

Brain sterol dysregulation in sporadic AD and MCI: relationship to heme oxygenase-1

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Abstract

The objective of this study was to ascertain the impact of aging and Alzheimer's disease (AD) on brain cholesterol (CH), CH precursors, and oxysterol homeostasis. Altered CH metabolism and up-regulation of heme oxygenase-1 (HO-1) are characteristic of AD-affected neural tissues. We recently determined that HO-1 over-expression suppresses total CH levels by augmenting liver X receptor-mediated CH efflux and enhances oxysterol formation in cultured astroglia. Lipids and proteins were extracted from postmortem human frontal cortex derived from subjects with sporadic AD, mild cognitive impairment (MCI), and no cognitive impairment ($n = 17$ per group) enrolled in the Religious Orders Study, an ongoing clinical-pathologic study of aging and AD. ELISA was used to quantify human HO-1 protein expression from brain tissue and gas chromatography–mass spectrometry to quantify total CH, CH

precursors, and relevant oxysterols. The relationships of sterol/oxysterol levels to HO-1 protein expression and clinical/demographic variables were determined by multivariable regression and non-parametric statistical analyses. Decreased CH, increased oxysterol and increased CH precursors concentrations in the cortex correlated significantly with HO-1 levels in MCI and AD, but not no cognitive impairment. Specific oxysterols correlated with disease state, increasing neuropathological burden, neuropsychological impairment, and age. A model featuring *compensated* and *de-compensated* states of altered sterol homeostasis in MCI and AD is presented based on the current data set and our earlier *in vitro* work.

Keywords: Alzheimer's disease, cholesterol, cholesterol precursors, heme oxygenase-1, lipids, multivariable analysis, oxysterols, Religious Orders Study.

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Alzheimer's disease (AD) is an aging-related dementing illness that afflicts ~5–10% of North Americans over the age of 65 and ~30–50% of those who survive to the end of their ninth decade (Hendrie 1998). AD is characterized by progressive neuronal degeneration, gliosis, and the accumulation of intracellular inclusions (neurofibrillary tangles; NFT) and extracellular deposits of β -amyloid (senile plaques) in discrete regions of the basal forebrain, hippocampus, and association cortices (Selkoe 1991). The role(s) of sterol homeostasis, particularly cholesterol (CH) metabolism, is currently the subject of intense interest vis-à-vis the etiopathogenesis of AD. CH subserves a variety of biological roles in mammalian tissues and is of particular importance in the CNS, being a major component of cellular membranes and a determinant of membrane fluidity. CH may act as a cell signaling molecule, influence gene transcription,

and is an essential precursor of all bioactive steroids. The blood–brain barrier (BBB) (Pfrieger 2003; Bjorkhem and Meaney 2004) is largely impermeable to circulating CH and

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Abbreviations used: 24-OH, 24-hydroxycholesterol; 7keto-CH, 7-ketocholesterol; 7 α -OH, 7 α -hydroxycholesterol; 7 β -OH, 7 β -hydroxycholesterol; AD, Alzheimer's disease; APOE, apolipoprotein; BBB, blood–brain barrier; CH, cholesterol; CO, carbon monoxide; GC-MS, gas chromatography–mass spectrometry; HO-1, heme oxygenase-1; K–W test, Kruskal–Wallis test; MCI, mild cognitive impairment; NCI, no cognitive impairment; NFT, neurofibrillary tangles; NIA, National Institute on Aging; Post-Est, post-estimation; SPC, Spearman's correlation.

in the adult CNS CH is synthesized and regulated within glial cells. The astrocytic compartment meets neuronal CH demands by secreting CH-apolipoprotein (APOE) complexes (Bjorkhem and Meaney 2004).

Epidemiological studies have suggested an association between circulating CH and the pathogenesis of AD (Simons *et al.* 2001; Shobab *et al.* 2005; Panza *et al.* 2006). However, this relationship remains largely uncharacterized and ill-defined because of several factors: First, most human studies have correlated peripheral serum or plasma CH levels with the incidence of AD; few have focused on CNS CH which is regulated independently of the peripheral CH pool. Second, most human studies have relied on retrospective epidemiological data which often lack critical subject information germane to both AD and CH homeostasis. This includes factors such as APOE4 status, exposure to pharmacological CH lowering agents (e.g. statins), neuropathology scores, neuropsychological testing and disease progression rates, all of which may impact the interpretation of sterol-related findings. Finally, most human studies have emphasized select components of the CH regulatory pathway, rather than evaluating the pathway as a whole. Given the numerous biological factors and conditions which may impact CH and its regulatory mechanisms, there is much to be gained by conducting comprehensive evaluations of the pathways impacting CH levels/turnover within the CNS and determining their relationships to brain aging and AD.

Heme oxygenase-1 (HO-1) is a 32-kDa stress protein that mediates the catabolism of heme to biliverdin, free iron, and carbon monoxide (CO). The ho-1 gene is susceptible to up-regulation by a host of noxious stimuli (e.g. β -amyloid, hydrogen peroxide, and Th1 cytokines) and is induced in CNS tissues affected by AD, Parkinson's disease, and other neurological conditions (Schipper 2004). The up-regulation of HO-1 may confer cytoprotection by enhancing the breakdown of pro-oxidant heme to the radical scavenging bile pigments, biliverdin and bilirubin (Nakagami *et al.* 1993; Llesuy and Tomaro 1994; Dore *et al.* 1999; Baranano and Snyder 2001). Under certain conditions, however, heme-derived iron and CO may exacerbate intracellular oxidative stress and substrate damage by provoking free radical generation within mitochondria and other subcellular organelles (Piantadosi *et al.* 2006). In AD, increased oxidative stress may intensify neurodegeneration, increase lipid peroxidation and engender the formation of oxidized CH (oxysterols) (Bjorkhem and Diczfalusy 2002; Arca *et al.* 2007), events which may directly or indirectly promote amyloid precursor protein processing in favor of insoluble β -amyloid deposition (Nelson and Alkon 2005), tau hyperphosphorylation and NFT formation, mitochondrial insufficiency and neuronal cell death (Melov *et al.* 2007).

Our laboratory has previously established a link between HO-1 over-expression/over-activity and sterol dysregulation in cultured astroglia, characterized by suppression of total

CH concentrations and increased enzymatic and non-enzymatic oxysterol formation (Vaya *et al.* 2007). Additionally, we have recently delineated the mechanisms whereby HO-1 mediates these effects on CH homeostasis (Hascaloivici *et al.* 2009). To ascertain the link between HO-1 and altered sterol regulation in aging and AD, we now extend our investigation to postmortem brain tissues derived from individuals with no cognitive impairment (NCI), mild cognitive impairment (MCI), and AD enrolled in the Religious Orders Study.

Materials and methods

Postmortem brain tissue

We evaluated 51 postmortem brain tissue samples of frontal cortex derived from subjects with sporadic AD, MCI, and NCI ($n = 17$ per group). However, low-resolution output from one specimen limited most analyses to 50 subjects. All subjects were older Catholic clergy enrolled in the Religious Orders Study, a longitudinal clinical-pathologic study of aging and AD at Rush University Medical Center (Chicago, IL, USA). Each subject signed an informed consent and an anatomical gift act donating his/her brain to Rush investigators at the time of death. The Religious Orders Study was approved by the Institutional Review Board of Rush University Medical Center. Each subject underwent a uniform structured clinical evaluation that included a medical history, neurologic examination, neuropsychological performance testing, and review of neuroimaging when available, as previously described (Bennett *et al.* 2002). The diagnosis of AD was made according to criteria established by the National Institute of Neurologic and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS/ADRDA)(McKhann *et al.* 1984). The diagnosis of MCI refers to persons who are rated as impaired on neuropsychological tests but were not found to have dementia by the examining physician (Bennett *et al.* 2002). Cognitive function tests were selected to assess a broad range of abilities commonly affected by aging and AD as previously reported (Wilson *et al.* 2002). Subject information germane to AD and sterol metabolism included clinical diagnoses at the time of death, gender, age, Mini-Mental State Examination (MMSE), years of education, APOE genotype, statin exposure (Arvanitakis *et al.* 2008), time from diagnosis to death, postmortem interval, and neuropathology data [Braak's staging and classification according to National Institute on Aging (NIA)-Regan and Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria].

Lipid extraction and measurements

Tissue preparation

Brain samples were manually homogenized in a solution containing 0.5 mL of phosphate-buffered saline, with 0.45 mmol/L of butylated hydroxytoluene (an antioxidant present throughout the entire workup procedure to obviate tissue oxidation), one protease inhibitor tablet per 10 mL of solution, and 49.6 μ mol/L solution of 19-hydroxycholesterol as internal standard (in 1.5 mL Eppendorf tubes). Samples were transferred to 15 mL conical tubes and phosphate-buffered saline was added to each sample to obtain a final volume of 2 mL. Brain homogenate was sonicated on ice three times for 20 s at 80 W. Samples

(15 μ L) were removed for total protein concentration measurements by the RC DC protein assay based on the Lowry protocol (Bio-Rad Laboratories, Hercules, CA, USA). Sodium chloride (0.2 g, 684.5 mmol/L) was added to the extracts followed by 3 mL hexane : 2-propanol (3 : 2 v/v) with vigorous vortexing between steps. The resulting two phases were separated by centrifugation (20 000 g 10 min at 4°C) and the upper phase was collected. The extraction procedure was repeated with 3 mL hexane : 2-propanol. The collected organic solvents were combined, dried (0.3 g anhydrous sodium sulfate), filtered, and evaporated to dryness with purging nitrogen. Sample extracts were stored at -20°C until analysis.

Gas chromatography/mass spectrometry analysis of cholesterol, oxysterols, and cholesterol precursors

Cholesterol, oxysterols, 7 α -hydroxycholesterol (7 α -OH), 7 β -hydroxycholesterol (7 β -OH), 4 β -hydroxycholesterol, β -epoxide, α -epoxide, 7-ketocholesterol (7keto-CH), 3,5,6-trihydroxy cholesterol, 24-hydroxycholesterol (24-OH), 27-hydroxycholesterol (27-OH) and CH precursors, lathosterol, lanosterol, and desmosterol (hereafter termed Metabolites) were analyzed by gas chromatography–mass spectrometry (GC–MS). We employed an HP Model 5890 Series II gas chromatograph (Waldbronn, Germany) fitted with an HP-5 trace analysis capillary column (0.32 mm i.d., 0.25 mm film thickness, 5% phenyl methyl silicone) and a Model 5972 mass selective detector (HP) linked to an HP ChemStation data system. Samples were first subjected to hydrolysis to convert all sterol esters into their free form. The dry residue of the extracted samples was dissolved in 0.5 mL KOH solution (20% KOH in a mixture of MeOH : DDW 70 : 30) and mixed for 3 h at 20°C. Two volumes of diethyl ether were added and the pH was adjusted to 5 with 0.5 mL of citric acid (20% in DDW). The upper organic phase was removed and the liquid phase was extracted with 2 mL diethyl ether. The organic layers were combined, treated with sodium sulfate, and evaporated to dryness. Dried extracts were subjected to a silylating reagent *N,O*-bis(trimethyl-silyl) acetamide, using 1,4-dioxane (dried on 4 Å molecular sieves and passed through aluminum oxide) as solvent and heated to 80°C for 60 min. The GC was operated in a splitless mode for 0.8 min and then at a split ratio of 1 : 1. Helium was used as the carrier gas at a flow rate of 0.656 mL/min, with 10.4 psi pressure and at a linear velocity of 31 cm/s. The MS transfer line was maintained at 280°C. The injector was set at 300°C and the detector at 330°C. The column was gradient-heated starting at 200°C, increasing to 250°C at 10°C/min, followed by 5°C/min to 300°C, and then maintained for an additional 15 min at 300°C. Samples were injected in total and single ion modes, selecting characteristic 2–4 *m/z* fragmentation values for each compound. A calibration curve of CH, individual oxysterols, and CH precursor standards was run with each set of analyses. The recovery and reproducibility of this method was verified using standards of cholesteryl linoleate and three oxysterols – β -epoxide, 7keto CH, and 3,5,6-trihydroxycholesterol. These standards were subjected to identical hydrolysis, extraction, and analysis conditions as samples to verify recovery and possible artifactual autoxidation of the sterols (Vaya *et al.* 2001). The *m/z* values for each sterol was deduced by injecting a standard of the specific sterol in total ion mode and two to four of the most representative fragments were selected for re-injection in single ion mode to enhance the limit of detection (which was 0.1 ppm). The specific *m/z* values selected for each sterol used were identical to those previously reported (Vaya *et al.* 2001).

Heme oxygenase-1 protein assay

Sample preparation

Approximately 50–100 mg of human frontal cortex was manually homogenized in 10 volumes of 1x extraction buffer with protease inhibitor tablets (Roche Diagnostics, Laval, QC, Canada) 20–30 times at 4°C. Samples were vortexed and centrifuged at 15 000 g at 4°C for 15 min. Supernatants were aliquoted and stored at -80°C. Lysates were assayed for protein concentration by the RC DC protein assay based on the Lowry protocol (Bio-Rad Laboratories).

ELISA

A Human HO-1 ELISA kit (Assay Designs, Inc. Ann Arbor, MI, USA) was used to quantify HO-1 expression in human brain samples according to the manufacturer's protocol. Briefly, samples were balanced with 1x extraction buffer to 2 mg/mL and were individually diluted 1/10 in sample diluent. Recombinant HO-1 standard was serially diluted in sample diluent ranging from 0.78 to 25 ng/mL. One hundred microliter of prepared standards and samples were loaded in duplicate wells of the anti-HO-1 immunoassay plate, incubated at 21°C for 30 min and washed six times with 1x washing buffer. One hundred microliter of anti-human HO-1 was added to each well for 1 h, followed by six washes with 1x washing buffer. One hundred microliter of horseradish peroxidase conjugate was added to each well for 30 min and washed for six times. One hundred microliter of tetramethylbenzidine substrate was added to each well and the plate was incubated at 21°C for 15 min with light shielding. One hundred microliter of stop solution was added to each well and the absorbance at 450 nm was recorded using a microplate reader. HO-1 standard curves were plotted. HO-1 sample concentrations were calculated by interpolating concentrations from the standard curve, multiplying by the dilution factor, and dividing by the protein concentration, yielding a ratio of nanograms (ng) of HO-1/mg protein for each sample.

Statistical analyses

Statistical analyses were performed using GRAPHPAD Prism version 3.02 (GraphPad Software Inc., San Diego, CA, USA) and Intercooled Stata 8.2 statistical software (StataCorp, College Station, TX, USA). Demographic and clinical variables, such as age, years of education, and MMSE, were described by disease state (NCI, MCI, and AD). Comparisons of mean metabolite levels by disease state were examined using ANOVA. Non-parametric tests of the equality of distributions of metabolite levels across disease states were conducted using a Kruskal–Wallis (K–W) test. If an overall significant difference was found for an ANOVA or K–W test of three groups ($p < 0.05$), statistical significance levels for multiple pairwise comparisons were determined using a Bonferroni correction to the *p*-value equal to $0.05/3 = 0.017$. Two-way associations between categorical variables were determined by a chi-square test (for large numbers) or a Fisher's exact test (if any expected table cell count was less than 5).

The primary objectives were to survey sterols in the AD brain and to evaluate the association between HO-1 and all considered metabolites using linear regression analysis (Reg). Because there were some outlier values of HO-1, a natural log transformation for HO-1 was used to reduce the influence of these large values in regression models. Interaction terms were created to represent

separate slopes for Ln HO-1 and age within each disease state. Comparison of Ln HO-1 and age slopes between disease states were conducted using a Wald's test for post-estimation comparison of regression coefficients (Post-Est). Multivariable linear regression models were constructed separately for the outcomes: total CH, total oxysterols, and total precursors. Final multivariable models were selected based on a backward elimination procedure using covariates Ln HO-1 and available subject characteristics (disease state, gender, E4 allele, statin exposure, neuropathology score, age, education, and MMSE). Correlations between metabolites were determined by non-parametric Spearman's rank correlation coefficients and *p*-values.

Analyses between the 11 measured metabolites by disease state and subject characteristics were exploratory. As many possible associations were examined, the *p*-values should be interpreted cautiously. Results are presented in the context of our research hypotheses, and both positive and negative findings are reported.

Results

Associations between HO-1 and metabolites that are presented herein were analyzed separately (stratified) by disease state; overall unstratified results were inconclusive. Associ-

ations were examined for groups of metabolites which were categorized to represent three distinct components of sterol biosynthesis: Total CH precursors (sum of lanosterol, lathosterol, and desmosterol), total CH, and total oxysterols (sum of 7 α -OH, 7 β -OH, 4 β -OH, α -epoxide, β -epoxide, 24-OH, and 7keto-CH). These analyses were extended by examining associations between HO-1 and individual metabolites.

Distribution of subject characteristics by disease state

Subject characteristics were compared across the disease states NCI, MCI, and AD, using a Fisher's exact test for categorical characteristics and a non-parametric K-W test for continuous numerical characteristics (Table 1). The brain samples were carefully selected, by Religious Orders Study investigators (ZA and DB), for equal distribution of gender across disease state (eight males and nine females per group). AD and MCI subjects had a higher proportion of APOE4 alleles compared with NCI controls, although the difference was not statistically significant. As the current study focuses on lipid neurochemistry, subjects prescribed statins were

Table 1 Demographic, clinical, and neuropathological characteristics of the study population

Group	Total	Gender		E4		Statins							
		Male	Female	(+)	(-)	Yes	No						
General information													
NCI	17	8	9	3	14	4	13						
MCI	17	8	9	7	9	4	13						
AD	17	8	9	8	9	2	15						
Fisher's exact		1		0.173		0.742							
Group	Total	Age		Education		MMSE		Postmortem Interval		Ln HO-1			
		Mean \pm SD	Median	Mean \pm SD	Median	Mean \pm SD	Median	Mean \pm SD	Median	Mean \pm SD	Median		
Continuous variables													
NCI	17	85 \pm 6	86	19 \pm 4	18	28 \pm 3	28	6 \pm 5	5	2.88 \pm 0.53	2.71		
MCI	17	86 \pm 5	86	17 \pm 3	18	27 \pm 3	27	5 \pm 3	4.8	2.89 \pm 0.18	2.91		
AD	17	86 \pm 6	86	19 \pm 4	19	14 \pm 10	14	6 \pm 5	5	2.85 \pm 0.38	2.80		
K-W <i>p</i> -value		0.97		0.458		0.0001		0.957		0.475			
Group	Total	Braak's staging					NIA-Reagan			CERAD			
		I	II	III	IV	V	1	2	3	1	2	3	4
Neuropathology													
NCI	17	4	1	6	4	4	0	11	6	0	13	0	4
MCI	17	0	1	5	6	5	2	9	6	4	7	0	6
AD	17	2	1	2	5	7	7	7	3	11	2	2	0
Fisher's exact		0.272					0.035			< 0.001			

AD, Alzheimer's disease; MCI, mild cognitive impairment; NCI, no cognitive impairment; K-W, Kruskal-Wallis; MMSE, Mini-Mental State Examination.

NIA-Reagan – (1) high likelihood AD; (2) intermediate likelihood AD; and (3) low likelihood AD.

CERAD – (1) definite AD; (2) probable AD; (3) possible AD; and (4) no AD.

identified. Analysis of this variable revealed that although several subjects were using lipid-lowering agents, the proportions of subjects across disease state were less than 25% and were not significantly different (Exact $p = 0.742$).

Neuropathologic data used in analyses included Braak's staging and classification of cases according to NIA-Reagan and Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria. Of these, the NIA-Reagan criteria were the most consistent, evidencing greatest neuropathology (indicated by lowest score) in AD, moderate values in MCI and least pathology in NCI (Exact $p < 0.05$). No statistical differences were observed in subject age, years of education, postmortem time interval to autopsy, and level of Ln HO-1 expression across disease state. MMSE scores for AD subjects were significantly lower than scores for MCI and NCI subjects (K-W $p = 0.0001$), as expected.

Associations between sterols and disease state

Mean levels of CH, oxysterols, and CH precursors were compared across disease state. In the majority of samples, 27-hydroxycholesterol levels were below the limit of detection of our assay and thus excluded from analysis. One-way ANOVA of individual metabolites revealed no significant differences by disease. These associations are summarized in Table 2, along with descriptive statistics of all metabolite levels. Of note, a correlation among the AD subjects of elevated total CH and diminishing MMSE scores fell just short of statistical significance ($p = 0.0533$). If substantiated,

these findings would imply a possible association between cognitive impairment and brain CH retention in AD. In addition, levels of α -epoxide in AD brain, a marker of oxidative stress (Ferderbar *et al.* 2007), exhibited a trend (K-W $p = 0.09$) towards increasing concentrations with greater neuropathological burden (disease progression) as measured by Braak's score (data not shown).

Associations between age and sterols

Age is a risk factor for AD and was thus considered as a continuous variable for linear regression analysis of all metabolites. A positive association between age and total precursors was observed (Reg $p < 0.05$) independent of disease state. Age and total oxysterols were marginally associated (Reg $p = 0.09$), while the association between age and total CH was not significant. To investigate further the association between sterols and age, we controlled the analysis for disease state by estimating different age slopes for each disease state. There were no observed differences between the age slopes within disease states for the separate outcomes: total CH, total oxysterols, and total CH precursors. There was a greater association between age and β -epoxide for MCI subjects relative to NCI (Post-Est $p < 0.05$) and AD (Post-Est $p = 0.10$) (Fig. 1a). Additional analyses (Fig. 1b) revealed a positive association between age and the enzymatic formation of 24-OH in NCI and MCI. However, in this association, AD subjects exhibited a decrease in 24-OH with age relative to NCI (Post-Est $p < 0.05$). A

Table 2 Summary statistics and p -values for separate one-way ANOVA representing comparisons between each metabolite as outcome and disease state (AD/MCI/NCI) as covariate

	n	Mean	SD	Median	Min	Max	ANOVA p -Value AD/MCI/NCI
7 α -OH	50	0.0106	0.0124	0.00584	0	0.0674	0.8995
7 β -OH	50	0.0160	0.00991	0.0135	0.00058	0.0436	0.5693
4 β -OH	50	0.0117	0.0115	0.00908	0	0.0480	0.9179
β -Epoxide	50	0.0548	0.0745	0.0294	0	0.374	0.5286
α -Epoxide	50	0.0328	0.0408	0.0216	0.00499	0.210	0.9179
24-OH	50	0.0593	0.0343	0.0512	0.00480	0.124	0.9748
7keto-CH	50	0.111	0.0877	0.0825	0	0.404	0.6533
Desmosterol	50	0.298	0.124	0.308	0	0.547	0.6008
Lathosterol	50	0.0764	0.0304	0.0732	0.0248	0.145	0.1295
Lanosterol	50	0.176	0.141	0.141	0.0224	0.571	0.5441
Total CH	51	1842.005	1571.994	1456.155	0	8505.3	0.8992
Total precursors	50	0.00362	0.00262	0.00275	0.000969	0.0137	0.3064
Total oxysterols	50	0.00666	0.00355	0.00592	0.00120	0.0197	0.9994

AD, Alzheimer's disease; CH, cholesterol; MCI, mild cognitive impairment; NCI, no cognitive impairment.

All values are presented as % of Total CH ($\mu\text{mol/L}$).

Total oxysterols is equal to the sum of 7 α -hydroxycholesterol (7 α -OH), 7 β -hydroxycholesterol (7 β -OH), 4 β -hydroxycholesterol (4 β -OH), β -epoxide, α -epoxide, 7-ketocholesterol (7keto-CH), and 24-hydroxycholesterol (24-OH). Total precursors is equal to the sum of lathosterol, lanosterol, and desmosterol.

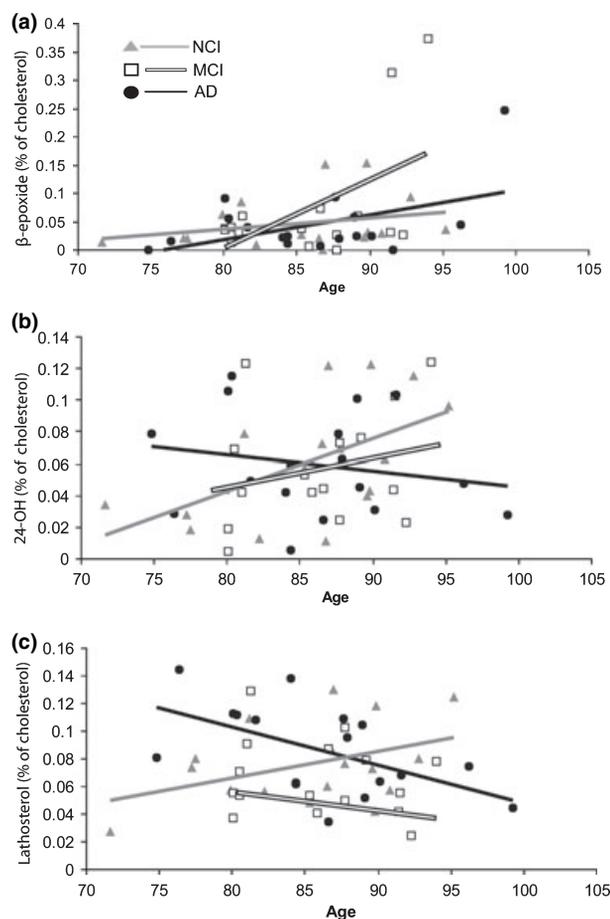


Fig. 1 Linear regressions of oxysterols and CH precursors (% of total cholesterol; $\mu\text{mol/L}$) as a function of age are depicted within disease state stratifications.

similar relationship for the CH precursor lathosterol was observed (AD vs. NCI Post-Est $p < 0.005$) (Fig. 1c), where lathosterol also decreases with age in AD, increases with age in NCI and decreases in MCI (non-significantly relative to NCI).

Associations between HO-1 and sterols

The primary objective was to examine the associations between HO-1 protein expression and all metabolites using linear regressions and disease state stratification. No significant difference in Ln HO-1 protein expression was observed amongst disease states (Table 1). In MCI, there was a significant decrease in total brain CH as Ln HO-1 increased. This relationship was not significant for the NCI and AD samples (Fig. 2c). Decreased CH was also observed when comparing the slopes of NCI to AD subjects, but this did not reach statistical significance (Post-Est $p = 0.16$). A positive association between Ln HO-1 and total oxysterols was observed in AD and was significantly different from the slope of this association in NCI (Post-Est $p < 0.005$)

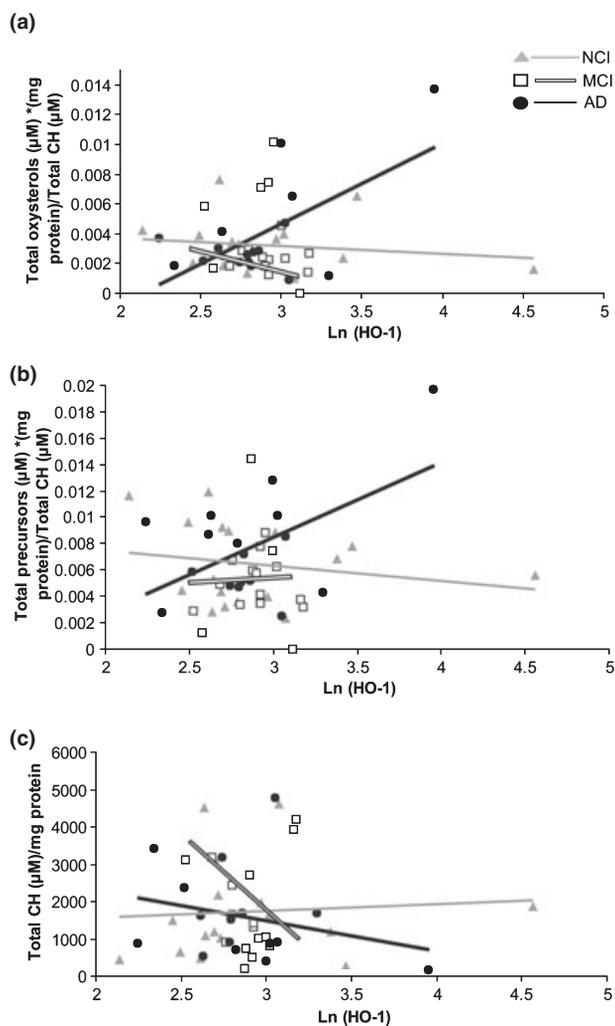


Fig. 2 The regressions illustrate the differential impact of HO-1 protein levels on total oxysterols (a), total CH precursors (b), and total CH (c) in AD, MCI, and NCI.

(Fig. 2b). Additionally, a significant association between total CH precursors and Ln HO-1 was observed in AD that was significantly different from the slope of this association in NCI (Post-Est $p = 0.01$) (Fig. 2c).

In an analysis of individual oxysterol metabolites and Ln HO-1, the influence of HO-1 expression on total oxysterols in AD was primarily driven by the positive associations of $7\alpha\text{-OH}$ ($p < 0.05$), $7\beta\text{-OH}$ ($p = 0.07$) and $\beta\text{-epoxide}$ ($p = 0.08$), all of which have positive slopes in AD relative to those of NCI. No significant associations were observed for Ln HO-1 with respect to $\alpha\text{-epoxide}$ ($p = 0.7$), 7keto-CH ($p = 0.13$) and 24-OH ($p = 0.9$) and $4\beta\text{-hydroxycholesterol}$ ($p = 0.8$) among AD subjects.

Metabolite-to-metabolite correlations by disease state

To determine the interrelationship between oxysterols, CH precursors, and CH for disease state, non-parametric Spear-

man's correlations (SPC) were calculated for all possible outcome combinations (Table 3). For the CH autoxidation products (non-enzymatically generated oxysterol species: 7 β -OH, α -epoxide, β -epoxide, and 7keto-CH), we observed significant non-parametric SPC between α -epoxide and 7 β -OH in AD (SPC = 0.639; $p < 0.005$) and MCI (SPC = 0.726; $p < 0.001$) and no correlation for NCI (SPC = 0.183; $p = 0.48$). For oxysterols formed by enzymatic reactions (7 α -OH, 24-OH), we obtained a positive correlation between 24-OH and 7 α -OH in AD (SPC = 0.529; $p < 0.05$) and MCI (SPC = 0.841; $p < 0.001$), but no correlation in NCI (SPC = -0.127; $p = 0.62$). Finally, comparing CH autoxides and enzymatic oxysterols, we observed positive correlations between β -epoxide and 24-OH for NCI (SPC = 0.713; $p < 0.001$) and MCI (SPC = 0.597; $p < 0.01$), but not for AD (SPC = 0.283; $p = 0.27$).

Multivariable models for total CH, total CH precursors, and total oxysterols

Multivariable models were constructed to represent three distinct components of sterol biosynthesis: total CH precursors, total CH, and total oxysterols (Table 4). Because associations between Ln HO-1, age, and metabolites were found when data were stratified by disease type, interaction terms were used to represent separate slopes for Ln HO-1 and age within each disease state. A graphical representation of the regression lines corresponds to the plotted lines in Fig. 2 when only the intercept terms and slope coefficient terms for Ln HO-1 within disease state are in the model for each outcome variable. In addition to these variables, presence of E4 allele, age and gender were also important predictors in specific models.

The best predictors of total brain CH content were Ln HO-1 among MCI subjects (Reg $p = 0.08$) and gender (Reg $p = 0.09$); total CH was higher for men versus women and values decreased as Ln HO-1 increased only among MCI subjects. The model for total CH precursors revealed that the latter significantly increased as Ln HO-1 protein levels increased among AD subjects (Reg $p = 0.01$), and age and E4 allele were also minor predictors of precursor levels ($0.05 < \text{Reg } p < 0.10$). Total oxysterols also significantly increased as Ln HO-1 increased among AD subjects (Reg $p = 0.001$) and oxysterols were lower for subjects with an E4 allele (Reg $p = 0.01$).

Discussion

The role of CH in AD is the subject of considerable controversy and literature surveys of this field have revealed little consistency (Simons *et al.* 2001; Puglielli *et al.* 2003; Bjorkhem and Meaney 2004; Shobab *et al.* 2005; Bjorkhem *et al.* 2006; Panza *et al.* 2006). Several unique features were incorporated into the current experimental design in an attempt to overcome limitations inherent to prior work and

derive a more direct and comprehensive understanding of deranged sterol homeostasis in AD. First, rather than focusing on select components of the CH regulatory pathway, we evaluated the cascade as a whole by assessing levels and interactions among relevant CH precursors, key oxysterol species (CH end products), and CH itself. Second, we made these determinations in affected human brain tissue in contradistinction to blood sterol/clinical correlations investigated in earlier reports. Third, the brain tissue samples were procured from the Religious Orders Study (described in Materials and methods) permitting meticulous correlation of neural sterol concentrations with a range of salient demographic, clinical and neuropathological variables. Finally, we queried, for the first time, the impact of HO-1 protein expression on human brain sterol homeostasis, guided by novel hypotheses generated in the course of our *in vitro* experimentation (Vaya *et al.* 2007; Hascalovici *et al.* 2009). An initial analysis comparing concentrations of all metabolites in respective disease states (NCI, MCI, and AD) did not reveal statistically significant relationships (Table 2) suggesting that 'disease state' may simply be too crude a stratification for lipid neurochemical analyses, or that associations differed depending on where in the course of disease the metabolites were assayed. Three multivariable models were constructed to determine if and to what extent relevant AD predictors impact the chief components of the CH regulatory pathway within the CNS, *viz.*, total CH precursors, CH, and oxysterols. In our first model, gender and disease state (MCI) emerged as the only reliable predictors of total brain CH. A second model constructed for the outcome of total oxysterols revealed that the E4 allele, increased HO-1 protein levels and disease state were robust predictors of this class of sterol metabolites. These findings are consistent with our previous observation of augmented oxysterol production in cultured rat astroglia engineered to over-express the human *ho-1* gene by transient transfection (Vaya *et al.* 2007) at levels akin to those documented in astrocytes residing within AD-affected cerebral cortex and hippocampus (Schipper *et al.* 1995). Furthermore, the relationship of total brain oxysterols to the E4 allele and increased HO-1 protein levels is commensurate with a recent report by Jofre-Monseny *et al.* indicating that lipopolysaccharide induces HO-1 expression in macrophages transfected with the E4 allele, but not those expressing E3 (Jofre-Monseny *et al.* 2007). A third model constructed for total CH precursors supported our prediction that the latter would be impacted (augmented) by HO-1 and disease state. This prediction was based on our observation that HO-1 over-expression facilitates *de novo* CH biosynthesis in cultured astroglia, an event contingent upon the enhanced availability of CH precursors (Hascalovici *et al.* 2009).

In the current study, age, an important risk factor for AD (Tyas *et al.* 2001; Lindsay *et al.* 2002), exhibited complex interactions with brain sterol homeostasis. In an analysis of

Table 3 Metabolite-to-metabolite interrelationships (non-parametric SPC) for disease state

Interrelationship	NCI		MCI		AD	
	SPC	<i>p</i> -Value	SPC	<i>p</i> -Value	SPC	<i>p</i> -Value
7α-OH						
7 β -OH	0.343	0.177	0.732	0.0013***	0.495	0.0433*
24-OH	-0.127	0.625	0.841	< 0.0001***	0.529	0.0289*
α -Epoxide	0.188	0.468	0.711	0.002**	0.661	0.003**
β -Epoxide	-0.095	0.715	0.517	0.040*	0.54	0.025*
7keto-CH	0.561	0.019*	0.529	0.035*	0.791	0.0002***
Lathosterol	-0.237	0.358	0.326	0.217	0.093	0.7222
Desmosterol	0.480	0.051	0.732	0.001**	0.676	0.003**
Lanosterol	0.321	0.209	0.482	0.059	0.703	0.002**
Total precursors	0.240	0.353	0.600	0.014*	0.825	< 0.0001***
Total CH	-0.144	0.578	-0.514	0.041*	-0.781	0.0002***
Total oxysterol	0.157	0.548	0.535	0.033*	0.792	0.0002***
7β-OH						
24-OH	-0.196	0.450	0.691	0.003**	0.414	0.098
α -Epoxide	0.183	0.480	0.726	0.001***	0.639	0.005**
β -Epoxide	-0.274	0.286	0.605	0.012*	0.687	0.002**
7keto-CH	0.411	0.100	0.605	0.012*	0.732	0.0008***
Lathosterol	-0.085	0.743	0.494	0.051	0.259	0.313
Desmosterol	0.324	0.205	0.724	0.002**	0.515	0.035*
Lanosterol	0.556	0.020*	0.585	0.017*	0.887	< 0.0001***
Total precursors	0.098	0.708	0.564	0.022*	0.610	0.009**
Total CH	-0.210	0.416	-0.670	0.004**	-0.686	0.002**
Total oxysterol	-0.137	0.599	0.603	0.013*	0.618	0.0082***
24-OH						
α -Epoxide	0.129	0.619	0.597	0.014*	0.340	0.180
β -Epoxide	0.713	0.001***	0.597	0.014*	0.283	0.270
7keto-CH	-0.058	0.822	0.411	0.113	0.580	0.0145*
Lathosterol	0.519	0.032*	0.567	0.021*	0.480	0.05*
Desmosterol	0.353	0.165	0.685	0.003**	0.598	0.011*
Lanosterol	0.282	0.273	0.509	0.044**	0.591	0.013*
Total precursors	0.517	0.033*	0.485	0.056	0.578	0.015*
Total CH	-0.495	0.043*	-0.523	0.037*	-0.607	0.009**
Total oxysterol	0.588	0.013*	0.412	0.113	0.529	0.029*
α-Epoxide						
β -Epoxide	0.036	0.888	0.470	0.065	0.472	0.055
7keto-CH	0.502	0.039*	0.450	0.080	0.705	0.001**
Lathosterol	0.058	0.822	0.355	0.176	-0.051	0.844
Desmosterol	0.277	0.282	0.521	0.039*	0.576	0.016*
Lanosterol	0.324	0.205	0.344	0.192	0.708	0.002**
Total precursors	0.235	0.363	0.358	0.172	0.649	0.004**
Total CH	-0.414	0.098	-0.450	0.080	-0.649	0.004**
Total oxysterol	0.505	0.039*	0.715	0.002**	0.664	0.004**
β-Epoxide						
7keto-CH	-0.041	0.873	0.291	0.273	0.595	0.011*
Lathosterol	0.297	0.248	0.405	0.118	0.193	0.456
Desmosterol	0.189	0.468	0.541	0.030**	0.288	0.262
Lanosterol	0.336	0.188	0.374	0.154	0.649	0.005**
Total precursors	0.225	0.384	0.338	0.200	0.705	0.001**
Total CH	-0.311	0.223	-0.497	0.050*	-0.542	0.024*
Total oxysterol	0.412	0.101	0.585	0.017*	0.781	0.0002***
7keto-CH						
Lathosterol	-0.056	0.829	0.414	0.110	0.088	0.736

Table 3 Continued

Interrelationship	NCI		MCI		AD	
	SPC	<i>p</i> -Value	SPC	<i>p</i> -Value	SPC	<i>p</i> -Value
Desmosterol	0.103	0.694	0.700	0.003**	0.738	0.0007***
Lanosterol	0.640	0.006**	0.750	0.0008***	0.858	< 0.0001***
Total precursors	0.174	0.504	0.800	0.0002***	0.825	< 0.0001***
Total CH	-0.372	0.140	-0.694	0.002**	-0.840	< 0.0001***
Total oxysterol	0.292	0.256	0.535	0.033*	0.865	< 0.0001***
Lathosterol						
Desmosterol	0.225	0.384	0.335	0.204	0.005	0.985
Lanosterol	0.358	0.159	0.615	0.011*	0.355	0.162
Total precursors	0.705	0.001***	0.502	0.047*	0.142	0.586
Total CH	-0.762	0.0004***	-0.741	0.001***	-0.335	0.187
Total oxysterol	0.475	0.054	0.429	0.097	0.127	0.626
Desmosterol						
Lanosterol	0.343	0.178	0.765	0.0006***	0.716	0.001**
Total precursors	0.625	0.007**	0.747	0.0009***	0.765	0.0003***
Total CH	-0.436	0.080	-0.674	0.004**	-0.770	0.0003***
Total oxysterol	0.333	0.191	0.391	0.134	0.608	0.010**
Lanosterol						
Total precursors	0.446	0.073	0.785	0.0003***	0.836	< 0.0001***
Total CH	-0.725	0.001***	-0.829	< 0.0001***	-0.922	< 0.0001***
Total oxysterol	0.324	0.205	0.279	0.295	0.806	< 0.0001***
Total precursors						
Total CH	0.705	0.001***	-0.829	< 0.0001***	-0.870	< 0.0001***
Total oxysterol	0.767	0.0003***	0.547	0.028*	0.936	< 0.0001***
Total CH						
Total Oxysterol	-0.706	0.002**	-0.535	0.033*	-0.826	< 0.0001***

SPC, Spearman's correlation with associated *p*-value; AD, Alzheimer's disease; CH, cholesterol; MCI, mild cognitive impairment; NCI, no cognitive impairment. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

4 β -Hydroxycholesterol, a housekeeping oxysterol, was included in this analysis but not depicted here. Correlation values with a magnitude greater than 0.5 were considered of interest.

individual metabolites, we determined that 24-OH and lathosterol (CH precursor) decrease as a function of age in AD brain, but increase in relation to age in NCI and MCI (Fig. 1b and c). 24-OH is the product of an enzymatic hydroxylation of CH at the 24th carbon yielding a more hydrophilic sterol capable of traversing the BBB (Leoni *et al.* 2003; Bjorkhem *et al.* 2006; Vaya and Schipper 2007). To examine the possibility that the observed decrease in 24-OH might be because of neuronal loss in AD and an attendant decrease in the levels and activity of CYP46A1 (cholesterol 24-hydroxylase), we standardized the levels of these sterols to mg of cellular protein (rather than total CH) and plotted 24-OH/mg protein as a function of total CH/mg protein. Disease state did not impact this relationship suggesting that the decrease in 24-OH may be the result of an increase in total free CH (likely derived from widespread myelin and cell membrane collapse) that exceeds the brain's capacity for conversion to 24-OH. Linkages between augmented pools of

free CH and degeneration of neuronal perikarya and synapses in AD brain have been previously reported (Vaya and Schipper 2007).

There is currently considerable interest in the role(s) CH may play in the etiopathogenesis of AD. CH promotes β -secretase activity (Xiong *et al.* 2008) and β -amyloid deposition (Burns *et al.* 2003) and may influence the stability of lipid rafts (Simons and Ehehalt 2002) and NFT (Distl *et al.* 2003). Acting through these (and possibly other) mechanisms, curtailment of CH export from the aging AD brain may exacerbate the neurodegenerative process and account for the inverse relationship of brain CH and MMSE scores observed in these subjects. Sequestration of CH in aging AD brain may also be responsible for the concomitant decline in the CH precursor, lathosterol (Fig. 1), a widely accepted marker of CH biosynthesis (Kempen *et al.* 1988), as CH inhibits the latter via feedback servomechanisms (Goldstein and Brown 1990). A similar phenomenon may

Regression model	Total CH Coefficient (<i>p</i> -value)	Total precursors Coefficient (<i>p</i> -value)	Total oxysterols Coefficient (<i>p</i> -value)
Slope coefficient terms			
Ln HO-1 among NCI	356 (0.6)	-0.000864 (0.6)	-0.000587 (0.6)
Ln HO-1 among MCI	-3719 (0.08)*	0.00462 (0.3)	-0.000235 (0.9)
Ln HO-1 among AD	-660 (0.5)	0.00552 (0.01)**	0.005447 (0.001)**
Age	-	0.000140 (0.09)*	-
Gender (1 male and 0 female)	752 (0.09)*	-	-
E4 Allele (1 yes and 0 no)	-	-0.00185 (0.07)*	-0.00192 (0.01)**
Intercept terms			
Among NCI	381 (0.9)	-0.00257 (0.8)	0.00527 (0.098)*
Among MCI	12 533 (0.06) *	-0.01889 (0.2)	0.00526 (1.0)
Among AD	3159 (0.4)	-0.01921 (0.03)**	-0.01072 (0.004)**
<i>R</i> ²	0.165	0.323	0.342
Overall model <i>p</i> -value	0.05	0.02	0.005

Table 4 Multivariable linear regression models for each of the outcomes: total CH, total CH precursors, and total oxysterols

AD, Alzheimer's disease; CH, cholesterol; MCI, mild cognitive impairment; NCI, no cognitive impairment; *0.05 < *p* < 0.10; ***p* < 0.05.

Each column represents a separate model with the following equation: Outcome level = Intercept term + Ln HO-1 coefficient × Ln HO-1 + age coefficient × age + gender coefficient × gender (1 if male; 0 if female) + E4 allele coefficient × E4 allele category (1 if E4 allele; 0 otherwise); *p*-values are reported for each estimated coefficient indicating whether the value is statistically different from 0. Overall *p*-values represent the significance of all variables in each model. The *R*² correlation coefficient is the proportion of total variance in the outcome variable that is explained by the regression equation; (-) represents a variable that was not significant in the model and was therefore not included.

explain the progressive diminution of brain lathosterol levels in the amyloid precursor protein 23 mouse model of AD (Lutjohann *et al.* 2002).

The strong association of age with β-epoxide in MCI (Fig. 1a), but not NCI or AD, may further inform current thinking concerning the role of oxidative stress in the pathogenesis of AD. β-epoxide is a metabolite of several potentially amyloidogenic sterol species derived from the autoxidation of CH (Wang *et al.* 2007) and a reliable oxidative stress reporter molecule (Ferderbar *et al.* 2007). Our findings argue that, in the course of AD, non-enzymatic oxidative modification of brain sterols is maximal in pre-clinical (MCI) stages of the illness. This formulation is compatible with earlier reports of (i) increased levels of 8,12-isoPF2α, an index of lipid peroxidation, in the CSF, plasma, and urine of subjects with MCI relative to persons without cognitive impairment (Pratico *et al.* 2002) and (ii) elevated brain 8-hydroxy-2'-deoxyguanosine and nitrotyrosine immunoreactivities, markers of oxidative nucleic acid and protein damage, respectively, in very early AD which decline with progressive disease severity (Nunomura *et al.* 2001).

The linear associations between HO-1 protein concentrations and CH, oxysterols and CH precursors (current study) are consistent with our earlier observations in HO-1 transfected rat astroglia (Vaya *et al.* 2007; Hascalovici *et al.* 2009). In both the latter and in AD-affected human brain tissue (Fig. 2), total CH decreases and oxysterols and CH

precursors/biosynthesis increase in proportion to HO-1 protein levels. Together, our findings suggest that, in AD astrocytes, enhanced CO signaling resulting from HO-1-mediated degradation of endogenous heme stimulates the CH biosynthetic pathway, while heme-derived iron and CO promote CH efflux via activation of the liver X receptor. Our *in vitro* data indicate further that the glial iron may indirectly contribute to CH efflux by provoking formation of oxysterols, natural ligands of the liver X receptor (*ibid.*). Conceivably, enhanced egress of CH from the astrocytic compartment provides essential sterols for neuronal repair and synaptogenesis (neuroplasticity) in MCI and initial stages of AD. Under the influence of HO-1, CH efflux may exceed biosynthesis such that net glial CH content is diminished (providing further stimulus for CH biosynthesis).

Correlations of oxysterol subtypes, enzymatic oxysterols and oxysterols derived from CH autoxidation were determined for all possible associations. Our analysis uncovered disease-specific changes in the associations of individual metabolites favoring a state of dysregulated sterol homeostasis in AD and MCI relative to NCI (Table 3). Strict regulation of these pathways is likely essential for normal brain function; while enzymatically generated oxysterols are indispensable for CH turnover in the CNS, CH autoxidation products, accruing from free radical attack on the CH molecule, have neurotoxic properties (Nelson and Alkon 2005).

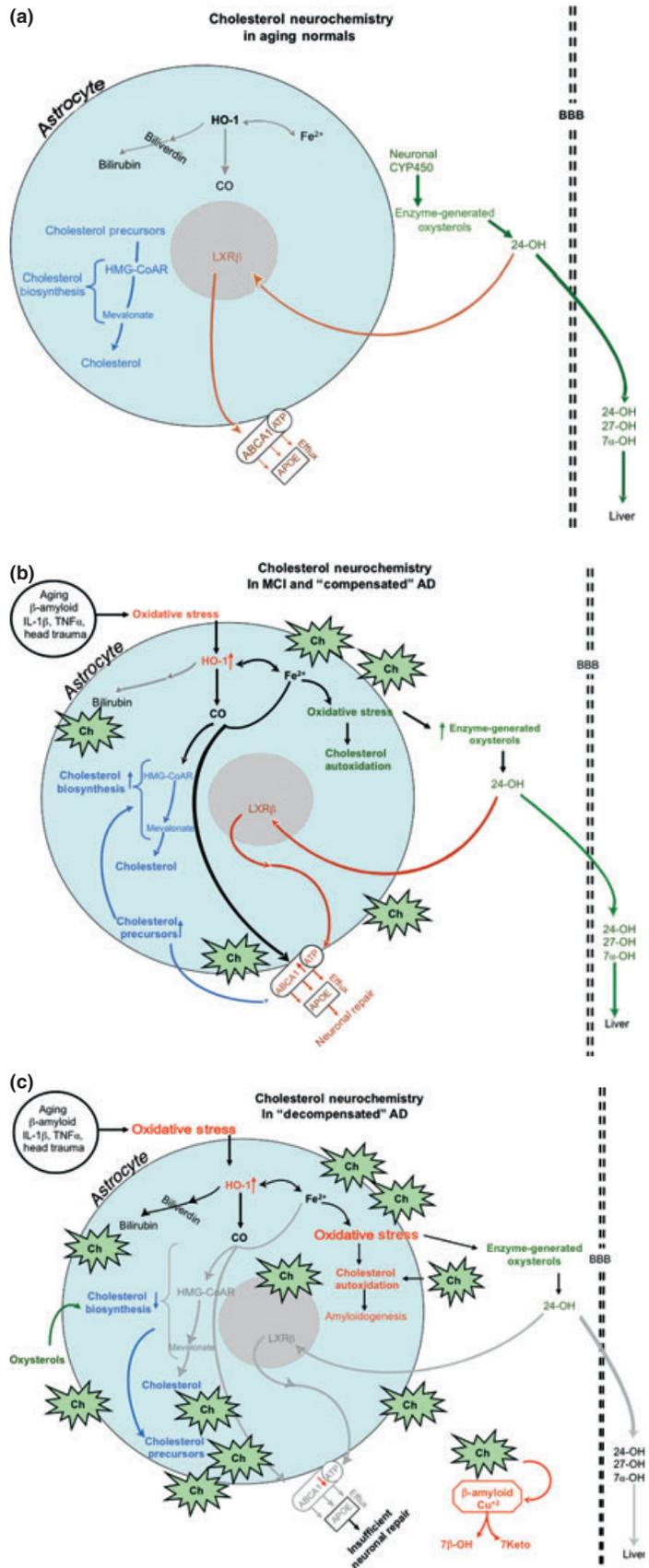


Fig. 3 A model for sterol dysregulation in AD brain. (a) *In the normal aging brain*, sterol homeostasis is maintained by pathways governing baseline CH biosynthesis, CH efflux, and oxysterol formation. Glial HO-1 expression and its influence on sterol metabolic pathways are *minimal*. (b) In initial, 'compensated' *MCI and AD*, enhanced HO-1 expression stimulates CH biosynthesis, oxysterol formation, and CH efflux by the astroglial compartment. Glial CH efflux and oxysterol formation (facilitating CH delivery for neuronal repair and egress across the blood–brain barrier) exceeds biosynthesis and CH levels in affected brain are *normal or diminished*. (c) In 'de-compensated' *AD*, massive uptake of CH (derived from widespread neuronal degeneration) overwhelms glial efflux pathways resulting in *increased* brain CH levels. The latter suppresses *de novo* CH biosynthesis (negative servomechanism as in peripheral tissues) and brain CH precursor levels decline. Enhanced free radical production in AD-affected tissues oxidizes the excess CH deposits to a host of oxysterols promoting further oxidative stress and disease progression.

On the basis of the current (human) data sets, our previous *in vitro* work on astroglial HO-1/sterol interactions (Vaya *et al.* 2007; Hascavici *et al.* 2009) and the available literature on the topic, we propose a model that organizes the findings in a mechanistic and testable fashion that should facilitate further investigation of sterol dysregulation in AD and MCI: In the *normal aging brain*, sterol homeostasis is maintained by pathways governing baseline CH biosynthesis, CH efflux, and oxysterol formation (Fig. 3a). Glial HO-1 activity/expression is present at physiological levels and its influence on sterol homeostasis is minimal. In *MCI and 'compensated' AD* (Fig. 3b), various pro-oxidant and inflammatory stressors (e.g. β -amyloid, hydrogen peroxide, and Th1 cytokines) induce the *HMOX1* gene in affected astroglia (Schipper 2004). Resulting products of heme catabolism (CO and ferrous iron) stimulate CH biosynthesis, oxysterol formation, and CH efflux by the astroglial compartment. Glial CH efflux facilitates CH delivery for neuronal repair and transport of surplus sterol (as 24-OH) across the BBB. CH egress exceeds biosynthesis and CH levels in affected brain are initially unchanged or diminished. Our data suggest that a threshold may be reached in the aging AD brain whereby the ability of HO-1 to impact the CH regulatory pathway is overwhelmed by excessive CH accumulation attending widespread neuronal degeneration and failure of sterol homeostatic mechanisms. In this 'de-compensated' AD state (Fig. 3c), glial efflux pathways cannot meet demand and brain CH progressively increases. The latter, in turn, suppresses *de novo* CH biosynthesis and brain CH precursor levels decline. In 'de-compensated' AD, the aberrant CH deposits may exacerbate the neurodegenerative process by (i) undergoing autoxidation to neurotoxic lipid species, (ii) accelerating β -amyloid deposition, and (iii) promoting tau hyperphosphorylation and NFT formation (Xiong *et al.* 2008).

In this study, we have provided direct evidence of deranged sterol homeostasis in the brains of persons with MCI and definite sporadic AD based on a comprehensive survey of total CH, CH precursors, and oxysterols. In conjunction with data derived from earlier cell culture studies, we propose a model whereby up-regulation of HO-1 favorably impacts sterol regulatory pathways in MCI and younger 'compensated' AD brains, whereas failure of this HO-1/sterol axis in later, 'de-compensated' AD exacerbates the degenerative process by facilitating neural CH deposition. Attempts to forestall deterioration to this 'de-compensated' state, by pharmacological or other means, may constitute a rationale neuroprotective strategy for the management of AD and possibly other aging-related neurodegenerative disorders.

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Disclosures

Hyman Schipper has served as consultant to Osta Biotechnologies, Molecular Biometrics Inc., TEVA Neurosciences, and Caprion Pharmaceuticals. He holds stock options in Osta and equity in Molecular Biometrics Inc. None of the authors has financial interests related to the material contained in this article.

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