Neurofibrillary Tangles Mediate the Association of Amyloid Load With Clinical Alzheimer Disease and Level of Cognitive Function

David A. Bennett, MD; Julie A. Schneider, MD; Robert S. Wilson, PhD; Julia L. Bienias, ScD; Steven E. Arnold, MD

Objective: To test the hypothesis that the association of amyloid load with clinical Alzheimer disease (AD) and cognitive impairment is mediated through neurofibrillary tangles.

Design: Longitudinal clinicopathologic cohort study.

Participants and Setting: Forty-four individuals with clinically diagnosed AD and 53 without dementia who participated in the Religious Orders Study underwent a uniform structured clinical evaluation for AD and cognitive testing about 8 months prior to death, and brain autopsy at death.

Methods: The percent area occupied by amyloid-β/H9252 and the density of neurofibrillary tangles were quantified from 6 brain regions and averaged to yield summary measures of amyloid load and neurofibrillary tangles. Multivariate regression analyses were used to simultaneously examine the effects of amyloid load and neurofibrillary tangles on clinically diagnosed AD and level of cognition.

Main Outcome Measures: Clinically diagnosed AD and level of global cognitive function proximate to death.

Results: In separate logistic regression analyses, each 1% increase in amyloid load was associated with about a 50% increase in the odds of clinical AD ($P = .002$), and each neurofibrillary tangle was associated with a greater than 20% increase in the odds of clinical AD ($P < .001$). When a term for tangles was added to the regression model with amyloid, the association of amyloid load with clinical disease was reduced by more than 60% and was no longer significant, whereas the association of tangles with clinical disease was essentially unchanged. Similar results were found in analyses of global cognitive function.

Conclusion: These findings are consistent with a sequence of pathologic events whereby the effect of amyloid deposition on clinical disease is mediated by neurofibrillary tangles.

Arch Neurol. 2004;61:378-384

AMYLOID-β PEPTIDE PLAQUES and hyperphosphorylated paired helical filament tau protein–rich neurofibrillary tangles are the principal pathologic lesions of Alzheimer disease (AD).1 Determining how these 2 pathologic indices are related to each other and to the clinical manifestations of AD has important implications for strategies to treat and ultimately to prevent AD. Several lines of evidence suggest that deposition of amyloid-β peptide is the first step in a sequence of events that ultimately leads to clinical disease.2 By contrast, some have argued that these lesions are not directly related but are a consequence of a third underlying variable.3 It had been hoped that clinicopathologic studies would further elucidate this important controversy. However, few clinicopathologic studies have quantified amyloid load and tangles in persons with and without dementia,4,6,8 and only 3 small studies used multivariate statistical techniques to simultaneously examine the relationship of amyloid load and tangles to clinical status.6,8 In general, these studies suggest that neurofibrillary tangles correlate better with the presence and severity of dementia than do plaques. However, the results are also consistent with a sequence of events in which tangles are an intermediate step linking amyloid deposition to clinical disease. We used clinical data and postmortem tissue from older persons participating in the Religious Orders Study to test the hypothesis that the association of amyloid load with clinical AD and cognitive impairment is mediated through neurofibrillary tangles.

METHODS

SUBJECTS

Subjects were older Catholic nuns, priests, and brothers participating in the Religious Orders Study (see the “Acknowledgment” section).9,10
Each participant agreed to an annual clinical evaluation and signed an informed consent form and an Anatomical Gift Act, donating his or her brain to Rush investigators at the time of death. The study was approved by the institutional review board of Rush University Medical Center (Chicago, Ill). Since January 1994, more than 900 persons have enrolled in the study and completed the baseline evaluation. Participation in the annual follow-up evaluations exceeds 95% of survivors, and the autopsy rate exceeds 90%.10

CLINICAL EVALUATION

Subjects underwent a uniform structured clinical evaluation, including a medical history, neurologic examination, cognitive performance testing, and review of a brain scan when available. Twenty-one cognitive performance tests were administered that assessed a broad range of cognitive abilities commonly affected by aging and AD, as previously described.9,10 Cognitive test results were reviewed by a board-certified neuropsychologist, and participants were evaluated in person by a physician with expertise in the evaluation of older persons with and without dementia. On the basis of this evaluation, participants were classified with respect to AD and other common conditions with the potential to affect cognitive function. Details of the clinical evaluation have been previously described.11 Follow-up evaluations, identical in all essential details, were performed annually by examiners blinded to previously collected data. At the time of death, all available clinical data were reviewed, and a summary diagnostic opinion was rendered regarding the most likely clinical diagnosis at the time of death. The reviewers were blinded to all postmortem data.

A global cognitive summary measure was used to minimize floor and ceiling effects and other sources of measurement error. It was constructed by converting the raw scores from 19 individual tests to z scores, using the mean and standard deviation from the baseline evaluation of all participants and averaging the z scores. To have a valid summary score, at least half of the component scores must be present. The derivation of the summary measure has been previously reported in detail.9,11

TISSUE PREPARATION

The brains of deceased subjects were removed in a standard fashion as described previously.11–13 After the brain was weighed, it was cut coronally into 1-cm-thick slabs, immersion fixed in 4% paraformaldehyde for 48 to 72 hours, and then placed in 2% dimethylsulfoxide/2% glycero1.3 in phosphate-buffered saline for storage. Tissue from 6 regions of interest was dissected into 0.5-cm-thick blocks and embedded in paraffin: the entorhinal cortex (Brodmann area 20); the inferior parietal cortex or angular gyrus/supramarginal gyrus (Brodmann area 39/40), the primary visual cortex or calcarine cortex (Brodmann area 17). Two blocks were obtained from adjacent 1-cm slabs from the neocortex and cut into 20-mm sections. For the hippocampal formation, sections were cut from consecutive 1-cm slabs throughout its length (up to 6 blocks per case).

Paired helical filament tau was labeled with an antibody specific for phosphorylated tau, AT8 (1:800) in 4% horse serum, and amyloid-β was labeled with MO0872 (1:100), which identifies both the 1-40 and 1-42 length amyloid-β fragments (Figure 1). Immunohistochemical analysis was performed as previously described15 using diaminobenzidine as the reaction product contrast. All sections were run using identical incubation times on an automated immunohistochemical stainer in precisely timed runs. Control sections processed without primary antibodies were included in all runs and displayed no specific staining.

Quantification of tangle density per square millimeter was performed with a stereological mapping station, which included a Leica DMRBE microscope and a computer (Millenium Mme; Micron Electronic Inc, Boise, Idaho) equipped with Stereoinvestigator software version 5.00 (MicroBrightField Inc, Colchester, Vt). Briefly, after the region was delimited at low power, a grid of predetermined size was randomly placed over the entire region by the software program. Total magnification was raised to ×400, and the program was engaged to direct the motorized stage on the microscope to stop at each intersection point of the grid for sampling. The operator focused through the section depth as the fields were visualized on the video monitor within the superimposed counting frame. All objects within the 150 × 150-µm counting frame that did not touch the exclusion lines of the box (bottom and left sides) were counted. Approximately 50% of the area of a delimited region of interest was quantified. Neurofibrillary tangles labeled with AT8 have a characteristic appearance and location in the neuronal cell body or as ghost tangles.

A systematic random sampling scheme was used to capture video images of amyloid-β–stained sections for quantitative analysis of plaque deposition using a custom algorithm previously described.15 Briefly, after camera and illumination calibration, 24-bit color images obtained at each sampling site were converted to 8-bit gray scale images. Calculation of the percent area occupied by amyloid-β immunoreactive pixels was performed using the public domain Object-Image 1.62p15 (developed by Norbert Vischer, http://simon.bio.uva.nl/object-image.html). The analysis algorithm segmented each image labeled and background compartments using 1 of 2 histogram-dependent automatic thresholding procedures (Iterative Self-Organizing Data Analysis) and triangulation.16 The percent areas for each section were then averaged, and that number was used in the analyses.

We developed composite summary measures of the percent area occupied by amyloid-β and the density of neurofibrillary tangles by averaging the values for each lesion for all regions assessed. Summary measures were supported by examination of item-total correlations and by principal component factor analyses. The Cronbach α coefficient was .88 for amyloid load and .84 for neurofibrillary tangles, indicating a high degree of internal consistency. The 6 amyloid values, all loaded on a single factor, accounted for 66% of the variance. Tangles in 5 regions loaded on factor 1 and explained 51.1% of the variance, and tangles in the calcarine cortex loaded on factor 2 accounted for 18.1% of the variance. Because we wanted to include the same regions in both composite measures, and because the calcarine tangles correlated with tangles in the other 5 regions (Spearman p = 0.57–0.38; P < .001 to P = .003), we chose to include it in the composite.

STATISTICAL ANALYSIS

Linear regression was used to examine the relationship between amyloid load and tangles, controlling for age, sex, and education. To examine whether neurofibrillary tangles account for the association of amyloid load with clinical AD, we constructed multiple logistic regression models that examined the relationship of the amyloid load and tangles to clinical diagnosis, first separately and then simultaneously. We examined these models to see if the association of amyloid load with clinical diagnosis was reduced when a term for tangles was added to the model. A similar set of analyses used multiple linear regression to examine the relationship of amyloid load and tangles to level of global cognitive function proximate to death. We have used this analytic approach previously to examine potential events in a causal chain.12,13 All analyses controlled for...
Ninety-seven deceased participants who underwent brain autopsy between August 28, 1996, and May 5, 2002, were included in these analyses; 44 of these 97 had met clinical criteria for probable AD and 53 did not have dementia. The median interval from the last clinical evaluation to brain autopsy was 8.2 months. Persons with AD were older, had slightly fewer years of education, and had lower scores on the Mini-Mental State Examination and the global measure of cognition (Table 1).

### RELATIONSHIP OF AMYLOID LOAD TO TANGLES

The crude association between amyloid load and tangle density is shown in Figure 2. In a regression analysis adjusted for age, sex, and education, each 1% increase in amyloid load was associated with about 2.30 (SE=0.40) additional tangles (P<.001) and contributed 24.7% to the variance of tangles.

### RELATIONSHIP OF AMYLOID LOAD AND TANGLES TO CLINICAL AD

Persons with AD had about twice as much amyloid deposition as persons without dementia and had about 4 times as many tangles (Table 1, Figure 3A and B). A series of regression analyses was performed to see if tangles accounted for the relationship of amyloid load to the clinical diagnosis of AD. In separate analyses controlling for age, sex, and education and were carried out using SAS/STAT software version 8 (SAS Institute Inc, Cary, NC) on a Sun-UltraSparc (SUN Microsystems Inc, Santa Clara, Calif) workstation. Models were validated graphically and analytically.

---

**Figure 1.** Inferior temporal cortex immunolabeled for amyloid scattered plaque deposits (A), abundant plaque deposits (B), paired helical filament tau rare neurofibrillary tangles (C), and abundant neurofibrillary tangles (B) (bar=10 µm).

**Table 1. Selected Characteristics of Persons With Alzheimer Disease and Persons Without Dementia***

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No Dementia (n = 53)</th>
<th>Alzheimer Disease (n = 44)</th>
<th>Total (N = 97)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, No. (%)</td>
<td>24 (45.3)</td>
<td>17 (38.6)</td>
<td>41 (42.3)</td>
</tr>
<tr>
<td>Age at death, y</td>
<td>82.4 ± 6.5</td>
<td>88.1 ± 5.3</td>
<td>85.0 ± 6.6</td>
</tr>
<tr>
<td>Education, y</td>
<td>18.7 ± 3.3</td>
<td>17.3 ± 3.0</td>
<td>18.1 ± 3.2</td>
</tr>
<tr>
<td>MMSE score</td>
<td>27.66 ± 1.89</td>
<td>16.07 ± 7.38</td>
<td>22.4 ± 7.75</td>
</tr>
<tr>
<td>Global cognition</td>
<td>−0.07 ± 0.53</td>
<td>−1.82 ± 0.83</td>
<td>−0.87 ± 1.11</td>
</tr>
<tr>
<td>Median interval, mo</td>
<td>8.0</td>
<td>8.6</td>
<td>8.2</td>
</tr>
<tr>
<td>Amyloid load, %</td>
<td>1.83 ± 1.84</td>
<td>3.81 ± 2.47</td>
<td>2.73 ± 2.35</td>
</tr>
<tr>
<td>Tangles/mm²</td>
<td>3.51 ± 3.64</td>
<td>14.22 ± 12.38</td>
<td>8.37 ± 10.23</td>
</tr>
</tbody>
</table>

Abbreviation: MMSE, Mini-Mental State Examination. Data are given as mean ± SD unless otherwise indicated.
age, sex, and education, each 1% increase in amyloid load was associated with about a 50% increase in the odds of clinical AD (Table 2, model 1), and the odds of clinical AD increased more than 20% for each additional tangle (Table 2, model 2). When tangles were added to the regression analysis with amyloid, the association of amyloid load with clinical disease was reduced by more than 60% and was no longer significant, whereas the association of tangles with clinical disease was essentially unchanged (odds for amyloid, 1.18; 95% confidence interval [CI], 0.87-1.60; odds for tangles, 1.21; 95% CI, 1.08-1.36) (Table 2, model 3). Figure 4A shows how the relationship between amyloid load and the probability of having AD is modified by the inclusion of a term

![Figure 2. Relationship between the percentage of area occupied by amyloid and the density of tau-positive tangles.](image)

![Figure 3. Box plots of the percentage of area occupied by amyloid (A) and the density of tau-positive tangles (B) for persons with and without Alzheimer disease (AD).](image)

![Figure 4. The probability of having Alzheimer disease (AD) as a function of amyloid in a logistic regression model without controlling for tau-positive tangles (amyloid-only model) and in a model controlling for tau-positive tangles (amyloid and tangles model) (A); and density of tau-positive tangles in a logistic regression model without controlling for amyloid (tangles-only model) and in a model controlling for amyloid (amyloid and tangles model) (B). All models controlled for age, sex, and education.](image)

### Table 2. Multiple Logistic Regression Models* Examining the Odds of Clinical AD

<table>
<thead>
<tr>
<th>Terms</th>
<th>Model 1</th>
<th></th>
<th>Model 2</th>
<th></th>
<th>Model 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P Value</td>
<td>OR (95% CI)</td>
<td>P Value</td>
<td>OR (95% CI)</td>
<td>P Value</td>
</tr>
<tr>
<td>Amyloid</td>
<td>1.49 (1.15-1.92)</td>
<td>.002</td>
<td>NA</td>
<td>NA</td>
<td>1.18 (0.87-1.60)</td>
<td>.29</td>
</tr>
<tr>
<td>Neurofibrillary tangles</td>
<td>NA</td>
<td>NA</td>
<td>1.23 (1.10-1.38)</td>
<td>&lt;.001</td>
<td>1.21 (1.08-1.36)</td>
<td>.006</td>
</tr>
</tbody>
</table>

Abbreviations: AD, Alzheimer disease; CI, confidence interval; NA, not applicable; OR, odds ratio.

*Model 1 = amyloid; model 2 = neurofibrillary tangles; and model 3 = amyloid and neurofibrillary tangles. All models controlled for age, sex, and education.
for tangles in the model. The solid line shows the relationship between amyloid load and the probability of having AD from a model adjusted for age, sex, and education. The dotted line represents the relationship after including a term for tangles. Note that the relationship represented by the dotted line slope is much less steep, indicating a weaker association between amyloid load and AD after controlling for tangles. By contrast, the solid line representing the relationship between tangles and the probability of AD is essentially unchanged after including a term for amyloid (Figure 4B).

**RELATIONSHIP OF AMYLOID LOAD AND TANGLES TO LEVEL OF COGNITIVE FUNCTION**

To ensure that our results did not depend on the diagnostic classification approach used in the study, we conducted a similar set of analyses with level of global cognition assessed proximate to death. The crude association between amyloid load and tangles to level of cognition is shown in Figure 5. In separate analyses controlling for age, sex, and education, each 1% increase in amyloid load was associated with a 0.16 (P = .001) standard unit lower cognitive score (Table 3, model 1), and each tangle was associated with a 0.06 (P < .001) standard unit lower cognitive score (Table 3, model 2). When a term for tangles was added to the regression analysis with amyloid, the association of amyloid load with clinical disease was reduced by more than 80% and was no longer significant, whereas the association of tangles with clinical disease was essentially unchanged (amyloid = −0.028 units; P = .46; tangles = −0.057 units; P < .001) (Table 3, model 3). Figure 6A shows how the relationship between amyloid load and global cognition changes as a function of amyloid and tangles load.

![Figure 5.](image)

**Figure 5.** Relationship between global cognition and the percentage of area occupied by amyloid (A) and the density of tau-positive tangles (B).

![Figure 6.](image)

**Figure 6.** Level of global cognition as a function of amyloid in a linear regression model without controlling for tau-positive tangles (amyloid only model) and in a model controlling for tau-positive tangles (amyloid-and-tangles model) (A); and density of tau-positive tangles in a linear regression model without controlling for amyloid (tangles-only model) and in a model controlling for amyloid (amyloid and tangles model) (B). All models controlled for age, sex, and education.

**Table 3. Multiple Linear Regression Models* as a Function of a Global Measure of Cognitive Function**

<table>
<thead>
<tr>
<th>Terms</th>
<th>Model 1</th>
<th>P Value</th>
<th>Model 2</th>
<th>P Value</th>
<th>Model 3</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloid</td>
<td>−0.16 (0.040)</td>
<td>&lt;.001</td>
<td>NA</td>
<td>NA</td>
<td>−0.028 (0.039)</td>
<td>.46</td>
</tr>
<tr>
<td>Neurofibrillary tangles</td>
<td>NA</td>
<td>NA</td>
<td>−0.060 (0.007)</td>
<td>&lt;.001</td>
<td>−0.057 (0.009)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable.

*Model 1 = amyloid; model 2 = neurofibrillary tangles; and model 3 = amyloid and neurofibrillary tangles. All models controlled for age, sex, and education.
laid load and level of cognition is modified by inclusion of a term for tangles in the model. The solid line shows the relationship between amyloid load and level of cognition from a model adjusted for age, sex, and education. The dotted line represents the relationship after including a term for tangles. Note that the dotted line is nearly straight. By contrast, the solid line representing the relationship between tangles and level of cognition is essentially unchanged after including a term for amyloid (Figure 6B).

**COMMENT**

We quantified extracellular deposits of amyloid-β peptide plaque deposits and phosphorylated tau-immunoreactive neurofibrillary tangles from multiple brain regions from persons with and without clinically diagnosed AD. Both lesions were related to the presence of clinical AD and to the level of cognitive impairment when examined separately. However, when a term for tangles was added to the regression model with amyloid, the association of amyloid load with clinical disease was markedly attenuated and no longer significant, whereas the effect of tangles was essentially unchanged. These data are consistent with a sequence of events whereby neurofibrillary tangles mediate the association of amyloid deposition with clinical disease.

These data are in agreement with and extend the results of 3 small prior studies that used immunocytochemical techniques to assess plaques and tangles from persons with and without dementia and employed multivariate analytic techniques to examine their relative contribution to clinical status proximate to death. Two of those studies relied on semiquantitative measures of distribution to clinical status proximate to death. Two of these studies reported that plaques and tangles were related to a single cognitive measure proximate to death but that the plaques were no longer significant after controlling for tangles.

Our findings are most consistent with a sequence of pathologic events whereby the effect of amyloid deposition on cognitive impairment is mediated primarily through the formation of neurofibrillary tangles. The precise molecular mechanisms linking amyloid deposition with tangle formation remains to be elucidated. However, recent data suggest that amyloid deposition may be required for tangles to develop and that both lesions may be required for neurotoxicity. For example, double transgenic mice overexpressing both human amyloid precursor protein and human tau appear to develop more tangle abnormalities than single transgenics with tau alone. Similarly, injecting amyloid into tau transgenic mice appears to result in enhanced tau changes. Finally, recent in vitro studies suggest that amyloid can promote tau aggregation and phosphorylation, perhaps through the cleavage of caspase.

There are several strengths to this study. Amyloid load and tangles were linked to both clinical diagnosis and level of global cognitive function assessed proximate to death. All analyses were performed on comparable persons from a single cohort, with high rates of follow-up participation and, finally, brain autopsy. This cohort controls for other potentially confounding variables such as occupation and lifestyle, and the sample size was sufficiently large to control for other important and potentially confounding variables such as age, sex, and education. Finally, uniform structured procedures were followed, examiners were blinded to previously collected data, and all postmortem data were collected by personnel blinded to clinical data, further reducing the potential for bias.

The study has several limitations. Most important, clinicopathologic analyses are inherently cross-sectional. Therefore, a sequence can be inferred but cannot be proved. Thus, although we tested an a priori hypothesis supported by the literature, we need to be cautious about the inferences that can be made from the analytic approach in this study. Likewise, one must also be cautious when drawing inferences regarding mechanisms of disease to humans from in vitro studies and animal models. A complete understanding of disease will come from the convergence of findings from several types of studies, all with their particular strengths and weaknesses. There are other limitations. We only quantified the fibrillar form of amyloid-β and paired helical filament tau–positive tangles identified by antibodies to a single epitope. Different epitopes of amyloid-β or tau or soluble amyloid-β or tau may be important to the development of clinical disease. To simplify the analyses, we combined data from multiple brain regions into a single measure of each pathologic index. It is possible that region-specific analyses would further illuminate the association of amyloid-β and tau with clinical status. Other pathologic findings in the brains of older persons could affect the relationship between amyloid-β and tau or modify their relationship to clinical disease. Finally, participants are not representative of the US population as a whole in terms of education and lifestyle. It is possible that these factors could alter the relationship of amyloid load and tau to clinical disease. Therefore, the findings will need to be replicated in similar studies of laypersons.

**Accepted for publication November 20, 2003.**

**Author contributions:** Study concept and design (Drs Bennett, Wilson, and Arnold); acquisition of data (Drs Bennett, Schneider, Wilson, Arnold); analysis and interpretation of data (Drs Bennett, Wilson, Arnold, and Bienias); drafting of the manuscript (Dr Bennett); critical revision of the manuscript for important intellectual content (Drs Schneider, Wilson, Arnold, and Bienias); statistical expertise (Drs Bennett and Bienias); obtained funding (Dr Bennett); administrative, technical, and material support (Drs Bennett, Schneider, Wilson, and Arnold); study supervision (Drs Bennett and Arnold).

This study was supported by grants AG15819 and AG10161 from the National Institute on Aging, Bethesda, Md.

We are indebted to the hundreds of nuns, priests, and brothers from the following groups participating in the Religious Orders Study: Chicago, Ill, Dubuque, Iowa, and Milwaukee, Wis: Archdiocesan priests of Chicago, Dubuque, and Milwaukee; Lisle, Ill: Benedictine Monks; Benedictine Sisters of the Sacred Heart; Collegeville, Minn: Benedictine...
REFERENCES


