects on cognition: amyloid- β dimers, amyloid- β trimers and A β *56. There is strong evidence that amyloid- β dimers and A β *56 impair brain function when injected into young, healthy rats (Lesné *et al.*, 2006; Shankar *et al.*, 2008; Reed *et al.*, 2011). In contrast, the evidence for deleterious effects of amyloid- β trimers is less clear. Amyloid- β trimers appear to be the compositional assembly unit of A β *56 because A β *56 dissociates into trimers before monomers (Lesné *et al.*, 2006). It has been difficult to conclude that amyloid- β trimers disrupt cognition, because they are present in mice before memory decline (Lesné *et al.*, 2006), and do not convincingly impair cognition when injected into the cerebral ventricles of rats (Reed *et al.*, 2011). These amyloid- β oligomers have been studied in animals, but we do not yet know their relevance to human disease. In particular, we know neither the order in which these amyloid- β oligomers appear nor the stages of Alzheimer's disease in which they are present. Characterizing amyloid- β dimers, amyloid- β trimers and A β *56 in human brain tissue from cognitively intact individuals spanning a wide age range as well as from impaired subjects may provide clues about the temporal sequence and effects of amyloid- β oligomers in the amyloid cascade.

Our measurements of amyloid- β oligomers in human brain tissue used techniques that under some conditions artificially generate or degrade synthetic amyloid- β oligomers. There has, therefore, been concern that A β *56 and other oligomers found in the brain are methodological artefacts, related to exposing amyloid- β to non-native conditions (Benilova *et al.*, 2012). However, this would not be the case if they were identified in CSF. Amyloid- β dimers were previously described in human CSF (Klyubin *et al.*, 2008). In the present study, we identified A β *56 and amyloid- β trimers in human CSF.

Several studies of amyloid- β oligomers in human brain have focused on subjects with clinically diagnosed Alzheimer's disease (Shankar *et al.*, 2008; Noguchi *et al.*, 2009; Mc Donald *et al.*, 2010; Pham *et al.*, 2010). Recent developments in the field suggest that therapeutic interventions may be more effective in the asymptomatic phase of Alzheimer's disease (Zahs and Ashe, 2010; Golde *et al.*, 2011); understanding how amyloid- β is involved very early in its pathogenesis may lead to better preventive treatments. One approach is to examine amyloid- β oligomers in cognitively intact individuals, some of whom may be in the asymptomatic phase of Alzheimer's disease. Therefore, in addition to measuring amyloid- β dimers, amyloid- β trimers and A β *56 in 58 subjects with mild cognitive impairment or probable Alzheimer's disease, and five subjects with non-Alzheimer dementia, we also studied 75 neurologically intact individuals between the ages of 1 and 96 years.

Materials and methods

Human brain tissue

Religious Orders Study

Brain tissue from the inferior temporal gyrus (Brodmann area 20) from 90 subjects underwent biochemical analyses. Cognitive status was assessed with the Mini-Mental Status Examination and 19 other tests summarized as a global measure of cognition and five cognitive domains (Boyle *et al.*, 2006). Selected cases were chosen to ensure that the three groups would not differ significantly from the whole Religious Orders Study cohort (283 subjects at the start of this study). The characteristics of the three clinical diagnostic groups are summarized in Supplementary Table 1.

Each participant had undergone a uniform structured baseline clinical evaluation and annual follow-up evaluation until death (Bennett *et al.*, 2012). Briefly, both dementia and an Alzheimer's disease diagnosis required evidence of meaningful decline in cognitive function and impairment in at least two cognitive domains (for Alzheimer's disease, one domain had to have been episodic memory), based on the results of 21 cognitive performance tests and their review by a clinical neuropsychologist and expert clinician. Mild cognitive impairment refers to participants with cognitive impairment as assessed by the neuropsychologist but without a diagnosis of dementia, as determined by the clinician. No cognitive impairment refers to those individuals without dementia or mild cognitive impairment. At death, a neurologist blinded to all post-mortem data reviewed all available clinical data and rendered a summary diagnostic opinion regarding the clinical diagnosis at the time of death (Bennett *et al.*, 2006).

Following death, each case was assigned, by examiners blinded to all clinical data, a Braak score based on neuronal neurofibrillary tangle pathology (Braak and Braak, 1991), a neuritic plaque score based on the modified Consortium to Establish a Registry for Alzheimer Disease criteria (excluding age and clinical diagnosis), and an Alzheimer's pathological diagnosis based on the National Institute on Aging–Reagan Institute Working Group criteria (Bennett *et al.*, 2005). Neuritic plaques, diffuse plaques, and neurofibrillary tangles in the inferior temporal cortex were counted after Bielschowsky silver staining, as previously described (Bennett *et al.*, 2003). Amyloid load and the densities of plaques and tangles were quantified in six brain regions (hippocampus, entorhinal cortex, midfrontal gyrus, inferior temporal gyrus, inferior parietal gyrus and calcarine cortex) (Bennett *et al.*, 2004). For amyloid load, amyloid- β was labelled with an N-terminus directed monoclonal antibody (MO0872, Dako, 1:100), using diaminobenzidine as the reporter with 2.5% nickel sulphate to enhance immunoreaction product contrast. Video images of amyloid- β stained sections were captured for a random sample for quantitative analysis of amyloid deposition using StereoInvestigator software version 9 (MicroBrightfield Inc) and an Olympus BX-51 microscope with an attached motorized stage.

Exclusion criteria

Cases with post-mortem intervals >12 h (four with no cognitive impairment, one with Alzheimer's disease) were excluded from the original selection, because after 12 h levels of the postsynaptic markers drebrin and NR2B declined with increasing post-mortem intervals. One study participant with a clinical diagnosis of mixed dementia (Alzheimer's disease plus stroke) was also excluded. One tissue sample showed pronounced protein degradation of all proteins tested and was excluded from all analyses.

National Institute of Child Health and Human Development Brain and Tissue Bank for developmental disorders brain specimens

The inferior temporal gyrus (Brodmann area 20) from 49 individuals with post-mortem intervals <12 h was obtained from the Brain and Tissue Bank at the University of Maryland, Baltimore.

Human cerebrospinal fluid specimens

After the calvarium but before the brain was removed, a needle was inserted into the occipital horn of the lateral ventricle and ~20 ml of ventricular CSF was removed, centrifuged and stored in cryogenic tubes at -80°C , as previously reported (Hensley *et al.*, 2011).

Transgenic animals

Mice used in this study included heterozygous transgenic mice and non-transgenic littermates from Tg2576 (Hsiao *et al.*, 1996) and J20 (Mucke *et al.*, 2000), which express human amyloid precursor protein with the Swedish (Lys670Arg, Met671Leu) and Indiana (Val717Phe) mutations, respectively, driven by the prion protein and platelet-derived growth factor promoters, respectively. All Tg2576 mice were offspring of mice backcrossed successively to B6SJL/F1 breeders while J20 mice were crossed for at least 10 generations with C57Bl/6J mice. Genotype was analysed by PCR and confirmed using immunoblots probed with 6E10 antibodies.

Protein extractions

We used two extraction protocols, as previously described (Lesne *et al.*, 2006; Shankar *et al.*, 2008; Sherman and Lesne, 2011). All supernatants were ultracentrifuged for 20 min at 100 000g. Protein amounts were determined using a bicinchoninic acid protein assay (BCA Protein Assay, Pierce). Finally, before analysis, fractions were immunodepleted by incubating for 1 h at room temperature with 50 μl of 1:1 slurry Protein A-Sepharose, Fast Flow[®] followed by 50 μl of 1:1 slurry Protein G-Sepharose, Fast Flow[®] (GE Healthcare Life Sciences).

Immunoprecipitation of amyloid- β from human cerebrospinal fluid

For immunoblots probed with 6E10 antibodies, aliquots of human CSF (500 μl) were pre-cleared with 50 μl of 1:1 slurry of Protein-G Fast Flow Sepharose[®] (GE Healthcare Life Sciences) for 1 h at 4°C , then centrifuged at 9300g for 5 min. Subsequently, 250 μl of immunoglobulin-depleted CSF was incubated with 5 μg 6E10 antibodies and 50 μl Protein-G coated magnetic beads (Life Technologies) overnight at 4°C . The beads were washed with immunoprecipitation buffer A [50 mM Tris-HCl, 300 mM NaCl, 0.1% Triton[®] X-100 (v/v), 1 mM EDTA, pH 7.4] and immunoprecipitation buffer B [50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton[®] X-100 (v/v), 1 mM EDTA, pH 7.4] for 20 min under gentle agitation at 4°C and captured proteins were eluted by boiling in 30 μl of SDS-PAGE loading buffer.

For immunoblots probed with A11 antibodies, aliquots of human CSF (1 ml) were pre-cleared with 50 μl of 1:1 slurry Protein A-Sepharose, Fast Flow[®] (GE Healthcare Life Sciences) for 1 h at 4°C . Following centrifugation at 9300g for 5 min, supernatants were incubated with 5 μg of 6E10 antibodies and 50 μl of 1:1 slurry Protein A-Sepharose, Fast Flow[®] overnight at 4°C . The beads were washed sequentially with immunoprecipitation buffer A [50 mM Tris-HCl, 300 mM NaCl, 0.1% Triton[®] X-100 (v/v), 1 mM EDTA, pH 7.4] and immunoprecipitation buffer B [50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton[®] X-100 (v/v), 1 mM EDTA, pH 7.4] for 20 min under gentle agitation at 4°C and captured proteins were eluted by boiling in 25 μl of SDS-PAGE loading buffer.

Western blotting and quantification

Gel electrophoresis

Depending upon the targeted protein, 2–100 μg of protein were aliquoted, resuspended with $4\times$ Tricine loading buffer, and size fractionated by PAGE using pre-cast 10–20% SDS polyacrylamide Tris-Tricine gels, or 10.5–14% or 7.5% Tris-HCl gels (Bio-Rad).

Transfer

Proteins were transferred to a 0.45 μm polyvinylidene difluoride membrane (Immobilon P membrane, Millipore) or 0.2 μm nitrocellulose membrane (Bio-Rad).

Blotting

Nitrocellulose membranes were boiled twice in 50 ml PBS by microwaving first for 25 s and then, after 3 min, for 15 s. Membranes were blocked in Tris-buffered saline-0.1% Tween[®]20 containing 5% bovine serum albumin (Sigma) for 2 h at room temperature, and probed with appropriate antisera/antibodies diluted in blocking buffer. Primary antibodies were detected with anti-IgG immunoglobulins conjugated with either biotin or horseradish peroxidase. When biotin-conjugated

secondary antibodies were used, horseradish peroxidase-conjugated Neutravidin® (Pierce) or ExtrAvidin® (Sigma) was added to amplify the signal. All blots were developed with an enhanced chemiluminescence western blotting detection system (Supersignal Pico Western system, Pierce).

Stripping

Membranes were stripped using Restore™ Plus Stripping buffer (Pierce) for 30–180 min at room temperature, depending on antibody affinity.

Quantification

Densitometry was performed using OptiQuant software (Packard Bioscience). Pilot experiments for each protein were run to determine the experimental conditions that produced signals within the linear range of detection. This method produced a dynamic range of ~100-fold above the background level of 10^4 densitometry light units. The level of each protein was the mean of triplicate measurements. The 138 brain specimens were each extracted using two methods, yielding five soluble fractions or extracts per specimen; >600 samples were assayed for each protein studied. We did not normalize protein level values to α -tubulin or β III-tubulin, because these tubulins correlated inversely with some amyloid- β species.

Antibodies

The following primary antibodies were used in this study: 6E10 (1:2500) against the amino-terminus of amyloid- β , 4G8 (1:2500) against the mid-region of amyloid- β , biotinylated-6E10 (1:2500) (Covance), A11 (1:2000) against large oligomers (generous gift of R. Kaye and C. Glabe), A β_{42} - and A β_{40} -end specific monoclonal antibodies Mab2.1.3 and Mab13.1.1 (1:1000; generous gift of Pritam Das) and A β_{42} and A β_{40} carboxyl end-specific monoclonal antibodies 8G7 and 5C3 (1:1000; EMD Biosciences), amyloid- β amino end-specific monoclonal antibody 82E1 (1:2000; IBL), APP_{Cter}-C17 (1:5000) against APP C-terminus (generous gift of A. Delacourte and N. Sergeant), anti- α -tubulin (Sigma; 1:100 000), anti- β III-tubulin (Sigma; 1:50 000), anti-drebrin (1:4000; Sigma), anti-NeuN (Invitrogen; 1:5000), anti-synaptophysin (Chemicon; 1:25 000), CP13/PG5/PHF1/Alz50 (generous gift of P. Davies; 1:1000), Tau-5 (Biosource; 1:2000) and T14 (Invitrogen; 1:2000), anti-PSD95 (1:200; Santa Cruz Biotechnology), anti-Fyn (1:1000; BD Biosciences) and anti-NR1CT (1:1000; Millipore).

Statistical analyses

When dependent variables were not normally distributed, non-parametric statistics were used (Spearman rank correlations, Kruskal-Wallis one-way ANOVA by ranks followed by Bonferroni-corrected two-group *post hoc* Mann-Whitney U tests). Relationships between amyloid- β trimers or amyloid- β dimers and age were determined using the Pearson correlation test. Curvilinear relationships between A β *56 levels and age were estimated by quadratic polynomial regression analysis.

To control for the increased probability of type I errors due to multiple comparisons, the criterion for statistical significance was set at $P=0.01$. All analyses were performed using StatView software, version 5.0.1 (SAS Institute).

Results

Detection of amyloid- β dimers, amyloid- β trimers and A β *56 in human cerebrospinal fluid and brain tissue

Our analysis focused on three oligomers—amyloid- β dimers, amyloid- β trimers and A β *56—whose effects on cognition have been studied in animals. To ascertain whether these oligomers exist under native conditions in humans, we immunocaptured proteins from human CSF with 6E10 antibodies, and found 6E10-immunoreactive bands with molecular masses consistent with amyloid- β trimers and A β *56 (Fig. 1A), and 4G8-immunoreactive bands with molecular masses consistent with amyloid- β dimers (not shown). Immunocaptured A β *56 also reacted with A11 antiserum (Fig. 1B), which specifically detects soluble amyloid- β oligomers larger than tetramers that are distinct from amyloid- β fibrils (Kayed *et al.*, 2003). In a separate study (unpublished data), we obtained additional evidence supporting the existence of amyloid- β dimers, amyloid- β trimers and A β *56 in human CSF: (i) proteins with the expected molecular masses were immunocaptured with 4G8 antibodies and detected with 6E10 antibodies, excluding the possibility that they were fragments of soluble amyloid precursor protein; (ii) proteins with the expected molecular masses were not immunoprecipitated by amyloid precursor protein antibodies flanking amyloid- β , arguing against the possibility that they were cleavage or degradation products of the amyloid precursor protein recognized by 6E10; and (iii) 6E10-immunoreactive proteins with the expected molecular masses were separated under native conditions using size-exclusion chromatography, arguing against the possibility that they were artificially generated by detergents.

To measure A β *56, amyloid- β trimers and amyloid- β dimers in brain tissue, we used two protein extraction protocols previously used to detect soluble amyloid- β oligomers in brains from humans and Tg2576 mice overexpressing human amyloid- β (Lesné *et al.*, 2006; Shankar *et al.*, 2008). One noteworthy difference between the two extraction protocols is that the extracellular- and intracellular-enriched fractions are devoid of membrane-associated proteins, while Tris-buffered saline extracts contain small amounts of membrane-associated proteins (Supplementary Fig. 1). The presence of membrane-associated proteins in Tris-buffered saline extracts precludes using these extracts to measure amyloid- β trimers, because they could potentially be confused with C-terminal fragments, which associate with membranes and have similar molecular masses. We estimate that in our brain extracts 50–100 μ g protein contains 20–1000 pg of A β *56 or amyloid- β trimers, and may therefore escape detection using standard immunoblot protocols in which the limit of detection is ~1000 pg of amyloid- β . By employing high-sensitivity, high-specificity immunoblots, which detect as few as ~10 pg of amyloid- β , in combination with a broad panel of antibodies, we completed a biochemical analysis confirming that these proteins were comprised of amyloid- β .

We examined the inferior temporal gyrus (Brodmann area 20) because this brain region shows metabolic abnormalities in the

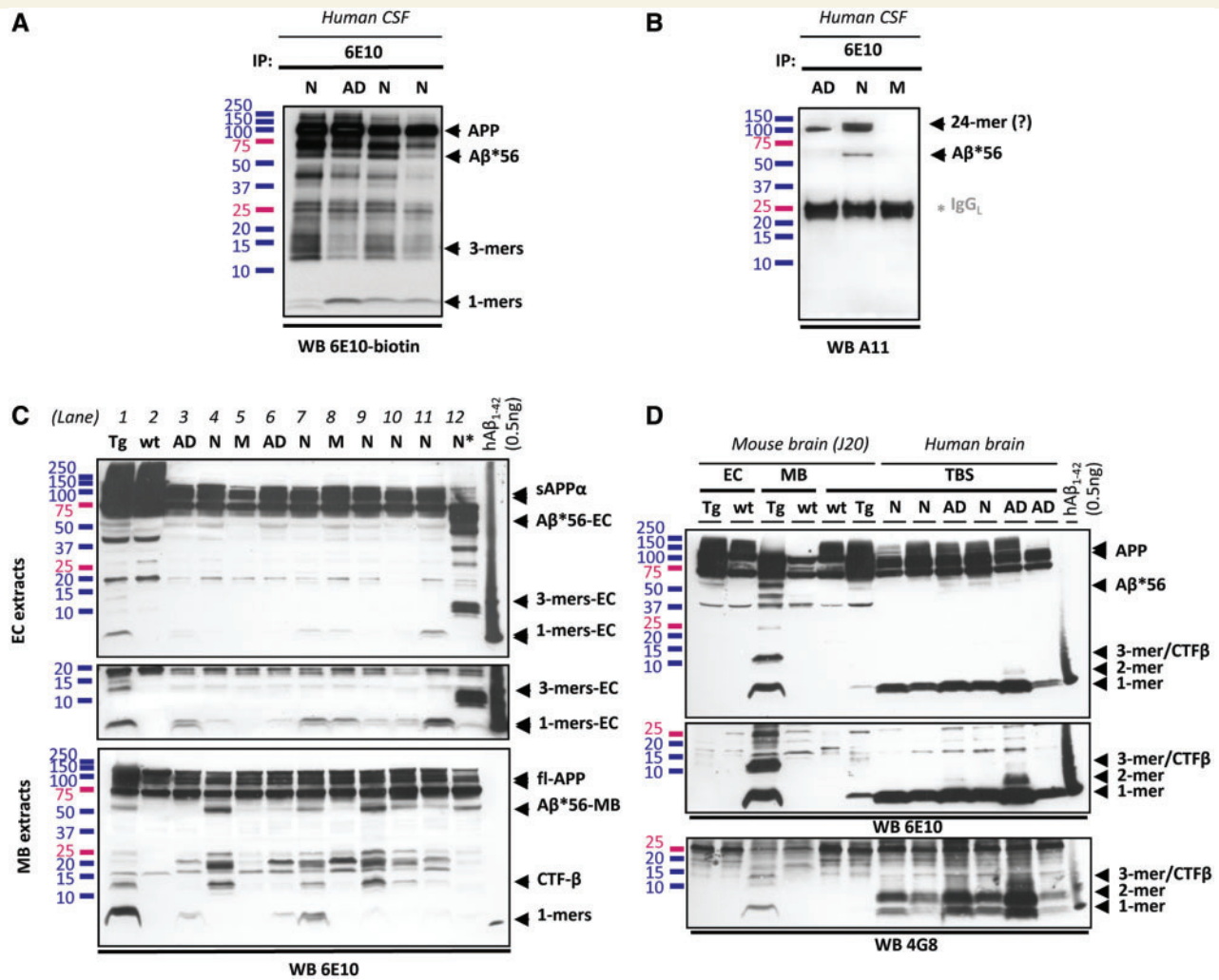


Figure 1 Detection of amyloid- β dimers, amyloid- β trimers and A β *56 in CSF and brain. (A) 6E10-immunoreactive soluble amyloid- β and soluble amyloid precursor proteins in 6E10-immunocaptured proteins from human CSF. The middle band in the triplet running at molecular masses between 13–16 kDa is an amyloid- β oligomer; the upper and lower bands are fragments of the amyloid precursor protein (unpublished data). (B) A11-immunoreactive amyloid- β oligomers in 6E10-immunocaptured proteins from human CSF. (C) 6E10-immunoreactive proteins in extracellular (EC)- or membrane (MB)-enriched fractions from human and mouse brain. Lanes 1 and 2: J20 transgenic (Tg) and non-transgenic (wt) mice; Lanes 3–12: human brain from subjects with no cognitive impairment (N), mild cognitive impairment (M) or probable Alzheimer's disease (AD). *The sample in Lane 12 shows protein degradation and was excluded from all analyses. The middle panel represents a longer exposure of the lower portion of the full panel above. (D) 6E10- and 4G8-immunoreactive proteins in extracellular-enriched and membrane-enriched fractions as well as Tris-buffered saline (TBS) extracts from human and mouse brain. *Middle*: A longer exposure of the lower portion of the full panel above. Amyloid- β dimers are better detected using 4G8 than 6E10 antibodies. A synthetic human amyloid- β_{1-42} loading standard (0.5 ng) was included on all blots.

asymptomatic phase of Alzheimer's disease (Small *et al.*, 2000; Reiman *et al.*, 2004; Petrie *et al.*, 2009), and its neuropathological characteristics, defined by plaque load and tangle density, were highly representative of the six regions sampled. In the inferior temporal gyrus, plaques correlated with the average plaque load of the six regions (Spearman $\rho = 0.95$; $P < 0.01$) and tangles also correlated with the average tangle load of the six regions (Spearman $\rho = 0.77$; $P < 0.01$). To assess the possibility that amyloid- β oligomer levels might be influenced by neuron loss, we performed measurements of the neuronal protein NeuN and a general cellular marker, α -tubulin (Supplementary Fig. 2A). We detected no significant changes in the levels of α -tubulin (not

shown) or NeuN with respect to clinical status or Braak stage (Supplementary Fig. 2B). However, there was a trend toward lower levels of NeuN in subjects with mild cognitive impairment or Alzheimer's disease [Kruskal-Wallis followed by Mann-Whitney U test with Bonferroni corrections, $P(\text{no cognitive impairment versus mild cognitive impairment}) = 0.12$ and $P(\text{no cognitive impairment versus Alzheimer's disease}) = 0.12$].

We identified soluble A β *56 in extracellular- and membrane-enriched fractions (Fig. 1C), and also in Tris-buffered saline extracts (Fig. 1D). To confirm that A β *56 is not a cleavage product of the amyloid precursor protein, we showed that antibodies recognizing the amyloid precursor protein but not amyloid- β ,

including APP^{Cter}-C17 and 22C11, do not detect a 56kDa protein (Supplementary Fig. 3). We also showed that A β *56 is recognized by the mid-region antibody 4G8 and another amino-region antibody, 82E1 (Supplementary Fig. 4). We could not detect A β *56 using A β ₄₀ or A β ₄₂ end-specific antibodies 5C3 and 8G7, presumably because its folding structure buries the carboxyl-terminus of amyloid- β (Supplementary Fig. 3). Importantly, the A11 antiserum detected A β *56 (Supplementary Fig. 5), providing additional evidence that it is an oligomer comprised of amyloid- β .

We found amyloid- β dimers in Tris-buffered saline extracts (Fig. 1D). Although both 6E10 and 4G8 detected amyloid- β dimers, we used 4G8 antibodies to quantify amyloid- β dimers, because 4G8 showed greater affinity for dimers than 6E10 antibodies, consistent with a previous report (Kawarabayashi *et al.*, 2004). We also showed that some amyloid- β dimers are recognized by A β ₄₀ and A β ₄₂ end-specific antibodies (Supplementary Fig. 6). Thus, we detected amino-, mid- and carboxyl regions of amyloid- β , confirming the composition of amyloid- β dimers.

We measured amyloid- β trimers in extracellular-enriched extracts (Fig. 1C). We avoided membrane-enriched extracts because C-terminal fragments of the amyloid precursor protein can mask potential trimers, which have equivalent molecular masses. We detected amyloid- β trimers with 6E10, 4G8 and 8G7. Hence, we detected amino-, mid- and carboxyl regions of amyloid- β , confirming the composition of amyloid- β trimers.

In summary, using amyloid- β -specific antibodies, we detected the amino-, mid- and carboxyl-regions of amyloid- β in amyloid- β trimers and amyloid- β dimers. We detected the amino- and mid-regions of amyloid- β in A β *56, and showed that it was recognized by A11, an oligomer-specific antiserum. We found no immunoreactivity of these proteins using antibodies that detect regions of the amyloid precursor protein outside of the amyloid- β sequence. We eliminated the possibility that the amyloid- β oligomers were artificially generated through exposure to detergents by showing that they fractionated separately in CSF under non-denaturing conditions. Collectively, the data show that the oligomers are comprised of amyloid- β , and support the existence of amyloid- β dimers, amyloid- β trimers and A β *56 in the human CNS.

Amyloid- β oligomers increase with age in intact subjects

To study the accumulation of amyloid- β dimers, amyloid- β trimers and A β *56 in neurologically intact individuals, we measured these amyloid- β oligomers in the inferior temporal gyrus (Brodmann area 20) in 75 neurologically intact subjects between the ages of 1 and 96 years. Forty-nine specimens were obtained from the Brain and Tissue Bank for Developmental Disorders and 26 specimens from the Religious Orders Study Tissue Bank. All measurements were performed blind to the age of the subjects. Analyses were performed on the mean of triplicate measurements.

We detected amyloid- β trimers at all ages (Fig. 2A and B). This finding is consistent with previous observations in mice overexpressing amyloid- β , in which amyloid- β trimers appear to be the

basic assembly unit and are present prior to memory decline (Lesné *et al.*, 2006). Their levels rose gradually and became significantly higher relative to those in children and adolescents beginning in subjects in their 70s.

We found amyloid- β dimers in a single individual <60 years of age; their levels increased markedly thereafter (Fig. 2C and D).

We found that A β *56 rose with age in a curvilinear manner (Fig. 2E and F). A β *56 was undetectable in children and adolescents. It remained at low or undetectable levels until middle-age, increasing significantly above baseline levels in subjects in their 40s. It is noteworthy that on average, significant increases in A β *56 occurred at least two decades before the increases in amyloid- β dimers and amyloid- β trimers became significant. These observations raised the possibility that A β *56 may play a pathogenic role in the asymptomatic phase of Alzheimer's disease.

A β *56 correlates positively with pathological soluble tau proteins and negatively with postsynaptic proteins

To explore the possibility that A β *56 may play a pathogenic role in the asymptomatic phase of Alzheimer's disease, we first examined its relationship to pathological tau molecules. Recent *in vitro* and *in vivo* studies have shown that insoluble tau in neurofibrillary tangles exerts negligible neurotoxicity compared with soluble tau (Santacruz *et al.*, 2005; Oddo *et al.*, 2006; Roberson *et al.*, 2007; Hoover *et al.*, 2010; Ittner *et al.*, 2010). The recent finding that unspecified forms of soluble amyloid- β appear to induce pathological changes in soluble tau in mice (Chabrier *et al.*, 2012) raises the questions of whether this also occurs in humans and which forms of amyloid- β are responsible. To address these questions, we measured two forms of soluble tau in membrane-enriched extracts, total tau and tau-Alz50, a pathological tau conformer (Wolozin *et al.*, 1986). Total soluble tau remained stable (Fig. 3A and B), but soluble tau-Alz50 rose significantly with age (Fig. 3C and D). Interestingly, both soluble tau-Alz50 and A β *56 began to increase in subjects in their 40s (Figs 2F, 3C and D). Consistent with this coincidental increase in the levels of both proteins, we found strong correlations between A β *56 and soluble tau-Alz50 (Spearman $\rho = 0.71$, $P < 0.01$; Fig. 3F), but not between A β *56 and total soluble tau (Fig. 3E). We also evaluated our data using a polynomial regression approach to control for the possibility that the significant associations between A β *56 and soluble tau-Alz50 occurred because both dependent variables correlated with age. Even after adjusting for age, we found a very strong relationship between A β *56 and soluble tau-Alz50 (adjusted multiple $R^2 = 0.74$, ANOVA, $P < 0.01$; significance of A β *56 as a predictor of tau-Alz50, $P < 0.01$), indicating that A β *56 predicts soluble tau-Alz50, independent of age.

To obtain additional support for our hypothesis that A β *56 may play a pathogenic role in the asymptomatic phase of Alzheimer's disease, we examined the relationships between A β *56 and two additional pathological forms of soluble tau, tau-CP13 and

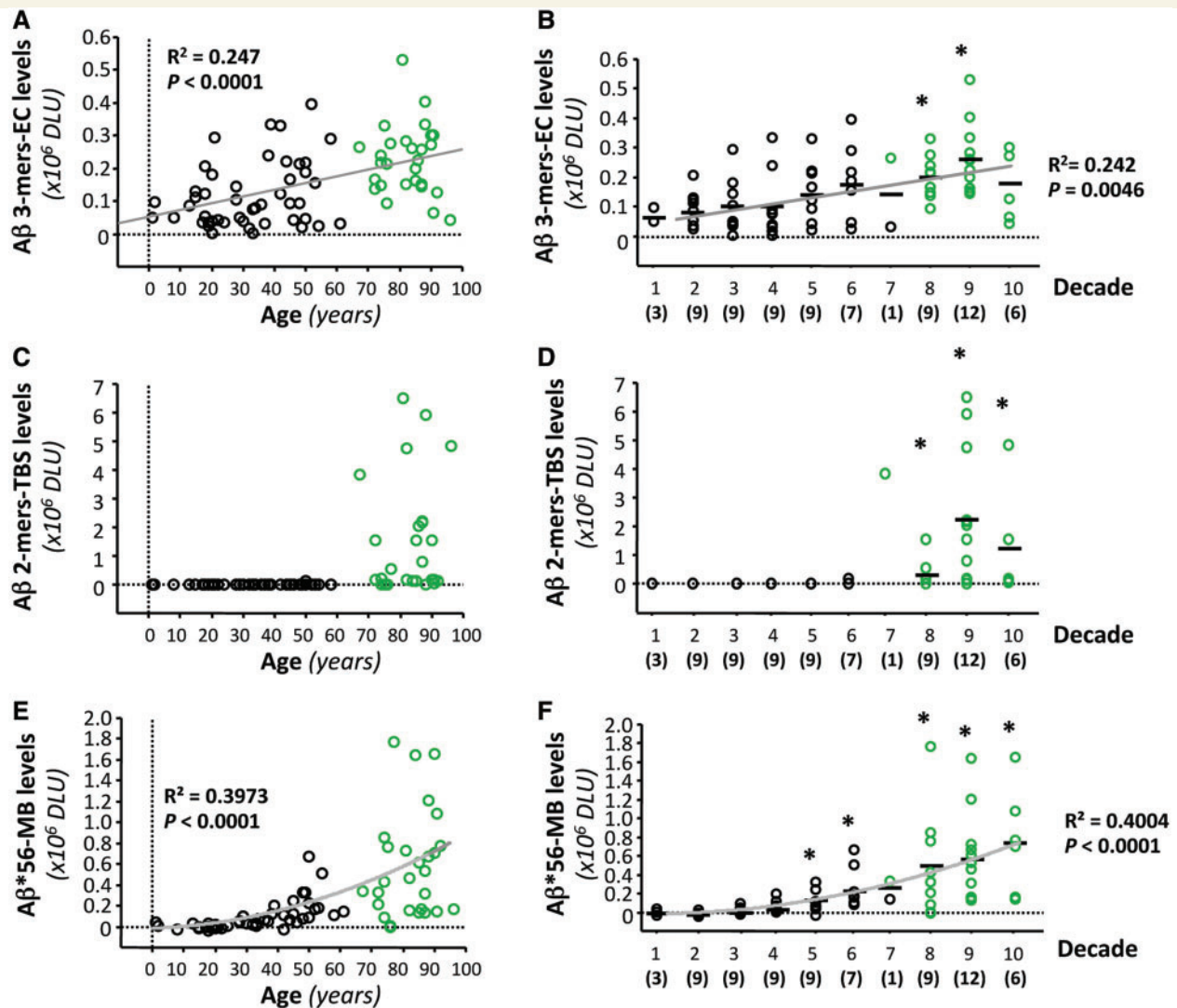


Figure 2 Specific amyloid- β oligomers in the brain rise at different ages in cognitively intact subjects. (A–F) Forty-nine brain specimens from the Brain and Tissue Bank for Developmental Disorders and 26 brain specimens from the Religious Orders Study Tissue Bank (shown in black and green, respectively) were analysed as one group ($n = 75$) to evaluate soluble amyloid- β dimers, amyloid- β trimers and A β^*56 in cognitively intact subjects ranging from 1 to 96 years of age. The relationships between age and amyloid- β trimers in extracellular (EC)-enriched fractions (A and B), amyloid- β dimers in Tris-buffered saline (TBS) extracts (C and D) and A β^*56 in membrane (MB)-enriched fractions (E and F) expressed as a function of age (A, C and E) or grouped by decade (B, D and F). Curves show the best fit quadratic regression functions. * $P < 0.01$, compared with individuals < 20 years of age, using Kruskal-Wallis test followed by Mann-Whitney U test with Bonferroni corrections. DLU = densitometry light units.

tau-PG5. These phospho-specific tau antibodies detect Ser202 and Ser409, respectively. In this study we focused on subjects in the Religious Orders Study without cognitive impairment. We found strong positive correlations between A β^*56 and two pathological forms of soluble tau (tau-CP13 and tau-Alz50) (Table 1). The positive correlations with tau-CP13 and tau-Alz50 are consistent with A β^*56 inducing specific pathological abnormalities in tau in humans. We also measured five synaptic proteins: the presynaptic protein, synaptophysin, and four postsynaptic proteins, PSD-95, drebrin, the NR1 subunit of NMDA receptors, and fyn kinase, because A β^*56 appears to impair long-lasting synaptic plasticity (Chapman *et al.*, 1999). We found strong negative correlations

between A β^*56 and two postsynaptic proteins (drebrin and fyn kinase) (Table 1). The negative correlations between A β^*56 and the two postsynaptic proteins are consistent with the possibility that A β^*56 disrupts synaptic function in humans. Since these relationships were found in unimpaired subjects, they might underlie the reductions in glucose utilization and neuropsychological test performance observed in subjects at risk of Alzheimer's disease. Taken together, the data support our hypothesis that A β^*56 may play a pathogenic role in the asymptomatic phase of Alzheimer's disease.

In contrast, we found no correlations between amyloid- β dimers or amyloid- β trimers and either total soluble tau or soluble

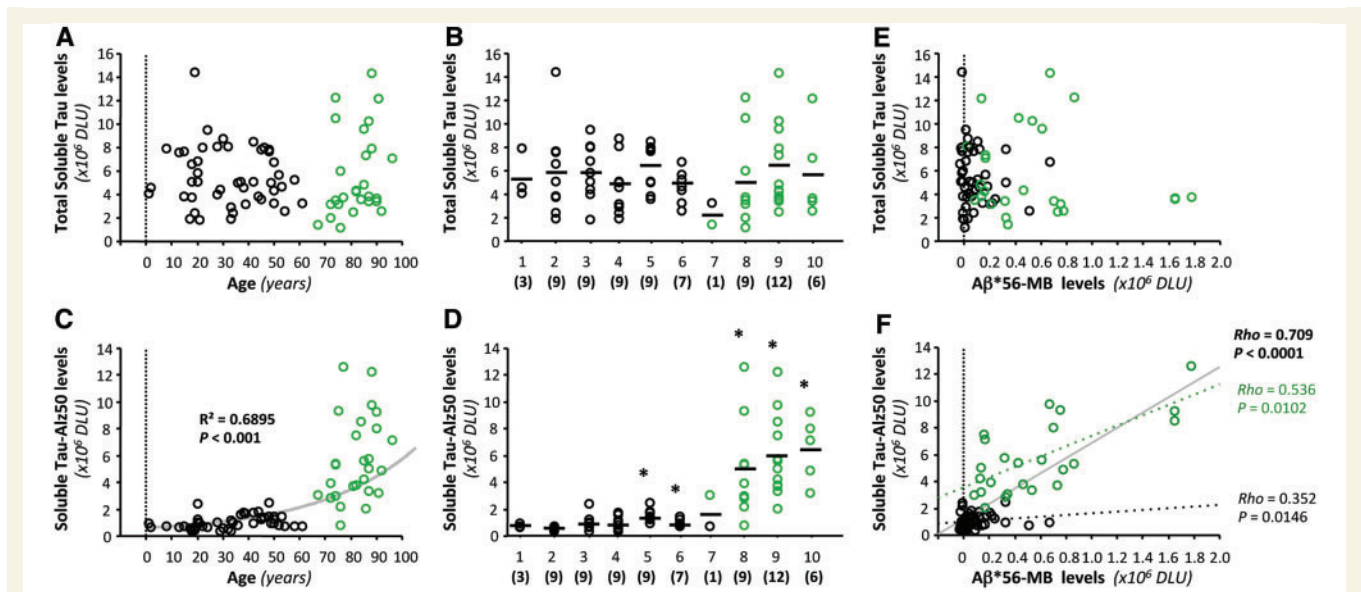


Figure 3 Brain Aβ*56 correlates with pathological soluble tau in cognitively intact subjects. Forty-nine brain specimens from the Brain and Tissue Bank for Developmental Disorders and 26 brain specimens from the Religious Orders Study Tissue Bank (shown in black and green, respectively) were analysed as one group ($n = 75$) to evaluate total soluble tau and soluble tau-Alz50 as a function of age (A and C) or decade (B and D). (A and B) Total soluble tau, detected with Tau-5 antibodies, did not change with age. (C and D) The function that best defines the relationship between soluble tau-Alz50 and age is an exponential curve. Soluble tau-Alz50 increased with age, starting in subjects in their 40s, compared with individuals <20 years of age (Kruskal-Wallis followed by Mann-Whitney U test with Bonferroni corrections, $*P < 0.01$). (E and F) Relationships (Spearman rank correlations) between Aβ*56, total soluble tau and soluble tau-Alz50 in membrane (MB)-enriched fractions. We found no correlation between total soluble tau and Aβ*56 (E), but a strong correlation between Aβ*56 and soluble tau-Alz50 (F). The solid line is the regression for the pooled samples and the dotted lines are for the separate Religious Orders Study and Brain and Tissue Bank for Developmental Disorders samples, respectively. DLU = densitometry light units.

Table 1 Correlations between amyloid-β dimers, amyloid-β trimers, Aβ*56 and synaptic and soluble pathological tau proteins in cognitively intact elderly subjects^a

Soluble amyloid-β species ^b	Amyloid-β dimers	Amyloid-β trimers	Aβ*56
Synaptic proteins			
Presynaptic			
Synaptophysin	0.01, 0.85	-0.17, 0.40	0.02, 0.91
Postsynaptic			
PSD-95	0.28, 0.17	-0.20, 0.33	-0.14, 0.48
Drebrin	-0.01, 0.97	-0.30, 0.13	-0.52, <0.01
Fyn	0.19, 0.34	-0.25, 0.22	-0.53, <0.01
NMDAR1	-0.10, 0.64	0.15, 0.46	0.02, 0.92
Soluble pathological tau proteins			
Tau-CP13	-0.00, 0.99	0.35, 0.08	0.62, <0.01
Tau-Alz50	0.08, 0.71	0.04, 0.85	0.44, <0.01
Tau-PG5	-0.06, 0.75	-0.07, 0.75	0.10, 0.64

^a26 subjects in the Religious Orders Study with no cognitive impairment.

^bAmyloid-β dimers, amyloid-β trimers and Aβ*56 were measured in Tris-buffered saline, extracellular-enriched and membrane-enriched fractions, respectively. Table entries show (*rho*, *P*-value) for Spearman rank correlations.

Significant correlations are indicated in bold.

tau-Alz50 in the intact full cohort (Supplementary Fig. 7), and none between these two amyloid-β oligomers and the three pathological tau or five synaptic proteins we examined from subjects in the Religious Orders Study without cognitive impairment

(Table 1). The lack of significant relationships between amyloid-β dimers or amyloid-β trimers and the tau or synaptic proteins attenuates the possibility that these oligomers are pathogenic in the asymptomatic phase of Alzheimer’s disease.

Differential expression patterns of amyloid- β oligomers between clinical groups

We also characterized amyloid- β dimers, amyloid- β trimers and A β *56 in brain tissue from impaired subjects from the Religious Orders Study with mild cognitive impairment, probable Alzheimer's disease and non-Alzheimer's dementia, and compared them with subjects without cognitive impairment. The clinical statuses of these individuals were assigned before autopsy, and the cases with non-Alzheimer dementia were confirmed by neuropathological inspection after autopsy. Education, post-mortem interval, gender, and interval of time between last exam and autopsy did not differ among the clinical diagnostic groups (Supplementary Table 1). Global cognitive status, episodic memory, plaque load, and neurofibrillary tangle density differed significantly between the clinical diagnostic groups (Fig. 4). The pathological characteristics of the clinical diagnostic groups selected for this study were similar to those of the entire cohort, whether assessed by plaque load or tangle density (Fig. 4). All measurements were performed blind to the clinical status of the subjects. Analyses were performed on the mean of triplicate measurements.

We measured amyloid- β dimers, amyloid- β trimers and A β *56 in each protein fraction, and found distinct but overlapping levels of these oligomers in the different clinical groups (Fig. 5). Amyloid- β dimers in Tris-buffered saline extracts were elevated in subjects with probable Alzheimer's disease compared with subjects with no or mild cognitive impairment, but the latter two groups did not differ from each other (Fig. 5A).

Amyloid- β trimers in extracellular-enriched fractions peaked in subjects with mild cognitive impairment and fell in probable Alzheimer's disease. Amyloid- β trimers in subjects with probable Alzheimer's disease were lower than those in subjects with either no or mild cognitive impairment (Fig. 5B).

A β *56 in extracellular-enriched fractions in subjects without cognitive impairment and mild cognitive impairment did not differ significantly, but was significantly lower in subjects with probable Alzheimer's disease (Fig. 5C). A β *56 in membrane-enriched fractions was significantly lower in subjects with both mild cognitive impairment and probable Alzheimer's disease compared with subjects without cognitive impairment (Fig. 5D).

Five brain specimens from individuals with non-Alzheimer's dementia showed very low levels of all three amyloid- β oligomers. This is a small sample size, but nonetheless suggests that

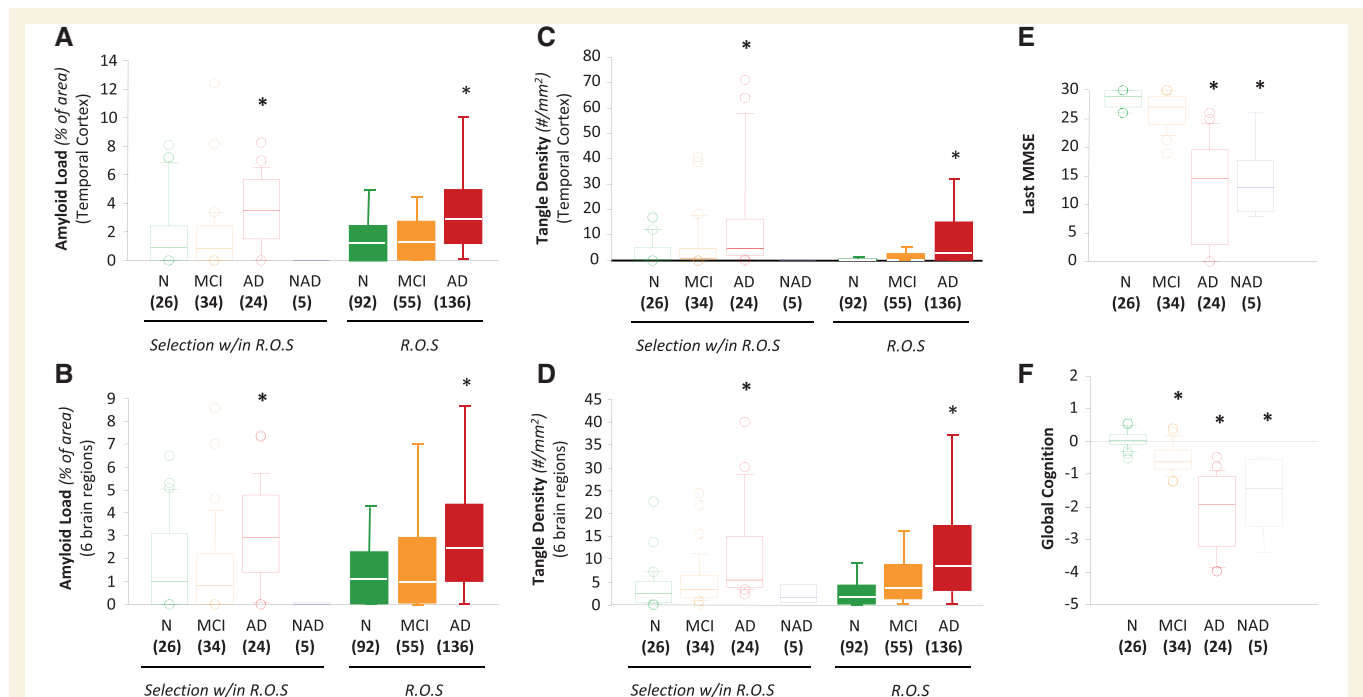


Figure 4 Neuropathological features and cognitive profiles of the Religious Orders Study (R.O.S.) subjects in this study compared with those in the complete cohort. Amyloid- β plaques and neurofibrillary tangles were assessed using unbiased stereological methods. (A and B) Plaque loads were quantified in the inferior temporal gyrus (A) and as the mean of six brain regions (B). (C and D) Neurofibrillary tangle densities (Bielschowsky-positive tangles/mm²) were quantified in the inferior temporal gyrus (C) and as the mean of six brain regions (D). (E and F) Box plots illustrating last Mini-Mental Status Examination (MMSE) (E) and Global Cognition (F) among the clinical groups. Group sizes are shown in parentheses. Green indicates no cognitive impairment (N), orange mild cognitive impairment (MCI), red probable Alzheimer's disease (AD) and blue non-Alzheimer's dementia (NAD). Lines within box plots denote median values, upper and lower box boundaries represent the 75th and 25th percentiles, respectively, and short bars flanking boxes represent the 95th and 5th percentiles. * $P < 0.01$, compared with subjects with no cognitive impairment.

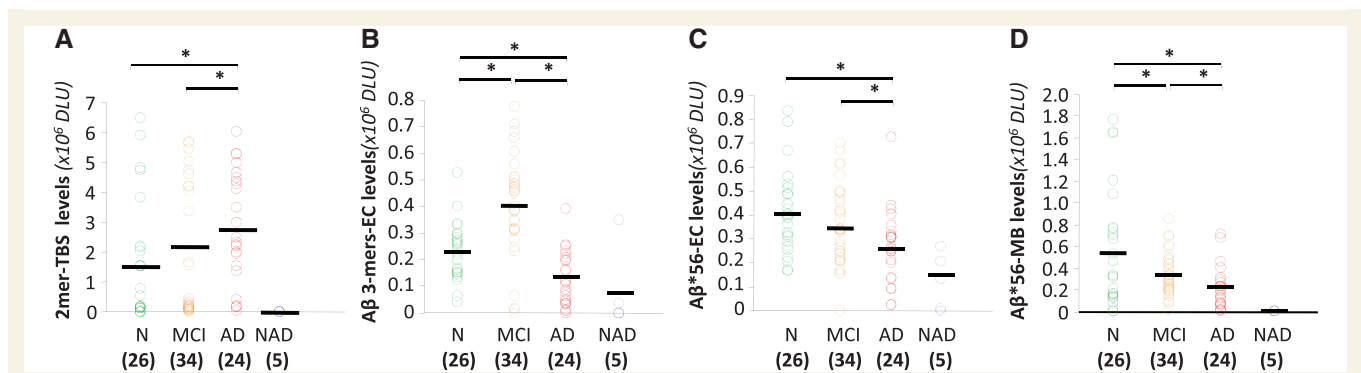


Figure 5 Association between clinical status and specific amyloid- β oligomers in the brain. (A) Amyloid- β dimer levels in Tris-buffered saline (TBS) extracts overlapped between groups but were significantly higher in subjects with probable Alzheimer's disease (AD) than in subjects with mild cognitive impairment (MCI) or no cognitive impairment (N). (B) Amyloid- β trimer levels in extracellular (EC)-enriched fractions were highest in the group with mild cognitive impairment. (C and D) A β *56 levels in both in extracellular (EC)-enriched and membrane (MB)-enriched fractions were highest in subjects with no cognitive impairment. Group sizes are shown in parentheses. * $P < 0.01$; Kruskal-Wallis test followed by Mann-Whitney U test with Bonferroni corrections. Green symbols indicate no cognitive impairment, orange symbols mild cognitive impairment, red symbols probable Alzheimer's disease, and blue symbols non-Alzheimer dementia. DLU = densitometry light units.

amyloid- β oligomers may play more important roles in Alzheimer's disease than in other forms of dementia (Fig. 5).

Amyloid- β dimers reflect plaque load

Previous studies of amyloid- β oligomers have focused on amyloid- β dimers and on clinically and pathologically diagnosed Alzheimer's disease. Shankar *et al.* (2008) found amyloid- β dimers specifically in Alzheimer's disease but not in control brains devoid of Alzheimer-related pathology. However, we did not exclude subjects with plaques or tangles from the group without cognitive impairment. We found amyloid- β dimers in subjects with and without cognitive impairment. Although the discrepancy between previously published results and ours may stem from differences in the sensitivity of detection methods, we believe it is more likely to be related to the exclusion of Alzheimer-related pathology in the control brains in the former study.

To explore the relationship between plaque load and the three amyloid- β oligomers, we calculated correlation coefficients between these variables. We found a strong correlation between amyloid- β dimers and plaque load in pooled samples (Spearman $\rho = 0.68$, $P < 0.01$, Fig. 6A), consistent with a recent report (Villemagne *et al.*, 2010), and significant correlations within each clinical group (Fig. 6A). In contrast, neither amyloid- β trimers nor A β *56 correlated with plaque load (Fig. 6B and C).

Amyloid- β monomers and dimers correlate

We also examined the relationships between amyloid- β monomers and amyloid- β dimers, amyloid- β trimers or A β *56. We found that amyloid- β monomers and amyloid- β dimers in Tris-buffered saline extracts correlated strongly (Spearman $\rho = 0.87$, $P < 0.01$, Fig. 7A). We also found strong correlations between amyloid- β monomers and plaque load (Spearman $\rho = 0.74$, $P < 0.01$), in

keeping with the correlation between amyloid- β dimers and plaque load described above.

In contrast, we found no significant associations between amyloid- β monomers and amyloid- β trimers in extracellular-enriched fractions (Fig. 7B) or A β *56 in extracellular-enriched fractions (Fig. 7C) or membrane-enriched fractions (Fig. 7D).

Amyloid- β and neurofibrillary tangle pathology

Finally, we searched for relationships between neurofibrillary tangle pathology and amyloid- β dimers, amyloid- β trimers and A β *56. We used Braak staging to catalogue the extent of neurofibrillary tangles (Braak and Braak, 1991). As expected, Braak stages represented tangle density well (Fig. 8A), but plaque load to a lesser extent (Fig. 8B and C). Amyloid- β monomers in both extracellular-enriched fractions and Tris-buffered saline extracts were elevated in Braak stages IV and V (Fig. 8D and E), as were amyloid- β dimers in Tris-buffered saline extracts (Fig. 8F). In contrast, amyloid- β trimers in extracellular-enriched fractions (Fig. 8G) and A β *56 in extracellular-enriched and membrane-enriched fractions (Fig. 8H and I) showed no significant changes across Braak stages.

Discussion

Here we report the results of an extensive analysis of amyloid- β dimers, amyloid- β trimers and A β *56 in humans. In rodents, there is more evidence supporting the biological activity of amyloid- β dimers and A β *56 than amyloid- β trimers (Lesné *et al.*, 2006; Shankar *et al.*, 2008; Reed *et al.*, 2011). In cognitively intact humans, all three amyloid- β oligomers increased with age and the levels of A β *56, amyloid- β dimers and amyloid- β trimers became significantly greater than those in children and adolescents

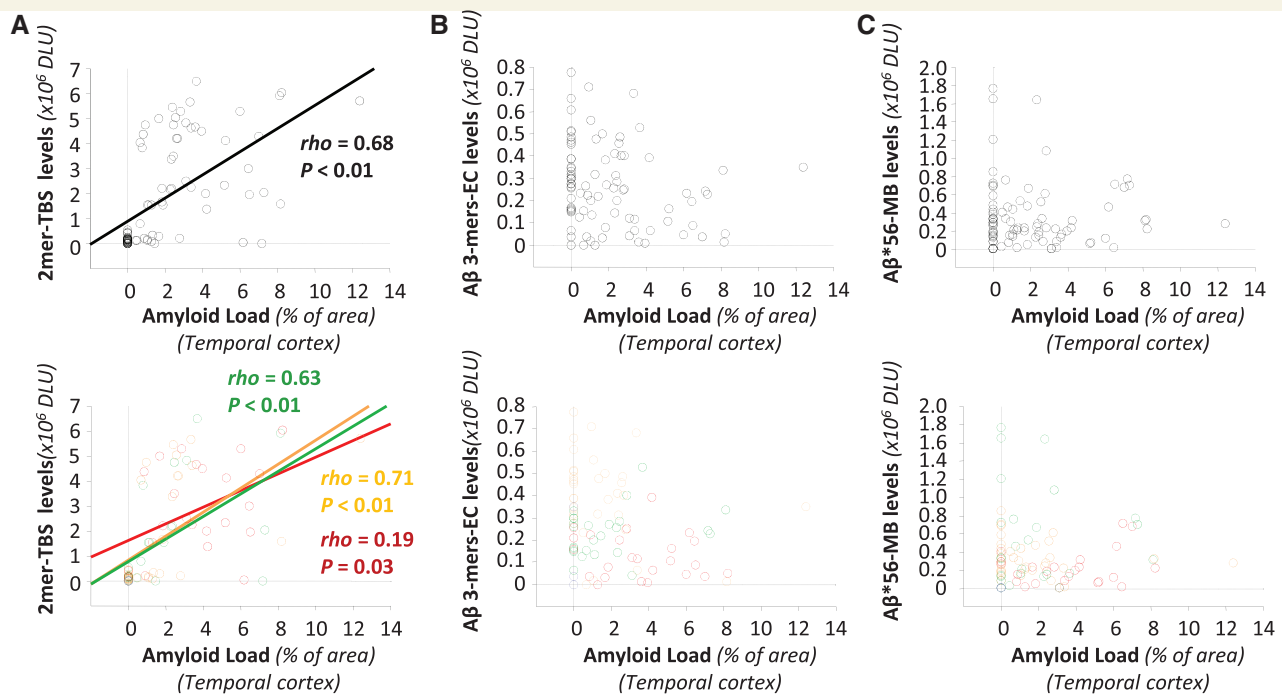


Figure 6 Relationships between specific amyloid- β oligomers in the brain and amyloid plaque load. *Top*: The relationships (Spearman rank correlations) between plaque load and amyloid- β dimers, amyloid- β trimers or A β *56, and *lower panels* illustrate these relationships stratified by clinical status. (A) Positive correlations between amyloid load and amyloid- β dimers in Tris-buffered saline (TBS) extracts. (B) No correlations between amyloid load and amyloid- β trimers in extracellular (EC)-enriched fractions. (C) No correlations between amyloid load and A β *56 in membrane (MB)-enriched fractions. Green symbols indicate no cognitive impairment, orange symbols mild cognitive impairment, red symbols probable Alzheimer's disease, and blue symbols non-Alzheimer dementia. DLU = densitometry light units.

in subjects in their 40s, 60s and 70s, respectively. In these subjects A β *56, but not amyloid- β dimers or amyloid- β trimers, correlated positively with soluble pathological tau proteins and negatively with the postsynaptic proteins, drebrin and fyn kinase. We speculated that the relationships we found between A β *56, tau and synaptic proteins may underlie the subtle cognitive deficits and impaired brain metabolism that have been observed in cognitively normal individuals who are at risk of Alzheimer's disease (Amieva *et al.*, 2008; Jack *et al.*, 2010; Sperling *et al.*, 2011; Bateman *et al.*, 2012). Based upon the temporal sequence of increases in the levels of specific amyloid- β oligomers, their correlations with tau and synaptic proteins, and our previous knowledge from rodent studies about the biology of these amyloid- β oligomers, we placed A β *56 ahead of amyloid- β trimers and amyloid- β dimers in the molecular sequence of events in Alzheimer's disease.

In mice, A β *56 appears to impair long-lasting synaptic plasticity and disrupt cognition independently of neuron loss or plaque deposition (Chapman *et al.*, 1999; Lesne *et al.*, 2006). Consistent with studies in rodents, the associations with A β *56 in humans were found in the absence of neuron loss and independently of plaque deposition.

We found significant increases in A β *56 in subjects in their 40s, when age-associated memory impairment, including subtle difficulties in declarative memory function, first appears (Youngjohn and Crook, 1993). However, we do not know whether the simultaneity of these events is coincidental or causal. Because A β *56

is sufficient to cause impairment on memory tasks in rodents, it is reasonable to speculate that the rising levels of this oligomer in middle and advanced ages might be associated with age-associated memory impairment. Another interesting question is whether A β *56 levels predict the development of dementia. The average age of our intact subjects in the Religious Orders Study was 83 years and the annual incidence of Alzheimer's disease increases from \sim 1% to \sim 4% between the ages of 80 and 90 years (Rocca *et al.*, 2011); therefore, some would probably have developed Alzheimer's disease had they lived longer. We can now measure A β *56 and amyloid- β trimers in CSF from living subjects (Handoko *et al.*, 2013), and are undertaking studies to define the relationships between these oligomers and both Alzheimer's biomarkers and risk for Alzheimer's disease.

The actual existence of A β *56 and other amyloid- β oligomers in the brain was eloquently called into question recently (Benilova *et al.*, 2012). An important, legitimate cause for concern about quantifying the levels of specific amyloid- β oligomers is that currently no method exists that does not involve exposing proteins to non-native conditions, which could theoretically lead to the dissociation or self-assembly of amyloid- β molecules that would invalidate estimates of molecular mass. In one study, A β *56 in extracellular-enriched brain extracts fractionated by size-exclusion chromatography appeared at the interval that was appropriate for its molecular mass (Lesne *et al.*, 2006), but a potential confound was the presence of detergents in the extraction buffer.

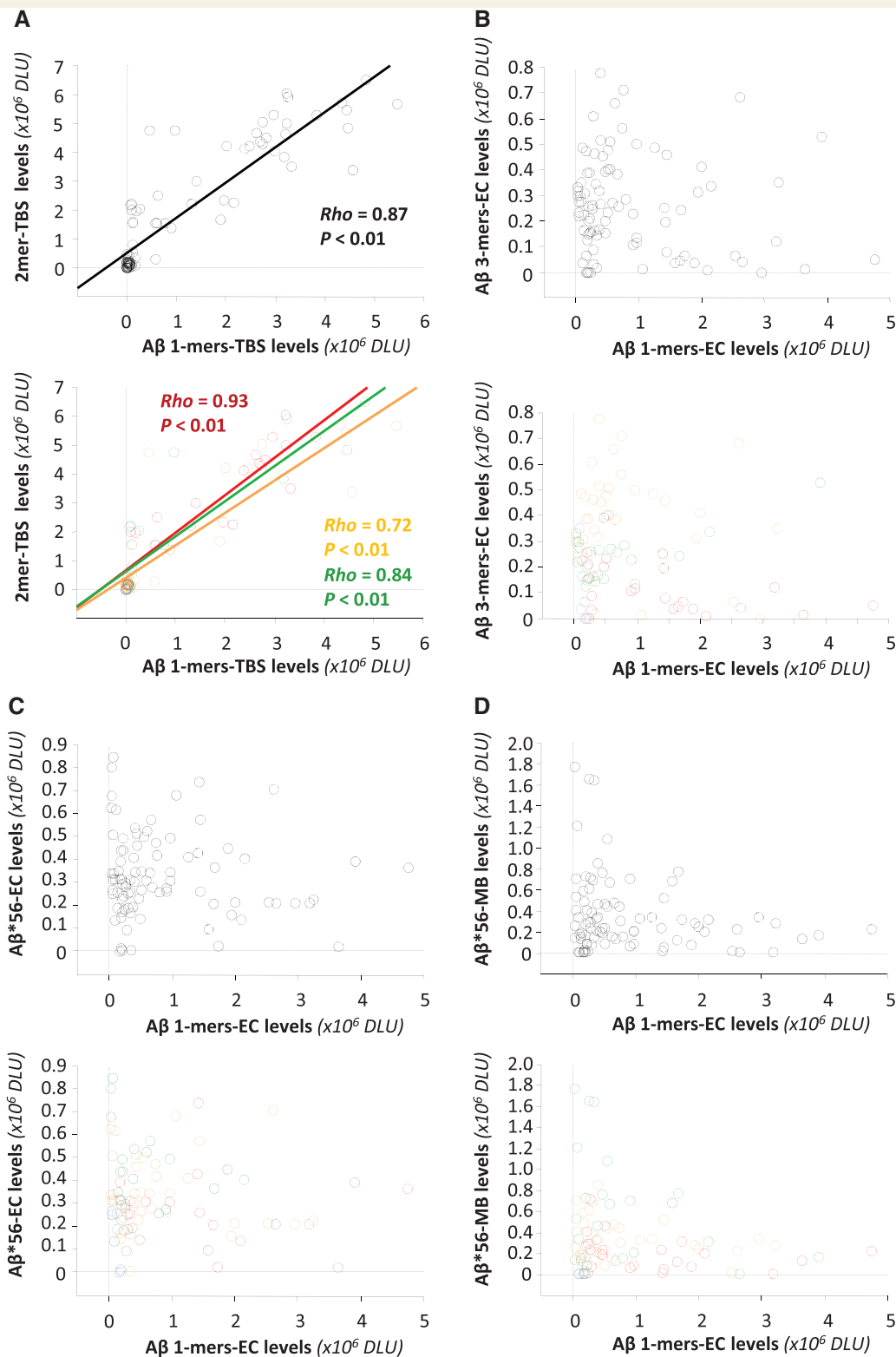


Figure 7 Relationships between specific amyloid- β oligomers and amyloid- β monomers in the brain. In each panel, the *upper* graphs show the relationships (Spearman rank correlations) between amyloid- β monomers and amyloid- β dimers, amyloid- β trimers or A β *56, and the *lower* graphs show these relationships stratified by clinical status. (A) Very strong correlations between amyloid- β monomers and amyloid- β dimers in Tris-buffered saline (TBS) extracts. (B–D) No correlations between amyloid- β monomers and amyloid- β trimers in extracellular (EC)-enriched fractions (B), A β *56 in extracellular (EC)-enriched fractions (C) or A β *56 in membrane (MB)-enriched fractions (D). Green symbols indicate no cognitive impairment, orange symbols mild cognitive impairment, red symbols probable Alzheimer's disease, and blue symbols non-Alzheimer dementia. DLU = densitometry light units.

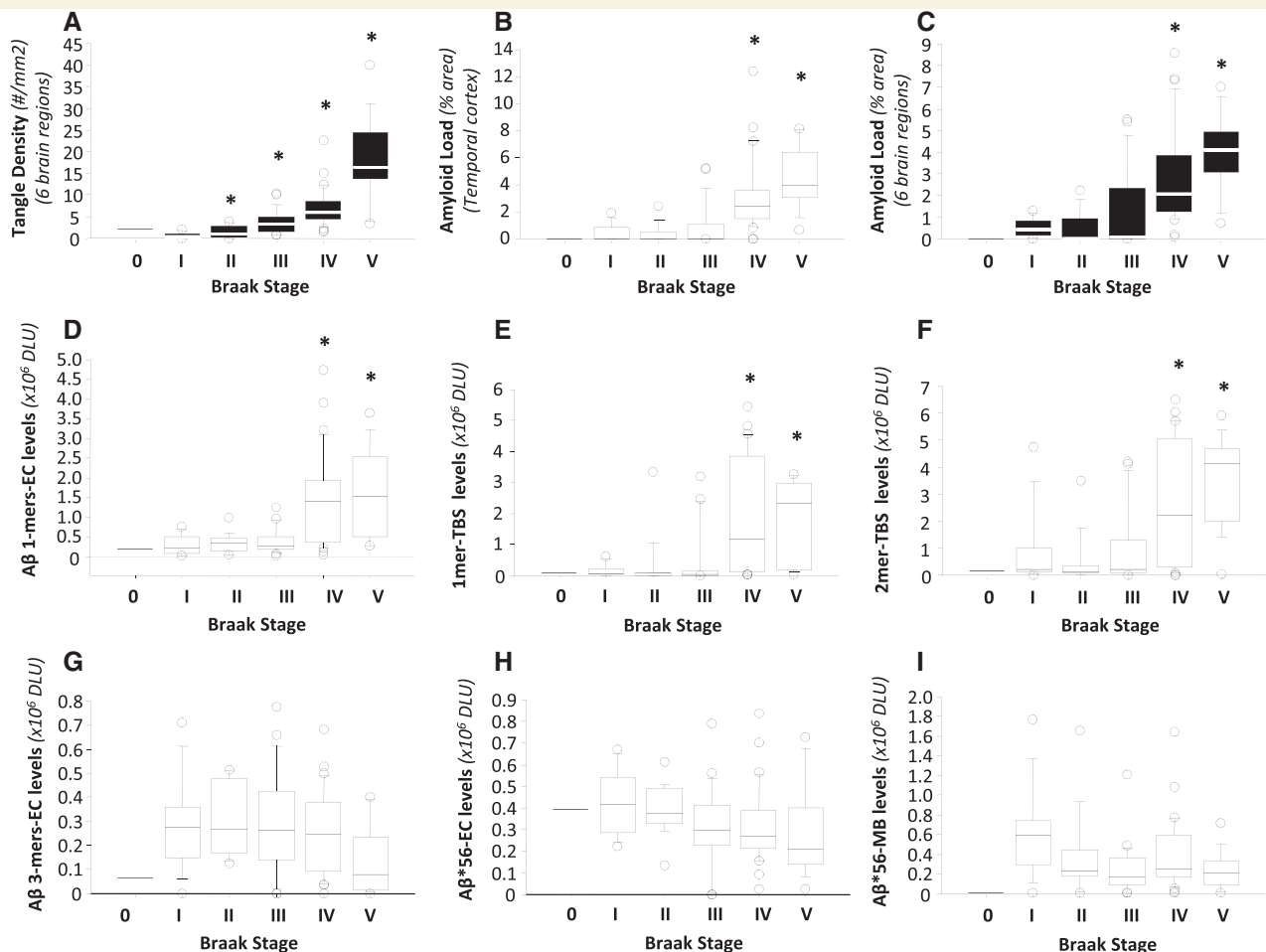


Figure 8 Relationships between specific amyloid- β oligomers in the brain and Braak stages. (A–C) The relationships between Braak stages and neurofibrillary tangle density (A), amyloid load in temporal cortex (B) and average amyloid load in six brain regions (C). (D–I) The relationships between Braak stages and the levels of various amyloid- β species, including amyloid- β monomers in extracellular (EC)-enriched fractions (D) and Tris-buffered saline (TBS) extracts (E), amyloid- β dimers in Tris-buffered saline extracts (F), amyloid- β trimers in extracellular-enriched fractions (G), and A β *56 in extracellular-enriched (H) and membrane (MB)-enriched (I) fractions. * $P < 0.01$; each of Braak stages II to V is compared with Braak stage I using Kruskal-Wallis test followed by Mann-Whitney U test with Bonferroni corrections. DLU = densitometry light units.

The observations that A β *56 can be immunocaptured directly from CSF and that A β *56 elutes separately from other amyloid- β oligomers when CSF is fractionated by size-exclusion chromatography mitigate but do not fully overcome this objection. A finite possibility remains that our current estimates of molecular mass may not reflect the native state of some or all the A β *56 molecules in the brain or CSF. The resolution of this problem will require the development of new techniques, including highly specific capture reagents. That said, it was difficult for us to ascribe the definitive temporal and correlative associations that we found for A β *56 to an entirely fictitious molecule 'in need of clothes'.

In this study we did not measure two other soluble amyloid- β oligomers that have been described in brain tissue, amylospheroids and annular protofibrils, both of which were first detected in synthetic preparations and later found in Alzheimer's brains. Annular protofibrils exhibit structural and immunospecific properties distinct from amyloid- β fibrils and globular oligomers such as A β *56 (Kayed *et al.*, 2009). Immunoblots of synthetic annular protofibrils

reveal a band at ~ 55 kDa that stains with antisera to annular protofibrils, but is A11-negative and therefore differs from A β *56. Amylospheroids are high molecular weight (>100 kDa), A11-negative assemblies concentrated in diffuse and compact plaques (Noguchi *et al.*, 2009). Here, we noted with great interest a novel ~ 110 kDa A11-immunoreactive band in 6E10-immunocaptured proteins in CSF (Fig. 1B) and brain (Supplementary Fig. 5), which was obscured by full-length or soluble amyloid precursor proteins in our standard protocol. Additional studies will be needed to determine its biological properties and relevance to Alzheimer's disease, as well as whether there is a stoichiometric relationship between it and A β *56.

In our cross-sectional analysis of Religious Orders Study subjects, the mean levels of amyloid- β dimers, amyloid- β trimers and A β *56 were highest in subjects with probable Alzheimer's disease, mild cognitive impairment and no cognitive impairment, respectively. However, their levels overlapped to such an extent that clinical status could not be predicted on the sole basis of any

single amyloid- β species. Interestingly, A β *56 and amyloid- β trimers were lowest in subjects with probable Alzheimer's disease, and exhibited distinctive fluctuating patterns (Larson and Lesné, 2012). Protein aggregates in neurodegenerative diseases are believed to accumulate as the neurological condition worsens. To our knowledge, our results are the first to show that some protein aggregates (A β *56 and amyloid- β trimers) decrease as clinical status worsens. We do not know the mechanism by which this occurs. There was a trend toward lower levels of NeuN in subjects with mild cognitive impairment and probable Alzheimer's disease that might have contributed to lowering A β *56, but does not explain the increase in amyloid- β trimers in the group with mild cognitive impairment. The diversion of amyloid- β monomers from a shared substrate pool to form amyloid- β fibrils and amyloid plaques might provide another explanation. Evidence supporting this possibility was observed in two transgenic mouse models in which the levels of A β *56 and amyloid- β trimers fell when the rate of plaque deposition increased (Cheng *et al.*, 2007; Lesné *et al.*, 2008). A version of this mechanism has been invoked to explain the inverse relationship between A β ₄₂ in the CSF and amyloid- β deposition in the brain (reviewed in de Leon *et al.*, 2007).

Our measurements of amyloid- β dimers, amyloid- β trimers and A β *56 enabled us to demonstrate a clear distinction between amyloid- β -derived diffusible ligands and A β *56. Amyloid- β -derived diffusible ligands are strikingly higher in subjects with Alzheimer's disease than in age-matched control subjects (Lacor *et al.*, 2004; Georganopoulou *et al.*, 2005; Bao *et al.*, 2012). In contrast, A β *56 is higher in control subjects than in subjects with Alzheimer's disease. The pattern of amyloid- β -derived diffusible ligands matched those of plaque load and amyloid- β dimers in our study.

Our data about amyloid- β oligomers provide biochemical support for an emerging consensus about phase-specific responses to therapies in Alzheimer's disease (Zahs and Ashe, 2010; Golde *et al.*, 2011), and may guide future studies in humans, animals and cells. First, A β *56, but not amyloid- β dimers or amyloid- β trimers, correlated with two pathological soluble tau proteins in intact subjects. Understanding how A β *56 is linked to tau abnormalities is an important question that might be elucidated by delineating the molecular pathways that are specifically triggered by A β *56 *in vitro* and *in vivo*. Second, A β *56, but not amyloid- β dimers or amyloid- β trimers, correlated inversely with postsynaptic proteins in intact subjects. Determining how A β *56 induces synaptic dysfunction may reveal new drug targets. Finally, A β *56 levels peaked in cognitively normal older adults and is the only oligomer we found that appears to participate in the amyloid cascade before the onset of symptoms. A key practical implication of these observations is that anti-amyloid- β therapies administered in the asymptomatic phase of Alzheimer's disease may be more likely to succeed if they block or reduce A β *56.

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Supplementary material

Supplementary material is available at *Brain* online.

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