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Research Report

Sphingolipids in Cerebrospinal Fluid and Plasma Lipoproteins of *APOE4* Homozygotes and Non-*APOE4* Carriers with Mild Cognitive Impairment versus Subjective Cognitive Decline

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Abstract.
Background: Alzheimer's disease (AD) patients display alterations in cerebrospinal fluid (CSF) and plasma sphingolipids. The *APOE4* genotype increases the risk of developing AD.
Objective: To test the hypothesis that the *APOE4* genotype affects common sphingolipids in CSF and in plasma of patients with early stages of AD.

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Methods: Patients homozygous for *APOE4* and non-*APOE4* carriers with mild cognitive impairment (MCI; $n = 20$ versus 20) were compared to patients with subjective cognitive decline (SCD; $n = 18$ versus 20). Sphingolipids in CSF and plasma lipoproteins were determined by liquid-chromatography-tandem mass spectrometry. $A\beta_{42}$ levels in CSF were determined by immunoassay.

Results: *APOE4* homozygotes displayed lower levels of sphingomyelin (SM; $p = 0.042$), SM(d18:1/18:0) ($p = 0.026$), and $A\beta_{42}$ ($p < 0.001$) in CSF than non-*APOE4* carriers. CSF-$A\beta_{42}$ correlated with Cer(d18:1/18:0), SM(d18:1/18:0), and SM(d18:1/18:1) levels in *APOE4* homozygotes ($r > 0.49$; $p < 0.032$) and with Cer(d18:1/24:1) in non-*APOE4* carriers ($r = 0.50$; $p = 0.025$). CSF-$A\beta_{42}$ correlated positively with Cer(d18:1/24:0) in MCI ($p = 0.028$), but negatively in SCD patients ($p = 0.019$). Levels of Cer(d18:1/22:0) and long-chain SMs were inversely correlated with Mini-Mental State Examination score among MCI patients, independent of *APOE4* genotype ($r < –0.47$; $p < 0.039$). Nevertheless, age and sex are stronger determinants of individual sphingolipid levels in CSF than either the *APOE* genotype or the cognitive state. In HDL, ratios of Cer(d18:1/18:0) and Cer(d18:1/22:0) to cholesterol were higher in *APOE4* homozygotes than in non-*APOE4* carriers ($p = 0.048$ and 0.047, respectively).

Conclusion: The *APOE4* genotype affects sphingolipid profiles of CSF and plasma lipoproteins already at early stages of AD. ApoE4 may contribute to the early development of AD through modulation of sphingolipid metabolism.

Keywords: Alzheimer's disease, amyloid-β peptides, Apolipoprotein E4, ceramides, cerebrospinal fluid, cognitive dysfunction, lipoproteins, sphingolipids

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. AD is characterized by a long preclinical phase with pathogenic alterations [1, 2]. Unfortunately, one of the first symptoms of AD is unawareness of the developing memory impairment. During this preclinical phase, patients may develop mild cognitive impairment (MCI), which is considered a stage of cognitive functioning intermediate between normal and any type of dementia [3]. Currently, cognitive decline is defined by performance on cognitive tests. A range of cerebrospinal fluid (CSF) and blood biomarkers for cognitive decline, including amyloid-β (Aβ) and phospho-Tau (pTau) are being used or under investigation [4, 5]. Early detection of AD development during the long preclinical phase provides opportunities for intervention.

The strongest genetic risk factor for developing AD is the ε4 allele of the *APOE* gene encoding apolipoprotein (Apo)E [6–8]. Compared with the other two common alleles of the *APOE* gene, ε2 and ε3, the risk of developing AD is ∼3 times and ∼15 times higher in heterozygous and homozygous *APOE4* carriers, respectively [9–12]. ApoE4 may contribute to the progression of AD in multiple manners, including modulation of lipid trafficking, cholesterol in particular, within the brain, blood-brain barrier functioning [13–15] and clearance of $A\beta_{42}$ from the brain [16]. In particular, ApoE-expressing lipoprotein-like particles transport $A\beta_{42}$ into CSF, with ApoE4 being less efficient than ApoE3 or ApoE2 [13]. In the circulation, ApoE is associated with lipoproteins, with ApoE3 and ApoE2 displaying a preference for high density lipoproteins (HDL) and ApoE4 for very low density lipoproteins (VLDL) [17]. Brain ApoE and circulating HDL facilitate the transport of $A\beta_{42}$ across bioengineered cerebrovascular vessels, ApoE4 being less effective than ApoE2 [16]. Brain ApoE predominantly secreted by astrocytes is associated with HDL-like particles involved in intercellular lipid trafficking within the central nervous system [18, 19]. In comparison with ApoE2 and ApoE3, ApoE4 secreted from astrocytes or for example choroid plexus epithelial cells is less lipidated, which may affect its role in lipid trafficking and thereby contribute to the risk of AD [20, 21].

Sphingolipids are among the lipids that together with cholesterol are carried by the ApoE-containing lipoproteins [22, 23]. Alterations in sphingolipids in the brain, CSF, and in plasma have been associated with the development of AD and mild cognitive impairment (MCI), its prodromal stage [24–29]. Sphingolipids are essential for cell membrane structure and also function as bioactive molecules controlling cellular growth, differentiation, proliferation, and apoptosis [30–33]. Therefore, imbalances in sphingolipid homeostasis may play a key role in disease pathogenesis, and consequently, ApoE4 may contribute to the development of AD and MCI through its effect on sphingolipid homeostasis. In plasma, there are indications for increased concen-

trations of Cer(d18:1/16:0) and Cer(d18:1/24:1) for patients with AD, but data on other sphingolipids in plasma as potential biomarkers for AD are inconsistent [34]. *APOE4* genotype, however, was not taken into account.

In this pilot study, we first tested the hypothesis that the *APOE4* genotype affects sphingolipid homeostasis in CSF and in the circulation in the prodromal stages of AD. Secondly, we tested the hypothesis that the effect of the *APOE4* genotype in patients with MCI differed from patients with subjective cognitive decline (SCD). We therefore determined concentrations of sphingosine-1-phosphate and a number of the most abundant ceramides and sphingomyelins in CSF, plasma and in circulating lipoproteins of patients with MCI and SCD who either carried two or no *APOE4* alleles.

MATERIALS AND METHODS

Participants

CSF and nonfasted plasma was obtained from patients in the ongoing Amsterdam Dementia Cohort project [35]. All patients visited the memory clinic of the Free University Medical Center between February 2004 and December 2016 for extensive dementia screening that consisted of neurological, physical, and neuropsychological evaluation, biomarker analyses in CSF obtained by lumbar puncture, electroencephalography, and brain magnetic resonance imaging [35]. Venous blood was collected in EDTA and centrifuged at 2000 g within 60 min (room temperature) [36]. Aliquots of plasma and CSF were stored at –80°C until further analysis [36]. $A\beta_{42}$ and pTau181 concentrations in CSF were determined by Innotest enzyme immune assay (Fujirebio, Ghent, Belgium) by technicians who were blinded for clinical diagnosis [37, 38].

Patients were selected based on *APOE* genotype and diagnosis as having either MCI or SCD. The diagnosis of MCI was based on Petersen's criteria until 2012 and the National Institute on Aging-Alzheimer's Association criteria thereafter [39]. Subjects were labeled as SCD upon multidisciplinary consensus when no abnormalities on clinical or cognitive testing were observed and criteria for MCI, dementia, and other medical conditions potentially causing cognitive decline were not met (i.e., no psychiatric diagnosis) [40]. Patients were either homozygous for the ε4 allele (*APOE4* homozygotes) or had no copies of the ε4 allele (non-*APOE4* carriers). CSF was obtained from 39 patients with MCI or SCD and plasma was obtained from 39 other patients with MCI or SCD. Written informed consent to use medical data and biomaterials for research purposes was in place, in accordance with the ethical consent by the Free University Amsterdam and with the Helsinki Declaration act of 1975.

APOE genotyping

Genomic DNA was isolated from EDTA blood. DNA was PCR-amplified and subsequently analyzed using QIAxcel DNA Fast Analysis kit (Qiagen, Venlo, The Netherlands) to verify amplimer size, and Sanger sequenced on the ABI130XL to determine *APOE* genotype. Non-*APOE4* participants were either *APOE2/E3* heterozygotes (plasma group 3/20 (15%), CSF group 7/20 (35%)) or *APOE3* homozygotes.

Lipoprotein isolation

Lipoproteins were isolated from 0.3–0.6 mL plasma by density-gradient ultracentrifugation (DGUC) as previously described [41]. In short, plasma samples were brought to 1 mL volume with physiological salt. Subsequently, potassium bromide (KBr) (0.35 g) was added to obtain a density of 1.26 g/mL. Of this plasma-KBr mix, 1 ml was placed in an ultracentrifuge tube (Beckmann Coulter) and 1.9 mL of 1.21, 1.10, 1.063, 1.04, and 1.02 g/mL KBr in physiological salt was layered on top, followed by 1 mL of water. Samples were centrifuged at 207,000 g for 18 h at 4°C using an SW41 rotor in a Optima XPN-80 Beckman ultracentrifuge (Beckman Instruments, Indianapolis, IN, USA). Thereafter, 40–45 fractions were collected from the gradient starting from the bottom of the tube. Lipoproteins were distinguished as high-density lipoproteins (HDL: 1.062–1.21 g/mL), low-density lipoproteins (LDL: 1.019–1.063 g/mL), and intermediate and very-low density lipoproteins (VLDL: <1.019 g/mL) [42]. Area under the curve of the obtained cholesterol profile was calculated using the trapezoidal rule to obtain the lipid content of each lipoprotein class.

Cholesterol and triglyceride measurements

All biochemical analyses were performed blinded for clinical information. Cholesterol, triglycerides, and ApoB100 were measured in plasma and

DGUC-fractions by an enzymatic method using the Selectra E (DDS Diagnostic system, Istanbul, Turkey).

Analysis of sphingosine-1-phosphate, selected ceramides, and selected sphingomyelins

Lipid extraction

Sphingolipids were extracted from CSF by a modified Bligh and Dyer procedure, as previously described [43]. In short, 100 μl CSF was combined with 10 μL 10% triethanolamine solution (TEA (10/90, v/v) in methanol/dichloromethane (MeOH/DCM; 50:50; by volume) and 5 μl internal standard mix (IS; 2 μg/mL Ceramide (Cer) (d18:1/17:0), 2 μg/mL Cer(d17:0/24:1), 10 μg/mL sphingomyelin (SM) (d18:1/17:0), and 0.2 μg/mL sphingosine-1-phospate (S1P) (d18:1)-D7 in MeOH; Avanti Polar Lipids). After thorough mixing, 450 μL MeOH/DCM (50:50, by volume) was added to extract the lipids. Samples were incubated under constant agitation for 30 min at 4°C. After incubation, samples were centrifuged at 18,500 g for 20 min at 4°C (Hettich mikro 200R, Geldermalsen, the Netherlands). The bottom layer was transferred to a glass vial and another 450 μL MeOH/DCM was added to the remaining solution for a second extraction step. The supernatant was transferred to the vial with the bottom layer, the extract was freeze dried and reconstituted in 100 μL MeOH before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses.

For lipid extraction from plasma or isolated lipoproteins, 10 μl plasma or 20 μl DGUC-fraction was combined with 10 μL 10% TEA solution and 10 μl IS. Only the second extraction step described above was performed. Supernatants were transferred to glass vials, freeze dried and reconstituted in 100 μL MeOH before LC-MS/MS analyses.

LC-MS/MS analysis

Sphingolipid analysis was performed according to Mielke et al. [44] and described previously [45]. Of the lipid extracts from CSF, plasma and DGUC fraction, 5, 5 and 10 μl were injected by an autosampler (Shimadzu, Kyoto, Japan) into a Shimadzu HPLC system (Shimadzu) equipped with a Kinetex C8 column (50 × 2.1 mm, 2.6 μm, Phenomenex, Maarssen, the Netherlands) at 30°C. Components were separated using a gradient, starting from 95% mobile phase A (MilliQ water/MeOH (50/50, v/v) containing 1.5 mM ammonium formate and 0.1% formic acid) and 5% mobile phase B (MeOH containing 1 mM ammonium formate and 0.1% formic acid) for 2 min and increased to 7% mobile phase A and 93% mobile phase B at 5.5 min. After 10 min the column was flushed with 99% mobile phase B for 2 min followed by a 2 min re-equilibration. The flow rate was set at 0.25 ml/min and total run time was 14 min. The effluent was directed to a Sciex Qtrap 5500 quadruple mass spectrometer (AB Sciex Inc., Thornhill, Ontario, Canada) and analyzed in positive ion mode following electrospray ionization using multiple reaction monitoring. The following settings were applied: curtain gas at 27.5; collision gas was set at low; ion spray voltage at 4000; temperature at 600°C; ion source Gas 1 at 30; ion source Gas 2 at 60; declustering potential at 100; entrance potential at 14, and collision cell exit potential at 14. Detailed LC-MS/MS settings for each sphingolipid species are given in Supplementary Table 1.

We quantified S1P(d18:1)), seven of the most abundant ceramide species, and seven of the most abundant SM species for which standards were commercially available (S1P(d18:1/), Cer(d18:1/14:0), Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0), Cer(d18:1/24:1), Cer(d18:1/24:0), SM(d18:1/16:0), SM(d18:1/18:1), SM(d18:1/18:0), SM(d18:1/24:1), and SM(d18:1/24:0) (Avanti polar lipids, Alabaster, AL, USA), as well as SM(d18:1/20:0) and SM(d18:1/22:0) (Matreya LLC, PA, USA)). To enable analysis of all sphingolipid subspecies in a single LC-MS/MS run, the highly abundant SM species were analyzed at m+3 instead of m+1. For each sphingolipid, nine-point calibration curves were constructed by plotting analyte to internal standard peak area ratios versus the corresponding analyte concentration. Linear regression correlation coefficients (r^2) were >0.99. Sphingolipid levels were determined from these calibration curves using S1P(d18:1)-D7 as internal standard for S1P, Cer(d18:1/17:0) for ceramides with acyl chains 14:0 till 22:0, Cer(d17:0/24:1) for ceramides with acyl chains 24:0 and 24:1, and SM(d18:1/17:0) for the SMs. Instrument control and quantification of spectral data was performed using MultiQuant software (AB Sciex Inc.). CSF, plasma, and lipoprotein sphingolipid data were displayed as mean ± SD in μM. Total ceramide and sphingomyelin levels were calculated as the sum of the individual sphingolipid measured, respectively. HDL sphingolipids were also expressed as ratio to HDL cholesterol, whereas LDL sphingolipids were also expressed as ratio to LDL apoB100.

Table 1
Characteristics of non-*APOE4* carriers and *APOE4* homozygotes with SCD or MCI

	Non-*APOE4* carriers		*APOE4* homozygotes		*p*	
	SCD	MCI	SCD	MCI	*APOE4* versus non-*APOE4*	MCI versus SCD
N	20	20	18	20		
apoE2/E3	5	5				
Female (%)	6 (30)	10 (50)	7 (39)	10 (50)	0.696	0.174
Age (y)	56.6 ± 10.6	69.5 ± 8.8	59.5 ± 7.0	66.4 ± 5.8	0.964	**<0.001**
MMSE#	28.4 ± 1.6	23.2 ± 2.0	29.0 ± 1.0	24.0 ± 1.9	0.226	**<0.001**
CSF Aβ_{42} (pg/mL)#	1162 ± 285	789 ± 263	883 ± 338	605 ± 110	**<0.001**	**<0.001**
CSF pTau (pg/mL)#	50 ± 24	71.2 ± 40.0	56.4 ± 20.9	63.2 ± 25.0	0.964	0.107
BMI (kg/m^2)#	26.1 ± 5.0	25.3 ± 4.5	26.4 ± 3.0	24.4 ± 3.7	0.853	0.062
Smoking, ever (%)#	10 (50)	8 (40)	7 (39)	6 (30)	0.477	0.222
Smoking, current (%)#	3 (15)	4 (20)	2 (11)	1 (5)	0.228	0.370
Statin use (%)#	2 (10)	2 (10)	4 (22)	5 (25)	0.309	0.083

Data are displayed as mean ± SD, or as n (%); #General Linear model with age squared, sex, *APOE* genotype, and cognitive category; contribution of age squared and sex to variance of the dependent variable was only significant for CSF pTau ($p = 0.025$; $\eta^2 = 0.14$); MMSE, Mini-Mental State Examination; BMI, body mass index.

Statistical analyses

All outcome parameters were analyzed with IBM SPSS Statistics version 24.0. For lipid parameters Z-values were calculated and individual values that corresponded to a Z-value that deviated more than 4 from the center were treated as outliers (0.20% of all data points). Normal distribution of the data was confirmed by the Shapiro-Wilk test.

Age, sex, and clinical variables among the four groups were displayed as mean ± SD or as *n* (%). To test effects of *APOE* genotype and cognitive state for cholesterol, triglyceride, ApoB100, and S1P(d18:1) levels, a univariate two-way ANOVA was used after adjustment for age and sex. Statistical analysis of lipid parameters was done by general multivariate regression modeling using sex (0: male; 1: female), age squared, *APOE* genotype (0: non-*APOE4*; 1: *APOE4*), and cognitive category (0: SCD; 1: MCI) as independent variables. Age squared was chosen over age, as incidence of dementia has been shown to increase exponentially with age, and underlying neuropathology may show a similar relationship with age [46, 47]. Effect sizes were given by paired eta squared (η^2), with a value of 0.14 and above being considered as a large effect. Spearman's correlation analysis was used to assess the correlation between CSF sphingolipid levels and CSF Aβ_{42}. Differences between correlation coefficients were analyzed after Fisher's r-to-z transformation. Since this is a hypothesis generating study, all *p*-values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics of the combined patient cohort are shown in Table 1, and of the patients that provided either CSF or plasma separately are shown in Supplementary Table 2. *APOE4* homozygotes and non-*APOE4* carriers did not differ significantly with respect to age and sex. In line with expectations, Aβ_{42} levels in CSF were lower in *APOE4* homozygotes than in non-*APOE4* carriers, and lower in MCI patients than in SCD patients. Among the MCI patients and among the SCD patients, Aβ_{42} levels were also lower in the *APOE4* homozygotes ($p = 0.007$ and $p = 0.009$, respectively). *APOE4* homozygotes showed higher LDL-C and VLDL-C levels. Patients with MCI were older than those with SCD and had a lower Mini-Mental State Examination (MMSE) score, but higher LDL-ApoB100 levels. pTau levels in CSF were not different among the groups, nor were the other tested parameters. Qualitatively similar results were obtained when the heterozygous non-*APOE4* carriers were excluded from the analysis, but differences among groups were no longer significant (data not shown).

Sphingolipids in CSF

In CSF, total SM and SM(d18:1/18:0) levels were lower in individuals homozygous for *APOE4* than in those without *APOE4* ($p = 0.042$ and 0.026, respectively; Table 2). No significant differences among the groups were observed for the other sphingolipids. In

Table 2
Sphingolipid levels in CSF from non-*APOE4* carriers and *APOE4* homozygotes with SCD or MCI

CSF	Non-*APOE4* carriers		*APOE4* homozygotes		*effect size (partial eta squared)*[a]			
	SCD (n-10)	MCI ($n = 10$)	SCD ($n = 9$)	MCI ($n = 10$)	Age^2	*Sex*	MCI versus SCD	*APOE4* versus non-*APOE*4
S1P(d18:1)		<LLOD*						
Ceramides	1.70 ± 0.20	1.63 ± 0.38	1.74 ± 0.51	1.47 ± 0.40	0.01	0.05	0.03	0.01
Cer(d18:1/14:0)	0.05 ± 0.01	0.05 ± 0.02	0.06 ± 0.03	0.05 ± 0.02	0.01	**0.18**[$]	0.01	0
Cer(d18:1/16:0)	0.08 ± 0.04	0.08 ± 0.05	0.09 ± 0.06	0.07 ± 0.07	0	**0.13**[$]	0	0
Cer(d18:1/18:0)	0.87 ± 0.12	0.79 ± 0.19	0.77 ± 0.26	0.65 ± 0.14	0.01	0	0.05	0.10
Cer(d18:1/20:0)	0.14 ± 0.02	0.13 ± 0.04	0.15 ± 0.05	0.13 ± 0.04	0	0.04	0	0.01
Cer(d18:1/22:0)	0.21 ± 0.04	0.21 ± 0.08	0.26 ± 0.11	0.22 ± 0.07	0.02	**0.11**[$]	0	0.05
Cer(d18:1/24:0)	0.23 ± 0.08	0.24 ± 0.13	0.29 ± 0.14	0.24 ± 0.11	0.07	0.09	0.01	0.02
Cer(d18:1/24:1)	0.12 ± 0.02	0.12 ± 0.03	0.12 ± 0.03	0.11 ± 0.04	0.09	0.01	0.04	0.01
Sphingomyelins	269.48 ± 64.31	268.21 ± 46.55	270.04 ± 83.63	235.64 ± 63.18	0.11	0.02	0.06	0.01
SM(d18:1/16:0)	70.10 ± 18.66	71.54 ± 15.80	69.25 ± 17.10	59.61 ± 18.97	**0.18**[$]	0.02	0.08	0.03
SM(d18:1/18:0)	62.86 ± 8.91	58.68 ± 9.53	55.90 ± 16.49	49.17 ± 9.20	0.01	0	0.07	**0.12**[$]
SM(d18:1/18:1)	14.97 ± 3.06	16.21 ± 4.34	15.17 ± 4.73	12.43 ± 3.01	0.04	0	0.04	0.05
SM(d18:1/20:0)		<LLOD*						
SM(d18:1/22:0)	66.48 ± 8.91	58.68 ± 9.53	55.90 ± 16.49	49.17 ± 9.20	0.09	0.01	0.04	0,01
SM(d18:1/24:0)	6.30 ± 2.74	6.32 ± 1.52	6.60 ± 3.20	5.79 ± 2.14	**0.11**[$]	0.04	0.03	0
SM(d18:1/24:1)	48.76 ± 64.31	48.50 ± 10.16	49.13 ± 17.63	42.83 ± 11.55	0.10	0.07	0.04	0.01

Data are mean ± SD in μM. [a]General Linear model with age squared, sex, *APOE* genotype, and cognitive category; [$]$p < 0.05$. *LLOD, lower limit of detection was 0.11 μM and 0.05 μM for S1P and SM(d18:1/20:0), respectively.

Table 3
Correlation between MMSE score and sphingolipid levels in CSF from patients without *APOE4* and patients homozygous for *APOE4* and from patients with either SCD or MCI

CSF	non-*APOE4* carriers ($n=20$)	*APOE4* homozygotes ($n=19$)	SCD ($n=19$)	MCI ($n=20$)	p^*
	r	*r*	*r*	*r*	
Total Ceramide	0.12 [–0.36; 0.54]	0.12 [–0.38; 0.56]	0.03 [–0.45; 0.50]	–0.38 [–0.71; 0.09]	
Cer(d18:1/14:0)	0.12 [–0.35; 0.54]	0.01 [–0.47; 0.49]	–0.36 [–0.72; 0.14]	–0.28 [–0.65; 0.19]	
Cer(d18:1/16:0)	–0.10 [–0.53; 0.37]	0.11 [–0.39; 0.56]	–0.02 [–0.50; 0.46]	–0.44 [–0.75; 0.13]	
Cer(d18:1/18:0)	0.42 [–0.04; 0.48]	0.28 [–0.23; 0.67]	0.39 [–0.11; 0.73]	–0.11 [–0.54; 0.36]	
Cer(d18:1/20:0)	0.03 [–0.43; 0.62]	–0.05 [–0.52; 0.44]	–0.37 [–0.72; 0.14]	–0.37 [–0.70; 0.10]	
Cer(d18:1/22:0)	–0.06 [–0.50; 0.40]	–0.01 [–0.49; 0.47]	–0.22 [–0.63; 0.29]	**–0.48 [–0.76;–0.03]&**	0.195
Cer(d18:1/24:0)	–0.02 [–0.47; 0.44]	–0.01 [–0.48; 0.47]	–0.22 [–0.62; 0.29]	–0.32 [–0.68; 0.16]	
Cer(d18:1/24:1)	0.06 [–0.40; 0.50]	0.02 [–0.47; 0.49]	0.18 [–0.33; 0.60]	–0.42 [–0.74; 0.04]	
Total SM	0.04 [–0.42; 0.49]	0.10 [–0.40; 0.55]	0.28 [–0.23; 0.67]	–0.44 [–0.74; 0.20]	
SM(d18:1/16:0)	0.03 [–0.43; 0.48]	0.20 [–0.31; 0.62]	0.34 [–0.17; 0.70]	–0.40 [–0.72; 0.07]	
SM(d18:1/18:0)	0.27 [–0.21; 0.64]	0.18 [–0.32; 0.61]	0.28 [–0.23; 0.67]	–0.21 [–0.60; 0.27]	
SM(d18:1/18:1)	–0.10 [–0.53; 0.37]	0.22 [–0.28; 0.63]	0.46 [–0.02; 0.77]	–0.43 [–0.74; 0.03]	
SM(d18:1/22:0)	–0.12 [–0.54; 0.36]	–0.13 [–0.57; 0.37]	0.09 [–0.41; 0.54]	**–0.47 [–0.76;–0.02]&**	**0.043**
SM(d18:1/24:0)	–0.04 [–0.49; 0.42]	–0.13 [–0.57; 0.38]	0.22 [–0.28; 0.63]	**–0.52 [–0.79;–0.09]&**	**0.011**
SM(d18:1/24:1)	–0.05 [–0.49; 0.42]	–0.03 [–0.50; 0.46]	0.10 [–0.40; 0.55]	**–0.50 [–0.78;–0.07]&**	**0.031**

Data are Spearman's correlation coefficients r [95% CI]. &significant correlation ($p<0.05$; in bold); *between-group difference in r after transformation to z (one-tailed).

multivariate analysis, *APOE* genotype significantly contributed to the variance of the SM(d18:1/18:0) levels, but not to any of the other sphingolipids measured. Cognitive state did not contribute significantly to any of the sphingolipid levels. In contrast, sex contributed significantly to the variance of the levels of the ceramides d(18:1/14:0), d(18:1/16:0), and d(18:1/22:0) whereas age contributed significantly to the levels of the sphingomyelins d(18:1/16:0) and d(18:1/24:0) (Table 2). Qualitatively similar results were obtained when the heterozygous non-*APOE4* carriers were excluded from the analysis, but contributions were no longer significant (data not shown).

Levels of none of the sphingolipids measured showed a significant correlation with the MMSE score among the non-*APOE4* carriers, the *APOE4* homozygotes or the SCD patients (Table 3). However, among the patients with MCI, levels of Cer(d18:1/22:0), SM(d18:1/22:0), SM(d18:1/24:0), and SM(d18:1/24:1) negatively correlated with the MMSE score, indicating that these long-chain sphingolipids are higher when cognitive ability is lower. For these long-chain sphingolipids, interactions between MMSE score, APOE4 genotype, and cognitive category were not significant (data not shown).

Because ApoE is thought to be involved in transport of $A\beta_{42}$ into CSF, we next assessed within-group correlations between $A\beta_{42}$ and sphingolipids levels in CSF (Table 4). $A\beta_{42}$ levels positively correlated with Cer(d18:1/24:1) levels in non-*APOE4* carriers, but not in *APOE4* homozygotes. $A\beta_{42}$ positively correlated with Cer(d18:1/18:0), SM(d18:1/18:0), and SM(d18:1/18:1) levels in *APOE4* homozygotes but not in non-*APOE4* carriers. Between-group differences in correlation coefficients were not significant. $A\beta_{42}$ levels negatively associated with Cer(d18:1/24:0) in SCD patients, but positively in MCI patients. $A\beta_{42}$ and Cer(d18:1/18:0) positively correlated in SCD patients, but not in MCI patients. On the other hand, $A\beta_{42}$ positively correlated with Cer(d18:1/24:1), SM(d18:1/16:0), SM(d18:1/24:0), and SM(d18:1/24:1) in MCI patients, but not in SCD patients. Between-group differences in correlation coefficients were significant for Cer(d18:1/18:0), Cer(d18:1/24:0) and SM(d18:1/24:0) for MCI versus SCD patients, but not for *APOE4* homozygotes versus non-*APOE4* carriers (Table 4; Supplementary Figure 1).

One of the SCD patients among the non-*APOE4* carriers had a very low $A\beta_{42}$ (431 pg/mL) and very high pTau level (125 pg/mL) in CSF resulting in a $A\beta_{42}$/pTau ratio of 3.45, which was more than 2SD less than the group mean ($27.36 \pm 10{,}69$; $n=19$). Excluding this patient from the analysis yielded qualitatively similar correlations between CSF $A\beta_{42}$ levels and CSF sphingolipid levels.

Table 4
Correlation between CSF $A\beta_{42}$ and sphingolipid levels in CSF from patients without *APOE4* and patients homozygous for *APOE4* and from patients with either SCD or MCI

CSF	non-*APOE4* carriers (*n* = 20)	*APOE4* homozygotes (*n* = 19)	*p**	SCD (*n* = 19)	MCI (*n* = 20)	*p**
	r	*r*		*r*	*r*	
Total Ceramide	0.39 [–0.05; 0.73]	0.40 [–0.08; 0.78]		0.24 [–0.28; 0.70]	0.28 [–0.28; 0.68]	
Cer(d18:1/14:0)	0.25 [–0.27; 0.63]	0.17 [–0.31; 0.56]		–0.10 [–0.57; 0.44]	0.18 [–0.28; 0.57]	
Cer(d18:1/16:0)	0.42 [–0.03; 0.75]	0.31 [–0.22; 0.71]		0.22 [–0.29; 0.68]	0.20 [–0.31; 0.60]	
Cer(d18:1/18:0)	0.44 [–0.03; 0.82]	**0.56 [0.09; 0.90]**[&]	0.322	**0.73 [0.30; 0.94]**[&]	–0.04 [–0.52; 0.44]	**0.003**
Cer(d18:1/20:0)	0.35 [–0.08; 0.62]	0.17 [–0.29; 0.56]		0.07 [–0.46; 0.52]	0.18 [–0.33; 0.62]	
Cer(d18:1/22:0)	0.30 [–0.15; 0.68]	0.11 [–0.40; 0.61]		–0.26 [–0.65; 0.25]	0.30 [–0.17; 0.65]	
Cer(d18:1/24:0)	0.13 [–0.36; 0.58]	0.06 [–0.40; 0.53]		**–0.53 [–0.81; –0.05]**[&]	**0.49 [0.04; 0.78]**[&]	**0.001**
Cer(d18:1/24:1)	**0.50 [0.09; 0.75]**[&]	0.30 [–0.11; 0.64]	0.246	0.30 [–0.20; 0.69]	**0.51 [0.05; 0.81]**[&]	0.234
Total SM	0.22 [–0.33; 0.67]	0.35 [–0.10; 0.69]		0.20 [–0.34; 0.6]	0.40 [–0.11; 0.75]	
SM(d18:1/16:0)	0.27 [–0.25; 0.67]	0.37 [–0.07; 0.66]		0.23 [–0.29; 0.67]	0.44 [–0.00; 0.75]	
SM(d18:1/18:0)	0.35 [–0.15; 0.81]	**0.49 [–0.06; 0.83]**[&]	0.312	**0.50 [0.17; 0.85]**[&]	0.24 [–0.25; 0.63]	0.191
SM(d18:1/18:1)	0.27 [–0.17; 0.63]	**0.51 [0.03; 0.79]**[&]	0.206	**0.54 [0.06; 0.81]**[&]	0.33 [–0.15; 0.67]	0.227
SM(d18:1/22:0)	0.02 [–0.47; 0.50]	0.11 [–0.34; 0.49]		–0.14 [–0.57; 0.36]	0.37 [–0.10; 0.76]	
SM(d18:1/24:0)	0.17 [–0.35; 0.64]	0.11 [–0.31; 0.54]		–0.03 [–0.48; 0.48]	**0.55 [0.10; 0.85]**[&]	**0.031**
SM(d18:1/24:1)	0.13 [–0.37; 0.59]	0.22 [–0.21; 0.58]		–0.02 [–0.52; 0.49]	**0.47 [0.01; 0.79]**[&]	0.064

Data are Spearman's correlation coefficients r [95% CI]. [&]significant correlation ($p < 0.05$ in bold); *between-group difference in r after transformation to z (one-tailed).

Sphingolipids in the circulation

Compared to non-*APOE4* carriers, the *APOE4* homozygotes had higher plasma LDL levels, expressed either as LDL-apoB100 or LDL-C, and higher VLDL-C levels (Supplementary Table 3). As expected, the *APOE* genotype significantly contributed to the variance in LDL-apoB100, LDL-C, and LDL-TG levels. In addition, LDL-apoB100 and LCL-C levels were significantly affected by age, and LDL-apoB100 levels by cognitive category. In plasma, the *APOE* genotype and cognitive category did not contribute to the variance in the levels of individual and total sphingolipids. (Supplementary Table 4). In contrast, sex contributed significantly to the variance in the levels of most sphingomyelins, whereas the levels of several ceramide species and some sphingomyelin species were associated with age (Supplementary Table 4).

Next, we analyzed sphingolipids contained in circulating HDL. Individual and total sphingolipids were similar in all four patient groups (Supplementary Table 5). *APOE* genotype and cognitive category hardly affected the levels of the individual sphingolipid levels. However, the levels of most individual sphingomyelins were associated with age. In addition, the levels of all sphingomyelins and some of the ceramides were associated with sex, in parallel with HDL cholesterol (Supplementary Table 5). When expressed as a ratio to HDL-cholesterol, Cer(d18:1/18:0) and Cer(d18:1/22:0) levels were associated with the *APOE* genotype (Table 5). Among the MCI patients, both ceramide species were slightly more abundant in the homozygotes ($p = 0.014$ and $p = 0.004$, respectively). There was an antagonistic interaction between *APOE* genotype and cognitive state determining Cer(d18:1/14:0), Cer(d18:1/16:0), and Cer(d18:1/24:0) levels: *APOE4* homozygotes with MCI displayed higher levels of these ceramides than non-*APOE4* carriers with MCI ($p = 0.017$, $p = 0.048$, and $p = 0.049$, respectively), but not in patients with SCD.

In circulating LDL, the *APOE* genotype significantly contributed to the levels of S1P and SM(d18:1/18:1), whereas cognitive state contributed to total ceramide levels (Table 6). LDL-ApoB100. LDL-cholesterol and LDL-TG levels were higher in the *APOE4* homozygotes than in the non-*APOE4* carriers. When expressed as a ratio to LDL-apoB100 levels thus reflecting lipid content per LDL particle, however, sphingolipid as well as cholesterol levels were similar in all four groups (Supplementary Table 6). The variance in the relative levels of individual sphingolipids in LDL were not explained by *APOE* genotype nor by cognitive category. There was antagonistic interaction between *APOE* genotype and cognitive state in determining relative Cer(d18:1/20:0) levels in LDL: *APOE4* homozygotes

Table 5
Sphingolipid/cholesterol ratios in plasma HDL from non-*APOE4* carriers and *APOE4* homozygotes with SCD or MCI

HDL	Non-*APOE4* carriers		*APOE4* homozygotes		effect size (partial eta squared)[a]			
	SCD ($n = 10$)	MCI ($n = 10$)	SCD ($n = 9$)	MCI ($n = 10$)	Age^2	Sex	*APOE4* versus non-*APOE4*	MCI versus SCD
Cholesterol (mM)	1.09 ± 0.31	1.28 ± 0.27	1.12 ± 0.40	1.07 ± 0.36	0.08	**0.25**[$]	0.05	0
Triglycerides (mM)	0.16 ± 0.02	0.14 ± 0.04	0.16 ± 0.05	0.16 ± 0.03	0.03	0.02	0	0
S1P(d18:1) (μM)*	1.37 ± 0.60	1.29 ± 0.64	1.62 ± 0.73	1.70 ± 1.00	0	0.03	0.06	0
Ceramides (μM)*	0.97 ± 0.26	0.80 ± 0.12	0.88 ± 0.20	1.08 ± 0.28	0	0.03	0.06[#]	0 [#]
Cer(d18:1/14:0)*	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.01	0.05	0.05[#]	0.01[#]
Cer(d18:1/16:0)*	0.10 ± 0.04	0.08 ± 0.02	0.08 ± 0.02	0.11 ± 0.04	0.02	0.11	0.01[#]	0.01[#]
Cer(d18:1/18:0)*	0.06 ± 0.03	0.05 ± 0.02	0.06 ± 0.03	0.09 ± 0.04	0.01	**0.12**[$]	**0.11**[$]	0.04
Cer(d18:1/20:0)*	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.02	0.07 ± 0.03	0	0.05	0.08	0
Cer(d18:1/22:0)*	0.24 ± 0.08	0.19 ± 0.05	0.25 ± 0.06	0.28 ± 0.07	0.01	0	**0.12**[$]	0.01
Cer(d18:1/24:0)*	0.31 ± 0.08	0.23 ± 0.03	0.26 ± 0.08	0.29 ± 0.06	0.01	0.03	0[#]	0.01[#]
Cer(d18:1/24:1)*	0.18 ± 0.04	0.18 ± 0.04	0.17 ± 0.04	0.22 ± 0.06	0.02	0	0.01	0.03
Sphingomyelins (μM)*	138.17 ± 58.03	134.94 ± 25.53	140.75 ± 44.19	138.06 ± 28.05	0.07	0.04	0.02	0.01
SM(d18:1/16:0)*	37.89 ± 8.38	40.99 ± 5.51	38.51 ± 10.75	42.91 ± 7.60	0.02	0	0.01	0.02
SM(d18:1/18:0)*	7.07 ± 2.03	7.93 ± 1.19	7.30 ± 1.31	8.55 ± 1.68	0.01	0.01	0.02	0.06
SM(d18:1/18:1)*	4.64 ± 1.24	4.86 ± 1.44	4.95 ± 1.34	5.89 ± 1.31	0.02	0.07	0.05	0.01
SM(d18:1/20:0)*	17.73 ± 5.83	18.15 ± 3.79	19.74 ± 5.42	19.41 ± 3.49	0.02	0	0.03	0.01
SM(d18:1/22:0)*	19.49 ± 8.07	19.41 ± 4.94	24.05 ± 8.76	18.35 ± 5.47	0.01	0.08	0.02	0.04
SM(d18:1/24:0)*	4.97 ± 1.37	4.83 ± 1.84	5.83 ± 2.65	5.02 ± 1.32	0.06	0.03	0.02	0.06
SM(d18:1/24:1)*	32.48 ± 11.12	38.76 ± 13.01	40.37 ± 19.10	37.93 ± 13.29	**0.12**[$]	0.05	0.01	0.02

Data are mean ± SD. [a]General Linear model with age squared, sex, *APOE* genotype, and cognitive category; [$]$p < 0.05$; [#]significant interaction between *APOE* genotype and cognitive category ($p < 0.05$); *expressed relative to HDL cholesterol.

Table 6
Sphingolipid levels in plasma LDL from non-*APOE4* carriers and *APOE4* homozygotes with SCD or MCI

LDL	Non-*APOE4* carriers		*APOE4* homozygotes		effect size (partial eta squared)			
	SCD ($n = 10$)	MCI ($n = 10$)	SCD ($n = 9$)	MCI ($n = 10$)	age^2	sex	*APOE4* versus non-*APO*E4	MCI versus SCD
ApoB100 (g/L)	0.54 ± 0.11	0.55 ± 0.11	0.65 ± 0.17	0.67 ± 0.13	**0.30**$^{\$}$	0	**0.23**$^{\$}$	**0.16**$^{\$}$
Cholesterol (mM)	2.33 ± 0.57	2.35 ± 0.71	2.73 ± 0.73	2.94 ± 0.78	**0.14**$^{\$}$	0.05	**0.15**$^{\$}$	0.07
Triglycerides (mM)	0.22 ± 0.03	0.20 ± 0.02	0.24 ± 0.09	0.28 ± 0.07	0.03	0.02	**0.15**$^{\$}$	0.05
S1P(d18:1) (μM)	0.17 ± 0.09	0.22 ± 0.14	0.29 ± 0.19	0.32 ± 0.15	0.02	0.01	**0.13**$^{\$}$	0.05
Ceramides (μM)	3.70 ± 1.35	3.63 ± 1.32	2.60 ± 1.44	4.97 ± 1.60	0.03	**0.20**$^{\$}$	0$^{\#}$	**0.17**$^{\$,\#}$
Cer(d18:1/14:0)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	0.04 ± 0.01	0.02	0	0.06	0.01
Cer(d18:1/16:0)	0.18 ± 0.06	0.17 ± 0.05	0.16 ± 0.06	0.21 ± 0.05	0.04	0.02	0.02	0.04
Cer(d18:1/18:0)	0.10 ± 0.04	0.11 ± 0.04	0.10 ± 0.04	0.17 ± 0.07	0	0.02	0.10	0.08
Cer(d18:1/20:0)	0.10 ± 0.03	0.10 ± 0.04	0.10 ± 0.04	0.15 ± 0.05	0	0.03	0.10	0.06
Cer(d18:1/22:0)	0.77 ± 0.27	0.77 ± 0.29	0.82 ± 0.50	1.20 ± 0.41	0.02	**0.12**$^{\$}$	0.10	0.10
Cer(d18:1/24:0)	1.98 ± 0.84	1.85 ± 0.88	1.69 ± 0.83	2.41 ± 0.97	0.06	0.08	0.01	0.07
Cer(d18:1/24:1)	0.54 ± 0.22	0.61 ± 0.23	0.54 ± 0.23	0.71 ± 0.28	0	**0.12**$^{\$}$	0.05	0.08
Sphingomyelins (μM)	227.83 ± 77.77	244.16 ± 73.16	270.14 ± 157.83	302.95 ± 83.44	0.01	0.10	0.07	0.02
SM(d18:1/16:0)	70.14 ± 21.14	78.75 ± 17.83	78.43 ± 47.43	95.40 ± 27.71	0.01	0.07	0.05	0.06
SM(d18:1/18:0)	13.05 ± 4.80	15.40 ± 4.94	15.20 ± 6.50	19.42 ± 5.13	0	0.06	0.09	0.09
SM(d18:1/18:1)	5.61 ± 2.22	6.25 ± 2.39	6.87 ± 3.12	8.40 ± 1.92	0.01	**0.15**$^{\$}$	**0.13**$^{\$}$	0.07
SM(d18:1/20:0)	30.70 ± 28.76	23.61 ± 6.15	27.20 ± 10.70	31.37 ± 8.06	0	0.06	0	0.01
SM(d18:1/22:0)	38.32 ± 14.25	38.33 ± 14.28	45.92 ± 26.68	48.37 ± 20.23	0.05	0.10	0.07	0.02
SM(d18:1/24:0)	11.88 ± 4.83	11.78 ± 5.87	16.47 ± 11.60	16.49 ± 6.21	0.02	0.07	0.11	0.01
SM(d18:1/24:1)	58.13 ± 23.91	70.03 ± 26.75	80.03 ± 54.08	83.94 ± 28.21	0	0.05	0.07	0.01

Data are mean ± SD. [a]General Linear model with age squared, sex, *APOE* genotype, and cognitive category; $^{\$}p < 0.05$; $^{\#}$significant interaction between *APOE* genotype and cognitive category ($p < 0.05$).

with MCI had higher Cer(d18:1/20:0) levels than non-*APOE4* carriers with MCI ($p = 0.026$), but not those with SCD.

VLDL-apoB100 levels were similar in the four patient groups (Supplementary Table 3). Levels of none of the sphingolipids in VLDL were different among the four groups whether or not expressed relative to VLDL-apoB100 (Supplementary Table 7). Neither *APOE* genotype nor cognitive status significantly contributed to the variance in the relative levels of individual sphingolipid levels in VLDL.

When normalized to the Cer(d18:1/24:0) concentration, levels of Cer(d18:1/16:0), Cer(d18:1/18:0), and Cer(d18:1/24:1) have recently been shown to predict cognitive state [48, 49]. However, in our study population these ratios differed among the groups neither in whole plasma, nor in HDL, LDL, or VLDL (Supplementary Table 8).

DISCUSSION

We assessed whether ceramides, SMs and S1P levels in CSF, plasma and circulating lipoproteins are associated with the presence of *APOE4* in patients with MCI or SCD, which are early stages of cognitive impairment. Our data shows lower sphingomyelin levels in CSF of *APOE4* homozygous patients than in non-*APOE4* carriers. Levels of long-chain sphingomyelins as well as Cer(d18:1/22:0) inversely correlated with the MMSE score in patients with MCI, independent of the *APOE4* genotype. Moreover, $A\beta_{42}$ levels in CSF decreased in parallel with, and correlated to, most ceramide and SM species independent of the *APOE* genotype and cognitive state, except for Cer(d18:1/24:0), which increased with decreasing $A\beta_{42}$ levels in CSF in SCD patients. Relative levels of Cer(d18:1/18:0) and Cer(d18:1/22:0) in HDL were higher in MCI patients homozygous for *APOE4* than non-*APOE4* carriers. As *APOE4* is a well-established risk factor for the development of AD, our data support the hypothesis that ApoE4 may modulate early AD development by affecting sphingolipid metabolism. Consequently, detected changes in sphingolipid levels may be associated with early signs of cognitive decline or could contribute to the development of cognitive decline. Nevertheless, age and sex appear to be stronger determinants of individual sphingolipid levels in CSF than either the *APOE* genotype or the cognitive state. We recently showed that sphingolipid levels in the mouse hippocampus is also predominantly determined by sex rather than by *APOE* genotype or by the presence of common AD mutations in the *APP* and *PS1* genes [45]. Interestingly, the *APOE* genotype and cognitive state interacted in determining HDL concentrations of Cer(d18:1/14:0), Cer(d18:1/16:0), and in particular of Cer(d18:1/24:0), showing that *APOE4* has a different impact on these ceramides in HDL in MCI patients compared to SCD patients.

Association between APOE genotype, cognitive state, and CSF sphingolipids

$A\beta_{42}$ concentrations in CSF were lower in patients with MCI compared to patients with SCD, an effect that was exacerbated by the presence of *APOE4*. That CSF-$A\beta_{42}$ levels are already markedly lower in patients with MCI before the development of AD has been well established [50, 51], and is taken to reflect reduced removal of Aβ from the brain. Part of Aβ removal from the brain interstitial fluid is mediated by brain ApoE, a process in which ApoE4 is less effective than the other ApoE isoforms [14, 16]. ApoE4 has been shown to exacerbate $A\beta_{42}$ deposition as amyloid plaques when compared with apoE3 [52]. Total measured sphingomyelins and SM(d18:1/18:1) were also lower in CSF of *APOE4* homozygotes than of non-*APOE4* carriers, and SM(d18:1/18:1) as well as SM(d18:1/18:0) and Cer(d18:1/18:0) levels decreased in parallel with $A\beta_{42}$ among the *APOE4* homozygotes. In contrast to the other measured sphingomyelins and ceramides, these three species are strongly enriched in CSF relative to plasma levels (Table 2 and Supplementary Table 4), suggesting that they mainly originate from brain cells rather than from plasma. ApoE can be lipidated in the interstitial fluid or in the CSF at the choroid plexus. Lipidated ApoE from the interstitial fluid may enter the CSF and vice versa [53]. Since $A\beta_{42}$ released in the interstitial fluid binds to ApoE, and is being degraded or cleared in parallel with ApoE [20], it is likely that $A\beta_{42}$ enters the CSF bound to ApoE. Since ApoE4 is less lipidated than ApoE3 or ApoE2, our results support the hypothesis that ApoE4 leads to reduced transport of sphingolipids and $A\beta_{42}$ and to reduced entrance into the CSF. Alternatively, ApoE4 might suppress $A\beta_{42}$ clearance (apoE4 > apoE3) by either competing with $A\beta_{42}$ for receptor binding, by retaining $A\beta_{42}$ from its clearance through the blood-brain barrier, or by being less stable than ApoE3 and ApoE2 and be more easily degraded [20, 52]. Recently, ApoE4 in comparison with ApoE3 was found to reduce cholesterol turnover and induce cholesterylester accumulation in oligo-

dendrocytes, resulting in reduced myelination [15]. Moreover, this may affect synaptogenesis [18] and in parallel it may affect the production and the trafficking of sphingolipids and consequently their entrance into the CSF. The reduced concentrations of sphingolipids is already evident in SCD patients without any signs of AD, suggesting that this is a mechanism by which ApoE4 contributes to the early development of AD.

Inconsistent data have been reported on sphingolipid levels in CSF of patients with MCI or AD. In contrast with our data, sphingomyelin levels were found to be increased in a population of MCI patients similar to our study, but the *APOE* genotype was not taken into account [54]. Fonteh et al. [55] found lower sphingolipid levels in CSF of AD patients compared to cognitively normal controls, but Koal et al. [56] reported increased SM(d18:1/18:0) levels in CSF of AD patients with pathologically low levels of Aβ_{42} in CSF. We did not observe any significant difference in total sphingolipids or levels of individual species between CSF of MCI and SCD patients. Possible reasons for these inconsistent results may be differences in CSF collection procedures, methods used for lipid extraction, sphingolipid separation and quantitation, or to the limited number of patients included in the studies. Our data shows a decrease in CSF-Aβ_{42} levels in parallel with Cer(d18:1/24:0), Cer(d18:1/24:1), SM(d18:1/24:0), and SM(d18:1/24:1) in CSF among MCI patients, while among SCD patients Cer(d18:1/24:0) levels increased simultaneously, and independently of the *APOE4* genotype. The correlation with CSF-Aβ_{42} differed significantly between MCI and SCD patients for Cer(d18:1/18:0), Cer(d18:1/24:0), and SM(d18:1/24:0). This suggests involvement of other differential non-ApoE4 related mechanisms for Aβ_{42} clearance. Alternative mechanisms proposed involve LDL-receptor-related protein- and clusterin-related transport, which also may involve sphingomyelins. Conspicuously, CSF-Aβ_{42} levels in SCD patients negatively correlated with Cer(d18:1/24:0) levels, while they positively correlated in MCI patients. This ceramide species is relatively much more abundant in plasma than in CSF (Table 2 and Supplementary Table 4) [22]. Therefore, Cer(d18:1/24:0) may be transported from plasma into the CSF at the choroid plexus. CSF sphingolipids may be affected by changes in plasma sphingolipids that are associated with various metabolic disorders, including diabetes mellitus and cardiovascular disease, both independent risk factors for the development of AD [23]. How this relates to CSF-Aβ_{42} levels in SCD patients remains to be established. Increased levels of intracellular Cer(d18:1/24:0) have been found to induce apoptosis in cultured neutrophils [57]. Reduced removal of Cer(d18:1/24:0) from the brain may therefore lead to neuronal loss due to increased apoptosis. Short chain ceramides are also involved in autophagy, but whether this also holds for the very long chain ceramides is not clear [58]. Sphingomyelins are recently implicated in triggering inflammasome activation, thus explaining a possible role in neuroinflammation, but unfortunately only SM(d18:1/14:0) was tested (E. Latz, 2022, oral presentation, European Atherosclerosis Society, Milano). Nevertheless, measuring Cer(d18:1/18:0), Cer(d18:1/24:0), and SM(d18:1/24:0) levels in CSF may contribute to distinguishing between MCI and SCD in the early stages of cognitive decline.

Effects of APOE genotype and cognitive state on plasma and lipoprotein sphingolipids

In our study population, we did not observe any differences in plasma sphingolipids between *APOE4* homozygotes and non-*APOE4* carriers, and not between MCI and SCD patients. Plasma ceramide levels have been reported to be lower in patients with MCI [25] and patients with AD compared to cognitively normal individuals [26, 59–61], and higher plasma concentrations of selected SM species were consistently associated with AD progression across prodromal and preclinical stages [62]. It has therefore been speculated that plasma sphingolipid levels are linked to cognitive state and that levels change with dementia stage. Perhaps, at the very early stage of cognitive decline, patients may not yet show detectable alterations in plasma sphingolipids levels. Alternatively, the discrepancies could have been due to differences in sample collection, since fasting and type of blood collection, i.e., serum or plasma and type of anticoagulants, are known to impact levels of circulating sphingolipids [22]. So far, the only study addressing sphingolipid profiles of lipoproteins in relation to dementia showed increased ceramide levels in LDL and VLDL from AD, but not MCI patients [63]. In our study, total ceramides measured were higher in LDL from patients with MCI than SCD, which was due to higher number of circulating LDL particles as indicated by the higher LDL-apoB100 levels. For five out of seven ceramide species measured, concentrations in HDL relative to its cholesterol content were higher in *APOE4* homozy-

gotes with MCI than in non-*APOE4* carriers with MCI. In our study, the relative level of three ceramide species in HDL was determined by antagonistic interaction of the *APOE* genotype and cognitive state. This interaction was strongest for Cer(d18:1/24:0), one of the most abundant ceramides in plasma, indicating that the relationship with *APOE* genotype differs between MCI and SCD patients. Interestingly, for this ceramide in CSF the relationship between $A\beta_{42}$ and *APOE* genotype also depended on the cognitive state of the patients. Brain ApoE and circulating HDL have been shown to synergistically facilitate $A\beta_{42}$ transport across bioengineered cerebral blood vessels, in which ApoE4 is less effective than ApoE2 [16]. It is therefore tempting to speculate that the ceramides in brain ApoE-containing HDL-like particles and in circulating HDL have a role in this process. Lipoprotein-sphingolipids may affect proper functioning of the brain vasculature [64] and changes in sphingolipid metabolism may contribute to the pathogenesis of neurodegenerative diseases. High ceramide levels were observed in astrocytes surrounding the brain capillary vasculature in cerebral amyloid angiopathy type 1, which is characterized by a high Aβ plaque load [65, 66]. Several studies have shown that increased ceramide levels are implicated in the induction of neural cell death, oxidative stress, and pro-inflammatory gene expression [67, 68]. The effect of high ceramide levels and the effect on capillary Aβ deposition and consequently neuroinflammation and cognitive decline [69, 70] on HDL function in *APOE4* homozygotes, and the direction of causality, remain to be investigated.

Strengths and limitations

The strength of this study is the inclusion of patients from the well-characterized Amsterdam Dementia Cohort, who either carry no or two copies of the *APOE4* allele. As the aim of our study was to assess the association between sphingolipid levels at very early stages of cognitive decline, we did not include patients diagnosed with AD for comparison. All participants visited a memory clinic, a normal healthy control group is missing. Since all participants were included well before 2018 and some even before 2011, diagnostication of SCD and MCI was not performed according to the 2018 [71] or 2011 [72] NIA-AA guidelines, respectively. In addition, follow-up data on disease progression for the participants were not available. The SCD group may have included participants with an MCI-like phenotype but without objective cognitive decline. We have identified one such candidate in our SCD group, but excluding this participant from the analysis had only limited effect on the outcome. Other limitations of this study are the relatively small sample size and the multiple statistical comparisons for which no corrections were made, which increases the risk of false negative and false positive results. The patient characteristics of the different subsets did not completely overlap, and sphingolipids were determined in CSF and plasma of different subsets of patients with SCD and MCI. Nevertheless, we did observe alterations in CSF and lipoprotein sphingolipid levels and strong correlations between CSF $A\beta_{42}$ and levels of specific sphingolipids. Blood was collected from non-fasting participants, which may have affected plasma and lipoprotein sphingolipid.

Conclusion

In conclusion, patients homozygous for *APOE4* displayed lower sphingomyelin and $A\beta_{42}$ levels in CSF than non-*APOE4* carriers. For *APOE4* homozygotes, non-*APOE4* carriers as well as for SCI and MCD patients, within-group correlations with $A\beta_{42}$ levels were found for several sphingolipid species. This suggests that sphingolipid and $A\beta_{42}$ transport from the brain into the CSF is reduced by ApoE4, which is further affected by cognitive decline in the very early stages of AD development. HDL-ceramides were higher in *APOE4* homozygotes than in non-*APOE4* carriers with MCI, which may affect HDL function in lipid transport across the cerebral vasculature, thereby increasing the risk of AD. Manipulation of sphingolipid levels, for example by targeting enzymes involved in sphingolipid turnover, may have therapeutic potential to prevent or retard early cognitive decline and development of AD.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

SUPPLEMENTARY MATERIAL

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