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Increased apolipoprotein D dimer formation in Alzheimer's disease hippocampus is associated with lipid conjugated diene levels

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Abstract

Previous studies indicate that apolipoprotein D (apoD) may have a lipid antioxidant function in the brain. We have shown that apoD can reduce free radical-generating lipid hydroperoxides to inert lipid hydroxides in a reaction that involves conversion of surface exposed apoD methione-93 (Met93) residue to Met93-sulfoxide (Met93-SO). One consequence of this reaction is the formation of a stable dimerized form of apoD. As cerebral lipid peroxidation is associated with Alzheimer's disease (AD), in the present study we aimed to assess the possible presence of apoD dimers in postmortem hippocampal and cerebellar tissues derived from a cohort of pathologically defined cases ranging from control to late stage AD. Both soluble and insoluble (requiring guanidine HCl extraction) fractions of tissue homogenates were analyzed for apoD and its dimerized form. We also assessed amyloid-β levels by ELISA and levels of lipid peroxidation by lipid conjugated diene and F2-isoprostane analysis. Our studies reveal a significant association between soluble apoD levels and AD Braak stage whereas apoD dimer formation appears to increase predominantly in the advanced stages of disease. The formation of apoD dimers is closely correlated to lipid conjugated diene levels and occurs in the hippocampus but not in the cerebellum. These results are consistent with the hypothesis that apoD acts as a lipid antioxidant in the brain.

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Increased apolipoprotein-D dimer formation in Alzheimer's disease hippocampus is associated with lipid conjugated diene levels

Abbreviated title: ApoD dimerization in Alzheimer's disease

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Key words: apolipoprotein-D, protein-dimerization, lipid-peroxidation, oxidative-stress

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ABSTRACT

Previous studies indicate that apolipoprotein-D (apoD) may have a lipid antioxidant function in the brain. We have shown that apoD can reduce free radical-generating lipid hydroperoxides to inert lipid hydroxides in a reaction that involves conversion of surface exposed apoD methione-93 (Met93) residue to Met93-sulfoxide (Met93-SO). One consequence of this reaction is the formation of a stable dimerized form of apoD. As cerebral lipid peroxidation is associated with Alzheimer's disease (AD), in the present study we aimed to assess the possible presence of apoD dimers in postmortem hippocampal and cerebellar tissues derived from a cohort of pathologically defined cases ranging from control to late stage AD. Both soluble and insoluble (requiring guanidine HCl extraction) fractions of tissue homogenates were analyzed for apoD and its dimerized form. We also assessed amyloid-beta levels by ELISA and levels of lipid peroxidation by lipid conjugated diene and F2-isoprostane analysis. Our studies reveal a significant association between soluble apoD levels and AD Braak stage whereas apoD dimer formation appears to increase predominantly in the advanced stages of disease. The formation of apoD dimers is closely correlated to lipid conjugated diene levels and occurs in the hippocampus but not in the cerebellum. These results are consistent with the hypothesis that apoD acts as a lipid antioxidant in the brain.

Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia as it accounts for 60-80% of all dementia cases. According to the recent World Alzheimer's Report, the population of patients suffering from AD is expected to reach 115.4 million by 2050. The disease is characterized by the presence of senile plaques, neurofibrillary tangles and loss of neurons that together result in cognitive impairment and memory loss [1-3]. The multifactorial nature of AD presents significant challenges, both in terms of understanding pathological mechanisms and for the development of therapeutics [4]. The oxidative stress hypothesis of AD identifies lipid and protein oxidation as potential initiators and propagators of the pathways contributing to AD [5-7]. Interestingly, oxidative stress is one of the early events in AD pathology and may even precede amyloid plaque formation [8, 9].

Oxidative stress involves oxidation of proteins, lipids, nucleic acids and sugars. Evidence from an increasing number of studies indicates biochemical markers of oxidative stress are significantly increased in the AD brain [10-12]. The brain is suggested to be highly susceptible to lipid peroxidation due to high levels of polyunsaturated fatty acids, high metabolic oxidative rate, high level of transition metal ions and relatively low levels of antioxidants [13]. It has been reported that the levels of lipid peroxidation increase before amyloid plaque formation in AD [9, 14, 15]. Products of lipid peroxidation that are found to be elevated in several brain regions of AD patients compared to control subjects include lipid conjugated dienes, 4-hydroxynonenal (4-HNE), 4-hydroxyhexenal (4-HHE), acrolein, thiobarbituric acid reactive-substances (TBARS), and F_2-isoprostanes [9, 16-20].

Apolipoprotein D (ApoD) is a multifunctional, phylogenetically conserved glycoprotein with widely distributed expression in mammalian tissues [21-23]. Although it is mainly synthesized by glial cells in the brain, apoD may also be expressed in neurons in association with aging and in several pathological conditions [24, 25]. ApoD is upregulated in the brain under oxidative stress conditions including in aging, AD, Parkinson's disease and Schizophrenia [26-31]. Several lines of evidence including the increase in apoD expression with AD progression [26, 29, 32], colocalisation of apoD with diffuse amyloid plaques in AD brain [33], and induction of apoD synthesis in hippocampal cells treated with Aβ 25-35 [34], raise the possibility that apoD may be upregulated as a neuroprotective response in AD.

ApoD has been highlighted as a brain lipid antioxidant in previous studies. ApoD null (apoD$^{-/-}$) mice show increased sensitivity to oxidative stress whereas transgenic mice over-expressing human apoD (h-apoDtg) show a reduction in brain lipid peroxidation levels when treated with paraquat as an inducer of oxidative stress [35]. Similarly, deletion of glial lazarillo, a homolog of apoD, reduced oxidative stress resistance in Drosophila [36], whereas over-expression of apoD was associated with reduced accumulation of aldehydic end-products of lipid peroxidation in an aged Drosophila model [37]. In addition, apoD provides protection against kainic acid lesion in rat hippocampal neurons by inhibiting lipid peroxidation as assessed by reduced levels of F_2-isoprostanes, 7-ketocholesterol and 4-HNE [38]. It is therefore plausible that apoD may act as a neuroprotectant in AD by reducing brain lipid peroxidation.

In our previous studies we demonstrated that apoD acts as a lipid antioxidant by reducing lipid hydroperoxides to lipid hydroxides in a 2-electron reduction that is dependent on a single surface-exposed Met residue at position 93 [23]. As a consequence of this reaction,

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apoD Met$_{93}$ is converted to Met sulfoxide (MetSO). Our in vitro and in silico studies revealed that this modification of apoD Met$_{93}$ to MetSO was found to destablilize the protein structure and promote self association that resulted in the formation of a stable (but non-covalent) homodimer [23, 39]. Interestingly, such apoD dimers were also observed in the insoluble fractions of postmortem hippocampal tissue homogenates derived from a small set (n=3) of late stage AD cases [23].

The objective of the current study was to assess a larger cohort of age-matched control and AD subjects (classified according to Braak staging) to determine the stage of disease at which apoD dimers may be formed and to examine the relationship between apoD dimer formation and measures of brain lipid peroxidation.

Material and Methods

Human brain tissue

Human brain tissues were received from the New South Wales Tissue Resource Centre and the Sydney Brain Bank. Patient data and brains were collected for research purposes as approved by institutional Human Ethics Committees. The use of human brain tissue for this project was approved by the University of Wollongong Human Research Ethics Committee (HE10/327).). Cases were screened using standardized protocols to confirm the presence of AD and exclude coexisting cerebrovascular and degenerative pathologies. Tissue pH was determined on a sample of cerebellum from each case. Braak staging of AD type pathology was performed on sections of the hippocampal formation and temporal cortex according to published criteria [40]. We confirm that all procedures involving experiments on human

subjects are done in accord with the Helsinki Declaration of 1975. The *APOE* genotype of all samples was determined by PCR as described previously [41]. Briefly, genomic *APOE* was amplified using the forward: TCCAAGGAGCTGCAGGCGGCGCA and reverse: ACAGAATTCGCCCCGGCCTGGTACACTGCCA primers and double digested with HaeII and AflIII. The presence of an ε4 allele was indicated by resistance to AflIII digestion, whilst the presence of an ε2 allele was indicated by resistance to HaeII digestion.

Protein analysis - sample preparation

Brain tissue homogenates were prepared by homogenizing ~60 mg tissue in 6 volumes of ice cold Tris-buffered saline (TBS) extraction buffer containing protease and phosphatase inhibitors using a Precellys 24 (Bertin Technologies) automatic homogenizer with 1.4 mm ceramic beads and a setting of 6000 rpm for 2 x 30 sec. After centrifugation at 20,000 *g* for 15 min at 4°C, the TBS soluble fraction was collected as the supernatant. The pellet was re-homogenized in 4 volumes of 6M guanidine HCl (gHCl) in the Precellys homogenizer as above. The homogenized mixture was further incubated on a rotator at 22°C for 16 h. It was then centrifuged at 20,000 *g* for 15 min at 4°C and the supernatant was collected as the gHCl fraction of the samples.

Western blotting

Sample protein concentrations were determined using BCA assay and equal amounts of protein were separated on 12% SDS PAGE gels at 140 V for 80 min, followed by transfer at 100 V for 30 min onto 0.45 μm nitrocellulose membranes. The membranes were blocked in 5% skim milk in phosphate-buffered saline (PBS) for 1 h at 22°C and then probed with anti apoD mouse monoclonal antibody (Sapphire Bioscience 36C6 clone, 1/2000 dilution) at 4°C for 16 h followed by incubation with horseradish-conjugated rabbit anti mouse (Dako 1/2000

dilution) polyclonal antibody for 1 h at 22°C. Membranes were washed and protein was detected using enhanced chemiluminescence (ECL, Amersham Biosciences) and X-ray film. Membranes were stripped and re-probed for β-actin to confirm equal protein loading. The signal intensity was quantified using NIH ImageJ software. All samples were analysed by western blotting with mean values of the resulting data presented in the histograms and representative samples selected for display in the western blot panels.

ELISA

The concentration of Aβ40 and Aβ42 in brain homogenates was determined using Beta Mark x-40 and 42 ELISA kits (following the manufacturer's instructions). The samples derived from gHCl soluble fractions were diluted 1:10 and 1:2000 for Aβ40 and for Aβ42 quantification, respectively. For quantification of Aβ40 and Aβ42 in TBS soluble fractions, dilutions of 1:10 and 1:5, respectively, were used.

Lipid analysis - sample preparation

Samples for lipid analysis were prepared by homogenizing 20 mg of frozen tissue in 0.6 ml methanol, 0.01% (v/v) butyalted hydroxytoluene (BHT) with internal standards for GC-MS analysis. Homogenized fractions were divided into aliquots that were processed separately for analysis of conjugated dienes by HPLC or for F_2-isoprostanes by GC-MS. To the aliquots (0.4 ml) to be used for conjugated diene analysis, (methyl-*tert*-butyl ether) MTBE was added so that the methanol to MTBE ratio was 3:10 (v/v). Sodium hydroxide (final concentration 0.7 M) was added for hydrolysis of ester bonds and the samples were vortex mixed and incubated at 4°C for 16 h in a rotator. This was followed by the addition and mixing of 0.15 M ammonium acetate (0.19 ml / ml solvent). Samples were centrifuged at 2000 *g* for 10 min at 4°C and the upper phase removed. The lower phase was re-extracted with MTBE:

methanol: 0.15 M ammonium acetate (20: 6: 5, v/v/v). Upper phases were pooled, dried under nitrogen gas and re-suspended in 200 µl methanol. Sample preparation for F_2-isoprostane analysis (200 µl) was achieved by addition of 100 µl 1M NaOH with 16 h incubation at 22°C on a sample mixer to hydrolyze ester bonds. This was followed by addition of 328 µl 1M formic acid and 2.2 ml H_2O. The samples were then allowed to flow under gravity through solid phase extraction (SPE) columns, preconditioned with 2 ml methanol and followed by 2 ml 40 mM formic acid (pH 4.5). The samples were washed with 2 ml 40 mM formic acid (pH 4.5) containing 40% (v/v) methanol, eluted from the SPE columns with MTBE containing 20% (v/v) methanol and 1% (v/v) formic acid and dried under nitrogen gas for 1-2 min.

Analysis of lipid oxidation markers by HPLC and GC-MS

Conjugated dienes and free fatty acids (40 µl aliquot) were analyzed using a 5µm, 25 x 0.46 cm, C18 reversed phase column at a flow rate of 1 ml / min at 22°C. The mobile phase used was 0.1% acetic acid in H_2O-acetonitrile-tetrahydrofuran (45:45:10 v/v/v) with UV234 nm absorbance detection. Lipid conjugated dienes are formed as a consequence of free radical-mediated hydrogen abstraction from lipid methylene groups and subsequent molecular rearrangement to stabilize the resulting carbon-centered radical [42]. Lipid conjugated dienes are a relatively stable marker of lipid peroxidation that have been shown to be increased in postmortem human Alzheimer's disease brain tissues compared to controls [43] and are also induced in animal models of neurodegeneration and injury [44].

The solvent extracted isoprostane fraction (see "Lipid sample preparation" above) was dried down under nitrogen at 37°C and derivatized with 30 µl of pentafluorobenzylbromide (PFBBr, 10% in acetonitrile) and 15 µl of N,N-diisopropylethylamine (DIPEA, 10% in

acetonitrile) at 37℃ for 30 min. Excess reagents were evaporated under N_2. The F_2-isoprostane PFBenzyl esters were then derivatized with 15 µl acetonitrile + 30 µl BSTFA + 1% TMCS for 1 h at 37℃. After drying under N_2 and reconstitution in 30 uL toluene, derivatized samples were analyzed by an Agilent 7000B triple quadrupole mass selective detector interfaced with an Agilent 7890A GC system gas chromatograph, equipped with an automatic sampler and a computer workstation. The injection port and GC-MS interface were kept at 270℃. Separations were carried out on a fused silica capillary column (20 m x 0.18 mm i.d. x 0.18 µm film thickness, Restek Rxi-5ms). Helium was the carrier gas with a flow rate of 0.8 ml/min (average velocity = 59 cm/sec). Selected-reaction monitoring (SRM) was performed using the NCI mode (70eV) with Ar as the reagent gas (1.25 ml/min) and the collision gas (0.6 ml/min). The ion source was maintained at 150°C and the quadrupoles at 150℃. For F_2-isoprostane analysis derivatized samples (1 µl) were injected splitless into the GC injection port. Column temperature was increased from 180°C to 280℃ at 40℃ / min after 1 min at 180℃, then temperature was raised to 290℃ at 2℃ / min, then at 40℃ / min to 305℃ with a final hold for 4 min. Quantification of F_2-isoprostanes was calculated by comparison of specific SRM transitions with their corresponding heavy isotope internal standards.

Statistics

Group comparisons were made using ANOVA with Fisher's least significant difference (LSD) post-hoc analysis. Where specific comparisons were made between two groups a t-test was applied. Pearson correlations were used to assess relationships between biochemical parameters and linear regression analysis was used to assess the strongest correlates of apoD dimer formation. Variance inflation factor (VIF) values were assessed in order to quantify multicolinearity. VIF values greater > 5 were considered as an indicator of high

multicolinearity [45]. Statistical testing was performed using SPSS Statistics (version 19) software, IBM Company).

Results

Characterisation of AD brain samples

Basic clinical and demographic data for the samples used in this study are provided in Table 1. The samples were stratified into four groups of disease severity using clinically and pathologically defined National Institute on Aging-Reagan criteria as described previously [46, 47]. Thirty four samples were obtained in total including nine cases of normal control brain. The "severity of disease" groupings according to Braak pathology are provided in Table 1. There were no significant group effects for age, postmortem interval (PMI) or sample pH as assessed by ANOVA. Post-hoc analysis using Fisher's LSD test revealed a lower average PMI in Group V/VI samples compared to both Group I/II and Group III/IV (both $p < 0.05$). There were no significant correlations between PMI and any of the demographic or biochemical parameters measured (data not shown) in any of the groups and it is therefore unlikely that PMI is a confounding factor in this study.

Braak staging of disease progression is based on the distribution of neurofibrillary tangles. The formation of neurofibrillary tangles is a tau-mediated process and is not unique to AD; hence tissue concentrations of Aβ were measured as an additional biochemical marker for late stage AD status. An ELISA method was used to measure Aβ40 and Aβ42 in TBS-soluble fractions of the tissue homogenates and in the TBS-insoluble fractions (that required treatment with 6M gHCl in order to solubilize).

The levels of insoluble and Aβ42 and Aβ40 in the hippocampus were significantly different across the stages of disease as assessed by ANOVA (both p = 0.001) as shown in Figure 1. Post-hoc analysis by Fisher's LSD test indicated that hippocampal insoluble Aβ42 and Aβ40 levels were significantly higher (p = 0.001 and p = 0.0004, respectively) in the late stage AD (Group V/VI) samples compared to the controls (Group 0). Levels of hippocampal soluble Aβ42 were also significantly different across the stages of disease as assessed by ANOVA (p = 0.007), where post-hoc analysis by Fisher's LSD revealed a significant increase (p = 0.001) in hippocampal soluble Aβ42 levels in the late stage AD (Group V/VI) samples compared to the controls (Group 0). There were no significant differences detected for either Aβ42 or Aβ40 in the cerebellum (Fig. 1). Levels of hippocampal soluble Aβ40 did not change significantly when the different groups were analyzed (Fig. 1).

The lack or correlation between the Braak staging and Aβ levels across AD stages is most likely due to differences in topographic distributions of neurofibrillary tangles and plaque pathology in AD. Neurofibrillary tangles (which form the basis of the Braak staging used in the current study) begin to appear in the allocortex of the medial temporal lobes (including the hippocampus) and only in the late stages of disease do they appear in the isocortical areas [48]. In contrast, Aβ accumulates mainly in the isocortex whereas the allocortex (including the hippocampus) is generally involved to a lesser extent until the final stages of disease [48, 49]. The changes we observed in Aβ40 levels are very minor when compared to Aβ42; in agreement with previous studies indicating Aβ42 as the predominant pathological Aβ species [1].

Overall, these data confirm the diagnosis of the late stage AD cases and also confirm that deposition of insoluble Aβ is more pronounced in the hippocampus compared to the cerebellum. These two regions can therefore be used to assess relative changes in affected versus non-affected brain regions, respectively.

Hippocampal apoD expression is associated with AD Braak stage

Previous studies have shown that apoD levels are increased in human AD brain tissues compared to controls [23, 26, 32, 33]. One study has also shown that the expression of apoD in the hippocampus increases with disease severity as assessed by Braak staging [29]. In the current study we first assessed apoD levels in different stages of AD in both the hippocampus and cerebellum. In agreement with previous observations, hippocampal TBS-soluble apoD levels were significantly associated (ANOVA $p = 0.038$) with AD Braak stage (Fig 2A). Post-hoc analysis by Fisher's LSD revealed a significant increase ($p = 0.006$) in hippocampal apoD levels in the late stage AD (Group V/VI) samples compared to the controls (Group 0). There were no significant differences detected for apoD in the cerebellum (Fig. 2B).

One of the earlier studies showing that apoD is increased in the AD hippocampus reported that the increase was dependent on *APOE* genotype, whereby AD cases who carried at least one ε4 allele did not show significant variations in hippocampal apoD expression [29]. Intriguingly, another study published in the same year showed that the increase in apoD levels detected in the prefrontal cortex from AD cases was not related to *APOE* genotype [32]. In our current study, the numbers of subjects expressing each of the *APOE* genotypes was not sufficiently powered to examine the impact of genotype on apoD expression across all groups; however, a t-test analysis comparing the four *APOE* ε3/4 heterozygotes in the control group (Group 0) samples to the five *APOE* ε3/4 heterozygotes in the late stage AD

(Group V/VI) samples indicated a significant increase in relative hippocampal apoD levels (Group 0, 1.00 ± 0.16 versus Group V/VI, 1.90 ± 0.12; values are means $\pm$ SE; $p = 0.003$). This suggests that increased hippocampal apoD expression in AD is not influenced by the presence of a single ε4 allele.

Hippocampal apoD dimerization increases in late stage AD

In our previous study we revealed that apoD exists as a dimer in the gHCl soluble fraction of a relative small number ($n = 3$) of late stage AD hippocampal samples compared to controls [23]. We therefore next examined the formation of apoD dimers in association with AD stages. Western blot analysis revealed that apoD dimers were detected at increased levels (ANOVA $p = 0.0164$) in the AD cases (Fig. 3A). Although the ~50kDa apoD dimer band was occasionally detected in less severe AD cases, post-hoc analysis revealed a significant increase only in the Group V/VI samples ($p = 0.009$, $p = 0.012$, $p = 0.012$ compared to Group 0, Group I/II and Group III/IV, respectively). We could detect only very low levels of apoD in the gHCl soluble fractions of the cerebellum samples and apoD dimers were also not reliably detected in the cerebellum (Bhatia S. and Garner B., unpublished observations).

It is interesting to note that overall hippocampal apoD expression appears to increase in proportion to disease severity (Fig 2A), whereas the levels of apoD dimer appear to significantly increase only in the later stages of AD (Fig. 3A). It is also noteworthy that neither apoD nor apoD dimer levels appear to increase in the cerebellum (a region that is relatively unaffected in AD). This suggests that the altered apoD expression may be related to AD pathological processes.

Lipid peroxidation markers increase in AD hippocampus

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To investigate possible associations between apoD and lipid peroxidation in AD, we measured levels of lipid-associated conjugated dienes (by absorbance at UV234 nm) and levels of F_2-isoprostanes (by GC-MS). Both are established methods for assessing lipid peroxidation in brain tissues [42]. We detected a significant increase (ANOVA p = 0.002) in conjugated dienes in late stage AD (Fig. 4A). Post-hoc analysis revealed a significant increase only in the Group V/VI samples (p = 0.001, p = 0.001, p = 0.006 compared to Group 0, Group I/II and Group III/IV, respectively). There were no changes in conjugated dienes in relation to AD status in any of the cerebellum samples (Fig 4B). Levels of F_2-isoprostanes did not differ significantly in relation to AD status in either the hippocampus or cerebellum as assessed by ANOVA (Fig. 5). Post-hoc analysis revealed a significant increase in hippocampal F_2-isoprostane levels only in the Group III/IV samples (p = 0.025) compared to Group 0 (Fig. 5).

Previous studies have suggested that markers of brain lipid peroxidation may increase in specific brain regions with extended PMI [50, 51]. In the present study cohort the median PMI was 17.5 h (20.5 $\pm$ 2.8 h, mean $\pm$ SE, n = 34). Importantly, PMI was not significantly associated with increased conjugated diene or F_2-isoprostane levels (Pearson correlation coefficients r^2 = -0.178 and 0.004, respectively). It is therefore unlikely that PMI was a confounding factor in our assessment of lipid peroxidation as a function of AD stage; however, we cannot rule out a possible global contribution of PMI to overall levels of conjugated dienes or F_2-isoprostanes across the study cohort.

Associations between hippocampal apoD dimer formation and markers of lipid peroxidation
The data presented above indicate that apoD dimer formation is significantly higher in late stage AD, although clearly there are cases in other groups where the dimer was detected (Fig.

3). In order to understand which of the factors we have assessed correlates with apoD dimer levels, Pearson correlations were examined. The data presented in Table 2 indicates that lipid conjugated diene concentration (UV234 absorbance) was the only variable signficiantly correlated (p = 0.006) with apoD dimer levels. Interestingly, soluble apoD and soluble $A\beta 42$ were also correlated (p = 0.025 and p = 0.004, respectively) with conjugated diene concentration (Table 2). Levels of both insoluble and soluble $A\beta 42$ and $A\beta 40$ were all significantly intercorrelated (Table 2), whereas there were no significant correlates of F_2-isoprostanes in this analysis (Table 2). To gain additional insights into the strengths of these correlations, we performed linear regression analysis.

Using apoD dimer as the dependent variable, the other seven variables measured (apoD monomer, insoluble $A\beta 42$, soluble $A\beta 42$, insoluble $A\beta 40$, soluble $A\beta 40$, conjugated dienes and F_2-isoprostanes) were analyzed in the linear regression model. The results indicated that conjugated dienes were a strong predictor of apoD dimer levels (t = 3.481, p = 0.002). Both insoluble and soluble $A\beta 42$ were also significant in this model (t = 2.422, p = 0.024 and t = -2.785, p = 0.011, respectively). We measured variance inflation factor values (VIF), that quantify the severity of multicolinearity for these variables, and found them to be 5.861 and 7.034, respectively. VIF values greater > 5.0 are considered as an indicator of high multicolinearity [45]. We therefore removed the soluble $A\beta 42$ and soluble $A\beta 40$ variables from the regression analysis to resolve the potential confounding affects of multicolinearity [45]. The revised model revealed that conjugated dienes were the only significant predictor of apoD dimer levels (t = 2.151, p = 0.041). All VIF values were < 2.6 in this analysis. Furthermore, when conjugated diene concentration was assigned as the dependent variable in this model, only apoD dimer levels were significantly correlated (t = 2.237, p = 0.034).

Overall this analysis suggests that conjugated diene levels are the strongest predictor of apoD dimer levels in the AD hippocampus. In addition, the association between apoD dimer levels and Aβ deposition appears to be secondary to the conjugated diene marker of lipid peroxidation. The F_2-isoprostane marker of lipid peroxidation does not appear to correlate with either Aβ accumulation or apoD dimerization in this cohort of post-mortem samples.

Discussion

The data presented in this study show that soluble apoD levels increase as the severity of AD pathology increases. This change in apoD expression occurs in the hippocampus but not in the cerebellum; the latter representing an area of the brain that is relatively spared in AD. Interestingly, the level of the dimerized form of apoD, that we have previously shown is promoted as a consequence of the interaction between apoD Met_{93} with L-OOHs [23, 39], is predominantly increased in the latest stages of AD pathology. Moreover, this late increase in apoD dimerization appears to be more closely related to lipid conjugated diene levels (a lipid peroxidation marker) than it is to insoluble Aβ levels. While such associations cannot be used to define a precise mechanism for the increased apoD dimerization in AD, one plausible explanation is that during the course of AD progression lipid peroxidation begins to increase. This may be related to the radical formation induced by Aβ42 [12, 52] as well as several other pathways [53, 54]. We speculate that along with other small molecule (e.g. ascorbate, glutathione) and enzymatic (e.g. superoxide dismutase, glutathione peroxidase) antioxidant defenses, apoD upregulation represents a homeostatic response that helps to combat lipid peroxidation.

The hydrophobic patch located close to the entrance of the apoD ligand binding pocket contains an exposed Met_{93} side chain that can directly reduce radical-propagating L-OOHs [23, 39]. So long as this crucial Met residue can be maintained in a native state via the action of methionine sulfoxide reductase (MSR), the lipid antioxidant function of apoD can be theoretically maintained. However, if the levels of lipid peroxidation in the hippocampus surpass the antioxidant capacity of apoD and other antioxidant defenses, we predict that the steady state concentration of apoD containing Met_{93} sulfoxide ($Met_{93}SO$) will be increased such that its propensity to self-associate is increased and the presence of apoD dimers in the insoluble fraction of hippocamapal homogenates will also increase. In this respect, apoD dimerization may be considered as a *de facto* marker of the extent of hippocampal lipid peroxidation.

The factors that may trigger the increase in lipid peroxidation (assessed as lipid conjugated dienes) and apoD dimerization in late stages of AD are not clear. This could be related to higher levels of both soluble and insoluble Aβ42 we have detected in the Group V/VI samples (which could promote radical production [52]), or it may be related to an impaired capacity for redox cycling of apoD $Met_{93}SO$ back to Met_{93}. In line with this latter suggestion, MSR levels are decreased by ~50% in the AD hippocampus but unchanged in the cerebellum [55].

Another intriguing finding in our current study relates to the apparent discrepancy when the lipid conjugated diene levels and F_2-isoprostane levels were compared. Unexpectedly, these markers of lipid peroxidation were not significantly correlated either in the hippocampus (Table 2) or across the whole data set including the cerebellum and hippocampus (data not shown). The reasons for this lack of correlation are not clear. It should be noted that F_2-

isoprostanes are derived from arachidonic acid whereas lipid conjugated dienes are derived from all unsaturated acyl chains that have been subjected to radical attack; i.e. the methods are measuring different products as indices of lipid peroxidation. It is clear, however, that F_2-isprostane levels are increased in the Braak stage III/IV cases in the hippocampus while there were no changes in the cerebellum of the same cases (Fig 5A and Fig 5B, respectively). This is in general agreement with previous findings that suggest cerebrospinal fluid F_2-isoprostane levels may reflect cerebral lipid peroxidation and thus be a useful biomarker for subjects who may proceed to AD [54, 56].

In summary, our studies reveal that soluble apoD levels are associated with AD Braak stage, whereas apoD dimer formation appears to increase predominantly in the advanced stages of disease. The formation of apoD dimers is closely correlated to lipid conjugated diene levels and occurs in the hippocampus but not in the cerebellum. These results are consistent with the hypothesis that apoD acts as a lipid antioxidant in the brain until lipid peroxidation overwhelms antioxidant capacity, at which point apoD may aggregate and accumulate in a dimeric form in amyloid plaques or other insoluble deposits.

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References

[1]   Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* **81**, 741-766.

[2]   Haass C, Selkoe DJ (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol* **8**, 101-112.

[3]   Masliah E (2008) Neuropathology: Alzheimer's in real time. *Nature* **451**, 638-639.

[4]   Iqbal K, Grundke-Iqbal I (2011) Opportunities and challenges in developing Alzheimer disease therapeutics. *Acta Neuropathol* **122**, 543-549.

[5]   Butterfield DA, Lauderback CM (2002) Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. *Free Radic Biol Med* **32**, 1050-1060.

[6]   Pratico D (2008) Evidence of oxidative stress in Alzheimer's disease brain and antioxidant therapy: lights and shadows. *Ann N Y Acad Sci* **1147**, 70-78.

[7]   Butterfield DA, Galvan V, Lange MB, Tang H, Sowell RA, Spilman P, Fombonne J, Gorostiza O, Zhang J, Sultana R, Bredesen DE (2010) In vivo oxidative stress in brain of Alzheimer disease transgenic mice: Requirement for methionine 35 in amyloid beta-peptide of APP. *Free Radic Biol Med* **48**, 136-144.

[8]   Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA (2001) Oxidative damage is the earliest event in Alzheimer disease. *J Neuropathol Exp Neurol* **60**, 759-767.

[9]   Pratico D, Uryu K, Leight S, Trojanoswki JQ, Lee VM (2001) Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis. *J Neurosci* **21**, 4183-4187.

[10]   Markesbery WR (1997) Oxidative stress hypothesis in Alzheimer's disease. *Free Radic Biol Med* **23**, 134-147.

[11]   Christen Y (2000) Oxidative stress and Alzheimer disease. *Am J Clin Nutr* **71**, 621S-629S.

[12]   Butterfield DA, Castegna A, Lauderback CM, Drake J (2002) Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiol Aging* **23**, 655-664.

[13]   Floyd RA, Hensley K (2002) Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases. *Neurobiol Aging* **23**, 795-807.

[14]   Drake J, Link CD, Butterfield DA (2003) Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid beta-peptide (1-42) in a transgenic Caenorhabditis elegans model. *Neurobiol Aging* **24**, 415-420.

[15]   Markesbery WR, Kryscio RJ, Lovell MA, Morrow JD (2005) Lipid peroxidation is an early event in the brain in amnestic mild cognitive impairment. *Ann Neurol* **58**, 730-735.

[16]   Bradley MA, Xiong-Fister S, Markesbery WR, Lovell MA (2012) Elevated 4-hydroxyhexenal in Alzheimer's disease (AD) progression. *Neurobiol Aging* **33**, 1034-1044.

[17]   Lovell MA, Xie C, Markesbery WR (2001) Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures. *Neurobiol Aging* **22**, 187-194.

[18]   Markesbery WR, Carney JM (1999) Oxidative alterations in Alzheimer's disease. *Brain Pathol* **9**, 133-146.

[19]   Pratico D, V MYL, Trojanowski JQ, Rokach J, Fitzgerald GA (1998) Increased F2-isoprostanes in Alzheimer's disease: evidence for enhanced lipid peroxidation in vivo. *FASEB J* **12**, 1777-1783.

[20]   Karelson E, Bogdanovic N, Garlind A, Winblad B, Zilmer K, Kullisaar T, Vihalemm T, Kairane C, Zilmer M (2001) The cerebrocortical areas in normal brain aging and in Alzheimer's disease: noticeable differences in the lipid peroxidation level and in antioxidant defense. *Neurochem Res* **26**, 353-361.

[21]   Milne RW, Rassart E, Marcel YL (1993) Molecular biology of apolipoprotein D. *Curr Opin Lipidol* **4**, 100-106.

[22]   Rassart E, Bedirian A, Do Carmo S, Guinard O, Sirois J, Terrisse L, Milne R (2000) Apolipoprotein D. *Biochim Biophys Acta* **1482**, 185-198.

[23]   Bhatia S, Knoch B, Wong J, Kim WS, Else PL, Oakley AJ, Garner B (2012) Selective reduction of hydroperoxyeicosatetraenoic acids to their hydroxy derivatives by apolipoprotein-D: Implications for lipid antioxidant activity and Alzheimer's disease. *Biochem J* **442**, 713-721.

[24]   Navarro A, Tolivia J, Astudillo A, del Valle E (1998) Pattern of apolipoprotein D immunoreactivity in human brain. *Neurosci Lett* **254**, 17-20.

[25]   Navarro A, del Valle E, Juarez A, Martinez E, Ordonez C, Astudillo A, Tolivia J (2010) Apolipoprotein D synthesis progressively increases in frontal cortex during human lifespan. *Age (Dordr)* **32**, 85-96.

[26]   Terrisse L, Poirier J, Bertrand P, Merched A, Visvikis S, Siest G, Milne R, Rassart E (1998) Increased levels of apolipoprotein D in cerebrospinal fluid and hippocampus of Alzheimer's patients. *J Neurochem* **71**, 1643-1650.

[27]   Belloir B, Kovari E, Surini-Demiri M, Savioz A (2001) Altered apolipoprotein D expression in the brain of patients with Alzheimer disease. *J Neurosci Res* **64**, 61-69.

[28]   Thomas EA, Dean B, Pavey G, Sutcliffe JG (2001) Increased CNS levels of apolipoprotein D in schizophrenic and bipolar subjects: implications for the pathophysiology of psychiatric disorders. *Proc Natl Acad Sci U S A* **98**, 4066-4071.

[29]   Glockner F, Ohm TG (2003) Hippocampal apolipoprotein D level depends on Braak stage and APOE genotype. *Neuroscience* **122**, 103-110.

[30]   Ordonez C, Navarro A, Perez C, Astudillo A, Martinez E, Tolivia J (2006) Apolipoprotein D expression in substantia nigra of Parkinson disease. *Histol Histopathol* **21**, 361-366.

[31]   Kim WS, Wong J, Weickert CS, Webster MJ, Bahn S, Garner B (2009) Apolipoprotein-D expression is increased during development and maturation of the human prefrontal cortex. *J Neurochem* **109**, 1053-1066.

[32]   Thomas EA, Laws SM, Sutcliffe JG, Harper C, Dean B, McClean C, Masters C, Lautenschlager N, Gandy SE, Martins RN (2003) Apolipoprotein D levels are elevated in prefrontal cortex of subjects with Alzheimer's disease: no relation to apolipoprotein E expression or genotype. *Biol Psychiatry* **54**, 136-141.

[33]   Desai PP, Ikonomovic MD, Abrahamson EE, Hamilton RL, Isanski BA, Hope CE, Klunk WE, DeKosky ST, Kamboh MI (2005) Apolipoprotein D is a component of compact but not diffuse amyloid-beta plaques in Alzheimer's disease temporal cortex. *Neurobiol Dis* **20**, 574-582.

[34]   Martinez E, Navarro A, Ordonez C, Del Valle E, Tolivia J (2012) Amyloid-beta25-35 induces apolipoprotein D Synthesis and growth arrest in HT22 hippocampal cells. *J Alzheimers Dis* **30**, 233-244.

[35]   Ganfornina MD, Do Carmo S, Lora JM, Torres-Schumann S, Vogel M, Allhorn M, Gonzalez C, Bastiani MJ, Rassart E, Sanchez D (2008) Apolipoprotein D is involved in the mechanisms regulating protection from oxidative stress. *Aging Cell* **7**, 506-515.

[36]   Sanchez D, Lopez-Arias B, Torroja L, Canal I, Wang X, Bastiani MJ, Ganfornina MD (2006) Loss of glial lazarillo, a homolog of apolipoprotein D, reduces lifespan and stress resistance in Drosophila. *Curr Biol* **16**, 680-686.

[37]   Walker DW, Muffat J, Rundel C, Benzer S (2006) Overexpression of a Drosophila homolog of apolipoprotein D leads to increased stress resistance and extended lifespan. *Curr Biol* **16**, 674-679.

[38]   He X, Jittiwat J, Kim JH, Jenner AM, Farooqui AA, Patel SC, Ong WY (2009) Apolipoprotein D modulates F2-isoprostane and 7-ketocholesterol formation and has a neuroprotective effect on organotypic hippocampal cultures after kainate-induced excitotoxic injury. *Neurosci Lett* **455**, 183-186.

[39]   Oakley AJ, Bhatia S, Ecroyd H, Garner B (2012) Molecular dynamics analysis of apolipoprotein-D-lipid hydroperoxide interactions: mechanism for selective oxidation of Met-93. *PLoS One* **7**, e34057.

[40]   Harding AJ, Kril JJ, Halliday GM (2000) Practical measures to simplify the Braak tangle staging method for routine pathological screening. *Acta Neuropathol* **99**, 199-208.

[41]   Elliott DA, Tsoi K, Holinkova S, Chan SL, Kim WS, Halliday GM, Rye KA, Garner B (2011) Isoform-specific proteolysis of apolipoprotein-E in the brain. *Neurobiol Aging* **32**, 257-271.

[42] Halliwell B, Gutteridge JMC. (1999) Free Radicals in Biology and Medicine. Oxford University Press, Oxford

[43] Chia LS, Thompson JE, Moscarello MA (1984) X-ray diffraction evidence for myelin disorder in brain from humans with Alzheimer's disease. *Biochim Biophys Acta* **775**, 308-312.

[44] Rosenthal RE, Chanderbhan R, Marshall G, Fiskum G (1992) Prevention of post-ischemic brain lipid conjugated diene production and neurological injury by hydroxyethyl starch-conjugated deferoxamine. *Free Radic Biol Med* **12**, 29-33.

[45] Kutner MH, Nachtsheim CJ, Neter J. (2004) Applied Linear Regression Models, 4th edition. McGraw-Hill Irwin, Chicago

[46] NIA (1997) Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease. *Neurobiol Aging* **18**, S1-2.

[47] Gregory GC, Macdonald V, Schofield PR, Kril JJ, Halliday GM (2006) Differences in regional brain atrophy in genetic forms of Alzheimer's disease. *Neurobiol Aging* **27**, 387-393.

[48] Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT (2011) Neuropathological alterations in Alzheimer disease. *Cold Spring Harb Perspect Med* **1**, a006189.

[49] Thal DR, Rub U, Orantes M, Braak H (2002) Phases of A beta-deposition in the human brain and its relevance for the development of AD. *Neurology* **58**, 1791-1800.

[50] Fitzmaurice PS, Tong J, Yazdanpanah M, Liu PP, Kalasinsky KS, Kish SJ (2006) Levels of 4-hydroxynonenal and malondialdehyde are increased in brain of human chronic users of methamphetamine. *J Pharmacol Exp Ther* **319**, 703-709.

[51]   Harish G, Venkateshappa C, Mahadevan A, Pruthi N, Srinivas Bharath MM, Shankar
         SK (2011) Glutathione metabolism is modulated by postmortem interval, gender
         difference and agonal state in postmortem human brains. *Neurochem Int* **59**, 1029-
         1042.

[52]   Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC,
         Cuajungco MP, Gray DN, Lim J, Moir RD, Tanzi RE, Bush AI (1999) The A beta
         peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion
         reduction. *Biochemistry* **38**, 7609-7616.

[53]   Butterfield DA, Bader Lange ML, Sultana R (2010) Involvements of the lipid
         peroxidation product, HNE, in the pathogenesis and progression of Alzheimer's
         disease. *Biochim Biophys Acta* **1801**, 924-929.

[54]   Pratico D (2010) The neurobiology of isoprostanes and Alzheimer's disease. *Biochim
         Biophys Acta* **1801**, 930-933.

[55]   Gabbita SP, Aksenov MY, Lovell MA, Markesbery WR (1999) Decrease in peptide
         methionine sulfoxide reductase in Alzheimer's disease brain. *J Neurochem* **73**, 1660-
         1666.

[56]   Montine TJ, Quinn J, Kaye J, Morrow JD (2007) F(2)-isoprostanes as biomarkers of
         late-onset Alzheimer's disease. *J Mol Neurosci* **33**, 114-119.

Table 1. Human brain sample basic clinical and demographic data

Sample ID No.	Group assignment	Braak stage	Age (y)	APOE genotype	Gender	PMI (h)	Cause of death	Tissue pH
1	0	0	85	3/3	F	23	Pneumonia	6.44
2	0	0	93	2/3	F	21	Cardiac failure	6.96
3	0	0	84	3/4	F	6	Respiratory arrest	6.51
4	0	0	66	3/4	M	23	Cardiac	6.74
5	0	0	68	3/4	M	45	Cardiac	6.12
6	0	0	69	3/3	M	52	Cardiac	6.95
7	0	0	57	3/4	M	18	Cardiac	6.39
8	0	0	64	3/3	M	17	Cardiac	6.55
9	0	0	79	2/3	M	8	Pulmonary embolism	6.65
			74 (4)			**24 (5)**		**6.59 (0.09)**
10	I/II	I	104	2/3	F	27	Respiratory	5.89
11	I/II	I	78	3/4	F	45	Toxicity	6.05
12	I/II	I	63	3/3	M	24	Cardiac	6.94
13	I/II	I-II	62	2/3	M	46	Cardiac	6.95
14	I/II	II	85	3/3	F	10	Respiratory	6.63
15	I/II	II	69	3/3	M	19	Cardiac	6.34
16	I/II	II	91	2/3	M	16	Pneumonia	6.52
17	I/II	II	103	3/3	M	20	Myocardial infarct	6.06
			82(6)			**26 (5)**		**6.42 (0.09)**
18	III/IV	III	81	3/3	F	28	Respiratory	6.20
19	III/IV	III	73	3/3	F	45	Cardiac	6.86
20	III/IV	III	92	3/3	F	14	Cancer	5.60
21	III/IV	III	67	3/4	M	25	Cardiac	6.70
22	III/IV	II-IV	83	2/4	F	64	Stroke	6.31
23	III/IV	IV	98	3/3	F	6	Cardiac/Respiratory	6.70
24	III/IV	IV	92	3/3	F	5	Pancytopaenia	6.08
			84 (4)			**27 (8)**		**6.35 (0.17)**
25	V/VI	V	94	3/3	F	7	Cardiac arrest	6.08
26	V/VI	V	83	3/3	F	3	Uraemia	5.88
27	V/VI	V	100	3/4	F	3	Aspiration pneumonia	6.44
28	V/VI	V	98	3/3	F	11	Cerebrovascular occlusion	6.11
29	V/VI	VI	84	3/4	F	6	Aspiration pneumonia	6.32
30	V/VI	VI	80	3/4	F	32	Cardiorespiratory failure	6.54
31	V/VI	VI	85	3/4	F	10	Cardiorespiratory failure	5.91
32	V/VI	VI	68	3/4	M	23	Cardiorespiratory failure	6.00
33	V/VI	VI	69	3/3	M	3	Colon cancer	6.73
34	V/VI	VI	67	3/3	M	9	Cardiorespiratory failure	6.52
			83 (4)			**11 (3)***		**6.25 (0.09)**

F, female; M, male; PMI, postmortem interval. Mean values and standard error (in parentheses) are listed for each group. * $p < 0.05$ compared with Group I/II and Group II/IV assessed by Fisher's LSD test post-hoc analysis.

Table 2. ApoD dimer correlations

	ApoD	Insol. Aβ42	Insol. Aβ40	Sol. Aβ42	Sol. Aβ40	UV234	F2-Isop.
ApoD dimer	.263	.253	.182	.136	.038	*.482*	-.140
	.146	.162	.318	.458	.834	*.006*	.444
ApoD		.344	.172	.305	.069	*.403*	.259
		.054	.348	.090	.708	*.025*	.152
Insol. Aβ42			*.755*	*.868*	*.440*	.317	.219
			<.0001	*<.0001*	*.009*	.073	.214
Insol. Aβ40				*.743*	*.560*	.313	.242
				<.0001	*<.0001*	.076	.169
Sol. Aβ42					*.623*	*.487*	.129
					<.0001	*.004*	.469
Sol. Aβ40						.268	-.002
						.131	.991
UV234							.099
							.583

Pearson correlations are presented as the upper value in each cell. Significance (2-tailed) is presented as the lower value in each cell.

Figure Legends

Fig 1. *Amyloid-β levels in the hippocampus and cerebellum.* The levels of insoluble (A-B) and soluble (C-D) Aβ42 and Aβ40 were measured in hippocampal (black bar) and cerebellar (grey bar) regions of postmortem brain. Both soluble and insoluble (solubilized with 6M guanidine HCl) protein fractions were analyzed by ELISA. **$p < 0.01$ by Fischer's LSD post hoc analysis between stage 0 and stage V/VI.

Fig 2. *Expression of apoD in soluble protein fractions.* Western blotting was used to measure soluble apoD levels in hippocampal (A) and cerebellar (B) regions of postmortem brain. Representative western blots of each brain region show apoD and β-actin levels in the different stages of the disease. ==Hippocampal apoD expression is significantly associated with Braak stage (ANOVA $p < 0.05$)==. ** $p < 0.01$ by Fischer's LSD post hoc analysis between stage 0 and stage V/VI.
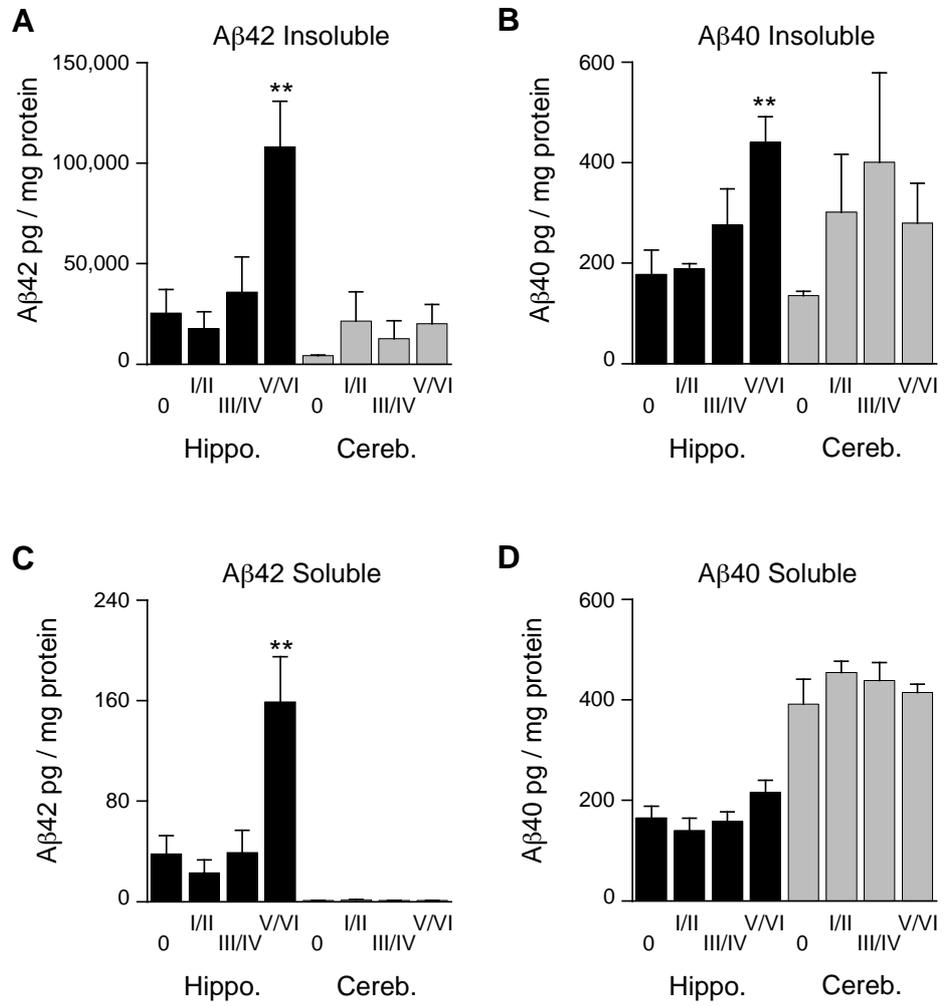
Fig 3. *Expression of apoD in insoluble (guanidine HCl-extracted) protein fractions.* Western blotting was used to measure insoluble (gHCl-solublized) apoD levels in the hippocampal region of postmortem brain tissues. Representative western blots show apoD and β-actin levels in the different stages of the disease. ** $p < 0.01$ by Fischer's LSD post hoc analysis between stage 0 and stage V/VI. Arrows indicate position of apoD dimer.

Fig 4. *Tissue lipid peroxidation levels assessed as conjugated dienes.* Hippocampal (A) and cerebellar (B) tissue samples of postmortem brain were solvent extracted and the fatty acids anlaysed for total conjugated diene content by HPLC with spectrophotometric absorption monitoring at UV 234 nm. The values represent HPLC peak area units at 234 nm / mg tissue.
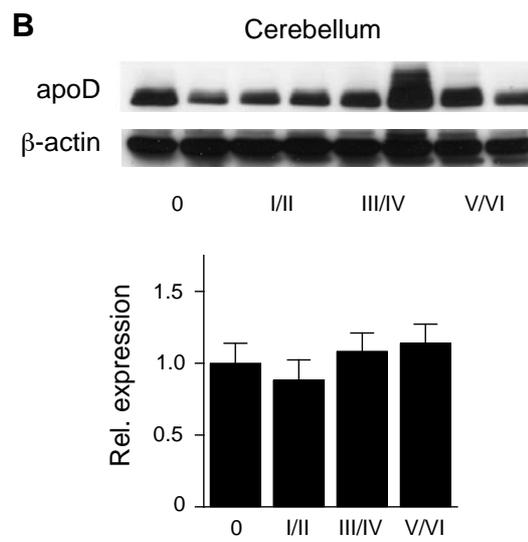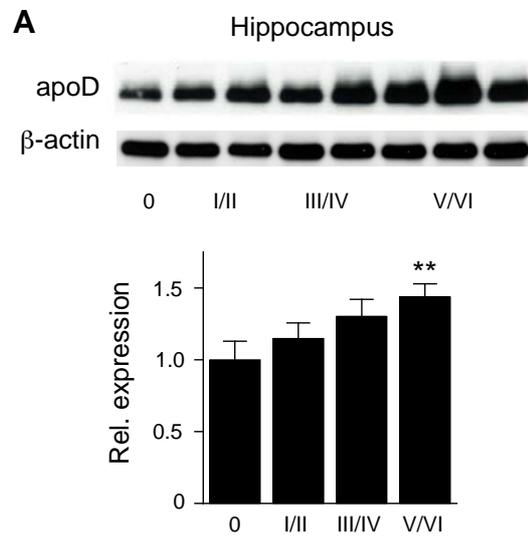
Error bars represent ± SE. ** $p < 0.01$ by Fischer's LSD post hoc analysis between Group 0 and Group V/VI.

Fig 5. *Tissue lipid peroxidation levels assessed as F_2-isoprostanes*. Hippocampal (A) and cerebellar (B) tissue samples of postmortem brain were solvent extracted and analyzed for F_2-isoprostane by GC-MS. The values are means and the error bars represent ± SE. ** $p < 0.05$ by Fischer's LSD post hoc analysis between Group 0 and Group III/IV.

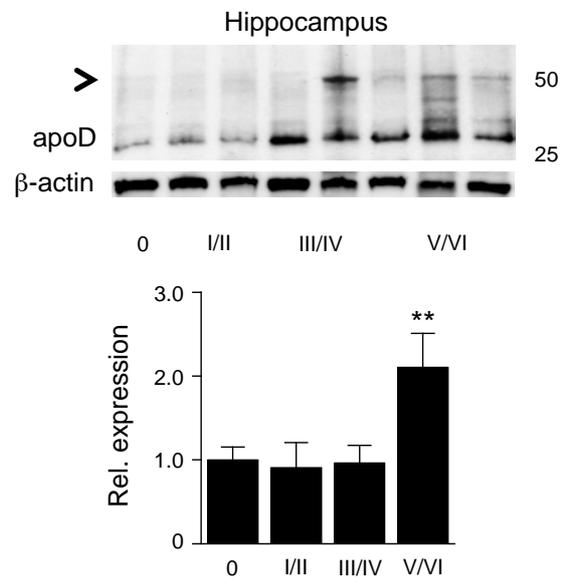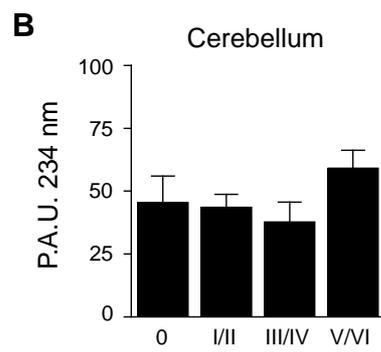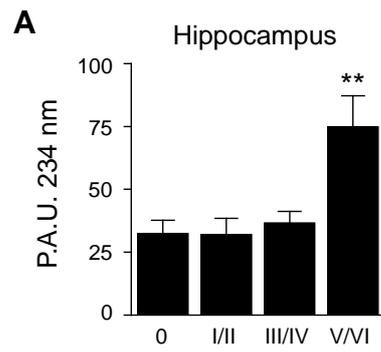**A** Aβ42 Insoluble

B Aβ40 Insoluble

C Aβ42 Soluble

D Aβ40 Soluble

A

Hippocampus

apoD

β-actin

0   I/II   III/IV   V/VI

B

Cerebellum

apoD

β-actin

0   I/II   III/IV   V/VI