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Journal
Journal of Alzheimer's Disease, 22(1)

ISSN
1387-2877

Authors
Murphy, M Paul
Morales, Jacqueline
Beckett, Tina L
et al.

Publication Date
2010

DOI
10.3233/jad-2010-100639

Journal of Alzheimer's Disease 22 (2010) 135–150
DOI 10.3233/JAD-2010-100639
IOS Press

Changes in Cognition and Amyloid-β Processing with Long Term Cholesterol Reduction using Atorvastatin in Aged Dogs

M. Paul Murphy[a,b,*], Jacqueline Morales[e], Tina L. Beckett[a], Giuseppe Astarita[e], Daniele Piomelli[e], Adam Weidner[a,b], Christa M. Studzinski[a], Amy L.S. Dowling[a], Xiaohong Wang[a], Harry LeVine, III[a,b], Richard J. Kryscio[a,c], Yushun Lin[a,c], Edward Barrett[f] and Elizabeth Head[a,d,*]
[a]*Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY, USA*
[b]*Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY, USA*
[c]*Department of Statistics, University of Kentucky, Lexington, KY, USA*
[d]*Department of Molecular and Biomedical Pharmacology, University of Kentucky, Lexington, KY, USA*
[e]*University of California Irvine, Irvine, CA, USA*
[f]*Lovelace Respiratory Research Institute, Albuquerque, NM, USA*

Handling Associate Editor: Gary Arendash

Accepted 8 June 2010

Abstract. Human studies suggest either a protective role or no benefit of statins against the development of Alzheimer's disease (AD). We tested the hypothesis that statin-mediated cholesterol reduction in aged dogs, which have cognitive impairments and amyloid-β (Aβ) pathology, would improve cognition and reduce neuropathology. In a study of 12 animals, we treated dogs with 80 mg/day of atorvastatin for 14.5 months. We did not observe improvements in discrimination learning; however, there were transient impairments in reversal learning, suggesting frontal dysfunction. Spatial memory function did not change with treatment. Peripheral levels of cholesterol, LDLs, triglycerides, and HDL were significantly reduced in treated dogs. Aβ in cerebrospinal fluid and brain remained unaffected. However, β-secretase-1 (BACE1) protein levels and activity decreased and correlated with reduced brain cholesterol. Finally, lipidomic analysis revealed a significant decrease in the ratio of omega-6 to omega-3 essential fatty in temporal cortex of treated aged dogs. Aged beagles are a unique model that may provide novel insights and translational data that can predict outcomes of statin use in human clinical trials. Treatment with atorvastatin may be beneficial for brain aging by reducing BACE1 protein and omega6:omega3 ratio, however, the potential adverse cognitive outcomes reported here should be more carefully explored given their relevance to human clinical outcomes.

Keywords: Amyloid-β protein precursor (AβPP), β-secretase (BACE1), canine, cholesterol, dog, LRP-1, statin

INTRODUCTION

Alzheimer's disease (AD) is a progressive dementia characterized by the presence of senile plaques and neurofibrillary tangles [1]. Several early cross sectional or case control epidemiological studies have revealed a striking link between cholesterol-lowering drugs (statins and others) and up 70% reduction in the development of AD in the general population [2–9]. However some [10–12], but not all [13,14], prospective studies have reported no benefits of statin use with

*Correspondence to: Elizabeth Head, M.A., Ph.D., Department of Molecular and Biomedical Pharmacology, Sanders-Brown Center on Aging, 203 Sanders-Brown Building, 800 South Limestone St., Lexington, KY, 40536, USA. Tel.: +1 859 257 1412 ext 481; Fax: +1 859 323 2866; E-mail: elizabeth.head@uky.edu. Or M. Paul Murphy, M.A., Ph.D., Department of Molecular and Cellular Biochemistry, University of Kentucky, 211 Sanders-Brown Center on Aging, 800 S. Limestone, Lexington, KY, 40536-0230, USA. Tel.: +1 859 257 1412 x490; Fax: +1 859 257 9479; E-mail: mpmurp3@email.uky.edu.

ISSN 1387-2877/10/$27.50

respect to protection against dementia. Further, more recent studies with large sample sizes have not found a risk reduction for AD [15–17]. Differential reports of the positive effects of statins on the development of AD may be due to the cohort studied, sample size, confounds by indication, prescription bias, the types of statins used, the age group studied, and whether cross-sectional/case control studies or prospective study approaches were applied [18,19].

One mechanism by which statins may reduce the risk of incident AD is through the reduction of the amyloid-β (Aβ) peptide [20–22]. Aβ is the major protein constituent of senile plaques observed in the AD brain and may be a causative factor in disease pathogenesis [23, 24]. High dietary cholesterol in transgenic mouse models of AD leads to increases in brain Aβ [25,26]. Further, reducing cholesterol [27] by treatment with inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) or statins can reduce Aβ [28]. However, rodents respond to statin treatment by massively upregulating HMG-CoA reductase levels in the liver, preventing any stable, long term reduction in cholesterol levels [29–32]. This leads to difficulties in conducting long term studies in rodents and physiologically excessive statin doses relative to human clinical trials.

Due to similarities to humans in terms of responsiveness, drug tolerance and metabolism, the dog can be considered to be a useful model for chronic statin treatment [29,33]. Indeed, dogs are unique in that they were used to establish efficacy and safety in the majority of statins currently on the market and have been evaluated in chronic studies of over 2 years in length at physiological doses relevant for humans. Dogs naturally develop Aβ deposits with age [34–36] and the sequence of Aβ in dogs is identical to humans [37,38]. In addition, aged dogs develop learning and memory impairments in cognitive domains sensitive to age and dementia in humans [39–41]. Thus the dog model is particularly useful for testing the link between cholesterol, Aβ, and cognition, can provide data that complement and extend the existing rodent literature, and can provide treatment data that can be translated to human clinical trials. In the current study of aged beagles, we hypothesized that atorvastatin (Lipitor®) would reduce circulating levels of cholesterol, improve learning and memory, and reduce Aβ neuropathology.

MATERIALS AND METHODS

Subjects

Twelve beagles ranging in age from 8.9–13.2 yrs were obtained from either a colony at the Lovelace Respiratory Research Institute or from Harlan (Indianapolis, IN). Based upon our previous work, dogs of this age show cognitive decline and significant amounts of brain Aβ [36,41]. All animals had documented dates of birth and comprehensive medical histories, and a veterinary examination ensured that animals were in good health prior to the start of the study. At the end of the study, all but one control animal had received treatment for 14.5 months. At the end of the study, animals ranged in age from 10.1–14.6 yrs. All research was conducted in accordance with approved IACUC protocols. To determine the minimum and sufficient number of animals necessary for each treatment group we conducted a power analysis using estimated means and variances derived from Aβ load data and cognitive test scores collected from over 160 canines over the past 10 years. PASS 6.0 was used for all power analyses with an alpha level of 0.05. All analyses indicate that 6 animals/group and a two-fold reduction in Aβ or error scores [8] can be detected with nominal power levels of up to 0.9.

Cognitive testing – baseline

Table 2 provides a summary of the timeline for individual tasks used to assess cognition throughout the study. The behavioral testing apparatus has been described previously [39]. For 5 days a week, animals were tested on individual tasks with each task consisting of 10 presentation trials per day. For baseline testing, animals were given a reward and object approach learning problem to teach them to respond in the test apparatus and to learn to manipulate stimulus objects to reveal hidden food rewards [39]. Animals were trained until they correctly selected either 8/10 correct 2 days in a row or 9/10 correct in a single day. After reaching these criteria, animals were given an additional 3 days of testing (30 trials) and were required to maintain an average score of 7 out of 10 correct. Subsequently, an object discrimination test was used where animals were shown two different stimulus objects, one of which consistently associated with food reward [39]. The next stage of testing consisted of object reversal learning where the same two objects were presented, but the reward was now placed under the previously unrewarded object [39]. Spatial memory was examined by using a 2-choice delayed non matching to position procedure [42]. Briefly, animals were shown a single red plastic block covering either the left or right food well. After a 5 s delay, animals were shown two identical blocks and the reward was hidden under the block on

Table 1
Animals used in the study

Dog#	Gender	Group	Age at Start (Yrs)	Age at death (Yrs)	Time on treatment (Mos)
1	F	Control	10.4	11.6	14.5
2	F	Control	10.4	11.6	14.5
3	M	Control	10.0	10.6	7.2
4	F	Control	9.5	10.7	14.5
5	F	Control	10.4	11.6	14.5
6	M	Control	13.4	14.6	14.5
Mean			10.7	11.8	13.2
1	F	Atorvastatin	8.9	10.1	14.5
2	F	Atorvastatin	10.8	12.0	14.5
3	F	Atorvastatin	9.3	10.5	14.5
4	M	Atorvastatin	10.5	11.7	14.5
5	F	Atorvastatin	10.8	12.0	14.5
6	M	Atorvastatin	11.8	13.0	14.5
Mean			10.3	11.5	14.5

Table 2
Study timeline

Task	Time on treatment (months)
Baseline-Reward Approach Learning	0.0
Baseline-Object Approach Learning	0.0
Baseline – Discrimination Learning	0.0
Baseline – Reversal Learning	0.0
Baseline – Spatial Memory – 2 Choice	0.0
Baseline-Spatial Memory – 3 Choice	0.0
Start of Treatment	0.0
Baseline – Spatial Memory – 2 Choice	1.2
Baseline-Spatial Memory – 3 Choice	4.4
Size Discrimination	5.8
Size Reversal	6.0
Baseline – Spatial Memory – 2 Choice	8.4
Black/White Discrimination	11.0
Black/White Reversal	12.0
Baseline-Spatial Memory – 3 Choice	13.4
End of Study	14.5

the side opposite to that seen previously. During the acquisition phase of the test, animals were required to remember the location of a hidden food reward over a short 5 s delay interval. Animals were tested with this 2-choice spatial task until criterion was met. Subsequently, dogs were sequentially tested with a 10 s, 20 s, 30 s, and 50 s delay intervals after reaching criterion on a shorter delay for a maximum of 50 days. The maximal delay that individual animals reached in 50 days of testing was used as one measure of memory. A second measure of spatial memory involved giving dogs a variable delay test procedure. For this test dogs were given 12 trials per day with 3 possible delay intervals of 20 s, 70 s, or 110 s (counterbalanced across trials). Animals were tested for 20 days using this procedure and accuracy was calculated as a function of delay to established memory curves.

Group assignments and study timeline

Animals were ranked based on cognitive test scores and placed into 2 equivalent groups, with 2 males and 4 females per/group. These groups were randomly designated as either the placebo-treated control group or the atorvastatin-treated group.

Drug treatment

Atorvastatin (Atorvastatin Calcium or Lipitor^{R-} 40 mg tablets) and placebo tablets were kindly provided by Pfizer Inc (New York, NY). Atorvastatin-treated animals received daily dose of 80 mg per day and control animals received placebo tablets. Atorvastatin was chosen for the study as long term studies of 80 mg doses in dogs do not lead to adverse events including cataracts [43,44].

Blood samples

At regular intervals prior to and during the treatment study, serum and plasma samples were collected for measurement of Aβ, blood lipids, and biochemistry. For plasma samples, whole blood was collected in 10 cc tubes containing EDTA. For serum samples, whole blood was collected in tubes without anti-coagulant. Fresh samples were immediately provided to a commercial laboratory for measures of basic biochemistry (e.g., liver function), cholesterol, triglycerides, low density lipoproteins (LDL), and high density lipoproteins (HDL). Remaining samples were aliquotted and frozen at -80°C for measurements of plasma Aβ and other lipid markers.

Cognitive testing – treatment

After treatment initiation, animals were regularly retested over the next 14.25 months and assessed for learning and memory ability (Table 2). Animals were tested for spatial acquisition after 1.2 months of treatment and for spatial memory after 4.4 months. Testing was identical to that described for baseline assessment. After 5.8 months of treatment, dogs were given a size discrimination and reversal learning problem [45,46]. This task simultaneously shows animals two objects that differ only in size [46]. Once animals reach criterion levels the reward contingencies were reversed and animals were required to select the previously negatively-rewarded object. After 8.4 months of treatment, dogs were reassessed for spatial learning. After 11 months of treatment, animals were given a black/white discrimination and reversal learning problem [45]. Animals were shown two objects that were similar in all aspects except that one object was black and the other was white. Once animals learned to discriminate these two objects by responding only to one consistently, the reward contingencies were reversed. Last, after 13.4 months of treatment, dogs were given a final spatial memory assessment. The study was concluded at 14.5 months of treatment.

Tissue collection

Twenty minutes before induction of general anesthesia, animals were sedated by subcutaneous injection with 0.2-mg/kg acepromazine. General anesthesia was induced by inhalation with 5% isoflurane. While maintained under anesthesia, a cerebrospinal fluid sample was taken, dogs were exsanguinated by cardiac puncture and blood samples were collected. Within 15 minutes, the brain was removed from the skull and sectioned midsagitally. The intact left hemisphere was immediately placed in 4% paraformaldehyde for 48–72 hr at 4°C prior to long term storage in phosphate buffered saline containing 0.02% sodium azide at 4°C. The right hemisphere was coronally sectioned ($\sim$1 cm) and flash frozen at -80°C. The dissection procedure was completed within 20 min yielding a 35–45 minute postmortem interval.

Aβ ELISAs

Frozen CSF and cortical samples were taken from the contralateral hemisphere and included the dorsolateral prefrontal, posterior parietal, temporal, and occipital cortex. Grey matter was carefully dissected from the white matter for the assays. Measurement of Aβ is routinely performed in our lab [47]. Diethylamine (DEA, 0.2%), detergent (SDS, 2%) and formic acid (FA, 70%) soluble pools of Aβ were measured in tissue samples using a standard, well characterized sandwich ELISA as described previously [48]. Briefly, each well of the Immulon 4HBX plate was coated with 1.0 μg of antibody, and blocked with a solution of Synblock (Serotec), as per the manufacturer's instructions. To detect Aβ_{40}, capture was performed using monoclonal antibody Ab9 (against the N-terminal end of Aβ), and detection was performed using horseradish peroxidase conjugated 13.1.1 (end-specific for Aβ_{40}). To detect Aβ_{42}, capture was performed using monoclonal antibody 2.1.3 (end-specific for Aβ_{42}), and detection was performed using horseradish peroxidase conjugated Ab9 (as above). Formic acid extracted material was initially neutralized by a 1:20 dilution in TP buffer (1 M Tris base, 0.5 M Na_2HPO_4), followed by a further dilution as needed (1:100 to 1:400) in AC buffer. SDS soluble fractions were diluted (1:20) in AC buffer alone. A peptide standard curve of Aβ was run on the same plate for comparison, and standards and samples were run at least in duplicate; Aβ values were determined by interpolation relative to the standard curve. Plates were washed between steps with standard PBS containing 0.05% Tween-20 (2-4x) followed by PBS (2-4x). Plates were developed with TMB reagent (Kirkegaard & Perry Laboratories), stopped with 6% *o*-phosphoric acid, and read at 450 nm using a BioTek multiwell plate reader.

Oligomer assay

PBS samples were loaded onto a G-75 column (10 mL bed), run in PBS $+$0.1% Tween-20 and 400 μL fractions were collected. A 96-well plate was coated with 50 μl of an N-terminal Aβ specific antibody (6E10) at 3 μg/mL in 10mM NaPi 5 buffer (pH 7.5) and incubated overnight at 4°C. The plate was blocked with PBS $+$2 mg/mL BSA for two hr. Wells were washed 3X with TBST (20 mM Tris-HCl, 34 mM NaCl, 0.1% Tween-20). Samples from each fraction were added (100 μl) and incubated for two hr. Wells were washed 3X with TBST. Fifty μl of 1 μg/mL biotinylated detection antibody (4G8) in TBST was then added and incubated for 2 hr. Wells were washed 3X with TBST. Fifty μl of 50 ng/mL streptavidin in TBST was added and incubated for 1 hr. Finally, a solution of TMB/H_2O_2 was added and allowed to develop (time

based on concentration). The reaction was stopped with 1% H_2SO_4 solution and the OD at 450 nm was recorded using a plate reader. All samples were compared to a standard curve of Aβ_{40} run concurrently using the same method.

Aβ Immunohistochemistry

Free floating sections containing the prefrontal cortex were selected from the coronal sections. Blocks of fixed tissue were sectioned by Vibratome at 50 μm. The selected region was a components of cortical circuits responsible for impaired function on the size reversal learning task used in the study [46]. This region was also described in a previous study establishing the pattern of Aβ deposition as a function of age in canines [36]. Aβ was detected with anti-Aβ_{1-17} (6E10 antibody, mouse monoclonal human Aβ protein, 1:5000, Signet Laboratories Inc., Dedham, MA) and plaque "loads" obtained using previously published methods [49]. Briefly, ten images (525 $\times$ 410 μm each) were captured at a 20X objective using a high-resolution video camera and NIH Image 1.59b5 for each animal. Sampling consisted of five images from the superficial and five from the deep cortical layers. The cross-sectional area occupied by Aβ in each individual image was quantified using gray scale thresholding, which separated positive staining from background and calculated the percentage of area occupied by Aβ immunoreactivity or "Aβ load". This entire experiment was replicated with a second set of coronal sections (at least 200 μm away from the first set) to confirm the results.

Lipid analyses

Frozen tissues were weighed and homogenized in methanol (1 ml/100 mg tissue) containing the following internal standards: heptadecanoic acid (Nu-Chek Prep, Elysian, MN) and cholesterol-D7 (Avanti Polar Lipids, Alabaster, AL). Lipids were extracted with 2 volumes of chloroform and washed with 1 volume of water. Organic phases were collected and dried under liquid N_2. Lipids were reconstituted in chloroform/methanol (1:4, vol/vol, 0.1 ml) for liquid chromatography/mass spectrometry (LC/MS) analyses.

Non-esterified fatty acids were analyzed using an 1100-LC system coupled to a 1946D-MS detector (Agilent Technologies, Inc., Santa Clara, CA) equipped with an electrospray ionization (ESI) interface. Fatty acids were separated on a reversed-phase XDB Eclipse C18 column eluted with a linear gradient (from 90% to 100% of methanol in water containing 0.25% acetic acid and 5 mM ammonium acetate in 2.5 min) at a flow rate of 1.5 ml/min with a column temperature of 40°C. ESI was in the negative mode, capillary voltage was 4 kV and fragmentor voltage was 100 V. Nitrogen was used as drying gas at a flow rate of 13 liters/min and a temperature of 350°C. Nebulizer pressure was set at 60 PSI. We used commercially available fatty acids as reference standards and monitored deprotonated molecular ions [M-H]$^-$ in the SIM mode and heptadecanoic acid (m/z269.3) as an internal standard. Sterol lipids were analyzed using an 1100-LC system coupled to an ion trap XCT MS detector (Agilent Technologies, Inc., Santa Clara, CA). Sterol lipids were separated on a Poroshell 300 SBC18 column (2.1 $\times$ 75 mm i.d., 5 μm, Agilent Technologies) maintained at 50°C. A linear gradient of methanol in water containing 5 mM ammonium acetate and 0.25% acetic acid (from 85% to 100% of methanol in 4 min) was applied at a flow rate of 1 ml/min. We used atmospheric chemical pressure-ionization (ACPI) in positive ion-scanning mode with corona discharge needle voltage at 4000 V. Nitrogen was used as drying gas at a flow rate of 10 liters/min, temperature of 350°C, nebulizer pressure of 50 PSI and vaporization temperature at 400°C. Helium was used as collision gas. Lipids were identified by comparison with retention times and tandem MS fragmentation patterns of authentic standards. Cholesterol was detected at m/z 369.3, [M+H-H_2O]$^+$, 24-hydroxycholesterol was detected at m/z 367.3 [M+H-2H_2O]$^+$ and desmosterol at m/z 367.3 [M+H-H_2O]$^+$.

Western blots

Frozen samples from the prefrontal cortex, temporal cortex or cerebellum were homogenized in 2% SDS in PBS (150 mg/ml) plus protease inhibitors (ICN Pharmaceuticals, Costa Mesa, CA). Proteins were separated on a 4–20% SDS-PAGE Criterion gel (Bio-Rad Laboratories, Hercules, CA) and transferred to polyvinylidene difluoride (PVDF) or nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were probed with anti-AβPP (22C11, Millipore, Temecula, CA), anti-AβPP CTFs (CT20 [47]), or anti-BACE1 (MAB931, R&D Systems, Minneapolis, MN). Anti-GAPDH (Abcam Inc., Cambridge, MA), Anti-β-actin (AC15, Sigma, St. Louis, MO) or anti-tubulin (Abcam Inc., Cambridge, MA) were used as protein loading controls. Protein was detected by incubating membranes in either anti-rabbit (1:2500–1:10000), or

anti-mouse (1:2500–1:10000) horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence (ECL, Pierce, Rockford, IL). For densitometry, a subset of samples was pooled and each membrane included 4 lanes with increasing protein concentrations (2.5–20 μg or 10–60 μg) to ensure that optical densities were within the linear range of detection. Membranes were scanned and proteins of interest were quantified by measuring the intensity using Scion Image software.

β-Secretase assay

$\beta-$Secretase activity was assayed using a commercial kit (BioVision, Mountain View, CA) according to manufacturer's instructions. Briefly, parietal cortex samples were homogenized in extraction buffer and protein concentration measured. Subsequently, β-secretase substrate conjugated to EDANS and DABCYL was added and relative fluorescence was measured at 495–510 nm. A positive and a negative control were included in the assay, and the value from the negative control was subtracted from all measurements.

Data analysis

Cognitive test scores, serum and brain lipids, CSF and brain Aβ, immunohistochemistry "loads" and protein levels (optical densities) were compared across groups using either independent t-tests, repeated measures analysis of variance (ANOVA) or a linear mixed model with a compound symmetry covariance structure (brain Aβ). When multiple comparisons were used, a Bonferonni correction was applied. Pearson correlations were used to compare brain with plasma levels of lipids. All statistics were conducted using SPSS for Windows and PC-SAS.

RESULTS

During baseline testing, 2 animals that did not respond consistently in the testing apparatus were identified. These animals were maintained in the study for biological, but not cognitive outcome measures and each assigned randomly to the two treatment groups. Therefore, each group contained 5 animals for cognitive comparisons. At baseline, there were no significant group differences for object discrimination and reversal learning. For spatial testing, the number of errors individual animals made when initially learning the problem with a 5 second delay and the maximal memory scores were matched between groups. Accuracy as a function of delay during the variable delay memory testing procedure was also similar across groups at 20 s, 70 s, and 110 s.

Peripheral lipid and cholesterol measures

Measures of HDL, triglycerides, LDL and cholesterol were used to determine the effect of atorvastatin on peripheral lipid profiles throughout the study. HDLs significantly decreased over time ($F(11,77) = 4.8\ p < 0.0005$) and atorvastatin treated dogs showed an 18% reduction in HDLs with treatment ($F(1,7) = 45.77\ p < 0.0005$). For LDLs, a significant effect of time by treatment ($F(9,63) = 2.41\ p = 0.02$) and a significant main overall effect of treatment ($F(1,7) = 6.70\ p = 0.03$) was observed in atorvastatin treated dogs. Treated animals had lower levels of LDLs ($\sim$48% during months 7–11). Triglycerides were measured at 12 time points including at baseline. Triglycerides were increased in both groups over time ($F(11,77) = 4.1\ p < 0.0005$). Although there was no treatment by time interaction, atorvastatin treated dogs had $\sim$27% lower triglycerides ($F(1,7) = 5.45\ p = 0.05$). Cholesterol was measured at 10 time points after the start of treatment and showed a significant $\sim$24% reduction in cholesterol ($F(1,7) = 45.38\ p < 0.0005$).

Cognition as a function of treatment

After the start of treatment, dogs were give 2 discrimination and reversal learning tasks (size and black/white) and 2 retests of spatial memory. One animal in the control group was euthanized due to mammary tumors after 6 months on study and could not complete the black/white discrimination tasks and tissue from this animal was also not included in the final neuropathology studies. A repeated measures analysis of variance was used to determine if the two groups differed in discrimination learning or reversal learning over time. These analyses all included baseline measures of discrimination or reversal. Given changes in peripheral measures of cholesterol and other lipids we predicted learning and memory improvements in our treated animals. For discrimination learning, there was no main effect of treatment or a time by treatment (Fig. 1A, C). Reversal learning over time showed a significant time by treatment interaction effect ($F(2,14) = 4.24\ p = 0.04$). This was primarily due to atorvastatin treated animals showing poorer size reversal learning

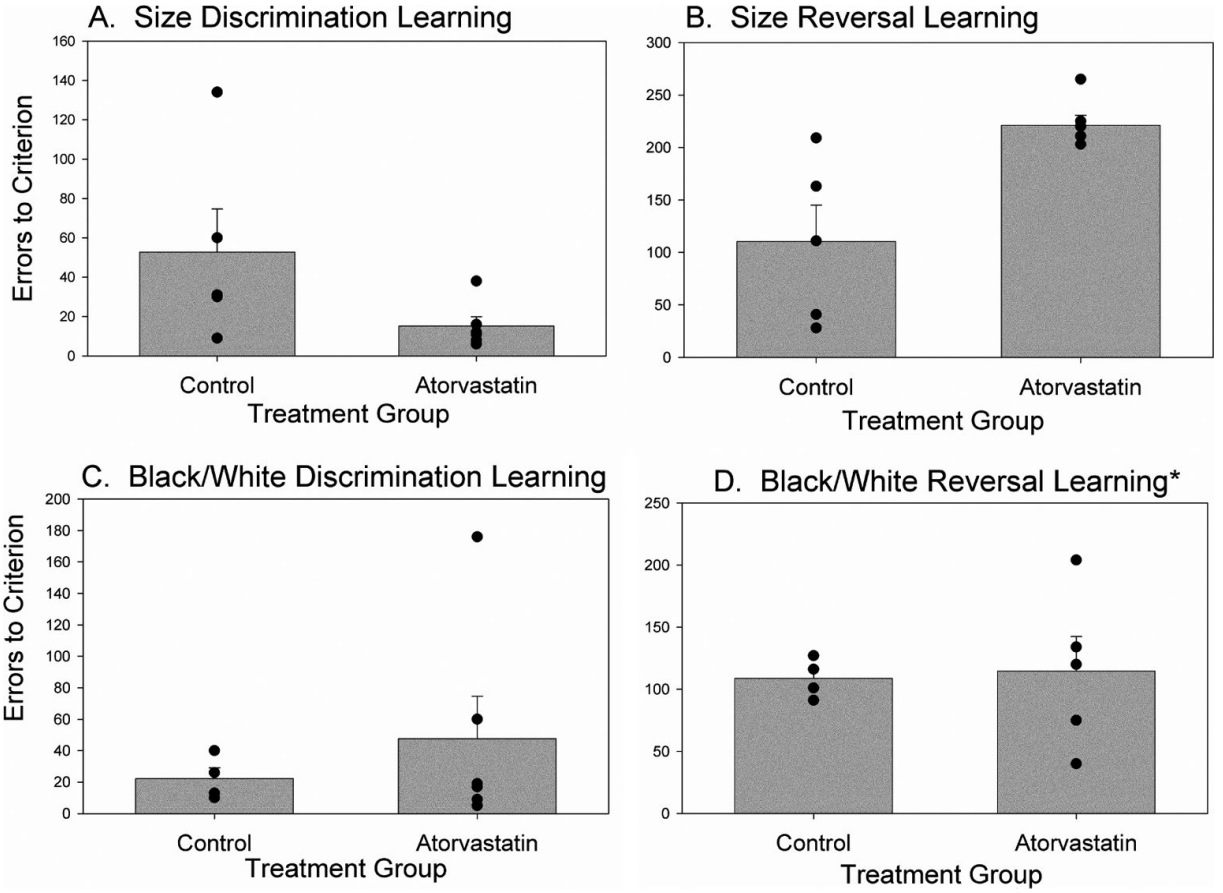

Fig. 1. Effects of atorvastatin on learning in aged beagles. After 5.8 months of treatment animals began testing on a size discrimination and reversal task. A) Animals treated with atorvastatin showed a trend towards reduced error scores on discrimination learning that were not statistically significant. B) Size reversal learning was significantly impaired in atorvastatin treated dogs. C) After 11 months of treatment, the two treatment groups performed similarly on a black/white discrimination task. D) There was no significant difference in average error scores between the two groups on a size reversal learning problem.

than control animals (Fig. 1B) but performing equivalently to control animals on black/white reversal learning (Fig. 1D).

Spatial learning was assessed at 3 time points (baseline, 1.2 months and 8.4 months of treatment; Fig. 2A). A repeated measures univariate ANOVA indicated a significant effect of time on error scores ($F(2,16) = 13.05$ $p < 0.0001$), but no main effect of either treatment or time by treatment interaction. Figure 2A shows that all groups showed improved spatial learning (i.e., lower error scores) with repeated testing and atorvastatin treated animals did not show additional benefits. Maximal memory scores were analyzed similarly (Fig. 2B) and we observed a significant main effect of time on maximal memory ($F(2,16) = 5.38$ $p = 0.02$), but neither a main effect of treatment nor a time by treatment interaction.

To assess spatial working memory, we used two approaches. First, for each time point after the start of treatment, we compared the two groups across all 3 delay intervals (Fig. 2C, 2D, 2E). After 4.4 months of treatment, we observed a significant main effect of delay interval on accuracy ($F(2,18) = 51.3$ $p < 0.0005$) but neither a main effect of treatment nor a treatment by delay interaction. In the second measure of spatial working memory after 13.4 months of treatment, similar effects were observed. There was a significant difference in accuracy across delays ($F(2,16) = 96.72$ $p < 0.0005$) but no main effect of treatment nor treatment by delay interaction. A second analysis compared changes in accuracy across the 3 time points on treatment and a repeated measures analysis of accuracies at 20 s, 70 s, and 110 s were conducted separately. For all delays, (20 s, 70 s, 110 s) dogs performed reliably over time but neither an effect of the treatment overall nor

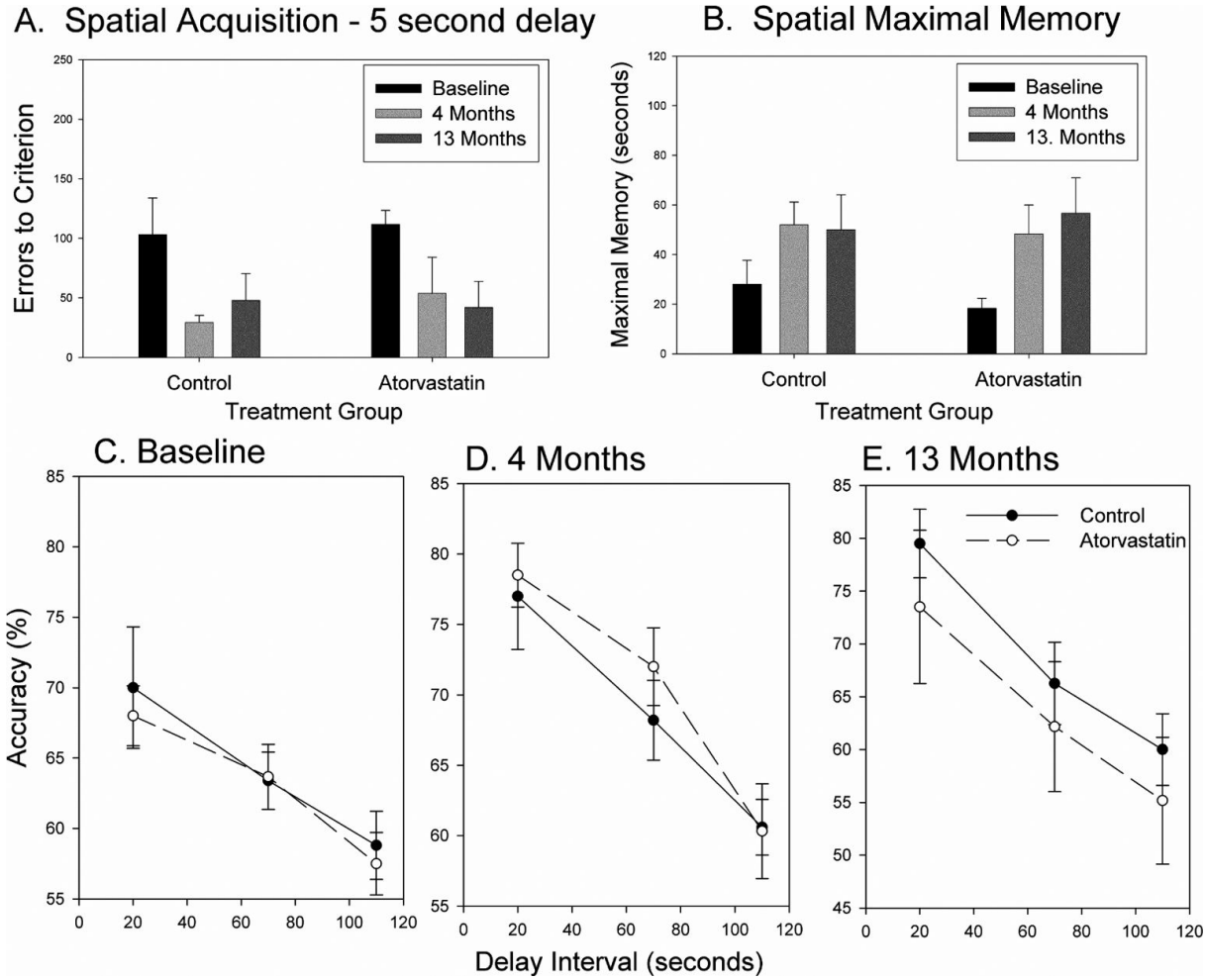

Fig. 2. Spatial learning and memory as a function of atorvastatin treatment in aged dogs. A) Spatial learning at baseline, 4 months, and 13 months of treatment was similarly improved in both treatment groups. B) Spatial maximal memory increased with repeated assessments at 4 months and 13 months of treatment and was not different between the control and atorvastatin groups. Spatial working memory was not significantly different in the groups at C) baseline, and did not improve in treated dogs at D) 4 months or E) 13 months.

a delay by treatment interaction was observed. Thus, atorvastatin did not improve spatial memory.

CSF and brain Aβ

We next hypothesized that CSF and brain levels of Aβ may be reduced in response to atorvastatin treatment based upon the existing literature in rodent models. In CSF, total Aβ was similar in the two groups. Aβ_{1-42}, specifically, was similar in the two groups. Thus, atorvastatin did not decrease CSF Aβ (Fig. 3A). Interestingly, CSF Aβ was positively correlated with HDLs in blood (Spearman $r = 0.750$ $p = 0.02$).

A linear mixed model with a compound symmetry covariance structure was used to test for group differences in DEA (Fig. 3B), RIPA (Fig. 3C), or formic acid extracted (Fig. 3D) Aβ_{1-40} and Aβ_{1-42} for each of 5 brain regions sampled (prefrontal, parietal, entorhinal cortex, hippocampus, and cerebellum). There were no overall decreases in Aβ_{1-40} or Aβ_{1-42} in any fraction measured as a function of treatment group or as a treatment group by brain region interaction. To extend and confirm the lack of treatment effect of atorvastatin on extracted Aβ, the prefrontal cortex was immunostained for Aβ_{1-16} (6E10) and loads were obtained. We observed no significant differences in Aβ load in the prefrontal cortex (t(10) $= 0.18$ $p = 0.86$) (Fig. 3E).

Total Aβ remained unchanged in response to atorvastatin treatment, as did Aβ deposited within diffuse plaques in the brain. However, other assembly states

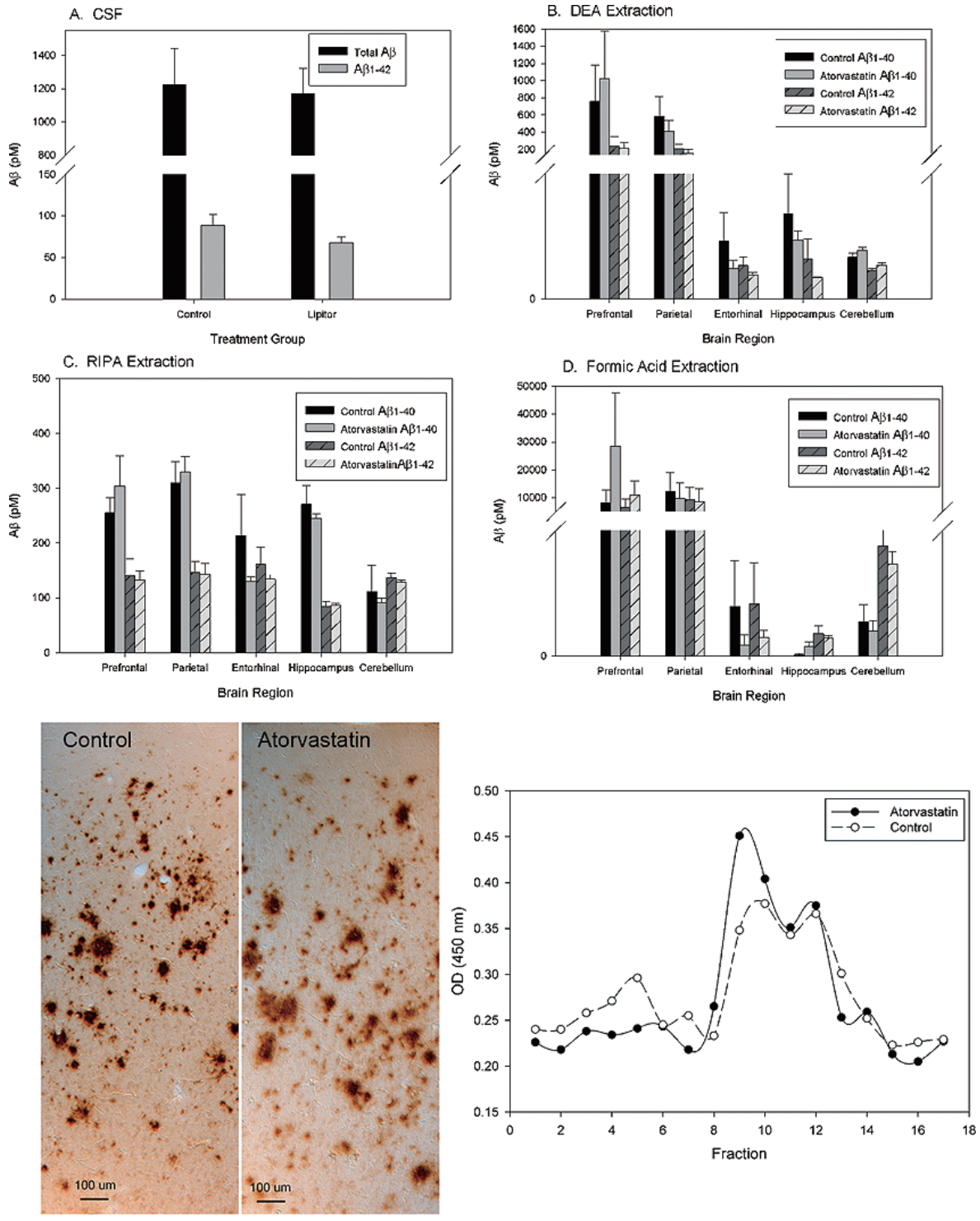

Fig. 3. CSF and brain Aβ in response to atorvastatin in aged dogs. A) CSF Aβ did not change in response to atorvastatin treatment although a small decrease was observed in Aβ_{1-42}. In brain, B) DEA extracted, C) RIPA extracted and D) formic acid extracted Aβ_{1-40} and Aβ_{1-42} did not vary as a function of treatment condition in brain regions examined. E) Similarly, Aβ diffuse plaques in the prefrontal cortex remained unchanged in response to treatment as seen by immunohistochemistry. F) The amount of Aβ oligomers measured in temporal cortex was not decreased in atorvastatin treated dogs.

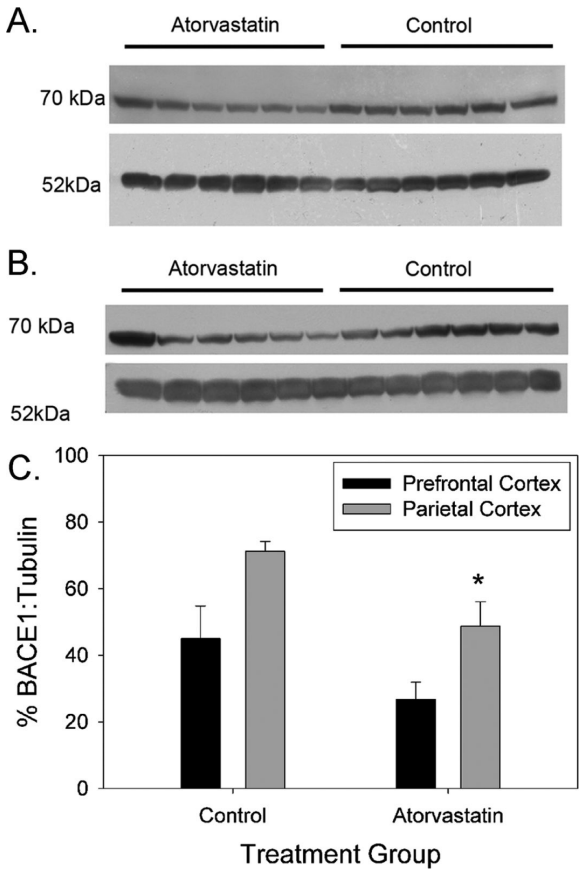

Fig. 4. BACE1 in atorvastatin treated dogs. A) BACE1 protein level in the parietal cortex was decreased in atorvastatin treated dogs (∼28.7%). B) In the prefrontal cortex, similar effects were seen but the changes did not reach statistical significance (∼32.1). C) Quantification of BACE1 protein shows a statistically significant decrease in the parietal cortex of treated animals compared to control. $^{*}p < 0.05$.

of Aβ including oligomers may change in response to treatment. Using gel filtration in combination with ELISAs, we tested this hypothesis but did not observe significant differences between atorvastatin and placebo treated dogs in total oligomeric Aβ (Fig. 3F). Results of the gel filtration assay for Aβ oligomers were also confirmed using dot blots with the M204 antibody, showing no differences in total oligomer accumulation when comparing treated versus untreated animals (data not shown).

Aβ pathways

Aβ is derived from the proteolytic cleavage of the amyloid-β protein precursor (AβPP) by sequential processing by β-secretase (BACE) and γ-secretase [50]. In previous *in vitro* reports, AβPP and C-terminal fragments (CTFs) of AβPP measures of secretase activity increase in response to statins [51,52]. Thus, we measured total endogenous AβPP and CTFs in the prefrontal and parietal cortex using Western blot. No change in total AβPP or in CTFs was detected (data not shown). However, in parietal cortex we observed a significant decrease in protein level of BACE1 (when co-varying for α-tubulin loading; (F(1,9) $= 5.07$ $p =$ 0.05) or when comparing the ratio of BACE1 to tubulin (t(10) $= 2.85$ $p = 0.017$) (Fig. 4A, 4C) in statin treated dogs. A similar decrease in BACE1 protein was observed in the prefrontal cortex, but did not reach statistical significance (Fig. 4B, 4D). We hypothesized that BACE1 protein level in the parietal cortex reflected beta-secretase activity. Higher BACE1 protein was significantly correlated with beta-secretase activity (Spearman $r = 0.67$, $p = 0.02$).

Lipid/cholesterol brain measures

We next hypothesized that the lack of change in brain Aβ and little effect on memory may be due to the limited ability of atorvastatin to reduce brain cholesterol levels. To address this question, we compared the brain lipid profile of treated versus untreated aged animals (Table 3). We determined that brain cholesterol levels were not reflective of peripheral cholesterol measures and did not find a significant correlation between blood cholesterol and either brain cholesterol ($r = -0.21$ $p =$ 0.56) or 24OH-cholesterol ($r = 0.026$ $p = 0.94$). However, we observed that the treated animals have small but not statistically significant decreases in the levels of cholesterol (13.8% decrease), the cholesterol biosynthetic precursor desmosterol (22% decrease), and the cholesterol metabolite 24-hydroxycholesterol (20.1% decrease) (Table 3). Further lipidomic analysis revealed a significant decrease in the ratio of omega-6 (C20:4 and C18:2) to omega-3 (C18:3 and C22:6) essential fatty acids in temporal cortex of dogs provided with atorvastatin (t(9) $= 2.3$ $p = 0.05$) (Table 3).

BACE1 protein in the parietal cortex was selectively correlated with both brain cholesterol (Spearman $r =$ 0.65 $p = 0.02$) and 24OH-cholesterol (Spearman $r =$ 0.650 $p = 0.02$) but not with desmosterol, DHA or arachidonic acid. Similarly, beta-secretase activity was also correlated only with brain cholesterol (Spearman $r = 0.61$ $p = 0.04$) and with 24OH-cholesterol (Spearman $r = 0.681$ $p = 0.02$) and not with desmosterol, DHA or arachadonic acid. These results suggest that lower brain cholesterol measures are associated with lower BACE1 protein and β-secretase activity.

Table 3
Temporal cortex lipid outcomes

	Treatment group	
Lipid measure	Control	Atorvastatin
24OH-Cholesterol	31.3 ± 13.8	25.0 ± 7.8
Desmosterol	179.2 ± 134.0	139.8 ± 48.0
Cholesterol	10951.2 ± 7219.2	9440.4 ± 3367.7
Alpha-linolenic acid (C18:3, omega-3)	0.56 ± 0.15	0.61 ± 0.06
Docosahexaenoic acid (C22:6, omega-3)	98.0 ± 99.8	73.9 ± 16.0
Linoleic acid (C18:2, omega-6)	10.61 ± 0.74	9.63 ± 1.35
Arachidonic Acid (C20:4, omega-6)	416.0 ± 355.4	231.2 ± 55.1
Omega-6:Omega-3	18.38 ± 0.78	14.67 ± 1.3*

Lipids measures are expressed in nmol/g. Means ± SE, $^*p < 0.05$.

DISCUSSION

Statins are thought to play a protective role against the development of AD, and clinical trials have shown cognitive benefits in moderately demented patients [53]. However, more recent studies and reviews of several prospective studies suggest that statin use provides no protection against the development of dementia [16]. Thus, whether statins are of benefit to the aging brain is still somewhat controversial. Indeed, there is some evidence to suggest in normal aging that statin use may lead to cognitive decrements [54,55]. In the current study, aged beagles that naturally accumulate human brain Aβ and develop progressive cognitive decline were treated for over a year with atorvastatin with physiologically relevant doses of atorvastatin (80 mg/day) [56]. Dogs are uniquely well suited for statin studies because they do not require increasingly higher doses to counteract compensatory upregulation of HMG-CoA reductase, a problem that is typically observed in rodent models [57].

In the current study in aged beagles, we did not observe improvements in medial temporal lobe associated discrimination learning. However, prefrontal cortex-dependent reversal learning was impaired. Learning decrements were not observed on a second test after ∼11 months of treatment; atorvastatin dogs performed similarly to placebo treated controls. However, on our second measures of cognitive function, the number of dogs still on study had dropped to 5 atorvstatin animals and 4 control animals. Although there was significant overlap in the error scores from the two groups, it is possible that our sample size was too small to detect significant differences, and should be interpreted more conservatively. There have been no systematic studies of behavioral changes in rodent models in response to statin treatment and transient impairments in reversal learning contrast with human clinical trials with atorvastatin [56]. In particular, treatment of AD patients with atorvastatin (80 mg/day) with mild to moderate dementia leads to improvements in the Alzheimer's disease Assessment Scale-cognitive subscale at 6 months but smaller, nonsignificant benefits at 12 months [58]. In combination, these results suggest that even with long term treatment in aged dogs with cognitive impairments, no benefits were observed and short term impairment in prefrontal function was detected. This is in contrast to previous canine work showing significant improvements in learning and memory in response to antioxidants or behavioral enrichment using the same tasks [59]. However, impaired cognition in treated aged dogs is consistent with reports in elderly people [54, 55] suggesting that the canine model mimics human responses.

The lack of beneficial cognitive effects of atorvastatin in aged beagles is not due to a lack of drug effect on peripheral levels of lipids. Increased circulating HDLs (18%) and decreased LDLs (48%), triglycerides (27%) and cholesterol (24%) are all within ranges reported in clinical trials in normocholesteromic individuals e.g., between a 25–40% reduction in cholesterol in response to atorvastatin [56], simvastatin [60,61], lovastatin [61], or pravastatin [61]). However, an unexpected outcome in our study was reductions in HDL in treated dogs but this is consistent with some previous studies in humans. Several papers report an increase in HDLs in humans in response to atorvastatin [62,63]. Further, the range in increase may be between 5 and 9%, relatively modest compared with the reductions in LDLs observed. In animal models of AD, there is one report of a reduction in HDLs in mice [64] and in AD patients treated with atorvastatin, HDLs were also decreased [65]. One possible explanation is that animals are normocholesteremic, whereas most human clinical trial reports are in hypercholesteremic subjects. Second, we used the highest administered atorvastatin dose as used in humans. Given our results with circulating levels of lipids were consistent with the AD clinical

trials, we feel our study in combination with previously published data raises an interesting issue regarding the use of chronic atorvastatin as a prevention approach in normocholestermic individuals. Interestingly, HDL in plasma was correlated with CSF Aβ in our study suggesting this may not be a beneficial outcome of the atorvastatin treatment.

Although atorvastatin is lipophilic and has the potential to cross the blood brain barrier (BBB), evidence suggests that it is distributed to peripheral tissues [66]. Further, the brain synthesizes its own cholesterol independently from its peripheral availability [67]. Brain cholesterol is produced primarily by astrocytes [68] and given even a relatively low permeability of atorvastatin into the brain, it may be sufficient to reduce cholesterol synthesis. Reduced cholesterol in the brain might be expected and also lead to less availability of cholesterol to neuron membranes, leaving neurons more vulnerable to excitotoxic insults [69], glutamate homeostasis [70] among other deleterious effects. Thus it was not surprising that aged beagles treated with atorvastatin showed small but nonsignificant reductions in brain lipids (including cholesterol), consistent with previous studies in rodent models [22,71,72]. Further, reduced brain cholesterol may be responsible for poorer cognition, although these impairments were transient. However, atorvastatin treatment in aged dogs did result in a significant reduction of the brain omega-6:omega-3 ratio, which suggests possible anti-inflammatory effects [73] and has implications for protective mechanisms mediated by statins [74,75].

The protective effects of statins on the development of AD may be related to a link between cholesterol and Aβ [76]. For example, New Zealand white rabbits fed a diet high in cholesterol [77,78] show increased Aβ in the brain, whereas rabbits fed even low-levels of cholesterol have Aβ plaques detectable by MRI [79]. Further, transgenic AD mice (tg2576) fed a hypercholesteromic diet show significantly decreased secreted AβPPα, increased CTFβ [25] and increased Aβ [25, 26]. Thus, one would predict that cholesterol lowering drugs, such as atorvastatin, would reduce Aβ. The majority of preclinical research on the effects of statins on Aβ has been in rodent models. In rats, treatment with low doses of simvastatin or atorvastatin leads to reduced peripheral levels of cholesterol but no change in total brain Aβ levels [72]. However, in high dose studies in guinea pigs, treatment with simvastatin leads to reduced brain Aβ [22]. Similarly, in transgenic mouse models of AD, either diet-induced hypercholesterolemia [25,26] or pharmacologically-induced hypocholesterolemia [27,28] leads to increased or decreased Aβ accumulation, respectively. However, in female Tg2576 mice treated with lovastatin, an increase in brain Aβ was observed, although the mechanisms underlying this effect are yet unknown [80]. It is important to point out that in rodent studies reporting a reduction of brain Aβ in response to statin treatment, doses are typically between 200 and 400 fold higher than that used in humans, leading to concern regarding the translation of these outcomes to AD clinical trials. In the current canine study, dose levels were more comparable to those used in human treatment studies.

There were substantial differences in the amount of Aβ_{40} and Aβ_{42} across different brain regions in our study, similar to what has been reported previously [36, 81]. These differences parallel known differences in the relative abundance of different forms of Aβ that can be found within different soluble fractions with the human brain, and that vary by disease state [47]. Differential extraction of Aβ into increasingly insoluble pools yields important information regarding the transition of the Aβ peptide through the soluble monomer, oligomers, and fibrillar states, a transition roughly corresponding to the relative abundance of diffuse and neuritic plaques in the human brain, and to similar structures in genetically modified mice [47]. Low ionic strength alkaline solutions (e.g., DEA; aqueously soluble and membrane associated Aβ) and various SDS-containing detergents (e.g., RIPA; diffuse amyloid deposits) can extract fractions of Aβ of high to intermediate solubility, with harsher conditions required to extract the remainder (e.g., 70% FA; fibrillar material, deposited either in the vasculature or in neuritic plaques) [47,82,83]. This approach is believed to quantitatively extract all of the Aβ in the brain [84].

In aged beagles, no decreases in brain Aβ were observed using human doses of atorvastatin (80 mg/day). In an 8–12 kg dog, this dose translates into $\sim$6–10 mg/kg, whereas in humans it may be less than 1 mg/kg. Similarly, no decreases in CSF or plasma Aβ were noted in response to long term treatment with atorvastatin, consistent with human clinical trials [85–87], including individuals who were hypercholesterolemic [88]. A lack of effect of atorvastatin on Aβ levels in the aged dog brain was also reflected in no changes in total AβPP or AβPP CTFs, in contrast with previous *in vitro* work using neuronal and/or glial cultures [20,51,52,65,71,89]. A lack of reduction of brain Aβ in treated dogs may reflect the atorvastatin dose, as decreased Aβ in rodents is typically only observed with higher doses which may not be physiologically relevant to humans.

Interestingly, in the canine study described here, BACE1 in the parietal cortex was reduced in atorvastatin treated dogs, although there was no effect on Aβ. BACE1 reduction was associated with $\sim$13% decrease in brain cholesterol in treated dogs, which may be consistent with the reduction in BACE1 observed in neuronal cultures reported previously [90]. Further, brain cholesterol measures were selectively associated with both BACE1 protein level and β-secretase activity, suggesting a link between brain cholesterol and AβPP processing. Results in the current study are consistent with experiments in rabbits fed a high cholesterol diet; BACE1 protein levels were increased [91]. Tg2576 transgenic mice show increased CTFβ [25] or reduced CTFβ [27] when cholesterol is increased or decreased, respectively. *In vitro* studies confirm these effects as decreasing cholesterol leads to decreased CTFβ [20]. However, statin treatment may not decrease BACE activity *per se* but rather prevent dimerization and stabilization [92] thereby reducing BACE's ability to access AβPP. Further, moderate membrane cholesterol reduction ($\sim$30%) can lead to decreased BACE1 from detergent-resistant membrane microdomains or rafts but increased BACE1-AβPP co-localization [90]. Thus, reducing brain cholesterol may lead to reduced Aβ or, paradoxically, to enhanced amyloidogenesis. However, reduced BACE1 without changes in Aβ may indicate that pre-existing Aβ pathology cannot be reversed with atorvastatin treatment, but strongly suggests that it may prevent pathology.

Statins inhibit cholesterol synthesis through reduction of HMG-CoA reductase activity, although there are many downstream modifications to other molecular pathways leading to pleiotropic effects [93,94]. Interestingly, many pathways modified by statins could have direct effects on AD pathogenesis and Aβ associated neuropathology [95]. Furthermore, our results indicate that atovarstatin lowers the omega-6:omega-3 ratio, which is a marker of inflammation [73]. By lowering this ratio, atorvastatin may shift the equilibrium between the omega-6-derived pro-inflammatory mediators (e.g., prostaglandins) and the omega-3-derived anti-inflammatory and anti-apoptotic lipids (e.g., neuroprotectins) [96]. This evidence suggests a potential anti-inflammatory benefit of atorvastatin on the aging brain, which should be further studied.

The lack of change in Aβ in canines treated for over one year, in spite of substantial changes in circulating cholesterol and lipid profiles, strongly suggests that changes in pre-existing Aβ pathology may not be the mechanism related to some reports of clinical benefits of statin treatment. It is possible that reductions in the activity of BACE1, or alterations in brain lipids (e.g., the omega 3:omega 6 ratio), may lead to reduced risk in dementia through another mechanism. Perhaps the reduction in AD risk associated with long term statin use in human epidemiological studies reflects a chronic reduction in Aβ production (via BACE1 reduction) over an extended period of time, a long term reduction in neuroinflammation (reflected by a decrease in omega3:omega6), or both. This supports the idea that mid-life reductions in cholesterol may have far greater benefit than those pursued at later ages [97]. Further, this study suggests that targeting the reduction of cholesterol may only yield a benefit as a preventative measure against the onset of dementia. However, it is important to note that adverse effects, albeit short-lasting, did occur on a frontal-dependent task and it is worthwhile pursuing this outcome and the possible mechanisms, such as a trend towards lowered brain cholesterol, given previous reports of a decrement in cognition in statin treated aged people [54,55].

ACKNOWLEDGMENTS

Funded by the Alzheimer's Association (IIRG 03-5673 to EH and MPM) and the Agilent Foundation (DP). The contributions of the Agilent Technologies/UCI Analytical Discovery Facility and the Center of Drug Discovery at UCI are gratefully acknowledged.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=485).

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