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Evaluation of Apolipoprotein E Fragmentation as a Biomarker for Alzheimer's Disease

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Abstract

Recent studies have supported a role for the proteolytic cleavage of apolipoprotein *E4 (APOE4)* as a potential mechanism for the enhanced dementia risk associated with Alzheimer's disease. To determine whether *APOE4* fragmentation is correlated with AD, ELISA assays were performed with cerebral spinal fluid (CSF) and plasma samples utilizing an antibody that specifically detects a 17 kDa amino-terminal fragment (p17) of *APOE* (nApoECF antibody). In CSF samples, levels of *APOE* fragmentation were minimal in both neuropathological normals (NPNs) and AD cases and there were no significant differences between the two cohorts across *APOE* genotypes. Similar results were found in plasma samples where the p17 *APOE* fragment comprised only 8.4% of the total level of identified APOE. As with CSF, there were no significant differences found between NPNs and AD cases in terms of the amount of nApoECF quantified. Taken together, these results suggest that the p17 amino-terminal fragment of *APOE* is not correlated with AD or *APOE* genotype in the plasma or CSF.

Keywords: Apolipoprotein E; Cerebral spinal fluid; Alzheimer's disease; Plasma; Biomarker; Proteolysis

Introduction

The apolipoprotein E (APOE) allele is polymorphic leading to the expression of three proteins (*APOE2*, *E3*, and *E4*) that differ by single amino acid substitutions of cysteine to arginine at positions 112 and 158, respectively, of the full-length form of the protein [1]. Harboring the *APOE3* allele is believed to neither increase nor decrease one's risk of AD, while having the *E2* form may decrease one's risk. In contrast, inheritance of one copy of the *APOE4* allele increases disease risk fourfold, while two copies raises the risk tenfold [2]. It has been well established that harboring the *APOE4* allele enhances dementia associated with Alzheimer's disease (AD), and several studies have supported a role of *APOE4* proteolysis as an important factor that may contribute to this risk [2,3-10]. Thus, the high susceptibility of *APOE4* to proteolytic cleavage may be the driving force behind the enhanced risk for AD in individuals who are either heterozygous or homozygous for the *APOE4* allele. Recently, we reported the presence of a 17 kDa fragment (p17) of *APOE4* in the AD brain (corresponding to amino acids 1-151) that localized intracellularly, predominantly within the nucleus of microglia and neurons and the levels of this fragment was significantly higher as compared to neuropathological normals (NPNs) [11]. These findings were accomplished using a site-directed cleavage antibody to *APOE* that is highly specific for a 17 kDa amino-terminal fragment of *APOE3* or *E4* but does not react with the mature, full-length form of *APOE* [11]. In the present study, we sought to examine the utility of this antibody (termed nApoECFp17 antibody) to detect the p17 fragment in either cerebrospinal fluid or plasma samples. The results indicated the levels of the p17 fragment were very low in both fluids and no significant differences between the NPN and AD cohorts were observed. Therefore, the 17 kDa amino-terminal fragment of *APOE* does not appear to be a viable biomarker for predicting AD.

Materials and Methods

Materials

The in house, rabbit polyclonal nApoECFp17 antibody was synthesized and extensively characterized as previously described and detects the amino-terminal fragment of *APOE* corresponding to amino acids 1-151 [11]. The nApoECFp17 antibody was

synthesized using the 7-mer peptide C-RKRLLRD, which represents the N-terminal upstream neoepitope fragment of *APOE4* and *E3* that would be generated following cleavage after the terminal aspartic acid residue at position D151.The anti-*APOE4* N-terminal rabbit polyclonal antibody was purchased from Aviva Systems Biology Corp. (San Diego, CA). Peroxidase-conjugated AffiniPure goat anti-rabbit (IgG) was purchased from Jackson ImmunoResearch Labs (West Grove, PA). This antibody is known to recognize the extreme N-terminal region of human *APOE*. The TMB microwell peroxidase substrate system was purchased from KPL, Inc. (Gaithersburg, MD). 96-well, clear, flat bottom, high binding, polystyrene plates were purchased from Corning (Kennebunk, ME).

CSF and Plasma Samples

CSF and plasma samples from NPNs as well as AD subjects were provided by the Institute for Brain Aging and Dementia Tissue Repositories at the University of California, Irvine. Approval from the Boise State University Institutional Review Board was not obtained due to the exemption granted that all samples were received from University of California, Irvine. Fluid samples obtained from University of California, Irvine were anonymized and never identified except by case number. Donors or their next of kin provided informed signed consents to the Institute for Memory Impairments and Neurological Disorders for the use of their biological samples in research (IRB 2014-1526). Case demographics are presented in Table 1.

Group	Age (yrs)	Sex	*APOE* Genotype
NPN	90	F	3/3
NPN	93	F	3/4
NPN	79	M	3/4
NPN	82	M	3/3
NPN	92	M	N/A
NPN	87	F	4/4
NPN	96	M	2/3
NPN	97	M	3/3
NPN	83	M	¾
NPN	90	F	3/3
NPN	79	F	N/A
NPN	86	F	3/3
NPN	82	M	3/3
NPN	88	M	4/4
NPN	87	F	3/4
NPN	77	M	4/4
AD	79	F	4/4
AD	84	F	N/A
AD	86	F	N/A
AD	90	F	3/3
AD	79	M	4/4
AD	82	M	3/4
AD	90	M	3/3
AD	92	M	3/3
AD	95	M	3/4
AD	93	F	2/4
AD	93	F	3/4
AD	92	M	3/3
AD	82	F	2/3
AD	81	M	2/3
AD	83	F	3/3
AD	77	M	2/4
AD	83	F	3/3
AD	80	M	3/3
AD	87	M	3/3
AD	87	F	3/4

Group	Age (yrs)	Sex	*APOE* Genotype
AD	90	F	3/4
AD	90	F	3/4
AD	92	F	3/4
AD	86	F	3/4
AD	87	M	3/4
AD	87	M	3/4
AD	78	M	4/4

NPN = Neuropathologically normal (average age, 86.8±6.1)
AD = Alzheimer's disease (average age, 86.1±5.2)
Table 1: Case demographics

ELISA Assays

Protein content for CSF and plasma samples was determined using the BCA method (Pierce) to ensure equal protein loading between samples. In 96-well plates, 50 µl of CSF and/or 25 µl of plasma diluted with 100 mM carbonate coating buffer (pH, 9.7) to a final volume of 100 µl and were incubated overnight at 4 °C on a slow rocker. Wells were washed 3X in tween, tris-buffered saline (TBS, 10 mM TRIS, 100 mM NaCl, and .05% Tween 20). Following this wash step, 300 µl of blocking solution (3% non-fat dried milk in TBS) was added and the plate was incubated for 1 hour at 37 °C. Following a wash step (3X), wells were then incubated for 2 hours at 37 °C with the nApoECFp17 antibody at a 1:500 dilution in TBS containing 0.3% non-fat dried milk. The two hour incubation time and temperature of incubation at 37 °C were determined to be optimal parameters based on our previous studies [11]. Following the wash step (3X), wells were then incubated for 1 hour at 37 °C with anti-rabbit secondary HRP antibody at a 1:5,000 dilution in TBS containing 0.3% non-fat dried milk. The plate was then washed (3X) in TBS followed by the development of color utilizing the TMB microwell peroxidase substrate system. Color was allowed to develop for 4 minutes before the addition of a stop solution. The relative absorbance at 450 nm was recorded using a BioTek Synergy Mx microplate reader. All samples were evaluated in duplicate and data are representative of three independent experiments ±S.E.M. To calculate the percent of total nApoECFp17 fragment present in either CSF or plasma, we used the overall OD value obtained using the full-length *APOE* antibody as representing the total amount of both full-length and any associated amino-terminal fragments of *APOE*. The value for nApoECF was then subtracted by this value and then divided by the value obtained by full-length *APOE*. For example, to calculate the percent of nApoECF found in CSF samples, 1.57 (averaged OD for full-length *APOE*)-.090 (averaged OD for nApoECF/1.57 (averaged OD for full-length *APOE*), which was equal to 94.2%. This number was then subtracted from 100% to give a value of 5.8% nApoECF fragment present in the CSF samples. A similar calculation was undertaken to determine the percent of nApoECF found in plasma samples.

Statistical Analysis

Statistical differences in this study were determined using Student's two-tailed T-test employing Microsoft Office Excel to determine significant differences between samples. P-values greater than 0.05 were considered non-significant.

Results

In a previous study, we designed and characterized a site-directed cleavage antibody directed towards position D151 of the mature form of *APOE*. This antibody (termed the nApoECFp17 antibody) does not react with full-length *APOE* and specifically detects a 17 kDa fragment in AD cases that localizes predominantly within the nucleus of microglia [11]. Immunohistochemical analysis of AD cases indicated staining of nApoECFp17 was exclusively intracellular with no evidence of immunoreactivity in extracellular, plaque-rich regions. However, we did observe staining along blood vessels and therefore hypothesized that the p17 fragment of *APOE* may serve as a biomarker in AD. To test this hypothesis, we first examined whether this amino-terminal fragment of *APOE* could be detected in CSF in both AD and neuropathological normal (NPN) cases and compared these results to those obtained using an antibody that detects full-length *APOE*. It is important to note that the nApoECFp17 antibody does not distinguish between the various isoforms of *APOE* and will readily detect amino-terminal fragments of *APOE3* and *E4* [11].

As shown in Figure 1, ELISA analysis of CSF using the nApoECFp17 antibody showed no significant differences between NPNs and AD subjects (Figure 1A). Indeed, there was a trend for lower levels of the p17 fragment in AD cases as compared to NPNs. It is noteworthy that all data were expressed in relative units due to lack of any control antigen that could possibly provide for a standard curve.

Previous studies have supported the hypothesis that *APOE4* is more susceptible to proteolytic cleavage than *APOE2* and *E3* (for a recent review see [5]). Therefore, we sought to determine whether there were any differences in the levels of the p17 fragment based on *APOE* genotype alone (both AD and NPN subjects). Due to the rarity of the *APOE2* allele, we analyzed all heterozygous *APOE2/X* together for this experiment. There were no significant differences across *APOE* genotypes following this analysis (Figure 1B). In contrast to these results, the use of a positive control consisting of the immunogen used to generate the

nApoECFp17 antibody consisting of the 7-mer peptide C-RKRLLRD, resulted in significantly higher OD values (green bar labeled positive control, Figure 1A).

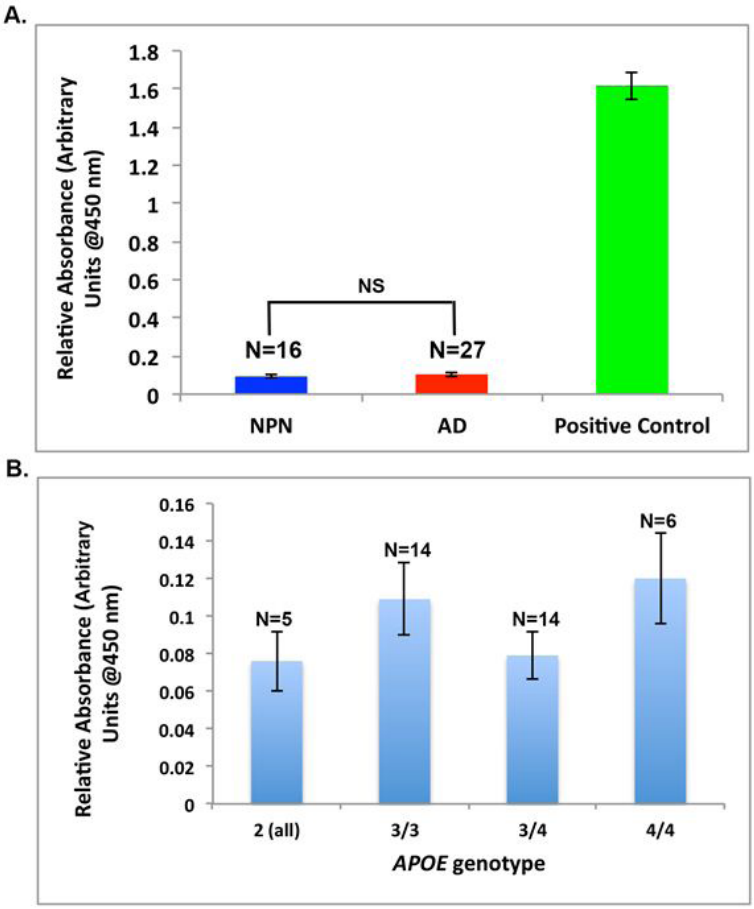
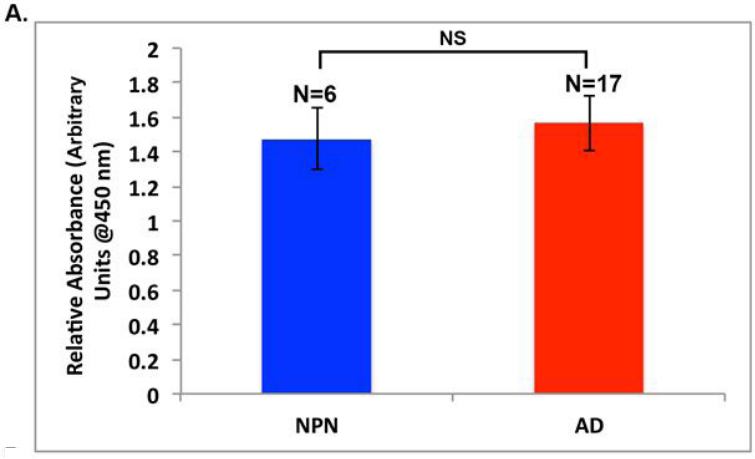


Figure 1: Examination of CSF for *APOE* fragmentation by ELISA reveals no significant difference between NPNs and AD subjects. **(A):** CSF samples were assayed by ELISA utilizing the antibody, nApoECFp17, which is specific for an amino-terminal fragment of *APOE*. Data are representative of three independent experiments ±S.E.M. N denotes the number of cases tested and NS denotes non-significant statistical difference ($p>0.05$). The use of a positive control consisting of the immunogen used to generate the nApoECFp17 antibody consisting of the 7-mer peptide C-RKRLLRD resulted in much higher relative OD values (green bar). **(B):** Determination of *APOE* fragmentation according to *APOE* genotype following ELISA utilizing the nApoECFp17 antibody. Due to the rarity of the *APOE2* genotype, heterozygous cases consisting of at least 1 *APOE2* allele were combined together and are depicted as 2(all) in the bar graph. No significant differences were found across any of the for genotyping groups ($p>0.05$)

The results from Figure 1 indicated not only little difference in the levels of the p17 fragment between cohorts, but also very little fragment was identified as compared to background controls (typically in the range of .035-.045 Relative OD units). To confirm this finding, we repeated ELISAs using a commercially available antibody to the extreme amino-terminus of *APOE* that detects full-length *APOE* (299 amino acids) as well as any putative amino-terminal fragments. As Figure 2A indicates, robust levels of full-length *APOE* were detected under these conditions, however, like the p17 fragment, no significant differences were found between the NPN and AD cohorts. Comparison of the two antibodies indicated that approximately 94.2% of the *APOE* detected in the AD cohorts was full-length, while only 5.8% was equated to the p17 amino-terminal fragment of *APOE* (Figure 2B).



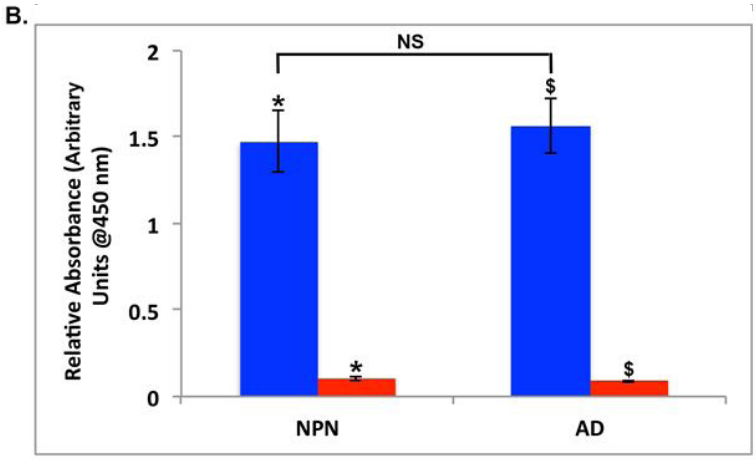


Figure 2: Comparison of full-length APOE to *APOE1-151* in CSF samples from neuropathological NPNs and AD cases by ELISA. **(A):** ELISA analysis of CSF samples was performed in an identical manner as in Figure 1 with the exception that an antibody that recognizes full-length *APOE* was employed. No significant difference between the two groups was noted. **(B):** Combined presentation of data from Figure 1A and Figure 2A indicated a significantly greater level of full-length *APOE* in CSF samples in both NPNs and AD cases (blue bars) as compared to *APOE1-151* (red bars). Data are representative of three independent experiments ±S.E.M. N denotes the number of cases tested and NS denotes non-significant statistical difference (p>0.05). *Denotes significant difference between full-length APOE and *APOE1-151* in the NPN cohort (p<0.001), while $ denotes significant difference (p<0.001) in the AD cohort

We also assessed plasma samples for the p17 fragment of *APOE* and obtained similar results as those reported for CSF (Figure 3). In this regard, no significant difference was observed between the NPN and AD cohorts (Figure 3A), and overall the p17 fragment made up only 8.4% of the total *APOE* detected in plasma AD samples (Figure 3B). There was a trend for lower levels of full-length *APOE* in the AD cohort but the data did not reach statistical significance (Figure 3B).

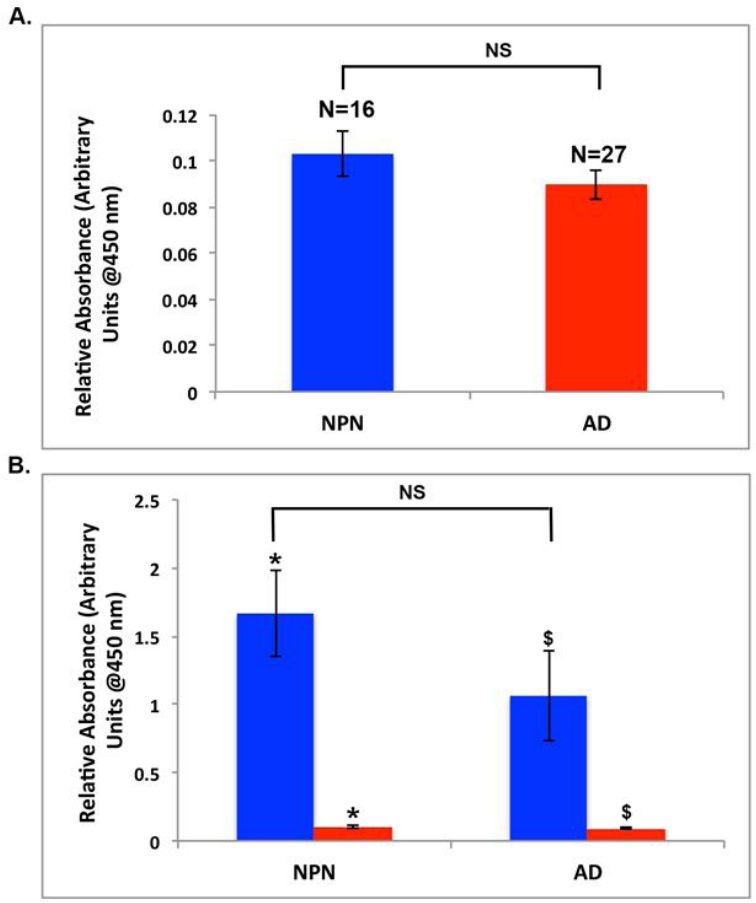


Figure 3: Comparison of full-length *APOE* to *APOE1-151* in plasma samples from neuropathological NPNs and AD cases by ELISA. **(A):** ELISA analysis of plasma samples was performed in an identical manner as in Figure 1 utilizing the nApoECFp17 antibody. No significant difference between the two groups was noted. **(B):** Comparison of full-length APOE and APOE1-151 by ELISA indicated a significantly greater level of full-length *APOE* in plasma samples in both NPN and AD cases (blue bars) as compared to *APOE1-151* (red bars). Data are representative of three independent experiments ±S.E.M. N denotes the number of cases tested and NS denotes non-significant statistical difference (p>0.05). *Denotes significant difference between full-length *APOE* and *APOE1-151* in the NPN cohort (p<0.05), while $ denotes significant difference (p<0.05) in the AD cohort

Discussion

Human APOE is polymorphic with three major isoforms, *APOE2*, *APOE3* and *APOE4*, which differ by single amino acid substitutions involving cysteine-arginine replacements at positions 112 and 158 [1]. Harboring the *APOE3* allele is believed to neither increase nor decrease one's risk of AD, while having the *E2* form may decrease one's risk. In contrast, inheritance of one copy of the *APOE4* allele increases disease risk fourfold, while two copies raises the risk tenfold [2]. It is noteworthy that 65-80% of all AD patients have at least one *APOE4* allele [12,13].

Numerous studies have supported a role for apoE4 proteolysis as a putative mechanism linking *APOE4* to the dementia risk associated with AD [3-10]. Thus, proteolysis of *APOE4* may promote AD pathogenesis through a loss of function (*i.e.*, inability to shuttle cholesterol in the CNS) or due to a toxic-gain of function whereby the cleaved amino-terminal domain becomes neurotoxic [5]. We recently characterized a novel antibody that specifically detects a 17 kDa fragment (p17) of *APOE2*, *E3* and *E4*. The antibody shows no reactivity to full-length APOE but readily identifies the p17 fragment by Western blot in the AD brain [11]. In addition, by immunohistochemistry it was demonstrated that the fragment preferentially localizes to the nucleus of microglia cells in the AD brain [11].

The purpose of the current study was to examine whether the p17 fragment identified by nApoECFp17 antibody could serve as a useful biomarker in AD. Identifying protein biomarkers of AD could help uncover novel treatment approaches by allowing for early diagnosis and treatment initiation. As an initial approach, we screened CSF samples for the presence of the p17 fragment utilizing the nApoECFp17 antibody and ELISA. Very little of the p17 fragment was identified, and there were no significant differences between controls and AD cohorts or across *APOE* genotypes. A comparison of the amount of full-length *APOE* versus the p17 fragment revealed 89% and 11%, respectively, in AD CSF samples. Similar findings were obtained following analysis of plasma samples: little of the p17 fragment was identified (5% as compared to full-length *APOE*), and there were no significant differences between NPN and AD cohorts. Interestingly, for full-length *APOE* in the plasma, there appeared to be a trend for lower levels in the AD cohort as compared to NPNs, but this did not reach statistical significance ($p=0.27$). These data do suggest that proteolysis of *APOE* within the plasma compartment may be occurring to a greater degree in AD perhaps generating a unique fragment distinct from the p17 fragment.

Conclusion

In conclusion, we attempted to verify the presence of an amino-terminal fragment of *APOE* in fluid samples in the AD brain and to determine whether it could serve as a potential biomarker for this disease. Our results support the conclusion that there are very low levels of the p17 fragment in extracellular fluids of the AD brain including the CSF and plasma suggesting that this proteolytic product is not a viable biomarker for AD. These data support our previous findings that the labeling of nApoECFp17 antibody in the AD brain is exclusively intracellular [11]. Future studies will be necessary to determine if other, unique proteolytic fragments of *APOE4* are present in AD plasma samples and could serve as potential biomarkers for this neurodegenerative disorder.

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