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Dietary Patterns, Ceramide Ratios, and Risk of All-Cause and Cause-Specific Mortality: The Framingham Offspring Study

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ABSTRACT

Background: Prior evidence suggests that diet modifies the association of blood ceramides with the risk of incident cardiovascular disease (CVD). It remains unknown if diet quality modifies the association of very long-chain-to-long-chain ceramide ratios with mortality in the community.

Objectives: Our objectives were to determine how healthy dietary patterns associate with blood ceramide concentrations and to examine if healthy dietary patterns modify associations of ceramide ratios (C22:0/C16:0 and C24:0/C16:0) with all-cause and cause-specific mortality.

Methods: We examined 2157 participants of the Framingham Offspring Study (mean age = 66 y, 55% women). Blood ceramides were quantified using a validated assay. We evaluated prospective associations of the Dietary Guidelines Adherence Index (DGAI) and Mediterranean-style Diet Score (MDS) with incidence of all-cause and cause-specific mortality using Cox proportional hazards models. Cross-sectional associations of the DGAI and MDS with ceramides were evaluated using multivariable linear regression models.

Results: The C22:0/C16:0 and C24:0/C16:0 ceramide ratios were inversely associated with all-cause, CVD, and cancer mortality; multivariable-adjusted HRs (95% CIs) were 0.73 (0.67, 0.80) and 0.70 (0.63, 0.77) for all-cause mortality, 0.74 (0.60, 0.90) and 0.69 (0.55, 0.86) for CVD mortality, and 0.75 (0.65, 0.87) and 0.75 (0.64, 0.88) for cancer mortality, respectively. Inverse associations of the C22:0/C16:0 and C24:0/C16:0 ceramide ratios with cancer mortality were attenuated among individuals with a higher diet quality (DGAI or MDS above the median, all *P*-interaction $\leq$0.1). The DGAI and MDS had distinct associations with ceramide ratios (DGAI: lower C22:0/C16:0 across quartiles; MDS: higher C24:0/C16:0 across quartiles; all *P*-trend $\leq$0.01).

Conclusion: In our community-based sample, ceramide ratios (C22:0/C16:0 and C24:0/C16:0) were associated with a lower risk of all-cause and cause-specific mortality. Further, we observed that a higher overall diet quality attenuates the association between blood ceramide ratios and cancer mortality and that dietary patterns have distinct relations with ceramide ratios. *J Nutr* 2020;150:2994–3004.

Keywords: dietary pattern, diet quality, Mediterranean, ceramide, sphingolipid, cancer, cardiovascular disease, mortality

Introduction

Ceramides are a class of sphingolipids that are synthesized de novo from the condensation of palmitate and serine or are generated by the catabolism of complex sphingolipids (1). Ceramides are bioactive lipids involved in insulin resistance, inflammation, and the pathogenesis of atherosclerosis (2). Current evidence indicates that ceramides are a heterogeneous class of lipids with the length of their acyl chains conferring distinctive physiological properties upon each subspecies

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(3, 4). Associations between ceramides quantified in plasma and risk of chronic diseases (including coronary artery disease, cancer, and Alzheimer's disease) differ by acyl chain length (3, 5). Hence, prior work has focused on examining the relative proportion of ceramides. Ratios comparing the relative proportion of very long (carbon chain length ≥20) and long-chain ceramides (carbon chain length 14–18) are associated with cardiovascular death (6–8). A ratio <1 is directly associated with cardiovascular death. A previous meta-analysis involving data from the Framingham Offspring Study and the Study of Health in Pomerania observed that higher ratios of very long to long-chain ceramide circulating concentrations are associated with a lower risk of mortality in a generally healthy sample of middle-aged adults (9). Thus, alterations in ceramide metabolism that modify the relative concentrations of ceramide species (of varying length) may represent a mechanism to reduce the risk of death related to chronic diseases.

Prospective cohort studies demonstrate that adherence to healthy dietary patterns is associated with reduced risk of cardiometabolic diseases, cancer, and mortality (10–13). Prior work has demonstrated that diet is a modifier of the plasma lipidome and that diet-induced modifications of the lipidome may be a mechanism by which diet influences cardiometabolic risk (14). Intake of dietary fat and foods that influence de novo lipogenesis have the potential to influence ceramide metabolism. Analysis in the PREDIMED (Prevention with Mediterranean Diet) study suggested that Mediterranean diet interventions modified the direct association between a blood ceramide score (comprised of individual ceramides and ceramide ratios) and risk of incident cardiovascular disease (CVD) (15). However, whether a healthy dietary pattern modifies associations between blood ceramide ratios and risk of all-cause and cause-specific mortality in a free-living population is unknown. Further, associations between different healthy dietary patterns and concentrations of circulating ceramides are unclear.

To address these knowledge gaps, the present investigation determined cross-sectional associations of complementary dietary patterns, a healthy American dietary pattern, or a Mediterranean-style dietary pattern, with concentrations of 3 circulating ceramides (C16:0, C22:0, and C24:0) and ceramide ratios (C22:0/C16:0 and C24:0/C16:0). We additionally sought to evaluate if the respective dietary patterns modify the associations of blood C22:0/C16:0 and C24:0/C16:0 ceramide ratios and the risk of all-cause and cause-specific mortality in heathy free-living middle-aged adults.

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Abbreviations used: CVD, cardiovascular disease; DGA, US Dietary Guidelines for Americans; DGAI, Dietary Guidelines Adherence Index; hsCRP, high-sensitive C-reactive protein; MDS, Mediterranean-style Diet Score; PREDIMED, Prevention with Mediterranean Diet; RERI, relative excess risk due to interaction.

Methods

Study sample

The Framingham Offspring Study was initiated in 1971 with the enrollment of 5124 adults and has been described elsewhere (16). In the present investigation, we evaluated participants of the Framingham Offspring Study who attended the eighth examination cycle (2005–2008) and had available data on 3 circulating ceramides (n = 2843). We excluded participants from the present analysis who did not have complete dietary assessment data at examination cycles 5 (1991–1995) and 8 (n = 638). Of participants with complete blood ceramide and dietary data (n = 2205), we further excluded participants with no follow-up (n = 6) and participants who had missing covariate data (n = 42), resulting in a final sample size of 2157 participants (**Supplementary Figure 1**). The Boston University Medical Center and Tufts University Health Sciences Institutional Review Boards approved the study protocol and all participants provided written informed consent.

Dietary assessment

FFQ.

Dietary assessment was completed using the Harvard semiquantitative FFQ to measure food and nutrient exposures required for the calculation of the dietary pattern scores. The Harvard FFQ measures the consumption of 126 food items to indicate usual frequency of consumption over the past year (17). Frequency categories range from none or <1 serving per month to ≥6 servings per day. The validity of the Harvard FFQ has previously been assessed using 7-d dietary records (17, 18). In the present investigation, we only used FFQs that were considered valid (<13 blank items and estimated daily caloric intake was ≥600 kcal/d and <4000 kcal/d for women or <4200 kcal/d for men) (19). We computed the cumulative intake of food and nutrient exposures to reduce potential error in our analyses (20). We calculated the cumulative dietary intake as the average consumption among participants who had complete dietary data at the fifth and eighth examination cycles.

Dietary pattern scores

Dietary Guidelines Adherence Index.

The 2010 Dietary Guidelines Adherence Index (DGAI) measures conformity to the US Dietary Guidelines for Americans (DGA). The 2010 DGAI has been described in detail elsewhere (21). Briefly, the DGAI is composed of 2 subscores: food group and healthy choice. The food group subscore measures intake of 14 food groups identified in the DGA (fruit; dark green vegetables; orange and red vegetables; starchy vegetables; other vegetables; grains; dairy; meat, proteins, and eggs; seafood; nuts; legumes; sugar, variety in protein choices; and variety of fruits and vegetables). The healthy choice subscore measures conformity to 11 consumption recommendations regarding intake levels of food groups and nutrients (amount of total fat, saturated fat, *trans* fat, cholesterol, sodium, fiber, alcohol; and percentage of protein that is lean, dairy that is low fat, grains that are whole grain, and fruits that are whole fruits). Conformity to recommendations for each subscore component is scored as a proportion on a continuous scale of 0–1 (food group maximum = 14 and healthy choice maximum = 11). To obtain the final DGAI score, component scores are summed and scaled to a range of 0–100 (maximum DGAI score = 100). A higher DGAI score indicates higher conformity to the 2010 DGA. A unique feature of the DGAI is a penalization on component scores of energy-dense foods (foods that provide >50 kcal per serving) for overconsumption (21). The penalty prevents individuals from obtaining a higher DGAI score by overconsumption and is in agreement with recommendations in the 2010 DGA that focus on weight management.

Mediterranean-style Diet Score.

Components of the Mediterranean-style Diet Score (MDS) (22, 23) included vegetables, fruits, nuts, legumes, whole grains, fish, red meat, ratio of MUFAs to SFAs, and alcohol. With the exception of alcohol,

component scores are based on sex-specific quartile categories of intake for our respective sample. Positive scoring was used for all components except red and processed meats, for which negative scoring was used. Possible component scores were 0, 1, 2, or 3; participants in the fourth quartile category (Q4; Q1 for red and processed meats) had the maximum score of 3. For the alcohol component, participants received a score of 1 if consumption was ≥10 g/d and ≤25 g/d for men or ≥5 g/d and ≤15 g/d for women, and a score of 0 otherwise. Component scores were summed to obtain the final MDS score (maximum MDS = 25), with a higher score indicating greater conformity to a Mediterranean-style diet.

Blood ceramide concentrations

Absolute plasma concentrations of C16:0, C22:0, and C24:0 ceramides were assayed previously using a validated LC-MS/MS assay at the eighth Framingham Offspring Study examination cycle (9). Briefly, internal standards containing known amounts of the respective ceramides were added to plasma samples (50 μL). A 9:1 isopropanol-chloroform solution was then used for lipid extraction. Ceramide data was analyzed on a Shimadzu HPLC system coupled with an Applied Biosystems/MDS Sciex 4000QTRAP mass spectrometer using multiple reaction monitoring (MRM). Analyst software (version 1.5.2) was used for data acquisition and analysis.

All-cause and cause-specific mortality

For mortality analyses, participants were followed from examination cycle 8 (2005–2008) until date of death, loss to follow-up, or 31 December 2016 (whichever occurred first). Mortality outcomes included CVD death (death due to fatal myocardial infarction, coronary artery disease death, sudden cardiac death, fatal ischemic stroke, or other CVD), cancer death, and all-cause mortality [CVD, cancer, and other (nonspecified)]. All deaths were verified using death certificate records. In addition to death certificate records, cause of death was determined by a detailed review of all available medical records, information provided by attending physicians, medical examiners, and/or family members.

Covariate assessment

We used the following covariates for this investigation: age, sex, smoking, total energy intake, BMI, physical activity, use of lipid-lowering medication, prevalent CVD, non-HDL-C, and high-sensitive C-reactive protein (hsCRP). We assessed all covariates at the eighth examination cycle of the Framingham Offspring Study (2005–2008). At each examination cycle, participants underwent a physical examination and medical history using standardized protocols. Standard phlebotomy procedures and assays were used for the assessment of fasting blood lipid concentrations. The quantification of circulating hsCRP is described elsewhere (24). For blood pressure, we used the average of 2 measurements (taken 5 min apart) obtained by a physician using a mercury column sphygmomanometer and a cuff of appropriate size on the participant's left arm. Height and weight were measured using standardized protocols and BMI was calculated as weight divided by height (kg/m^2). Use of antihypertensive medications and use of lipid-lowering medications was based on self-reported use in the year preceding the Heart Study examination. Individuals with fasting plasma glucose concentrations ≥7 mmol/L (126 mg/dL) or who self-reported use of antidiabetic medications were considered to have diabetes mellitus. We classified participants who smoked regularly in the year preceding the Heart Study examination as current smokers. Physical activity status was calculated based on self-reported time and intensity of activities in a day (25). Total energy intake was calculated from the aforementioned semiquantitative FFQ.

Statistical analyses

We categorized participants by quartile categories of the DGAI and MDS. We estimated age- and sex-adjusted Pearson's correlation coefficients between the DGAI and MDS. Additionally, we estimated Pearson's correlation coefficients among individual blood ceramides adjusting for age, sex, and use of lipid-lowering medications.

We used multivariable linear regression models to determine cross-sectional associations of the DGAI and MDS (independent variables) with concentrations of circulating ceramides and ceramide ratios (C16:0, C22:0, C24:0, C22:0/C16:0, and C24:0/C16:0; dependent variables, separate model for each diet score and ceramide). We examined 2 multivariable regression models; an initial model (model 1) adjusted for total energy intake, age, sex, smoking status, use of lipid-lowering medications, physical activity, BMI, and prevalent CVD; and a second model (model 2) that additionally adjusted for concentrations of non-HDL-C and hsCRP.

The DGAI and MDS were modeled as quartile categories and as continuous variables. When modeling the DGAI and MDS as continuous independent variables, we set both scores to a scale of 0–25 for comparability. Additionally, we tested for a linear trend across quartile category of the respective diet scores using the median quartile category scores as continuous values in linear regression models. Data are displayed pictorially as least square means and their 95% CIs for ceramide concentrations across quartile categories of the DGAI and MDS. Multivariable regression analyses (model 2) were repeated to examine associations of individual DGAI and MDS components with concentrations of ceramides and ceramide ratios (separate model for each diet component and each ceramide). For the MDS and 2 DGAI subscores (food group and healthy choice), we modeled individual components by tertile categories with mutual adjustment for other components of the respective score. We considered a Bonferroni adjusted 2-tailed $P \leq 0.01$ (0.05/5) statistically significant in cross-sectional analysis of the DGAI and MDS with ceramides. Cross-sectional analyses of the individual diet score components was exploratory and $P \leq 0.05$ was considered statistically significant.

Consistent with prior work (9), we used multivariable-adjusted Cox proportional hazards regression models to relate the C22:0/C16:0 and C24:0/C16:0 ceramide ratios to all-cause and cause-specific mortality (separate model for each ceramide ratio and mortality outcome) adjusting for the same covariates (9). Covariates in the models included age, sex, BMI, systolic blood pressure, diabetes mellitus, smoking status, use of antihypertensive medications, the ratio of total/HDL cholesterol, triglycerides, use of lipid-lowering medication, and prevalent CVD. We tested associations of the C22:0/C16:0 and C24:0/C16:0 ceramide ratios with all-cause and cause-specific mortality outcomes for effect modification by the DGAI and MDS. To determine the significance of an additive interaction we calculated the relative excess risk due to interaction (RERI). In the present study, the RERI would indicate the proportion of the multivariable HRs, among individuals with both exposures (higher ceramide ratio and higher diet quality), that is due to an interaction between diet quality and ceramide ratios. Deviations from additivity are implied when the null hypothesis (RERI = 0) is rejected. We estimated the RERI and its 95% CIs using the delta method (26). We considered a 2-sided $P \leq 0.1$ as the statistical significance level for the interactions and a 2-sided $P \leq 0.05$ statistically significant in all other prospective analyses. All statistical analyses were completed using SAS statistical software (version 9.4; SAS Institute).

Results

Sample characteristics

The average age of participants in our sample was 66 y and 55% of participants were women. Across increasing quartile categories (from Q1 to Q4) of both the DGAI and MDS, participants with higher scores were more likely to be older, less likely to smoke, slightly more physically active, had slightly lower BMIs, and had lower concentrations of hsCRP (**Table 1**). Higher DGAI quartiles were comprised of a higher proportion of women. Individuals in higher MDS quartile categories had lower concentrations of non-HDL-C and, on average, consumed more total calories per day.

TABLE 1 Characteristics across extreme quartile categories of the Dietary Guidelines Adherence Index and the Mediterranean-style Diet Score in older men and women of the Framingham Offspring Study[1]

	DGAI Q1 ($n = 541$)	DGAI Q4 ($n = 540$)	MDS Q1 ($n = 532$)	MDS Q4 ($n = 564$)
Age, y	65 ± 9	68 ± 9	64 ± 8	68 ± 9
Women, *n* (%)	203 (38)	401 (74)	301 (57)	302 (54)
Current smoker, *n* (%)	77 (14)	15 (3)	72 (14)	15 (3)
Physical activity score	34.9 ± 5.9	35.7 ± 4.9	34.8 ± 5.7	36.0 ± 4.9
BMI, kg/m^2	29.0 ± 5.6	27.1 ± 5	28.6 ± 5.9	27.2 ± 4.8
Lipid-lowering medications, *n* (%)	243 (45)	243 (45)	226 (42)	238 (42)
Plasma non-HDL-C, mg/dL	127 ± 33.8	128 ± 32.4	130 ± 33.5	126 ± 31.3
Plasma HDL-C, mg/dL	54.8 ± 18.4	59.7 ± 17.6	57.5 ± 18.7	58.4 ± 18.4
Serum hsCRP, mg/L	1.7 [0.9, 3.3]	1.4 [0.7, 3.1]	1.7 [0.9, 3.4]	1.3 [0.7, 2.8]
Prevalent CVD	93 (17)	72 (13)	86 (16)	72 (13)
Diabetes	76 (14)	63 (12)	64 (12)	76 (13)
Energy intake, kcal/d	1829 ± 733	1879 ± 494	1591 ± 565	2150 ± 592
DGAI (0–100)	47 ± 5	73 ± 4	51 ± 8	70 ± 7
MDS (0–25)	9 ± 3	16 ± 3	7 ± 2	17 ± 2
Plasma ceramide C16:0, μg/mL	0.164 ± 0.036	0.163 ± 0.036	0.168 ± 0.037	0.159 ± 0.034
Plasma ceramide C22:0, μg/mL	0.632 ± 0.180	0.591 ± 0.170	0.629 ± 0.177	0.596 ± 0.176
Plasma ceramide C24:0, μg/mL	2.30 ± 0.64	2.22 ± 0.59	2.24 ± 0.60	2.28 ± 0.61
Plasma ceramide C22:0/C16:0	3.89 ± 0.86	3.64 ± 0.77	3.78 ± 0.85	3.77 ± 0.87
Plasma ceramide C24:0/C16:0	14.2 ± 3.55	13.8 ± 3.03	13.6 ± 3.23	14.5 ± 3.27

[1] Values presented as mean ± SD, *n* (%), or median [Q1, Q3]. DGAI, 2010 Dietary Guidelines Adherence Index; hsCRP, high-sensitive C-reactive protein; MDS, Mediterranean-style Diet Score.

Correlations between diet scores and circulating ceramides

The age- and sex-adjusted Pearson's correlation coefficient between the DGAI and MDS was r = 0.77 (P <0.0001). Additionally, we estimated Pearson's correlation coefficients among ceramides, adjusting for age, sex, and use of lipid-lowering medications. The 2 very long-chain ceramides were highly and directly correlated with each other (r = 0.84), as were the ceramide ratios (r = 0.76, all P <0.0001). The C16:0 ceramide had moderate direct associations with the C22:0 (r = 0.63) and C24:0 (r = 0.55) ceramides (all P <0.0001).

Associations of the DGAI with concentrations of ceramides

Concentrations of the C16:0, C22:0, C24:0, and C22:0/C16:0 ceramide ratio were lower across increasing quartile categories of the DGAI (all P-trend ≤0.01; **Figure 1**A–D). Compared with the lowest quartile category, the highest DGAI quartile category was associated with 0.007 μg/mL (95% CI: –0.011, –0.003) lower concentrations of the C16:0 ceramide, 0.054 μg/mL (–0.071, –0.036) lower concentrations of the C22:0 ceramide, 0.107 μg/mL (0.171, –0.043) lower concentrations of the C24:0 ceramide, and a 0.185 unit (–0.286, –0.083) lower C22:0/C16:0 ceramide ratio (model 2, **Table 2**). We found no statistically significant associations between the DGAI and the C24:0/C16:0 ceramide ratio. When further adjusting for non-HDL-C and hsCRP, results were similar. Additionally, results were similar when the DGAI was modeled as a continuous variable (Table 2).

Associations of the MDS with concentrations of ceramides

Similar to the DGAI, concentrations of the C16:0 and C22:0 ceramides were significantly lower with increasing quartile categories of the MDS (all P-trend ≤0.01; Figure 1A and B). However, we observed that concentrations of the C24:0 ceramide and the C24:0/C16 ceramide ratio were directly associated with quartile categories of the MDS (Figure 1C and E). We found no significant associations between the MDS and the C22:0/C16:0 ratio. Compared with the lowest quartile category of the MDS, the highest quartile category was associated with 0.009 μg/mL (–0.012, –0.005) lower concentrations of the C16:0 ceramide and 0.024 μg/mL (–0.042, –0.006) lower concentrations of the C22:0 ceramide. In contrast, the highest MDS quartile category of the MDS was associated with a 1.135 unit (0.727, 1.543) higher C24:0/C16:0 ceramide ratio (model 2, **Table 3**) compared with the lowest quartile category. Of note, the direct associations between MDS (modeled by quartile or as a continuous variable) and C24:0 concentrations did not remain statistically significant following adjustment for multiple testing (Table 3).

Associations of DGAI and MDS components with concentrations of ceramides

To better understand the observed differences in associations between the respective diet scores and ceramides (C24:0/C16:0 and C22:0/C16:0), we related individual components of the diet scores (DGAI and MDS) to ceramides and ceramide ratios (**Supplementary Figures 2–4**). Seven components (whole fruit, lean protein, whole grains, total fat, saturated fat, sodium, and cholesterol) from the DGAI healthy choice subscore and 5 DGAI food group subscore components (red and orange vegetables, total fruit and vegetables, grains, dairy, and sugar) had statistically significant associations with ≥1 ceramide or ceramide ratio (all P ≤0.05). The directionality of the DGAI component and ceramide associations differed based on the specific dietary component and the specific ceramide species evaluated. The strongest association between DGAI components and the C22:0/C16:0 ratio was an inverse association between this ceramide ratio and the component measuring adherence to the recommendation for saturated fat. This represents a direct association with the intake of saturated fat. Additionally, the C22:0/C16:0 ratio had direct associations with total fat, sugar (inverse associations with the total fat and sugar components), and alcohol intake. Six components (vegetables, fruit, nuts, red/processed meat, MUFA to SFA ratio,

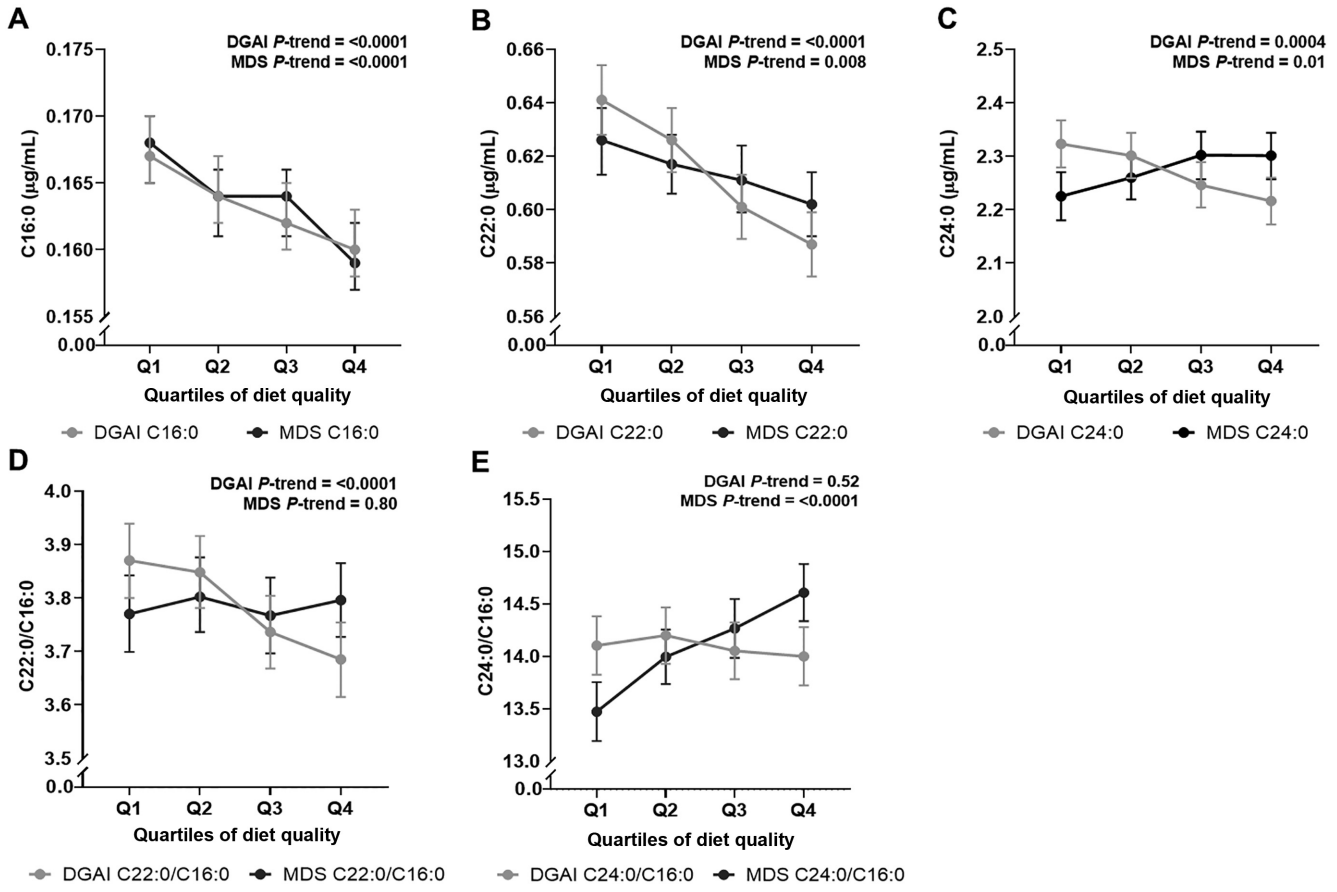

FIGURE 1 Values are adjusted least square means and 95% CIs of circulating ceramide concentrations (A, C16:0; B, C22:0; and C, C24:0) and ceramide ratios (D, C22:0/C16:0 and E, C24:0/C16:0) by quartile categories of the Dietary Guidelines Adherence Index and the Mediterranean-style dietary pattern score in a sample of 2157 participants (DGAI Q1 $n = 541$, Q2 $n = 539$, Q3 $n = 537$, Q4 $n = 540$; MDS Q1 $n = 532$, Q2 $n = 569$, Q3 = 492, Q4 = 564) of the Framingham Offspring Study, men and women with a mean age of 66 y. Q1 is indicative of poor conformity to the DGAI or MDS and Q4 is indicative of high conformity to the DGAI or MDS. Multivariable regression models with diet scores as the independent variable and ceramides as the dependent variable. Models are adjusted for age, sex, smoking, total energy intake, BMI, physical activity, use of lipid-lowering medication, prevalent CVD, non-HDL, and high-sensitive C-reactive protein. *P* values are for a test for linear trend across consumption categories (*P*-trend). CVD, cardiovascular disease; DGAI, 2010 Dietary Guidelines Adherence Index; MDS, Mediterranean-style Diet Score.

and alcohol) of the MDS were associated with ≥1 ceramide or ceramide ratio (all $P \leq 0.05$). The strongest association was the positive relation of the C24:0 ceramide with nut consumption (an MDS component). In addition, the MUFA to SFA ratio was directly related to C24:0, whereas fruit intake was inversely associated. The nut intake, MUFA to SFA ratio, and vegetable intake components were directly associated with the C24:0/C16:0 ratio.

Dietary patterns, ceramide ratios, and risk of mortality

Over a mean follow-up of 10 y, there were 469 deaths, 101 of which were attributable to CVD and 172 of which were attributable to cancer. In agreement with prior work in the Framingham Offspring Study, the C22:0/C16:0 and C24:0/C16:0 ceramide ratios were associated with a lower risk of all-cause and CVD mortality (**Table 4**). Additionally, we observed that the C22:0/C16:0 and C24:0/C16:0 ceramide ratios were associated with a lower risk of cancer mortality. Multivariable-adjusted HRs (95% CIs) for associations of the C22:0/C16:0 ceramide ratio with all-cause mortality, CVD mortality, and cancer mortality were HRs (95% CIs) of 0.73 (0.67, 0.80), 0.74 (0.60, 0.90), and 0.75 (0.65, 0.87), respectively (Table 4). Associations of the C24:0/C16:0 ceramide ratio with all-cause mortality, CVD mortality, and cancer mortality were similar to that for the C22:0/C16:0 ratio, with multivariable-adjusted HRs (95% CIs) of 0.70 (0.63, 0.77), 0.69 (0.55, 0.86), and 0.75 (0.64, 0.88), respectively.

We observed significant effect modification of the associations of the C22:0/C16:0 ceramide ratio with cancer mortality by the MDS (*P*-interaction = 0.08, Table 4). Effect modification by the DGAI was statistically significant for the association of C22:0/C16:0 with all-cause and cancer mortality (all *P*-interaction ≤0.1, Table 4). For the C24:0/C16:0 ceramide ratio, we observed significant effect modification by the MDS and DGAI of its association with all-cause mortality and cancer mortality (all *P*-interaction ≤0.1, Table 4). Notably, we observed no statistically significant effect modification for the associations of the ceramide ratios with CVD mortality with either dietary index.

In analyses stratified using median cut-points of the DGAI and MDS, we examined associations of the C22:0/C16:0 and C24:0/C16:0 ceramide ratios with all-cause and cancer mortality. In participants with a higher diet quality (DGAI or MDS), the inverse associations of the C22:0/C16:0 and C24:0/C16:0 ceramide ratios with all-cause mortality were maintained but were slightly attenuated (**Figure 2**A and B).

TABLE 2 Associations of the Dietary Guidelines Adherence Index with concentrations of circulating ceramides and ceramide ratios in older men and women of the Framingham Offspring Study[1]

$n = 2157$	DGAI Q1 541 β (95% CI)	DGAI Q2 539 β[2] (95% CI)	DGAI Q3 537 β (95% CI)	DGAI Q4 540 β (95% CI)	DGAI 2157 β[3] (95% CI) per 5-unit increase
Ceramide C16:0, μg/mL					
Model 1	Ref.	−0.002 (−0.006, 0.002)	−0.004 (−0.009, −0.000)	−0.008 (−0.012, −0.003)[4]	−0.007 (−0.010, −0.004)[4]
Model 2	Ref.	−0.003 (−0.006, 0.001)	−0.005 (−0.008, −0.001)[4]	−0.007 (−0.011, −0.003)[4]	−0.006 (−0.008, −0.003)[4]
Ceramide C22:0, μg/mL					
Model 1	Ref.	−0.010 (−0.031, 0.011)	−0.036 (−0.057, −0.015)[4]	−0.056 (−0.078, −0.034)[4]	−0.045 (−0.061, −0.030)[4]
Model 2	Ref.	−0.015 (−0.031, 0.002)	−0.040 (−0.057, −0.022)[4]	−0.054 (−0.071, −0.036)[4]	−0.042 (−0.055, −0.030)[4]
Ceramide C24:0, μg/mL					
Model 1	Ref.	−0.004 (−0.078, 0.070)	−0.060 (−0.135, 0.016)	−0.114 (−0.192, −0.036)[4]	−0.085 (−0.141, −0.030)[4]
Model 2	Ref.	−0.022 (−0.082, 0.039)	−0.076 (−0.138, −0.014)	−0.107 (−0.171, −0.043)[4]	−0.076 (−0.121, −0.030)[4]
Ceramide C22:0/16:0					
Model 1	Ref.	−0.010 (−0.108, 0.088)	−0.119 (−0.219, −0.019)	−0.185 (-0.288, −0.081)[4]	−0.142 (−0.215, −0.068)[4]
Model 2	Ref.	−0.021 (−0.117, 0.075)	−0.134 (−0.232, −0.035)[4]	−0.185 (−0.286, −0.083)[4]	−0.141 (−0.214, −0.069)[4]
Ceramide C24:0/16:0					
Model 1	Ref.	0.140 (−0.249, 0.529)	0.022 (−0.377, 0.421)	−0.091 (−0.502, 0.321)	−0.001 (−0.293, 0.290)
Model 2	Ref.	0.096 (−0.288, 0.479)	−0.050 (−0.443, 0.343)	−0.103 (−0.508, 0.303)	−0.013 (−0.300, 0.274)

[1] Values are β estimates (95% CIs). Multivariable regression models with diet scores as the independent variable and ceramides as the dependent variable. Q1 is indicative of poor conformity to the DGAI and Q4 is indicative of high conformity to the DGAI. Model 1 adjusted for age, sex, smoking, total energy intake, BMI, physical activity, use of lipid-lowering medication, and prevalent CVD. Model 2 additionally adjusted for non-HDL and high-sensitive C-reactive protein. CVD, cardiovascular disease; DGAI, 2010 Dietary Guidelines Adherence Index.
[2] β estimates represent the difference in ceramide concentration in the top diet score quartile category (Q4) relative to the lowest quartile category (Q1).
[3] β estimates represent the difference in ceramide concentration per 5-unit increase in the DGAI. The DGAI was set to a scale of 0–25.
[4] P value ≤0.01.

In contrast, a higher diet quality (DGAI or MDS) resulted in the attenuation of the inverse associations of both ceramide ratios with cancer mortality, rendering them statistically nonsignificant in the groups with ceramide ratios below the medians (Figure 2A and B). Compared to participants with a lower C22:0/C16:0 ratio (below the median) and a lower MDS, participants with a higher C22:0/C16:0 ratio and a lower MDS had a 48% lower risk [HR (95% CI): 0.52 (0.33, 0.83)] of cancer mortality (**Supplementary Table 1**). However, participants with a higher C22:0/C16:0 ratio and a higher MDS

TABLE 3 Associations of the Mediterranean-style Diet Score with concentrations of circulating ceramides and ceramide ratios in older men and women of the Framingham Offspring Study[1]

$n = 2157$	MDS Q1 532 β (95% CI)	MDS Q2 569 β[2] (95% CI)	MDS Q3 492 β (95% CI)	MDS Q4 564 β (95% CI)	MDS 2157 β[3] (95% CI) per 5-unit increase
Ceramide C16:0, μg/mL					
Model 1	Ref.	−0.004 (−0.008, 0.000)	−0.005 (−0.009, −0.000)	−0.011 (−0.016, −0.007)[4]	−0.005 (−0.007, −0.003)[4]
Model 2	Ref.	−0.004 (−0.008, −0.001)	−0.004 (−0.008, −0.001)	−0.009 (−0.012, −0.005)[4]	−0.004 (−0.006, −0.002)[4]
Ceramide C22:0, μg/mL					
Model 1	Ref.	−0.007 (−0.028, 0.013)	−0.014 (−0.036, 0.008)	−0.034 (−0.056, -0.012)[4]	−0.020 (−0.030, −0.010)[4]
Model 2	Ref.	−0.010 (−0.027, 0.007)	−0.015 (−0.032, 0.003)	−0.024 (−0.042, −0.006)[4]	−0.015 (−0.023, −0.006)[4]
Ceramide C24:0, μg/mL					
Model 1	Ref.	0.043 (−0.031, 0.116)	0.086 (0.009, 0.164)	0.046 (−0.033, 0.125)	0.013 (−0.023, 0.049)
Model 2	Ref.	0.030 (−0.030, 0.090)	0.080 (0.017, 0.143)	0.076 (0.011, 0.141)	0.028 (−0.002, 0.059)
Ceramide C22:0/16:0					
Model 1	Ref.	0.036 (−0.062, 0.130)	0.007 (−0.096, 0.109)	0.024 (−0.082, 0.129)	−0.014 (−0.063, 0.035)
Model 2	Ref.	0.024 (−0.072, 0.119)	-0.006 (−0.107, 0.095)	0.026 (−0.077, 0.129)	−0.012 (−0.060, 0.036)
Ceramide C24:0/16:0					
Model 1	Ref.	0.546 (0.164, 0.929)[4]	0.871 (0.469, 1.274)[4]	1.164 (0.751, 1.578)[4]	0.495 (0.303, 0.688)[4]
Model 2	Ref.	0.488 (0.111, 0.865)	0.796 (0.399, 1.193)[4]	1.135 (0.727, 1.543)[4]	0.483 (0.294, 0.673)[4]

[1] Values are β estimates (95% CIs). Multivariable regression models with diet scores as the independent variable and ceramides as the dependent variable. Q1 is indicative of poor conformity to the MDS and Q4 is indicative of high conformity to the MDS. Model 1 adjusted for age, sex, smoking, total energy intake, BMI, physical activity, use of lipid-lowering medication, and prevalent CVD. Model 2 additionally adjusted for non-HDL and high-sensitive C-reactive protein. CVD, cardiovascular disease; MDS, Mediterranean-style Diet Score.
[2] β estimates represent the difference in ceramide concentration in the top diet score quartile category (Q4) relative to the lowest quartile category (Q1).
[3] β estimates represent the difference in ceramide concentration per 5-unit increase in the MDS. The MDS was set to a scale of 0–25.
[4] P value ≤0.01.

TABLE 4 HRs of all-cause and cause-specific mortality according to ceramide ratios in older men and women of the Framingham Offspring Study[1]

$n = 2157$	HR[2] (95% CIs)	MDS *P*-interaction[3]	DGAI *P*-interaction[4]
Ceramide C22:0/C16:0			
All-cause	0.73 (0.67, 0.80)	0.11	0.003
CVD	0.74 (0.60, 0.90)	0.16	0.77
Cancer	0.75 (0.65, 0.87)	0.01	0.03
Ceramide C24:0/C16:0			
All-cause	0.70 (0.63, 0.77)	0.08	0.003
CVD	0.69 (0.55, 0.86)	0.38	0.75
Cancer	0.75 (0.64, 0.88)	0.01	0.04

[1] Values are HRs (95% CIs). Multivariable models with ceramide ratios as the independent variables and mortality outcomes as the dependent variables. Models were adjusted for age, sex, current smoking, BMI, systolic blood pressure, antihypertensive medication, diabetes mellitus, total cholesterol/HDL-C ratio, triglycerides, use of lipid-lowering medication, prevalent CVD. CVD, cardiovascular disease; DGAI: 2010 Dietary Guidelines Adherence Index; MDS, Mediterranean-style Diet Score.
[2] HRs and corresponding 95% CIs are reported for a 3-unit increase in the C24:0/C16:0 ratio and a 0.7-unit increase in the C22:0/C16:0 ratio.
[3] *P* values for a ceramide ratio and MDS interaction (dichotomous; above or below the median MDS of 12).
[4] *P* values for a ceramide ratio and DGAI interaction (dichotomous; above or below the median DGAI of 62).

had a 35% lower risk [0.65 (0.42, 0.99)] of cancer mortality, compared to participants with a lower C22:0/C16:0 ratio and lower MDS. Results were similar for the C24:0/C16:0 ratio and MDS, C22:0/C16:0 ratio and DGAI, and C24:0/C16:0 ratio and DGAI (**Supplementary Tables** 1 and **2**). Calculation of the RERI and estimated corresponding 95% CIs indicated deviation from additivity for the associations between the C24:0/C16:0 ceramide ratio and cancer mortality with all dietary pattern interactions (**Figure 3**B). Deviation from additivity was also suggested for effect modification by both dietary patterns for the association of the C22:0/C16:0 ceramide ratio and cancer mortality (Figure 3A).

Discussion

Building on a prior investigation of ceramide remodeling and mortality in the Framingham Offspring Study, the present analysis confirmed that the C22:0/C16:0 and C24:0/C16:0 ceramide ratios were associated with a lower risk of all-cause and CVD mortality after an extended follow-up period (mean 10 y). We added to these findings by observing that the respective ceramide ratios were associated with a lower risk of cancer mortality. In addition, we observed that the inverse associations of ceramide ratios with all-cause and cancer mortality were modified by overall diet quality. In particular, a higher overall diet quality, as indicated by a higher MDS or DGAI score, attenuated the inverse associations of the respective ceramide ratios with cancer mortality. Lastly, our findings indicate that the DGAI and MDS were differentially associated with blood C22:0/C16:0 and C24:0/C16:0 ceramide ratios. Further evaluation of individual dietary components suggests that differences in scoring of dietary fat quality and food sources by the 2 scores likely contributed to the varying associations.

In the PREDIMED study, effect modification by the Mediterranean diet interventions was observed for the direct association between a deleterious ceramide score (individual ceramides and ceramide ratios) and the risk of incident CVD (15). Participants with a higher ceramide score assigned to either Mediterranean diet intervention had an incidence of CVD similar to participants with a lower ceramide score (15). We observed inverse associations of the ceramide ratios (C22:0/C16:0 and C24:0/C16:0) with CVD mortality, irrespective of high or low MDS or DGAI score. However, both dietary patterns (the DGAI or MDS) attenuated the inverse association of these ratios with cancer mortality. The nature of effect modification by diet is similar in that a higher quality diet attenuates blood ceramide–disease relations. The PREDIMED study examined a blood ceramide score that was directly associated with incidence of CVD, whereas our investigation examined the exposure of ratios reflecting potentially benign ceramides to the deleterious C16:0 ceramide. Such ratios have previously been found to inversely associate with mortality (9); whereas ratios of deleterious ceramides to the very long-chain ceramides directly associate with CVD mortality (6). We note several important differences between the PREDIMED study and the Framingham Offspring Study that may explain, in part, the inconsistencies in results; these include the intervention versus observational study designs, the measurement of ceramides, the examination of high-risk individuals (PREDIMED) as opposed to our relatively healthier ambulatory sample, and the difference in outcomes (incident CVD and CVD mortality). In particular, our study examined CVD mortality and is in agreement with prior work demonstrating inverse associations between C24:0 concentrations and CVD death (6, 9). In contrast, relations between very long-chain ceramides and incidence of CVD are less defined.

To date, a growing body of evidence links distinct circulating ceramides and ceramide ratios with the risk of CVD death (6–8). However, ceramides are implicated in numerous other disease states, including cancer (5, 27). Serum lipidomic profiling studies have reported distinct differences in the blood concentrations of sphingolipids (including ceramides) among individuals with ovarian cancer and individuals with colorectal cancer, compared with controls (28–32). Ceramides play a role in a diverse array of biological processes including apoptosis (33–35), endoplasmic reticulum stress (36, 37), oxidative stress (38), inflammation (39), and insulin resistance (40). Such biological processes, in particular, regulation of apoptosis, are thought to underlie associations between blood ceramide concentrations and cancer (41). It is plausible that a higher quality diet may affect the relative proportion of ceramides by altering circulating fatty acid profiles. This may reduce circulating concentrations of ceramides implicated in tumor suppression and ultimately mitigate the effects of excess ceramides involved in apoptosis or tumor suppression (27, 42). Further, shared biological or metabolic pathways may mediate the respective associations of circulating ceramides and diet quality with cancer. The observed attenuation in the relation

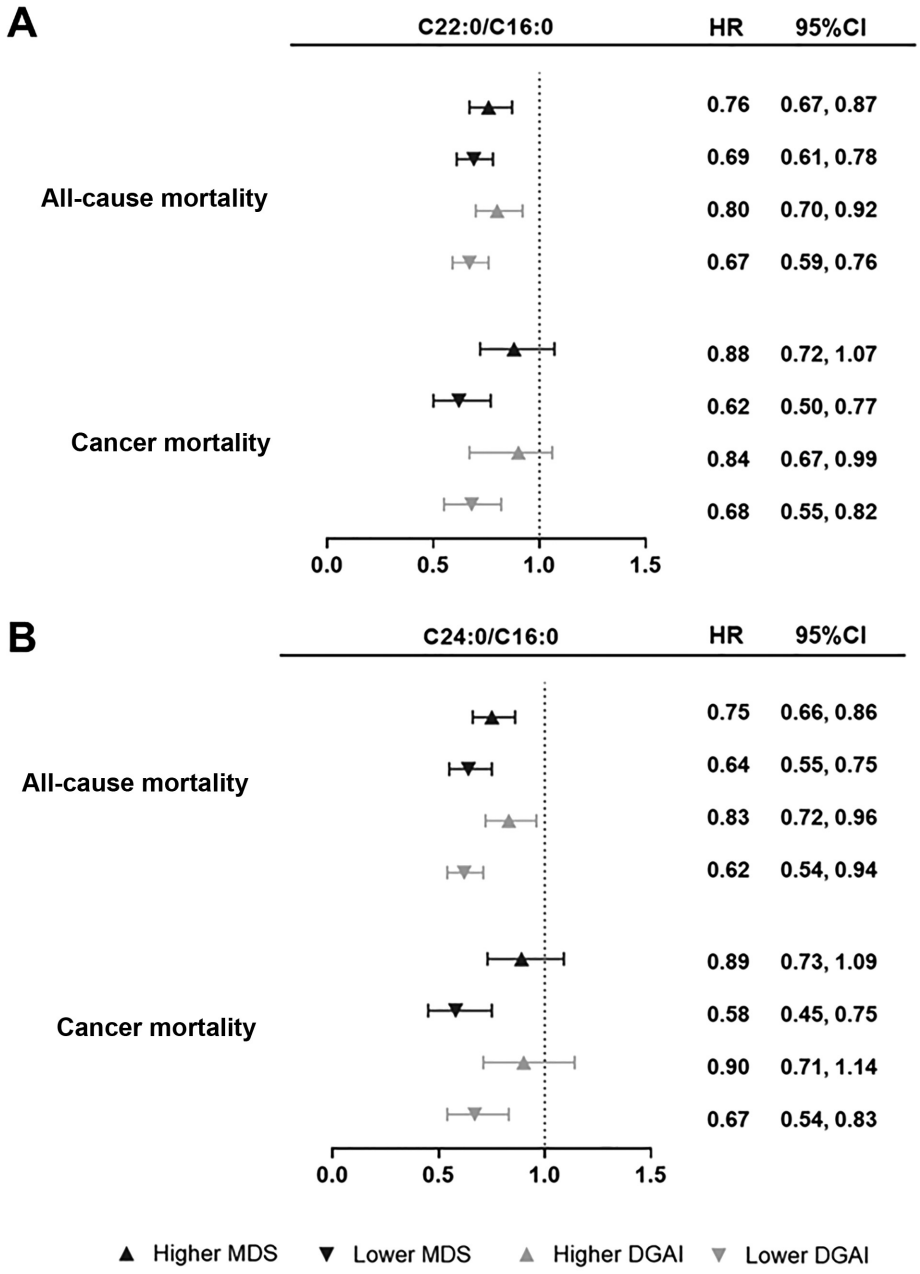

FIGURE 2 Values are HRs and 95% CIs of all-cause and cancer mortality according to C22:0/C16:0 (A) and C24:0/C16:0 (B) ceramide ratios stratified by median cut-points of the DGAI (below median $n = 1075$, above median $n = 1082$) and the MDS (below median $n = 1001$, above median $n = 1156$) in participants of the Framingham Offspring Study, men and women with a mean age of 66 y. Multivariable models with ceramide ratios as the independent variables and all-cause and cancer mortality as the dependent variables. Models are adjusted for age, sex, current smoking, BMI, systolic blood pressure, antihypertensive medication, diabetes mellitus, total cholesterol/HDL cholesterol ratio, triglycerides, use of lipid-lowering medication, prevalent CVD. HRs and corresponding 95% CIs are reported for a 3-unit increase in the C24:0/C16:0 ratio and a 0.7-unit increase in the C22:0/C16:0 ratio. CVD, cardiovascular disease; DGAI, 2010 Dietary Guidelines Adherence Index; MDS, Mediterranean-style Diet Score.

between ceramide ratios and cancer mortality by diet quality warrants further investigation in additional studies.

In agreement with our analysis of the DGAI, a cross-sectional study of 96 middle-aged adults observed that concentrations of the C22:0 ceramide were significantly associated with the 2015 Healthy Eating Index (HEI-2015) and had direct significant associations with the saturated fat and added sugar intakes (indicating inverse associations with components) (43). A randomized clinical trial examining a healthy Nordic diet (limiting saturated fat and increasing fiber, fruits, vegetables, and fish) led to statistically significant reductions in concentrations of very long-chain ceramides (C22:0, C23:0, and C24:0) after 12 wk (44). In contrast, after a year of follow-up in the PREDIMED study, no differences were observed in the concentrations of ceramides (C16:0, C22:0, C24:0, and C24:1) in the 2 Mediterranean diet intervention groups compared with the control diet group (15). It is worth noting that all 3 diets tested in PREDIMED were high in total fat and had similar proportions of saturated fat (45). Despite the limited evidence regarding dietary patterns and blood ceramide concentrations,

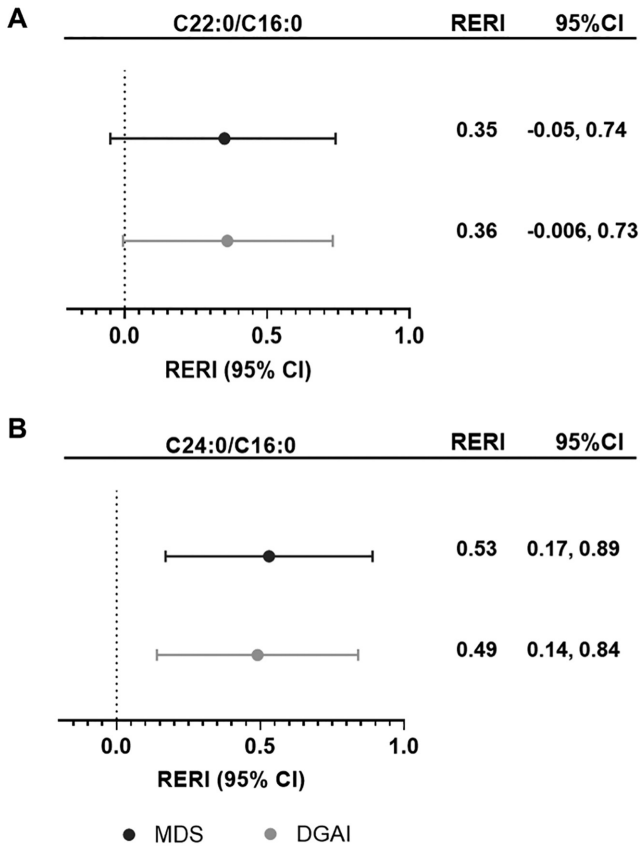

FIGURE 3 Values represent the relative excess risk due to interaction and estimated 95% CIs in multivariable models with the C22:0/C16:0 (A) and C24:0/C16:0 (B) ceramide ratios as the independent variables and cancer mortality as the dependent variable (separate model for each ceramide ratio) in participants of the Framingham Offspring Study, men and women with a mean age of 66 y. Models are adjusted for age, sex, current smoking, BMI, systolic blood pressure, antihypertensive medication, diabetes mellitus, total cholesterol/HDL cholesterol ratio, triglycerides, use of lipid-lowering medication, and prevalent CVD. Analyses were conducted in a sample of 2157 participants. The RERI indicates the proportion of the multivariable HRs, among individuals with both exposures (higher ceramide ratio and higher diet quality), due to an interaction between diet quality and ceramide ratios. A RERI of 0.53 for the interaction by the MDS in the association between the C24:0/C16:0 ratio and cancer mortality indicates that the multivariable HR among individuals with both exposures was 0.53 higher than expected from the additive effects of the MDS and C24:0/C16:0 ratio on cancer mortality. CVD, cardiovascular disease; DGAI, 2010 Dietary Guidelines Adherence Index; MDS, Mediterranean-style Diet Score; RERI, relative excess risk due to interaction.

our work is in agreement with prior studies (43, 44) that suggest a healthy dietary pattern, that includes limiting saturated fat intake, is associated with lower concentrations of very long-chain ceramides.

Our findings suggest a role for dietary fat quality, and perhaps quantity, in the observed differences of the associations of the DGAI and MDS with blood ceramide ratios. Human feeding studies have demonstrated the differential effects of overfeeding unsaturated and SFAs on circulating ceramide concentrations (46–48). In a cross-sectional analysis of nut composition and plasma metabolites, the C24:0 ceramide and very long-chain sphingomyelins (C22:0 and C24:0) were directly associated with nut intake (49). This is in agreement with our finding that the concentration of the C24:0 ceramide was specifically associated with the nut intake and MUFA to SFA ratio components of the MDS. Very long-chain SFA concentrations are influenced by both endogenous metabolism and diet, with peanuts and peanut butter serving as primary dietary sources (50, 51). These particular SFAs are integral components of very long-chain ceramides and experimental evidence suggests that they may induce ceramide synthesis in vivo (52). Relative to long-chain SFAs (e.g., palmitate), very long-chain SFAs appear to have beneficial associations with health outcomes including atrial fibrillation, heart failure, diabetes mellitus, and mortality (53–56). As our investigation and others suggest that higher proportions of very long-chain ceramides relative to the long-chain C16:0 ceramide may be beneficial, additional work should be done to determine how the differential associations of dietary patterns, or dietary fat type, on circulating very long-chain ceramides and ceramide ratios may affect health or risk of adverse outcomes.

The present investigation has several strengths including its prospective study design, the large sample size, and the robust collection of clinical, lifestyle, and dietary data. Further, blood ceramides were measured by a validated, FDA compliant assay which provided absolute quantification of the 3 ceramides (9). Lastly, we used 2 complementary dietary patterns to reflect overall diet quality. For both dietary patterns we used the average score from 2 examination cycles, which may reduce analytical error and better reflect longer-term usual dietary intake (20). However, the present investigation is not without limitations. First, we used single occasion measurements of select ceramide species; we did not assay additional sphingolipid species that may relate to chronic disease states and may be affected by overall diet quality. Second, there are difficulties associated with the use of self-reported dietary data such as biases and measurement errors, which may lead to misclassification and attenuation of observed associations. Third, relations between ceramides and cancer mortality may differ by cancer type, which was not evaluated in this investigation. Fourth, our study sample was predominantly white and of European descent. Hence, our results may not be generalizable to more racially diverse populations. Lastly, given the observational nature of our study, we are unable to evaluate causality of associations, and cannot rule out the possibility of uncontrolled or residual confounding in our study.

In the present investigation, we report that overall diet quality modifies the prospective association between ceramide ratios and cancer mortality in a sample of free-living middle-aged adults. In addition, we identified distinct cross-sectional associations between the DGAI and MDS diet quality scores with ceramide ratios. These findings warrant additional investigation in larger and more ethnically diverse study samples.

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The authors' contributions were as follows—MEW and PFJ: designed the research; MEW: analyzed the data; MEW: wrote the manuscript; VX, LRP, MSD, JL, JM, SB, LLM, PAQ, and RSV: critically revised the manuscript; MEW and PFJ: had primary responsibility for the final content; and all authors: read and approved the final manuscript.

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