Extreme Cerebrospinal Fluid Amyloid β Levels Identify Family with Late-Onset Alzheimer's Disease Presenilin 1 Mutation

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Objective: Aggregation and deposition of amyloid beta (Aβ) in the brain is thought to be central to the pathogenesis of Alzheimer's disease (AD). Recent studies suggest that cerebrospinal fluid (CSF) Aβ levels are strongly correlated with AD status and progression, and may be a meaningful endophenotype for AD. Mutations in presenilin 1 *(PSEN1)* are known to cause AD and change Aβ levels. In this study, we have investigated DNA sequence variation in the presenilin *(PSEN1)* gene using CSF Aβ levels as an endophenotype for AD.

Methods: We sequenced the exons and flanking intronic regions of *PSEN1* in clinically characterized research subjects with extreme values of CSF Aβ levels.

Results: This novel approach led directly to the identification of a disease-causing mutation in a family with late-onset AD.

Interpretation: This finding suggests that CSF Aβ may be a useful endophenotype for genetic studies of AD. Our results also suggest that *PSEN1* mutations can cause AD with a large range in age of onset, spanning both early- and late-onset AD.

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Amyloid β (Aβ) peptides that are 40 and 42 amino acids in length (Aβ40 and Aβ42, respectively) are normal products of amyloid precursor protein (APP) processing and can be detected in both plasma and cerebrospinal fluid (CSF). It has been suggested that the aggregation of Aβ peptide into insoluble forms (eg, oligomers and plaques) in the brain is a central feature of Alzheimer's disease (AD) pathology and pathogenesis.[1] This hypothesis is supported by the biological effects of known genetic risk factors for AD. Although rare, mutations causing familial AD (FAD) involve genes in the APP processing pathway (*APP,* presenilin 1 *[PSEN1],* and presenilin 2 *[PSEN2]*) and generally result in increased Aβ42/40 ratios. In addition, the confirmed genetic risk factor for late-onset AD (LOAD), the apolipoprotein E *(ApoE)* gene, has been shown to affect the aggregation of Aβ, in mouse models of Aβ deposition, in an isoform-dependent manner (ApoE2 < E3 < E4).[2,3] For some disorders, such as Parkinson's disease, prion disease, and frontotemporal dementia, variation in the levels of expression of the genes that cause autosomal dominant forms of these diseases have been shown to be associated with risk for sporadic forms of the same disease.[4–12] It has been proposed that LOAD may have a similar cause.[7]

Despite many studies of associations between polymorphisms in *PSEN1* and AD (for a summary see Bertram and colleagues[13]), little progress has been made in identifying specific polymorphisms in *PSEN1* or other candidate genes that show consistent evidence for association.[14] One possible factor is that the clinical diagnoses are not always correct. Diagnosis of AD relies on clinical judgment, which may introduce error at the level of ascertainment.[15,16] One way to overcome this problem is the use of intermediate traits, or endophenotypes.[17] The use of an endophenotype for studying complex disease may confer several advantages, providing greater power because it is less heterogeneous than clinical diagnoses and more directly affected by genetic variation. Bearden and Freimer[18] suggest that a meaningful endophenotype must be heritable, associated with the causes of the disorder, reliably measured, normally distributed in the population, and associated with risk for the disorder of interest.

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The biological effects of known AD risk factors have led to studies of the viability of Aβ levels as an endophenotype for AD. Plasma Aβ levels have been reported to increase in some individuals with FAD.[19] Several studies suggest that plasma Aβ levels are related to LOAD status and progression.[20–22] However, other studies suggest that plasma Aβ levels are not correlated with LOAD.[23,24] As a result, it is unclear whether plasma Aβ levels are meaningful biomarkers for LOAD. In contrast, there is more consistent evidence that levels of Aβ42 in CSF correlate with LOAD status.[24–35] Recently, Fagan and colleagues[36] showed that CSF Aβ42 levels vary inversely with Aβ deposition as measured by positron emission tomography using the amyloid imaging tracer Pittsburgh compound B. A recent review of these findings suggests that CSF Aβ levels are one of the most promising endophenotypes for AD.[37] Given that the known genetic risk factors for AD affect Aβ processing, variation in CSF Aβ levels may be a potentially useful endophenotype for genetic studies of AD risk.

In this study, we have attempted to identify variation in the *PSEN1* gene and test for a correlation with CSF Aβ levels. We hypothesize that the use of CSF Aβ levels as a quantitative endophenotype will provide an alternative and powerful approach for the identification of novel genetic risk factors for AD.

Subjects and Methods

Samples

Our sample consists of 191 volunteers participating in studies of aging and dementia at the Alzheimer's Disease Research Center at Washington University School of Medicine from July 1998 to December 2005.[38] Sixty-four percent of these volunteers have a positive family history of AD (one or more first-degree relatives with AD; see Table 1). This figure is similar to the general population of "sporadic" AD cases in which approximately 40% are "family history–positive."[39] The percentage of AD cases who are positive for the ApoE4 allele in this sample is 56%, similar to that reported for other research samples of AD cases (eg, 55% for amnestic mild cognitive impairment and 69% for mild AD in the Alzheimer's Disease Cooperative Study sample).[40] Our sample includes 121 female and 70 male subjects between the ages of 45 and 95 years. Our sample includes 143 subjects with Clinical Dementia Rating (CDR) of 0 (nondemented), 33 subjects with CDR of 0.5 (very mild dementia), and 15 subjects with CDR of 1.0 (mild dementia).[41] Eighty-one of the 191 individuals carry at least one *ApoE4* allele.

Plasma and CSF were collected from all participants. CSF and plasma collection, processing, and Aβ measurements were performed as Fagan and colleagues[36] described. All studies were approved by the Washington University School of Medicine Human Studies Committee, and informed consent was obtained from all subjects.

Statistical Analyses

Levels of Aβ40, Aβ42, and the ratio of Aβ42 to Aβ40 were tested for normality using the Shapiro–Wilk test. Values were then log transformed to approximate a normal distribution and tested again for normality. CSF Aβ values were stratified by sex. The log-transformed values for each sex were tested for association with age, CDR, and the presence or absence of an *ApoE4* allele (*ApoE4*+) using linear regression. Age was also tested for nonlinear effects using a locally weighted polynomial regression (LOESS) fitted curve. After adjustment for correlated traits, the residuals were tested for normality using the Shapiro–Wilk test. We calculated Pearson's correlation coefficients to evaluate the correlation of CSF and plasma Aβ40 and Aβ42 levels. Plasma Aβ levels were tested for association with age using linear regression, with sex and *ApoE4*+ using *t* tests, and with CDR using analysis of variance.

Sequencing and Genotyping

To maximize the probability of detecting functional genetic variation in our sequencing efforts, we selected the top and bottom 5% from the distributions of Aβ40, Aβ42, and Aβ42/40 ratio residuals for each sex. This resulted in 42 unique individuals (some individuals appear in multiple extremes, eg, high Aβ42 and high Aβ42/40 ratio). Primers were designed to target each exon of *PSEN1* and at least 50bp of 3′ and 5′ flanking intronic sequence. Primers for sequencing were designed using consensus sequence from Ensembl and PRIMER3 (primer sequences will be provided on request).[42] A total of nearly 5,600bp were sequenced in each individual in the *PSEN1* gene region. Sequencing was performed using ABI Big Dye version 3.1 (Applied Biosystems, Foster City, CA). Sequence analysis was done using Sequencher (Gene Codes, Ann Arbor, MI). Common single nucleotide polymorphisms (SNPs) that were detected in this sequencing effort were genotyped in the full sample using Sequenom genotyping technology (Sequenom, San Diego, CA; assay details are available on request). These SNPs were tested for association with CSF Aβ levels using analysis of variance.

Plasmids, Transfection, and Amyloid β Enzyme-Linked Immunosorbent Assay

To test the effects of the A79V mutation, we transfected A79V mutant PSEN1 into cell lines and measured the secreted Aβ40 and Aβ42. The complementary DNA constructs for *wtPSEN1* and *APPΔNL* have been described previously.[43] The QuickChange II site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) was used to introduce the *PSEN1* A79V point mutation into *wtPSEN1*. The construct was confirmed by sequence analysis. *PSEN1* and *PSEN2* double-knock-out mouse embryonic fibroblasts were transfected with *APPΔNL* and *wtPS1* or A79V, conditioned medium was collected, and secreted Aβ40 and Aβ42 were measured with a sandwich enzyme-linked immunosorbent assay, as described previously.[43]

Results

There is a more than 10-fold difference between the lowest and highest raw values in both Aβ40 and Aβ42 levels in CSF in our sample (Aβ40: mean = 9,794pg/

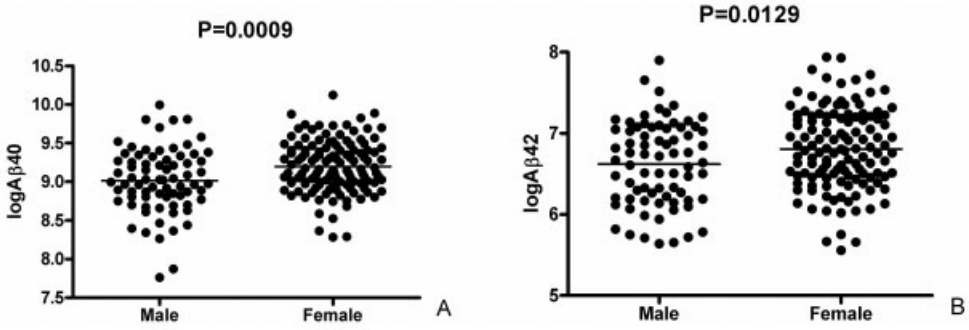

Fig 1. Sex and cerebrospinal fluid (CSF) amyloid β (Aβ) levels. (A) Log-transformed (log) Aβ40 compared between male and female subjects. p = *0.0009. (B) LogAβ42 compared between male and female subjects.* p = *0.0129.* p *values are from a* t *test to compare the means.*

ml, range = 2,355–24,899pg/ml; Aβ42: mean = 940pg/ml, range = 259–2,802pg/ml). The raw Aβ42/40 ratio values have a mean of 0.1 and range from 0.026 to 0.30. Although CSF Aβ40 and Aβ42 levels and Aβ42/40 ratios were not normally distributed, log transformation of each trait produced a distribution that was not inconsistent with normality, and thus could be analyzed using standard parametric statistics. The log-transformed CSF Aβ40 and Aβ42 levels were significantly greater in women than in men (p = 0.0009 and 0.0129, respectively; Fig 1), but Aβ42/40 ratios were similar in both sexes. To account for the possibility of sex-specific effects, we stratified Aβ40 and Aβ42 values by sex for subsequent analyses.[44] Whereas Aβ40 levels were significantly associated with sex, we failed to detect age-, CDR-, or *ApoE4*+-dependent differences in Aβ40 levels (Table 2).

In contrast, Aβ42 levels were significantly decreased with increased age and increased CDR in both male and female subjects. Aβ42 levels were also significantly less in the presence of at least one *ApoE4* allele in both male and female subjects. The Aβ42/40 ratio was significantly decreased with increased age, increased CDR, and presence of the ApoE4 allele.

We detected no strong evidence for a nonlinear relation between age and CSF Aβ levels (Fig 2). After adjusting for age and the other associated traits using linear regression, we failed to reject normality for the residuals of Aβ40, Aβ42, and Aβ42/40 ratio, allowing for the use of analysis of variance for the genetic analyses. Whereas our sample is enriched for subjects with positive family histories, residual Aβ42 values are not significantly different between the groups with positive and negative family histories (p = 0.5; Table 1). Not surprisingly, the major difference between these two groups is that the positive family history group has a much greater frequency of the *ApoE4* allele (0.49 in the positive vs 0.31 in the negative group).

Aβ levels in plasma are not strongly correlated with CSF Aβ levels (Aβ40: n = 176, Pearson's r = 0.06,

Table 1. **Summary of the Characteristics of the Total Sample, as Well as Subsets with Positive (One or More First-Degree Relatives with Alzheimer's Disease) and Negative Family Histories**

Characteristics	Whole Sample (N = 191)	Positive Family History (n = 122)	Negative Family History (n = 69)
Mean age (range, SD), yr	66.9 (45–95, 12.2)	65.7 (45–91,11.5)	69.1 (46–95, 13.1)
Female, %	63	64	61
ApoE4+, %	42	49	31
CDR	0 = 75%	0 = 75%	0 = 75%
	0.5 = 17%	0.5 = 19%	0.5 = 15%
	1 = 8%	1 = 6%	1 = 10%
Mean Aβ42	0.0009	0.016	−0.013

Included are the number of subjects, age, percentage of female subjects, percentage of apolipoprotein E4 (*ApoE4*) allele carriers (*ApoE4*+), Clinical Dementia Rating (CDR), and the mean of amyloid beta peptide 42 amino acids in length (Aβ42) residuals after log transformation and adjustment for correlated traits (age, CDR, sex, and presence/absence of the *ApoE4* allele). The mean Aβ42 values between the positive and negative family history subsets were not significantly different when compared using a t test (p = 0.5).
SD = standard deviation.

***Table 2. p* Values for Linear Regressions of the Cerebrospinal Fluid LogAβ40, LogAβ42, and LogAβ42/40 Ratio Values with Age, Clinical Dementia Rating, and the Presence or Absence of the Apolipoprotein E4 Allele in Male and Female Subjects**

Trait	Sex	Age	CDR	*ApoE4*+
logAβ40	0.0009			
Male		0.5858	0.5677	0.103
Female		0.2576	0.5508	0.3182
logAβ42	0.0129			
Male		0.0077	0.0064	0.0071
Female		0.0064	<0.0001	0.0262
log(Aβ42/Aβ40 ratio)	0.2758	0.001	<0.0001	0.0012

CDR = Clinical Dementia Rating; *ApoE4*+ = apolipoprotein E4 allele carrier; Aβ42 = amyloid beta peptide 42 amino acids in length.

$p = 0.46$; Aβ42: n = 175, Pearson's $r = 0.08$, $p = 0.28$). Plasma Aβ40 levels had a mean of 177.9pg/ml and ranged from 75.57 to 399.99pg/ml. Plasma Aβ42 levels had a mean of 35.37pg/ml and ranged from 1.03 to 163.04pg/ml. Plasma Aβ40 and Aβ42 levels were not significantly associated with sex, CDR, or *ApoE4*. Plasma Aβ40 but not plasma Aβ42 was significantly associated with age (Fig 3).

We used normalized and adjusted values of CSF Aβ40, Aβ42, and Aβ42/40 ratio to choose individuals with extreme values (the top and bottom 5% for each phenotype, independently) for DNA sequencing. We did not detect significant differences in the ages or CDR scores of any extreme category when compared with the rest of the sample. We identified eight genetic variations in *PSEN1* in our sample. Five are previously reported SNPs (rs165932, rs1800839, rs362384, rs362385, and rs7523). rs1800839 is located 31bp upstream of exon 1. rs165932 is located 16bp downstream of exon 8 and has been investigated for association with AD in more than 30 published studies.[13] rs362384, rs362385, and rs7523 are all located in exon 12, which is not translated. For each of these common SNPs, the minor allele was more frequently observed in individuals from the upper end of the Aβ distribution, suggesting that they could be linked to functional variation affecting Aβ levels. To evaluate this further, we genotyped each of these SNPs in the entire CSF data set. We failed to detect evidence for association of these common SNPs with CSF Aβ levels.

The other three genetic variations in this sample are present in single heterozygous individuals. SNP1 and SNP2 are located in the untranslated exons 1 and 12, respectively. They do not appear to be localized to known splice sites. However, their location in untranslated exons makes it possible that they could alter the stability or regulation of *PSEN1* messenger RNA. The remaining variant is a missense mutation in exon 4, which changes codon 79 from alanine (GCC) to valine (GTC), or A79V. This is a known FAD mutation that has been reported previously in four families.[45,46] The carrier of this mutation in our sample is nondemented and has the fifth highest adjusted Aβ42 value and the third highest Aβ42/40 ratio value in our sample. Pittsburgh compound B imaging in this individual showed no evidence of β-amyloid deposition (data not shown).

This individual is a member of a large, multigenerational FAD kindred that had been previously ascertained at the Washington University School of Medicine Alzheimer's Disease Research Center. This family is classified as a LOAD family; the mean age at onset (AAO) of AD for the family is 69 years and varies widely (range, 55–78 years). We genotyped 20 additional family members for this variant, including four demented individuals, six at-risk individuals (offspring of demented individuals), five nondemented elderly siblings of demented individuals, and five offspring of nondemented individuals. Three of the four demented individuals were found to be heterozygous for the mutation, with AAO greater than 75 years and autopsy-confirmed AD. The fourth case (AAO of 78 years) lacked the mutation and had a large number of cortical Lewy bodies in addition to histopathological AD at autopsy. Three of the six at-risk individuals were heterozygous for the mutation. The remaining 10 individuals (5 nondemented elderly siblings and 5 offspring of nondemented individuals) had wild-type sequence. No other individuals from our CSF sample came from families with an established multigenerational AD inheritance pattern.

To investigate the consequences of this mutation on Aβ levels, we introduced the A79V point mutation into wild-type *PSEN1* sequence, then transfected

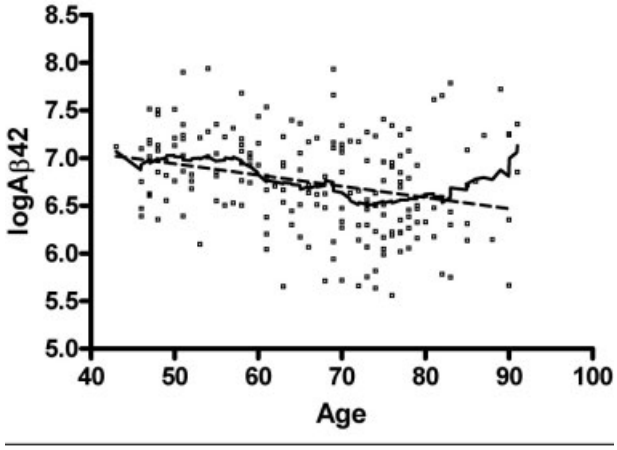

Fig 2. Age and cerebrospinal fluid (CSF) logAβ42. Dashed line *is a linear regression* ($r^2 = 0.08$; $p < 10^{-3}$). Solid line *is a LOESS fitted curve.*

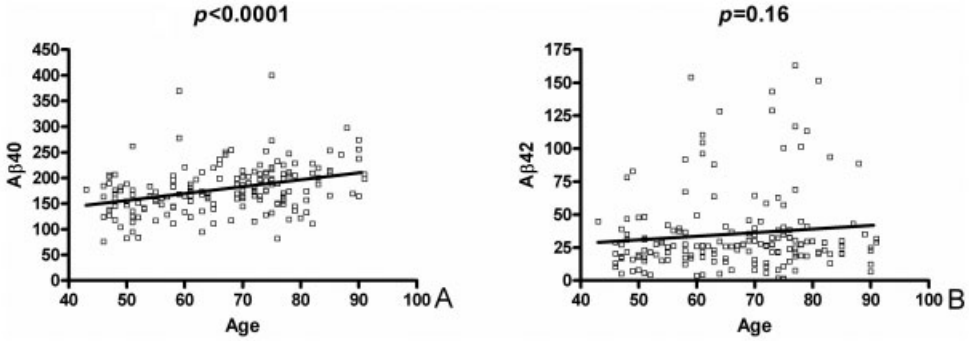

Fig 3. Age and plasma Aβ levels. (A) Plasma Aβ40 and age. p = *0.0001. (B) Plasma Aβ42 and age.* p = *0.16.* p *values are from linear regressions.*

into PSEN1/2 knock-out mouse embryonic fibroblasts. The Aβ42 levels and the Aβ42/Aβ40 ratio in the conditioned media from these cells were significantly greater than the wild-type *PSEN1* sequence (Fig 4). Aβ40 levels and total Aβ (combined Aβ40 and Aβ42) levels were not significantly different from the wild type.

Discussion

We have investigated variation in the exons and flanking intronic regions of *PSEN1* using CSF Aβ levels as an endophenotype for AD. This novel approach led directly to the identification of a single disease-causing mutation in a LOAD family. This finding suggests that CSF Aβ values may be a useful endophenotype for genetic studies of AD. Our results also suggest that *PSEN1* mutations can cause AD with a large range in AAO, spanning the ranges of both early-onset AD and LOAD. It appears that genetic variation in *PSEN1* explains the extreme Aβ levels in just 1 of the 42 samples we sequenced. This suggests that the other extreme values are due to variation in other genes or environmental factors.

CSF Aβ42 levels and Aβ42/40 ratios are associated

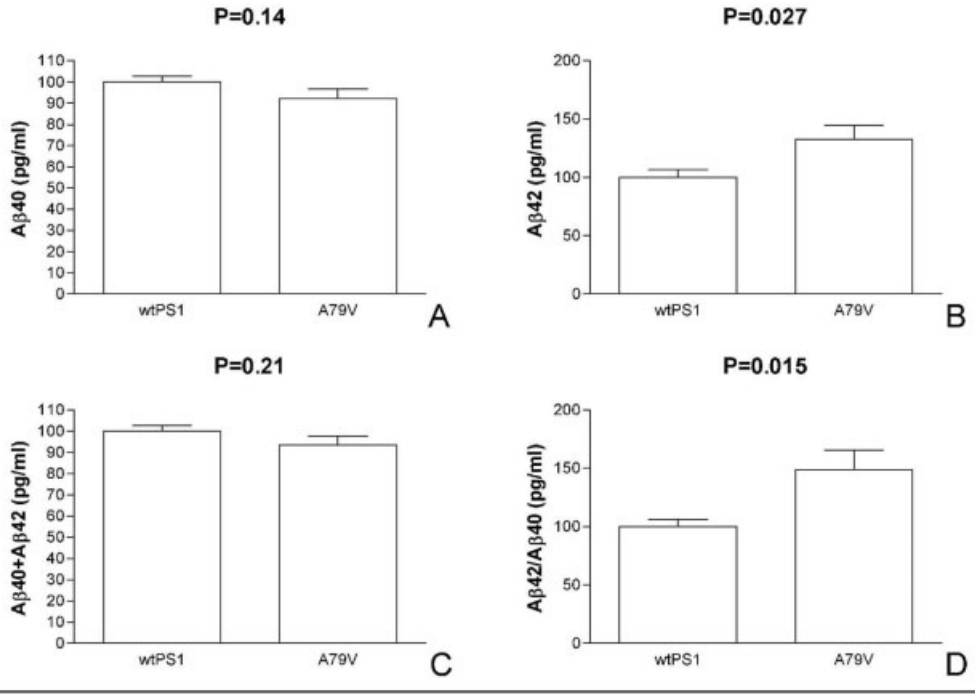

Fig 4. Amyloid β (Aβ) analysis of the A79V mutant. PS1/2 knock-out cells were transfected with APPΔNL and wtPS1 or the A79V mutant. Secreted Aβ40 and Aβ42 were measured. Each data set (in triplicates) was normalized to wtPS1 values. The amount of Aβ40 (A), Aβ42 (B), total Aβ (Aβ40 + Aβ42) (C), as well as Aβ42/Aβ40 ratio (D) was plotted. Error bars represent standard error of the mean. p *values from a Student's* t *test are provided for each plot. Shown are combined data from four independent experiments.* p = *0.14 (A); 0.027 (B); 0.21 (C); 0.15 (D).*

with dementia as measured by the CDR, as well as the known risk factors for AD, age, and presence of the *ApoE4* allele. These results confirm previous findings.[36,47–51] Maccioni and colleagues'[52] recent study reported no effect of sex on CSF Aβ42 in 93 individuals. In our larger sample, we detected a significant sex effect. Our findings are also consistent with analysis performed by Farrer and coworkers,[53] which showed that the effects of the *ApoE4* allele on risk for AD varied with age and sex. Plasma Aβ levels in our sample did not show strong correlation with CSF Aβ levels. This result, together with the strong association between CSF Aβ levels and CDR, suggests that CSF Aβ levels are a better candidate endophenotype for AD than plasma Aβ levels.

Recently, Fagan and colleagues[36] showed that CSF Aβ42 is inversely correlated with β-amyloid deposition. Another recent study, using samples from a large cohort of individuals with a broad age distribution, observed that the relation of CSF Aβ42 with age is complex, with the slope of a LOESS fitted curve showing a marked decrease in slope after the sixth decade of life.[51] Based on these studies, it might be hypothesized that Aβ42 levels might decrease more quickly as individuals reach the age at which Aβ42 deposition begins (50–60 years).[54,55] Peskind and colleagues'[51] data roughly fit this prediction.[51] However, the age range of their sample (21–88 years) is much larger than in our sample (45–95 years) and includes many younger individuals. In our sample, which includes only individuals that are at or near the age at which deposition is expected to begin, we detect a simple linear relation between Aβ42 and age.

The inclusion of subjects with positive family histories enriches our sample with individuals who carry genetic risk factors for AD. Such enrichment increases the probability of a few individuals with autosomal dominant mutations that cause disease (such as *PSEN1* mutations), or more likely with common genetic variations related to risk for AD, for example, *ApoE4*. Although we did not detect association with the five common polymorphisms that we identified, we did identify several individuals who are heterozygous for rare variants including one individual carrying the A79V mutation, a mutation known to segregate with FAD. The four families that have previously been found to carry this mutation had AAO ranging from 50 to 62 years. The mutation segregates with AD in the family we identified in this study, though AAO is much later in this family than those studied previously. One individual in this family had AD but was found not to carry the mutation. On autopsy it was evident that this individual had a more complicated pathological burden than the three carriers of the mutation. Given the large size of this pedigree and the late AAO (78 years) in this individual, it is probable that this person had "sporadic AD." The individual we first identified is near the age at which amyloid deposition would be expected to begin. This individual currently has no clinical symptoms of disease and no evidence of β-amyloid deposition using Pittsburgh compound B imaging. This individual had extreme values (though not the most extreme in our sample) for both CSF Aβ42 and Aβ42/40 ratio. This in vivo observation is consistent with our in vitro assays, which show increases in Aβ42 level and Aβ42/Aβ40 ratio in cells with the A79V mutation relative to control cells.

PSEN1 mutations are generally associated with early-onset AD. This may be due to ascertainment bias, because many families with strong histories of AD and late or highly variable onset may not have been screened for *PSEN1* mutations. Our findings and those of another recent report by Larner and coworkers[56] suggest that *PSEN1* mutations can result in much later onset than traditionally has been considered. Furthermore, the A79V mutation is associated with a broad range of AAO, suggesting the existence of genetic or environmental modifiers of disease onset. *ApoE* genotype does not appear to explain the variation in AAO in this family. In future studies we hope to determine the additional genetic or environmental factors, or both, that may be influencing AAO in this family.

This work demonstrates that CSF Aβ levels are a useful endophenotype for AD in the search for novel genetic risk factors. We anticipate that continued application of this approach will lead to the identification of additional genetic variation in other genes, which may explain the extreme Aβ values observed in other individuals in this sample.

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