Demographic and clinical variables affecting mid- to late-life trajectories of plasma ceramide and dihydroceramide species

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Introduction
Ceramides, the central molecular species of the sphingolipid pathway, function both as structural lipids and as second messengers for intra- and intercellular signaling affecting cellular growth, proliferation, differentiation, senescence, and apoptosis. At low levels, ceramides are important for injury-induced cytokine production and activating protein phosphatases and kinases, enzymes involved in stress signaling cascades (Hannun, 1996). However, at high levels, ceramides inhibit cell division and induce cellular dysfunction and apoptosis. Thus, the homeostasis of ceramide metabolism may be critical in regulating lifespan and age-associated diseases (Cutler & Mattson, 2001; Rao et al., 2007). Indeed, it has been increasingly recognized, from the cellular to human level, that perturbations in ceramide metabolism are associated with longevity (Yu et al., 2012; Gonzalez-Covarrubias et al., 2013; Cutler et al., 2014; Huang et al., 2014) and the development and progression of many age-related diseases including cancer (Alberg et al., 2013), atherosclerosis (Ichi et al., 2006), insulin resistance and diabetes (Holland et al., 2007; Holland & Summers, 2008; Boon et al., 2013), Alzheimer’s disease (Mielke et al., 2012), and Parkinson’s disease (Mielke et al., 2013).

Notably, each sphingolipid species has multiple chain lengths that are regulated by specific biochemical pathways and contribute to distinct cell functions (Hannun & Obeid, 2011). However, the majority of this research has been conducted at the cellular level. With improved understanding of ceramide chain lengths at the cellular level, and of their role in disease mechanisms, there is a need to quantitatively and translate this knowledge to clinical and population studies. Yet, compared to other lipids (e.g., cholesterol, triglycerides), there are little data on ceramides at the population level and the intra- and interindividual changes in levels of specific carbon chain lengths by age, sex, race, and disease status. Previous, carefully validated, studies have utilized small sample sizes of 10–15 individuals aged 20–40 years (Hammad et al., 2010; Bui et al., 2012) or have used pooled samples (Quehenberger et al., 2010). A recent population-based study performed plasma lipid profiling in over 1000 individuals, median age of 36, enrolled in the San Antonio Family Heart Study (Weir et al., 2013). This study showed important cross-sectional associations between ceramides and dihydroceramides (DHCer) and psychological and demographic factors. However, to further understand the relationship between individual ceramide species and disease risk, it is important to understand intra- individual changes with age and onset of disease in people over 50 years.

In this study, we quantified, by age and sex, the individual species of plasma ceramides and DHCer in 992 individuals, aged 55 and older, enrolled in the Baltimore Longitudinal Study of Aging (BLSA). Individuals were followed, with serial measures, up to six visits (mean of 4.1 visits, range 2–6) and 38 years, allowing the assessment of both interindividual variation and intra-individual changes over time in circulating ceramide concentrations. In addition, we assessed factors associated with variation and changes in these levels including demographics, diseases, medications, lifestyle factors, and other blood lipids.

Summary
It has been increasingly recognized at the basic science level that perturbations in ceramide metabolism are associated with the development and progression of many age-related diseases. However, the translation of this work to the clinic has lagged behind. Understanding the factors longitudinally associated with plasma ceramides and dihydroceramides (DHCer) at the population level and how these lipid levels change with age, and by sex, is important for the clinical development of future therapeutics and biomarkers focused on ceramide metabolism. We, therefore, examined factors cross-sectionally and longitudinally associated with plasma concentrations of ceramides and DHCer among Baltimore Longitudinal Study of Aging participants (n = 992; 3960 total samples), aged 55 years and older, with plasma at a mean of 4.1 visits (range 2–6). Quantitative analyses were performed on a high-performance liquid chromatography-coupled electrospray ionization tandem mass spectrometer. Linear mixed models were used to assess the relationships between plasma ceramide and DHCer species and demographics, diseases, medications, and lifestyle factors. Women had higher plasma concentrations of most ceramide and DHCer species and showed steeper trajectories of age-related increases compared to men. Ceramides and DHCer were more associated with waist-hip ratio than body mass index. Plasma cholesterol and triglycerides, prediabetes, and diabetes were associated with ceramides and DHCer, but the relationship showed specificity to the acyl chain length and saturation. These results demonstrate the importance of examining the individual species of ceramides and DHCer, and of establishing whether intra-individual age- and sex-specific changes occur in synchrony to disease onset and progression.

Key words: aging; ceramide; dihydroceramide; human; longitudinal; sex differences.
Plasma ceramide and dihydroceramide concentrations by age and sex at the first blood draw

Table 2 provides the cross-sectional raw means and SD of the individual ceramide and DHCer species at the first blood draw grouped in 10-year intervals and by sex. The cross-sectional relationships between the plasma concentrations, by age and sex, differed by ceramide class and species. For example, mean concentrations of ceramide C16:0 were lower in women than in men at ages 55–64, but higher in women at ages 65–74 years. In contrast, concentrations of ceramide C24:0 were cross-sectionally lower in women than in men over the entire age span tested (55–94). Dihydroceramides are precursors to ceramide, although they also appear to have independent biological functions. Two DHCer species were readily detectable in plasma (C20:0 and C24:0) and cross-sectionally increased with age in men and women.

Longitudinal intra-individual stability

The mean (SD) number of serial plasma samples available for each subject with measured ceramides and metabolites was 4.1 (0.7). We included 3960 total samples from the 992 participants that were collected over an average of 14.3 (6.7) years, with a range of 2.0–38.9 years. Storage time was not associated with ceramide or DHCer concentrations, suggesting that these classes of lipids were stable in long-term −80 °C storage. The intraclass correlation (ICC) of the various ceramide species, a measure of how well different molecular classes track over time, ranged from 0.14 to 0.22 (all P < 0.0001). The ICC of DHCer was similar and ranged from 0.14 to 0.20 (all P < 0.0001).

Variables cross-sectionally and longitudinally associated with ceramide and dihydroceramide concentrations

We next identified factors associated cross-sectionally and longitudinally, in a time-dependent manner, with each of the ceramide and DHCer species using linear mixed models. We identified the most parsimonious models and distinguished clinical and demographic variables most strongly associated with each ceramide class and individual ceramide species. Table 3 and Fig. 1 provide the model and model-fitted predicted values for the ceramides. Table 4 and Fig. 2 provide the model and model-fitted predicted values for the DHCer.

Associations with demographic variables

Examining the within-individual changes using linear mixed models and adjusting for covariates, all ceramide and DHCer species significantly increased with age in both men and women, but women showed a steeper trajectory of increase. The single exception was ceramide C26:0, which decreased in women and increased in men with age (Tables 3 and 4, predicted values are shown in Figs 1 and 2). Compared to Caucasians, African Americans exhibited lower plasma concentrations of most ceramide and DHCer species at the first blood draw, and also had less age-related increases after controlling for potentially confounding variables. More years of education were associated with greater age-related increases in ceramides C18:0 and C24:1 and DHCer C20:0.

Associations of anthropometric measures and diabetes

Both WHR and body mass index (BMI) were associated with higher concentrations of ceramides and DHCer at the first blood draw, but the relationship between baseline WHR and plasma ceramide and DHCer...
Table 2  Plasma ceramides and dihydroceramides by age and sex at the first blood draw

| Plasma lipids (ng mL⁻¹) | N | Mean (SD) | Range | N | Mean (SD) | Range | N | Mean (SD) | Range |
|-------------------------|---|-----------|-------|---|-----------|-------|---|-----------|-------|
| Ceramides               |   |           |       |   |           |       |   |           |       |
| C16:0                   |   |           |       |   |           |       |   |           |       |
| Women                   | 205 | 97.7 (60.6)c | 15.6–339 | 104 | 113.7 (65.8)a | 15–302 | 43 | 111.0 (70.4) | 25.9–348 |
| Men                     | 415 | 122.1 (64.8) | 10.8–344 | 118 | 109.9 (57.4) | 27.7–326 | 67 | 120.8 (64.8) | 30.9–326 |
| C18:0                   |   |           |       |   |           |       |   |           |       |
| Women                   | 211 | 74.9 (52.8)a | 12.9–307 | 105 | 80.3 (45.6) | 18.3–282 | 46 | 92.8 (54.4)a | 16.9–243 |
| Men                     | 419 | 83.1 (46.7) | 1.1–308 | 124 | 90.8 (57.9) | 13.3–302 | 69 | 86.6 (48.8) | 18.9–235 |
| C20:0                   |   |           |       |   |           |       |   |           |       |
| Women                   | 212 | 170.8 (95.1) | 21.1–579 | 105 | 177.1 (95.5)b | 33.5–507 | 48 | 212.7 (110.7)c | 53.9–606 |
| Men                     | 426 | 180.1 (90.5) | 34.1–625 | 127 | 196.0 (96) | 25.8–533 | 69 | 198.7 (104.7) | 34.7–562 |
| C22:0                   |   |           |       |   |           |       |   |           |       |
| Women                   | 210 | 1898.3 (929) | 176–5660 | 104 | 1903.1 (763.3)b | 587–3840 | 46 | 2114.6 (850.4)c | 854–4300 |
| Men                     | 426 | 1968.0 (852.5) | 274–5400 | 126 | 1931.8 (731.4) | 444–3790 | 69 | 2130.6 (993.2) | 698–5140 |
| C24:0                   |   |           |       |   |           |       |   |           |       |
| Women                   | 209 | 7537.0 (3735.3) | 1330–23300 | 104 | 7588.6 (3780.8)b | 1810–22300 | 47 | 8386.8 (3924.1)c | 2740–23200 |
| Men                     | 426 | 8155.8 (4025.8) | 1120–23700 | 126 | 8034.8 (3365.9) | 2220–18400 | 69 | 8707.0 (4468.7) | 2450–22000 |
| C26:0                   |   |           |       |   |           |       |   |           |       |
| Women                   | 207 | 119.0 (72.7)b | 19.0–376.0 | 104 | 122.4 (82.6)c | 23.3–389.0 | 46 | 137.1 (83.7) | 26.1–339.0 |
| Men                     | 421 | 116.5 (72.4) | 13.9–395.0 | 126 | 116.7 (67.4) | 21.5–347.0 | 65 | 128.8 (81.8) | 22.4–393.0 |
| C22:1                   |   |           |       |   |           |       |   |           |       |
| Women                   | 208 | 23.5 (15.4)c | 2.8–78.8 | 105 | 24.9 (15.7)c | 1.2–80.4 | 47 | 27.9 (15.2)c | 8.1–81.5 |
| Men                     | 422 | 21.3 (15.6) | 0.9–79.3 | 105 | 20.2 (13.1) | 1.7–70.0 | 68 | 24.3 (16.3) | 2.1–70.5 |
| C24:1                   |   |           |       |   |           |       |   |           |       |
| Women                   | 210 | 307.2 (237.4)c | 8.0–1610.0 | 105 | 362.8 (266.4)c | 13.0–1220.0 | 48 | 394.0 (268.4)c | 25.1–1090.0 |
| Men                     | 427 | 295.5 (247.2) | 2.8–1530.0 | 126 | 332.2 (288.3) | 8.5–1330.0 | 69 | 363.3 (278.6) | 12.1–1620.0 |
| DihydroCeramides        |   |           |       |   |           |       |   |           |       |
| C20:0                   |   |           |       |   |           |       |   |           |       |
| Women                   | 211 | 3.2 (1.8) | 0.2–11.2 | 104 | 3.2 (1.8)a | 0.4–8.6 | 47 | 3.8 (1.8)c | 0.9–9.3 |
| Men                     | 422 | 3.4 (1.7) | 0.1–11.0 | 126 | 3.7 (1.8) | 0.5–8.7 | 69 | 3.8 (1.9) | 0.4–11.0 |
| C24:0                   |   |           |       |   |           |       |   |           |       |
| Women                   | 211 | 29.2 (22.6) | 1.5–117.0 | 104 | 32.5 (22.9)c | 2.1–87.7 | 48 | 30.9 (20.6)c | 1–82.3 |
| Men                     | 427 | 28.7 (22.6) | 1.4–125.0 | 127 | 32.7 (22.7) | 0.8–105.0 | 69 | 29.5 (22.9) | 0.9–117.0 |

_Tests used to compare cross-sectional sex differences within each age group. a, P < 0.05; b, P < 0.01; c, P < 0.001._

concentrations was stronger. A higher WHR was also associated with a steeper trajectory of age-related increases in these ceramide species. As WHR is correlated with type II diabetes, we next determined the association of plasma ceramides and DHCer with diabetes. Prediabetes was cross-sectionally associated with higher ceramide C18:0 (b = 5.29, P < 0.01) and diabetes with higher C22:1 (b = 2.28, P < 0.05), and this association was maintained longitudinally (i.e., no interaction with time). There was an interaction between diabetes and ceramide C24:0 such that with increasing age, diabetes was associated with lower ceramide concentrations (b = −51.44, P < 0.05). There were no cross-sectional or longitudinal associations between DHCer and prediabetes or diabetes.

**Associations with blood cholesterol, triglycerides, statins, and APOE E4 genotype**

Higher plasma concentrations of cholesterol were cross-sectionally associated with higher concentrations of all ceramide and DHCer species (P < 0.001). However, higher cholesterol was only associated with a steeper age-related increase in ceramide C24:0 (b = 0.34, P < 0.05). Similarly, higher plasma concentrations of triglycerides were cross-sectionally associated with most ceramide species, with the exceptions of the very long chain ceramides C26:0 and C24:1 and DHCer C24:0. Higher triglycerides were associated with steeper age-related increases in ceramide C18:0, but not other chain lengths. Unexpectedly, we found that statin use was associated with higher ceramide C18:0, C20:0, and C24:1 and DHCer C20:0 levels at baseline, but the proportion of individuals taking statins at baseline was < 6%. There were no associations for any ceramides or DHCer with APOE E4 genotype.

**Associations with other age-related diseases**

There were essentially no associations between circulating plasma ceramides or DHCer and hypertension, myocardial infarction, or smoking status. Individuals with chronic kidney disease did have higher concentrations of all ceramides, with the exception of C26:0, and DHCer at the first study visit, and these elevated levels were maintained longitudinally (i.e., no interaction with time).

**Discussion**

Over the past two decades, the importance of ceramides and ceramide metabolites as mediators, and biomarkers, of complex disease processes has emerged. Indeed, it is increasingly recognized at the cellular level that the homeostasis of ceramide metabolism is critical in regulating...
Table 3 Variables cross-sectionally and longitudinally associated with specific carbon chain lengths of plasma ceramides in the Baltimore Longitudinal Study of Aging

| Covariates            | C16:0 Baseline | C16:0 Time-dependent | C18:0 Baseline | C18:0 Time-dependent | C20:0 Baseline | C20:0 Time-dependent | C22:0 Baseline | C22:0 Time-dependent | C24:0 Baseline | C24:0 Time-dependent | C26:0 Baseline | C26:0 Time-dependent | C22:1 Baseline | C22:1 Time-dependent | C24:1 Baseline | C24:1 Time-dependent |
|-----------------------|----------------|----------------------|----------------|----------------------|----------------|----------------------|----------------|----------------------|----------------|----------------------|----------------|----------------------|----------------|----------------------|----------------|----------------------|
| Age                   | 1.69 (0.41)    | 1.67 (0.37)          | 4.24 (0.66)    | 30.83 (6.56)         | 81.97 (28.12)  | 0.49 (0.20)          | 0.11 (0.05)    | 16.02 (1.99)         |
| Age²                  | −0.01 (0.02)   | 0.002 (0.01)         | −0.001 (0.02)  | −0.04 (0.15)         | 0.21 (0.67)    | 0.004 (0.01)         | −0.04 (0.02)   | −109.83 (15.48)      |
| Men                   | 8.97 (3.39)    | −1.41 (0.32)         | −4.85 (2.82)   | −1.30 (5.32)         | −27.78 (5.32)  | −1.76 (0.38)         | −164.55 (47.85) | −23.54 (4.78)        |
| Men                   | 3.99 (0.32)    | −1.09 (0.27)         | −15.29 (2.82)  | −0.91 (5.32)         | −32.16 (0.33)  | −1.96 (0.38)         | −311.66 (47.85) | −11.39 (2.82)        |
| Education             | 0.31 (0.40)    | 0.07 (0.04)          | 0.45 (0.36)    | 0.87 (0.71)          | 1.21 (0.71)    | 0.12 (0.07)          | 9.99 (2.87)    | 6.15 (2.87)          |
| APOE E4               | −0.20 (0.40)   | −0.50 (0.04)         | −0.41 (0.36)   | −0.77 (0.71)         | −4.19 (0.71)   | −0.31 (0.07)         | −3.77 (0.28)   | 0.39 (0.07)          |
| BMI                   | −0.94 (0.27)   | 0.95 (0.29)          | 1.50 (0.29)    | 10.55 (4.26)         | 3.77 (0.28)    | 0.31 (0.07)          | 0.03 (0.07)    | 2.64 (1.34)          |
| WHR                   | 7.65 (15.24)   | 4.45 (1.49)          | 52.70 (1.36)   | 4.47 (26.63)         | 119.96 (26.63)| 7.12 (23.83)         | 24.0 (1047.1)  | 181.70 (75.95)       |
| CKD                   | 18.58 (3.36)   | 22.45 (3.36)         | 35.84 (3.13)   | 1.14 (6.99)          | 296.15 (6.99)  | 1.06 (56.51)         | 796.78 (241.7) | 7.59 (9.11)          |
| Statin use            | 14.29 (4.28)   | 14.29 (4.28)         | 21.74 (4.28)   | 21.74 (4.28)         | 21.74 (4.28)   | 21.74 (4.28)         | 21.74 (4.28)   | 21.74 (4.28)         |
| Pre-diabetes          | 4.09 (2.08)    | 5.29 (2.28)          | −216.21 (23.23)| −18.56 (23.23)       | −0.28 (0.09)   | −0.05 (0.06)         | −0.28 (0.05)   | 0.05 (0.06)          |
| Diabetes              | −1.52 (2.24)   | 3.49 (3.01)          | −11.11 (5.01)  | 3.49 (15.11)         | −5.44 (15.11)  | −2.28 (0.09)         | −2.28 (0.09)   | −0.16 (0.09)         |
| Hypertension          | −3.81 (2.08)   | −5.68 (3.26)         | −55.68 (33.26) | −55.68 (33.26)       | −55.68 (33.26) | −55.68 (33.26)       | −55.68 (33.26) | −55.68 (33.26)       |
| Smoker                | 0.27 (0.02)    | 0.53 (0.02)          | 2.04 (0.02)    | 2.04 (0.02)          | 3.09 (0.02)    | 0.01 (0.004)         | 0.01 (0.004)   | 0.01 (0.004)         |
| Triglycerides         | −0.02 (0.01)   | 0.004 (0.01)         | 0.14 (0.01)    | 0.14 (0.01)          | 3.09 (0.01)    | 0.01 (0.004)         | 0.01 (0.004)   | 0.01 (0.004)         |
| Cholesterol           | 0.20 (0.03)    | 0.11 (0.03)          | 4.18 (0.41)    | 4.18 (0.41)          | 23.25 (1.74)   | 0.39 (1.71)          | 0.39 (1.71)    | 0.39 (1.71)          |

APOE E4, presence of at least one APOE E4 allele; BMI, body mass index; WHR, waist–hip ratio; CKD, chronic kidney disease. We forced age, age², sex, and BMI into all models then tested backward selection with P < 0.10 to determine which variables to include in the final model of each ceramide species and carbon chain length. a, P < 0.05; b, P < 0.01; c, P < 0.001.
lifespan and age-associated diseases (Cutler & Mattson, 2001; Rao et al., 2007; Huang et al., 2014). However, given the longer lifespan and greater complexity of sphingolipid metabolism in mammals, fewer studies have translated this cellular work to humans and large-scale clinical and epidemiological studies of these lipids are lacking. As the biological pathways and consequences of ceramide metabolism continue to be elucidated for specific age-associated diseases and treatments are developed, a more thorough understanding of the factors that affect ceramides in humans and how they change with age is needed. This information could be useful for identifying biomarkers of disease risk.

Table 4 Variables cross-sectionally and longitudinally associated with plasma dihydroceramides C20:0 and C24:0 in the Baltimore Longitudinal Study of Aging

| Covariates          | C20:0 Baseline | Time-dependent | C24:0 Baseline | Time-dependent |
|---------------------|----------------|----------------|----------------|----------------|
| Age                 | 0.08 (0.01)c   | 0.77 (0.16)c   | 0.77 (0.16)c   | 0.77 (0.16)c   |
| Age²                | 0.0001 (0.0003)| -0.01 (0.004)b | -0.01 (0.004)b | -0.01 (0.004)b |
| Men                 | -0.49 (0.10)c  | -0.04 (0.007)c | -0.75 (1.23)c  | -0.51 (0.11)c  |
| African American    | -0.56 (0.11)c  | -0.03 (0.01)b  | -6.61 (1.30)c  | -3.50 (0.15)a  |
| Education           | 0.02 (0.01)    | 0.003 (0.001)a | 0.50 (0.16)b   | 0.50 (0.16)b   |
| BMI                 | 0.02 (0.01)a   | 0.11 (0.11)    | 14.67 (6.25)a  | 14.67 (6.25)a  |
| WHR                 | 2.26 (0.51)c   | 0.74 (1.47)c   | 9.97 (1.47)c   | 9.97 (1.47)c   |
| Statin use          | 0.35 (0.08)c   | 2.07 (1.06)    | 2.07 (1.06)    | 2.07 (1.06)    |
| Triglycerides       | 0.002 (0.001)c | 0.09 (0.01)c   | 0.09 (0.01)c   | 0.09 (0.01)c   |
| Cholesterol         | 0.01 (0.001)c  | 0.09 (0.01)c   | 0.09 (0.01)c   | 0.09 (0.01)c   |

BMI, body mass index; WHR, waist-hip ratio; CKD, chronic kidney disease. We forced age, age², sex, and BMI into all models then used backward selection with P < 0.10 to determine which variables to include in the final model of each ceramide species and carbon chain length. a, P < 0.05; b, P < 0.01; c, P < 0.001.

Fig. 1 Plasma ceramides by age and sex. Concentrations are based on predicted values obtained in linear mixed models and controlling for additional factors specific to each model (see Table 3).
Plasma ceramides by age and sex, M. M. Mielke et al.

Fig. 2  Plasma dihydroceramides by age and sex. Concentrations are based on predicted values obtained in linear mixed models and controlling for additional factors specific to each model (see Table 4).

Ceramide metabolism has repeatedly been found, using both in vitro and in vivo models, to induce insulin resistance via the phosphatidylinositol-3-kinase-Akt pathway (Holland & Summers, 2008). Ceramide blocks the translocation of Akt/protein kinase B (PKB) to the plasma membrane, thus inhibiting insulin signaling (Stratford et al., 2001), and promotes the dephosphorylation of Akt/PKB by protein phosphatase 2A (Chavez et al., 2003). In rodent models of type 2 diabetes, increases in islet ceramides precede beta-cell dysfunction (Lee et al., 1994). Translating this work to humans, plasma ceramides have been reported to be higher in both patients with prediabetes and patients with diabetes. Elevated levels of ceramides C18:0, C20:0, and C22:0 were reported in adolescents and young adults with type 2 diabetes (Lopez et al., 2013). In the present study, individuals with prediabetes had higher levels of ceramide C18:0 and those with diabetes had higher C22:1. Notably, ceramide synthase 1 has specificity for C18 chain lengths and is primarily expressed in skeletal muscles. Ceramide muscle content has been associated with insulin resistance in both normal individuals and athletes, suggesting that elevated ceramides may be a mechanism by which insulin resistance is also associated with accelerated decline in muscle mass and strength (Amati et al., 2011; Dube et al., 2011; Kalyani et al., 2012).

The majority of studies examining sphingolipids and cardiovascular diseases have focused on sphingomyelins or the cardioprotective effects of sphingosine-1-phosphate. Plasma ceramides C16:0, C22:0, C24:0, and C24:1 were elevated in both spontaneously hypertensive rats and 19 treatment naïve patients with stage 1–3 hypertension compared to controls (Spijkers et al., 2011). In the present study, we did not find an association between any of the ceramides or DHCer and hypertension after adjusting for age, sex, BMI, diabetes, and other covariates. However, virtually all BLSA participants with high blood pressure were treated with antihypertensive medications, and the effect of the different classes of antihypertensives on plasma ceramide levels is not known.

Previous cellular studies have determined that high ceramide levels cause renal damage, for example (Itoh et al., 2006), but few have examined specific carbon chain lengths. One study reported higher absolute values of ceramides C16:0, C22:0, and C24:0 in the kidney extracted from CD-1 mice 2 and 18 h after ischemia–reperfusion injury (Kalhorn & Zager, 1999). Translating to humans, a recent study found higher levels of all serum ceramides, except C18:1, in children with chronic kidney disease (CKD; Mitsnepes et al., 2014). Consistently, we also found that individuals with CKD had high levels of several ceramides and DHCer, even after adjusting for diabetes. However, longitudinal research is needed to determine whether blood ceramides are risk factors for CKD or are markers of disease progression and severity.

The levels of plasma ceramides and DHCer did not vary by APOE E4 genotype in this study. Our findings are consistent with a genomewide association study of circulating sphingolipids (Demirkan et al., 2012), and with clinical (Han et al., 2011) and animal (Sharman et al., 2010) studies that have also not found blood ceramide levels to vary by APOE genotype. However, we previously found that levels of C5F very long chain ceramides with chain lengths of C20–C26 were higher in APOE E4 carriers compared with noncarriers (Mielke et al., 2014). Further, studies of Alzheimer (Bandaru et al., 2009) and HIV dementia brains (Cutler et al., 2004) and aortic tissue levels of APOE knockout mice (Kobayashi et al., 2013) have found APOE genotype alters ceramide levels in these compartments. Thus, the relationship between ceramides and APOE genotype likely varies depending on the compartment and disease state.

Limitations of the study warrant consideration. First, the BLSA is a community-dwelling volunteer cohort that is predominantly white, of...
upper-middle socioeconomic status, and with an above average educational level. While this may hinder generalizability, the relative homogeneity of the sample may be seen as an asset because the majority of individuals have good access to medical care and have remained relatively healthy over the follow-up interval. Second, there are multiple ways ceramide can be synthesized and metabolized and these functions are highly compartmentalized. Therefore, for some diseases, tissue-specific lipid measures (e.g., skeletal muscle for diabetes) may be a better biomarker. Because the collection of blood is noninvasive and more acceptable and feasible for serial measures, we initially focused on the characterization of ceramides and metabolites in this medium. Lastly, ceramides are hydrophobic, so they are carried on lipoproteins in the blood, with the greatest concentrations in VLDL and LDL (Hammad et al., 2010) and the ceramide transporter (CERT) (Mencarelli et al., 2010). The composition and quantification of the specific acyl chain lengths of ceramides and DHCer on lipoproteins or CERT may differ by age and with disease onset. However, to quantitate all of the lipids by specific lipoproteins and CERT would require many more runs and would take a significantly greater amount of time and effort. Thus, the present work is the first step in beginning to understand the relationship between plasma ceramides and DHCer with age and disease processes.

Experimental procedures

Participants

Initiated in 1958, the BLSA is a longitudinal cohort study of community-dwelling individuals aimed at examining the physiological and psychological aspects of aging (Shock et al., 1984). At each study visit, participants underwent an extensive medical examination, neuropsychological battery, blood draw, medical history, and medication review. Historically, BLSA visits occurred every 2 years. In 2003, the sampling times were modified because historical data indicated nonlinear changes at the oldest ages. To improve sampling of the epoch with accelerated physical and cognitive change, individuals aged 80 and older have been evaluated annually since 2003. The protocol was approved by the local Institutional Review Board, and written informed consent was obtained. Since its inception in 1958, over 3100 BLSA participants have contributed data on the aging process. Due to the cost and time constraints of measuring the plasma ceramides, the present analyses included 992 individuals. These individuals were randomly selected from the BLSA participants and had at least two blood draws (mean of 4.1, range 2–6) after the age of 55 years to allow for measurement of within-individual changes in the ceramide levels starting at mid-life. The individuals included in the current analyses are representative of the larger BLSA cohort with regard to demographics and health characteristics.

Blood samples were drawn at all visits from the antecubital vein between 7 and 8 AM after an overnight fast (Shock et al., 1984). Participants were not allowed to smoke, engage in physical activity, or take medications before the sample was collected. Plasma samples were immediately processed, catalogued, and stored at −80 °C.

Description of variables examined in relation to lipid levels

All variables were assessed at each visit using the same methods. Demographic variables included age, sex, race, and years of education. Height (in meters) and weight (in kilograms) were measured to calculate BMI. Waist–hip ratio was measured in the standing position using a flexible metal tape. Smoking status was obtained by a questionnaire, and individuals were classified as ever or never smokers. Medical history information included hypertension, myocardial infarction, atrial fibrillation, angina, chronic heart failure, and CKD. The diagnoses of diabetes and prediabetes at each visit were established by combining information on medications, fasting glucose, and glucose levels at 2 h of a standard glucose tolerance test. In particular, participants who were taking antidiabetes medication or had a fasting glucose > 126 mg dL$^{-1}$ and/or

\begin{table}[h]
\centering
\begin{tabular}{lcc}
\hline
\textbf{Ceramides} & \textbf{Molecular/fragment ion m/z} \\
\hline
d18:1/12:0 & 482.9/264.4\footnote{Internal standard.} \\
d18:1/16:0 & 538.9/264.4 \\
d18:1/18:0 & 566.3/264.4 \\
d18:1/20:0 & 594.8/264.4 \\
d18:1/22:0 & 622.5/264.4 \\
d18:1/24:0 & 650.9/264.4 \\
d18:1/26:0 & 678.9/264.4 \\
d18:1/16:1 & 536.9/264.4 \\
d18:1/18:1 & 564.3/264.4 \\
d18:1/20:1 & 592.8/264.4 \\
d18:1/22:1 & 620.5/264.4 \\
d18:1/24:1 & 648.9/264.4 \\
d18:1/26:1 & 676.9/264.4 \\
\hline
\textbf{Dihydroceramides} & \\
d18:0/16:0 & 540.9/266.4 \\
d18:0/18:0 & 568.3/266.4 \\
d18:0/20:0 & 596.8/266.4 \\
d18:0/22:0 & 624.5/266.4 \\
d18:0/24:0 & 652.9/266.4 \\
d18:0/26:0 & 680.9/266.4 \\
\hline
\end{tabular}
\caption{Multiple reaction monitoring transitions for molecular species of ceramide molecular and fragment ions}
\end{table}
a 2-h glucose > 200 mg dL⁻¹ were defined as diabetics. Among those who had no diabetes, participants with fasting glucose > 100 mg dL⁻¹ and/or a 2-h glucose > 140 mg dL⁻¹ were defined as having prediabetes.

Plasma total cholesterol and triglycerides were determined by an enzymatic method (Abbott Laboratories ABA-200 ATC Biochromatic Analyzer, Irving, TX, USA). APOE E4 genotype was determined using polymerase chain reaction amplification of leukocyte deoxyribonucleic acid followed by HhaI digestion and product characterization (Hixson & Vernier, 1990) or TaqMan, relying on several single nucleotide polymorphisms around the APOE gene (Koch et al., 2002).

**Lipid extraction and LC/ESI/MS/MS analysis**

A crude lipid extraction of plasma was conducted using a modified Bligh and Dyer procedure with ceramide C12:0 included as an internal standard (Avanti Polar Lipids, Alabaster, AL, USA) (Bandaru et al., 2013). Plasma extracts were dried in a nitrogen evaporator (Organomation Associates Inc., Berlin, MA, USA), and resuspended in pure methanol just prior to analysis. An autosampler (LEAP Technologies Inc., Carrboro, NC, USA) injected extracts into an HPLC (PerkinElmer, Waltham, MA, USA) equipped with a reverse-phase C18 column (Phenomenex, Torrance, CA, USA). Ceramide species were separated by gradient elution at the flow
rate of 400.0 μL min⁻¹. The mobile phase A consisted of 85% methanol, 15% H₂O, and 5 mM ammonium formate. Mobile phase B consisted of 99% methanol, 1% formic acid, and 5 mM ammonium formate. Gradient conditions were as follows: a gradual increase from 100% A to 100% B over 0.5 min, hold at 100% B for 4.5 min, then a decline from 100% to 0% B during the next 1 min.

Eluted sample was injected into an electrospray ion source coupled to a triple quadrupole mass spectrometer (API3000; AB Sciex Inc., Thornhill, ON, Canada) (Bandaru et al., 2013). Instrument parameters were as follows: ion spray voltage (V) 5500 at a temperature of 80 °C with a nebulizer gas of 8 psi, curtain gas 8 psi, and collision gas 4 psi. The declustering potential was 80 V, focusing potential 400 V, entrance potential 10 V, collision energy 30 V, and collision cell exit potential 18 V. Analysis was conducted by multiple reaction monitoring. MS/MS transitions (m/z) of individual ceramide molecular species for sphingomyelin precursor and product ions are provided in Table 5. Eight-point transitions (18 V. Analysis was conducted by multiple reaction monitoring. MS/MS potential 10 V, collision energy 30 V, and collision cell exit potential declustering potential was 80 V, focusing potential 400 V, entrance nebulizer gas of 8 psi, curtain gas 8 psi, and collision gas 4 psi. The

° with long-term scatter (Fig. 4), suggesting that ceramide content of plasma was stable over time. When arranged by date of visit, each species showed a random decreasing trends in ceramide concentrations coincident with storage continued enzymatic activity would be manifested by increasing or decreasing trends in ceramide concentrations coincident with storage time. When arranged by date of visit, each species showed a random scatter (Fig. 4), suggesting that ceramide content of plasma was stable with long-term −80 °C storage.

**Statistical analyses**

Sex differences in baseline demographic and health-related characteristics were examined using Fisher's exact test for categorical variables and t-tests or ANOVA for continuous variables. We examined all ceramide species for outliers. As normal ranges for plasma ceramides are not yet known, we conservatively defined an outlier as a concentration of more than three interquartile ranges (25th percentile–75th percentile) from the median; 3.9% of all obtained lipid species were excluded.

Some individuals had missing covariates for a specific visit. Missing values for the covariates described below ranged from 0% to 20.1% (for APOE genotype only) of the 3960 total visits. As the BLSA follows individuals for decades, we imputed the missing covariates for each individual and visit using the nonmissing values of neighboring visits. This allowed us to utilize the largest number of samples.

We used linear mixed models to account for the longitudinal nature of the data and to model the trajectories for individual ceramide class and species over time. Based on the literature, we initially examined the following predictors: age, age squared, sex, race (white vs. African American), education, presence of an APOE E4 allele, BMI, WHR, smoking (never vs. ever), triglycerides, cholesterol, medications (e.g., antidepressants, statins), and medical conditions (e.g., diabetes, hypertension, CKD), and the interaction term between these predictors and age. We forced age, age squared, sex, and BMI into all models and then used backward selection with P < 0.10 to determine which variables to include in the final model for each ceramide and DHCer species. Analyses were conducted using Stata version 11.0 (StataCorp LP, College Station, TX, USA). P < 0.05 was used as the threshold for statistical significance.

**Author contributions**

MMM conceived the analyses, contributed to the statistical analyses and interpretation of results, and wrote the initial draft of the manuscript. VVRB contributed to the mass spectrometry analyses and interpretation of the data. DH and YA contributed to the statistical analyses, interpretation of results, and writing of the manuscript. RS and LF contributed to the study design of the Baltimore Longitudinal Study on Aging, data collection, interpretation of results and writing of the manuscript. NH contributed to the mass spectrometry analyses, interpretation of results, and writing of the manuscript.

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**Conflict of interest**

The authors have no conflict of interests to declare.

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