# Reduction of β-Amyloid Peptide$_{42}$ in the Cerebrospinal Fluid of Patients with Alzheimer's Disease

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**In this clinical study, the cerebrospinal fluid (CSF) level of a novel form of the β-amyloid peptide (Aβ) extending to position 42 (Aβ$_{42}$) was determined in patients with Alzheimer's disease (AD) as well as controls. In addition to measurement of CSF Aβ$_{42}$ levels, total Aβ peptides, microtubule-associated protein τ, and apolipoprotein E (ApoE) genotype were also assessed. It is interesting that CSF Aβ$_{42}$ levels were found to be significantly lower in AD patients relative to controls, whereas total Aβ levels were not. Aβ$_{42}$ has recently been shown to preferentially deposit in the brain tissue of patients with AD, suggesting that diminished clearance may account for its reduction in CSF. As previously reported, τ levels were increased in AD patients; however, neither Aβ$_{42}$ nor τ levels were apparently influenced by the ApoE genotype.**

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Alzheimer's disease (AD) is the most common form of dementia in the elderly population [1]. Clinically, it is diagnosed primarily by exclusion of other dementing illnesses. Diagnosis is only definitive at autopsy, or at brain biopsy, and is confirmed by a high density of senile plaques, largely composed of the β-amyloid (Aβ) peptide, and neurofibrillary tangles comprised of the microtubule-associated protein τ [2]. It is established that apolipoprotein E (ApoE) is associated with senile plaques and that the ApoE ε4 allele frequency is increased in the AD population [3]. For these reasons, we and others have focused attention on measurements of τ, Aβ, and ApoE as AD brain pathology–related proteins that might be useful as diagnostic markers during life. In this regard, recent studies have shown that τ is elevated in AD cerebrospinal fluid (CSF) [4, 5]. In contrast, a recent study has shown that total Aβ levels in AD patients did not differ from controls; although early-onset AD patients had slightly higher Aβ levels than elderly controls [6]. However, the generation of specific Aβ antibodies, along with biochemical purification and structural analyses, have revealed that Aβ extending to position 42(Aβ$_{42}$) predominates in both diffuse and senile amyloid plaques in AD brain tissue [7, 8]. Conversely, the predominant form of Aβ found in CSF was shown to contain 40 amino acids [9]. Analysis of CSF Aβ$_{42}$ levels in AD patients, therefore, is of significant interest but has not yet been reported. To address this, and the question of the combined utility of τ and Aβ measurements in the diagnosis of AD, the present study was undertaken on well-characterized groups of AD patients, neurological disease control patients, and normal elderly individuals.

## Materials and Methods

All subjects enrolled in this study underwent detailed clinical and neurological evaluation at university medical centers by neurologists expert in the diagnosis of dementia. Informed consent was obtained from subjects, or their guardians, as appropriate. The patient evaluation included medical history,

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physical and neurological examinations, laboratory blood tests to exclude metabolic causes of dementia, a neuroimaging study (head computed tomography or magnetic resonance within the past 3 years for demented patients and neurological controls), and detailed psychometric testing (this varied between institutions). In addition, all subjects received the following assessment instruments: the Mini-Mental State Examination (MMSE) [10], the Hamilton Depression Inventory [11], and the Hachinski Ischemic Index [12]. Patients with more than one dementia diagnosis, recent stroke, head trauma, or significant peripheral nervous system disorders were excluded. Each of the clinical centers involved in this study have either reported a clinical accuracy for probable AD of 85% or more [13] or have established, but have not published, such findings. The following diagnostic criteria were used.

*Patients with Alzheimer's Disease*

AD patients (n = 37) met National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) guidelines for probable AD; those who met criteria for possible AD were excluded [14]. All patients were community dwelling and had mild-to-moderate dementia.

*Neurological Disease Controls*

Neurological disease controls (ND; n = 32) were patients with non-AD dementia or degenerative disorders affecting the central nervous system. For neurological controls, a summary of clinical records was also reviewed by a second neurologist (D.G.). Patients with frontal lobe dementia were diagnosed according to the criteria set forth by the Lund and Manchester groups [15].

*Nondemented Controls*

Nondemented controls (NC; n = 20) were subjects who were age 50 or older and lacked significant cognitive complaints, did not have functional impairment, had normal findings on neurological examination, and scored 28 to 30 on the MMSE. A subgroup of these controls had symptoms of depression that did not result in significant cognitive or functional impairment and were judged not to have AD or any organic neurological condition.

Lumbar punctures were performed in the mornings, after an overnight fast. The first 2 to 3 ml of CSF was analyzed for protein, glucose, and cells at the local medical center laboratory, and 4 to 5 ml were removed from original collection tubes and added to 8-ml Sarstedt tubes containing 500 μl of buffer (containing additives such that the final CSF solution composition included 20 mM sodium phosphate, 20 mM triethanolamine, 0.05% Triton X-100, 100 mM NaCl, 0.05% $NaN_3$, 1 mM diethylenetriaminepentaacetic acid [DTPA], 1 mM EGTA, pH 7.4) and frozen at −20°C until analysis. Assay operators were unaware of the subjects' diagnoses.

*Apolipoprotein E Genotyping*

ApoE genotyping was performed on available blood samples that had been collected into EDTA vacutainer tubes. Samples were prepared by the method of Kawasaki [16] and polymerase chain reaction analysis performed as described by Wenham and colleagues [17].

*Total Aβ Enzyme-linked Immunosorbent Assay*

Total Aβ was measured in a sequential double monoclonal antibody sandwich enzyme-linked immunosorbent assay (ELISA) as previously described [18]. In brief, Aβ in CSF was captured by monoclonal antibody 266 (specific for Aβ peptide, residues 13–28), which had been precoated in microtiter plate wells. Detection utilized a second, Aβ-specific, biotinylated monoclonal antibody, 6C6 (recognizing Aβ residues 1–16), followed by reaction with an alkaline phosphatase-avidin conjugate. After incubation with the fluorogenic substrate 4-methylumbelliferyl phosphate (MUP), the fluorescent product was measured using a Millipore Cytofluor 2350 fluorometer.

*$Aβ_{42}$ ELISA*

$Aβ_{42}$ was measured in a similarly formatted assay using 266 as the capture antibody. The reporter polyclonal antibody 277-2, was raised against a synthetic peptide that included Aβ residues 33 to 42 (GLMVGGVVIA), with cysteine-aminoheptanoic acid at the peptide amino terminus. It was conjugated through the cysteine to cationized bovine serum albumin (Pierce). The antibody 277-2, affinity purified using the synthetic peptide conjugated to Sulfo-link resin (Pierce), reacted strongly with $^{125}I$-$Aβ_{1–42}$ as detected by immunoprecipitation of tracer. It showed no detectable cross-reactivity with $Aβ_{1–40}$ by immunoprecipitation. Using the 266/277-2 ELISA directed against $Aβ_{42}$, a variety of peptides including $Aβ_{1–28}$, $Aβ_{1–38}$, $Aβ_{1–40}$, $Aβ_{1–41}$, $Aβ_{1–42}$, and $Aβ_{1–43}$ were assayed in a dose range of 1 to 4,000 pg/ml. The $Aβ_{1–42}$ peptide gave a linear dose–response signal, while peptides $Aβ_{1–28}$, $Aβ_{1–38}$, and $Aβ_{1–40}$ showed no reactivity. $Aβ_{1–41}$ and $Aβ_{1–43}$ showed less than 5% and less than 7.5% cross-reactivity, respectively (data not shown). $Aβ_{1–28}$, $Aβ_{1–40}$, and $Aβ_{1–42}$ were purchased from Bachem. $Aβ_{1–38}$, $Aβ_{1–41}$, and $Aβ_{1–43}$ were synthesized at Athena Neurosciences, Inc. The $Aβ_{1–42}$ used as the standard for quantitation of CSF levels was a gift from Dr Charles Glabe (University of California, Irvine, CA). Detection of the 277-2 reporter antibody was achieved using a donkey anti-rabbit IgG-alkaline phosphatase conjugate and the AMPPD chemiluminescent substrate with Emerald enhancer (Tropix) [5].

To eliminate interassay variability as a factor in the $Aβ_{42}$ analysis, all samples were run in duplicate on the same day with the same lot of standards. The intraassay variability was less than 10%. Prior to measurement, aliquots of CSF samples were heated to 100°C for 3 minutes and then stored at 4°C overnight before assay. The heating step was found to generally increase immunoreactivity in CSF samples, independent of diagnosis, and was therefore included. It should be noted that different lots of synthetic $Aβ_{42}$ generate slightly different standard values, despite being normalized by amino acid analysis (data not shown). Values listed are based upon a single standard used for the entire study. Studies involving addition of synthetic $Aβ_{42}$ to CSF demonstrated that measured recovery was 80 ± 5%.

*τ ELISA*

The τ ELISA has been described elsewhere [5]. It consists of a two-site sandwich ELISA using two monoclonal antibodies

(16B5 and 16G7). These monoclonal antibodies have been shown to bind to τ independent of its phosphorylation state.

*Statistical Analysis*

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) using InStat, version 1.21.

## Results

Comparison of the three patient groups (Table) showed that they were well matched for age and gender. The AD group had an average MMSE of 17.5 ± 7.1 indicating mild-to-moderate cognitive impairment. The neurological disease control group consisted of a variety of disorders including vascular dementia (4 patients), frontal lobe dementia (7), depression (6), Parkinson's disease (3), corticobasal ganglionic degeneration (2), cerebellar ataxia (2), progressive supranuclear palsy (1), normal pressure hydrocephalus (1), grand mal seizure (1), Bell's palsy (1), age-associated memory impairment (1), dementia with extrapyramidal signs (1), amnestic syndrome (1), and cerebellar degeneration (1).

Analysis of total CSF Aβ levels revealed no significant differences among the different patient groups (see Table). The mean values ranged from 19.0 ng/ml in the AD group to 17.9 ng/ml in the NC group. There was significant overlap with no statistically significant differences among the groups ($p > 0.05$).

*Summary of Patient Profiles and Measured Variables*

| Variable | Alzheimer's Disease (AD) | Neurological Controls (ND) | Normal Controls (NC) |
|---|---|---|---|
| n | 37 | 32 | 20 |
| Age (mean ± SD; yr) | 70 ± 9.1 | 66 ± 9.1 | 70 ± 6.2 |
| Sex (M%/F%) | 48.6/51.4 | 59.4/40.6 | 50/50 |
| MMSE (mean ± SD) | 17.5 ± 7.1 | 23 ± 8.2 | 29.5 ± 0.6 |
| CSF Aβ (mean ± SD; ng/ml) | 19.0 ± 6.9 | 17.9 ± 6.7 | 21.8 ± 6.9 |
| APOE ε4 frequency[a] | 0.58 | 0.26 | 0.21 |
| APOE ε3 frequency | 0.38 | 0.74 | 0.74 |
| APOE ε2 frequency | 0.03 | 0.00 | 0.06 |
| $Aβ_{42}$ (mean ± SD; pg/ml) | 383 ± 76[b] | 543 ± 177 | 632 ± 156 |
| τ (mean ± SD; pg/ml) | 407 ± 241[c] | 168 ± 63 | 212 ± 102 |

[a]Apolipoprotein E (ApoE) genotypes were determined on 30 of 37 AD, 19 of 32 neurological controls, and 17 of 20 normal controls.
[b]$p < 0.0001$, comparing AD group to either control group.
[c]$p < 0.001$, comparing AD group to either control group.

MMSE = Mini-Mental State Examination; CSF = cerebrospinal fluid; $Aβ_{42}$ = β-amyloid peptide extending to position 42.

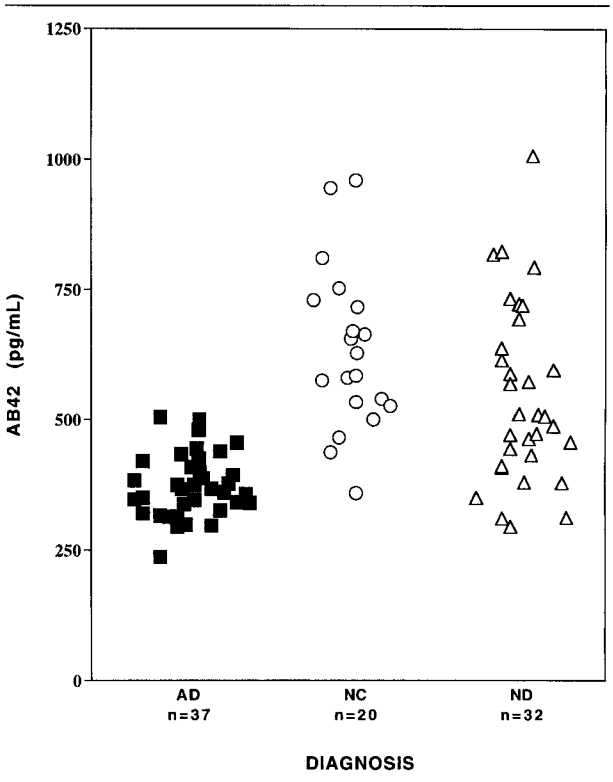


*Fig 1. Comparison of individual cerebrospinal fluid levels of β-amyloid peptide$_{42}$ ($Aβ_{42}$). $Aβ_{42}$ was measured as described in the text. All measures are the averages of duplicate determinations; variation was ≤10%. Samples were assigned randomly to plates and the operator was unaware of the subject diagnoses. Reference standards, present on each microtiter plate, were not significantly different between plates. A significant difference exists between the Alzheimer's disease (AD) and either control group (p < 0.0001). NC = nondemented controls; ND = neurological disease controls.*

Analysis of the $Aβ_{42}$ form of the peptide, however, demonstrated a reduction in the mean value in the AD group, relative to both the ND and NC subjects (383 versus 543 and 632 pg/ml, respectively), that was significant at the $p < 0.0001$ level (Fig 1). The relatively small standard deviation (76 pg/ml) of the AD group was particularly striking. A cutoff of 505 pg/ml best separated AD patients from ND and NC subjects. All AD patients, 15 of 32 ND, and 4 of 20 NC fell below this level. Therefore, an elevated $Aβ_{42}$ (>505 pg/ml) was an indicator of the likely absence of AD, accounting for 33 of 52 non-AD subjects. These initial data result in a calculated sensitivity of 100% and a specificity of 63%.

τ levels in the same subjects' CSF samples were also examined. Consistent with previous reports [4, 5], AD patients had a mean value of 407 pg/ml, which is significantly higher ($p > 0.001$) than the 168 and 212

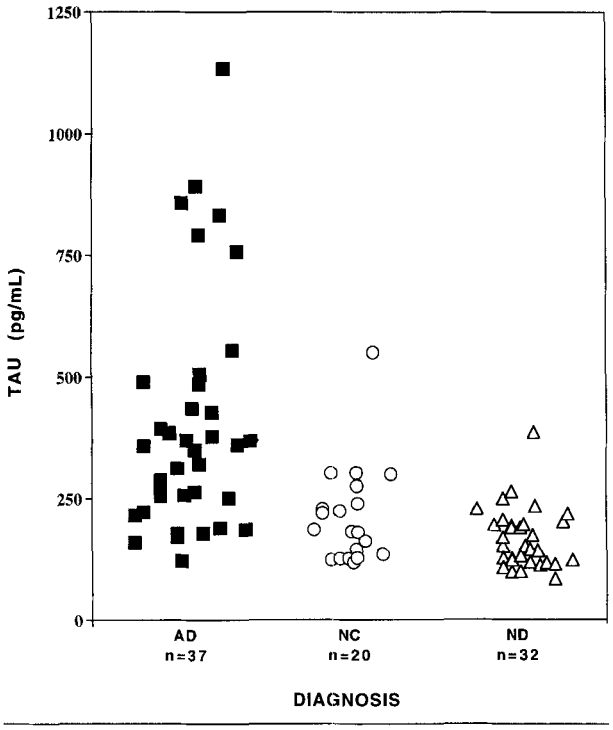


*Fig 2. Comparison of individual cerebrospinal fluid levels of τ. τ measurements were performed in duplicate. To ensure consistency, several samples from previous assays were included on subsequent plates and all samples were evaluated in at least replicate measure. Replicate measures were within 15% of original values. A significant difference exists between the Alzheimer's disease (AD) group and either control group ($p < 0.001$). Human brain–derived τ (provided by Innogenetics, Inc) was used as the reference standard. NC = nondemented controls; ND = neurological disease controls.*

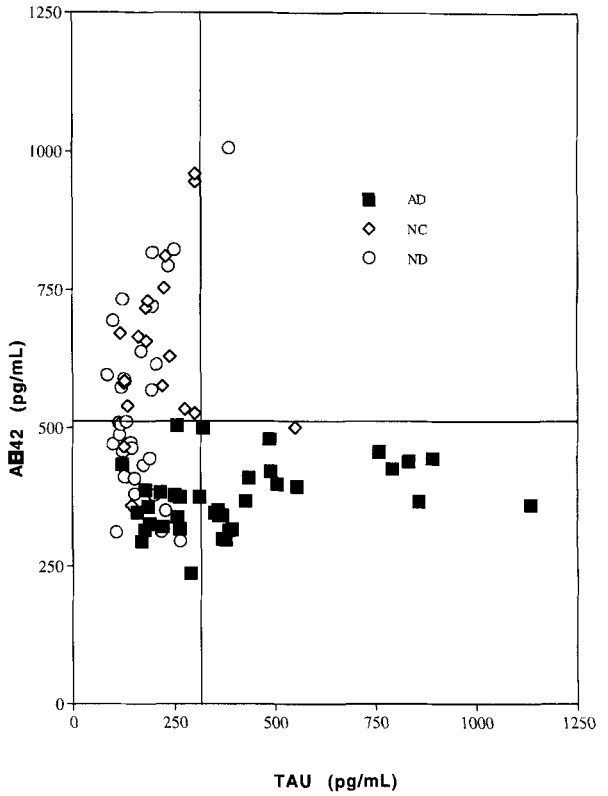


*Fig 3. Combined β-amyloid peptide$_{42}$ ($A\beta_{42}$) and τ measures in the diagnosis of Alzheimer's disease (AD). Data from Figures 1 and 2 are combined to illustrate the effect of simultaneous consideration of the two measures in discriminating the AD group. Lines indicate optimized cutoffs. The high τ/low $A\beta_{42}$ quadrant contains AD patients with only a single exception (21 of 22 patients), whereas the low τ/high $A\beta_{42}$ quadrant contains only control individuals.*

pg/ml observed in neurological and normal controls, respectively (Fig 2). Although previous studies of CSF τ levels have reported different cutoff values for distinguishing AD from control patients [4, 5], this appears to be due to differences in the τ standards employed in these studies (Van de Voorde A, personal communication). In this study, a cutoff of 312 pg/ml for τ best separated AD subjects from ND or NC. Of the 24 subjects with τ levels above this value, 22 were in the AD group (92% specificity), representing 22 of 37 AD patients (59.4% sensitivity).

CSF levels of Aβ, $A\beta_{42}$, and τ did not differ systematically between participating centers by diagnostic category. Also there were no significant correlations between levels of these markers and age, sex, or MMSE scores in the AD subjects (data not shown).

Of particular interest was the simultaneous analysis of $A\beta_{42}$ and τ measurements in the same CSF samples (Fig 3). Figure 3 is divided into four quadrants using the cutoffs for $A\beta_{42}$ and τ previously described. The presence of elevated τ and reduced $A\beta_{42}$ (lower right quadrant) was highly predictive of AD (22/23 = 96%). Conversely, high $A\beta_{42}$ and low τ (upper left quadrant) was observed only in control patients (see Fig 3). More than half (62%) of all the individuals in this study fell into one of these two quadrants. All but one of the remaining patients exhibited low $A\beta_{42}$ and low τ levels (lower left quadrant).

Analysis of the ApoE allele frequency present in the different patient groups demonstrated a clear overrepresentation of ApoE ε4 in the AD group (see Table). Analysis of the effect of ApoE alleles on τ and total Aβ, however, demonstrated no statistically significant effect on either τ, Aβ peptides, or $A\beta_{42}$ levels in any of the subject categories. Thus, when AD patients were segregated by ApoE genotype, average values of both $A\beta_{42}$ and τ CSF levels did not differ significantly among patients with 0, 1, or 2 ApoE ε4 alleles.

## Discussion

τ has previously been demonstrated to be elevated in AD CSF and this study is in agreement with these

findings (see Fig 2 and [4, 5]). The present study augments previous results by demonstrating that levels of τ in AD CSF do not correlate with age, MMSE, total Aβ, $A\beta_{42}$, or ApoE ε4 (data not shown). Although the precise reason for elevation of τ in AD remains unclear, it is likely due to the increased τ levels in AD brain tissue [19] combined with the ongoing degeneration of neurons in the disease.

The finding that $A\beta_{42}$ is consistently low in AD CSF (see Fig 1) is somewhat surprising since total Aβ levels appear to be unchanged (see Table). However, recent observations have shown that while the predominant form of Aβ in CSF and mixed brain-cell cultures is $A\beta_{1-40}$ [9, 18], plaques in AD brain tissue are composed primarily of $A\beta_{42}$ [7, 8]. A possible explanation consistent with these findings is that the $A\beta_{42}$ peptide preferentially deposits in AD brain tissue, leading to reduced levels in CSF. A finding analogous to this exists for another CNS amyloid disease, the Icelandic variant of cystatin C, where the level of the amyloid protein is also reduced in the CSF of affected individuals [20]. Alternative explanations for low $A\beta_{42}$ levels include decreased secretion or other forms of enhanced clearance of $A\beta_{42}$ from AD CSF.

Since plaque deposition most likely precedes cognitive symptoms in AD [21, 22], low CSF $A\beta_{42}$ in some ND subjects could indicate concomitant AD pathology that contributes to the dementia. The significance of low $A\beta_{42}$ in the small number of NC patients needs to be determined by longitudinal studies, ideally with autopsy examination.

The observation that the ApoE ε4 allele is overrepresented in the AD population in this study is consistent with numerous other reports [e.g., 3]. It has been proposed that ApoE ε4 might exacerbate AD through either physical interaction with Aβ [23] or τ [24]. It is curious that average values of these markers are equivalent in AD patients with or without the ApoE ε4 allele, suggesting any differential binding affinity for τ or Aβ by ApoE isoforms is not reflected in steady-state CSF levels of these markers.

Of interest is the combined analysis of CSF $A\beta_{42}$ and τ (see Fig 3). These data show that patients who exhibit high τ and low $A\beta_{42}$ had a strong likelihood of having AD (22/23; 96% specificity). Fifty-nine percent of the AD patients (i.e., sensitivity, 22/37) in this study fall into this category. Conversely, patients who exhibit low τ and elevated $A\beta_{42}$ were free of AD (100% 28/28, see Fig 3). Slightly over half of the non-AD subjects (32/52, 62%) fall into this category. Taken together, the combined analysis of CSF τ and $A\beta_{42}$ was informative of either the presence or the absence of AD in slightly over half of all individuals enrolled in this study. The combined CSF τ and $A\beta_{42}$ measurements, however, were not informative in those patients who fell into the low $A\beta_{42}$/low τ group. Possible reasons for neurological control patients exhibiting low $A\beta_{42}$ include nonspecificity of reduced $A\beta_{42}$ or the presence of plaques and the absence of clinical AD-type symptoms [25]. Autopsy confirmation on the cohort would be required to distinguish these possibilities. Nevertheless, the ability of any test to aid in the inclusion or exclusion of AD with good specificity and even moderate sensitivity is of potential diagnostic importance. This study suggests that the combined measurement of CSF $A\beta_{42}$ and τ might accomplish these goals.

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