Distinct Pools of β-Amyloid in Alzheimer Disease–Affected Brain

A Clinicopathologic Study

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Objective: To determine whether β-amloid (Aβ) 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 Aβ40 and Aβ42 were quantified in each biochemical compartment by enzyme-linked immunosorbent assay.

Results: The Aβ42 level in all biochemical compartments was significantly elevated in patients with AD vs controls (P<.01). The Aβ40 levels in the TRIS and formic acid fractions were elevated in patients with AD (temporal, P<.01; cingulate, P=.03); however, Triton and SDS Aβ40 levels were similar in patients with AD and in controls. Functional impairment proximal to death correlated with Triton Aβ42 (r=0.48, P=.02) and SDS Aβ42 (r=0.41, P=.04) in the temporal cortex. Faster cognitive decline was associated with elevated temporal SDS Aβ42 levels (P<.001), whereas slower decline was associated with elevated cingulate formic acid Aβ42 and SDS Aβ42 levels (P=.02 and P=.01, respectively).

Conclusion: Intracellular and membrane-associated Aβ, especially Aβ42 in the temporal neocortex, may be more closely related to AD symptoms than other measured Aβ species.

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Critical Role of the β-Amyloid (Aβ) Peptide in the Pathogenesis of Alzheimer Disease (AD) has been supported by human, animal, and in vitro studies. Most measures of Aβ are markedly elevated in the AD-affected brain, yet the extent of total Aβ accumulation tends to correlate poorly with AD severity. Because there is evidence that specific biochemical forms of Aβ (eg, Aβ40, soluble Aβ, and oligomeric Aβ) selectively lead to neuronal dysfunction and neurodegeneration and can be more reliably correlates of clinical status, identification and reliable measurement of these toxic Aβ species should enhance their utility as biologic markers of disease.

Clarifying the dynamics of Aβ production and compartmentalization is also necessary to explain AD pathogenesis. Specific Aβ 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 Aβ, distinguish Aβ pools, and measure changes in Aβ concentration and location over time.

The Aβ 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 Aβ40 and Aβ42 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.\textsuperscript{7} We hypothesized that these measures would differentially correlate with disease diagnosis, progression, and severity.

**METHODS**

**STUDY PARTICIPANTS**

The sample derives from the Predictors Study,\textsuperscript{15} 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 disease stage and examined every 6 months at 1 of 3 academic centers. The inclusion and exclusion criteria and disease stage and examined every 6 months at 1 of 3 academic centers. The inclusion and exclusion criteria and disease stage and examined every 6 months at 1 of 3 academic centers. The inclusion and exclusion criteria and disease stage and examined every 6 months at 1 of 3 academic centers.

**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.\textsuperscript{13} The tissue was first extracted in 14-µL/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 centrifuged (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. Lesne et al.\textsuperscript{16} demonstrated that the TRIS fraction was enriched for the extracellular proteins α-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 D-aspartate receptor subunit NR2, suggesting a membrane pro-

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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at death, y</td>
<td>78.4 (8.8) [57-89]</td>
</tr>
<tr>
<td>Male to female ratio</td>
<td>13:14</td>
</tr>
<tr>
<td>Educational level, y</td>
<td>14.3 (2.5) [8-20]</td>
</tr>
<tr>
<td>No. of APOE-e4 alleles</td>
<td>0 (Noncarrier) 9</td>
</tr>
<tr>
<td>Estimated age at symptom onset, y</td>
<td>68.3 (8.8) [48-83]</td>
</tr>
<tr>
<td>Illness duration, y</td>
<td>10.1 (4.7) [3.9-19.6]</td>
</tr>
<tr>
<td>Last Blessed DRS score</td>
<td>12.3 (4.0) [3-17]</td>
</tr>
<tr>
<td>Time from last examination to death, y</td>
<td>1.1 (1.7) [0.1-6.5]</td>
</tr>
<tr>
<td>mMMSE score at intake</td>
<td>40.2</td>
</tr>
<tr>
<td>No. of mMMSEs administered</td>
<td>7.6 (4.6) [1-17]</td>
</tr>
<tr>
<td>Time from last measured mMMSE, y</td>
<td>2.3 (2.4) [0.1-9.0]</td>
</tr>
</tbody>
</table>

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

\textsuperscript{a}Data are presented as mean (SD) [range] unless otherwise indicated.

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

<table>
<thead>
<tr>
<th></th>
<th>Temporal Neocortex, pmol/g\textsuperscript{a}</th>
<th>Cingulate Neocortex, pmol/g\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aβ\textsubscript{40}</td>
<td>Aβ\textsubscript{42}</td>
</tr>
<tr>
<td>TRIS</td>
<td>2.7 (3.5)</td>
<td>14.6 (7.5)</td>
</tr>
<tr>
<td>Patients with AD</td>
<td>0.8 (0.4)</td>
<td>1.6 (3.2)</td>
</tr>
<tr>
<td>Controls</td>
<td>.01</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Triton</td>
<td>13.3 (9.3)</td>
<td>7.0 (3.1)</td>
</tr>
<tr>
<td>Patients with AD</td>
<td>11.9 (5.2)</td>
<td>4.0 (1.6)</td>
</tr>
<tr>
<td>Controls</td>
<td>.63</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>SDS</td>
<td>55.4 (22.1)</td>
<td>53.9 (27.3)</td>
</tr>
<tr>
<td>Patients with AD</td>
<td>55.8 (34.9)</td>
<td>18.6 (12.7)</td>
</tr>
<tr>
<td>Controls</td>
<td>.98</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>FA</td>
<td>555.6 (817.2)</td>
<td>1240.2 (835.1)</td>
</tr>
<tr>
<td>Patients with AD</td>
<td>89.7 (52.9)</td>
<td>186.3 (343.4)</td>
</tr>
<tr>
<td>Controls</td>
<td>.01</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

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

\textsuperscript{a}All data are presented as mean (SD). Statistically significant case-control differences (P≤.01) are set in boldface type. These 10 Aβ values are included in subsequent analyses within the AD group.
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 Aβ42 and Aβ40 in each fraction were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using capture antibody BNT77 (anti-Aβ1-16) and detector antibodies BA27 (anti-Aβ42) and BC05 (anti-Aβ40), according to published protocols. 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. Modifications to the Folstein MMSE include the addition of digit span for-
and TRIS Aβ42 (P = .01) levels in the temporal cortex. Post hoc analysis revealed that these differences were most pronounced in the 4 APOE-e4 homozygotes (Figure 1). Homozygotes had greater temporal FA Aβ40 and TRIS Aβ30 when compared with either heterozygotes (P = .03) or non-carriers (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-e4 groups in Triton Aβ40, SDS Aβ40, or Aβ42 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 Aβ12 (r = 0.48, P = .02) and SDS Aβ12 (r = 0.44, P = .03) levels in the temporal cortex after adjusting for time from last assessment until death (i.e., worse terminal functional status was associated with higher Aβ12 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 Aβ30 level (r = 0.51, P = .007) and TRIS Aβ30 level (r = 0.57, P = .002) in the temporal cortex. No significant correlation was observed between illness duration and any Aβ42 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 Aβ 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 Aβ42 level in the temporal neocortex was associated with more rapid decline (P < .001). In the cingulate cortex, however, higher FA Aβ42 and SDS Aβ42 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 Aβ isoforms were elevated in patients with AD compared with controls; these fractions are predicted to contain extracellular soluble Aβ (TRIS) and insoluble Aβ 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 Aβ42 but not the Aβ40 level was elevated in the patients with AD. These findings are con-

![Table 3. Results of Cross-sectional and Longitudinal Analyses](image-url)
compartments may be enriched with intracellular and membrane-associated proteins. Raw data are shown, unadjusted for time from last assessment to death, for the SDS-extracted (A) and Triton-extracted (B) Aβ42 levels. The SDS and Triton compartments may be enriched with intracellular and membrane-associated proteins.

In our longitudinal analyses, elevated SDS Aβ42 levels in temporal neocortex correlated with decreased Modified MMSE scores. These studies indicate that intraneuronal Aβ accumulation in the membrane-associated intracellular compartments is closely tied to disease symptoms, such as cognitive changes early in the clinical course.

APOE-ε4 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-ε4 allele dose-related increase in Aβ40 in the TRIS and FA fractions. This finding is in agreement with previous findings; the pronounced increase among APOE-ε4 homozygotes was previously reported using methods similar to the current study. Although the molecular mechanism of the APOE-ε4 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 Aβ40 to be the strongest correlate of illness duration in our study. Thus, constitutive extracellular Aβ40 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 Aβ40 species to the clinical state of our study participants. It has been shown that mice that produce only Aβ40 do not produce cerebral amyloid deposits. Thus, additional factors, including Aβ42 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 Aβ. These studies were enabled by the development of antibodies that could differentiate Aβ40 and Aβ42 from the transmembrane amyloid precursor protein from which they derive. In human and mouse brain studies, intraneuronal Aβ has been detected before the emergence of extracellular plaques. In a recent animal study, appearance of intraneuronal Aβ coincided with the emergence of cognitive impairment, which was reversible with immunotherapy. Oligomerization of Aβ associated with increased neurotoxicity has been identified within neurons.
Although the sources of intraneuronal Aβ are not well defined, accumulation is known to occur at subcellular compartments of the endosomal pathway \(^{30,34}\) and is associated with impairment of intracellular protein trafficking after endocytosis. \(^{35}\) Recently, genetic studies \(^{36,37}\) have also implicated alterations of intraneuronal protein recycling and sorting mechanisms in the pathogenesis of AD. Elsewhere, it has been hypothesized that endocytosed Aβ\(_{42}\) is not degraded as efficiently as Aβ\(_{40}\). \(^{38}\)

The results of the present study support a selective accrual of intraneuronal Aβ\(_{42}\) with progression of AD. Additional work is necessary to elaborate the mechanisms of extracellular Aβ\(_{40}\) accumulation and their relation to the protein misprocessing that leads to intracellular Aβ\(_{42}\) 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 Aβ\(_{42}\) in the endosomal organelles and/or facilitated transfer of Aβ\(_{40}\) 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 homogenous biochemical compartment. In fact, future studies using serial extraction procedures on multiple brain regions may be suitable for analyzing regional covariance in toxic Aβ.

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 Aβ measures is a significant strength. We are not aware of other studies of human AD-affected brain that include biochemical compartments and brain regions and more detailed study of Aβ 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, Rau, Hyman, and Stern.

Acquisition of data: Irizarry, Scarmeas, Rau, Brandt, Albert, Blacker, and Stern.

Analysis and interpretation of data: Steinerman, Irizarry, Scarmeas, Rau, Hyman, and Stern.

Drafting of the manuscript: Steinerman.

Critical revision of the manuscript for important intellectual content: Steinerman, Irizarry, Scarmeas, Rau, Brandt, Albert, Blacker, Hyman, and Stern.

Statistical analysis: Scarmeas and Stern.

Obtained funding: Hyman and Stern.

Administrative, technical, and material support: Irizarry, Rau, Albert, Blacker, Hyman, and Stern.

Study supervision: Irizarry, Scarmeas, Albert, and Hyman.

Financial Disclosure: Dr Irizarry has shares and options holding in GlaxoSmithKline. The present study was completed before he joined GlaxoSmithKline. GlaxoSmithKline has drug development programs in AD unrelated to the present study. No GlaxoSmithKline funding was used to sponsor this research.

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Role of the Sponsor: The sponsors played no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.

REFERENCES


2. Giannakopoulos P, Herrmann FR, Bussiere T, et al. Tangle and neuron numbers, 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 Aβ ELISA is insensitive to certain biologically relevant species of cerebral amyloid. \(^{39}\) For example, it is expected to quantify monomers only and does not distinguish multimeric forms or N-terminal modifications of the Aβ peptide.

We expect that detailed biochemical fractionation of Aβ pools will significantly enhance future clinicopathologic investigations of AD. Our study confirms the relevance of Aβ\(_{42}\) in the intracellular and membrane-associated compartments to disease manifestations. Constitutive accumulation of extracellular Aβ\(_{40}\) appears to be an AD trait that correlates with illness duration and is accentuated among APOE-ε4-positive patients. Further study of the covariance of Aβ measures across biochemical compartments and brain regions and more detailed study of Aβ length and conformation within the intracellular and membrane-associated pools may contribute to updated models of amyloid dynamics.


