

# Distinct Pools of $\beta$ -Amyloid in Alzheimer Disease–Affected Brain

## A Clinicopathologic Study

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**Objective:** To determine whether  $\beta$ -amyloid ( $A\beta$ ) peptides segregated into distinct biochemical compartments would differentially correlate with clinical severity of Alzheimer disease (AD).

**Design:** Clinicopathologic correlation study.

**Participants:** Twenty-seven patients from a longitudinal study of AD and 13 age- and sex-matched controls without a known history of cognitive impairment or dementia were included in this study.

**Interventions:** Temporal and cingulate neocortex were processed using a 4-step extraction, yielding biochemical fractions that are hypothesized to be enriched with proteins from distinct anatomical compartments: TRIS (extracellular soluble), Triton (intracellular soluble), sodium dodecyl sulfate (SDS) (membrane associated), and formic acid (extracellular insoluble). Levels of  $A\beta_{40}$  and  $A\beta_{42}$  were quantified in each biochemical compartment by enzyme-linked immunosorbent assay.

**Results:** The  $A\beta_{42}$  level in all biochemical compartments was significantly elevated in patients with AD vs controls ( $P < .01$ ). The  $A\beta_{40}$  levels in the TRIS and formic acid fractions were elevated in patients with AD (temporal,  $P < .01$ ; cingulate,  $P = .03$ ); however, Triton and SDS  $A\beta_{40}$  levels were similar in patients with AD and in controls. Functional impairment proximal to death correlated with Triton  $A\beta_{42}$  ( $r = 0.48$ ,  $P = .02$ ) and SDS  $A\beta_{42}$  ( $r = 0.41$ ,  $P = .04$ ) in the temporal cortex. Faster cognitive decline was associated with elevated temporal SDS  $A\beta_{42}$  levels ( $P < .001$ ), whereas slower decline was associated with elevated cingulate formic acid  $A\beta_{42}$  and SDS  $A\beta_{42}$  levels ( $P = .02$  and  $P = .01$ , respectively).

**Conclusion:** Intracellular and membrane-associated  $A\beta$ , especially  $A\beta_{42}$  in the temporal neocortex, may be more closely related to AD symptoms than other measured  $A\beta$  species.

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**A** CRITICAL ROLE OF THE  $\beta$ -amyloid ( $A\beta$ ) peptide in the pathogenesis of Alzheimer disease (AD) has been supported by human, animal, and in vitro studies.<sup>1</sup> Most measures of  $A\beta$  are markedly elevated in the AD-affected brain, yet the extent of total  $A\beta$  accumulation tends to correlate poorly with AD

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severity.<sup>2-4</sup> Because there is evidence that specific biochemical forms of  $A\beta$  (eg,  $A\beta_{42}$ , soluble  $A\beta$ , and oligomeric  $A\beta$ ) selectively lead to neuronal dysfunction and neurodegeneration<sup>5-7</sup> and can be more reliable correlates of clinical status,<sup>8,9</sup> identification and reliable measurement of these toxic  $A\beta$  species should enhance their utility as biological markers of disease.

Clarifying the dynamics of  $A\beta$  production and compartmentalization is also necessary to explain AD pathogenesis. Specific  $A\beta$  species may preferentially exert toxic effects as a function of their cellular location. Although established histologic techniques identify primarily insoluble extracellular and vascular amyloid deposits, novel methods can enhance detection of intraneuronal  $A\beta$ , distinguish  $A\beta$  pools, and measure changes in  $A\beta$  concentration and location over time.<sup>10-14</sup>

The  $A\beta$  in the brain can be segregated into distinct biochemical compartments defined by sequential extraction procedures. In this study of brain autopsy samples from a well-characterized longitudinal cohort of patients with AD and matched controls, we quantified  $A\beta_{40}$  and  $A\beta_{42}$  in biochemical compartments defined by their solubility in 4 solutions. Proteins in these biochemical pools are predicted to derive from distinct anatomical compartments within the cerebral cortex: ex-

tracellular soluble, intracellular, membrane associated, and extracellular insoluble.<sup>7</sup> We hypothesized that these measures would differentially correlate with disease diagnosis, progression, and severity.

## METHODS

### STUDY PARTICIPANTS

The sample derives from the Predictors Study,<sup>15</sup> which consists of patients with AD recruited at the mild to moderate disease stage and examined every 6 months at 1 of 3 academic centers. The inclusion and exclusion criteria and examination procedures have been fully described elsewhere<sup>15</sup> and were approved by the respective institutional review boards. At entry, all patients met National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer’s Disease and Related Disorders Association crite-

ria for probable AD and had a modified Mini-Mental State Examination (mMMSE) score of 30 or higher (equivalent to  $\geq 16$  on the Folstein MMSE). Twenty-seven cases with autopsy-confirmed AD were included in the present study. Thirteen brains of individuals of similar age and sex who were free of neurodegenerative disease by clinical or pathologic criteria were selected as controls.

### BIOCHEMICAL COMPARTMENTALIZATION

At autopsy, coronal sections from 1 hemisphere and hemibrainstem were fresh frozen between dry ice–cooled aluminum plates. A 1-cm strip of cortex was dissected from frozen temporal neocortex and cingulate cortex and mechanically homogenized. A 4-step extraction was used.<sup>13</sup> The tissue was first extracted in 14- $\mu$ L/mg wet weight TRIS buffer, pH 7.2 (50mM TRIS, 200mM sodium chloride, 2mM EDTA, and complete protease inhibitors), with 2% protease-free bovine serum albumin. After centrifugation (15 000 rpm, 21 000g, 4°C, 5 minutes), the supernatant was retained as the *TRIS-soluble fraction*. The pellet was rehomogenized with TRIS extraction buffer that contained 0.1% Triton X-100 and spun (15 000 rpm, 21 000g, 4°C, 5 minutes), and the supernatant was retained as the *Triton-soluble fraction*. The remaining pellet was homogenized in 2% sodium dodecyl sulfate (SDS) and spun, and the supernatant was saved as the *SDS-soluble fraction*. The remaining pellet was homogenized in 70% formic acid (FA) and recentrifuged (22 000 rpm, 44 000g, 4°C, 5 minutes), and the resulting FA-extracted supernatant was neutralized with 1M TRIS buffer (pH 11.0), representing the *FA-extracted fraction*.

These fractions are defined by their biochemical properties; however, they are predicted to contain proteins from distinct cellular compartments: extracellular soluble (TRIS), intracellular soluble (Triton), membrane-associated (SDS), and insoluble (FA) proteins. Lesné et al<sup>7</sup> demonstrated that the TRIS fraction was enriched for the extracellular proteins  $\alpha$ -secretase cleavage product of the amyloid precursor protein and tissue plasminogen activator; the Triton fraction was enriched for intracellular proteins c-jun, tau, extracellular signal-regulated kinases, and jun amino-terminal kinase; and the SDS fraction was enriched for full-length amyloid precursor protein and N-methyl-D-aspartate receptor subunit NR2, suggesting a membrane pro-

**Table 1. Characteristics of the 27 Patients With Alzheimer Disease**

| Characteristics                        | Value <sup>a</sup>    |
|--|-----------------------|
| Age at death, y                        | 78.4 (9.8) [57-89]    |
| Male to female ratio                   | 13:14                 |
| Educational level, y                   | 14.3 (3.5) [8-20]     |
| No. of <i>APOE-ε4</i> alleles          |                       |
| 0 (Noncarrier)                         | 9                     |
| 1 (Heterozygous)                       | 14                    |
| 2 (Homozygous)                         | 4                     |
| Estimated age at symptom onset, y      | 68.3 (9.8) [48-83]    |
| Illness duration, y                    | 10.1 (4.7) [3.9-19.6] |
| Last Blessed DRS score                 | 12.3 (4.0) [3-17]     |
| Time from last examination to death, y | 1.1 (1.7) [0.1-6.5]   |
| mMMSE score at intake                  | 40.2                  |
| No. of mMMSEs administered             | 7.6 (4.6) [1-17]      |
| Time from last measured mMMSE, y       | 2.3 (2.4) [0.1-9.0]   |

Abbreviations: DRS, Dementia Rating Scale; mMMSE, modified Mini-Mental State Examination.

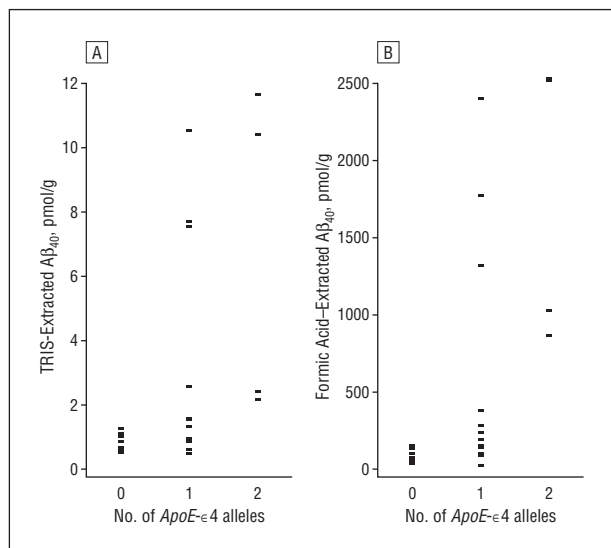
<sup>a</sup>Data are presented as mean (SD) [range] unless otherwise indicated.

**Table 2. Mean  $A\beta_{40}$  and  $A\beta_{42}$  Levels in the Biochemical Compartments of the Temporal and Cingulate Neocortex**

|                  | Temporal Neocortex, pmol/g <sup>a</sup> |                       | Cingulate Neocortex, pmol/g <sup>a</sup> |                       |
|------------------|---|-----------------------|--|-----------------------|
|                  | $A\beta_{40}$                           | $A\beta_{42}$         | $A\beta_{40}$                            | $A\beta_{42}$         |
| TRIS             |   |                       |  |                       |
| Patients with AD | <b>2.7</b> (3.5)                        | <b>14.6</b> (7.5)     | 1.7 (2.4)                                | <b>10.2</b> (7.3)     |
| Controls         | <b>0.8</b> (0.4)                        | <b>1.6</b> (3.2)      | 0.6 (0.5)                                | <b>1.1</b> (2.5)      |
| P value          | <b>.01</b>                              | <b>&lt;.001</b>       | .03                                      | <b>&lt;.001</b>       |
| Triton           |   |                       |  |                       |
| Patients with AD | 13.3 (9.3)                              | <b>7.0</b> (3.1)      | 12.4 (4.2)                               | <b>8.8</b> (3.0)      |
| Controls         | 11.9 (5.2)                              | <b>4.0</b> (1.6)      | 11.6 (1.5)                               | <b>5.0</b> (1.4)      |
| P value          | .63                                     | <b>&lt;.001</b>       | .50                                      | <b>&lt;.001</b>       |
| SDS              |   |                       |  |                       |
| Patients with AD | 55.4 (22.1)                             | <b>53.9</b> (27.3)    | 78.9 (28.4)                              | <b>25.6</b> (7.9)     |
| Controls         | 55.8 (34.9)                             | <b>18.6</b> (12.7)    | 73.6 (40.3)                              | <b>17.1</b> (9.1)     |
| P value          | .98                                     | <b>&lt;.001</b>       | .67                                      | <b>.004</b>           |
| FA               |   |                       |  |                       |
| Patients with AD | <b>555.6</b> (817.2)                    | <b>1240.2</b> (835.1) | 389.9 (597.6)                            | <b>1088.9</b> (520.9) |
| Controls         | <b>89.7</b> (52.9)                      | <b>186.3</b> (343.4)  | 120.4 (70.8)                             | <b>297.5</b> (698.5)  |
| P value          | <b>.01</b>                              | <b>&lt;.001</b>       | .03                                      | <b>.002</b>           |

Abbreviations: AD, Alzheimer disease; FA, formic acid; SDS, sodium dodecyl sulfate.

<sup>a</sup>All data are presented as mean (SD). Statistically significant case-control differences ( $P \leq .01$ ) are set in boldface type. These 10  $A\beta$  variables are included in subsequent analyses within the AD group.



**Figure 1.** Aβ<sub>40</sub> levels in the temporal neocortex according to APOE-ε4 genotype: TRIS-extracted Aβ<sub>40</sub> levels (A) and formic acid-extracted Aβ<sub>40</sub> levels (B). Markedly elevated Aβ<sub>40</sub> levels in the biochemical fractions predicted to contain extracellular proteins are seen in APOE-ε4 homozygotes and a subset of heterozygotes.

tein-enriched fraction. The FA fraction contained flotillin-2, suggesting that lipid raft domains and the insoluble proteins may be enriched in that fraction. Although the fractions are enriched for proteins from specific cellular compartments, they are unlikely to correspond precisely to these cellular compartments, and Aβ may spill over between biochemical and cellular compartments during the extraction procedure.

## Aβ QUANTIFICATION

Levels of Aβ<sub>40</sub> and Aβ<sub>42</sub> in each fraction were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using capture antibody BNT77 (anti-Aβ<sub>11-28</sub>) and detector antibodies BA27 (anti-Aβ<sub>40</sub>) and BC05 (anti-Aβ<sub>42</sub>), according to published protocols.<sup>13,16</sup> Thus, using 2 brain regions, 4 biochemical fractions, and 2 ELISAs, 16 Aβ variables were generated for each study participant.

## CLINICAL MEASURES

Cognition was assessed using the mMMSE.<sup>17</sup> Modifications to the Folstein MMSE<sup>18</sup> include the addition of digit span forward and backward,<sup>19</sup> 2 calculation items, recall of recent US presidents, 10 items from the Boston Naming Test,<sup>20</sup> 1 sentence to repeat, 1 written command, and 2 figures to copy. The mMMSE has a maximum of 57 points, with lower scores indicating poorer cognitive function. We used the Blessed Dementia Rating Scale parts I and II to assess patients' functional capacity. This is a 17-point scale, with higher scores indicating worse functional status.<sup>21</sup> Illness duration is the sum of the neurologist's estimate of duration of symptoms at intake and the time from study entry to death.

## STATISTICAL ANALYSIS

Cases and controls were compared for group differences in demographics. The means of the 16 Aβ variables were then compared in the 2 groups using *t* tests. Our approach to limiting the liabilities associated with multiple comparisons included applying a *P* value of .01 to identify Aβ variables that reliably differed between cases and controls. The 10 variables that met

this criterion are included in subsequent analyses. This approach also addressed the conceptual difficulty of interpreting the significance of an amyloid-related measure that is purported to relate to clinical features of AD but does not significantly differ from controls.

The Aβ variables were then compared in AD cases with 0, 1, or 2 APOE-ε4 alleles using 1-way analysis of variance and a post hoc least significant difference. For cross-sectional analysis, linear regression was used to relate the Aβ measures and clinical features of the AD cases. After log transformation of the data to better approximate normal distributions, the results of the analyses were essentially unchanged; we present the untransformed data.

Rates of cognitive decline were compared in groups of AD cases dichotomized at the median of each of the Aβ measures. Because we were interested in declines in mMMSE scores, which eventuated in high or low Aβ levels at autopsy, the time scale was reversed. Thus, date of death is defined as time 0, and preceding examinations have positive time values. Analyses of the longitudinal data were performed by applying the method of generalized estimating equations (GEE)<sup>22</sup>; GEE takes into account that each individual's multiple mMMSE measurements are likely to be correlated. In our model, time, Aβ level (high or low), and the time × Aβ interaction were included as independent variables. The mMMSE score was the dependent variable. As a result, all study participants had positive regression coefficients for the time; this finding corresponds to a decrease in cognition in chronological time, with higher coefficients indicating more rapid decline. A significant time × Aβ interaction term indicates a differential rate of decline in individuals with high or low Aβ fractions measured at autopsy. The cross-sectional and longitudinal analyses were performed again with sex and age at death included as covariates. The findings were nearly identical; the unadjusted analyses are presented.

## RESULTS

Clinical features of the 27 patients with autopsy-confirmed AD are summarized in **Table 1**. There were 5 male and 8 female control patients with a mean (SD) age at death of 70.1 (16.2) years; neither of these measures differed significantly from the patients with AD.

### CASES vs CONTROLS

Mean Aβ<sub>42</sub> and Aβ<sub>40</sub> levels in the biochemical compartments of temporal and cingulate neocortex are given in **Table 2**. Compared with controls, AD-affected brains had significantly higher mean concentrations of Aβ<sub>42</sub> in the TRIS and FA fractions of the temporal and cingulate cortex (*P* < .001) and higher mean concentrations of TRIS and FA Aβ<sub>40</sub> in the temporal (*P* = .01) and cingulate (*P* = .03) cortex. Mean Triton and SDS Aβ<sub>42</sub> fractions were higher in the AD temporal and cingulate cortex (*P* < .01). However, mean Triton and SDS Aβ<sub>40</sub> levels were similar in AD and control brains.

### APOE-ε4 GENOTYPE

Of the 27 patients with AD, 14 were heterozygous and 4 were homozygous for the APOE-ε4 allele. Individuals with 0, 1, or 2 APOE-ε4 alleles differed in FA Aβ<sub>40</sub> (*P* = .001)

**Table 3. Results of Cross-sectional and Longitudinal Analyses<sup>a</sup>**

|                                   | Temporal Neocortex      |             |                         |                 | Cingulate Neocortex     |            |
|-----------------------------------|-------------------------|-------------|-------------------------|-----------------|-------------------------|------------|
|                                   | A $\beta$ <sub>40</sub> |             | A $\beta$ <sub>42</sub> |                 | A $\beta$ <sub>42</sub> |            |
|                                   | r, F, or $\beta$        | P Value     | r, F, or $\beta$        | P Value         | r, F, or $\beta$        | P Value    |
| Blessed DRS score before death    |                         |             |                         |                 |                         |            |
| TRIS                              | -0.04                   | .85         | 0.25                    | .23             | 0.04                    | .84        |
| Triton                            | NA                      | NA          | <b>0.48</b>             | <b>.02</b>      | -0.18                   | .39        |
| SDS                               | NA                      | NA          | <b>0.44</b>             | <b>.03</b>      | 0.17                    | .43        |
| FA                                | -0.08                   | .73         | -0.06                   | .76             | -0.19                   | .38        |
| Illness duration                  |                         |             |                         |                 |                         |            |
| TRIS                              | <b>0.57</b>             | <b>.002</b> | 0.13                    | .52             | 0.12                    | .57        |
| Triton                            | NA                      | NA          | 0.29                    | .15             | -0.04                   | .85        |
| SDS                               | NA                      | NA          | -0.09                   | .67             | 0.20                    | .33        |
| FA                                | <b>0.51</b>             | <b>.007</b> | -0.10                   | .63             | 0.004                   | .99        |
| No. of APOE- $\epsilon$ 4 alleles |                         |             |                         |                 |                         |            |
| TRIS                              | <b>5.15</b>             | <b>.01</b>  | 0.23                    | .80             | 0.51                    | .61        |
| Triton                            | NA                      | NA          | 0.87                    | .43             | 0.60                    | .56        |
| SDS                               | NA                      | NA          | 1.27                    | .30             | 0.09                    | .92        |
| FA                                | <b>9.34</b>             | <b>.001</b> | 1.70                    | .21             | 0.28                    | .76        |
| Rate of cognitive decline         |                         |             |                         |                 |                         |            |
| TRIS                              | -1.24                   | .27         | 0.005                   | .10             | 2.07                    | .14        |
| Triton                            | NA                      | NA          | 1.75                    | .20             | -0.11                   | .94        |
| SDS                               | NA                      | NA          | <b>3.51</b>             | <b>&lt;.001</b> | <b>-2.75</b>            | <b>.01</b> |
| FA                                | -2.11                   | .07         | 1.02                    | .38             | <b>-2.48</b>            | <b>.02</b> |

Abbreviations: DRS, Dementia Rating Scale; FA, formic acid; NA, not applicable; SDS, sodium dodecyl sulfate.

<sup>a</sup>All analyses reflect unadjusted models using raw data, except for the Blessed DRS correlations, which include time from last examination until death as a covariate. Values are *r* for the Blessed DRS score and illness duration, *F* for APOE- $\epsilon$ , and  $\beta$  for the rate of cognitive decline. The reported  $\beta$  in the generalized estimating equation analysis is that of the time  $\times$  A $\beta$  interaction term; positive values indicate a more rapid cognitive decline for patients with measured A $\beta$  in the upper median. Statistically significant case-control differences ( $P < .05$ ) are set in boldface type.

and TRIS A $\beta$ <sub>40</sub> ( $P = .01$ ) levels in the temporal cortex. Post hoc analysis revealed that these differences were most pronounced in the 4 APOE- $\epsilon$ 4 homozygotes (**Figure 1**). Homozygotes had greater temporal FA A $\beta$ <sub>40</sub> and TRIS A $\beta$ <sub>40</sub> when compared with either heterozygotes ( $P = .03$ ) or noncarriers ( $P < .01$ ). Mean values were greater in the heterozygotes than noncarriers, but this finding did not reach statistical significance ( $P = .16$ ). No significant difference was found among the APOE- $\epsilon$ 4 groups in Triton A $\beta$ <sub>40</sub>, SDS A $\beta$ <sub>40</sub>, or A $\beta$ <sub>42</sub> levels within any biochemical compartment in either brain region.

#### CORRELATIONS WITH CLINICAL SEVERITY

As indicated in **Table 3**, significant correlations were observed between the last measured Blessed Dementia Rating Scale score and Triton A $\beta$ <sub>42</sub> ( $r = 0.48$ ,  $P = .02$ ) and SDS A $\beta$ <sub>42</sub> ( $r = 0.44$ ,  $P = .03$ ) levels in the temporal cortex after adjusting for time from last assessment until death (ie, worse terminal functional status was associated with higher A $\beta$ <sub>42</sub> levels in the fractions predicted to contain intracellular and membrane-associated proteins). The unadjusted scatterplots are shown in **Figure 2**.

#### ILLNESS DURATION

Significant correlations were observed between illness duration and FA A $\beta$ <sub>40</sub> level ( $r = 0.51$ ,  $P = .007$ ) and TRIS A $\beta$ <sub>40</sub> level ( $r = 0.57$ ,  $P = .002$ ) in the temporal cortex. No significant correlation was observed between illness dura-

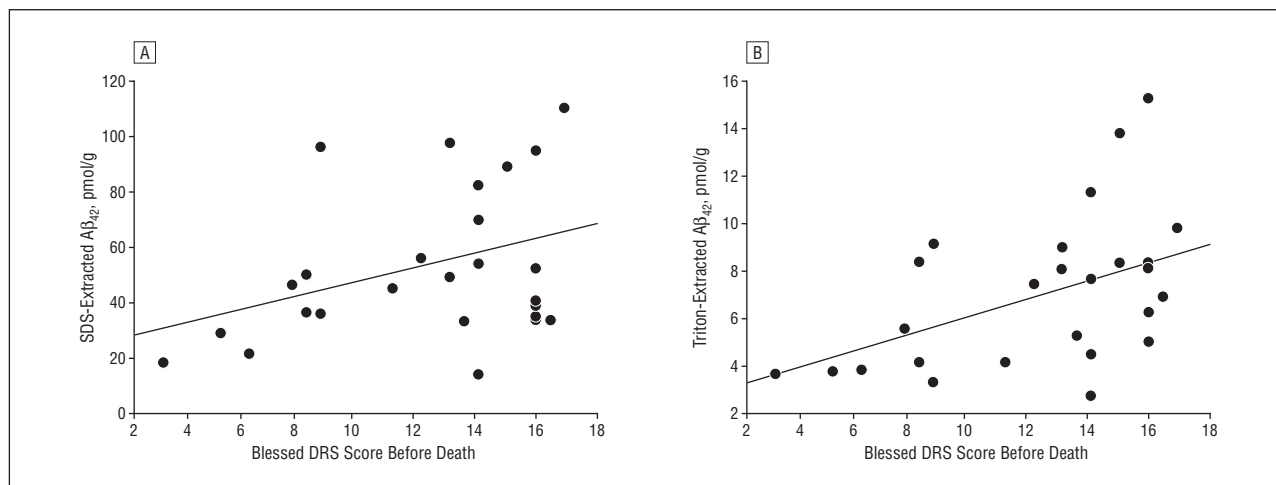
tion and any A $\beta$ <sub>42</sub> measurement. No significant correlation was found between illness duration and age at onset, age at death, or functional impairment at last examination (data not shown).

#### RATE OF COGNITIVE DECLINE

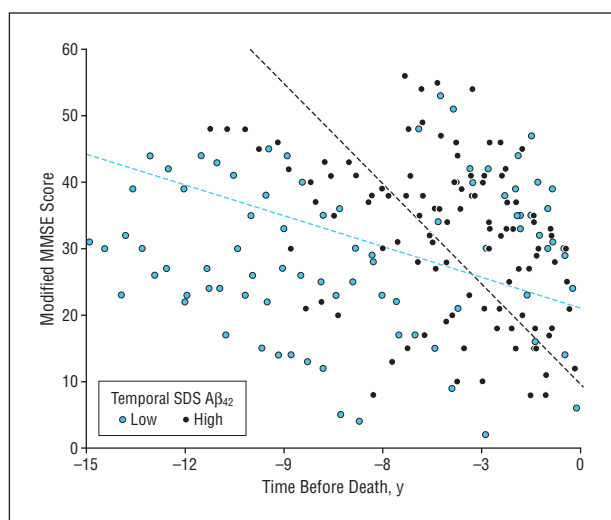
GEE was used to compare rates of cognitive decline in groups split at the median of measured A $\beta$  at death; results are given in Table 3 and **Figure 3**. The mean (SD) number of cognitive assessments was 7.6 (4.6) per study participant. An elevated SDS A $\beta$ <sub>42</sub> level in the temporal neocortex was associated with more rapid decline ( $P < .001$ ). In the cingulate cortex, however, higher FA A $\beta$ <sub>42</sub> and SDS A $\beta$ <sub>42</sub> levels were related to slower decline ( $P = .02$  and  $P = .01$ , respectively).

#### COMMENT

In this clinicopathologic correlation study, we observed that all TRIS- and FA-extracted A $\beta$  isoforms were elevated in patients with AD compared with controls; these fractions are predicted to contain extracellular soluble A $\beta$  (TRIS) and insoluble A $\beta$  associated with parenchymal and vascular amyloid deposition (FA). In contrast, in the biochemical compartments predicted to contain intracellular (Triton) and membrane-associated (SDS) protein pools, the A $\beta$ <sub>42</sub> but not the A $\beta$ <sub>40</sub> level was elevated in the patients with AD. These findings are con-



**Figure 2.** Relation between sodium dodecyl sulfate (SDS)- and Triton-extracted levels of  $A\beta_{42}$  in the temporal neocortex and functional disability before death. Raw data are shown, unadjusted for time from last assessment to death, for the SDS-extracted (A) and Triton-extracted (B)  $A\beta_{42}$  levels. The SDS and Triton compartments may be enriched with intracellular and membrane-associated proteins.



**Figure 3.** Generalized estimating equation (GEE)-derived models of estimated course of cognitive decline. Lines depict the differential rate of decline in modified Mini-Mental State Examination (MMSE) scores before death as predicted by the GEE model for study participants whose sodium dodecyl sulfate (SDS)-extracted  $A\beta_{42}$  level in the temporal cortex was greater than (black dashed line) or less than (blue dashed line) the median. Circles represent participants' modified MMSE scores at all times before death in the 2 groups. The raw data are consonant with the GEE models.

sistent with previous work that established that a range of extracellular  $A\beta$  measurements are elevated in the AD-affected brain. Our data suggest that intracellular and membrane-associated  $A\beta_{42}$  levels may be more closely related to the expression of symptoms in AD than other measured  $A\beta$  species.

Among AD cases, we found that only Triton- and SDS-extracted  $A\beta_{42}$  in temporal neocortex correlated with dementia severity at the last examination before death. These measures are predicted to be enriched with intracellular and membrane-associated  $A\beta_{42}$ . This finding lends support to the contention that accumulation of intracellular  $A\beta$  is not simply a marker of disease state but progresses with clinical severity.

In our longitudinal analyses, elevated SDS  $A\beta_{42}$  levels in the temporal cortex at autopsy were associated with

a more rapid cognitive decline observed during the mild to moderate stages of dementia. This finding was of greatest magnitude and statistical significance and supports the contention that  $A\beta_{42}$  accumulation in the membrane-associated intracellular compartments is closely tied to disease symptoms, such as cognitive changes early in the clinical course.

$APOE-\epsilon 4$  is a well-recognized genetic risk factor for AD, which is associated with younger age at symptom onset. In our study, there was an  $APOE-\epsilon 4$  allele dose-related increase in  $A\beta_{40}$  in the TRIS and FA fractions. This finding is in agreement with previous findings<sup>23,24</sup>; the pronounced increase among  $APOE-\epsilon 4$  homozygotes was previously reported using methods similar to the current study.<sup>25</sup> Although the molecular mechanism of the  $APOE-\epsilon 4$  effect is uncertain, as a genetic factor it likely exerts its influence for years before symptom emergence. Furthermore, we also found TRIS- and FA-extracted  $A\beta_{40}$  to be the strongest correlate of illness duration in our study. Thus, constitutive extracellular  $A\beta_{40}$  accumulation may be a trait of individuals destined to develop AD. Although their levels appear to increase as a function of genetic risk and illness duration in AD, we could not relate these  $A\beta_{40}$  species to the clinical state of our study participants. It has been shown that mice that produce only  $A\beta_{40}$  do not produce cerebral amyloid deposits.<sup>26</sup> Thus, additional factors, including  $A\beta_{42}$  production, appear necessary to generate toxic amyloid and clinical manifestation of disease.

Recent work has led to increased recognition of the presence and importance of intraneuronal  $A\beta$ . These studies<sup>27</sup> were enabled by the development of antibodies that could differentiate  $A\beta_{40}$  and  $A\beta_{42}$  from the transmembrane amyloid precursor protein from which they derive. In human and mouse brain studies,<sup>28-30</sup> intraneuronal  $A\beta$  has been detected before the emergence of extracellular plaques. In a recent animal study,<sup>31</sup> appearance of intraneuronal  $A\beta$  coincided with the emergence of cognitive impairment, which was reversible with immunotherapy. Oligomerization of  $A\beta$  associated with increased neurotoxicity has been identified within neurons.<sup>32,33</sup>

Although the sources of intraneuronal A $\beta$  are not well defined, accumulation is known to occur at subcellular compartments of the endosomal pathway<sup>30,34</sup> and is associated with impairment of intracellular protein trafficking after endocytosis.<sup>35</sup> Recently, genetic studies<sup>36,37</sup> have also implicated alterations of intraneuronal protein recycling and sorting mechanisms in the pathogenesis of AD. Elsewhere, it has been hypothesized that endocytosed A $\beta_{42}$  is not degraded as efficiently as A $\beta_{40}$ .<sup>38</sup>

The results of the present study support a selective accrual of intraneuronal A $\beta_{42}$  with progression of AD. Additional work is necessary to elaborate the mechanisms of extracellular A $\beta_{40}$  accumulation and their relation to the protein misprocessing that leads to intracellular A $\beta_{42}$  accumulation. A potential link can be sought at the retromer complex, which shuttles proteins from the endosomal system to the secretory pathway. Selective retention of A $\beta_{42}$  in the endosomal organelles and/or facilitated transfer of A $\beta_{40}$  to the secretory system could account for such findings.

In the longitudinal analysis, results from the cingulate cortex appear discrepant with those of the temporal cortex. We have focused on the temporal cortex data in the figures and discussion for several reasons. Temporal association cortex is more likely to be involved in our patients who were recruited at early stages. Beyond this, the factors that contribute to differential regional vulnerability and alternate patterns of disease progression in AD are poorly understood. Thus, different results by region can be expected and informative. As such, we caution against modeling the whole brain as a homogeneous biochemical compartment. In fact, future studies using serial extraction procedures on multiple brain regions may be suitable for analyzing regional covariance in toxic A $\beta$ .

A major contribution of the present analyses lies in the careful diagnosis and clinical follow-up that patients received. Clinical diagnosis took place via consensus conference in university hospitals with specific expertise in dementia. The patients were observed prospectively, which eliminates the potential biases of retrospective medical record reviews. Examinations were performed semiannually and included assessments closely proximate to death. Finally, the novelty of the A $\beta$  measures is a significant strength. We are not aware of other studies of human AD-affected brain that include biochemical pools predicted to contain intracellular and membrane-associated proteins.

Relative weaknesses of our study include the limited number of patients studied, which resulted in reduced statistical power; however, multiple data points per patient increased the power of our longitudinal analysis. We do not have detailed clinical information on the control subjects, who were not part of the predictors cohort. However, the control data were used only for between-group comparison and were not included in our cross-sectional or longitudinal analyses of AD patients. We recognize the exploratory nature of the investigation and the problems associated with multiple comparisons. We have attempted to mitigate these by only including in subsequent analyses A $\beta$  variables that, in the initial case-control comparison, satisfied a moderately conservative

correction for multiple comparisons. Nevertheless, the reported findings should be considered hypothesis generating and require replication and refinement in future studies. In addition, it is likely that our A $\beta$  ELISA is insensitive to certain biologically relevant species of cerebral amyloid.<sup>39</sup> For example, it is expected to quantify monomers only and does not distinguish multimeric forms or N-terminal modifications of the A $\beta$  peptide.

We expect that detailed biochemical fractionation of A $\beta$  pools will significantly enhance future clinicopathologic investigations of AD. Our study confirms the relevance of A $\beta_{42}$  in the intracellular and membrane-associated compartments to disease manifestations. Constitutive accumulation of extracellular A $\beta_{40}$  appears to be an AD trait that correlates with illness duration and is accentuated among APOE- $\epsilon$ 4-positive patients. Further study of the covariance of A $\beta$  measures across biochemical compartments and brain regions and more detailed study of A $\beta$  length and conformation within the intracellular and membrane-associated pools may contribute to updated models of amyloid dynamics.

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**Author Contributions:** Dr Stern had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Irizarry, Raju, Hyman, and Stern. *Acquisition of data:* Irizarry, Scarmeas, Raju, Brandt, Albert, Blacker, and Stern. *Analysis and interpretation of data:* Steinerman, Irizarry, Scarmeas, Raju, Hyman, and Stern. *Drafting of the manuscript:* Steinerman. *Critical revision of the manuscript for important intellectual content:* Steinerman, Irizarry, Scarmeas, Raju, Brandt, Albert, Blacker, Hyman, and Stern. *Statistical analysis:* Scarmeas and Stern. *Obtained funding:* Hyman and Stern. *Administrative, technical, and material support:* Irizarry, Raju, Albert, Blacker, Hyman, and Stern. *Study supervision:* Irizarry, Scarmeas, Albert, and Hyman.

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