Cerebrospinal Fluid C18 Ceramide Associates with Markers of Alzheimer's Disease and Inflammation at the Pre- and Early Stages of Dementia

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Abstract.

Background: Understanding how dysregulation in lipid metabolism relates to the severity of Alzheimer's disease (AD) pathology might be critical in developing effective treatments.

Objective: To identify lipid species in cerebrospinal fluid (CSF) associated with signature AD pathology and to explore their relationships with measures reflecting AD-related processes (neurodegeneration, inflammation, deficits in verbal episodic memory) among subjects at the pre- and early symptomatic stages of dementia.

Methods: A total of 60 subjects that had been referred to an Icelandic memory clinic cohort were classified as having CSF AD ($n = 34$) or non-AD ($n = 26$) pathology profiles. Untargeted CSF lipidomic analysis was performed using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) for the detection of mass-to-charge ratio (m/z) features. CSF proteins reflecting neurodegeneration (neurofilament light [NFL]) and inflammation (chitinase-3-like protein 1 [YKL-40], S100 calcium-binding protein B [S100B], glial fibrillary acidic protein [GFAP]) were also measured. Rey Auditory Verbal Learning (RAVLT) and Story tests were used for the assessment of verbal episodic memory.

Results: Eight out of 1008 features were identified as best distinguishing between the CSF profile groups. Of those, only the annotation of the m/z feature assigned to lipid species C18 ceramide was confirmed with a high confidence. Multiple regression analyses, adjusted for age, gender, and education, demonstrated significant associations of CSF core AD markers ($A\beta_{42}$: st.$\beta = -0.36$, $p = 0.007$; T-tau: st.$\beta = 0.41$, $p = 0.005$) and inflammatory marker S100B (st.$\beta = 0.51$, $p = 0.001$) with C18 ceramide levels.

Conclusion: Higher levels of C18 ceramide associated with increased AD pathology and inflammation, suggesting its potential value as a therapeutic target.

Keywords: Alzheimer's disease, biomarkers, cerebrospinal fluid, inflammation, lipidomics

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease that leads to progressive cognitive impairment and dementia. The pathology of AD, which starts decades before the appearance of clinical symptoms [1], is characterized by extracellular amyloid plaques containing amyloid-β (Aβ) and intracellular neurofibrillary tangles (NFTs) comprised of phosphorylated tau (P-tau) [2, 3]. The most widely accepted hypothesis regarding AD pathogenesis proposes that the deposition of Aβ leads to formation of NFTs, neuronal dysfunction, and dementia [4]. Although abundant evidence places Aβ and tau pathology at the center of AD pathogenesis, the mechanisms linking the two together and to eventual neuronal dysfunction and death are still unclear [4, 5]. As Aβ and tau pathology levels are relatively constant in the symptomatic stages of the disease, they are not considered suitable as progression markers [6–9]. Biomarkers reflecting other aspects of AD pathology are therefore needed. Genome-wide association studies have identified about 30 risk genes with a high proportion related to lipid metabolism, immune response, or both [10, 11]. These findings suggest that dysregulation of lipids and inflammatory proteins play an essential part in the pathogenesis of AD.

Lipids play various roles in the human body, both as structural components of cell membranes and in diverse biochemical processes, including membrane trafficking and cell signaling [12]. A dozen of major lipid classes are found within eukaryotic organisms, each comprising hundreds of individual molecular species [13]. Major classes, including glycerophospholipids, sphingolipids, fatty acids, and cholesterol [14], are abundantly expressed in the brain. These lipids are utilized in different compartments of glial cells and neurons [15]. In recent years, the role of lipid metabolism defects in AD pathogenesis has gained increased attention. Several molecular mechanisms have been identified, which connect membrane lipids to the generation and aggregation of Aβ. Pathological forms of Aβ proteins are formed by proteolytic cleavage of the transmembrane protein AβPP by β- and γ-secretases [16]. Lipid membrane structure and organization can affect the activity of these transmembrane enzymes, and thus AβPP processing and Aβ production [17]. Furthermore, secretases, AβPP, and its derivatives also appear to affect the activity of lipid metabolic enzymes and subcellular trafficking, thereby changing the membrane lipid composition.

Lipids might, therefore, play a role in the initiation and progression of AD pathogenesis [16].

Studies have established neuroinflammation as a contributing factor in the pathogenesis and progression of AD and other neurodegenerative diseases [18, 19]. Aβ plaques induce an immune response by activation of microglia and astrocytes [20–22], which in turn is thought to play a role in the formation of NFTs, contributing to neuronal dysfunction and loss [23]. The glial proteins chitinase-3-like-1 protein (YKL-40), calcium-binding protein S100B, and glial fibrillary acidic protein (GFAP) have been associated with AD pathology [24]. All proteins are expressed primarily (YKL-40 and S100B) [25, 26], or exclusively (GFAP) [27] in astrocytes within the central nervous system (CNS). YKL-40, a chitin-binding glycoprotein [28], has been reported to be a promising candidate biomarker of glial activation in AD. Previous studies have detected positive relationships between YKL-40 and the neurodegeneration markers tau [29–32] and neurofilament light (NFL) [33] in cerebrospinal fluid (CSF), demonstrating an association between glial activation and neurodegeneration [34]. NFL is mainly located in myelinated axons, with recent studies indicating a potential for this protein as both a diagnostic and a progression marker in AD and other neurodegenerative diseases [35, 36]. S100B, a calcium-binding protein, exerts both intracellular and extracellular functions and has been found to be upregulated in AD tissues [37, 38]. GFAP is an intermediate filament protein and a marker for astrocyte activation, which has both been associated with amyloid plaque load and the number of NFTs [39–41].

In recent years, a paradigm shift has occurred from clinical to biological definition of AD based on *in vivo* biomarkers measured in CSF or with positron emission tomography (PET) imaging [1]. The most recent research criteria base the diagnosis of AD partly [42] or primarily [1] on signature profiles defined by CSF/PET Aβ, CSF total tau (T-tau), and CSF P-tau levels. The International Working Group (IWG) [42], for example, defines typical AD as a combination of biomarker evidence (decreased CSF/PET Aβ$_{42}$ together with increased CSF T-tau or P-tau) and a specific phenotype (presence of a significant episodic memory impairment). Although the diagnostic accuracies of these core biomarkers are satisfactory [43], there is still a need to examine others. Understanding how biomarkers reflecting other processes could influence AD pathogenesis and severity is critical for the improvement of diagnosis and development of effective pharmacologic treatments. The first aim of
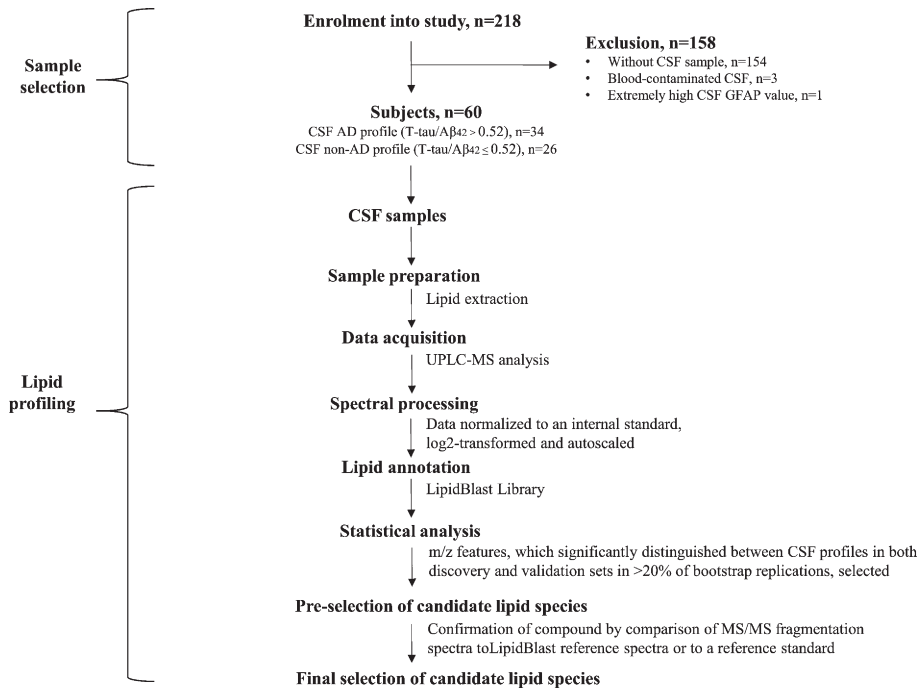
Fig. 1. Flow diagram of sample selection and lipid profiling techniques applied to the analysis of CSF samples.

this study was to identify CSF lipid species associated with CSF profile reflecting signature AD pathology in a cohort of subjects at the pre- and early symptomatic stages of dementia. The second aim was to explore the relationships between candidate lipid species and measures reflecting other AD-associated processes, including neuronal generation, inflammation and impairment in verbal episodic memory.

MATERIALS AND METHODS

Subjects

Subjects from The Icelandic MCI study cohort ($n = 218$), who had undergone lumbar puncture, were selected for this cross-sectional study ($n = 64$). The cohort was comprised of individuals who had been referred to Landspitali University Hospital (LUH) Memory Clinic over a four-year period (Fig. 1). The inclusion criteria for joining the cohort study were: 1) a score between 24–30 on the Mini-Mental State Examination (MMSE) [44] and 2) a score of 4.0 or less on the Informant Questionnaire on Cognitive Decline in the Elderly (IQCODE) [45]. The exclusion criteria were the following: 1) cognitive impairment caused by a pre-existing condition, 2) difficulties participating due to health or social issues, and 3)

residency outside the Reykjavík Capital Area. Each subject underwent various measurements at baseline, which included a medical assessment and a detailed neuropsychological assessment as well as brain magnetic resonance imaging (MRI) for the evaluation of medial temporal lobe atrophy (MTA) and white matter lesions. Lumbar puncture was carried out for the collection of CSF, but the intervention was optional required by the National Bioethics Committee. The final sample included 60 subjects as four were removed due to excessively high CSF GFAP value ($n = 1$) or blood-contamination in CSF samples ($n = 3$). Clinical diagnosis of AD was based on the criteria for probable AD dementia defined by the National Institute on Aging-Alzheimer's Association (NIA-AA) [46], with evidence of AD pathophysiological processes (based on MTA score or/and analysis of core CSF markers). Patients with Lewy body dementia (LBD) were diagnosed based on the consensus criteria of McKeith [47]. The diagnosis of mild cognitive impairment (MCI) required the fulfillment of the Winblad criteria [48]. Those without cognitive impairment were considered to have subjective cognitive impairment (SCI), as they had been referred to the Memory Clinic due to concerns of cognitive decline. Of the 60 participants in this study, 13 were diagnosed with SCI, 23 with MCI, 20 with AD, three with LBD, and one with Parkinson's disease.

The study has been approved by the National Research Ethics Committee of Iceland (VSN-14-028) and all subjects signed an informed consent. The study was conducted in accordance with the Helsinki Declaration latest revision of 2013.

CSF collection and analysis

Collection of CSF was done via lumbar puncture with a 22-gauge spinal needle at the L3/4 or L4/5 interspace. Samples, uncentrifuged, were frozen in 2 ml polypropylene tubes and stored at –80°C. Levels of all proteins were determined using commercially available sandwich enzyme-linked immunosorbent assays (ELISAs) and performed according to manufacturer's instructions. Levels of T-tau (IBL International, Hamburg, Germany), P-tau181 (INNO TEST, Gent, Belgium), and $A\beta_{42}$ (IBL International, Hamburg, Germany), were measured in the ISO 15189 accredited medical laboratory MVZ Labor P.D. Dr. Volkmann und Kollegen GbR (Karlsruhe, Germany). Levels of NFL (Uman Diagnostics, Umeå, Sweden), YKL-40 (Quantikine ELISA Human Chitinase-3–like 1; R&D systems, MN, USA), S100B (BioVendor GmbH, Heidelberg, Germany), and GFAP (BioVendor GmbH, Heidelberg, Germany) were measured in a laboratory at the University of Iceland. All assays had mean Intra-assay CV < 10% and Inter-assay CV < 15%.

Subject grouping based on CSF measures

Each subject was classified based on CSF T-tau and $A\beta_{42}$ values, independently of clinical diagnosis. A cut-off of 0.52 for T-tau/$A\beta_{42}$ ratio was selected based on results from a large memory clinic cohort study [49]. T-tau/$A\beta_{42}$ ratio > 0.52 was defined as a signature CSF AD profile. The CSF AD profile group had a total of 34 subjects (20 with a clinical diagnosis of AD dementia, 10 with MCI, three with SCI and one with Lewy body dementia) while the non-AD profile had a total of 26 subjects (13 with MCI, 10 with SCI, two with LBD, and one with Parkinson's disease). The same ratio cut-off point was also used as a part of the clinical diagnosis of AD, explaining full concordance with the CSF AD profile.

Neuropsychological tests

A detailed neuropsychological assessment, for the evaluation of different cognitive domains, was performed by licensed psychologists under the supervision of a clinical neuropsychologist. A significant impairment in episodic memory is commonly the earliest clinical symptom of AD [50], and therefore of specific interest here. Two tests were used for the evaluation of verbal episodic memory, The Rey Auditory Verbal Learning Test (RAVLT) [51], and a Story test based on the Logical Memory test of the Wechsler Memory Scale-Revised [52]. RAVLT consists of 15 nouns presented across five consecutive trials, with each trial followed by a free-recall test (immediate recall). A score for RAVLT immediate recall was calculated by summing up the number of words recalled from trials 1 through 5 (0 to 75 points). After a 30 min delay, subjects were required to recall the words without being reread the list (delayed recall). A point was given for each correct word (0 to 15 points). The second test was composed of an orally presented story, which included 25 ideas. Right after the presentation, the subject was asked to repeat what they remembered without being given any clues. After a 30 min delay, there was another recall without the story being reread to the subject. For both immediate and delayed recall, a point was given for each idea (0 to 25 points).

Sample preparation and scanning UPLC-MS analysis

CSF sample extraction was based on the method used in Bird et al. [53]. Briefly, C12 ceramide and C17 sphingomyelin (SM d18:1/17:0) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and added to 30 μl of CSF as internal standards prior to lipid extraction. Dried lipid extracts were resuspended in 300 μL of ACN/IPA/H2O (65:30:5 v/v/v) and stored at –80°C prior to analyses. C18 SM (d18:1/18:0) and C18 ceramide (d18:1/18:0) were run alongside samples for reference as both SMs and ceramides have consistently been associated with AD [54]. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) lipidomic analysis was carried out as described in Kotronoulas et al. [55]. The analytical instrumentation used was an ACQUITY UPLC system (UPLC ACQUITY, Waters Corporation, Milford, MA) coupled to a traveling-wave ion mobility (IMS) qTOF mass spectrometer (Synapt G2 HDMS, Waters Corporation, Manchester, UK). The chromatographic gradient separation was performed on an ACQUITY CSH C18 column (2.1 mm × 150 mm, 1.7 μm particle size, Waters Corporation) at 60°C (Supplementary Figure 1). Mobile phase A was H2O/2-isopropanol (80:20) and mobile phase B 2-isopropanol/ACN/H2O (90:9.1),

both with 0.05% of formic acid and 5 mM ammonium formate. Injection volume was 10 µL, flow rate was 0.4 mL/min, and the run time was 17 min. The following gradient pattern (solvent B) was used: 0 min, 40%B; 1 min, 40%B; 3 min, 60%B; 10 min, 100%B; 13.5 min, 100%B; 14 min, 40%B; 17 min, 40 %B. Both positive (+) and negative (-) electrospray ionization (ESI) modes were acquired. The capillary and cone voltage were 2.5 kV and 30 V, respectively. The source and desolvation temperature were 120 and 500°C and the desolvation gas flow was 800 L/h. During High Definition MS^E (HDMSE) experiments, the collision energy in the trap cell was off, and in the transfer cell, it ranged from 20 to 30 eV for the positive mode and from 25 to 40 eV for the negative mode. The resulting MS data were analyzed further within the MS-Dial program.

Spectral processing

Waters RAW files were converted to ABF format using the Reifycs Abf Converter tool. All patient ABF files and pooled samples from either positive or negative controls were loaded into the MS-Dial application [56]. MS-Dial was used for sample alignment and peak detection. Selected mass-to-charge ratio (m/z) peaks, hereafter referred to as m/z features, were manually curated to minimize the effect of sample drift. All m/z features were normalized to the internal standards. M/z feature annotation was performed with MS-Dial and compared to MS/MS spectra in the LipidBlast Library [57]. A total of 1013 m/z features were detected based on their peak mass. M/z features with more than 5% missing values were excluded from the analysis ($n = 5$), leaving 1008 features. The remaining missing data (5% or less) were imputed by the smallest observed value of that particular feature.

Statistical analysis

All CSF measures (detected m/z features and proteins) were log2-transformed to fit a Gaussian distribution. After autoscaling, each CSF measure had an average and a standard deviation of 0 and 1, respectively. Mann-Whitney U non-parametric tests were performed to compare levels of different variables between CSF profile groups. The sample ($n = 60$) was divided into the two sets (discovery and validation) using a bootstrap sampling method [58] for the selection of m/z features best distinguishing between CSF AD and non-AD profile groups. A discovery set

was created by drawing a random sample of equal size from the original sample ($n = 60$) with replacement. Approximately 63% of the sample (38 subjects) were selected into the discovery set, and those left out (22 subjects) made up the validation set. The levels of each m/z feature were compared between CSF profile groups in both sets using Mann-Whitney U tests with a significance level of 0.05. This procedure was repeated 1000 times for the purpose of enhancing the robustness of feature selection. The m/z features most frequently significant in both sets (> 20%) were selected for more detailed identification. The cut-off point of 20% was selected based on the distribution of m/z features after 1000 bootstrap replications (Supplementary Figure 2). A cut-off at 20% was considered optimal, as frequencies of significant m/z features started to slowly increase below that point. Pearson's correlations and linear regression models (both unadjusted and adjusted for gender, age, and years of education) were used for estimation of relationships between continuous measures and final selection of lipids. All statistical analyses were performed using R (version 3.6.1, The R Foundation for Statistical Computing).

RESULTS

Table 1 presents the demographic, pathophysiological, and cognitive characteristics of subjects, both within the whole sample and divided by CSF profile (AD and non-AD). No statistical differences ($p > 0.05$) were found between CSF profile groups by gender, age, education, CSF protein levels, or MMSE scores. Subjects with a CSF AD profile performed worse on tests assessing verbal episodic memory, both for immediate (RAVLT: $p = 0.009$; Story: $p = 0.003$) and delayed (RAVLT: $p < 0.001$; Story: $p = 0.002$) recall.

In order to relate changes in lipid species levels to typical AD pathology and related measures, we performed a lipidomic analysis of CSF from patients described in Table 1. The selection of the m/z features best distinguishing between CSF profile groups was done using Mann-Whitney U non-parametric tests. Of the 1008 features detected, eight significantly differed in levels between groups within both the discovery and validation sets in more than 200 of the 1000 (20%) bootstrap replicates (Fig. 2) and were selected for further identification. Eight m/z features reached significance in 10–20% of the replicates, 23 in 2–10% of the replicates and 969 in less than 2%

Table 1
Subject demographics, CSF protein levels and cognitive scores by CSF profile

	CSF profile			All
	Non-AD T-tau/ $A\beta_{42} \leq 0.52$ $n = 26$	AD T-tau/ $A\beta_{42} > 0.52$ $n = 34$	p^{a}	$n = 60$
Demographics				
Gender (M/F)	16/10	16/18	0.27	32/28
Age, y	68 (46–85)	70 (51–84)	0.19	70 (46–85)
Education, y	14 (9–20)	13 (6–20)	0.82	13 (6–20)
Clinical diagnosis				
SCI/MCI/AD/LBD/PD	10/13/0/2/1	3/10/20/1/0	N/A[d]	13/23/20/3/1
CSF measures[b]				
$A\beta_{42}$ (pg/ml)	770 (374–2332)	470 (140–977)	N/A[e]	555 (140–2332)
T-tau (pg/ml)	182 (100–722)	429 (132–1086)	N/A[e]	290 (100–1086)
P-tau (pg/ml)	45 (24–77)	84 (30–144)	N/A[e]	58 (24–144)
NFL (ng/ml)	2.0 (0.9–6.5)	2.5 (1.2–5.3)	0.052	2.2 (0.9–6.5)
YKL-40 (ng/ml)	165 (83–399)	189 (124–367)	0.35	183 (83–399)
S100B (pg/ml)	215 (132–335)	240 (129–509)	0.06	228 (129–509)
GFAP (ng/ml)	1.1 (0.1–7.1)	1.3 (0.5–21.3)	0.11	1.3 (0.1–21.3)
Global cognition				
MMSE, score	28 (24–30)	28 (24–30)	0.87	28 (24–30)
Verbal episodic memory				
RAVLT immediate recall, score[c]	34.5 (23–66)	27 (13–58)	0.009	30 (13–66)
RAVLT delayed recall, score[c]	4.5 (0–15)	1 (0–12)	<0.001	3 (0–15)
Story – immediate recall, score	13.5 (5–17)	8 (1–21)	0.003	10 (1–21)
Story – delayed recall, score	11.5 (1–19)	6 (0–19)	0.002	7 (0–19)

AD, Alzheimer's disease; CSF, Cerebrospinal fluid; LBD, Lewy body dementia; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; N/A, Not applicable; PD, Parkinson's disease; RAVLT, Rey Auditory Verbal Learning Test; SCI, subjective cognitive impairment. Values are shown as median (range) or as numbers per group. [a]Mann-Whitney U non-parametric test used for continuous variables and Chi-Square test for the categorical variable (gender). [b]Raw values (neither log2-transformed nor autoscaled). [c]Analysis based on 59 subjects, one missing value. [d]p-values not applicable for clinical diagnosis due to CSF profiles being part of the diagnostic criteria for AD. [e]p-values not applicable for $A\beta_{42}$ and tau due to their values used for defining CSF profiles.
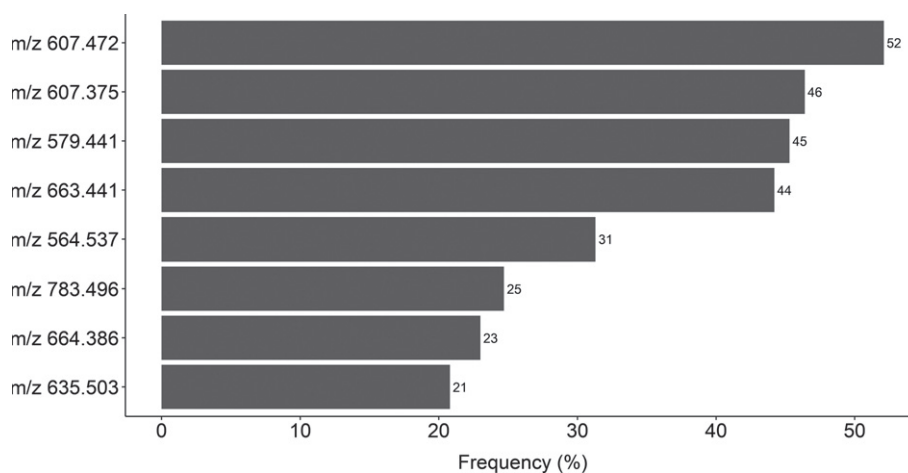

Fig. 2. Frequencies of m/z features significantly distinguishing between CSF profiles (AD and non-AD) in both discovery and validation sets ($p < 0.05$) after 1000 bootstrap replications. A total of eight features with frequencies higher than 200 (20%) were selected for more detailed identification.

of the replicates. The distribution of all m/z features after 1000 bootstrap replications is depicted in Supplementary Figure 2.

Table 2 presents the lipid species annotations that were automatically assigned to the eight selected m/z features using the LipidBlast spectral library. It

Table 2
LipidBlast annotations of the eight selected m/z features. Levels for each feature compared between CSF AD and non-AD profile groups

Feature/peak (m/z)	Average RT (min)	Ionization mode	Annotated lipid species	Proposed structure	CSF profile[a]		p[b]
					Non-AD	AD	
607.472	7.74	(-)	FAHFA 40:7	FAHFA (18:5/22:2)	–0.50	0.25	<0.001
607.375	6.00	(-)	MGDG 20:0	MGDG (10:0/10:0)	–0.54	–0.05	<0.001
579.441	7.24	(-)	FAHFA 38:7	FAHFA (18:4/20:3)	–0.42	0.27	<0.001
663.441	6.15	(-)	MGDG 24:0	MGDG (10:0/14:0)	–0.28	0.38	<0.001
564.537	7.50	(-)	Cer 36:1	C18 Cer (d18:1/18:0)	–0.48	0.31	0.002
783.496	6.65	(-)	PMeOH 42:9	PMeOH (20:3/22:6)	–0.36	0.29	0.003
664.386	6.21	(-)	PE 32:6e	PE (16:23/16:4)	–0.10	0.36	0.003
635.503	8.07	(-)	FAHFA 42:7	FAHFA (20:3/22:4)	–0.56	–0.11	0.005

m/z, mass-to-charge ratio; RT, retention time; Cer, ceramide; FAHFA, fatty acid ester of hydroxyl fatty acid; MGDG, monogalactosyldiacyl-glycerol; PE, phosphatidylethanolamines; PMeOH, phosphatidyl methanol. [a]Based on normalized peak area arbitrary units (A.U.), log2-transformed and autoscaled before analysis. [b]Mann-Whitney U non-parametric test used.

also includes comparisons in levels of each feature between CSF profiles. Annotations of compounds searching the library are based on similarity matching of elements, including m/z peaks and retention time (RT). The lipid species selected belonged to four different lipid categories; glycerophospholipids (PE 16:23/16:4, PMeOH 20:3/22:6), sphingolipids (ceramide d18:1/18:0), branched fatty acid esters of hydroxy fatty acids (FAHFA 18:5/22:2, 18:4/20:3 and 20:3/22:4), and monogalactosyldiacylglycerol (MGDG 10:0/10:0). Of the eight lipid species anno-tated via LipidBlast library search, a reference standard was only run for C18 ceramide (d18:1/18:0). A comparison of the MS/MS fragmentation spectra of the C18 ceramide standard confirmed the annotation of C18 ceramide in the CSF samples (Supplemen-tary Figure 3). The other seven annotated lipid species remain unconfirmed, as a comparison of each MS/MS fragmentation spectra to the LipidBlast reference spectra did not clearly confirm the correct chemical structure. All measured m/z features are listed in Sup-plementary Table 1A (positive ionization mode) and 1B (negative ionization mode).

As the structure for C18 ceramide was confir-med with high confidence, it was selected for fur-ther analysis. Figure 3 presents the levels of CSF C18 ceramide by different CSF profiles. As can be observed, levels of C18 ceramide were elevated in the CSF AD profile group compared to the non-AD group ($p = 0.002$).

Linear regression (unadjusted and adjusted for gender, age, and education) was performed to esti-mate the relationships of established AD ($A\beta_{42}$, T-tau, P-tau), inflammatory (YKL-40, S100B, GFAP) and neuronal degeneration (NFL) markers with CSF C18 ceramide (Table 3). Levels of $A\beta_{42}$
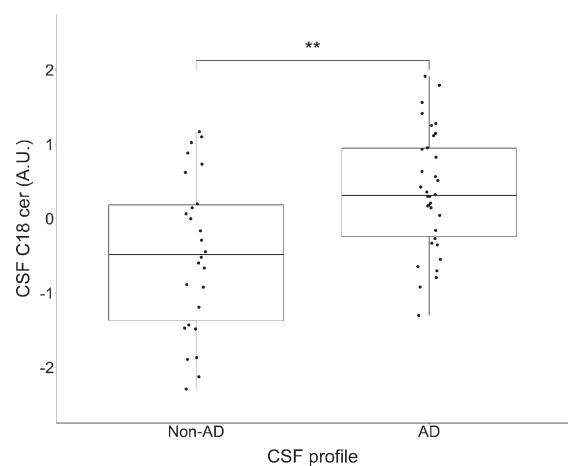

Fig. 3. Comparison in levels of CSF C18 ceramide by CSF profile (non-AD and AD). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significance according to Mann-Whitney U non-parametric test. Based on nor-malized peak area arbitrary units (A.U.), log2-transformed and autoscaled before analysis. The lower and upper horizontal lines of the boxplot correspond to the 25th and the 75th centiles and the middle line to the median. Levels of CSF C18 ceramide were significantly higher ($p = 0.002$) among participants with a CSF AD profile compared to those without.

were negatively associated with C18 ceramide when adjusted for age, gender, and education (st. $\beta = -0.36$, $p = 0.007$). T-tau (st. $\beta = 0.41$, $p = 0.005$) and S100B (st. $\beta = 0.51$, $p = 0.001$) positively associated with C18 ceramide when adjusted for the same covariates. Statistically significant, albeit weaker, associations were detected between the same measures when lin-ear regression was not adjusted for demographic variables. Levels of other CSF proteins (NFL, YKL-40, GFAP) and test scores presenting verbal episodic memory (RAVLT, Story) did not significantly asso-ciate with levels of C18 ceramide. One subject did

Table 3
Linear regression estimates (unadjusted and adjusted) for the association between various measures and levels of CSF C18 ceramide

| | CSF C18 ceramide (A.U.)[a] | | | |
| | Unadjusted model | | Adjusted model* | |
	St. β	p	St. β	p
Demographics				
Age, y	–0.02	0.902	–0.05	0.765
Education, y	–0.05	0.688	–0.07	0.596
CSF proteins[a]				
$A\beta_{42}$ (pg/ml)	–0.35	0.006	–0.36	0.007
T-tau (pg/ml)	0.30	0.018	0.41	0.005
P-tau (pg/ml)	0.17	0.188	0.21	0.129
$T\text{-tau}/A\beta_{42}$	0.44	<0.001	0.52	<0.001
NFL (ng/ml)	0.03	0.835	0.05	0.782
YKL-40 (ng/ml)	0.11	0.411	0.25	0.194
S100B (pg/ml)	0.37	0.004	0.51	0.001
GFAP (ng/ml)	0.22	0.087	0.26	0.087
Verbal episodic memory				
RAVLT immediate recall, score[b]	–0.02	0.893	–0.01	0.928
RAVLT delayed recall, score[b]	–0.18	0.339	–0.29	0.095
Story immediate recall, score	–0.13	0.356	–0.14	0.345
Story delayed recall, score	–0.13	0.305	–0.15	0.335

A.U., arbitrary units; RAVLT, Rey Auditory Verbal Learning Test. Numbers present standardized beta coefficients (st.β). *Adjusted for age, gender, and years of education. [a]Values log2-transformed and autoscaled before analysis. [b]Analysis based on 59 subjects, with one missing.

not take the RAVLT test, and therefore the analyses involving RAVLT immediate and delayed recall were only based on 59 subjects. This did not skew comparisons between RAVLT and other regression estimates, as results in Table 3 did not change when calculated again without that subject.

The statistically significant relationships between the levels of proteins and C18 ceramide ($p < 0.05$) from Table 3 are depicted in Fig. 4. The associations were estimated with Pearson's coefficients, which are equal to the unadjusted standardized beta coefficients (st. β) in Table 3. Pearson's coefficients were also calculated for the CSF markers within each CSF profile group (Table 4), but none reached statistical significance ($p > 0.05$).

DISCUSSION

We used an untargeted lipidomic approach to identify CSF lipid species associated with a signature CSF profile reflecting AD pathology. A total of 1008 m/z features were detected, with eight selected

as candidate markers. Out of these, one was fully confirmed as corresponding to the lipid species C18 ceramide. Our results showed that C18 ceramide levels were higher among subjects with a CSF AD profile. Relationships were also detected between established AD markers ($A\beta_{42}$, T-tau) and C18 ceramide. Higher levels of C18 ceramide associated with lower levels of $A\beta_{42}$ and higher levels of T-tau. In addition, levels of the inflammatory marker S100B positively related to C18 ceramide. Overall, our results indicate that CSF C18 ceramide levels could increase during pathological changes in the early stages of AD.

Ceramides, the core constituents of sphingolipid metabolism, are composed of a sphingosine backbone linked to a fatty acid chain of varying carbon atom length (C14-C26) [59]. Alterations in sphingolipid metabolism have been observed in healthy aging and in neurodegenerative diseases, including AD [60]. They play essential roles in the structural stability of membranes and as signaling molecules affect differentiation, proliferation, inflammation, and apoptosis [61, 62]. Ceramides are synthesized via two main pathways in eukaryotic cells [63]. In the salvage pathway, sphingomyelin is hydrolyzed through sphingomyelinase (SMase) to produce ceramide, which can be further metabolized to sphingosine by ceramidase. Ceramides can also be generated through the de novo pathway via anabolism of serine and palmitate. Results from cellular and animal studies suggest that both direct and indirect mechanisms by which ceramides can contribute to an increase in Aβ levels and AD pathogenesis [64]. Ceramides stimulate Aβ generation by stabilizing β-secretase enzyme BACE1 and increasing its half-life [65, 66]. Furthermore, soluble and fibrillar forms of Aβ can induce degradation of SM to ceramide by SMases [67, 68] through oxidative stress-mediated mechanisms [69]. This positive loop of ceramide production possibly contributes to immune activation and neuronal loss in AD. Long-chain ceramides, specifically C18 ceramide, have also been linked to tau phosphorylation through modulation of PP2A activity [70–74]. Several postmortem studies have found increased levels of ceramide in brain tissues of AD patients compared to healthy controls [60, 69, 75–77]. Two of the studies [60, 76] examined different ceramide species, with long-chain ceramide levels (C18 and C24) being significantly elevated in the AD group. Han et al. [75] observed the highest elevation of total ceramide in the brains of patients with mild AD, compared to those with severe AD and to controls,
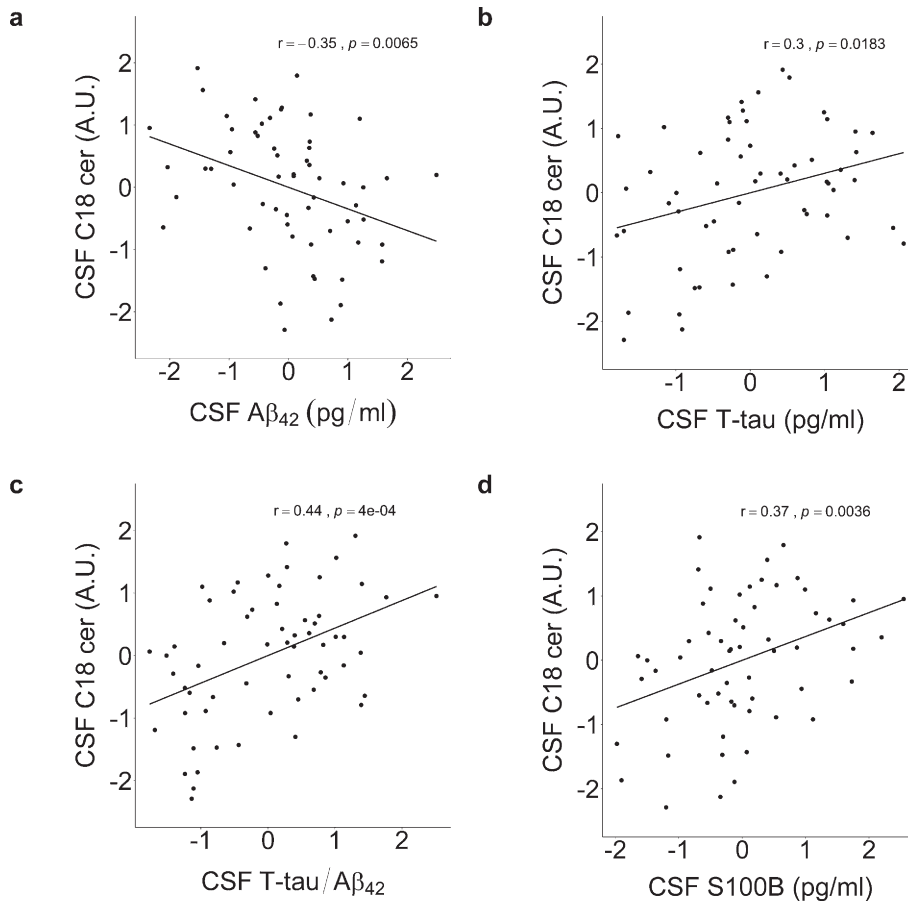
Fig. 4. Pearson's correlations between CSF levels of a) $A\beta_{42}$, b) T-tau, c) T-tau/ $A\beta_{42}$, d) S100B and CSF C18 ceramide. Normalized peak area arbitrary units (A.U.). All CSF measures were log2-transformed and autoscaled before analysis. C18 ceramide levels significantly correlated with levels of the core AD markers $A\beta_{42}$, T-tau, and inflammatory marker S100B.

Table 4
Pearson's correlations between selected CSF markers and levels of CSF C18 ceramide within CSF profiles

| | CSF C18 ceramide (A.U.)[a] | | | |
| | CSF non-AD profile $n = 26$ | | CSF AD profile $n = 34$ | |
	r	p	r	p
CSF proteins[a]				
$A\beta_{42}$ (pg/ml)	–0.04	0.86	–0.25	0.15
T-tau (pg/ml)	0.23	0.25	–0.20	0.25
P-tau (pg/ml)	–0.20	0.34	–0.13	0.47
T-tau/$A\beta_{42}$	0.32	0.11	0.08	0.67
NFL (ng/ml)	–0.09	0.68	–0.07	0.70
YKL-40 (ng/ml)	0.08	0.70	0.02	0.90
S100B (pg/ml)	0.39	0.052	0.21	0.24
GFAP (ng/ml)	0.16	0.43	0.17	0.34

A.U., arbitrary units. [a]Values log2-transformed and autoscaled before analysis.

indicating early changes in the pathological processes of AD. To further confirm the implications of ceramide metabolism in AD, upregulation [78] and increase in activity of enzymes [77] controlling ceramide synthesis have been observed in brain areas (temporal and frontal cortices) affected in the early stages of the disease.

While postmortem studies are essential, *in vivo* studies are the only way to ascertain the role of lipids in early AD pathogenesis and whether these lipids may be indicators or predictors of disease progression. The most informative medium for studying lipid changes in the brain is the CSF, since it is in direct contact with the brain interstitial fluid. Very few CSF studies have been published comparing ceramide levels in AD patients to other groups. Fonteh et al. [79] found slightly higher, albeit not significant, levels of ceramide in AD compared to MCI and control groups in supernatant fluid of CSF. In another study [80], patients with AD had significantly higher levels compared to controls with other neurological condi-

tions. A study by Mielke et al. [81], examined the relationship between different ceramide species in relation to Aβ and tau levels in CSF. A positive correlation was found between C18 ceramide and all Aβ species except Aβ$_{42}$ as well as tau levels in a cohort of cognitively healthy individuals aged 36–69 years ($n = 91$) with a parental history of AD. In contrast with cellular studies, no significant correlation was found between the lipid species and P-tau. The same study also found a negative association between the longer carbon chain species (C20, C22, and C26) and performance on cognitive tests reflecting verbal episodic memory (delayed recall) or working memory. Our results expand upon the findings from this study. The results we present show a significant positive relationship between levels of C18 ceramide and T-tau, but not with P-tau, among the cohort of patients at risk of or at the early stages of dementia. Association between C18 ceramide and verbal episodic memory (immediate or delayed recall) was also not detected. No significant relationship was found with NFL levels, which is interesting considering it is a marker for neurodegeneration and white matter changes, and SM, a precursor of ceramide, is enriched in the myelin sheath of neurons. A possible explanation is CSF C18 ceramide being a marker of apoptosis, rather than degradation of myelinated axons. Evidence supports the role of ceramides in neuronal apoptosis, initiating a cascade of biochemical alterations leading ultimately to neuronal death [64]. In contrast with the study by Mielke et al. [81], we showed higher levels of C18 ceramide associating with lower levels of Aβ$_{42}$, one of the primary toxic species of amyloid [82]. A possible reason could be that all subjects in our study were already at the symptomatic pre- or early stages of dementia, and it is not unlikely that the relationship between the analytes strengthens during the progression of AD. Our study also found a positive association between levels of C18 ceramide and inflammatory marker S100B, but not YKL-40 and GFAP. Our hypothesis is that the relationship could be due to higher concentrations of ceramides and S100B, as both have been associated with the induction of neuronal apoptosis [26, 64]. In high doses, extracellular S100B has been shown to cause neuronal death by activation of the receptor for advanced glycation end products (RAGE). The protein can, by excessive stimulation of RAGE expressed in neurons, hyperactivate the Ras/MEK/ERK pathway, which consequently leads to apoptosis [83, 84]. S100B can also, indirectly, lead to neu-

ronal death through the release of nitric oxide by astrocytes and microglia via RAGE-dependent activation [85, 86].

There are several limitations to our study. First, our sample size was relatively small, with a vast number of features compared to the number of subjects, resulting in little power and a considerable risk of type-II errors. Corrections for multiple comparisons were, therefore, not performed when comparing levels of features between groups in the discovery set. A more significant validation set would also have allowed for evaluation of accuracy in distinguishing between CSF profile groups using multiple lipid species simultaneously as predictors. Second, our study did not include a healthy control group or patients with moderate to severe AD. It also included very few participants with other dementias. It would be of interest to examine the relationships between ceramides and AD-related markers in CSF in a more diverse cohort, for a better evaluation of C18 ceramide as a marker of AD progression and severity, preferably in a longitudinal study. Third, information about the *APOE* genotype of subjects was not available, and therefore, its potential effect on results could not be adjusted for [87].

In summary, our results indicate that CSF C18 ceramide levels associate with established markers of AD pathology (Aβ$_{42}$ and T-tau) and inflammation (S100B) at the symptomatic pre- and early stages of dementia. These findings suggest that ceramide metabolism could influence the pathophysiological processes during the early stages of AD. Furthermore, ceramides could potentially serve as therapeutic targets, with strategies aiming at reducing ceramide levels to slow down the progression of the disease. Longitudinal studies are, however, needed to validate the pathological implications of these results.

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SUPPLEMENTARY MATERIAL

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