



## Cerebrospinal fluid sphingolipids, $\beta$ -amyloid, and tau in adults at risk for Alzheimer's disease



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### ABSTRACT

Cellular studies suggest sphingolipids may cause or accelerate amyloid-beta ( $A\beta$ ) and tau pathology but in vivo human studies are lacking. We determined cerebrospinal fluid levels of sphingolipids (ceramides and sphingomyelins), amyloid-beta ( $A\beta$ 1-42,  $A\beta$ X-38,  $A\beta$ X-40, and  $A\beta$ X-42) and tau (T-tau and p-tau181) in 91 cognitively normal individuals, aged 36–69 years, with a parental history of Alzheimer's disease. The 18-carbon acyl chain length ceramide species was associated with  $A\beta$ X-38 ( $r = 0.312$ ,  $p = 0.003$ ),  $A\beta$ X-40 ( $r = 0.327$ ,  $p = 0.002$ ), and T-tau ( $r = 0.313$ ,  $p = 0.003$ ) but not with  $A\beta$ X-42 ( $r = 0.171$ ,  $p = 0.106$ ) or p-tau ( $r = 0.086$ ,  $p = 0.418$ ). All sphingomyelin species correlated (most  $p < 0.001$ ) with all  $A\beta$  species and T-tau; many also correlated with p-tau. Results remained in regression models after controlling for age and APOE genotype. These results suggest in vivo relationships between cerebrospinal fluid ceramides and sphingomyelins and  $A\beta$  and tau levels in cognitively normal individuals at increased risk for Alzheimer's disease, indicating these sphingolipids may be associated with early pathogenesis.

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### 1. Introduction

As evidenced by the many failed treatment trials for Alzheimer's disease (AD), there appears to be minimal treatment benefit in the fully symptomatic stage of the disease. The pathogenesis of amyloid-beta ( $A\beta$ ) plaques and neurofibrillary tangles begins decades before the emergence of clinical symptoms (Jack et al., 2010; Reiman et al., 2012) and impacts multiple cellular pathways. Although much research has focused on  $A\beta$  as a biomarker and therapeutic target for AD, there is limited

understanding of the cellular pathways that contribute to, or are caused by,  $A\beta$  aggregation and deposition or tau phosphorylation. Understanding the early mechanisms associated with AD pathology may be especially important for identifying disease-modifying therapeutic targets.

Lipidomic and targeted approaches have identified pathways and products of sphingolipid metabolism that are altered early in the course of AD and contribute to AD neuropathology (Haughey et al., 2010; Mielke and Haughey, 2012). Sphingolipid metabolism is a dynamic process that modulates the formation of a number of bioactive metabolites. Sphingomyelins are among the most abundant lipids in many mammalian cells and tissues. They are central to the creation of lipid rafts and ordered membrane domains (Quinn and Wolf, 2009; Simons and Ikonen, 1997; Simons and Vaz, 2004), the functional regulation of membrane-spanning proteins (Contreras et al., 2012), act as important regulators of plasma membrane and cell cholesterol

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homeostasis (Gatt and Bierman, 1980; Slotte and Bierman, 1988), and are precursors for other sphingolipids such as ceramides. Ceramides, the central molecular species of the sphingolipids pathway, have important structural roles in cell membranes and function as second messengers for critical intra- and inter-cellular signaling affecting cellular growth, differentiation, proliferation, and apoptosis. Although ceramides are important for cell survival, injury-induced cytokine production, and activate stress-signaling protein phosphatases and kinases (Hannun, 1996), at high levels ceramides inhibit cell division and induce cellular dysfunction and apoptosis (Goodman and Mattson, 1996).

Several lines of evidence suggest both direct and indirect associations between ceramides and A $\beta$  levels at the cellular level (Mattson et al., 2005; Tamboli et al., 2011). Recent studies have also suggested that ceramides, particularly the 18-carbon acyl chain length species, modulate tau phosphorylation (Chalfant et al., 1999; Dobrowsky et al., 1993; Goedert et al., 1995; Gong et al., 1994; Mukhopadhyay et al., 2009). Although 1 postmortem study reported correlations among sphingomyelinase activity, A $\beta$ , and phosphorylated tau (He et al., 2010), cerebrospinal fluid (CSF) studies of sphingolipids in AD have not examined the in vivo relationship between sphingolipids and AD pathology (Kosicek et al., 2012; Sato et al., 2005). Therefore, we examined the association between CSF ceramides and sphingomyelins, A $\beta$ , and tau in cognitively normal individuals aged 36–69 years at increased risk for AD because of a parental family history.

## 2. Methods

### 2.1. Participants and recruitment

Participants included 91 cognitively normal middle-aged adults with parental history of AD enrolled in the ESPRIT (Evaluating Simvastatin's Potential Role in Therapy) trial who had available CSF A $\beta$ , tau, and sphingolipid measures at baseline, before randomization. The ESPRIT study is a prospective, randomized, placebo-controlled clinical trial that aimed to determine the effects of 9 months of simvastatin therapy on CSF biomarkers for AD including A $\beta$  and phospho-tau. Participants were recruited from the Wisconsin Registry for Alzheimer's Prevention (WRAP) (Sager et al., 2005) and also from the community. Before enrollment into the ESPRIT trial, the diagnosis of AD in 1 or both parents was confirmed for each participant by using the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association criteria through clinical evaluation and/or chart review by Wisconsin Alzheimer's Disease Research Center physicians and neuropsychologists. Exclusion criteria included current use of cholesterol-lowering medications or medications known to interact with simvastatin, contraindication to lumbar puncture, active liver disease, diabetes mellitus, past adverse reaction to a statin, or elevated creatine kinase. The study was approved by the Institutional Review Board of the University of Wisconsin. Subjects provided written informed consent before participating. This clinical trial was registered with [ClinicalTrials.gov](http://ClinicalTrials.gov) (NCT00486044).

### 2.2. Study procedures

CSF collection was completed in the morning, at approximately the same time at each visit. A Sprotte 22- or 25-gauge spinal needle was inserted into the L3–4 or L4–5 interspace and between 8 and 22 mL of CSF was removed. CSF samples were centrifuged (to remove red blood cells [RBCs]) at the University of Wisconsin Clinical Research Unit within 30 minutes of collection, aliquoted into 0.5 mL polypropylene storage tubes, and stored in a –80 °C freezer until

simultaneous analysis was conducted at the conclusion of the study. As ceramide and sphingomyelin levels can be elevated in RBCs, we also quantified RBCs in the CSF and found the concentrations to be low (mean: 15.55, SD: 82.5, median: 1.00). This suggests that remaining RBCs would have little impact on our results.

### 2.3. Cognitive testing

All participants underwent cognitive testing by a trained technician according to protocols established for each test. The cognitive test battery targeted 4 domains: memory and learning (Hopkins Verbal Learning Test [HVLT] [Brandt, 1991]; New York University Paragraph Recall [Kluger et al., 1999]; Modified Taylor Complex Figure delayed recall [Hubley, 1996]; Visual Spatial Learning Test [Malec et al., 1997]); language (Controlled Oral Word Association Test [Benton and Hamsher, 1989]); visual-motor skills (Modified Taylor Complex Figure–Copy [Hubley, 1996]; Grooved Pegboard [Kløve, 1963]); and executive functioning (Ruff 2 and 7 Selective Attention Test [Ruff et al., 1986]; Stroop Color-Word Test [Spreen and Strauss, 1998]; Color Trails Test [D'Elia et al., 1996]; Wechsler Memory Scale–Third Edition [WMS-III] Mental Control, Letter-Number Sequencing, and Spatial Span subtests [Wechsler, 1997]; Wechsler Adult Intelligence Scale [WAIS-III] Symbol Search and Digit Symbol Substitution Subtests [Wechsler, 1991]). The Mini-Mental State Exam was administered to assess global cognitive function (Folstein et al., 1975). To reduce multiple comparisons among cognitive measures, 6 cognitive variables were prospectively identified targeting memory, learning, and executive function: (1) HVLT total learning summary score (sum of trials 1–3); (2) HVLT delayed recall score; (3) WAIS-III Processing Speed Index; (4) WMS-III Mental Control total score; (5) WMS-III Working Memory score; and (6) Stroop Color-Word score.

### 2.4. Laboratory evaluation

Blood tests were collected following a 12-hour fast. Lipoproteins were analyzed by NMR spectroscopy (LipoScience, Inc, Raleigh, NC, USA) (Otvos, 2002). CK, AST, and ALT levels were measured using enzymatic precipitation techniques. APOE genotype was measured using standard polymerase chain reaction and DNA sequencing techniques.

### 2.5. Sphingolipid assays

A crude lipid extraction of CSF was conducted using a modified Bligh and Dyer procedure as previously described (Haughey et al., 2004). To control for slight differences in extraction efficiencies, and day to day variations in the efficiency of the mass spectrometer, purified standards of sphingomyelin C12:0 and ceramide C12:0 (10 nM each; Avanti Polar Lipids) were added directly to samples before extraction. Extractions were performed by the addition of 900  $\mu$ L methanol containing ammonium formate (53 mM) to 300  $\mu$ L of CSF. The mixture was vortexed, and 1200  $\mu$ L of chloroform was added. The mixture was centrifuged at 1000g for 10 minutes. The chloroform (bottom) layer was carefully removed and dried in a nitrogen evaporator (OASYS model 11,848; Organomation Associates, Inc). Dried extracts were sealed and stored at –80 °C. The dried extracts were resuspended in 100% methanol before analysis.

Analyses of sphingolipids were performed on a high-performance liquid chromatography coupled electrospray ionization tandem mass spectrometer (LC/ESI/MS/MS) (API3000s, Sciex Inc, Thornhill, Ontario, Canada) using methods similar to those described in previous studies (Haughey et al., 2004). Quantitation of sphingomyelins (d18:1/C16:0–C26:1) and simple and complex

ceramides (d18:1/C16:0-C26:1) were conducted by multiple reaction monitoring. Samples were injected using a CTC PAL autosampler (LEAP Technologies, Inc) into a PerkinElmer HPLC equipped with a 2.6  $\mu\text{m}$  C18 100 Å LC Column 50  $\times$  2.1 mm (Kinetex) and a guard column with identical packing material (Phenomenex). For a typical run, the LC column was first pre-equilibrated for 0.5 minutes with the first mobile phase consisting of 85% methanol, 15% H<sub>2</sub>O, and 5 mM ammonium formate. The column was then eluted with the second mobile phase consisting of 99% methanol, 1% formic acid, and 5 mM ammonium formate at the flow rate of 400.0  $\mu\text{L}/\text{min}$ . The eluted sample was injected into the ion source where the detection of each analyte was conducted by ESI/MS/MS using multiple reaction monitoring mode monitoring the precursor, and products by ion scan.

Area under the curve was used to quantitate each sphingomyelin and simple and complex ceramide species using MultiQuant (AB Sciex). The resulting data were normalized to the corresponding internal standard for sphingomyelin (C18:1/C12:0) or ceramides (C18:1/C12:0). Each molecular species was analyzed separately to determine whether there were chain-specific associations within each sphingolipid species. A summary variable of all species within each class (sphingomyelins, ceramides) was also examined. Quantitation was reported as counts per second (cps). As concentrations were skewed to the right, similar to previous studies (Mielke et al., 2010a, 2012), they were log-transformed before analyses.

## 2.6. CSF amyloid-beta and tau assays

Levels of  $\beta$ -amyloid1-42 (A $\beta$ 1-42), total-tau (T-tau), and phosphorylated tau181 (p-tau) in CSF were analyzed using the xMAP platform with the INNO-BIA AlzBio3 assay, as described previously in detail (Olsson et al., 2005). Levels of CSF A $\beta$ X-38, A $\beta$ X-40, and A $\beta$ X-42 were measured using Meso Scale Discovery (MSD) electrochemiluminescence detection technology and the MSD Human/Rodent (4G8) Abeta Triplex Assay on a SECTOR Imager 2400 instrument as described by the manufacturer (Meso Scale Discovery, Gaithersburg, MD, USA). This assay uses C-terminally specific antibodies to capture A $\beta$ X-38, A $\beta$ X-40, and A $\beta$ X-42, respectively, and a SULFO-TAG-labeled 4G8 antibody (directed against the A $\beta$  mid-domain) to quantify them. All analyses were performed batch-wise by board-certified laboratory technicians who were blinded to all clinical data.

## 2.7. Statistical analysis

The relationship between the CSF sphingolipids and demographics, health characteristics, cognitive performance, and CSF A $\beta$  and tau levels were examined using Spearman rank correlation for continuous variables and *t*-tests for dichotomous variables. Linear regression models were used to examine the relationship between the CSF sphingolipids and CSF A $\beta$  and tau, controlling for age and APOE E4 genotype. Linear regression models were also used to examine the relationship between the CSF sphingolipids and performance on each cognitive test, controlling for age, education, and APOE genotype. The a priori *p*-value was set at *p* < 0.05. All analyses were conducted using STATA Version 12.1 (StataCorp, College Station, TX, USA).

## 3. Results

Characteristics of the 91 individuals included in this study are described in Table 1. On average, participants were 53.4 (SD = 7.9) years of age, had 16 years of education, were overweight (mean BMI 27.8, SD = 5.5), and had normal levels of cholesterol, triglycerides, and blood glucose. There were 63 women (69.2%); 33 participants

(36.3%) had at least 1 APOE E4 allele. All individuals were considered cognitively normal and had levels of A $\beta$  and tau that are thought to be in the “normal” range. There was a significant, positive correlation between A $\beta$ 1-42 using the xMAP assay and A $\beta$ X-42 using the MSD assay (*r* = 0.766, *p* < 0.0001).

Levels of CSF very long chain ceramides with chain lengths of C20-C26 were higher in APOE E4 carriers compared with non-carriers (*p*-values range from 0.016 to 0.045). However, levels of other ceramide species (C16:0 and C18:0) or sphingomyelins (C16:0-C26:1) did not vary by APOE genotype (all *p* > 0.10, data not shown). There were positive correlations between all sphingomyelins (total and each species) and age (e.g., total CSF sphingomyelin: *r* = 0.246, *p* = 0.019), but not between ceramides and age. Neither sphingomyelin nor ceramide levels differed by sex. There were also no associations between any sphingomyelins nor ceramides and use of vitamins or blood pressure medications, body mass index, and fasting serum cholesterol, triglycerides, or glucose (data not shown).

### 3.1. Relationship between CSF sphingolipids and CSF A $\beta$ and tau

Correlations between total ceramides, sphingomyelins and each individual species within these lipid classes and CSF A $\beta$  and tau are shown in Table 2. Only ceramide C18:0 consistently correlated with A $\beta$  and tau (Table 2 and Fig. 1). Notably, there were positive, significant, correlations with A $\beta$ X-38 (*r* = 0.312, *p* = 0.003) and A $\beta$ X-40 (*r* = 0.327, *p* = 0.002), but not with A $\beta$ X-42 (*r* = 0.171, *p* = 0.106) or with A $\beta$ 1-42 (*r* = 0.117, *p* = 0.268). In contrast to the specific carbon-chain length findings for ceramides, virtually all CSF sphingomyelins were positively correlated with all CSF A $\beta$  measures, and also with tau, p-tau, and the tau to A $\beta$ 1-42 ratio (Table 2 and Fig. 2).

**Table 1**  
Characteristics of sample

Characteristics	N	Mean (SD) or n (%)	Range
Age, y	91	53.4 (7.9)	36–69
Female	91	63 (69.2%)	
Education, y	91	16.3 (2.9)	8–23
Any E4 allele	91	33 (36.3%)	
Body mass index, kg/m <sup>2</sup>	91	27.8 (5.5)	19.2–47.8
Vitamin C supplements	91	22 (24.2%)	
Vitamin E supplements	91	20 (22.0%)	
Blood pressure medications	91	11 (12.1%)	
Systolic blood pressure, mm Hg	91	121.6 (13.4)	94–161
Total cholesterol, mg/dL	91	190.7 (32.4)	114–299
HDL cholesterol, mg/dL	91	56.0 (16.5)	31–122
LDL cholesterol, mg/dL	91	118.8 (29.6)	41–215
Triglycerides, mg/dL	91	99.7 (43.8)	46–246
Glucose, mg/dL	90	83.4 (7.2)	72–109
MMSE, points of 30	90	29.5 (0.7)	27–30
HVLT-total learning score	91	29.6 (3.4)	20–35
HVLT-delayed recall score	91	10.5 (1.8)	3–12
Processing speed index	91	90.8 (12.6)	57–119
Mental control total score	91	30.3 (4.9)	19–40
Working memory composite score	91	26.4 (4.2)	14–37
Stroop color-word score	91	43.1 (7.7)	26–69
CSF A $\beta$ X-38 by MSD, ng/L	91	1575.7 (661.7)	405–4042
CSF A $\beta$ X-40 by MSD, ng/L	91	6662.9 (2015.8)	2710–13,235
CSF A $\beta$ X-42 by MSD, ng/L	91	524.6 (202.9)	155–1315
CSF A $\beta$ 1-42 by xMAP, ng/L	91	344.1 (69.0)	150–510
CSF tau by xMAP, ng/L	91	63.4 (25.0)	27–132
CSF p-tau by xMAP, ng/L	91	36.4 (18.6)	13–127
CSF nucleated cells, cells/ $\mu\text{L}$	91	2.05 (2.42)	

Key: AB, amyloid-beta; CSF, cerebrospinal fluid; HVLT, hopkins verbal learning test; MMSE, mini-mental state examination; MSD, meso scale discovery electrochemiluminescence; xMAP, INNO-BIA AlzBio3.

**Table 2**  
Correlation between CSF sphingolipids and CSF amyloid-beta and tau measures

Log CSF sphingolipid	A $\beta$ X-38 <sup>a</sup>		A $\beta$ X-40 <sup>a</sup>		A $\beta$ X-42 <sup>a</sup>		A $\beta$ 1-42 <sup>b</sup>		Tau <sup>b</sup>		p-tau181 <sup>b</sup>		Tau/A $\beta$ 1-42 <sup>b</sup>		p-tau181/ A $\beta$ 1-42 <sup>b</sup>	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
<b>Ceramides</b>																
d18:1-C16:0																
d18:1-C18:0	0.312	0.003	0.327	0.002					0.313	0.003			0.306	0.003		
d18:1-C20:0																
d18:1-C22:0																
d18:1-C24:0																
d18:1-C24:1			0.221	0.035												
d18:1-C26:0																
<b>Total ceramides<sup>c</sup></b>																
<b>Sphingomyelins</b>																
d18:1-C16:0	0.404	0.0001	0.412	<0.0001	0.301	0.004	0.259	0.013	0.410	<0.0001			0.311	0.003		
d18:1-C16:1	0.484	<0.0001	0.500	<0.0001	0.369	<0.001	0.253	0.016	0.441	<0.0001	0.277	0.008	0.331	0.001		
d18:1-C18:0	0.558	<0.0001	0.554	<0.0001	0.428	<0.0001	0.333	0.001	0.565	<0.0001	0.245	0.019	0.446	<0.0001		
d18:1-C18:1	0.552	<0.0001	0.559	<0.0001	0.419	<0.0001	0.281	0.007	0.535	<0.0001	0.306	0.003	0.426	<0.0001	0.214	0.042
d18:1-C20:0	0.652	<0.0001	0.642	<0.0001	0.505	<0.0001	0.358	0.001	0.627	<0.0001	0.332	0.001	0.483	<0.0001	0.208	0.048
d18:1-C20:1	0.421	<0.0001	0.456	<0.0001	0.334	0.001	0.266	0.011	0.359	<0.001			0.238	0.023		
d18:1-C22:0	0.613	<0.0001	0.611	<0.0001	0.488	<0.0001	0.354	<0.001	0.605	<0.0001	0.317	0.002	0.463	<0.0001		
d18:1-C22:1	0.386	<0.001	0.427	<0.0001	0.319	0.002	0.255	0.015	0.353	<0.001			0.239	0.023		
d18:1-C24:0	0.297	0.004	0.305	0.003	0.239	0.023	0.249	0.017	0.294	0.005			0.273	0.009	0.282	0.007
d18:1-C24:1	0.387	<0.001	0.421	<0.001	0.326	0.002	0.257	0.014	0.384	<0.001						
Total sphingomyelins <sup>c</sup>	0.595	<0.0001	0.598	<0.0001	0.462	<0.0001	0.34	0.001	0.580	<0.0001	0.29	0.005	0.443	<0.0001	0.271	0.010

Key: A $\beta$ , amyloid-beta; CSF, cerebrospinal fluid.

<sup>a</sup> Assays conducted using Meso Scale Discovery (MSD) electrochemiluminescence.

<sup>b</sup> Assays conducted using INNO-BIO AlzBio3 (xMAP).

<sup>c</sup> Total refers to the sum of all carbon-chain lengths within the specific lipid class.

### 3.2. Linear regression models and the effects of age and APOE genotype

Based on the previously described correlations, we focused on ceramide C18 in multivariate analyses because this compound was consistently associated with both CSF A $\beta$  and tau. In contrast, since all sphingomyelins species were significantly associated with CSF A $\beta$  and tau, linear regression models only examined total sphingomyelin levels. The associations between CSF ceramide C18:0 or sphingomyelins and CSF A $\beta$  and tau remained in linear regression models controlling for age and APOE genotype (Table 3). Notably, the association between ceramide C18 and A $\beta$ X-42 ( $b = 67.24$ ,  $p = 0.031$ ) and A $\beta$ 1-42 ( $b = 19.12$ ,  $p = 0.071$ ) were also stronger in the multivariate models.

In additional analyses, we separately stratified the previously mentioned models by median age ( $\geq 54$  vs.  $< 54$  years) and the presence of an APOE E4 allele to further determine how these factors affected the previously mentioned associations. Almost all relationships between CSF ceramide C18 or sphingomyelin and CSF A $\beta$  and tau were stronger among individuals aged  $\geq 54$  compared with those  $< 54$  years (Table 3). Although there were only 33 individuals with an APOE E4 allele, the associations between ceramide C18:0 and A $\beta$ X38 and A $\beta$ X40, and between total sphingomyelins and all measures were also stronger within this group compared with those without an E4 allele.

### 3.3. Relationship between CSF sphingolipids and cognitive performance

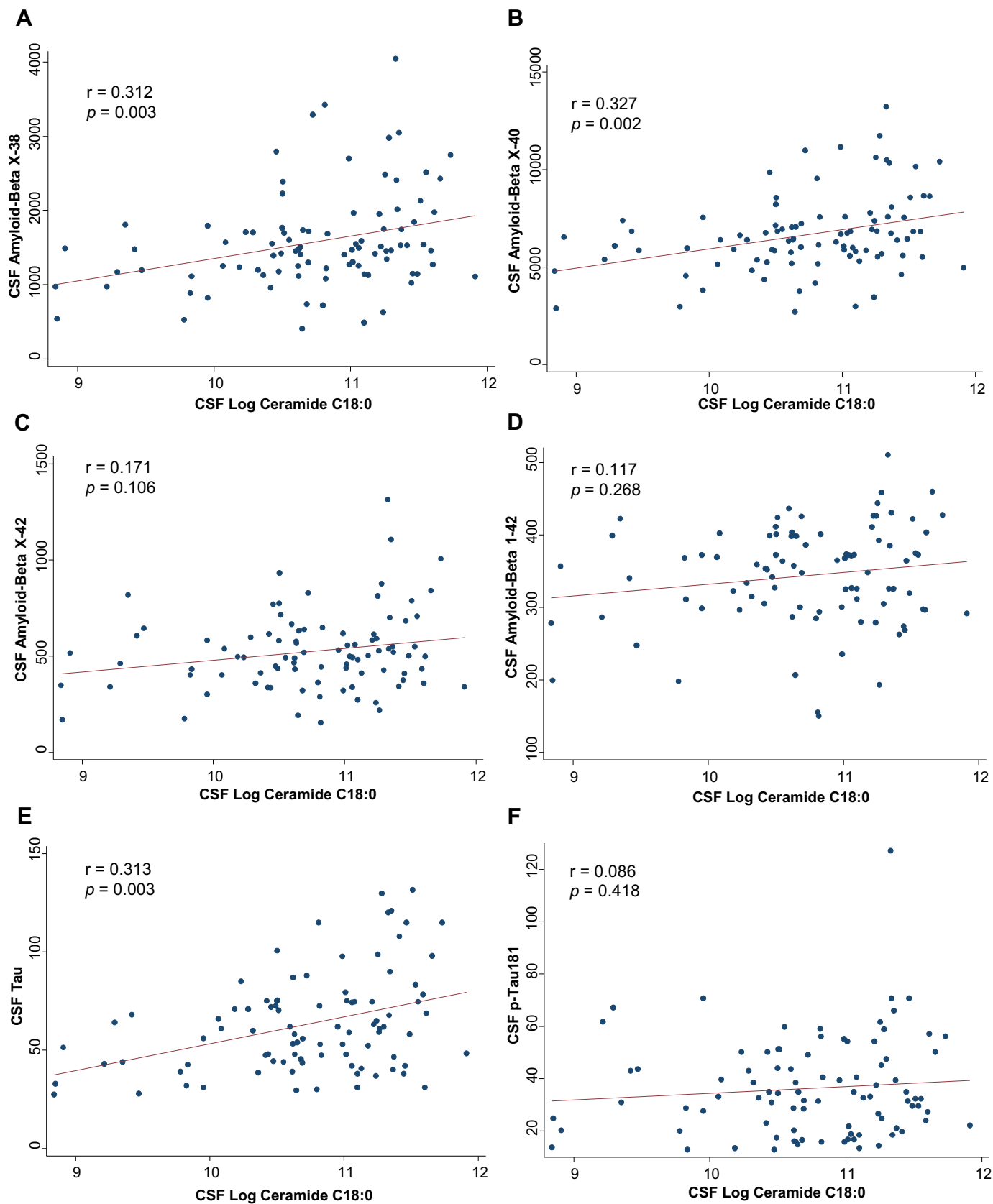
Although all participants were cognitively normal, we did see some associations between CSF ceramides and cognitive performance, specifically in domains of memory performance. Using linear regression models and controlling for age, education, and APOE E4 genotype, higher ceramide C20:0 ( $b = -0.67$ ,  $p = 0.034$ ) and C22:0 ( $b = -0.69$ ,  $p = 0.026$ ) were associated with worse performance on the HVLT-delayed recall. Higher ceramide C26:0

( $b = -1.32$ ,  $p = 0.049$ ) was also associated with worse performance on the Working Memory Composite Score. Examining A $\beta$  and tau measures and controlling for the same variables, CSF tau was associated with better performance on Working Memory ( $b = 0.04$ ,  $p = 0.042$ ), whereas p-tau was associated with worse performance on the HVLT-immediate recall ( $b = -0.04$ ,  $p = 0.047$ ).

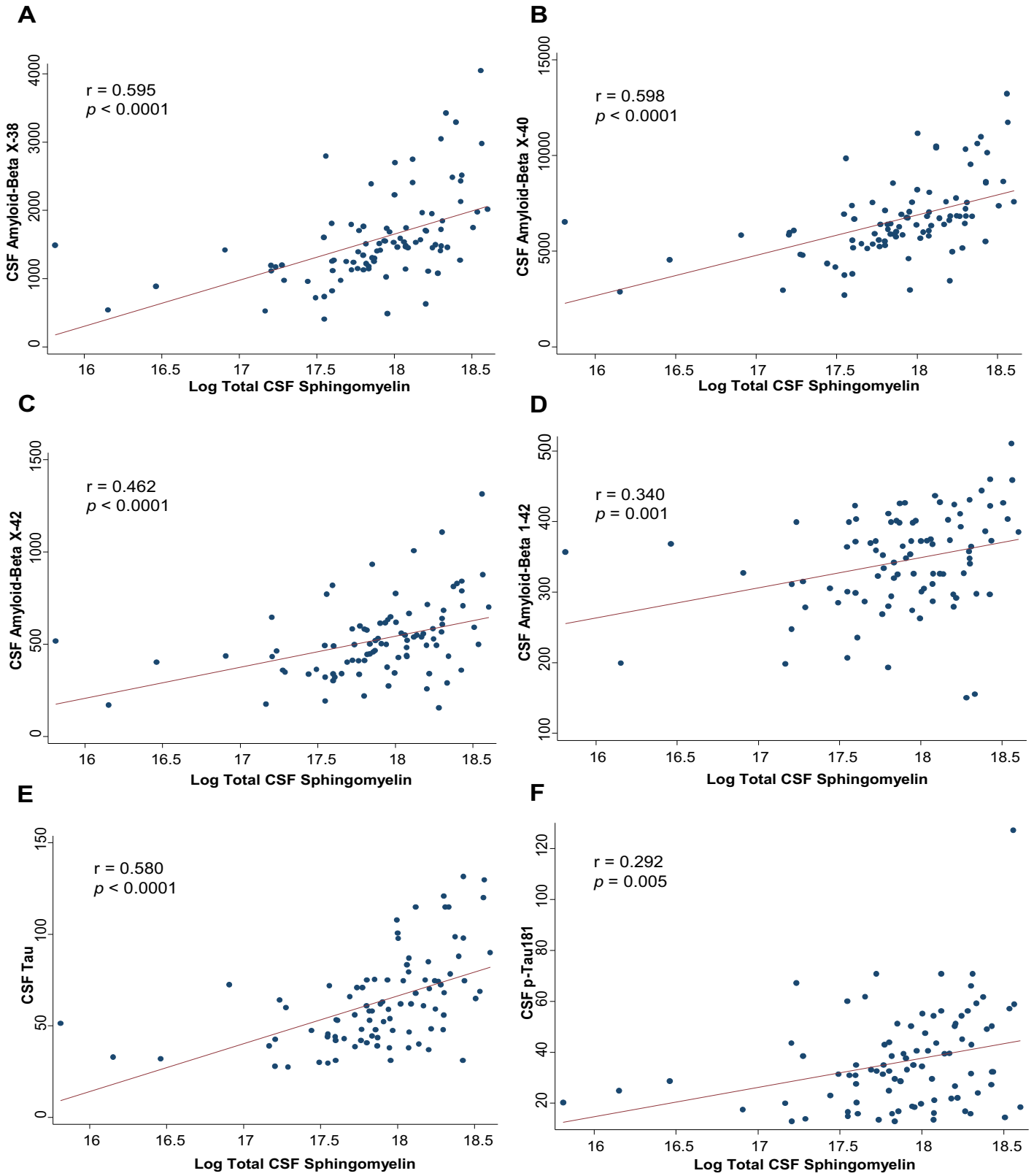
## 4. Discussion

In the present study, we found cross-sectional, positive associations between CSF ceramide C18:0 and total sphingomyelins and CSF A $\beta$  and tau levels in a cohort of cognitively normal individuals aged 36–69 years with a confirmed parental history of AD. These associations were stronger among individuals that were older than the median sample age (54 years) and among those with at least 1 APOE E4 allele. These results suggest in vivo associations between CSF sphingolipids and CSF A $\beta$  and tau levels, and although cross-sectional, may suggest new pathways for the treatment or prevention of AD.

Cellular and animal studies have shown direct and indirect associations between sphingolipids and A $\beta$  metabolism (Cutler et al., 2004; Grimm et al., 2005; Kalvodova et al., 2005; Lee et al., 2004; Mattson et al., 2005; Puglielli et al., 2003). Ceramides regulate A $\beta$ (1-42) production by modulating the physical location of APP with beta-secretase and stabilizing the activity of gamma-secretase (Kalvodova et al., 2005; Puglielli et al., 2003). Exposure of cultured neurons to A $\beta$ (1-42) increases ceramide levels by activating sphingomyelinase (Grimm et al., 2005; Lee et al., 2004); blocking this ceramide increase protects neurons from A $\beta$ -induced cell death (Cutler et al., 2004). Last, A $\beta$ (1-42) indirectly increases ceramides through an oxidative stress-mediated mechanism (Cutler et al., 2004; Mattson et al., 2005). Studies have also linked long-chain ceramides, specifically the 18-carbon acyl chain length species to tau phosphorylation through modulation of PP2A activity (Chalfant et al., 1999; Dobrowsky et al., 1993; Goedert et al., 1995; Gong et al., 1994; Mukhopadhyay et al., 2009). In this study, we extend these



**Fig. 1.** Correlations between CSF ceramide C18:0 and CSF levels of: (A) A $\beta$ X-38, (B) A $\beta$ X-40, (C) A $\beta$ X-42, (D) A $\beta$ 1-42, (E) tau, and (F) p-tau181. In F (correlation between ceramide C18:0 and p-tau181), excluding the one outlier with p-tau181 level >121 had little effect on the results (excluding outlier:  $r = 0.068$ ,  $p = 0.524$ ). Abbreviations: A $\beta$ , amyloid-beta; CSF, cerebrospinal fluid.



**Fig. 2.** Correlations between CSF total sphingomyelin and CSF levels of: (A) A $\beta$ X-38, (B) A $\beta$ X-40, (C) A $\beta$ X-42, (D) A $\beta$ 1-42, (E) tau, and (F) p-tau181. In F (correlation between total sphingomyelins and p-tau181), excluding the one outlier with p-tau181 level >121 slightly reduced the significance of the results (excluding outlier:  $r = 0.270$ ,  $p = 0.010$ ). Abbreviations: A $\beta$ , amyloid-beta; CSF, cerebrospinal fluid.

findings to humans to show in vivo correlations between CSF sphingolipids and CSF A $\beta$  and tau measures. Notably, only ceramide C18:0 was associated with A $\beta$  and tau levels, which is a direct in vivo translation of animal and cellular studies. However, further investigation is needed to determine why ceramide C18:0, the most abundant CSF ceramide species, was only associated with total tau and not phospho-tau.

An intriguing finding is that ceramide C18:0 was most strongly associated with A $\beta$ x-38 and A $\beta$ x-40. Although the associations with A $\beta$ x-42 or A $\beta$ 1-42 were stronger among older individuals (>54 years) and those with an APOE E4 allele, most associations still did not reach statistical significance. These results suggest that the relationship between ceramides and A $\beta$  may vary by A $\beta$  isomer length. Alternatively, as these individuals are all cognitively normal and A $\beta$ 42 is the most toxic species of amyloid, the relationship between ceramide C18:0 and A $\beta$ 42 may strengthen with increasing pathology. Additional research is needed to replicate these findings and to longitudinally examine this association in persons with mild cognitive impairment (MCI).

Interestingly, although much basic science research, as described previously, has focused on the associations between ceramides and A $\beta$  and tau, we observed the strongest correlations between CSF sphingomyelins and CSF A $\beta$  and tau. A previous study reported that sphingomyelin levels were elevated in prodromal AD and then decreased in mild to moderate AD (Kosicek et al., 2012). However, the reason for the mechanism behind this sphingomyelin increase is not currently clear. Kosicek et al. (2012) speculate that it is possible that the increased sphingomyelin could be the result of a cellular response to initially increased ceramide. As ceramide can be catabolized to sphingomyelin, levels of this lipid would increase. An alternative explanation is that sphingomyelin is elevated as a result of brain atrophy and cellular degeneration because sphingomyelin is primarily a structural lipid located in membranes.

Previous clinical and epidemiologic studies have reported associations of both CSF and plasma sphingolipids with AD severity. Two studies reported that CSF ceramide (Sato et al., 2005) and sphingomyelin levels varied by AD severity (Kosicek et al., 2012), but they did not examine associations between these lipids and markers of AD pathology, including CSF A $\beta$  or tau. High levels of plasma ceramides have also been associated with an increased risk of cognitive impairment and AD among cognitively normal individuals (Mielke et al., 2010a, 2012), memory decline and hippocampal volume loss among patients with MCI (Mielke et al., 2010b), and faster rates of cognitive decline among AD patients (Mielke et al., 2011). A shotgun lipidomic approach also reported differences between plasma sphingolipids in AD patients and controls (Han et al., 2011). The present analysis is one of the first studies to extend this research to examine associations between these sphingolipids and CSF biomarkers of AD. Examining the relationship between CSF sphingolipids and cognition, ceramides C20:0 and C22:0 were associated with worse performance on the HVLT-delayed recall and C26:0 with worse performance on the Working Memory Composite Score. There were also very few associations between A $\beta$  and tau levels and cognitive performance. These limited associations might be expected in this group of relatively young (aged 36–69 years) cognitively normal individuals. Notably, the associations between CSF sphingolipids and CSF A $\beta$  and tau were stronger in individuals aged  $\geq$ 54 years, at the time when AD pathology is thought to begin (Jack et al., 2010) and also in APOE E4 carriers. This may suggest, in line with cellular studies, that sphingolipids are involved early in the etiopathogenesis of AD and may be viable therapeutic targets.

Serial measures of CSF and brain imaging are needed to better understand the temporality of these associations in humans. Similarly, back translational research in transgenic animals to

**Table 3**  
Cross-sectional associations between CSF ceramides and sphingomyelins and CSF amyloid-beta and tau using multivariate linear regression

Log sphingolipid	All individuals (n = 91) <sup>a</sup>		Age <54 (n = 44)		Age $\geq$ 54 (n = 47)		No APOE E4 (n = 58)		APOE E4 (n = 33)		
	b (SE)	p	b (SE)	p	b (SE)	p	b (SE)	p	b (SE)	p	
Ceramide C18:0	ABX-38 <sup>b</sup>	302.75 (96.57)	0.002	163.11 (158.45)	0.309	418.91 (120.88)	0.001	286.68 (103.30)	0.008	358.88 (204.89)	0.090
	ABX-40 <sup>b</sup>	983.20 (291.75)	0.001	549.37 (474.95)	0.254	1349.24 (365.49)	0.001	868.16 (341.89)	0.014	1298.58 (549.95)	0.025
	ABX-42 <sup>b</sup>	67.24 (30.58)	0.031	21.21 (49.84)	0.522	90.07 (38.76)	0.025	66.01 (36.62)	0.077	69.02 (57.31)	0.238
	AB1-42 <sup>c</sup>	19.12 (10.44)	0.071	14.46 (14.37)	0.320	21.19 (15.10)	0.168	19.49 (11.88)	0.107	18.47 (21.07)	0.388
	Tau <sup>c</sup>	13.44 (3.49)	<0.001	7.61 (5.41)	0.167	18.18 (4.63)	<0.001	13.70 (4.03)	0.001	12.77 (6.91)	0.075
Total sphingomyelin	p-tau181 <sup>c</sup>	2.80 (2.84)	0.327	0.81 (4.77)	0.867	4.38 (3.55)	0.224	0.94 (2.92)	0.750	6.37 (6.24)	0.315
	Tau/AB1-42 <sup>c</sup>	0.03 (0.01)	0.039	0.01 (0.01)	0.244	0.04 (0.02)	0.062	0.03 (0.01)	0.025	0.03 (0.03)	0.391
	ABX-38 <sup>b</sup>	653.29 (128.14)	<0.001	3.89 (202.17)	0.021	816.50 (158.79)	<0.001	450.22 (149.83)	0.004	1152.83 (216.49)	<0.001
	ABX-40 <sup>b</sup>	2037.57 (387.32)	<0.001	1502.38 (605.20)	0.017	2553.43 (481.13)	<0.001	1455.48 (492.0)	0.005	3490.53 (548.05)	<0.001
	ABX-42 <sup>b</sup>	173.49 (41.01)	<0.001	158.65 (62.64)	0.015	184.46 (53.72)	0.001	129.12 (52.42)	0.017	266.29 (66.48)	<0.001
p-tau181 <sup>c</sup>	AB1-42 <sup>c</sup>	47.00 (14.40)	0.002	45.39 (21.68)	0.042	45.43 (21.68)	0.042	35.79 (17.17)	0.004	71.80 (26.93)	0.012
	Tau <sup>c</sup>	24.56 (4.73)	<0.001	18.69 (6.86)	0.009	30.68 (6.44)	<0.001	18.22 (6.01)	0.004	37.74 (7.58)	<0.001
	Tau/AB1-42 <sup>c</sup>	10.85 (3.92)	0.007	13.48 (6.06)	0.032	9.79 (5.11)	0.062	4.53 (4.24)	0.290	22.48 (7.91)	0.008
Tau/AB1-42 <sup>c</sup>	0.05 (0.02)	0.007	0.03 (0.01)	0.053	0.08 (0.03)	0.020	0.03 (0.02)	0.068	0.09 (0.04)	0.037	

Key: CSF, cerebrospinal fluid; SE, standard error.

<sup>a</sup> Models control for age and APOE E4 allele.

<sup>b</sup> Assays conducted using Meso Scale Discovery (MSD) electrochemiluminescence.

<sup>c</sup> Assays conducted using INNO-BIO AlzBio3 (xMAP).

understand the mechanism and timing by which CSF sphingomyelins and ceramide increase in relation to amyloid is also critical. This research is particularly important to understand alterations in sphingolipids in the context of the current biomarker ordering of AD, with the hypothesis that amyloid is deposited first, followed by tau accumulation, neurodegeneration, and then cognitive symptoms (Jack et al., 2013a). Although, recent research suggests that, among some individuals, neurodegeneration may appear before significant amyloid deposition (Jack et al., 2013b).

Limitations of the study warrant consideration. First, the sample size was relatively small. However, despite this, we still detected significant associations between CSF sphingolipids and CSF A $\beta$  and tau levels. Second, the sample consisted of cognitively normal individuals with “normal” levels of CSF A $\beta$  and tau. Although this is an advantage from the standpoint of understanding the earliest pathologic changes, it is not known whether these correlations will be as robust in patients with MCI and AD. Such knowledge will help to determine whether CSF sphingolipids levels could be used as diagnostic or prognostic markers of disease severity. Third, the study was cross-sectional and cannot generate causal inferences. Longitudinal studies to assess the relationships between changes in CSF sphingolipids and changes in AD pathology are also needed to validate the pathologic implications of these cross-sectional findings. Last, given the associations between plasma ceramides and cognitive decline across severities of AD (Mielke et al., 2010a, 2010b, 2012), further research should be directed to elucidate the mechanistic relationship between plasma sphingolipids and CSF A $\beta$  and tau.

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