Plasma Levels of HDL and Carotenoids are Lower in Dementia Patients with Vascular Comorbidities

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Abstract. Elevated serum cholesterol concentrations in mid-life increase risk for Alzheimer’s disease (AD) in later life. However, lower concentrations of cholesterol-carrying high density lipoprotein (HDL) and its principal apolipoprotein A1 (ApoA1) correlate with increased risk for AD. As HDL transports oxocarotenoids, which are scavengers of peroxynitrite, we have investigated the hypothesis that lower HDL and oxocarotenoid concentrations during AD may render HDL susceptible to nitration and oxidation and in turn reduce the efficiency of reverse cholesterol transport (RCT) from lipid-laden cells. Fasting blood samples were obtained from subjects with 1) AD without cardiovascular comorbidities and risk factors (AD); 2) AD with cardiovascular comorbidities and risk factors (AD Plus); 3) normal cognitive function; for carotenoid determination by HPLC, analysis of HDL nitration and oxidation by ELISA, and 3H-cholesterol export to isolated HDL. HDL concentration in the plasma from AD Plus patients was significantly lower compared to AD or control subject HDL levels. Similarly, lutein, lycopene, and zeaxanthin concentrations were significantly lower in AD Plus patients compared to those in control subjects or AD patients, and oxocarotenoid concentrations correlated with Mini-Mental State Examination scores. At equivalent concentrations of ApoA1, HDL isolated from all subjects irrespective of diagnosis was equally effective at mediating RCT. HDL concentration is lower in AD Plus patients’ plasma and thus capacity for RCT is compromised. In contrast, HDL from patients with AD-only was not different in concentration, modifications, or function from HDL of healthy age-matched donors. The relative importance of elevating HDL alone compared with elevating carotenoids alone or elevating both to reduce risk for dementia should be investigated in patients with early signs of dementia.

Keywords: Aging, Alzheimer’s disease, free radical scavenger, 3-nitrotyrosine, protein carbonyl formation, protein oxidation

INTRODUCTION

Aging is the major risk factor for dementia. Prevalence rates across the world are estimated to lie between 5.9–9.4% for people aged over 65 with a third of the >85 year old population affected. Correspondingly, absolute incidence of age-related pathologies particularly Alzheimer’s disease (AD) is increasing [1].
AD represents at least 70% of dementia cases and is characterized by progressive neurodegenerative alterations, gradually reducing cognitive performance with loss of memory, orientation, and judgment. Loss of synapses and cholinergic neurons, accumulation of extracellular amyloid-β (Aβ) plaques, and intraneuronal neurofibrillary tangles of hyperphosphorylated tau are major hallmarks of AD brain and are implicated in its pathogenesis. There is accumulating evidence that vascular pathology plays a central role in dementia onset and development, and some important information from recent reanalyses include data from a 75+ year-old community cohort in which 49% of the AD cases clinically diagnosed on the basis of the NINCDS-ADRDA criteria showed a possible vascular component [2]. The next most prevalent dementia, vascular dementia (VaD), is characterized by macroangiopathy and is often present as a post-stroke dementia with overlapping traditional hallmarks of AD including Aβ accumulation. This and other information is certainly very important to the clarification of vascular-targeted preventive strategies. Nevertheless, cholesterol transport and metabolism was associated with lower lutein and lycopene levels with mild AD or controls, and among AD patients a severe dementia have lower plasma levels of two major carotenoids, lutein and lycopene, compared to patients with mild AD or controls, and among AD patients a lower Mini-Mental State Examination (MMSE) score was associated with lower lutein and lycopene levels.
Carotenoids are lipophilic and are transported by lipoproteins, and the oxo-carotenoids are mainly associated with HDL [28]. They are potent scavengers of peroxynitrite and singlet oxygen, therefore their depletion in dementia may promote post-translational modifications to circulating proteins such as ApoA1 on HDL and impair RCT. We have shown previously that a decrease of circulating carotenoids and toco-pherols after correction for fruit and vegetable intake is associated with increased protein oxidation in patients with dementia [29]. Therefore we have investigated the hypothesis that oxocarotenoid depletion in dementia may render HDL more susceptible to nitration and modified HDL may reduce the efficiency of RCT from lipid-laden cells.

MATERIALS AND METHODS

Subject recruitment

Seventy seven community dwelling subjects were recruited from the Neurology Outpatient Clinic NeuroMed in Cologne, Germany. Patients were recruited after diagnosis with AD using NINCDS-ADRDA criteria either in the presence of vascular comorbidities and risk factors (elevated intima-media thickness of the common carotid artery and/or type 2 diabetes mellitus) (AD Plus group) or without cardiovascular comorbidities and risk factors (AD group) [9]. Control subjects showed no evidence of cognitive impairment and had no vascular comorbidities and risk factors. Informed consent was obtained from the patients or their caregivers according to severity of disease. Smokers as well as subjects taking medications and/or antioxidant/vitamin/iron supplements were excluded from the study. Patients with secondary dementias or with ongoing acute diseases were also excluded. The patient demographics are reported in Table 1.

Subjects underwent full physical/neurological examination as well as collection of medical history to assess clinical conditions and of nutritional status by means of a qualitative food-frequency questionnaire modified to assess daily intake of fruits and vegetables [27]. An ECG, two consecutive measurements of blood pressure, a carotid duplex sonography as well as the MMSE were performed in all subjects.

Blood sampling and measurements

This investigation conforms to the principles outlined in the Declaration of Helsinki and according to local ethical committee approval. After informed consent, fasting blood was collected from the antecubital vein into EDTA tubes and kept on ice until centrifugation within 2 h of collection. Plasma was frozen at −80°C until analysis. Plasma samples were coded and analysis was carried out in a blind fashion.

HDL isolation and quantitation

Cholesterol concentrations were measured enzymatically with CHOD-PAP kits from Randox, Ireland. Each assay included appropriate standards and calibrators. HDL was separated from the plasma by precipitation with dextran sulphate and magnesium chloride.

For RCT studies, HDL fractions were separated from plasma by density gradient gel electrophoresis according to the method of Chung et al. [30] and purity confirmed by agarose gel electrophoresis. Patient and control HDL nitration was determined by ELISA according to Weber et al. [31].

Plasma carotenoid analysis

Lycopene, β-carotene, α-carotene, lutein, zeaxanthin, and cryptoxanthin were determined by HPLC according to Stahl et al. [32].

| Table 1 | Demographic profile of dementia patients and controls |
|--------|-----------------|-----------------|
| Group  | Control (n = 33) | AD (n = 27) | AD Plus (n = 16) |
| Average age (mean ± SD) | 73.2 ± 8.3 | 80.5 ± 5.7 | 79.4 ± 4.7 |
| Gender (Female) | 33.3 | 37 | 37 |
| MMSE score (mean ± SD) | 29 ± 7 | 17 ± 8 | 19.5 ± 4.9 |
| ApoE ε2/ε2 | 0% | 0% | <1% |
| ApoE ε2/ε3 | 25% | 15% | <1% |
| ApoE ε2/ε4 | 10% | 23% | 19% |
| ApoE ε4/ε4 | 0% | 12% | 12% |
| % with hypertension | 24% | 29% | 38% |
| Body mass index (Bmi) | 24.1 ± 1.5 | 23.9 ± 2 | 25.5 ± 1.4 |
| LDL, cholesterol (mg/dL) | 140.9 ± 53.3 | 129.3 ± 40.6 | 119 ± 41.4 |
In vitro HDL nitration and analysis
HDL (200 μg/ml) was incubated with the NO donor, spermine NONOate (30 μM) in the presence or absence of homocysteine at 37°C in the absence or presence of 10 μM copper for 16 h. Oxidative reactions were terminated by the addition of DTT. Five micrograms of HDL was lyophilized to dryness, and dissolved in 20 μl of 8 M urea/25 mM ammonium bicarbonate, pH 8.0, diluted further to lower urea concentration and digested with trypsin (1:50 protease to protein ratio) at 37°C for 4 h. The tryptic peptide pool was lyophilized to dryness, dissolved in 0.5% acetic acid, and analyzed by RP-HPLC-MS/MS on a Surveyor HPLC system and a 0.075 x 100 mm C18 RP column (Proxeon), at ~1.5 μm/min, connected online via nano-electrospray with a LQX linear ion trap mass spectrometer (ThermoFisher). Solvents were 99.5% water and 0.5% acetic acid (buffer A) and 90% acetonitrile, 9.5% water, and 0.5% acetic acid (buffer B). Peptides were eluted at a 90-min gradient from 5% to 60% solvent B and monitored by data dependent analysis MS/MS scans for the masses corresponding to tyrosine nitration and methionine oxidation (+45, +16 respectively).

Western blot
HDL nitration and ABCA1 expression in differentiated THP-1 cells were assessed by western blotting. Protein samples from NO/Hcy/Cu-modified HDL or differentiated THP-1 cells (10 μg) were separated by 1D SDS-PAGE (12.5% gel) and electroblotted onto polyvinylidene fluoride (PVDF) membrane. After electroblotting at 20 mA for 16 h, membranes were blocked in Tween 20 (1%) Tris Buffered Saline (TTBS) containing 3% bovine serum albumin (BSA) for 2 h. Membranes were incubated with either mouse monoclonal antibody against 3-nitrotyrosine or anti-ABCA1 (1:1000) overnight. The membranes were washed with TTBS (6 x 15 min) before incubation with a peroxidase conjugated secondary antibody (Sigma Aldrich, Poole, UK) for 2 h (1:4000) peroxidase conjugated anti goat IgG (diluted in TTBS containing 0.3% BSA). Membranes were rinsed in TBS (6 x 15 min) and then a chemiluminescent substrate (ECL Plus, Little Chalfont, Amersham Biosciences) was used to visualize detected proteins. The images recorded using a GS 710 calibrated imaging densitometer. Bands were analyzed and quantified using Scion software (NIH, USA).

Carotene and 3-nitrotyrosine ELISAs
To determine protein oxidation and nitration in plasma proteins and in HDL isolated by density gradient centrifugation, proteins were diluted to 10 μg/ml carbonate buffer prior to analysis by ELISA as previously described [31, 33].

Differentiation protocol
Human THP-1 monocyte cells were seeded into six-well plate at 1.8 x 10⁶ cells per well in RPMI 1640 medium + GlutaMAX™I containing bovine fetal serum (10%, v/v), penicillin (50 unit/ml), and streptomycin (50 μg/ml). Cells were differentiated into macrophages in the presence of phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) for 5 days and medium was replaced at least every 48 h.

Reverse cholesterol transport
Macrophages were transformed into foam cells by incubation with oxidized LDL (50 μg/ml) in serum-free medium containing BSA (1.5%, w/v), for 48 h including [3H]-cholesterol (0.1 μCi/ml). The medium was then removed and the cells were washed and incubated in serum free medium with BSA (1.5%, w/v), ApoAI (10 μg/ml), or HDL (10 μg/ml) for 24h to determine efflux.

RESULTS
Patients in the AD Plus group had significantly higher body mass index (p<0.05) than control subjects and hypertension was twice as frequent; however, LDL cholesterol was not different between groups (Table 1). The APOE e4 allele was present in AD and AD Plus patients at three times the frequency of control subjects (Table 1). HDL cholesterol was lower in AD Plus patients but not in the plasma from patients with AD when compared with control subjects (Fig. 1A). Similarly, analysis of plasma carotenoid levels revealed that lutein, lycopene, and zeaxanthin concentrations were significantly lower in the AD Plus group but not in control subjects or AD patients (Figs. 1B-D). However, β-carotene, α-carotene, and cryptoxanthin were not significantly different between any of the patient groups and controls (data not shown). MMSE correlated positively with plasma HDL, lutein, and zeaxanthin but not lycopene, β-carotene, α-carotene, and cryptoxanthin. As HDL is the principal carrier of
Fig. 1. Plasma HDL and carotenoids are depleted in AD Plus. A) HDL cholesterol concentration (mg/dl) was analyzed in plasma from patients with AD, AD Plus, and age-matched control subjects by dextran sulfate-magnesium precipitation using Randox kit. Plasma lutein (B), lycopene (C), and zeaxanthin (D) were determined in plasma from patients in the AD group, AD Plus group, and age-matched control subjects by HPLC. Data are expressed as mean and 95% confidence interval and differences were evaluated by ANOVA where *p < 0.05; **p < 0.01; and ***p < 0.005.

specific carotenoids including lutein and zeaxanthin, it may be anticipated that lower plasma carotenoid concentrations mirror the reduction in plasma HDL concentration. However, the correlations of both zeaxanthin and lutein with MMSE were maintained after correcting the carotenoid concentration per mole of HDL cholesterol.

Nitration of ApoA1 and HDL was induced by homocysteine/nitric oxide and copper oxidation (Fig. 2A). HDL carbonyl content was elevated in the presence of homocysteine and copper, and was attenuated by the presence of spermine NONOate (Fig. 2B). Conversely, protein nitration was increased when HDL was co-incubated with homocysteine, spermine NONOate, and copper. By MS, three oxidized methionine containing peptides were present ETEGLRQEM*SKDLEEVK, KWQEEM*ELYR, LSPLGEEM*R corresponding to methionine 86, 112 and 148; and one nitrated peptide LAEY*HAKATEHLSTLSEK corresponding to tyrosine nitration at position 192 was identified. RCT from cholesterol-loaded macrophages was diminished after modification to HDL by homocysteine, copper with and without spermine nonoate (Fig. 2C). However, for HDL isolated from patient and control plasma, RCT was not significantly different whether HDL was isolated from patients with and without dementia and controls on a mol/mol basis (Fig. 3A). When cholesterol efflux was corrected to HDL concentration found in the plasma, RCT was significantly lower in the AD Plus group (Fig. 3B).

There was a tendency for HDL to be nitrated to a greater extent in the AD Plus group, however, there was no significant difference in HDL nitration or oxidation (Fig. 3C, F) nor were total plasma levels of 3-nitrotyrosine or protein carbonyl different between any of the subject groups studied (Fig. 3D, E).

DISCUSSION

Here we have shown for the first time that plasma HDL, lutein, and zeaxanthin concentrations are lower in the AD Plus patient group and thus their effective capacity in plasma to mediate RCT is lowered. MMSE
Fig. 2. Oxidized and nitrated HDL is less effective in reverse cholesterol transport. A) HDL treated with 30 μM spermine NONOate (SNO), 10 μM copper, and 50 μM homocysteine (HCy) show the greatest increase in 3-nitrotyrosine level as determined by western blotting. B) HDL carbonyl content determined by ELISA was increased significantly by HCy/Cu and this was attenuated in part by spermine NONOate. C) RCT measured as 3H-cholesterol efflux is impaired to nitrated and oxidized HDL. Differences were evaluated by ANOVA where *p < 0.05; **p < 0.01.

correlated positively with plasma HDL, lutein, and zeaxanthin suggesting that increasing HDL cholesterol and increasing carotenoids may also improve cognitive outcomes for patients.

HDL plays a critical role in RCT within the systemic circulation, removing cholesterol from peripheral tissues for hepatic degradation. Disturbances in cholesterol metabolism are common to a number of pathologies which increase the risk for development of dementia in later life [34]. The present study demonstrates that HDL function and nitration levels are not different between patients with cognitive impairment and age-matched control subjects, but that in patients with AD and vascular comorbidities/risk factors the HDL levels are significantly lower.

Previous work has shown in a longitudinal study that lowering of HDL over five years associates with a significant decline in memory [35]. The InChianti study showed that among community-dwelling older people, those with dementia had significantly lower total cholesterol, non-HDL-cholesterol (HDL-C), and HDL-C levels; however, using multivariate analysis only low HDL-C was associated with dementia [16]. These results are consistent with our findings and suggest the existence of an independent relationship between the pathologic vascular component of AD and low HDL-C levels.

Peripheral cholesterol metabolism is considered to be completely separate from brain cholesterol metabolism, however, HDL can cross the blood-brain barrier [36]. The mechanisms by which HDL may exert a protective effect include the assembly with ApoA1 to mediate RCT [37], RCT from tissue via ABCA1-dependent pathways, and through delivery of carotenoids and tocopherols [35].

Many of the recently described functions of HDL are not strictly attributed to its capacity to promote cholesterol flux, but by the other molecules it transports. These include proteins, small RNAs, bioactive lipids, hormones, vitamins, and carotenoids [38, 39]. In chickens with ABCA1 deficiency, circulating HDL and carotenoid concentrations were low and tissue deposition of lutein was reduced to 5% of control animal tissue levels [40]. Another carotenoid, astaxanthin, has been described as an important inducer of ABCA1 expression in macrophages and plays an important role in movement of cholesterol to HDL [41]. A large percentage of the polar oxocarotenoids are found on HDL (lutein, 53%; zeaxanthin and cryptoxanthin, 39%) but while HDL only transports a small percentage of total plasma non-polar carotenoids (e.g., lycopene, 17%; α-carotene, 26%; and β-carotene, 22%) [38], it has been suggested that the content of β-carotene per unit liquid is greater in HDL than in LDL; hence are likely to play an important role in protecting HDL from modification by free radicals [28]. Lowered HDL concentration is associated with reduced carotenoid transport which may influence the expression of proteins involved in RCT, e.g., ABCA1 [42].

In the present work, we show that the oxocarotenoids, lutein and zeaxanthin, and lycopene are significantly depleted in the plasma of patients in the
Fig. 3. HDL modification and function were not different between AD (n=28), AD plus (n=16), and controls (n=33). A) RCT measured as 3H-cholesterol efflux to equivalent molar HDL concentrations isolated from plasma. B) RCT measured as 3H-cholesterol efflux to HDL in plasma isolated from patients with and without dementia and controls. C) 3-nitrotyrosine (3NT) in HDL was determined by ELISA. D) 3-nitrotyrosine levels in plasma proteins were determined by ELISA. E) Plasma protein carbonyl levels were determined by ELISA. F) HDL carbonyl levels determined by ELISA. Differences were evaluated by ANOVA where \( p<0.05 \).

AD Plus group and that their concentrations correlate with MMSE. This deficit remains for zeaxanthin and lutein even when adjusted for HDL cholesterol concentration. Hence either a decrease in HDL concentration or carotenoid concentration is expected to impact RCT independently. Carotenoids are powerful scavengers of singlet oxygen and so can prevent protein oxidation and nitration in vivo and in vitro [43–46]; there is a wealth of evidence for protein oxidation and nitration in the periphery and brain during cognitive impairment [29]. ApoA1, the major apoprotein in HDL, is susceptible to oxidation forming a bis-sulfoxide at methionines 112 and 148. This modification renders the molecule more susceptible to denaturation,
reduces the formation of discoid dimeric and tetrameric aggregates which are essential for reverse cholesterol function, and favors formation of amyloid like fibrils [47, 48]. HDL from atherosclerotic lesions and peripheral blood of patients with coronary artery disease also contain chlorinated, nitrated, and oxidized residues most likely arising from myeloperoxidase catalyzed oxidation. Others have shown that oxidation of Met\(^{148}\) to methionine sulfoxide was associated with loss of HDL RCT activity and Tyr\(^{192}\) was found to be the predominant site for nitration and chlorination when MPO or ONOO\(^{-}\) were used to oxidize APOA1, tyrosine chlorination and methionine oxidation markedly reduced the cholesterol efflux activity of APOA1 [22, 23]. In the present study, we have shown that oxidation and nitration of HDL occur in parallel in the presence of spermine NONOate, homocysteine, and copper resulting in methionine oxidation at 86, 112, and 148, nitration of Tyr\(^{177}\) and that RCT to HDL was impaired after modification. To explore whether the depletion of carotenoids in AD Plus patients was associated with tyrosine nitration or protein carbonyl formation, plasma HDL and total plasma proteins were analyzed by 3-nitrotyrosine and protein carbonyl ELISAs respectively. There was no significant increase in nitration of HDL in plasma from any of the patient groups with dementia when compared to control subjects. This differentiates dementia from general vascular diseases where HDL nitration is prevalent [19]. A recent study has shown that RCT to AD HDL is impaired when compared to age-matched control HDL [49]. Our present study does not confirm this earlier observation despite the sample size of AD and age-matched control groups being similar between the two studies. Unlike our AD Plus group, HDL concentrations in the plasma of AD patients in our study were not different from control subject serum HDL. However, in Khalil’s study, HDL concentrations were lower in AD patients [49]. It is probable that the previous study did not discriminate between AD with and without vascular pathology.

In our AD Plus patients, 3-nitrotyrosine per HDL was 50% higher than for AD or control subjects; conversely carotenoids and HDL concentrations were significantly lower than in control subjects. Cholesterol export from tissue to plasma HDL, and carotenoid transport to tissue is likely to be impaired in AD Plus based on plasma concentration alone. The importance of HDL as transporter of small regulatory molecules suggest that its depletion in dementia may have wider significance than for cholesterol metabolism alone [38].

Despite the wealth of evidence from cross-sectional and observational studies for an association between elevated serum cholesterol in mid-life and later development of AD, there is insufficient understanding to recommend statin interventions to reduce disease risk, probably due to the significant role of individual lifestyle at particular ages and the lack of investigation in well-characterized subcohorts, e.g., AD Plus [10]. In some studies, statin therapy has been effective in modifying plasma lipids levels, but not adequate as a monotherapy to normalize the HDL subclass distribution phenotype [50]. In the field of coronary heart disease, interest is growing to elevate HDL cholesterol rather than just reducing total cholesterol to reduce risk of cardiovascular events [51]. The present data suggest that increasing HDL cholesterol and increasing carotenoids may also improve cognitive outcomes for patients. This effect may be achieved through increasing transport of carotenoids to tissues and promoting export of Aβ and cholesterol from tissues. The relative importance of elevating HDL alone compared with elevating carotenoids alone or elevating both to reduce risk for dementia should be investigated in patients with early signs of dementia.

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I.H.K. Dias et al. / HDL and Carotenoids in Alzheimer’s Disease


