

# **Elevated plasma ceramide levels in post-menopausal women**

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# 25 ABSTRACT

26 Circulating ceramide levels are abnormally elevated in age-dependent pathologies such as  
 27 cardiovascular diseases, obesity and Alzheimer’s disease. Nevertheless, the potential impact of age on  
 28 plasma ceramide levels has not yet been systematically examined. In the present study, we quantified a  
 29 focused panel of plasma ceramides and dihydroceramides in a cohort of 164 subjects (84 women) 19 to  
 30 80 years of age. After adjusting for potential confounders, multivariable linear regression analysis  
 31 revealed a positive association between age and ceramide (d18:1/24:0) ( $\beta$  (SE) = 5.67 (2.38);  $p$  =  
 32 .0198) and ceramide (d18:1/24:1) ( $\beta$  (SE) = 2.88 (.61);  $p$  < .001) in women, and between age and  
 33 ceramide (d18:1/24:1) in men ( $\beta$  (SE) = 1.86 (.77);  $p$  = .0179). In women of all ages, but not men,  
 34 plasma ceramide (d18:1/24:1) was negatively correlated with plasma estradiol ( $r$  = -0.294;  $p$  = .007).  
 35 Finally, *in vitro* experiments in human cancer cells expressing estrogen receptors showed that  
 36 incubation with estradiol (10 nM, 24 h) significantly decreased ceramide accumulation. Together, the  
 37 results suggest that aging is associated with an increase in circulating ceramide levels, which in post-  
 38 menopausal women may be at least partially dependent on lower estradiol levels.

## INTRODUCTION

The ceramides are key lipid constituents of mammalian cells. They regulate the structural properties of the lipid bilayer [1] along with its interaction with cellular proteins [2], and control many signalling processes, including cell survival [3], growth and proliferation [4], differentiation [5], senescence [6] and apoptosis [7,8]. Dysfunctions in ceramide-mediated signalling may contribute to the initiation and progression of a variety of age-dependent diseases. Human studies have shown the existence of abnormal plasma levels of various ceramide species – including ceramide (d18:1/18:0), (d18:1/22:0), (d18:1/24:0) and (d18:1/24:1) – in several conditions such as obesity [9], type-2 diabetes [10], hypertension [11], atherosclerosis [12] and other cardiovascular diseases [13]. Furthermore, elevated serum levels of long-chain ceramides have been linked to the increased risk of memory deficits [14] and may be predictive of hippocampal volume loss and cognitive decline in patients affected by Mild Cognitive Impairment [15]. Other studies have reported the existence of sex-dependent differences in circulating ceramides, albeit with apparently contrasting results [16-18]. For example, in a study of a large cohort of Mexican-Americans of median age 35.7 years, plasma ceramides were found to be higher in men than in women [17]. By contrast, in the Baltimore Longitudinal Study of Aging, whose participants were aged 55 or older, plasma ceramide concentrations were shown to be higher in women than in men [18].

Aging in rats and mice is associated with sexually dimorphic changes in the sphingolipid composition of several brain structures, including the hippocampus [19]. Age-dependent sphingolipid alterations have also been documented in peripheral rodent tissues [20]. In the present study, we hypothesized that aging in humans might be similarly associated with changes in ceramide levels. To test this idea, we profiled six ceramide and dihydroceramide species in lipid extracts of plasma from 164 subjects (84 women) of age 19 to 80 years, using liquid chromatography/mass spectrometry (LC-MS/MS). A

63 primary criterion for subject selection was the absence of major medical illnesses and, particularly, of  
64 conditions that had been previously linked to ceramide alterations.

## MATERIALS AND METHODS

### Study subjects

We recruited 164 Italian subjects (84 women) from 19 to 80 years of age (Table 1). There were no significant differences between male and female subjects with regard to age, years of education, ethnic background, cognitive status, as assessed by the Mini-Mental State Examination (MMSE), obesity and diabetes, hypertension and use of anti-hypercholesterolemic drugs. By contrast, there was difference in smoking status (20.23% women versus 7.5% men). Moreover, 6 of 44 pre-menopause women used contraceptives at time of enrollment. None of the post-menopause women was under hormone replacement therapy (HRT).

**Table 1.** Sociodemographic and clinical characteristics of men and women included in the study.

|                        | Whole sample<br>(n=164) | Men<br>(n=80)           | Women<br>(n=84)         | <i>p</i> -value |
|------------------------|-------------------------|-------------------------|-------------------------|-----------------|
| Characteristic         |                         |                         |                         |                 |
| Age<br>(years)         | 49.3±16.6 (range 19-80) | 49.6±16.8 (range 19-80) | 49.0±16.6 (range 20-78) | .46             |
| Education<br>(years)   | 14.4±3.8 (range 5-25)   | 14.6±4.0 (range 5-25)   | 14.2±3.7 (range 5-24)   | .85             |
| MMSE                   | 29.4±1.0 (range 26-30)  | 29.4±1.0 (range 26-30)  | 29.4±1.0 (range 26-30)  | .82             |
| Ethnicity<br>(% white) | 100.00                  | 100.00                  | 100.00                  |                 |
| Obesity<br>(%)         | 0.00                    | 0.00                    | 0.00                    |                 |

|                 |       |       |       |      |
|-----------------|-------|-------|-------|------|
| Diabetes        | 0.00  | 0.00  | 0.00  |      |
| (%)             |       |       |       |      |
| Current smokers | 14.02 | 7.50  | 20.23 | .02  |
| (%)             |       |       |       |      |
| Former smokers  | 17.07 | 21.25 | 13.10 | .21  |
| (%)             |       |       |       |      |
| Hypertension    | 19.51 | 20.00 | 19.05 | 1.00 |
| (%)             |       |       |       |      |
| AHT             | 7.32  | 11.25 | 3.57  | .07  |
| (%)             |       |       |       |      |
| Contraceptives  | 3.66  | 0.00  | 7.14  | .03  |
| (%)             |       |       |       |      |

*Notes.* MMSE: Mini Mental State Examination. AHT: Anti-hypercholesterolemic therapy. Data are expressed as mean  $\pm$  standard deviation (SD) or as %. Differences between groups are considered statistically significant at  $p < .05$ ; unpaired Student's  $t$ -test for continuous variables; Fisher's exact test for categorical variables.

Exclusion criteria were: (i) suspicion of cognitive impairment or dementia based on MMSE [21] (score  $\leq 26$ , consistent with normative data collected in the Italian population) and confirmed by a detailed neuropsychological evaluation using the Mental Deterioration Battery [22] and clinical criteria for Alzheimer's dementia [23] or Mild Cognitive Impairment [24]; (ii) subjective complaints of memory difficulties or other cognitive deficits, regardless of whether or not these interfered with daily life; (iii) vision and hearing loss that could potentially influence testing results; (iv) major medical illnesses (i.e., unstable diabetes; obesity; obstructive pulmonary disease or asthma; hematological and oncological

disorders; pernicious anemia; significant gastrointestinal, renal, hepatic, endocrine, or cardiovascular system diseases; recently treated hypothyroidism); (v) current or reported psychiatric disease, as assessed by the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR SCID) [25] or neurological disease, as assessed by clinical evaluation; and (vi) known or suspected history of alcoholism or drug addiction. Finally, because ceramides have been previously involved in the pathogenesis of neurodegenerative disorders [15,26,27], we excluded subjects who showed brain abnormalities or vascular lesions as determined by using a recently published semi-automated method [28]. The menopausal status was prospectively assessed during clinical interviews. Women were defined as post-menopausal when showing 12 consecutive months of amenorrhea that was not due to other obvious pathological or physiological causes.

Blood collection and analyses were approved by the Santa Lucia Foundation Ethics Committee and complied with the ethical principles set out in the Declaration of Helsinki. A written consent form was signed by all participants after they received a full explanation of the study procedures.

#### **Variables examined in relation to ceramide levels.**

All variables were assessed for each patient using the same methods. Demographic variables considered in linear regression analysis included age and sex. Medical history covariates included hypertension, use of anti-hypercholesterolemic agents and use of contraceptives. Current and former smoking status was ascertained by an oral interview.

#### **Chemicals**

Solvents and chemicals were purchased from Sigma Aldrich (Milan, Italy). Ceramide standards were from Avanti Polar Lipids (Alabaster, AL, USA).

#### **Blood collection**

110 Blood was drawn by venipuncture in the morning after an overnight fast, and collected into 10-ml tubes  
111 containing spray-coated EDTA (EDTA Vacutainer, BD Biosciences, San Diego, CA, USA). Plasma  
112 was obtained by blood centrifugation at  $400 \times g$  at  $4^\circ\text{C}$  for 15 min. The plasma divided into aliquots  
113 was stored at  $-80^\circ\text{C}$  until analyses.

#### 114 **Lipid extraction**

115 Lipids were extracted using a modified Bligh and Dyer method [29]. Briefly, plasma samples (50  $\mu\text{L}$ )  
116 or cell pellets were transferred to glass vials and liquid-liquid extraction was carried out using 2 mL of  
117 a methanol/chloroform mixture (2:1 vol/vol) containing the odd-chain saturated ceramide (d18:1/17:0)  
118 as an internal standard. After mixing for 30 s, lipids were extracted with chloroform (0.5 mL), and  
119 extracts were washed with liquid chromatography-grade water (0.5 mL), mixing after each addition.  
120 The samples were centrifuged for 15 min at  $3500 \times g$  at room temperature. After centrifugation, the  
121 organic phases were collected and transferred to a new set of glass vials. To increase overall recovery,  
122 the aqueous fractions were extracted again with chloroform (1 mL). The two organic phases were  
123 pooled, dried under a stream of  $\text{N}_2$  and residues were dissolved in methanol/chloroform (9:1 vol/vol,  
124 0.07 mL). After mixing (30 s) and centrifugation (10 min,  $5000 \times g$ , room temperature) the samples  
125 were transferred to glass vials for analyses.

#### 126 **Ceramide quantification**

127 Ceramides were analyzed by LC-MS/MS using an Acquity® ultra-performance liquid chromatography  
128 (UPLC) system coupled to a Xevo triple quadrupole mass spectrometer interfaced with electrospray  
129 ionization (Waters, Milford, MA, USA), as previously described [29]. Lipids were separated on a  
130 Waters Acquity® BEH C18 column ( $2.1 \times 50$  mm,  $1.7 \mu\text{m}$  particle size) at  $60^\circ\text{C}$  and eluted at a flow  
131 rate of 0.4 mL/min. The mobile phase consisted of 0.1% formic acid in acetonitrile/water (20:80  
132 vol/vol) as solvent A and 0.1% formic acid in acetonitrile/isopropanol (20:80 vol/vol) as solvent B. A  
133 gradient program was used: 0.0–1.0 min 30% B, 1.0–2.5 min 30 to 70% B, 2.5–4.0 min 70 to 80% B,

4.0–5.0 min 80% B, 5.0–6.5 min 80 to 90% B, and 6.6–7.5 min 100% B. The column was reconditioned to 30% B for 1.4 min. The injection volume was 3  $\mu$ L. Detection was done in the positive electrospray ionization mode. Capillary voltage was 3.5 kV and cone voltage was 25 V. The source and desolvation temperatures were set at 120 °C and 600 °C respectively. Desolvation gas and cone gas (N<sub>2</sub>) flow were 800 L/h and 20 L/h, respectively. Plasma and cell-derived ceramides were identified by comparison of their LC retention times and MS/MS fragmentation patterns with those of authentic standards (Avanti Polar Lipids). Extracted ion chromatograms were used to identify and quantify the following ceramides and dihydroceramides (d18:1/16:0) ( $m/z$  520.3 > 264.3), (d18:1/18:0) ( $m/z$  = 548.3 > 264.3), (d18:1/24:0) ( $m/z$  = 632.3 > 264.3), (d18:1/24:1) ( $m/z$  = 630.3 > 264.3), (d18:0/24:0) ( $m/z$  = 652.5 > 634.5) and (d18:0/24:1) ( $m/z$  = 650.5 > 632.5). Data were acquired by the MassLynx software and quantified using the TargetLynx software (Waters, Milford, MA, USA).

#### **Estradiol quantification**

Plasma 17- $\beta$ -estradiol (E2) levels were quantified using a competitive binding immunoassay kit (Human E2 ELISA kit, Invitrogen, Milan, Italy) following manufacturer's instructions. Briefly, plasma samples, controls and standard curve samples (50  $\mu$ L) were incubated with E2-horseradish peroxidase conjugate (50  $\mu$ L) and anti-estradiol antibody (50  $\mu$ L) in a 96-well plate for 2 h, at room temperature, on a shaker set at 700  $\pm$  100 rpm. Washing was carried out by completely aspirating the liquid, filling the wells with diluted wash buffer (0.4 mL) provided in the kit and then aspirating again. After repeating this procedure 4 times, chromogen solution (200  $\mu$ L) was added to each well; reactions were run for 15 min and stopped adding 50  $\mu$ L of the stop solution provided in the kit. Absorbance was measured at 450 nm and estradiol concentrations were calculated by interpolation from the reference curve.

#### **Cell cultures and treatment**

157 The MCF7 human breast cancer cell line [30,31] was a kind gift of Dr. Gennaro Colella (Mario Negri  
158 Institute, Milan, Italy). Cells were cultured in phenol red-free Dulbecco's Modified Eagle's Medium  
159 (DMEM) (Gibco by Life Technologies, Carlsbad, CA, USA) supplemented with charcoal-stripped fetal  
160 bovine serum (10%) (Sigma Aldrich, Milan, Italy) to starve cells from steroid hormones (starvation  
161 medium), L-glutamine (2 mM), and penicillin/streptomycin (100 µg/mL), in a humidified atmosphere  
162 (5% CO<sub>2</sub>, 37 °C). Cells were seeded in 6-well plates (3 x 10<sup>5</sup> cells/well) and cultured for 24 h. Estradiol  
163 (Sigma Aldrich, Milan, Italy) was dissolved in dimethyl sulfoxide (DMSO) and diluted in phenol red-  
164 free DMEM to a final concentration of 10 nM (0.1% final DMSO concentration). After 24 h  
165 incubation, the media were removed, cells were washed with phosphate-buffered saline, scraped and  
166 centrifuged (800 x g, 4 °C, 10 min). Protein concentrations were measured using the bicinchoninic  
167 acid assay (Pierce, Rockford, IL, USA) and cell pellets were stored at -80 °C until analyses.

#### 168 **Statistical analyses**

169 Results are expressed as mean ± SEM (standard error of the mean). Sex differences in baseline  
170 demographic and health-related characteristics were examined using Fisher's exact test for categorical  
171 variables and unpaired Student's *t*-test for continuous variables. Data were analyzed by unpaired  
172 Student's *t*-test or 2-way ANOVA followed by Bonferroni post-hoc test. Pearson's correlation  
173 coefficient assessed the pairwise correlation between estradiol and ceramide levels. Significant outliers  
174 were excluded using the Grubbs' test. Multivariable linear regression method was used to assess the  
175 association between ceramides and patients' characteristics. Differences between groups were  
176 considered statistically significant at values of  $p < .05$ . The GraphPad Prism software (GraphPad  
177 Software, Inc., La Jolla, CA, USA) and SAS 9.4 (SAS Institute, Cary, NC, USA) were used for  
178 statistical analyses.

179

## RESULTS

### Plasma ceramide levels are positively correlated with age

The scatter plot reported in Fig 1A illustrates the total ceramide levels in plasma of individual women aged 20-78 years. Pearson's analysis of the data revealed a statistically significant positive correlation between ceramide levels and age ( $r = 0.378$ ;  $p = .0004$ ). Because the largest accrual in plasma ceramides occurred between the age of 40 and 50 years, which is coincident with menopause, in a secondary analysis we grouped the data according to the subjects' menopausal status. We found a statistically detectable difference between pre-menopausal women (20-54 years) and post-menopausal women (47-78 years) (Fig 1B). In particular, the levels of long-chain ceramide (d18:1/18:0) ( $p = .0035$ , unpaired Student's *t*-test), very long-chain ceramides (d18:1/24:0) ( $p = .0012$ ) and (d18:1/24:1) ( $p < .0001$ ), and dihydroceramide (d18:0/24:1) ( $p = .0340$ ) were higher in post-menopausal relative to pre-menopausal women (Fig 1B).

**Fig 1.** Scatter plot of plasma ceramide concentrations in women aged 20 to 78 years.

(A) Total ceramide levels in 84 female subjects included in the study. Pearson's correlation is considered statistically significant at  $p < .05$ . (B) Average levels of individual ceramide species in pre-menopausal women (20-54 years,  $n = 44$ , open bars) and post-menopausal women (47-78 years,  $n = 40$ , closed bars). Results are expressed as mean  $\pm$  SEM.  $*p < .05$ ,  $**p < .01$ ,  $***p < .001$ ; unpaired Student's *t*-test.

No differences were found in the levels of ceramide (d18:1/16:0) ( $p = .3526$ ) and dihydroceramide (d18:0/24:0) ( $p = .3633$ ). In contrast with these findings in women, men showed no significant age-dependent increases in plasma ceramides ( $r = 0.143$ ;  $p = .208$ ) (Fig 2A). Male subjects in the age groups 19-54 and 55-80 years displayed comparable levels of circulating ceramide (d18:1/18:0) ( $p =$

.7112), (d18:1/24:0) ( $p = .7895$ ), (d18:1/24:1) ( $p = .0847$ ) and dihydroceramide (d18:0/24:1) ( $p = .9014$ ). However, dihydroceramide (d18:0/24:0) was significantly lower in men >55 years, compared to younger men ( $p = .0003$ ) (Fig 2B).

**Fig 2.** Scatter plot of plasma ceramide concentrations in men aged 19 to 80 years.

(A) Total ceramide levels in 80 male subjects included in the study. Pearson's correlation is considered statistically significant at  $p < .05$ . (B) Average levels of individual ceramide species in men aged 19-54 years ( $n = 48$ , open bars) and 55-80 years ( $n = 32$ , closed bars). Results are expressed as mean  $\pm$  SEM. \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ ; unpaired Student's  $t$ -test.

In an additional analysis, we compared total ceramide levels in plasma of pre- and post-menopausal women with those measured in age-matched men (Fig 3). The results show that pre-menopausal women had significantly lower levels of circulating ceramides ( $p < .05$ , 2-way ANOVA followed by Bonferroni post-hoc test) relative to men of the same age (Fig 3A). The difference disappeared after menopause ( $p > .05$ ) (Fig 3A).

**Fig 3.** Plasma ceramide and estradiol concentrations in men and women.

(A) Plasma ceramide levels in, left, pre-menopausal women (20-54 years,  $n = 44$ ) and age-matched men (19-54 years,  $n = 48$ ) and, right, post-menopausal women (47-78 years,  $n = 40$ ) and age-matched men (55-80 years,  $n = 32$ ). (B) Plasma estradiol levels in, left, pre-menopausal women (20-54 years,  $n = 44$ ) and age-matched men (19-54 years,  $n = 48$ ) and, right, post-menopausal women (47-78 years,  $n = 40$ ) and age-matched men (55-80 years,  $n = 32$ ). \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ ; 2-way ANOVA followed by Bonferroni post-hoc test (women 20-54 years versus men 19-54 years). #  $p < .05$ , ##  $p < .01$ , ###  $p < .001$ .

.01, ###  $p < .001$ ; 2-way ANOVA followed by Bonferroni post-hoc test (women 20-54 years versus women 47-78 years).

### Variables associated with plasma ceramides

Next, we used multivariable linear regression models to test the association between age and ceramides and adjust for potential covariates for which data had been collected (Table 1). These factors included hypertension (32/164 subjects, 16 women), tobacco smoking (23/164 subjects, 17 women), use of anti-hypercholesterolemic (12/164 subjects, 3 women) or contraceptive agents (6/164 subjects, 6 women), obesity (0/164 subjects) and diabetes (0/164 subjects). We did not take into account the number of cigarettes smoked as a variable of multivariable linear regression analysis. The adjusted linear regression analysis confirmed that ceramide (d18:1/24:0) ( $\beta$  (SE) = 5.67 (2.38);  $p = .0198$ ) and ceramide (d18:1/24:1) ( $\beta$  (SE) = 2.88 (0.61);  $p < .0001$ ) were positively associated with age in women and also, unexpectedly, revealed an opposite, albeit weaker, trend with ceramide (d18:1/16:0) ( $\beta$  (SE) = -.08 (.04);  $p = .0285$ ) (Table 2A).

**Table 2A.** Multivariable linear regression analysis to assess the association between individual ceramide species and variables (age, smoke, hypertension, contraceptive use, anti-hypercholesterolemic therapy) among women.

|            | Ceramide<br>(d18:1/16:0)     |            | Ceramide<br>(d18:1/18:0)     |            | Ceramide<br>(d18:1/24:0)     |            | Ceramide<br>(d18:1/24:1)     |            |
|------------|------------------------------|------------|------------------------------|------------|------------------------------|------------|------------------------------|------------|
|            | $\beta$ -coefficient<br>(SE) | $p$ -value | $\beta$ -coefficient<br>(SE) | $p$ -value | $\beta$ -coefficient<br>(SE) | $p$ -value | $\beta$ -coefficient<br>(SE) | $p$ -value |
| Covariates |                              |            |                              |            |                              |            |                              |            |
| Age        | -0.08 (0.04)                 | .0285      | 0.01 (0.02)                  | .7805      | 5.67 (2.38)                  | .0198      | 2.88 (0.61)                  | <.0001     |

|                |              |       |              |       |                  |       |                |       |
|----------------|--------------|-------|--------------|-------|------------------|-------|----------------|-------|
| Smoke          | -0.22 (0.98) | .8237 | 0.01 (0.67)  | .9846 | 94.84 (65.56)    | .152  | 8.91 (16.74)   | .5963 |
| Hypertension   | 4.78 (1.42)  | .0012 | 2.48 (0.97)  | .0126 | -41.14 (94.85)   | .6657 | -20.60 (24.22) | .3978 |
| Contraceptives | 0.26 (1.86)  | .8896 | -0.22 (1.27) | .8606 | -200.79 (124.34) | .1104 | -36.64 (31.76) | .2521 |
| AHT            | 5.14 (2.63)  | .0544 | 2.76 (1.80)  | .1298 | -144.71 (76.10)  | .4137 | -51.18 (44.98) | .2586 |

244 Notes. SE: standard error. AHT: Anti-hypercholesterolemic therapy. N = 84. Differences are  
 245 considered statistically significant at  $p < .05$ .

246

247 In men (Table 2B), the analysis unmasked a statistically detectable association between age and  
 248 ceramide (d18:1/24:1) ( $\beta$  (SE) = 1.86 (.77);  $p = .0179$ ).

249 **Table 2B.** Multivariable linear regression analysis to assess the association between individual  
 250 ceramide species and variables (age, smoke, hypertension, contraceptive use, anti-hypercholesterolemic  
 251 therapy) among men.

|              | Ceramide<br>(d18:1/16:0)     |            | Ceramide<br>(d18:1/18:0)     |            | Ceramide<br>(d18:1/24:0)  |            | Ceramide<br>(d18:1/24:1)     |            |
|--------------|------------------------------|------------|------------------------------|------------|---------------------------|------------|------------------------------|------------|
|              | $\beta$ -coefficient<br>(SE) | $p$ -value | $\beta$ -coefficient<br>(SE) | $p$ -value | $\beta$ -coefficient (SE) | $p$ -value | $\beta$ -coefficient<br>(SE) | $p$ -value |
| Covariates   |                              |            |                              |            |                           |            |                              |            |
| Age          | -0.05 (0.03)                 | .1797      | -0.03 (0.03)                 | .3624      | 2.37 (2.50)               | .3456      | 1.86 (0.77)                  | .0179      |
| Smoke        | -0.74 (1.14)                 | .5199      | 0.25 (0.99)                  | .7989      | -1.46 (82.89)             | .986       | 18.25 (25.46)                | .4758      |
| Hypertension | 0.77 (1.35)                  | .5685      | 2.13 (1.14)                  | .0659      | 132.70 (97.13)            | .176       | 42.58 (29.80)                | .1573      |
| AHT          | -2.08 (1.75)                 | .2388      | -0.79 (1.52)                 | .6064      | -189.47 (128.14)          | .1435      | -77.42 (39.38)               | .053       |

252 Notes. SE: standard error. AHT: Anti-hypercholesterolemic therapy. N = 80. Differences are  
 253 considered statistically significant at  $p < .05$ .

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Interestingly, the analysis also pointed to a significant association, found only in women, between hypertension and ceramide (d18:1/16:0) ( $\beta$  (SE) = 4.78 (1.42);  $p$  = .0012) and (d18:1/18:0) ( $\beta$  (SE) = 2.48 (.97);  $p$  = .0126) (Table 2A). Of note, 15 out of 16 women affected by hypertension were in the post-menopausal group. Finally, we found no associations, in either sex, between ceramide levels and any other variable, including smoking, contraceptive use or anti-hypercholesterolemic agents, obesity and diabetes (Table 2A-B).

#### **Plasma ceramide levels are negatively correlated with estradiol in women, but not in men**

Next, we investigated a possible correlation between plasma levels of estradiol, which are known to fall significantly at menopause, and ceramides. Estradiol was measured with a competitive binding immunoassay. As expected, plasma estradiol was higher in pre-menopausal women (<55 years) compared to men of the same age ( $p$  < .001, 2-way ANOVA followed by Bonferroni post-hoc test) (Fig 3B). After menopause, estradiol levels sharply decreased in both sexes (Fig 3B). Fig 4 illustrates the results of Pearson's analyses of ceramide levels in female subjects of all ages. The results show a statistically significant negative correlation between estradiol and ceramide (d18:1/24:1) ( $r$  = -0.294;  $p$  = .007), a non-significant negative trend between estradiol and ceramide (d18:1/24:0) ( $r$  = -0.202;  $p$  = .066) and no correlations between estradiol and other ceramide species.

**Fig 4.** Pearson's correlation analysis between estradiol and levels of various ceramide species in plasma from 84 women aged 20 to 78 years.

(A) Ceramide (d18:1/24:1); (B) Ceramide (d18:1/24:0); (C) Ceramide (d18:1/16:0); (D) Ceramide (d18:1/18:0). Correlation is considered statistically significant at  $p$  < .05.

By contrast, in men, no correlation was observed between estradiol and any ceramide species, including ceramide (d18:1/24:1) ( $r = -0.034$ ;  $p = .763$ ) (Fig 5), which was found to be correlated with aging ( $\beta$  (SE) = 1.86 (.77);  $p = .0179$ ) (Table 2B).

**Fig 5.** Pearson's correlation analysis between estradiol and level of various ceramide species in plasma from 80 men aged 19 to 80 years.

(A) Ceramide (d18:1/24:1); (B) Ceramide (d18:1/24:0); (C) Ceramide (d18:1/16:0); (D) Ceramide (d18:1/18:0). Correlation is considered statistically significant at  $p < .05$ .

### **Estradiol suppresses ceramide accumulation *in vitro***

Sphingolipid-derived mediators regulate steroidogenesis [32], but it is still unknown whether estrogen hormones influence sphingolipid metabolism. To gain insight into the causality of the negative correlation observed between estradiol and ceramide in women, we asked whether the sex hormone might regulate ceramide mobilization (i.e. formation and/or degradation) in human MCF7 breast cancer cells, a cell line that expresses the estrogen receptor  $\alpha$  (ER $\alpha$ ) and  $\beta$  (ER $\beta$ ) [31]. MCF7 cells were treated with estradiol (10 nM) for 24 h and ceramides quantified in lipid extracts by LC-MS/MS. Results indicate that the exposure to estradiol causes a substantial reduction in ceramide (d18:1/16:0) ( $p = .02$ , unpaired Student's  $t$ -test), (d18:1/24:0) ( $p = .0002$ ) and (d18:1/24:1) ( $p = .0006$ ) (Table 3), thereby suggesting that estradiol causes a downward regulation in the mobilization of these ceramides.

**Table 3.** Effects of estradiol on ceramide levels in MCF7 human breast cancer cells. Cells were treated for 24 h with vehicle (0.1% DMSO in phenol-free DMEM) or 17- $\beta$ -estradiol (10 nM) and ceramide levels were measured by LC-MS/MS.

|              | Vehicle           | Estradiol         | <i>p</i> -value |
|--------------|-------------------|-------------------|-----------------|
| Ceramide     | (pmol/mg protein) | (pmol/mg protein) |                 |
| (d18:1/16:0) | 57.4 ± 6.2        | 41.8 ± 0.8        | .02             |
| (d18:1/18:0) | 16.9 ± 1.3        | 13.3 ± 1.6        | .12             |
| (d18:1/24:0) | 209.4 ± 14.1      | 134.6 ± 7.4       | .0002           |
| (d18:1/24:1) | 265.6 ± 13.4      | 184.6 ± 12.6      | .0006           |

## DISCUSSION

In the present study, we investigated the age- and sex-dependent trajectories of plasma ceramides in 80 men and 84 women aged 19-80 years. The subjects were not affected by any major illness or medical condition known to be linked to alterations in circulating ceramide levels. The results show that, in women, plasma levels of two ceramide species – (d18:1/24:0) and (d18:1/24:1) – increased with age, and that this change cannot be ascribed to confounding factors such as obesity, diabetes, hypertension, tobacco smoking or ongoing anti-cholesterol and contraceptive therapy. In men, the analysis revealed a statistically detectable association between age and ceramide (d18:1/24:1). Further analyses identified a significant negative correlation between circulating levels of estradiol and ceramide (d18:1/24:1) in women of all ages, but not in men. Finally, *in vitro* experiments in a human cell line expressing estrogen ER- $\alpha$  and ER- $\beta$  receptors showed that treatment with exogenous estradiol produced a significant decrease in ceramide accumulation. These findings suggest that aging is accompanied, in humans, by an increase in the plasma concentrations of two ceramide species, (d18:1/24:0) and (d18:1/24:1), which are also known to be elevated in age-dependent pathologies, such as atherosclerosis and cardiovascular disease [33,34]. The results also point to the intriguing possibility that estradiol might control circulating ceramide levels in a sexually dimorphic manner.

Previous studies have reported sex-dependent differences in plasma ceramides, but with somewhat contrasting results. In a small investigation of blood serum samples from 10 Caucasian volunteers (5 males aged 27-33 years and 5 females aged 26-33 years), ceramide (42:1) was found to be higher in women compared to men [16]. Another study, performed on a much larger cohort of young Mexican Americans (1,076 individuals, 39.1% males, median age 35.7 years) [17], uncovered an association between plasma ceramides and sex after adjusting for age and body mass index: ceramide levels were lower in women than in men. These disparities were mostly driven by long-chain ceramide species, such as (d18:1/22:0), (d18:1/24:0) and (d18:1/24:1). Finally, in a multiethnic population sample of 366

325 women and 626 men aged over 55 years, enrolled in the Baltimore Longitudinal Study of Aging,  
 326 plasma ceramide concentrations were found to be higher in women compared to men [18].  
 327 These studies did not focus on age as a variable. By contrast, in the present work we set out to address  
 328 the impact of aging on ceramide levels and excluded subjects with disease conditions that had been  
 329 previously shown to affect ceramides, such as diabetes [35], cancer [36], renal disease [37],  
 330 cardiovascular disease [38], and obesity [39]. We did not exclude subjects with hypertension, however,  
 331 because the association of this disease state with altered ceramides remains to be fully established  
 332 [11,18]. In our sample, we were able to confirm the presence of age- and sex-dependent differences in  
 333 the plasma concentrations of certain ceramide species, but not others. A multivariable analysis showed  
 334 that, in women, aging is accompanied by increased levels of ceramide (d18:1/24:0) and (d18:1/24:1). In  
 335 men, the analysis also unmasked a statistically detectable association between age and ceramide  
 336 (d18:1/24:1). These age-dependent changes could not be ascribed to obesity, diabetes, tobacco cigarette  
 337 smoking and use of anti-hypercholesterolemic or contraceptive agents. Interestingly, secondary data  
 338 analyses found that ceramide levels were significantly lower in female than male subjects aged 20-54, a  
 339 difference that disappeared after menopause. In their 2015 study, Mielke and collaborators did not  
 340 specifically include menopause as a variable but suggested that menopause and estradiol may influence  
 341 ceramide levels [18]. Two sets of results presented here support this prediction. First, we showed that,  
 342 in women of all ages, plasma estradiol was negatively correlated with ceramide (d18:1/24:1) and  
 343 displayed a trend toward correlation with ceramide (d18:1/24:0). No such relationship was detectable in  
 344 men. Second, we found that incubation with exogenous estradiol (10 nM) lowered the levels of various  
 345 ceramides, including ceramide (d18:1/24:0) and (d18:1/24:1), in human estrogen-sensitive MCF7 cells.  
 346 The findings outlined above suggest that age-dependent changes in estradiol may affect ceramide  
 347 metabolism differentially in men and women. The correlation between estradiol and ceramide raises the  
 348 possibility that changes in the availability of certain ceramide species [e.g. ceramide (d18:1/24:1)]

might be implicated in the reported cardioprotective, antihypertensive and neuroprotective effects of estradiol [40-42]. In this context, it is important to point out that increased ceramide levels have been consistently linked to heightened risk of myocardial infarction and stroke [43]. Moreover, recent evidence indicates that alterations of ceramide levels may mirror, indirectly, ongoing neurodegenerative processes and might be used as a biomarker for Alzheimer's disease development and progression [44]. Interestingly, the close association between alterations in ceramide levels and the likelihood of developing cognitive impairment and Alzheimer-related pathology may be particularly strong in women, thereby indicating that sex-related mechanisms might participate in shaping the Alzheimer phenotype. Of note, elderly women show changes in plasma ceramide levels at the onset of their memory impairment [15]. In the past few years, the neurobiology of memory disorders has received increasing attention [45]. Memory deficits are often reported by women in temporal proximity of menopause [46], and recent findings indicate distinct changes in memory processing that appear to be linked more to the premenopausal status than to chronological aging: in peri-menopausal women, the onset and progression of cognitive decline are often associated with a menopause-related decrease in estradiol levels [47]. Thus, the interplay between estradiol and ceramides, described here, may potentially be of broad significance for a variety of age-dependent disorders, including cardiovascular disease and cognitive impairment or any intersection of the two conditions [48].

The present study has several limitations. First, even though we excluded persons affected by obesity and diabetes, we did not collect information on metabolic factors that could potentially impact ceramide mobilization – such as adiposity, physical activity and levels of cholesterol and glucose in blood [18,49-51]. Second, our study was focused on a specific subset of ceramides that we had previously found to be altered in the hippocampus of aged male and female mice [19]. This group of ceramides has been proposed as potential biomarker for cardiovascular risk [52], but is still only a small fraction of the vast number of ceramides produced by the body. Third, we measured total

circulating levels of ceramides and did not attempt to separate ceramide pools bound to specific plasma lipoproteins [53]. Because the distribution of ceramides among lipoproteins may change with obesity and diabetes [50], it is possible that aging might exert a similar effect.

Our findings also raise a number of relevant questions, which should be addressed in future work. First, even though the results suggest that estradiol modulates ceramide mobilization in women, the precise mechanism and functional significance of this effect remain to be determined. One possibility is that activation of estrogen receptors results in the down-regulation of *de novo* ceramide biosynthesis, for example by suppressing the expression of key enzymes such as serine-palmitoyltransferase and ceramide synthase [54]. Alternatively, estradiol might stimulate the expression of ceramide-hydrolyzing enzymes such as acid or neutral ceramidase. Probing the link between estradiol and ceramides will require additional experimentation, which may include measuring circulating ceramide levels in women throughout the menstrual cycle or assessing the impact of endogenous and exogenous estradiol on sphingolipid metabolism in female animals. At the functional level, studies are needed to correlate ceramide and estrogen levels to a broad panel of biomarkers (e.g., lipoprotein profile, C-reactive protein) and clinical outcome measures (e.g., future adverse cardiovascular events and cognitive impairment) [52]. Without such information, the clinical significance of our findings remains speculative. Second, the lack of correlation between estradiol and ceramide (d18:1/18:0) and lack of association with age implies that this ceramide species, though elevated after menopause, may be subjected to a different regulation than ceramide (d18:1/24:1), whose levels are correlated with estradiol and are statistically associated with age. Third, we observed a substantial age-dependent decrease in plasma dihydroceramide (d18:0/24:0) in men aged 54-80 years. The significance of this finding is presently unclear, but warrants further attention. Fourth, we unexpectedly uncovered an association between hypertension in post-menopausal women and elevated levels of ceramide (d18:1/16:0) and (d18:1/18:0). While this result is consistent with previous reports [11], caution is

397 warranted until studies with a larger cohort of pre- and post-menopausal women are performed. Finally,  
 398 as mentioned above, the relation between ceramide levels and premorbid changes in cardiovascular,  
 399 metabolic or cognitive function was not investigated in our study and deserves further investigations.  
 400 Despite these unanswered questions, our results reveal the existence of a link between age, estradiol,  
 401 and ceramides, which might contribute to age-dependent pathologies in post-menopausal women.  
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