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Alteration of High-Density Lipoproteins functionality in Alzheimer’s disease patients

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Abstract

The aims of the present study were to determine whether High-Density Lipoproteins functionality (HDL)-mediated cholesterol efflux is altered in Alzheimer’s disease (AD) and to investigate the role and effect of amyloid-beta (Aβ) in the regulation of the anti-atherogenic activity of HDL. Eighty-seven elderly subjects were recruited, of whom 27 were healthy, 27 had mild cognitive impairment (MCI), and 33 had mild Alzheimer’s disease (mAD). Our results showed that total cholesterol levels are negatively correlated with the Mini Mental State Examination (MMSE) score ($r = –0.2602, p = 0.0182$). HDL from the mAD patients was less efficient at mediating cholesterol efflux from J774 macrophages ($p<0.05$) than HDL from the healthy subjects and MCI patients. While HDL from the MCI patients was also less efficient at mediating cholesterol efflux than HDL from the healthy subjects, the difference was not significant. Interestingly, the difference between the healthy subjects and the MCI and mAD patients with respect to the capacity of HDL to mediate cholesterol efflux disappeared when ATP-binding cassette transporter A1 (ABCA1)-enriched J774 macrophages were used. HDL fluidity was significantly inversely correlated with the MMSE scores ($r = –0.4137, p<0.009$). In vitro measurements of cholesterol efflux using J774 macrophages showed that neither Aβ$_{1-40}$ nor Aβ$_{1-42}$ stimulate cholesterol efflux from unenriched J774 macrophages in basal or ABCA1-enriched J774 macrophages.

**Keywords:** HDL, cholesterol efflux, amyloid beta, Alzheimer’s disease
Introduction

Alzheimer's disease (AD) is the principal cause of dementia worldwide, affecting approximately 35 million people. Since the major risk factor for AD is age, the aging of the population means that the number of people suffering from AD will only increase. The mechanism underlying AD is not clearly understood. As such, no treatments are currently available to prevent, slow, or arrest this disease.

The two main pathological characteristics of AD are the accumulation of amyloid-beta (Aβ) peptides in senile plaques and the presence of neurofibrillary tangles in the brain. Senile plaques are formed by the aggregation of Aβ peptides, mainly Aβ40 and Aβ42. These peptides are derived from the two-step proteolytic cleavage of the amyloid precursor protein (APP). During the first step, APP is cleaved either by an α-secretase (non-amyloidogenic pathway) or by a β-secretase (BACE) (amyloidogenic pathway). During the second step, γ-secretase induces the production of Aβ if APP has been cleaved by BACE. Aβ are also produced in healthy aged people. However, the physiological role of Aβ is not clear. Some studies have shown that the Aβ are involved in cholesterol transport by forming High density lipoproteins (HDL)-like particles (Michikawa et al. 2001; Nomura et al. 2013; Umeda et al. 2010).

Cholesterol is an essential component of the brain. Cholesterol in the brain accounts for 25% of all cholesterol in the body and plays an important role in neuronal plasticity and function. High cholesterol levels in cells, particularly in lipid rafts, induce the amyloidogenic cleavage of APP, resulting in enhanced Aβ formation (Xiong et al. 2008). Conversely, low cholesterol levels induce the non-amyloidogenic cleavage of APP and a decrease in β-secretase activity. Cholesterol metabolism, especially cholesterol transport, may thus be involved in the development and progression of AD.

Epidemiological and experimental studies have shown that hypercholesterolemia is a risk factor for AD (Refolo et al. 2000; Refolo et al. 2001; Shie et al. 2002; Umeda et al. 2012; Wolozin 2001). Moreover, total cholesterol levels in the AD brain are higher than those in the
healthy brain (Xiong et al. 2008). These studies suggest that cholesterol metabolism, especially cholesterol efflux, may be altered in AD. Alterations of cholesterol efflux can also induce an increase in cholesterol levels in the brains, contributing to an increase in Aβ levels.

Cholesterol efflux is mediated by apolipoproteine A-1 (apoA-1) and HDL particles and allows the transport of cholesterol from cells to the circulation for elimination by the liver. Three cholesterol efflux pathways have been identified: passive diffusion, Scavenger receptor class B type 1 (SR-B1) receptor mediated-cholesterol efflux, and ATP-binding cassette transporter A1 and G1 (ABCA1/ABCG1)-mediated cholesterol transport. Cholesterol efflux is initiated by the efflux of phospholipids and cholesterol to apoA-1 via its interaction with the ABCA1 transporter. We previously showed that ApoA-1 purified from the plasma of AD patients is less effective at mediating cholesterol efflux from J774 macrophages via the ABCA1 pathway than apoA-1 purified from healthy plasma and have suggested that the interaction between apoA-1 and the ABCA1 transporter may be altered in AD (Khalil et al. 2012).

Given the importance of cholesterol efflux in the regulation of membrane cholesterol and the modulation of Aβ formation, the aims of the present study were to determine whether HDL-mediated cholesterol efflux is altered in AD and to investigate the role and effect of Aβ in the regulation of HDL-mediated cholesterol efflux.
Materials and methods

Reagents and Cell Line

\( n \)-butanol and methanol were from Fisher (Canada). 1,1,3,3-Tetraethoxypropane, ethylenediaminetetraacetic acid (EDTA), 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium salt (cpt-cAMP), sodium hydroxide (NaOH), polyethylene glycol (PEG), Lipopolysaccharide (LPS), butylated hydroxytoluene (BHT), 2-thiobarbituric acid, 1,6-diphenyl-1,3,5-hexatriene (DPH), DNPH, and DTNB were from Sigma (USA). The J774 cells were from the American Type Culture Collection (ATCC, USA). RPMI 1640, Dulbecco’s-modified medium (DMEM), penicillin/streptomycin, sodium pyruvate, PBS, bovine serum albumin (BSA), and fetal bovine serum (FBS) were from Wisent Inc. (Canada). M-CSF was from Peprotech (Canada). \[^{3}H\]-cholesterol was from Perkin Elmer. A\( \beta \)40 and A\( \beta \)42 were from Tocris Bioscience (USA).

Subjects

Eighty-seven elderly subjects were recruited, of whom 27 were healthy (mean age 71 ± 5.39 years), 27 had mild cognitive impairment (MCI) (mean age 74 ± 5.02 years), and 33 had mild ADimer’s disease (mAD) (mean age 79 ± 4.7 years). The healthy elderly subjects were living at home and were functionally independent and cognitively intact. They were selected based on the SENIEUR (Senior Europeans) protocol recognized by the EURAGE Program on Aging of the European Community as the standard selection protocol for immuno-gerontological studies(Ligthart et al. 1984). The MCI and mAD patients were selected from the records of the Memory Clinic of the Sherbrooke University Geriatric Institute. The mAD patients had been diagnosed with probable AD based on the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) published by the American Psychiatric Association in 1994 and the criteria of the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer’s Disease and Related Disorders Association (NINCDS_ADRDA)(McKhann et al. 1984). All the healthy subjects and MCI and AD patients underwent the Folstein Mini-Mental State Examination (MMSE) test (Folstein et al. 1975). All other dementias and neurological and psychiatric disorders were excluded. The AD patients had no vascular problems, no kidney or liver disease, and were only slightly hypertensive. None had cancer or a history of cerebral vascular accident, and none was taking a statin or lipid-lowering agent. The study
protocol was approved by the Ethics Committee of the University of Sherbrooke. Informed, written consent was obtained from all the participants and/or their representatives. Their characteristics are summarized in Table 1.

**Blood collection**

After overnight fasting, 80 ml of blood was collected in heparin-containing vacuum tubes. The plasma was separated by low speed centrifugation (260 x g, 15 min, 22°C) and was stored at –80°C until used. The pellet was used to isolate monocytes.

**Human monocyte isolation and differentiation into macrophages**

Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation on Ficoll-Paque™ Plus columns (GE Healthcare Life Sciences). They were resuspended in RPMI 1640 medium containing penicillin and streptomycin.

Six-well or twelve-well plates were pre-coated with heat-inactivated AB human serum (Invitrogen) for 30 min. The PBMC were plated in twelve-well (3 x 10^6 cells per well) or six-well (10 x 10^6 cells per well) plates and were allowed to attach for 30 min. Unattached cells were removed, and adherent monocytes were washed twice with RPMI 1640 medium. The monocytes were cultivated in RPMI 1640 medium containing 10% heat-inactivated FBS, penicillin/streptomycin, and 50 ng/ml of M-CSF to allow differentiation into monocyte-derived macrophages (MDM). The medium was changed every 2-3 days. Differentiation into macrophages was complete after 7 days. The MDM were stimulated with 100 ng/mL of LPS for 24 h to direct the macrophages to the M1 route and to induce them to overexpress the ABCA1 transporter. These macrophages were used to measure cholesterol efflux and ABCA1 expression.

**Measurement of ABCA1 expression on MDM**

The MDM were incubated in 1% fixation buffer (BioLegend, USA) for 10 min and were then centrifuged at 5000 rpm for 5 min at 22°C. Non-specific sites were blocked for 15 min with 1% BSA in PBS. The cells were permeabilized for 15 min in Permwash (BD Bioscience, Canada) and were incubated for 30 min at room temperature with an anti-ABCA1 primary antibody (ab18180, 3 µg/10^6 cells; Abcam). The MDM were washed three times with PBS and were then incubated for 30 min at room temperature with goat anti-mouse IgG H&L (Alexa Fluor 488) antibody (ab150113, 1:500; Abcam). Following three washes with PBS, the cells were
resuspended in 200 µL of PBS, sorted by fluorescence activated cell sorting (FACS), and analyzed using FlowJo software.

**Isolation of HDL**

HDL were isolated by using the polyethylene glycol (PEG) precipitation method. Briefly, the plasma was mixed with an equal volume of PEG solution (20%, MW 5000-7000), and the mixture was incubated for 20 min at room temperature. The mixture was then centrifuged at 2000 x g for 20 min at 4°C, and the supernatant was collected (Izzo et al. 1981).

**Cholesterol efflux measurements**

Cholesterol efflux was measured as described previously (Berrougui et al. 2012). Briefly, MDM and J774 macrophages were loaded with [³H]-cholesterol (1 µCi/ml) for 24 h. Labeled macrophages were washed and were equilibrated in serum-free medium containing 1% BSA for an additional 12 h.

To produce ABCA1-enriched cells, [³H]-cholesterol-loaded J774 macrophages were equilibrated in DMEM containing 1% BSA and 0.3 mM cAMP (8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium salt) for 12 h (Bortnick et al. 2000). The macrophages were then washed before being used for the cholesterol efflux measurements.

Labeled MDM were incubated for 4 h with plasma obtained from the same patient. J774 macrophages and ABCA1-enriched J774 macrophages were incubated for 4 h with HDL obtained from each patient. The medium was recovered and was centrifuged at 17 000 x g for 15 min at 4°C to eliminate cell debris. The cells were washed in PBS and were lysed in PBS containing 1 M NaOH. The radioactivity in the medium and the cell lysate was measured using a liquid scintillation counter (LS6500 multi-purpose scintillation counter; Beckman, USA). The percentage of cholesterol efflux was calculated using the following formula: [radioactivity of medium / (radioactivity of medium + radioactivity of lysate of cells)] x 100. The radioactivity is expressed in counts per minute (cpm).
Measurement of sulfhydryl groups

Free sulfhydryl (SH) groups on proteins in the plasma and HDL were measured using the DTNB method as described by Hu (Hu 1994).

Measurement of carbonyl groups

Plasma and HDL protein carbonyl levels were assayed as described previously (Reznick et al. 1994). Briefly, carbonyl levels were determined by dinitrophenylhydrazine (DNPH) derivatization. DNPH reacts with carbonyl groups to form hydrazones, and the change in absorbance is measured at 360 nm ($\epsilon = 22,000 \text{ M}^{-1} \text{ cm}^{-1}$).

Measurement of lipid peroxidation

Lipid peroxidation was determined in the plasma and HDL by measuring malondialdehyde (MDA) and conjugated diene levels, respectively.

Plasma MDA levels were measured by high-performance liquid chromatography (HPLC) using a 5 µm ODS 100 mm x 4.6 mm HP Hypersil column, a 5 µm ODS guard column, and a methanol-buffer (40 : 60, v:v) mobile phase. The buffer was 50 mM potassium monobasic phosphate. The pH was adjusted to 6.8 using 5 M KOH (Agarwal et al. 2002). The fluorescence detector was set at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. Plasma samples were treated with BHT and were heat derivatized at 100°C for 1 h with thiobarbituric acid at acidic pH. Plasma MDA was extracted with n-butanol, and 10 µL was injected on the column. A standard curve of 1,1,3,3-tetraethoxypropane (TEP) was prepared to determine the concentration of MDA.

HDL lipid peroxidation was evaluated by measuring conjugated diene formation. Briefly, the absorbance was measured at 234 nm in a 1 cm path length cuvette using a spectrophotometer (UH5300; Hitachi, Canada).

Measurement of HDL fluidity

HDL fluidity was based on measurements of the steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) as described previously (Helal et al. 2013). Briefly, 25 µg of HDL was incubated with 1 µM DPH for 15 min at 37°C. The steady-state fluorescent polarization of DPH was measured using a fluorescence spectrophotometer (model F-4500; Hitachi, Canada). Fluidity is the inverse value of anisotropy and is expressed as [1/$r$: steady-state fluorescence anisotropy]. $r$
was calculated using the following formula: \[ \frac{(I_v - G_{lp})}{(I_v - 2G_{lp})} \], where \( I_v \) and \( I_p \) are the parallel and perpendicular polarized fluorescence intensities, respectively, and \( (G) \) is the monochromator grating correction factor.

**Measurement of plasma Aβ42 levels**

Plasma Aβ42 levels were measured by ELISA using SensoLyte® Anti-Human βeta-Amyloid (1-42) Quantitative ELISA colorimetric kits (Anaspec) according to the manufacturer's instructions.

**Statistical analysis**

Values are expressed as means ± SEM. A one-way ANOVA was used for multiple comparisons, followed by a Bonferroni and the Pearson correlation was calculated to assess the association between two continuous variables. All results were interpreted at an alpha level of 0.05. The statistical analyses were performed using GraphPad Prism 6.0 software.
Results

The baseline characteristics of the healthy and patient groups are summarized in Table 1. There was a significant difference between the healthy subjects and the MCI and mAD patients with respect to age (71 ± 5.39, 74 ± 5.02 and 79 ± 4.70 years, respectively). There was also a significant difference between the MMSE scores of the healthy subjects (MMSE = 29.67 ± 0.73) and the MCI (MMSE = 27.26 ± 1.95, \( p < 0.001 \)) and mAD (MMSE = 23.36 ± 3.85, \( p < 0.0001 \)) patients. However, there were no significant differences for biochemical parameters, including lipid profiles (triglycerides, total cholesterol, Low Density Lipoproteins (LDL) and HDL cholesterol), apoAS1 concentrations, and apoA-1/HDL ratios.

Measurement of plasma-mediated cholesterol efflux

In order to investigate the difference between the healthy subjects and the MCI and mAD patients with respect to the efflux of cholesterol from macrophages to the plasma, MDM from each patient were separately loaded with \(^{3}H\)-cholesterol for 24 h and were then incubated with 5\% plasma from the same patient for 4 h. Cholesterol efflux from the MDM to the plasma was significantly lower for the MCI and mAD patients than for the healthy subjects (\( p < 0.05 \)) (Figure 1A). However, efflux was identical for the MCI and AD patients, suggesting that the alterations had already occurred in the MCI patients prior to AD development.

The level of cholesterol efflux depends on the capacity of macrophages to liberate cholesterol and on apoA-1/HDL to accept cholesterol. The process is initiated by an interaction between apoA-1 and the ABCA1 transporter on macrophages. To determine which factor is involved in the decrease in cholesterol efflux from the MDM to the plasma of the MCI and mAD patients, we measured ABCA1 expression on the MDM obtained from each patient and from the healthy subjects. There were no significant differences between ABCA1 expression on the MDM from the healthy subjects and from the MCI and mAD patients (Figure 1B), indicating that disease-associated differences did not reside at the level of ABCA1 transporter expression.

Alteration in the capacity of HDL to mediate cholesterol efflux in Alzheimer's disease

To further investigate why cholesterol efflux is altered in AD, the capacity of HDL from each patient to mediate cholesterol efflux from macrophages was measured. J774 macrophages were used to compare the cholesterol efflux capacity of HDL as a function of health status (healthy, MCI, and mAD). HDL was incubated for 4 h with unenriched J774 and ABCA1-enriched J774
macrophages (pre-incubated with 0.3 mM cAMP to induce ABCA1 expression) previously loaded with [\(^3\)H]-cholesterol. The capacity of HDL from the mAD patients to mediate cholesterol efflux from unenriched J774 macrophages was relatively low \((p < 0.05)\) (Figure 2) compared to the healthy and MCI groups. The capacity of HDL from MCI patients to mediate cholesterol efflux from enriched J774 macrophages was lower than that of HDL from the healthy subjects, but the difference was not significant (Figure 2).

**Measurement of the oxidation of proteins and lipids in the plasma and HDL**

In order to understand the alteration in the capacity of HDL to mediate cholesterol efflux, protein oxidation and lipid peroxidation levels were measured in the plasma and HDL. We previously showed that the age-related reduction of HDL-mediated cholesterol efflux is due, in part, to oxidative modifications to apoA-1 (Berrougui et al. 2007). Oxidative modifications to proteins are determined by measuring free sulphhydryl (SH) and carbonyl group content. Interestingly, our results showed that AD patients possess high plasma SH group content compared to the healthy subjects \((p < 0.05)\) (Figure 3A). On the other hand, there was no significant difference among the three groups in terms of plasma carbonyl group content and MDA content (Figures 3B and 3C). While, the MCI and mAD patients had a slightly lower plasma carbonyl group content than the healthy subjects, the difference was not significant (Figures 3B and 3C).

We then measured the free SH group, carbonyl group, and conjugated diene content of HDL from the three groups of subjects. The SH group content of the HDL from the two groups of patients decreased progressively with the progression of the disease. The decrease was significant in the mAD patients compared to the healthy subjects \((p < 0.05)\) (Figure 4A). While the carbonyl group content of HDL from the MCI and mAD patients increased with the progression of the disease, there was no significant difference between these two groups (Figure 4B). In addition, the conjugated diene content of the HDL from the MCI and mAD patients was significantly higher than that of the healthy subjects (Figure 4C).

**Alteration of HDL fluidity during cognitive decline**

In addition to oxidative modifications to HDL, the biophysical properties of HDL may help explain the decrease in the capacity of HDL from mAD patients to mediate cholesterol efflux. The fluidity of the lipid fraction of HDL is a major determinant of the capacity of HDL to
mediate cholesterol efflux. Interestingly, despite the oxidative alterations to HDL as measured by conjugated diene formation, the fluidity of HDL from the mAD patients was higher than that of HDL from the healthy subjects (Figure 5A). The fluidity of HDL from the MCI patients was also higher, but the difference was not significant. In addition, HDL fluidity was significantly inversely correlated with the MMSE scores ($r = -0.4137$, $p < 0.009$, Figure 5B). The increase in the fluidity of HDL from the mAD patients suggests that the HDL particles had undergone biochemical changes.

**Effect of Aβ1-42 on HDL fluidity**

We next investigated the relationship between Aβ$_1$-42 levels and cholesterol homeostasis. A previous study by Umeda et al. had shown that Aβ$_1$-42 mediates cholesterol transport from the brain to the circulation, suggesting that Aβ is involved in the maintenance of cellular cholesterol homeostasis (Umeda et al. 2010). Based on this, we measured the Aβ$_1$-42 content of plasma from the healthy subjects and the MCI and mAD patients. Our results showed that that MCI patients had significantly higher levels of plasma Aβ$_1$-42 than the healthy subjects (Figure 6A). The mAD patients also had higher levels of plasma Aβ$_1$-42 than the healthy subjects although the difference was not significant (Figure 6A). Since Aβ can be transported by HDL (Koudinov et al. 1996), we hypothesized that Aβ$_1$-42 associates with and modifies the structure of HDL, altering cholesterol efflux. To investigate this possibility, we measured HDL fluidity in the presence of Aβ$_1$-42. Our results showed that incubating pooled purified HDL with Aβ$_1$-42 for 4 h significantly reduced HDL fluidity (Figure 6B), depending on the origin of the HDL (Figure 6B). While no changes were observed in HDL fluidity when Aβ$_1$-42 was incubated with HDL from the healthy subjects, Aβ$_1$-42 significantly decreased the fluidity of HDL from the mAD patients (Figure 6C; $p < 0.05$). It should be noted that this decrease resulted in a return of the fluidity of HDL from MCI and mAD patients to the normal level seen in healthy subjects.

**Effect of Aβ$_1$-42 and Aβ$_1$-40 on cholesterol efflux**

We measured the capacity of Aβ$_1$-42 and Aβ$_1$-40 to stimulate HDL-mediated cholesterol efflux in vitro. Our results showed that Aβ$_1$-42 and Aβ$_1$-40, alone or in the presence of HDL, had no effect on cholesterol efflux from unenriched J774 macrophages (Figure 7). However, when ABCA1-enriched J774 macrophages were used, Aβ$_1$-42 significantly reduced the capacity of HDL to
mediate cholesterol efflux compared to HDL alone or to HDL incubated in the presence of \( \text{A}\beta_1 \). \( \text{A}\beta_{1-42} \) appeared to alter HDL-mediated cholesterol efflux via the ABCA1 transporter by interacting with the transporter or by altering the structure of the HDL.
Discussion

While the exact cause of AD is unknown, Aβ plaques contribute to AD and are correlated with disease progression. The production of Aβ is influenced by the cholesterol content of neuron membranes (Abad-Rodriguez et al. 2004) and increases in parallel with the increase in cholesterol content (Simons et al. 1998; Xiong et al. 2008). Cellular cholesterol levels are strictly regulated by apoA-1/HDL-mediated cholesterol efflux via membrane ABCA1/ABCG1 transporters and the SR-BI receptor (Phillips 2014; Zannis et al. 2006). We previously showed that ABCA1-mediated cholesterol efflux is altered in AD (Khalil et al. 2012). In the present study, we examined cholesterol efflux in MCI and AD and found that changes to the oxidative status and fluidity of HDL also have an impact on cholesterol efflux, with a putative very early protective role followed by the deleterious participation of Aβ. We also investigated these changes in MCI, which is the prodromal state of AD.

The lipid measurements showed that total cholesterol levels tended to be higher in the MCI and mAD patients than in healthy subjects. In addition, total cholesterol levels were negatively correlated with the MMSE scores ($r = -0.2602$, $p = 0.0182$). LDL levels also tended to be higher in the mAD patients than in the healthy subjects, but not significantly so; reciprocally, HDL levels were lower in the mAD patients than in the healthy subjects, but again this difference was not statistically significant. These results suggest that cholesterol metabolism may be altered in MCI and AD, albeit not markedly. However, in biological terms, this could have an important impact over the long term. Indeed, some studies have shown that a decrease in HDL levels is a risk factor for dementia (Singh-Manoux et al. 2008; Zuliani et al. 2010) and that an increase of HDL levels can lower the risk of developing AD (Reitz et al. 2010). Together, these data suggest that cholesterol efflux may influence the development and progression of AD.

Our results showed that the apoA-1 levels of the MCI and mAD patients and the healthy subjects did not change. A study by Bergt et al. showed that the plasma levels of apo-A1 were the same in AD patients and in healthy subjects (Bergt et al. 2006). In contrast, some studies have shown that apo-A1 levels decrease during the progression of cognitive decline (Kawano et al. 1995). Collectively, these findings suggest that cholesterol transport may be altered in AD and that a decrease in apo-A1 levels does not explain this alteration.

To determine whether cholesterol efflux is altered in AD, we measured this parameter using macrophages isolated from the patients, and their plasma as a cholesterol acceptor. Our results
showed that cholesterol efflux was significantly lower in the MCI and mAD patients than in healthy subjects, suggesting that cholesterol efflux is disrupted in MCI and mAD but that the disruption cannot be explained by a decrease in apo-A1 levels. PBMCs of Alzheimer’s patients accumulate more cholesterol and are less effective at mediating cholesterol efflux than those of healthy subjects (Pani et al. 2009). Our results also showed that HDL levels and cholesterol efflux were lower in mAD patients than in the healthy subjects. A recent study by Voloshyna et al. (Voloshyna et al. 2013) showed that the plasma of patients with rheumatoid arthritis manifested disturbed cholesterol homeostasis in macrophages due to decreased expression of cholesterol transporters (ABCA1 or ABCG1). So, unknown deleterious substances in the plasma could reduce the expression of cholesterol transporters on macrophages and result in a reduction of the cholesterol efflux. Besides, the lower cholesterol efflux observed in AD could be due to a decrease in the capacity of HDL to mediate cholesterol efflux.

The present study showed that cholesterol efflux was altered in the MCI and the mAD patients compared to the healthy subjects. Because there were no significant difference between the three groups in terms of ABCA1 expression on MDM, it is unlikely that the plasma-mediated decrease in cholesterol efflux observed in the MCI and mAD patients can be explained by a decrease in the expression of ABCA1 expression or a decrease in apo-A1 levels. Mandas et al. recently reported that ABCA1 transporter mRNA levels in macrophages are the same in Alzheimer patients and aged healthy subjects (Mandas et al. 2012). A study by Malik et al. showed that amyloid beta peptides can increase the expression of ABCA1 by neurons (Malik et al. 2012). Interestingly, we showed a trend towards higher expression of ABCA1 in the MCI patients relative to healthy subjects. On the other hand, Aβ42 levels were significantly higher in the MCI patients than in the healthy subjects. The slight increase in ABCA1 expression was thus likely not entirely due to increased Aβ42 levels but these may have contributed to some extent to the increase.

We also hypothesized that alterations in cholesterol efflux observed with the mAD patients could be explained by a decreased capacity of HDL to mediate cholesterol efflux or by a decrease in ABCA1/apo-A1 interactions. We used unenriched J774 and ABCA1-enriched J774 macrophages to measure cholesterol efflux and showed that HDL from the mAD and MCI patients was less efficient at mediating cholesterol efflux from unenriched J774 macrophages than HDL from the healthy subjects. However, this difference disappeared when ABCA1-enriched J774 macrophages were used. These results showed that the interaction between apoA-1
and the ABCA1 transporter is not altered in AD and that the reduction in cholesterol efflux in the MCI and mAD patients compared to the healthy subjects was not due to a change in the ABCA1/apoA1 pathway. As such, the decrease in cholesterol efflux observed in the mAD patients may be caused by an alteration in cholesterol efflux via the SR-B1 receptor and/or the ABCG1 transporter. The results of a study by Koudinov et al., which are in agreement with this assumption, showed that the composition of different subclasses of HDL is altered in Alzheimer patients (Koudinov et al. 2001). However, there was an apparent discrepancy between the capacity of plasma and purified HDL to mediate cholesterol efflux in the MCI patients, suggesting that changes to HDL may be less important in MCI patients than in mAD patients and that the major determinant of reduced cholesterol efflux may be other components of the plasma such as increased Aβ levels.

Some studies have shown that oxidized HDL are less efficient at mediating cholesterol efflux (Girona et al. 1997). AD has been associated with stresses encompassing oxidation of proteins, lipids, and nucleic acids (Reddy et al. 2009). Accordingly, we measured oxidative modifications to proteins and lipids in plasma and HDL obtained from each patient and healthy subject. Our results showed that there was a slight but significant increase in SH groups in the plasma of MCI and mAD patients relative to the healthy subjects. On the other hand, there was a slight decrease in carbonyl groups in the plasma of the MCI and mAD patients compared to the plasma of the healthy subjects. A study by Russell and Siedlak (Russell et al. 1999) showed that there are more SH groups in the plasma of AD patients than in the plasma of healthy subjects. They suggested that the increase in SH groups in AD is due to a compensatory response to an oxidative stress. The increase in the SH group content observed in the plasma from the MCI and mAD patients might thus be explained by an increase in oxidative stress. The compensatory mechanism that is activated in the presence of AD may explain the reduction in the formation of carbonyl groups in these patients compared to healthy subjects as previously shown by Aldred et al. (Aldred et al. 2010). The fact that MDA levels were same in the plasma of the MCI and mAD patients and the healthy subjects lends support to this finding.

Interestingly, our measurements of the oxidative modifications of HDL indicated that there was an increase in oxidized lipids and proteins in the HDL particles. Indeed, our results showed that there were significantly fewer SH groups in the HDL from the mAD patients than in the HDL from the healthy subjects and that the carbonyl levels were slightly higher in the HDL from
the MCI and mAD patients than in the HDL from the healthy subjects. In a previous study, we showed that the HDL from AD patients were more oxidized than the HDL from healthy subjects (Khalil et al. 2012). Moreover, a study by Schippling et al. (Schippling et al. 2000) showed that AD lipoproteins in the cerebrospinal fluid are more sensitive to oxidation than healthy lipoproteins. All these studies appear to suggest that HDL are oxidized in MCI and AD.

The oxidation of HDL particles leads to an alteration of the composition and structure of HDL, which modifies their functionality (Ferretti et al. 2006). Our results showed that there was a significant increase in the fluidity of HDL from the mAD patients compared to the HDL from healthy subjects. We also showed that HDL fluidity is inversely correlated with cognitive decline as measured by MMSE scores. Sibmooh et al. showed that the oxidative modification of HDL is accompanied by an increase in fluidity as measured by fluorescence anisotropy (Sibmooh et al. 2004). Our results thus confirmed that the alterations to the HDL structure caused by oxidative modification of proteins and lipids may explain the reduction of the capacity of the HDL from the mAD patients to mediate cholesterol efflux.

Aβ plasma levels may also influence the capacity of HDL to mediate cholesterol efflux. Several studies have shown that Aβ associates with HDL in the plasma and the cerebrospinal fluid through an interaction with apolipoproteins (Biere et al. 1996; Koudinov et al. 1996; Kuo et al. 1999). The association of Aβ with HDL in AD may affect the structure and functionality of HDL particles, leading to a decrease in their functionality and especially in their capacity to mediate cholesterol efflux. Our results showed that Aβ1-42 levels were significantly higher in the plasma of MCI patients and slightly higher in the plasma of mAD patients compared to healthy subjects. Since the increase in Aβ1-42 levels did not appear to modify the capacity of HDL to mediate cholesterol efflux ($r = -0.1606, p = 0.2808$), the increase cannot not fully explain the reduction in cholesterol efflux in MCI and mAD. Umeda et al. used a mouse model to show that Aβ40 and Aβ42 can stimulate cholesterol efflux via ABCA1 by inducing the formation of HDL-like particles and that Aβ40 can transport cholesterol from the brain to the periphery (Umeda et al. 2010). In the present study, the in vitro measurements of cholesterol efflux showed that neither Aβ1-40 nor Aβ1-42 stimulates cholesterol efflux from unenriched J774 or ABCA1-enriched J774 macrophages. Our results also showed that Aβ1-42-enriched HDL from healthy subjects significantly affected their capacity to mediate cholesterol efflux from ABCA1-enriched J774 macrophages. This suggested that Aβ1-42 can alter cholesterol efflux by interacting with the
ABCA1 transporter or by altering the structure of HDL. Measurements of HDL fluidity showed that the effect of Aβ₁-₄₂ on the structure HDL depends on the origin of the HDL. While no effect was observed with HDL from the healthy subjects, Aβ₁-₄₂ slightly or significantly decreased the fluidity of HDL from the MCI or mAD patients, respectively. Treating HDL from mAD patients with Aβ₁-₄₂ reduced the fluidity of HDL to levels comparable to those seen in healthy subjects, suggesting that Aβ₁-₄₂ can restore the structure but not the functionality of HDL. A study by Kontush et al. showed that 0.1 nM to 1 nM of Aβ₁-₄₀ and Aβ₁-₄₂ have an antioxidant effect on plasma and cerebrospinal fluid lipoproteins (Kontush et al. 2001), suggesting that the beneficial effect of Aβ₁-₄₂ with respect to restoring the fluidity of HDL from the mAD patients may be due to the antioxidant activity of Aβ₁-₄₂. However, we suggest that the increase in Aβ₁-₄₂ levels in MCI and AD patients will ultimately decrease HDL functionality.

In conclusion, the loss of HDL functionality in mAD is likely due to the oxidation of specific proteins that are rich in SH groups and to the concomitant alteration in the structure of HDL. Even though Aβ₁-₄₂ restores HDL fluidity, it is unable to maintain functionality because of these oxidative changes. Because such oxidative changes are less marked in MCI, Aβ₁-₄₂ might be able to restore HDL functionality by restoring fluidity but, on the other hand, it would alter the capacity of the plasma to sustain cholesterol efflux, most likely by interacting with the ABCA1 receptor. There is thus a clear difference in the mechanisms mediating cholesterol efflux in MCI and mAD. Further studies are needed to identify other plasma components that may interfere with HDL functionality and alter cholesterol efflux in MCI. This will be important for more precisely understanding why the functionality of HDL is altered in AD and for developing effective therapeutic strategies, which may be different in MCI than in AD, where HDL functionality per se is also altered.
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References


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Legends

Figure 1: Alteration of cholesterol efflux in patients with cognitive decline is not caused by a decrease in the expression of the ABCA1 transporter

(A) Monocytes were differentiated into macrophages, loaded with \(^{3}\text{H}\)-cholesterol for 24 h, and incubated for 4 h in efflux medium containing 5% plasma. The radioactivity in the medium and the cells was measured. The percentage of cholesterol efflux was calculated using the following formula: \((\text{cpm in medium} / \text{cpm in medium} + \text{cpm in cells}) \times 100\). The results are expressed as means ± SEM. A one-way ANOVA test was used to compare the means of the patient groups with the mean of the healthy group. * \(p < 0.05\) vs. healthy subjects. \(n = 30\) (\(n = 8\) healthy, \(n = 10\) MCI, \(n = 13\) mAD). (B) Monocytes were differentiated into macrophages for 7 days. ABCA1 expression was measured by FACS. The results are expressed as means ± SEM. A one-way ANOVA test was used to compare the means of the patient groups with the mean of the healthy group.

Figure 2: Alteration of HDL-mediated cholesterol efflux in Alzheimer Disease patients

J774 macrophages were loaded with \(^{3}\text{H}\)-cholesterol for 24 h and were then equilibrated for 12 h in efflux medium containing cAMP (0.3 mM) to induce the expression of ABCA1. They were washed and were incubated for 4 h in efflux medium containing 5% HDL. The radioactivity in the medium and the cells was measured. The percentage of cholesterol efflux was calculated using the following formula: \((\text{cpm in medium} / \text{cpm in medium} + \text{cpm in cells}) \times 100\). The measurements were performed in triplicate for each subject. The results are expressed as means ± SEM. A one-way ANOVA test was used to compare the means of the patient groups with the mean of the healthy group. * \(p < 0.05\) vs. healthy subjects. \(n = 57\) (\(n = 17\) healthy, \(n = 15\) MCI, \(n = 25\) mAD).
Figure 3: Effect of cognitive decline on the oxidation states of proteins and lipids in the plasma

(A) Free sulfhydryl groups were measured in the plasma using the DTNB method. \( n = 59 \) (\( n = 15 \) healthy, \( n = 15 \) MCI, \( n = 29 \) mAD). (B) Carbonyl groups were measured in the plasma. \( n = 60 \) (\( n = 16 \) healthy, \( n = 15 \) MCI, \( n = 29 \) mAD). (C) Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) levels. \( n = 59 \) (\( n = 15 \) healthy, \( n = 15 \) MCI, \( n = 29 \) mAD). The results are expressed as means ± SEM. A one-way ANOVA test was used to compare the means of the patient groups with the mean of the healthy group. * \( p < 0.05 \) vs. healthy subjects.

Figure 4: Effect of cognitive decline on the oxidation states of proteins and lipids in the HDL

(A) Free sulfhydryl groups were measured in the HDL using the DTNB method. \( n = 59 \) (\( n = 15 \) healthy, \( n = 15 \) MCI, \( n = 29 \) mAD). (B) Carbonyl groups were measured in the HDL. \( n = 36 \) (\( n = 9 \) healthy, \( n = 9 \) MCI, \( n = 18 \) mAD). (C) Lipid peroxidation was evaluated by measuring conjugated diene levels. \( n = 29 \) (\( n = 10 \) healthy, \( n = 9 \) MCI, \( n = 10 \) mAD). The results are expressed as means ± SEM. A one-way ANOVA test was used to compare the means of the patient groups with the mean of the healthy group. * \( p < 0.05 \) vs. healthy subjects and ** \( p < 0.01 \) vs. healthy subjects.

Figure 5: Correlation of cognitive decline with HDL fluidity

Fluidity was measured by fluorescence polarization using the 1,6-diphenyl-1,3,5-hexatriene probe. Fluidity is the inverse of anisotropy and is expressed as \( 1/r \) (\( r \): steady-state fluorescence anisotropy). The measurements were performed in duplicate or triplicate for each subject. (A) Comparison of HDL fluidity among the groups. The results are expressed as means ± SEM. A one-way ANOVA test was used to compare the means of the patient groups with the mean of the healthy group. **** \( p < 0.0001 \) vs. healthy subject. \( n = 40 \) (\( n = 10 \) healthy, \( n = 10 \) MCI, \( n = 20 \) mAD). (B) Correlation between HDL fluidity and the level of cognitive decline. The Pearson correlation was used to study the relationship between HDL fluidity and the MMSE score (cognitive decline). \( n = 38, r = -0.4137, p = 0.0098 \).
Figure 6:

Effect of Aβ42 on HDL fluidity

(A) Measurement of Aβ42 levels in the plasma. \( n = 47 \) (\( n = 13 \) healthy, \( n = 13 \) MCI, \( n = 21 \) mAD). The results are expressed as means ± SEM. A one-way ANOVA test was used to compare the means of the groups. * \( p < 0.05 \) vs. healthy. (B and C) HDL particles were isolated from the plasma using the PEG method. The HDL were incubated for 4 h at 37°C with Aβ42 (0.5 nM). After 4 h, HDL fluidity was measured by fluorescence polarization using the 1,6-diphenyl-1,3,5-hexatriene probe. Fluidity was equal to \( 1/r \) (\( r \): steady-state fluorescence anisotropy). The measurements were performed in triplicate. The results are expressed as means ± SEM. A one-way ANOVA test was used to compare the means of the groups. * \( p < 0.05 \).

Figure 7: Effect of Aβ on cholesterol efflux

J774 macrophages were loaded with [\(^{3}\)H]-cholesterol for 24 h and were then equilibrated for 12 h in efflux medium containing cAMP (0.3 mM) to induce ABCA1 expression. They were washed and incubated for 4 h in efflux medium (control), efflux medium containing 5% HDL (HDL), efflux medium containing Aβ40 or Aβ42 (0.5 nM), or efflux medium containing 5% HDL and Aβ40 or Aβ42 (0.5 nM). The radioactivity in the medium and the cells was measured. The percentage of cholesterol efflux was calculated using the following formula: (cpm in medium / cpm in medium + cpm in cells) x 100. The measurements were performed in triplicate. The results are expressed as means ± SEM. A one-way ANOVA test was used to compare the means of the groups. * \( p < 0.05 \), ** \( p < 0.01 \), **** \( p < 0.0001 \).
Figure 2: HDL-mediated cholesterol efflux (%) from J774.

- Healthy
- MCI
- mAD
- Healthy
- MCI
- mAD

cAMP (0.3 mM): - - - + + + +
Figure 3

A

Free SH groups (μmol/mg of proteins)

Healthy MCI mAD

B

Plasma carbonyl content (nmol/mg of proteins)

Healthy MCI mAD

C

MDA (μM)

Healthy MCI mAD
Figure 4

A

Free SH groups
(µmol/mg of proteins)

Healthy | MCI | mAD

B

Carbonyl content
(nmol/mg of proteins)

Healthy | MCI | mAD

C

Conjuged dienes
(OD 234 nm)

Healthy | MCI | mAD
Figure 5

A

HDL Fluidity (1/Fluorescence anisotropy)

ratios

healthy  MCI  mAD

B

Fluidity (1/Fluorescence anisotropy)

r = -0.4137, p = 0.0098
Table 1: Fasting plasma lipid parameters and Mini Mental State Examination (MMSE) scores of participating individuals.

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>MCI</th>
<th>mAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>71 ± 5.39</td>
<td>74 ± 5.02</td>
<td>79 ± 4.7  ****</td>
</tr>
<tr>
<td>Sex, n (Male/Female)</td>
<td>8/19</td>
<td>5/22</td>
<td>7/26</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>2.75 ± 1.93</td>
<td>2.19 ± 1.59</td>
<td>2.19 ± 1.53</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>4.00 ± 1.47</td>
<td>4.43 ± 1.57</td>
<td>4.51 ± 1.39</td>
</tr>
<tr>
<td>LDL cholesterol (mM)</td>
<td>2.15 ± 0.94</td>
<td>2.45 ± 1.17</td>
<td>2.53 ± 1.00</td>
</tr>
<tr>
<td>HDL cholesterol (mM)</td>
<td>1.57 ± 0.48</td>
<td>1.54 ± 0.51</td>
<td>1.39 ± 0.41</td>
</tr>
<tr>
<td>ApoA-1 (g/L)</td>
<td>1.63 ± 0.30</td>
<td>1.58 ± 0.31</td>
<td>1.61 ± 0.28</td>
</tr>
<tr>
<td>HDL/LDL</td>
<td>0.91 ± 0.59</td>
<td>0.79 ± 0.57</td>
<td>0.63 ± 0.29</td>
</tr>
<tr>
<td>Cholesterol/HDL</td>
<td>2.65 ± 0.95</td>
<td>3.05 ± 1.10</td>
<td>3.53 ± 1.57</td>
</tr>
<tr>
<td>ApoA-1/HDL</td>
<td>2.89 ± 0.95</td>
<td>2.79 ± 0.69</td>
<td>3.2 ±0.87</td>
</tr>
<tr>
<td>MMSE scores</td>
<td>29.67 ± 0.73</td>
<td>27.26 ± 1.95 ***</td>
<td>23.36 ± 3.85 ****</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD. *** p < 0.001 vs. Healthy subjects and **** p < 0.0001 vs. Healthy subjects.