Plasma 24S-hydroxycholesterol (cerebrosterol) is increased in Alzheimer and vascular demented patients

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Abstract   Alzheimer's disease (AD) is characterized by the presence of senile plaques, neurofibrillary tangles, and neuronal cell loss associated with membrane cholesterol release. 24S-hydroxycholesterol (24S-OH-Chol) is an enzymatically oxidized product of cholesterol mainly synthesized in the brain. We tested the hypothesis that plasma levels of this oxysterol could be used as a putative biochemical marker for an altered cholesterol homeostasis in the brain of AD patients. Thirty patients with clinical criteria for AD, 30 healthy volunteers, 18 depressed patients, and 12 patients with vascular dementia (non-Alzheimer demented) were studied. Plasma concentrations of 24S-OH-Chol were assayed by isotope dilution–mass spectrometry, cholesterol was measured enzymatically, and apolipoprotein E (apoE) was genotyped by polymerase chain reaction and restricted fragment length polymorphism. The concentration of 24S-OH-Chol in AD and non-Alzheimer demented patients was modestly but significantly higher than in healthy controls and in depressed patients. There was no significant difference in the concentrations of 24S-OH-Chol between depressed patients and healthy controls nor between AD and non-Alzheimer demented patients. The apoE ε4 allele influences plasma 24S-OH-Chol. However, this influence could be completely accounted for by the elevated plasma cholesterol in apoE4 hetero- or homozygotes. Plasma 24S-OH-Chol levels correlated negatively with the severity of dementia. AD and vascular demented patients appear to have higher circulating levels of 24S-OH-Chol than depressed patients and healthy controls. We speculate that 24S-OH-Chol plasma levels may potentially be used as an early biochemical marker for an altered cholesterol homeostasis in the central nervous system.—Lütjohann, D., A. Papassotiropoulos, I. Björkhem, S. Locatelli, M. Bagli, R. D. Oehring, U. Schlegel, F. Jessen, M. L. Rao, K. von Bergmann, and R. Heun. **Plasma 24S-hydroxycholesterol (cerebrosterol) is increased in Alzheimer and vascular demented patients.** *J. Lipid Res.* 2000. **41:** 195–198.

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Alzheimer's disease (AD), the most common cause of dementia in old persons, is characterized by regional accumulation of senile plaques, neurofibrillary tangles, and extensive neuronal cell death (1). Of all the brain regions, the hippocampus, a region with a high density of neuronal cells, seems to be the first to be affected by AD (2). The mechanism causing neuronal cell death in AD has not yet been defined (3). In the course of the AD-associated neuronal degeneration, cell membrane degradation occurs releasing cholesterol (4). One of the most important mechanism for the elimination of brain-derived cholesterol is its conversion into the polar metabolite, 24S-OH-cholesterol (24S-OH-Chol) (5, 6).

There is hitherto no single satisfactory biological marker for AD and the definitive diagnosis had to be based on clinical symptomatology and confirmed by postmortem histology. Additionally, there is a considerable overlap in the phenomenology of disease between AD and other pathologic conditions. Depression is often accompanied by cognitive deficits similar to those occurring in AD (7) and has been discussed as a possible risk factor for developing AD (8). Moreover, there seems to be a significant comorbidity between depression and AD (9). This phenomenological relationship often causes difficulties in distinguishing between these two states especially in mild forms of dementia.

As most of the circulating 24S-OH-Chol in the human body originates in the brain (10), we hypothesized that the 24S-OH-Chol plasma concentration is a peripheral indicator of central neurodegeneration occurring in AD and possibly also in other neurological diseases. In order to assess whether 24S-OH-Chol could serve as a marker for

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Abbreviations: AD, Alzheimer's disease; 24S-OH-Chol, 24S-hydroxycholesterol; ICD 10, International Classification of Diseases, 10th revision; MMSE, Mini Mental State Examination; CI, 95% confidence interval of the mean.

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AD, we determined plasma concentrations of 24S-OH-Chol in AD patients, in age-matched healthy volunteers, depressed and non-Alzheimer vascular demented patients.

The apolipoprotein E (apoE) genotype is a well-established risk factor for AD (11, 12). Moreover, the apoE system is important for the distribution of cholesterol in the brain and is partially involved in its transfer from brain tissue to the cerebrospinal fluid and from there to the circulation (13). AD patients, healthy controls, depressed and non-Alzheimer demented patients were therefore genotyped with respect to apoE.

We observed that serum concentrations of 24S-OH-Chol were significantly higher in Alzheimer and vascular demented patients than in depressed patients and healthy controls.

MATERIALS AND METHODS

Participants

Thirty AD patients were recruited consecutively from the Department of Psychiatry and Psychotherapy, University of Bonn, after thorough clinical and neuropsychological examination, EEG, cranial CT, and sonography of extracranial and intracranial arteries. All patients were also evaluated by a structured psychiatric interview (Composite International Diagnostic Interview) (14). Wherever applicable, additional information was obtained from close relatives of the patients using semistructured interviews. All patients fulfilled the criteria of the International Classification of Diseases, 10th revision (ICD 10) (15) for the presence of dementia of the Alzheimer type. The severity of the dementia was measured by the Mini Mental State Examination (MMSE). MMSE scores can range between 0 and 30 with lower values indicating more severe dementia. Thirty age-matched healthy volunteers from the elderly general population were recruited from an epidemiological group. Exclusion of neurological or psychiatric disorders and dementia in this group was made after extensive interviews and neuropsychological testing. Depression represents one of the most important differential diagnoses for AD in the elderly. Therefore, 18 age-matched depressed hospitalized patients were selected as further controls. Depressed patients underwent the same diagnostic evaluation as AD patients. As a fourth group, 12 non-Alzheimer vascular demented patients were recruited consecutively from the Department of Neurology, University of Bonn. The description of all participants of the study is given in **Table 1.** The study was carried out in accordance with the principles of the Helsinki Declaration, the protocol was approved by the local ethics committee, and all subjects or their relatives gave informed consent.

Analysis of cholesterol and 24S-OH-Chol

Blood samples were taken after an overnight fast in EDTA-containing tubes; aliquots for analysis of total plasma cholesterol and 24S-OH-Chol were centrifuged at 2000 g for 10 min and the supernatant was stored at $-82°$C. Blood samples for apoE genotyping were immediately frozen at $-82°$C until analyzed. Plasma concentrations of cholesterol were measured by standard enzymatic procedures (CHOD-PAP Method, Boehringer, Mannheim, Germany) and plasma concentrations of 24S-OH-Chol were measured as described previously (16). Briefly, 10 μg tertiary butylated hydroxytoluene as antioxidant and 200 ng of [23.23.24.25-^{2}H$_4$]24S-OH-Chol in 50 μl methanol as internal standard were added to 500 μL of the plasma. After alkaline hydrolysis with 1 N

TABLE 1. Demographic data and apoE allele genotyping of patients and healthy subjects

	AD Patients	Healthy Controls	Depressed Patients	Vascular Dementia
Total number	30	30	18	12
Sex[a] (males/females)	11/19	16/14	4/14	5/7
Age (years)[b]				
Mean ± SD	73 ± 8	70 ± 10	70 ± 9	69 ± 8
Range	52–87	49–91	48–86	52–81
ApoE ε4 allele[c]				
(present/absent)	21/9	7/23	3/15	4/8
MMSE[d]				
Mean ± SD	17 ± 8	29 ± 1	25 ± 3	20 ± 4
Range	0–26	27–30	16–27	14–25

[a] $\chi^2 = 4.7$, df = 3, $P = 0.19$.
[b] F = 1.0, df = 3, $P = 0.38$.
[c] $\chi^2 = 19.2$, df = 3, $P = 0.006$.
[d] F = 13.7, df = 3, $P < 0.001$.

sodium hydroxide in 90% ethanol for 2 h at 50°C, the solution was neutralized with 100 μL phosphoric acid (50% v/v) and the sterols were extracted twice with 4.5 mL chloroform. The combined organic phases were dried with a gentle stream of nitrogen at 50°C, the residue was dissolved in 1 mL toluene and added to an isolute silica cartridge equilibrated with n-hexane. Cholesterol and other neutral serum sterols were eluted with 8 ml 0.5% 2-propanol–n-hexane (v:v) and the remaining oxysterols with 5 mL 30% 2-propanol–n-hexane (v:v). This fraction was dried with a jet of nitrogen and the oxysterols were silylated by addition of 1.0 mL of a silylation reagent (dry pyridine–hexamethyldisilazane–trimethylchlorosilane, 9:3:1, v/v/v). The solvent was evaporated with nitrogen at 50°C and the residue was dissolved in 40 μL n-decane. One μL was injected onto an Ultra-1 dimethylsilicone column (25 m × 0.2 mm internal diameter × 0.33 μm film thickness; Hewlett-Packard, HP) in a gas chromatograph (HP 5890) and 24S-OH-Chol was quantified by selected ion-monitoring on a mass selective detector (HP 5972) under the same conditions as described previously (5). Analysis of cholesterol and 24S-OH-Chol was carried out blindly without further information concerning diagnosis.

ApoE genotype

The apoE isoforms (ε2, ε3, ε4) were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) as described previously (17). Briefly, DNA was extracted from blood cells according to standard protocols and a 244 bp fragment was amplified by PCR. The PCR product was digested with *Hha*I and run on a 4% agarose gel. The bands were stained by ethidium bromide and the different alleles were distinguished as different band patterns.

Statistics

The hypothesis that patients with AD had higher plasma concentrations of 24S-OH-Chol was tested by ANCOVA (dependent variable: 24S-OH-Chol; independent variables: diagnosis, sex, and age). Plasma cholesterol concentrations served as covariate because of the known linear relationship between plasma concentrations of cholesterol and 24S-OH-Chol in healthy adults (10). Post-hoc comparisons were performed by the Bonferroni method. A second ANCOVA was carried out with an additional independent variable (i.e., the presence or absence of the apoE ϵ4 allele). Correlations between parameters were tested by partial correlation analysis (Pearson's). All calculations were done with the statistical software program of SPSS/Windows (SPSS Inc., Chicago, IL).

RESULTS

Demographic data and apoE genotypes in relation to 24S-OH-Chol and cholesterol

No significant difference in concentrations of 24S-OH-Chol was observed between different age strata nor between males and females. The apoE $\epsilon4$ allel was significantly more present in the AD patient group than in the other groups (Table 1). ANCOVA with apoE genotype as additional independent variable (i.e., presence vs. absence of the apoE $\varepsilon4$ allele) revealed that the apoE $\varepsilon4$ allele did not influence plasma 24S-OH-Chol (F = 2.9, df = 1, $P < 0.1$).

Cholesterol and 24S-OH-Chol

Plasma concentrations of cholesterol were similar in patients and controls (**Table 2**). In agreement with our hypothesis, a modest but highly significant difference in the plasma 24S-OH-Chol concentrations was observed among the four diagnostic groups (Table 2, **Fig. 1**). Bonferroni-adjusted post-hoc comparisons revealed that plasma 24S-OH-Chol concentrations in AD and non-Alzheimer demented patients were significantly higher than in healthy controls ($P < 0.001$ and $P < 0.004$, respectively) and depressed patients ($P < 0.001$ and $P < 0.002$, respectively) (Fig. 1). The levels of 24S-OH-Chol did not differ between depressed patients and healthy controls, nor between AD and non-Alzheimer demented patients. The 95% confidence intervals of the mean (CI) showed a good overlap for cholesterol within the four different groups whereas the corresponding intervals for 24S-OH-Chol were comparable between healthy volunteers and depressed patients (52 to 67 ng/mL for the healthy volunteers and 44 to 64 ng/ml for the depressed patients) and between patients with AD (69 to 81 ng/mL) and non-Alzheimer demented patients (67 to 89 ng/mL). None of the interactions between the independent variables (such as diagnosis, sex, and age) proved to be significant. The effect of the covariate (i.e., plasma cholesterol concentration) was highly significant (F = 26.6, df = 1, $P < 0.001$). In agreement, partial correlation analysis between plasma cholesterol and 24S-OH-Chol concentrations with the diagnostic group as control variable revealed a significant positive correlation ($r = 0.549$, $P < 0.001$).

Severity of disease and plasma 24S-OH-Chol

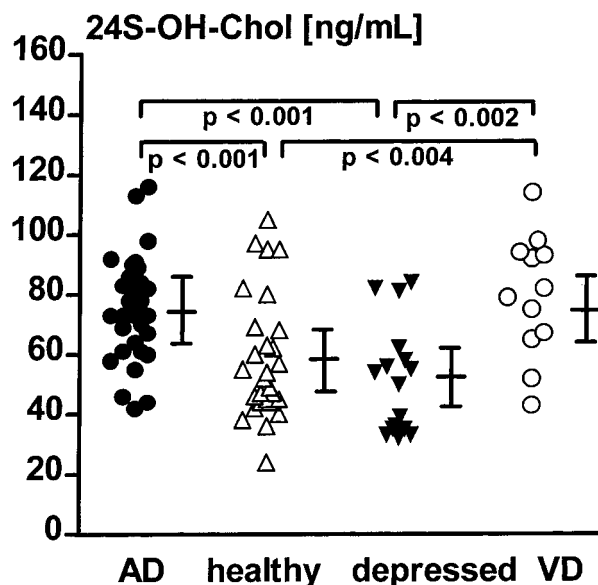The mean MMSE score was 17 ± 8 (range 0–26) and of the non-Alzheimer demented patients 20 ± 4 (range 14–

TABLE 2. Plasma concentrations of cholesterol and 24S-OH-cholesterol in patients and controls

	AD Patients	Healthy Controls	Depressed Patients	Vascular Dementia
Cholesterol [mg/dL][a]				
Mean ± SD	237 ± 35	228 ± 43	221 ± 44	243 ± 45
Range	170–347	119–316	139–281	178–326
24S-OH-Chol [ng/mL][b]				
Mean ± SD	75 ± 18	60 ± 21	54 ± 21	78 ± 20
Range	42–116	24–105	32–99	43–114

[a] F = 1.2, df = 3, $P = 0.31$.
[b] F = 7.9, df = 3, $P < 0.001$.

Fig. 1. Plasma concentrations of 24S-OH-Chol in patients with Alzheimer's disease (AD), healthy volunteers (healthy), depressed and non-Alzheimer vascular demented patients (VD).

25) (Table 1). Partial correlation analysis controlling for age and total cholesterol revealed that plasma 24S-OH-Chol concentration correlated positively with the MMSE score in demented Alzheimer ($r = 0.420$, $P < 0.03$), but not in healthy volunteers ($r = -0.447$, P < 0.2), depressed patients ($r = -0.152$, $P < 0.7$), and not in non-Alzheimer demented patients ($r = 0.077$, $P < 0.1$).

DISCUSSION

The main findings of the present study are significantly higher concentrations of 24S-OH-Chol in AD patients and non-Alzheimer vascular demented patients compared to depressed patients and healthy volunteers. The higher mean levels of 24S-OH-Chol in the circulation of AD and non-Alzheimer demented patients may reflect a higher flux of 24S-OH-Chol from the brain caused by increased release of membrane cholesterol occurring in the course of the neurodegenerative process (4).

Depression is one of the most important differential diagnoses of AD in elderly subjects and can be easily misdiagnosed, especially in mild forms of dementia. The difference in plasma 24S-OH-Chol concentrations between AD and depressed patients may be of some interest from a diagnostic point of view.

Cholesterol 24S-hydroxylase, an enzyme that is almost exclusively located in the brain, is involved in the stereospecific 24S-hydroxylation of brain cholesterol (5). 24S-OH-Chol passes the blood–brain barrier faster than cholesterol itself. In healthy subjects, about 6 mg of this oxysterol is transferred from the brain into the circulation during a 24-h period (6). The close correlation between circulating levels of 24S-OH-Chol and cholesterol may be

a consequence of the fact that cholesterol and 24S-OH-Chol are distributed similarly in the lipoproteins (18). The rapid transport of 24S-OH-Chol from the CNS into the periphery may be interpreted as a neuroprotective mechanism, as 24S-OH-Chol can exert strong neurotoxic effects in vitro (19).

In view of the fact that there exists a transport of cholesterol from the brain into the cerebrospinal fluid that is mediated by apoE (20), we investigated the possibility that the apoE genotype may be of importance for the circulating levels of 24S-OH-Chol. The apoE genotype did not contribute substantially to the observed elevation of plasma 24S-OH-Chol in AD patients: the ANCOVA revealed that the influence of apoE4 on plasma 24S-OH-Chol was completely accounted for by the elevated plasma cholesterol in apoE4 hetero- or homozygotes.

AD patients as well as patients with vascular dementia had higher circulating levels of 24S-OH-Chol, regardless of the apoE genotype. This is consistent with a central neurodegenerative process or an altered cholesterol homeostasis in the central nervous system. The significant negative correlation between plasma 24S-OH-Chol and the severity of AD and vascular dementia supports the hypothesis of 24S-OH-Chol being derived from degenerating neurons and explains the high levels of 24S-OH-Chol in the early stages of dementia, where the rate of neurodegeneration is high and CNS atrophy is minimal.

The results of the present investigation suggest that 24S-OH-Chol may be a useful diagnostic tool, especially in the early stages of dementia, where the differentiation between AD patients or age-related cognitively impaired subjects and depressed patients is difficult. However, it is important to emphasize that the extent of the 24S-OH-Chol elevation may change over time in different patients. Further longitudinal studies in larger AD populations are thus needed to evaluate whether 24S-OH-Chol is a suitable biochemical marker for the early detection of AD and its progression.

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