Diet Quality and Circulating Sphingolipids: The Strong Heart Family Study

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Abstract

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Sphingolipids, including ceramides (Cer) and sphingomyelins (SM), are involved in the development of chronic disease through stress response, inflammation, insulin sensitivity, and more. Plasma sphingolipids may be influenced by dietary factors, but studies linking the two are limited. Among 2025 non-diabetic participants in the Strong Heart Family Study, we used food frequency questionnaire responses to calculate diet quality scores [Diabetes Dietary Index (DDI; constructed from foods related to type 2 diabetes risk), and the Alternative Healthy Eating Index-2010 (AHEI)]. Associations of diet scores with 15 plasma sphingolipid species were assessed using linear mixed models. Higher DDI scores were associated with lower levels of Cer-18 (Geometric mean [GM] ratio: 0.93; 95%CI: 0.88-0.98) and higher levels of SM-20 (GM Ratio: 1.03; 95%CI: 1.000-1.065) when comparing the 90th percentile to the 10th percentile. Higher AHEI scores were associated with higher levels of SM-14 (GM Ratio: 1.07; 95%CI: 1.028-1.121), SM-20 (GM Ratio: 1.03; 95%CI: 1.003-1.06), and SM-24 (GM Ratio: 1.04; 95%CI: 1.013-1.074). BMI modified associations of DDI with SM-14, SM-16, SM-20, and SM-24; and age modified the association of DDI with SM-24. Higher diet quality is associated with lower Cer-18 and higher SM-14, SM-20, SM-24. BMI appears to modify associations of diet quality with plasma sphingomyelins. These studies need to be replicated in prospective studies and other populations.

Introduction

Sphingolipids are a family of lipids that include ceramides and sphingomyelins, both of which consist of several species that differ primarily by the composition of their fatty acid component (1). Ceramides contain a sphingoid base with a fatty acid attached via amide linkage; sphingomyelins are much the same in structure, but have an additional choline head group (Figure 1). The fatty acids in sphingolipids are often saturated and vary in length, with each species of sphingolipid referred to by its class and associated fatty acid. For example, the species Ceramide 16:0 has a sphingoid base with a saturated 16-carbon fatty acid (palmitic acid) attached; alternatively, sphingomyelin 16:0 also has the sphingoid base with a saturated 16-carbon fatty acid, although it has an additional choline head.

Figure 1. Structure of Ceramides and Sphingomyelins

R = H for ceramides; choline for sphingomyelins

Sphingolipids are membrane, intracellular, and circulating lipids. Those in circulation are biosynthesized in the liver and transported in lipoproteins such as VLDL, LDL, and HDL (2). Most cells in the body also *de novo* synthesize sphingolipids (3), and some ceramides may be released into circulation during hydrolysis of sphingomyelins by sphingomyelinases located in the cell membrane (4) (5). Circulating levels of sphingomyelins and ceramides may be a systemic metabolic reflection of overall homeostasis of these species (3).

Regulation of sphingolipid homeostasis is fundamental to the function and health of cells, as they are bioactive molecules whose accumulation or absence can greatly affect cell function. For this reason, the enzymes involved in sphingolipid production and breakdown are tightly controlled through transcriptional and posttranscriptional regulation, structural regulation, several signaling pathways, and protein phosphorylation (1). Endogenous formation of ceramides can be induced by different stimuli such as tumor necrosis factor-α, Fas ligand, phorbol ester, heat stress, and oxidative stress (especially oxidized LDL cholesterol) (6). Fatty acids play an important role as substrates in sphingolipid synthesis, but little is known of the association between dietary influences of these fatty acids on formation of sphingolipids in humans (1).

Sphingolipids are involved in several different biological processes or conditions implicated in the development of chronic disease, including cell cycle arrest, apoptosis, stress response, inflammation, insulin sensitivity, and hepatic steatosis (6). Through these processes, they have been implicated in the development of chronic diseases such as cancer, cardiovascular disease (CVD), Alzheimer's disease, multiple sclerosis, certain infectious diseases, and type 2 diabetes mellitus (T2DM) (7).

T2DM is a major public health problem in the United States, affecting roughly 21.3 million people as of 2014 (8). It is estimated that 9.3% of the general Unites States population has T2DM, with a higher burden of disease in minority groups. For American Indian and Alaska Natives, that number is estimated to be at 15.9%, which is more than double the 7.6% estimated for non-Hispanic whites (8). American Indian individuals have a 2.3 times greater risk of developing T2DM than non-Hispanic whites of the same age (9). Additionally, T2DM is a major risk factor for development of further chronic disease such as CVD, stroke, retinopathy, and kidney disease (10). Obesity, obstructive sleep apnea, advancing age, diet, intake of saturated and total fats, genetics, a sedentary lifestyle, and frequent consumption of processed meat and soft drinks are all risk factors for T2DM (11).

High plasma glucose due to insulin resistance is an early stage in development of T2DM. This disease is characterized by chronic hyperglycemia and impaired nutrient metabolism caused by complete or partial insufficiency of insulin secretion or insulin action (11). Sphingolipids have been emerging as potential additional risk factors for T2DM due to their role in insulin sensitivity (12) (13). They are involved in several different biological processes implicated in the development of T2DM, including stress response, inflammation, hepatic steatosis, and most of all, insulin sensitivity (6). *In vitro*, ceramides have been shown to inhibit phosphorylation and activation of Akt/protein kinase B, which is necessary for insulin action. Studies in rodents show that inhibition of ceramide or glucosylceramide biosynthesis is insulin sensitizing (13) (14) and global inhibition of *de novo* ceramide synthesis both improves insulin sensitivity and reduces lipotoxic responses associated with obesity-related chronic diseases or metabolic disorders (15) (16) (17) (18).

Unpublished personal communication with other Strong Heart Family Study (SHFS) researchers, Lemaitre et al. (19), shows that higher plasma levels of ceramides are associated with higher levels of fasting insulin, while higher levels of sphingomyelins with very long chain

saturated fatty acids (20 carbons or more) are associated with lower fasting insulin among participants with normal BMI. Other human studies report a negative association between plasma ceramides and insulin sensitivity (13) (14), supporting the effect of sphingolipids on insulin sensitivity in humans. While these studies show a class-effect of sphingolipids on metabolic function, it has been suggested that specific species of sphingolipids, rather than classes, are associated with obesity-related chronic diseases such as atherosclerosis and T2DM (20) (21) (22). Therefore, sphingolipids may be a potential target for reduction of insulin resistance as an early intervention to prevent T2DM development.

With fasting glucose and fasting insulin as commonly used markers of T2DM risk and severity, reducing insulin resistance is currently the main target of intervention for T2DM (11) (23). It is well known that some of the most common interventions to reduce insulin resistance are to reduce body weight, increase exercise, and improve dietary intake (11). For the prevention and management of T2DM, dietary recommendations include increased intake of fruits, vegetables, whole grains, non-fat dairy products, legumes, and lean meats with reduced intake of sugars, sweets, red meats, and overall energy (24). Overall dietary changes such as these have long been considered more effective for disease risk reduction, with dietary quality offering a more comprehensive intervention for food and nutrient consumption than focusing on alteration of individual foods and nutrients (25). Due to differing food preferences, habits, availability, and the fact that humans do not consume foods or nutrients in isolation, improving overall diet quality is more efficacious for disease risk management and overall health improvements than recommending any one specific food or food group. For this reason, diet quality is growing in popularity within the field of nutritional epidemiology as a more practical tool, easing the translation of findings into non-research, healthcare-oriented settings (25) (26), and aiding in protocol and policy development (27) (28). The suggested changes in overall dietary patterns to prevent T2DM are well demonstrated to be effective in disease risk reduction through improvements in both fasting glucose and fasting insulin (29) (30) (31) (32). What is unknown, however, is how dietary quality may influence sphingolipid levels in the circulation. While mice studies provide evidence that ceramides and sphingomyelins are influenced by dietary intake of foods and nutrients (13) (33) (34) (35) (36) (37), human studies focused on diet and plasma sphingolipids are very limited.

One way in which dietary patterns have been shown to alter circulating sphingolipid concentrations in animal studies is through altered endogenous synthesis of sphingolipids. Experimental rat studies have shown that increased dietary cholesterol causes decreased total sphingomyelin and increased ceramide concentrations in VLDL, possibly due to increased activity of sphingomyelinase (33) (34). Diet-induced hyperlipidemia has also been shown to increase levels of phytosphingosine and dihydrosphingosine, two important factors in sphingolipid biosynthesis and metabolism (35). When sphingomyelin synthase 2 knockout mice were fed a high fat diet, their SM concentrations declined while the wild-type control mice's plasma levels increased; suggesting that high fat diets lead to increased sphingomyelin synthesis (36). Experimental studies in mice and nonhuman primates have shown that an atherogenic diet higher in fat and cholesterol increases sphingomyelin concentrations (especially the stearoyl and palmitoyl species) in LDL, hepatic tissue, and peripheral tissues (38) (39). It is thought that the breakdown of sphingomyelin could yield significant amounts of ceramides (4) (5), which is a known inhibitor of insulin sensitivity (13). This effect on sphingomyelin may also hold true for diets high in saturated fat, although not for diets high in polyunsaturated fats (40) (41). Another mouse-model experimental study revealed that an atherogenic diet is associated with an increase in total plasma ceramides, while increased exercise is associated with a decrease (37).

As outlined above, there is some evidence from animal studies to suggest that dietary patterns or factors influence sphingolipid biosynthesis, but experiments researching the association between overall diet quality or dietary patterns with sphingolipid measures in humans very limited. Studies are also lacking in at-risk minority populations; there have been no studies researching the association between diet quality and sphingolipid species in American Indian communities. Ceramides and sphingomyelins were measured in the SHFS cohort as part of an NIH-funded study of sphingolipids, diabetes, and diabetes-related CVD, providing an opportunity to explore potential diet-sphingolipid associations. This study investigates whether circulating plasma sphingolipid species, ceramides and sphingomyelins with different fatty acids, are associated with diet quality.

Methods and Procedures

Setting and Study Population

The SHFS is a population-based longitudinal study of the genetics and risk factors for

CVD in American Indian communities in Arizona, North Dakota, South Dakota and Oklahoma; it is the only large, genetic study of CVD risk factors in American Indians. Details of the study design have been described previously (42). Briefly, this study includes participants from the Strong Heart Study (SHS) and their extended families who were recruited into the SHFS. In 1996, extended family members of those in the SHS aged 35-74 years were recruited, creating the SHFS with a larger cohort of approximately 90 families (43). These participants were added with the primary goal of identifying genes that influence risks of CVD, T2DM, and obesity. Our study was conducted using data from the phase 4 exam, which is the baseline for the study. This exam included a personal interview, a Food Frequency Questionnaire (FFQ), physical examination, medication review, and extensive laboratory work-up. For this exam, men and women from 93 large families (n=2780) completed a baseline examination. Institutional review boards from each Indian Health Service region and communities approved the study, and written informed consent was obtained from all participants.

Of the 2780 participants who were enrolled at phase 4 of the SHFS, those who were missing sphingolipid measures (n=57), had prevalent diabetes (n=513), did not complete the FFQ (n=141), or were missing adjustment covariate data (n=44) were excluded, leaving 2,025 participants eligible for the analyses.

Primary Exposure: Diet Quality Indices

Diet quality was assessed using responses to a culturally adapted Block FFQ that asked about usual dietary intake during the previous year. The 119 question Block FFQ is widely-used and well-established, and has demonstrated validity and reliability in previous studies (44) (45). It included questions on how often in the past year a specific food item was consumed, along with the participant's usual serving size for that food item. For the purposes of the SHFS, additional foods commonly consumed by these American Indian populations were added to the FFQ. The items added were Spam, flour and corn tortillas, red and green chilies, fry bread, Indian tacos, menudo, pozole, and guysava. These additional items were used to provide a better understanding of overall nutrient content, which is an important part of accurately assessing diet quality (46).

Responses to the FFQ were used to calculate dietary scores for two diet quality indices: the Diabetes Dietary Index (DDI), and the Alternative Healthy Eating Index-2010 (AHEI). These

indices were chosen to maintain continuity and build on a previous thesis by Kauffman, S.A.E. (29), which also studied participants in the SHFS and used the same diet quality indices.

The DDI was created by Nettleton, J. et al (47) and was developed using foods previously associated with diabetes risk. It was harmonized across multiple cohorts in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium Nutrition Working Group (48). This index is a composite score of healthy foods (whole grains, fish, fruits, vegetables, and nuts/seeds) and unhealthy foods (red/processed meats, sweets, sugared beverages, and fried potatoes), especially from the point of view of diabetes risk. In large meta-analyses, higher scores of this index are inversely associated with lower levels of metabolic risk factors including fasting blood glucose, fasting plasma insulin, total cholesterol, serum LDL, lipoprotein ApoB, Body Mass Index (BMI), and waist-to-hip ratio (Supplemental Table 1) (47) (29) (48).

In addition to the DDI, diet quality was evaluated using the AHEI, which is a score based on adherence to the Dietary Guidelines for Americans (30). It was built as an alternative to the Healthy Eating Index (HEI), and has since been shown to be more associated with chronic disease than the HEI, particlarly coronary heart disease and T2DM. This score is built using intake of vegetables, fruit, nuts, soy, white meat, red meat, fiber, trans-saturated fat, polyunsaturated fat, saturated fat, multivitamin use, and alcohol consumption. Previous studies have shown that higher AHEI scores are inversely related to risk of all-cause mortality, major chronic disease (CVD, T2DM, cancer, chronic obstructive pulmonary disease, anxiety, & depression), high blood lipids (total cholesterol, LDL, & serum lipoprotein ApoB), physical impairment, and hip fracture (Supplemental Table 1) (29) (30) (31) (32) (49) (50) (51) (52) (53) (54).

Primary Outcome: Plasma Sphingolipids

Sphingolipid species levels were measured by liquid chromatography and mass spectrometry in EDTA plasma samples from fasting blood, in the laboratory of Dr. Hoofnagle at the University of Washington. A total of 15 sphingolipid species with a coefficient of variation of less than 20% were included in this analysis: ceramides with 16:0 (Cer-16), 18:0 (Cer-18), 20:0 (Cer-20), 22:0 (Cer-22), and 24:0 (Cer-24); sphingomyelins with 14:0 (SM-14), 16:0 (SM-16), 18:0 (SM-18), 20:0 (SM-20), 22:0 (SM-22), 24:0 (SM-24); glucosylceramides with 16:0

(GC-16), 22:0 (GC-22), 24:0 (GC-24); and lactosylceramide with 16:0 (LC-16). Coefficients of variation can be found in Supplemental Table 2.

Statistical Analysis

To evaluate associations of each diet quality score (*e.g.* the DDI score) with the sphingolipid species of interest (*e.g.,* Cer-20), a linear mixed model was used. Levels of the sphingolipids were log transformed due to right skewed distributions. For each sphingolipid species, the subject-specific level of that sphingolipid species, Z_{ij} a matrix of subject-specific adjustment variables, DDI_{ij} the subject-specific DDI score, s_i a family-specific random-effect to account for possible familial aggregation, r_{ij} the random subject-specific effect due to possible polygenic inheritance of factors governing sphingolipid levels (correlation within families modeled based on kinship coefficients), and e_{ij} a subject-specific independent random effect for the j^{th} subject within the i^{th} pedigree, were used to fit the following model:

$$\text{Log(Cer-20)} = \beta_0 + \beta_1(DDI_{ij}) + \gamma Z_{ij} + s_i + r_{ij} + e_{ij}$$

The parameter of interest was β_1, which represents the geometric mean ratio (fold difference) in each sphingolipid level (e.g., Cer-20) associated with a 10-point higher DDI score, given a fixed set of adjustment parameters. We present results for a 10 point higher DDI, which corresponds approximatively to a difference between the 90^{th} and 10^{th} percentiles of DDI. Models included adjustment terms for: age, sex, study center, education, cigarette smoking (never, former, current), physical activity, BMI, and waist circumference. BMI was log-transformed due to a right-skewed distribution. For participants missing physical activity information, we used the variables age, sex, education, body fat, and triglycerides to impute physical activity with the Fully Conditional Expectation method implemented by the MICE package in R.(1)

We evaluated interactions of DDI and AHEI with BMI, age, and sex by including a multiplicative term between diet quality index and BMI, age, and sex in the equation above. To adjust for multiple comparisons, the Bonferroni method was used to set the p-value for significance (n=15; $p<0.05/15 = 0.0033$).

Results

Participant Characteristics

Characteristics of the 2025 included participants, stratified by DDI and AHEI quartiles

are presented in Tables 1 and 3. Of these participants, 41% were women, and the mean age at baseline was 38 years. Mean weight was 87 kg, mean BMI was 30 kg/m^2, with a mean waist circumference of 100cm and a mean body fat percentage of 35%. Mean baseline insulin was 15 uU/mL, mean hemoglobin A1C was 5.4%, and mean fasting glucose was 94 mg/dL. Of all participants, 2% had diagnosed coronary heart disease, 25% had hypertension, 13% used antihypertension medication at baseline, and 3% used lipid lowering medication. At the study baseline, 41% had never smoked, 21% were former smokers, and 37% were current smokers. Of all participants, 63% reported alcohol use, and the average consumption among those who drink was 1.5 drinks per week.

Diet Quality Indices

The DDI is a composite score of healthy foods (whole grains, fish, fruits, vegetables, and nuts/seeds) and unhealthy foods (red/processed meats, sweets, sugared beverages, and fried potatoes), while the AHEI is built using slightly different foods plus macronutrients including intake of vegetables, fruit, nuts, soy, white meat, red meat, fiber, trans-saturated fat, polyunsaturated fat, saturated fat, multivitamin use, and alcohol consumption. Despite the differences in construction, scores for the DDI and AHEI were positively correlated explaining an estimated 50% of each other's variation (r=0.71; see Supplemental Figure 1).

Diabetes Dietary Index and sphingolipids

DDI scores ranged from 2 to 24 out of a total possible 27, with a score of 13.22 as the mean. Participant characteristics, stratified by DDI quartile (Q1: 2-11, Q2: 12-13, Q3: 14-16, Q4: 17-24) are presented in Table 1. Compared to Q1, participants in Q4 were more likely to be older (46 vs 32 years), have some or more college education (43 vs 33%), have higher plasma triglycerides (153 vs 140mg/dL), have hypertension (35 vs 20%), use antihypertensive medications (25 vs 7%), and be former smokers (26 vs 15%).

Table 1: Descriptive Statistics Stratified by DDI Quartiles

	Total	Q1	Q2	Q3	Q4
	(2 - 24)	(2 - 11)	(12 - 13)	(14 - 16)	(17 - 24)
N	2025	662	419	567	377
Sex (% male)	41%	44%	42%	41%	32%
Age (years)	38 (16)	32 (13)	35 (15)	40 (16)	46 (17)
Highest level of education					
Less than HS	31%	37%	34%	28%	20%
HS grad	36%	36%	34%	38%	36%

At least some college	23%	19%	24%	24%	28%
BA/BS	7%	6%	5%	8%	11%
≥ graduate school	3%	2%	3%	2%	4%
Weight (kg)	86 (23)	85 (23)	87 (22)	88 (23)	86 (22)
BMI (kg/m2)					
< 25	23%	29%	24%	20%	17%
25-29.9	29%	28%	28%	28%	36%
30-34.9	24%	22%	22%	25%	27%
≥ 35	24%	21%	26%	26%	21%
Waist circumference (cm)	100 (17)	98 (17)	99 (17)	102 (18)	101 (17)
Body fat (%)	35 (10)	34 (11)	35 (10)	36 (10)	36 (9)
Total Cholesterol (mg/dL)	181 (36)	177 (36)	178 (37)	185 (37)	185 (33)
LDL (mg/dL)	53 (15)	52 (14)	52 (15)	53 (15)	55 (17)
HDL (mg/dL)	100 (30)	98 (30)	99 (32)	103 (31)	100 (28)
Plasma Triglycerides (mg/dL)	146 (89)	140 (75)	139 (87)	154 (108)	153 (84)
Fibrinogen (mg/dL)	369 (82)	365 (82)	368 (80)	371 (82)	375 (87)
Baseline Insulin (uU/mL)	15 (15)	15 (13)	15 (13)	15 (16)	15 (17)
HbA1C (%)	5.4 (0.5)	5.3 (0.4)	5.3 (0.4)	5.5 (0.6)	5.4 (0.5)
Fasting Glucose (mg/dL)	94 (10)	93 (10)	93 (10)	95 (10)	94 (11)
CHD (%)	2%	1%	1%	2%	4%
Hypertension	25%	20%	18%	28%	35%
Antihypertensive medication use	13%	7%	9%	14%	25%
Lipid-lowering medication	3%	1%	2%	4%	5%
Smoking Status					
Never	41%	40%	39%	41%	46%
Former	21%	15%	22%	24%	26%
Current	38%	45%	39%	34%	28%
Alcoholic drinks/week	0.6 (1.7)	0.7 (2.3)	0.6 (1.5)	0.6 (1.2)	0.3 (1.1)

Results from linear mixed models of DDI scores with sphingolipids are presented in Table 2. After adjustment for age, sex, study center, education, cigarette smoking, physical activity, BMI, and waist circumference, a DDI score in the 90th percentile was associated with 7.2% lower Cer-18 levels (Geometric mean [GM] ratio: 0.93; 95%CI: 0.88-0.98) and 3.2% higher SM-20 (GM Ratio: 1.03; 95%CI: 1.000-1.065) when compared with a DDI score in the 10th percentile. While $p<0.05$ for these results, none of these associations reached the pre-specified threshold of significance.

Table 2: DDI sphingolipid results

	Est.	SE	Ratio of GM*	95 CI	p-value
Cer-16	-0.0346	0.018	0.9659	(0.9324 , 1.0006)	0.0543
Cer-18	-0.0745	0.027	0.9282	(0.8804 , 0.9787)	0.0058

Cer-20	-0.0474	0.025	0.9537	(0.9081 , 1.0016)	0.058
Cer-22	-0.0289	0.022	0.9715	(0.9305 , 1.0143)	0.1891
Cer-24	-0.0185	0.019	0.9817	(0.9458 , 1.0189)	0.3304
SM-14	0.0386	0.024	1.0393	(0.9915 , 1.0894)	0.1083
SM-16	-0.002	0.012	0.9981	(0.9748 , 1.0218)	0.8709
SM-18	-0.0126	0.015	0.9874	(0.9588 , 1.0169)	0.3993
SM-20	0.0318	0.016	1.0323	(1.0004 , 1.0652)	0.047
SM-22	0.014	0.016	1.0141	(0.9828 , 1.0464)	0.3817
SM-24	0.0164	0.016	1.0165	(0.9851 , 1.0489)	0.3056
GC-181_160	-0.0338	0.018	0.9668	(0.9332 , 1.0015)	0.0605
GC-181_220	-0.0192	0.018	0.981	(0.947 , 1.0163)	0.2875
GC-181_240	-0.0236	0.019	0.9767	(0.941 , 1.0138)	0.2153
LC-181_160	-0.0187	0.016	0.9815	(0.9512 , 1.0128)	0.2427

*Fold change in geometric mean sphingolipid level associated with 10 point higher DDI score (difference between the 10th & 90th %iles)

Alternative Healthy Eating Index-2010 and sphingolipids

AHEI scores ranged from 20 to 78 out of a total possible 110, with a score of 44.58 as the mean. Participant characteristics, stratified by AHEI quartile are presented in Table 3. As observed with the DDI score, compared to Q1, participants in Q4 were more likely to be older (42 vs 32 years), have some or more college education (40 vs 25%), have higher plasma triglycerides (158 vs 137 mg/dL), have hypertension (30 vs 18%), use antihypertensive medications (19 vs 7%), and be former smokers (27 vs 17%).

Table 3: Descriptive Statistics Stratified by AHEI Quartiles

	Total	Q1	Q2	Q3	Q4
AHEI range	(20 - 78)	(20 - 38)	(38 - 44)	(44 - 51)	(51 - 78)
N	2069	507	506	506	506
Sex (% male)	41%	46%	41%	37%	39%
Age (years)	38 (16)	32 (14)	37 (16)	39 (16)	42 (17)
Highest level of education					
Less than HS	31%	38%	33%	28%	25%
HS grad	36%	37%	35%	37%	36%
At least some college	23%	18%	23%	24%	27%
BA/BS	7%	5%	8%	8%	9%
≥ graduate school	3%	2%	2%	3%	4%
Weight (kg)	86 (23)	86 (22)	87 (22)	86 (22)	87 (24)
BMI (kg/m^2; mean)					
< 25	23%	28%	21%	23%	21%
25-29.9	29%	26%	29%	30%	33%
30-34.9	24%	23%	24%	25%	23%
≥ 35	24%	22%	27%	23%	24%
Waist circumference (cm)	100 (17)	98 (18)	101 (18)	99 (16)	101 (18)

Body fat (%)	35 (10)	34 (10)	36 (10)	36 (10)	36 (10)
Total Cholesterol (mg/dL)	181 (36)	176 (38)	181 (36)	183 (35)	184 (36)
LDL (mg/dL)	53 (15)	51 (14)	52 (14)	53 (15)	54 (17)
HDL (mg/dL)	100 (30)	98 (32)	100 (30)	101 (30)	100 (30)
Plasma Triglycerides (mg/dL)	146 (89)	137 (74)	143 (83)	148 (91)	158 (105)
Fibrinogen (mg/dL)	369 (82)	363 (83)	373 (79)	370 (82)	369 (85)
Baseline Insulin (uU/mL)	15 (15)	15 (13)	15 (14)	16 (16)	15 (17)
HbA1C (%)	5.4 (0.5)	5.3 (0.4)	5.4 (0.4)	5 (1)	5 (1)
Fasting Glucose (mg/dL)	94 (10)	93 (10)	94 (11)	95 (10)	94 (10)
CHD (%)	2%	1%	3%	2%	2%
Hypertension	25%	18%	21%	29%	30%
Antihypertensive Medication Use	13%	7%	10%	14%	19%
Lipid-lowering medication	3%	1%	3%	3%	4%
Smoking Status					
Never	41%	42%	42%	42%	39%
Former	21%	17%	21%	20%	27%
Current	38%	41%	37%	38%	34%
Alcoholic drinks/week	0.6 (1.7)	0.6 (1.8)	0.5 (1.4)	0.6 (2.3)	0.5 (0.9)

Results from linear mixed models of AHEI scores with sphingolipids are presented in Table 4. After adjustment for age, sex, study center, education, cigarette smoking, physical activity, BMI, and waist circumference, an AHEI score in the 90th percentile was associated with a 7.3% higher SM-14 (GM Ratio: 1.07; 95%CI: 1.028-1.121), 3.1% higher SM-20 (GM Ratio: 1.03; 95%CI: 1.003-1.06), and 4.3% higher SM-24 (GM Ratio: 1.04; 95%CI: 1.013-1.074) when compared with those in the 10th percentile. While for these results $p<0.05$, only the association with SM-14 met the pre-specified threshold for significance.

Table 4: AHEI sphingolipid results

	Est.	SE	Ratio of GM*	95 CI	p-value
Cer-16	-0.0018	0.017	0.9982	(0.9654 , 1.032)	0.9134
Cer-18	-0.0372	0.025	0.9634	(0.9174 , 1.0118)	0.1363
Cer-20	-0.0219	0.023	0.9783	(0.9352 , 1.0234)	0.34
Cer-22	0.0023	0.02	1.0023	(0.9638 , 1.0424)	0.9085
Cer-24	0.0212	0.018	1.0214	(0.986 , 1.0581)	0.239
SM-14	0.0706	0.022	1.0732	(1.0279 , 1.1205)	0.0013
SM-16	0.0137	0.011	1.0138	(0.9922 , 1.0359)	0.2134
SM-18	-0.0082	0.014	0.9918	(0.965 , 1.0194)	0.5582
SM-20	0.0308	0.014	1.0313	(1.0034 , 1.06)	0.0279
SM-22	0.0228	0.0143	1.0231	(0.9949 , 1.0521)	0.1099
SM-24	0.0424	0.015	1.0433	(1.013 , 1.0744)	0.0048
GC-181_160	-0.0068	0.016	0.9932	(0.9625 , 1.0248)	0.6686
GC-181_220	-0.0102	0.016	0.9898	(0.9592 , 1.0213)	0.5219
GC-181_240	0.0048	0.017	1.0048	(0.9719 , 1.0389)	0.7777
LC-181_160	-0.012	0.014	0.9881	(0.9614 , 1.0156)	0.3934

*Fold change in geometric mean sphingolipid level associated with 24 point higher AHEI score (difference between the 10th & 90th %iles)

Interactions

We evaluated whether associations of the diet scores with sphingolipid levels differed by BMI, age, or sex (Tables 5-7). We found evidence that BMI influenced the association of DDI with four sphingomyelins (at $p< 0.003$), such that higher DDI was associated with higher SM-14, SM-16, SM-20, and SM-24 levels among participants with normal BMI, but not at higher BMIs (Table 5). In addition, we found evidence that higher DDI was associated with higher SM-24 levels among younger participants, and lower SM-24 levels among older participants (Table 6). In AHEI models, BMI appeared to influence the association between AHEI and two sphingolipids, including SM-14 and LC-16 (Table 7). All the interactions met the pre-specified threshold for significance. There was no evidence that sex influenced any associations.

Table 5: Geometric Mean Ratios of Sphingolipid Levels per 10-Point Higher DDI, by BMI

	20	25	30	35	p-value
Cer-16	1.01 (0.95 , 1.09)	0.99 (0.94 , 1.03)	0.96 (0.93 , 1.00)	0.94 (0.90 , 0.99)	0.0988
Cer-18	0.99 (0.89 , 1.09)	0.95 (0.89 , 1.02)	0.92 (0.88 , 0.98)	0.90 (0.84 , 0.96)	0.1443
Cer-20	1.02 (0.93 , 1.12)	0.98 (0.92 , 1.04)	0.95 (0.90 , 1.00)	0.93 (0.87 , 0.98)	0.103
Cer-22	1.03 (0.95 , 1.12)	1.00 (0.95 , 1.05)	0.97 (0.93 , 1.01)	0.95 (0.90 , 1.00)	0.0973
Cer-24	1.06 (0.98 , 1.14)	1.01 (0.97 , 1.06)	0.98 (0.94 , 1.02)	0.95 (0.91 , 1.00)	0.019
SM-14	1.22 (1.12 , 1.33)	1.11 (1.05 , 1.17)	1.03 (0.98 , 1.08)	0.97 (0.91 , 1.02)	<0.0001
SM-16	1.06 (1.01 , 1.10)	1.02 (0.99 , 1.05)	0.99 (0.97 , 1.02)	0.97 (0.94 , 1.00)	0.0024
SM-18	1.05 (1.00 , 1.11)	1.01 (0.98 , 1.05)	0.98 (0.96 , 1.01)	0.96 (0.92 , 1.00)	0.0081
SM-20	1.12 (1.06 , 1.19)	1.07 (1.03 , 1.11)	1.03 (1.00 , 1.06)	0.99 (0.96 , 1.03)	0.0008
SM-22	1.09 (1.02 , 1.15)	1.04 (1.01 , 1.08)	1.01 (0.98 , 1.04)	0.98 (0.95 , 1.02)	0.0073
SM-24	1.10 (1.04 , 1.17)	1.05 (1.01 , 1.09)	1.01 (0.98 , 1.05)	0.98 (0.94 , 1.02)	0.0029
GC-16	1.03 (0.97 , 1.10)	0.99 (0.95 , 1.04)	0.96 (0.93 , 1.00)	0.94 (0.90 , 0.98)	0.0211
GC-22	1.03 (0.97 , 1.10)	1.00 (0.96 , 1.05)	0.98 (0.94 , 1.01)	0.96 (0.92 , 1.00)	0.0748
GC-24	1.04 (0.97 , 1.12)	1.00 (0.96 , 1.05)	0.97 (0.94 , 1.01)	0.95 (0.91 , 0.99)	0.0273
LC-16	1.05 (0.99 , 1.11)	1.01 (0.97 , 1.05)	0.98 (0.95 , 1.01)	0.95 (0.92 , 0.99)	0.0084

Table 6: Geometric Mean Ratios of Sphingolipid Levels per 10-Point Higher DDI, By Age (In Years)

	20	40	60	80	p-value
Cer-16	0.98 (0.93 , 1.03)	0.96 (0.93 , 1.00)	0.95 (0.89 , 1.01)	0.93 (0.85 , 1.03)	0.4337
Cer-18	0.91 (0.84 , 0.98)	0.93 (0.88 , 0.98)	0.95 (0.87 , 1.04)	0.98 (0.85 , 1.13)	0.4298
Cer-20	0.95 (0.88 , 1.02)	0.95 (0.91 , 1.00)	0.96 (0.88 , 1.04)	0.96 (0.85 , 1.10)	0.8551
Cer-22	0.96 (0.90 , 1.02)	0.97 (0.93 , 1.02)	0.99 (0.92 , 1.06)	1.00 (0.89 , 1.12)	0.5815
Cer-24	1.00 (0.95 , 1.06)	0.98 (0.94 , 1.02)	0.96 (0.90 , 1.02)	0.94 (0.85 , 1.04)	0.355
SM-14	1.06 (0.99 , 1.13)	1.04 (0.99 , 1.09)	1.02 (0.94 , 1.09)	0.99 (0.88 , 1.12)	0.4468

SM-16	1.03 (1.00 , 1.07)	0.99 (0.97 , 1.02)	0.96 (0.92 , 1.00)	0.93 (0.87 , 0.98)	0.0088
SM-18	1.01 (0.97 , 1.05)	0.99 (0.96 , 1.02)	0.96 (0.92 , 1.01)	0.94 (0.87 , 1.02)	0.1809
SM-20	1.04 (0.99 , 1.09)	1.03 (1.00 , 1.06)	1.02 (0.97 , 1.08)	1.02 (0.94 , 1.10)	0.68
SM-22	1.04 (1.00 , 1.09)	1.01 (0.98 , 1.04)	0.98 (0.93 , 1.03)	0.95 (0.88 , 1.03)	0.111
SM-24	1.07 (1.02 , 1.12)	1.01 (0.98 , 1.04)	0.95 (0.90 , 1.00)	0.90 (0.82 , 0.98)	0.0018
GC-16	1.01 (0.96 , 1.06)	0.96 (0.93 , 1.00)	0.92 (0.87 , 0.97)	0.87 (0.80 , 0.96)	0.0184
GC-22	1.01 (0.96 , 1.06)	0.98 (0.94 , 1.01)	0.95 (0.89 , 1.00)	0.91 (0.83 , 1.00)	0.1066
GC-24	1.02 (0.96 , 1.07)	0.97 (0.94 , 1.01)	0.93 (0.88 , 0.99)	0.89 (0.81 , 0.98)	0.04
LC-16	1.02 (0.98 , 1.07)	0.98 (0.95 , 1.01)	0.93 (0.89 , 0.98)	0.89 (0.82 , 0.97)	0.0104

Table 7: Geometric Mean Ratios of Sphingolipid Levels per 10-Point Higher AHEI, by BMI

	20	25	30	35	p-value
Cer-16	1.04 (0.97 , 1.10)	1.01 (0.97 , 1.05)	1.00 (0.96 , 1.03)	0.98 (0.94 , 1.02)	0.1827
Cer-18	0.99 (0.90 , 1.09)	0.97 (0.92 , 1.03)	0.96 (0.92 , 1.01)	0.95 (0.89 , 1.01)	0.5125
Cer-20	1.01 (0.92 , 1.10)	0.99 (0.94 , 1.05)	0.98 (0.93 , 1.02)	0.97 (0.91 , 1.02)	0.4512
Cer-22	1.02 (0.95 , 1.10)	1.01 (0.96 , 1.06)	1.00 (0.96 , 1.04)	0.99 (0.94 , 1.04)	0.541
Cer-24	1.06 (0.99 , 1.14)	1.04 (1.00 , 1.08)	1.02 (0.98 , 1.06)	1.00 (0.96 , 1.05)	0.1645
SM-14	1.20 (1.11 , 1.31)	1.13 (1.07 , 1.18)	1.06 (1.02 , 1.11)	1.02 (0.96 , 1.07)	0.0011
SM-16	1.06 (1.01 , 1.10)	1.03 (1.00 , 1.06)	1.01 (0.99 , 1.03)	0.99 (0.97 , 1.02)	0.0222
SM-18	1.02 (0.97 , 1.08)	1.00 (0.97 , 1.04)	0.99 (0.96 , 1.02)	0.98 (0.94 , 1.01)	0.1581
SM-20	1.07 (1.01 , 1.13)	1.05 (1.01 , 1.08)	1.03 (1.00 , 1.06)	1.01 (0.98 , 1.05)	0.1157
SM-22	1.05 (1.00 , 1.11)	1.04 (1.00 , 1.07)	1.02 (0.99 , 1.05)	1.01 (0.97 , 1.05)	0.2047
SM-24	1.08 (1.02 , 1.15)	1.06 (1.02 , 1.10)	1.04 (1.01 , 1.07)	1.02 (0.99 , 1.06)	0.1278
GC-16	1.05 (0.99 , 1.12)	1.02 (0.98 , 1.06)	0.99 (0.96 , 1.02)	0.97 (0.93 , 1.01)	0.037
GC-22	1.03 (0.97 , 1.09)	1.01 (0.97 , 1.05)	0.99 (0.96 , 1.02)	0.97 (0.93 , 1.01)	0.151
GC-24	1.06 (0.99 , 1.13)	1.03 (0.99 , 1.07)	1.00 (0.97 , 1.04)	0.98 (0.94 , 1.02)	0.068
LC-16	1.06 (1.01 , 1.12)	1.02 (0.98 , 1.05)	0.98 (0.96 , 1.01)	0.95 (0.92 , 0.99)	0.0019

Discussion

In this study of American Indian participants, we provide evidence for the associations of diet quality with higher levels of SM-14. Additionally, our results suggest that BMI modifies the association of DDI scores with SM-14, SM-16, SM-20, and SM-24, as well the association of AHEI scores with SM-14 and LC-16. There is also evidence that age influences the interaction of DDI with SM-24, with differing results according to age.

Previous studies reported that higher diet quality is associated with lower fasting insulin (30) (31), although it has yet to be established how diet quality mechanistically influences fasting insulin and T2DM risk. Additionally, results within the SHFS reported via personal communication has shown that sphingomyelins (SM-18, SM-20, SM-22, SM-24) are associated

with lower fasting insulin in those with normal BMI (19). Our study connects these two findings by providing evidence that higher diet quality is associated with higher sphingomyelins in those with normal BMI, providing a possible mechanistic association between diet quality and fasting insulin. If higher diet quality is associated with higher plasma sphingomyelins, and higher plasma sphingomyelins are associated with reduced fasting insulin, this may show that diet has a possible effect on metabolism of sphingolipids and insulin sensitivity, offering a mechanism for diet's relation to insulin action and T2DM risk.

Past research shows that sphingomyelins containing saturated acyl-chains are associated with higher BMIs, and weight loss leads to reductions in serum levels of sphingomyelins only for those with a BMI of 25 or greater (12). Our findings are a valuable addition to this literature, providing evidence that BMI influences associations between diet quality and circulating sphingolipids, with greater influence seen at lower BMIs. Our study was ancilliary to another study of sphingolipids and T2DM in the SHFS by Lemaitre et al. (19). In this unpublished parent study, there is evidence of effect modification of the association between sphingomyelin and fasting insulin by BMI, using the same population and sphingolipid data as our study. Their results showed that higher levels of SM-18, SM-20, SM-22, and SM-24 are negatively associated with fasting insulin only for those with a BMI in the "normal" range; as BMI increased, the association between sphingomyelins and fasting insulin changed from negative, to null to positive. It is possible that at a higher BMI, endogenous production of sphingomyelins is altered, changing the effect diet may have on sphingolipid production. This may point to a greater need to tailor diet recommendations based on metabolic status if plasma sphingolipids are an intervention target. More research into the relationship between BMI and sphingolipids in the context of diet quality is needed to explore potential causes for these effects, their association with BMI, and what that might mean for disease prevention and treatment.

Our results indicate a relationship between diet quality and circulating sphingolipids in humans, providing evidence from a large population-based study to a body of evidence that is generally lacking. There are very few studies of diet and sphingolipids in humans; a small pilot study by Airhart et al. (55) using subjects with diagnosed T2DM (n=16) and sphingolipids as a secondary outcome, showed that those randomized to eat a diet high in medium-chain fatty acids had lower plasma levels of SM-12:0, SM-14:0, SM -15:0, SM -16:0, SM -20:0, and SM -23:1 than controls. Similarly, a randomized-controlled trial (n=200) by Laniken et al. (56), compared

participants assigned to receive a healthy Nordic diet (rich in low-fat dairy and meats, whole grains, and produce) to participants assigned to receive the control of an average Nordic diet (rich in low-fiber grains, high-fat meats and dairy, and limited produce). Their results showed that participants on the healthy Nordic diet had higher levels of SM-24 at 12 weeks, than those in the control group, although the researchers looked at many lipids and this difference was not observed at other time points, raising the possibility that their findings may be due to chance. These studies suggest an association between diet and sphingolipids, but are limited due to small sample size, secondary associations, transient results, and numerous tests. Our study brings evidence from a large observation study, as we also noted a positive association between diet and SM-14, SM-20, and SM-24, with SM-14 being the most notable result. Although it has been hypothesized that certain species of sphingolipids (*i.e.* Cer-16), rather than classes of sphingolipids (*i.e.* all ceramides) have different roles within the body (20) (21), the association trends we observed suggest that diet quality may affect plasma sphingolipids similarly based on their class instead of their fatty acid species. We observed that higher diet quality is negatively associated with plasma ceramides, while positively associated with plasma sphingomyelins. Research shows that higher ceramides are associated with higher fasting insulin, where sphingomyelins have the opposite association, supporting the concept of class-specific effects rather than species-specific effects (13) (14) (19) (20) (21). It is possible that these class effects are due in part to sphingolipid biosynthesis; in our measurements, all sphingolipids were correlated to sphingolipids of their same class (*i.e.* Cer-16 is associated with all other ceramides; Supplemental Table 3), suggesting possible coordinated synthesis and/or regulation.

While our results do show an association between diet quality and SM-14, they need to be confirmed by dietary trials to establish an effect of diet on sphingolipids. There are several possible mechanisms for the association between diet quality and plasma sphingolipids, the first of which being altered free-fatty acid availability due to altered fatty acid intake through dietary changes. Since sphingolipids consist of several species that differ by the composition of their fatty acid component (1), it is possible that the abundance of certain fatty acids leads to the production of more sphingolipids with that fatty acid component. The second possible mechanism is altered enzyme expression that results in altered production of sphingolipids. High-fat diet-induced hyperlipidemia in rats has been shown to increase levels of

phytosphingosine and dihydrosphingosine, which provide the backbone in sphingolipid biosynthesis and metabolism (35).

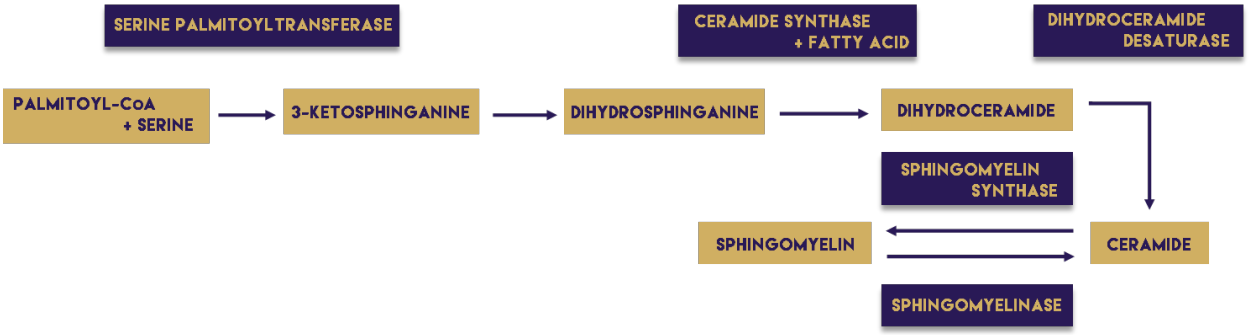
In our study, scores for the DDI and AHEI were positively correlated, accounting for 50% of their variability. They yielded differing results, which may be in part because the DDI was constructed using foods specifically related to T2DM risk, while the AHEI was constructed particularly for measuring adherence to the Dietary Guidelines for Americans. While both indices are built using intake of fruits, vegetables, and read meat, the rest of the components vary. For instance, the DDI includes foods such as sweets, sugared beverages, and fried potatoes (57) (58) (59). In contrast, the AHEI includes foods and food components such as trans-saturated fat, polyunsaturated fat, and saturated fat, in addition to supplement use and alcohol consumption (30). Between the diet quality scores, the only common association seen was with SM-20, although that did not meet the threshold for significance.

Strengths of this study include the sample size, examining nearly twice as many participants as the largest study that included both diet and plasma ceramides measures (60). This study includes participants from communities that are at a high risk of diabetes relative to the overall population (9) (23), possibly increasing our ability to detect associations of dietary intake with sphingolipids, if they are present. To better accommodate cultural food preferences and traditions, a modified FFQ was used to measure consumption of ethnic foods not usually included, which enabled a more accurate assessment of dietary intake in the study this population. Another major strength is the use of two diet quality indices that were constructed for different purposes, which may have allowed for greater insight into specific dietary patterns or components that are associated with plasma sphingolipids.

Our study also has limitations. The observational nature of this cross-sectional study prevents our ability to assess a causal relationship between dietary intake and sphingolipid levels. Due to potential misclassification of dietary intake with the FFQ, and measurement error in the sphingolipid assays, true associations may have been difficult to detect. Due to the number of sphingolipids investigated, the associations we observed may be due to chance; to account for this we used the Bonferroni method ($p<0.0033$), and with this criterion, the only significant primary finding is the association between the AHEI and SM-14. However, findings of interactions met the threshold for significance. Lastly, the study findings may not be generalizable to other populations.

Future studies are needed to replicate our findings in other populations and to establish temporality of the diet-sphingolipid association. Further, the role of DHA, potassium, fish consumption, fatty acid composition, and cholesterol intake should also be investigated, as these food and nutrients have been associated with plasma sphingolipids in the past (56). Differences between the DDI and AHEI should be explored further to determine if differences in results were due to index construction. Studies are needed to explore the influence of diet and diet quality on specific enzymes involved in sphingomyelin and ceramide synthesis (Figure 2), such as serine palmitoyltransferase, ceramide synthase, dihydroceramide desaturase, sphingomyelin synthase 2, and sphingomyelinase, as dietary changes may result in altered enzyme expression (12) (35) (36). Further exploration into the clinical relevance of BMI's effect on the associations between diet quality and circulating sphingolipids should be conducted.

Figure 2. De Novo Biosynthesis of Ceramides and Sphingomyelins



In summary, we obtained evidence that BMI modifies the association of diet quality with plasma levels of SM-14, SM-16, SM-20, SM-24, and LC-16. We also obtained evidence that suggests higher diet quality may be associated with higher circulating SM-14. Together, these results offer a hypothetical mechanism for the effects of diet on fasting insulin and T2DM risk for those with a "normal" BMI.

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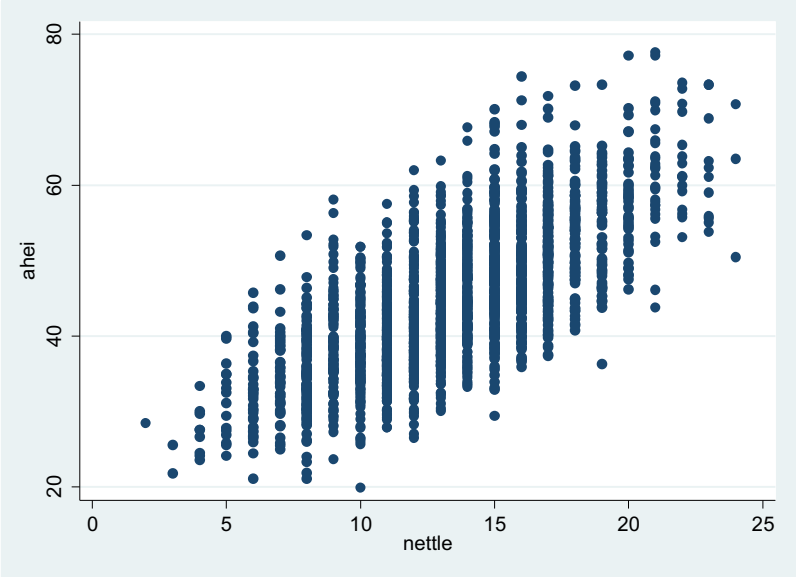
Supplemental Materials

Supplemental Table 1: Disease Risk Associations with Diet Quality Indices	
Index	Findings
DDI	- Higher scores are associated with lower overall fasting glucose and fasting insulin (47) - Higher scores are associated with lower BMI and BMI-adjusted waist-to-hip ratio (48) - Higher scores are associated with lower total cholesterol, serum LDL, and lipoprotein ApoB (29)
AHEI	- Higher scores associated with lower risk of: all-cause mortality, incident CVD, death from CVD, incident cancer, death from cancer, incident type 2 diabetes, heart failure, coronary heart disease, stroke, acute myocardial infarction (fatal and nonfatal), chronic obstructive pulmonary disease, hip fracture, physical impairment, anxiety, and depression (30) (31) (49) (50) (51) (52) (53) (54) - Higher scores associated with lower risk for colorectal, esophageal, pancreatic, prostate cancer, and estrogen-receptor-negative breast cancer (30) (31) - Higher scores are associated with waist circumference, total cholesterol, serum LDL, lipoprotein ApoB, and 10-year risk for coronary heart disease (29) (32) - More strongly associated with chronic disease risk than the Healthy Eating Index, particularly regarding coronary heart disease and diabetes (30)

Supplemental Table 2: Sphingolipid Coefficients of Variation	
Species	**%CV**
Cer-16	18.4%
Cer-18	21.2%
Cer-20	19.5%
Cer-22	13.9%
Cer-24 18:1_24	15.5%
Cer-24 18:2_24	12.1%
SM-14	18.2%
SM-16	11.5%
SM-18	12.2%
SM-20	12.6%
SM-22	12.4%
SM-24	13.3%
GC-18:1_16:0	13.4%
GC-18:1_22:0	13.9%
GC-18:1_24:0	16.0%
LC-18:1_16:0	14.4%

Supplemental Figure 1: Scatterplot of DDI (x-axis) and AHEI (y-axis)



Pearson correlation: r = 0.7101

Spearman correlation: rho= 0.6997

Supplemental Table 3: Correlations Between Sphingolipid Species*

	Cer-16	Cer-18	Cer-20	Cer-22	Cer-24	GC-16	GC-22	GC-24	LC-16	SM-14	SM-16	SM-18	SM-20	SM-22
Cer-16	1.0													
Cer-18	0.74	1.0												
Cer-20	0.72	0.74	1.0											
Cer-22	0.72	0.59	0.73	1.0										
Cer-24	0.72	0.58	0.61	0.87	1.0									
GC-16	0.51	0.39	0.29	0.31	0.40	1.0								
GC-22	0.36	0.20	0.24	0.44	0.44	0.67	1.0							
GC-24	0.40	0.23	0.23	0.37	0.48	0.72	0.85	1.0						
LC-16	0.40	0.27	0.26	0.29	0.32	0.59	0.51	0.49	1.0					
SM-14	0.59	0.47	0.48	0.62	0.66	0.48	0.42	0.45	0.38	1.0				
SM-16	0.70	0.49	0.48	0.55	0.62	0.69	0.56	0.60	0.63	0.71	1.0			
SM-18	0.57	0.71	0.54	0.49	0.53	0.55	0.40	0.41	0.49	0.65	0.75	1.0		
SM-20	0.48	0.44	0.55	0.65	0.59	0.35	0.48	0.40	0.40	0.78	0.66	0.72	1.0	
SM-22	0.51	0.37	0.52	0.70	0.59	0.34	0.51	0.43	0.42	0.66	0.67	0.60	0.89	1.0
SM-24	0.49	0.32	0.41	0.60	0.60	0.38	0.49	0.52	0.42	0.64	0.70	0.57	0.79	0.92

*Shaded areas represent correlations of species within same class

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