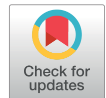

Circulating Sphingolipids, Insulin, HOMA-IR, and HOMA-B: The Strong Heart Family Study

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Experimental studies suggest ceramides may play a role in insulin resistance. However, the relationships of circulating ceramides and related sphingolipids with plasma insulin have been underexplored in humans. We measured 15 ceramide and sphingomyelin species in fasting baseline samples from the Strong Heart Family Study (SHFS), a prospective cohort of American Indians. We examined sphingolipid associations with both baseline and follow-up measures of plasma insulin, HOMA of insulin resistance (HOMA-IR), and HOMA of β-cell function (HOMA-B) after adjustment for risk factors. Among the 2,086 participants without diabetes, higher levels of plasma ceramides carrying the fatty acids 16:0 (16 carbons, 0 double bond), 18:0, 20:0, or 22:0 were associated with higher plasma insulin and higher HOMA-IR at baseline and at follow-up an average of 5.4 years later. For example, a twofold higher baseline concentration of ceramide 16:0 was associated with 14% higher baseline insulin (*P* < 0.0001). Associations between sphingomyelin species carrying 18:0, 20:0, 22:0, or 24:0 and insulin were modified by BMI (*P* < 0.003): higher levels were associated with lower fasting insulin, HOMA-IR, and HOMA-B among those with normal BMI. Our study suggests lowering circulating ceramides might be a target in prediabetes and targeting circulating sphingomyelins should take into account BMI.

The prevalence of type 2 diabetes is a global health issue with an estimated 347 million adults afflicted worldwide (1). Diabetes imparts a high burden of morbidity and comorbidities. Although overall diabetes incidence may have plateaued in the U.S., minority subgroups still experience high risk of diabetes with continued increases in incidence (2,3).

High levels of fasting plasma insulin, reflecting insulin resistance, are an early event in the progression to diabetes and suggested to be an underlying cause of type 2 diabetes (4–6). The search for metabolites that influence insulin levels in people without diabetes may lead to novel approaches to preventive efforts (7).

There is strong evidence from animal experimental studies and in vitro work that ceramides are implicated in insulin resistance (8,9). However, the influence of ceramides and related sphingolipids on insulin resistance in humans has received limited attention.

Experimental studies suggest that the saturated fatty acid that is acylated to ceramides influences ceramide biological activities (10). We have shown that circulating very long-chain saturated fatty acids (with 20 carbons or more) are associated with lower fasting insulin and lower risk of diabetes (11). Very long-chain saturated fatty acids are largely a component of sphingolipids, including sphingomyelins and ceramides. Whether circulating sphingolipids with very long-chain saturated fatty acids are associated with lower insulin resistance is not known.

The objective of the study was to determine the associations between circulating ceramide and sphingomyelin species with different saturated fatty acids and fasting plasma insulin and related markers of insulin homeostasis.

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See accompanying article, p. 1457.

We carried out this investigation in the Strong Heart Family Study (SHFS), a well-characterized cohort study conducted in a population at high risk of diabetes (12).

RESEARCH DESIGN AND METHODS

Study Design

We used plasma samples from the SHFS cohort to measure sphingolipids and investigate associations with insulin and other markers both cross-sectionally and prospectively.

Study Population

The SHFS is a family-based cohort study of risk factors for cardiovascular disease in several American Indian communities in Arizona, North Dakota, South Dakota, and Oklahoma. The SHFS includes a baseline examination conducted in 2001–2003 and a follow-up examination in 2007–2009. Details of the study design were previously reported (13). The institutional review boards (Indian Health Services of Rapid City, SD, Phoenix, AZ, and Oklahoma City, OK; MedStar and the University of Oklahoma; and the University of Washington) and each participating tribe approved the study, and written informed consent was obtained from all participants at enrollment. At baseline, the cohort consisted of 2,768 participants in 92 families. For this investigation, we excluded 55 participants without available baseline samples, 510 with baseline diabetes, 67 without plasma fasting insulin, and 50 with missing covariate information. The remaining 2,086 participants were included in the analyses of baseline data. For the analyses of follow-up data, we further excluded 201 participants with diabetes at the follow-up examination because we were interested in the potential role of sphingolipids in hyperinsulinemia preceding the progression to diabetes, 239 participants without follow-up examination, and 86 without insulin measurements.

Data Collection

The baseline and follow-up examinations included a physical examination, laboratory testing, medication review, a 1-week pedometer log, and an in-person interview to collect information on medical conditions, education, smoking, and alcohol consumption. BMI was calculated as body weight divided by height squared (kg/m^2). Diabetes was defined as use of insulin or oral antidiabetes medication or a fasting plasma glucose concentration ≥126 mg/dL. Blood samples were collected after a 12-h overnight fast and processed and stored at −70°C. Plasma insulin was measured using a modified version of the Morgan and Lazarow radioimmunoassay at both examinations (12).

Measurement of Sphingolipids

We focused on sphingolipids that carried a saturated fatty acid acylated to the sphingoid backbone, including palmitic acid (16:0 [16 carbons, 0 double bonds]), stearic acid (18:0), arachidic acid (20:0), behenic acid (22:0), and lignoceric acid (24:0). The sphingolipids were measured on baseline fasting plasma samples that had been stored at −70°C with the following method. Lipids were extracted using organic protein precipitation in a mixture of methyl *tert*-butyl ether, methanol, and isopropanol. For each sample, 10 μL was pipetted into the appropriate well of a 96–deep well polypropylene microtiter plate (Masterblock; Greiner Bio-One, cat. no. 780270). In a chemical fume hood, 190 μL of precipitation solvent was added to each well using a multichannel pipet. The plate was sealed with a MicroLiter silicone cap mat with sprayed-on PTFE barrier (Wheaton, cat. no. 07–0061N), placed in a plastic Ziploc bag, and mixed on a multitube vortex (VWR) for 5 min at speed 10. Subsequently, in a fume hood, a 10-μm glass filter plate (Captiva; Agilent, cat. no. A596401000) was placed above a new Masterblock plate. Using a multichannel pipet, the samples were transferred from the precipitation plate into the filter plate and allowed to flow through using gravity (approximately 50 μL flows through the membrane filter in each well). The filter plate was carefully removed and discarded. To each sample in the new Masterblock plate, 450 μL of 65% methanol/25% isopropanol (v:v) was added and mixed by pipetting up and down 10 times with an electronic multichannel pipet. The plate was sealed and then a volume of 5 μL was injected using an autosampler (samples were cooled at 8°C) and resolved using reversed-phase chromatography at 50°C on an Acquity UPLC Protein BEH C4 Column, 300Å, 1.7 μm, 2.1 mm × 50 mm analytical column (Waters, cat. no. 186004495) equipped with an Acquity UPLC Protein BEH C4 VanGuard Pre-column, 300Å, 1.7 μm, 2.1 mm × 5 mm guard column (Waters, cat. no. 186004623). Mobile phases were Optima water/0.2% formic acid (buffer A) and 60% acetonitrile/40% isopropanol/0.2% formic acid (buffer B). A linear gradient from 49% to 79% buffer B over 8.4 min at 0.4 mL/min was used to resolve the analytes. Analytes were introduced to the mass spectrometer (Sciex 6500) and analyzed using optimized mass spectrometric parameters for each compound.

Internal standards were included in the precipitation solvent at a concentration of 19.4 nmol/L (Ceramide/Sphingolipid Internal Standard Mixture I, 25 μmol/L; Avanti Polar Lipids, LM-6002), which controls for variability in extraction efficiency, pipetting, and ion suppression. Chromatographic peak areas of the endogenous analytes and the internal standards were quantified using SkyLine software (14). Each peak area for each endogenous sphingolipid was divided by the sum of the peak area of five internal standards (ceramide C12 [CerC12], CerC25, glucosyl ceramide C12 [GluCerC12], lactosyl ceramide C12 [LacCerC12], and sphingomyelin 12 [SM12]), which was called the peak area ratio. The peak area ratio for each sphingolipid was then divided by the mean peak area ratio in the single point calibrator in the batch (precipitated and analyzed 5 times in each batch, spread across the plate). The single point calibrator was a pooled EDTA-anticoagulated plasma sample made from discarded de-identified clinical samples from the clinical laboratory at the University of Washington Medical Center. Additional details on the sphingolipid measurements and quality control procedures are provided in the Supplementary Data. The coefficients of variation for each sphingolipid species are shown in Table 1.

Table 1—Correlations between the sphingolipid species, and coefficients of variation of the measurements

	Cer-16	Cer-18	Cer-20	Cer-22	Cer-24	GluCer-16	GluCer-22	GluCer-24	LacCer-16	SM-14	SM-16	SM-18	SM-20	SM-22	CV, %
Cer-16	1.0														18.4
Cer-18	0.74	1.0													21.2
Cer-20	0.72	0.74	1.0												19.5
Cer-22	0.72	0.59	0.73	1.0											13.9
Cer-24	0.72	0.58	0.61	0.87	1.0										15.5
GluCer-16	0.51	0.39	0.29	0.31	0.40	1.0									13.4
GluCer-22	0.36	0.20	0.24	0.44	0.44	0.67	1.0								13.9
GluCer-24	0.40	0.23	0.23	0.37	0.48	0.72	0.85	1.0							16.0
LacCer-16	0.40	0.27	0.26	0.29	0.32	0.59	0.51	0.49	1.0						14.4
SM-14	0.59	0.47	0.48	0.62	0.66	0.48	0.42	0.45	0.38	1.0					18.2
SM-16	0.70	0.49	0.48	0.55	0.62	0.69	0.56	0.60	0.63	0.71	1.0				11.5
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			12.2
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		12.6
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	12.4
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	13.3

CV, coefficient of variation.

In total we measured 22 sphingolipid species. This report is restricted to the 15 species with coefficient of variation ≤21% over the whole study period. It includes five ceramides: ceramide with 16:0 (Cer-16), 18:0 (Cer-18), 20:0 (Cer-20), 22:0 (Cer-22), and a composite concentration of Cer-24 computed as the sum of the concentrations of two species of ceramides with 24:0 having the distinct "d181" and "d182" sphingoid backbones. It also includes six sphingomyelins, SM-14, SM-16, SM-18, SM-20, SM-22, and SM-24; three glucosyl ceramides, GluCer-16, GluCer-22, and GluCer-24; and one lactosyl ceramide, LacCer-16. Simplified relationships between the sphingolipid classes that were measured are shown in Fig. 1.

Statistical Analysis

The statistical methods were identical for the outcomes of fasting plasma insulin, HOMA of insulin resistance (HOMA-IR), and HOMA of β-cell function (HOMA-B). The analyses are described using insulin as example. In the analyses, sphingolipid species concentrations and outcomes were log-transformed. We investigated the association of each sphingolipid with baseline plasma insulin, follow-up plasma insulin, and changes in insulin between baseline and follow-up examinations. Participants with diabetes at baseline were excluded from all analyses. For the prospective analyses and analyses of changes between baseline and follow-up, we further excluded participants with diabetes at the follow-up exam. The analyses of baseline and follow-up insulin used linear mixed models that included a family-specific random effects to account for familial aggregation and a subject-specific random effects with covariance among family members proportional to the kinship coefficient to account for genetic similarity among family members. Each model included prespecified adjustments for age, sex, geographic area, education, smoking, log(BMI), waist circumference, and physical activity. The analyses of change in insulin between baseline and follow-up did not include random effects with covariance proportional to the kinship matrix but were additionally adjusted for baseline insulin and used a random-effects longitudinal model to correct for measurement error in baseline insulin (15), as measurement error in an adjustment variable may introduce bias (16). To correct for multiple comparisons within each type of outcome model, we applied a Bonferroni correction and used a significance threshold of 0.0033 (0.05/15 sphingolipid species).

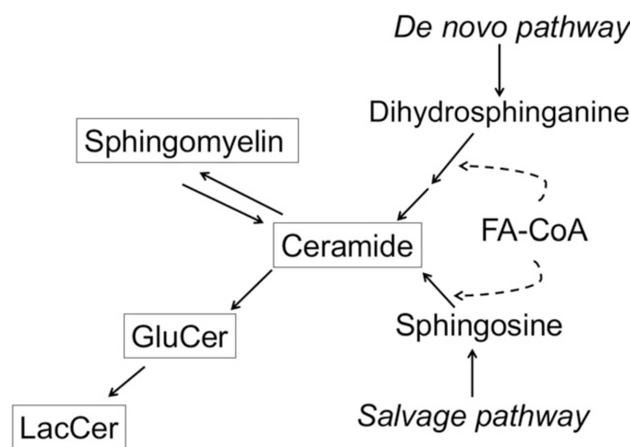

Figure 1—Synthesis of ceramide and other measured sphingolipids. Shown are simplified pathways leading to ceramide, sphingomyelin, glucosyl ceramide, and lactosyl ceramide, the four sphingolipids measured in the study. In the de novo synthesis pathway and the salvage pathway, ceramide is formed by acylation of a fatty acid (FA) to a "sphingoid" backbone, dihydrosphinganine and sphingosine, respectively. There are six ceramide synthases in humans with different fatty acid specificities, resulting in multiple ceramide species carrying different fatty acids. Synthesis of ceramide by the two pathways occurs in the endoplasmic reticulum. Ceramide can also be formed by sphingomyelinases on the plasma membrane. Sphingomyelin is synthesized by sphingomyelin synthase by addition of a choline head group to ceramide transported to the Golgi. Glucosyl ceramide is synthesized by addition of a glucose head group to ceramide, and lactosyl ceramide by further addition of galactose to glucosyl ceramide, also in the Golgi.

Missing values of baseline physical activity measures (n = 179) were multiply imputed (20 replicates) using information on age, sex, education, body fat, and triglycerides in models that accounted for possible family effect. The fully conditional expectation method implemented by the MICE package in R was used with predictive mean matching method (17). Variables included in the imputation were selected by minimizing the Bayesian information criterion in models predicting physical activity in the complete case data. Twenty imputed data sets were generated and model fitting results were pooled using standard methods (18).

We examined whether associations between sphingolipids and baseline insulin concentration were modified by differences in age, sex, and BMI by adding product interaction terms to the models above. To correct for multiple comparisons, we used a significance threshold of 0.0033 for interaction tests (0.05/15 sphingolipid species).

Multivariate model results are presented per twofold higher concentration of each sphingolipid. This twofold difference is comparable to the difference between the 90th and 10th percentiles of each sphingolipid species (Table 2). We saw no departure from linearity when modeling the sphingolipids with cubic splines (not shown).

RESULTS

Baseline characteristics of the cohort participants included in the study are shown in Table 3. Participants were on average 38 years old, 41% were men, and 24% had a BMI of 35 kg/m^2 or greater. Levels of the sphingolipid species, especially species from the same class, were correlated with each other (Table 1).

In multivariable linear mixed-effects models that included adjustment for potential confounders and accounted for the familial relationship between study participants, we observed that twofold higher concentrations of four ceramide species, Cer-16, Cer-18, Cer-20, and Cer-22, were each associated with 11–16% higher concentrations of baseline fasting plasma insulin and HOMA-IR (Table 4). Similar associations were observed with follow-up fasting plasma insulin and

Table 2—Sphingolipid concentrations, expressed in terms of normalized peak area ratios

Species	Log of normalized peak ratio, mean ± SD	Normalized peak ratio, median of Q1	Normalized peak ratio, median of Q5	Fold difference in normalized peak ratio: Q5 median/Q1 median
Cer-16	−0.61 ± 0.30	0.37	0.79	2.14
Cer-18	−0.81 ± 0.44	0.26	0.77	2.96
Cer-20	−0.31 ± 0.40	0.46	1.18	2.57
Cer-22	0.28 ± 0.36	0.85	2.10	2.46
Cer-24	0.79 ± 0.32	1.49	3.31	2.22
GluCer-16	−0.07 ± 0.28	0.66	1.31	1.98
GluCer-22	0.34 ± 0.29	0.97	2.04	2.10
GluCer-24	0.25 ± 0.31	0.86	1.88	2.19
LacCer-16	−0.12 ± 0.25	0.65	1.21	1.86
SM-14	−0.01 ± 0.39	0.60	1.59	2.65
SM-16	0.12 ± 0.19	0.89	1.44	1.62
SM-18	−0.18 ± 0.24	0.62	1.13	1.82
SM-20	0.08 ± 0.26	0.78	1.51	1.94
SM-22	0.36 ± 0.26	1.05	2.00	1.90
SM-24	0.31 ± 0.27	0.98	1.92	1.96

Q, quintile.

HOMA-IR measures, collected an average of 5.4 years after baseline (Fig. 2, middle estimates, and Supplementary Tables 1 and 2). Models of ceramides with baseline and follow-up HOMA-B resulted in associations that were similar to but slightly weaker than associations of ceramides with insulin and HOMA-IR (Table 4 and Supplementary Table 3). We did not observe significant associations of ceramides with change in insulin between baseline and follow-up, change in HOMA-IR, or change in HOMA-B (Fig. 2, bottom estimates, and Supplementary Tables 1–3).

In contrast to ceramides, higher concentrations of GluCer-24 and LacCer-16 were associated with lower baseline plasma insulin concentrations, lower HOMA-IR, and lower HOMA-B (Table 4). However, these ceramide derivatives were not associated with follow-up outcomes or change between baseline and follow-up (Supplementary Tables 1–3).

Sphingomyelin species concentrations were not associated with the outcomes at the prespecified significance threshold of 0.0033, although higher SM-16 concentrations showed marginal evidence of an association with lower baseline concentrations of insulin, HOMA-IR, and HOMA-B (Table 4). In sensitivity analyses, further adjustments for LDL and HDL cholesterol did not change the study results (Supplementary Table 4).

We did not observe any modification of the association between the sphingolipid species and outcomes by age or sex. However, we observed evidence of interactions between BMI and four of the sphingomyelin species, SM-18, SM-20, SM-22, and SM24, at the prespecified significance threshold of 0.0033 (Table 5). To illustrate the modification of sphingomyelin species associations with baseline insulin by BMI, we produced plots of the geometric mean insulin ratio corresponding to twofold higher sphingomyelin species as a function of BMI based on estimates from these interaction models (Fig. 3). At normal BMI, higher concentrations of the sphingomyelins were associated with lower insulin concentrations, whereas at very high BMIs, higher concentrations were associated with higher insulin. For example, a twofold higher concentration in SM-24 was associated with 24% lower geometric mean baseline insulin at BMI of 20 and with 14% higher insulin at BMI of 40 (P for interaction: 2.7×10^{-5}) (Table 5). Excluding BMI >45 kg/m^2 did not change the findings of interactions (not shown). Similar interactions were observed with the outcomes of HOMA-IR and HOMA-B and with each outcome at the follow-up exam (Table 5). For example, a twofold higher concentration in SM-24

Table 3—Baseline characteristics of the 2,086 SHFS participants in the study

	Mean or %
Age, years	37.88 ± 16.46
Male sex	40.70
Education, years	12.19 ± 2.28
BMI, kg/m^2	30.57 ± 7.22
Waist circumference, cm	99.98 ± 17.34
Smoking, current	37.44
LDL, mg/dL	99.90 ± 30.06
HDL, mg/dL	52.29 ± 14.83
Triglycerides, mg/dL	147.84 ± 92.24
Baseline outcomes	
Fasting insulin, μU/mL	15.62 ± 15.49
Fasting glucose, mg/dL	93.96 ± 10.40
HOMA-IR	3.75 ± 4.14
HOMA-B	178 ± 130

Table 4—Association of plasma sphingolipids with baseline fasting plasma insulin, HOMA-IR, and HOMA-B

	Outcome		
Sphingolipid	Insulin	HOMA-IR	HOMA-B
Cer-16	1.13 (1.07–1.20), 1.9×10^{-5}	1.15 (1.08–1.22), 4.7×10^{-6}	1.04 (0.99–1.11), 0.13
Cer-18	1.11 (1.06–1.15), 1.0×10^{-6}	1.12 (1.08–1.17), 6.3×10^{-8}	1.04 (1.00–1.09), 0.03
Cer-20	1.12 (1.07–1.17), 1.5×10^{-7}	1.13 (1.08–1.18), 1.3×10^{-7}	1.06 (1.02–1.11), 0.003
Cer-22	1.13 (1.08–1.19), 3.9×10^{-7}	1.15 (1.09–1.21), 1.0×10^{-7}	1.07 (1.02–1.12), 0.004
Cer-24	1.05 (0.99–1.11), 0.08	1.06 (1.00–1.13), 0.04	1.00 (0.94–1.05), 0.86
SM-14	1.00 (0.96–1.05), 0.91	1.01 (0.96–1.06), 0.79	0.97 (0.93–1.01), 0.19
SM-16	0.89 (0.81–0.97), 0.009	0.87 (0.79–0.96), 0.006	0.89 (0.82–0.98), 0.01
SM-18	0.95 (0.89–1.02), 0.19	0.96 (0.89–1.03), 0.24	0.93 (0.87–0.99), 0.03
SM-20	0.95 (0.89–1.02), 0.17	0.95 (0.89–1.02), 0.19	0.94 (0.88–1.00), 0.05
SM-22	1.00 (0.93–1.07), 0.97	1.00 (0.93–1.08), 0.91	0.96 (0.90–1.03), 0.30
SM-24	0.96 (0.90–1.02), 0.22	0.97 (0.90–1.03), 0.31	0.93 (0.87–0.99), 0.02
GluCer-16	0.96 (0.90–1.02), 0.16	0.95 (0.89–1.02), 0.15	0.95 (0.89–1.00), 0.07
GluCer-22	0.93 (0.87–0.98), 0.013	0.93 (0.87–0.98), 0.01	0.91 (0.86–0.97), 0.002
GluCer-24	0.92 (0.86–0.97), 0.0023	0.92 (0.86–0.97), 0.004	0.90 (0.85–0.95), 0.0002
LacCer-16	0.87 (0.82–0.94), 0.0001	0.86 (0.80–0.92), 3.7×10^{-5}	0.91 (0.85–0.98), 0.007

Data are ratio of geometric means associated with twofold higher sphingolipid (95% CI), *P* value.

was associated with 27% lower geometric mean follow-up HOMA-B at BMI of 20 kg/m^2 and with 24% higher HOMA-B at BMI of 40 kg/m^2 (*P* for interaction: 0.0002).

DISCUSSION

In this large family-based cohort of American Indians, higher levels of plasma ceramides were associated with higher fasting plasma insulin and HOMA-IR and less consistently with higher HOMA-B, cross-sectionally as well as prospectively. These associations were observed for most measured ceramide species, with saturated fatty acids of different length.

The association of circulating ceramides with higher plasma insulin complements a large body of evidence from animal experimental studies supporting a role of ceramides in insulin resistance and diabetes (8). For example, in mice fed a high-fat diet and in obese mice, inhibition of ceramide synthesis or enhanced degradation by genetic engineering or pharmacological means improves insulin sensitivity (19,20). Importantly, infusion of LDL-containing ceramides into lean mice reduces insulin-stimulated glucose uptake (21), suggesting that ceramides might be delivered from the circulation thereby influencing glucose homeostasis. In addition, in nonhuman primates, plasma ceramide levels increase in parallel with a reduction in insulin sensitivity in response to a high-fat, high-fructose diet (22).

In humans, the role of ceramide in insulin resistance is not as well established. Treatments that improve muscle insulin sensitivity, such as diet-induced weight loss and exercise training, lower muscle ceramides in some but not all studies (23,24). The evidence relating circulating ceramides and insulin resistance is limited. In small cross-sectional studies, with fewer than 50 subjects, plasma ceramide species correlate with insulin sensitivity (25,26). We show for the first time in a large population without diabetes that circulating ceramide species with saturated fatty acids of different lengths are associated with higher insulin levels and higher HOMA-IR.

Circulating dihydroceramide species, precursors of ceramides, were associated prospectively with diabetes risk in two nested case-control studies from the Data from an Epidemiological Study on the Insulin Resistance Syndrome (DESIR) cohort and the Cohorte Lausannoise (CoLaus) study (27). In addition, in a large prospective cohort in

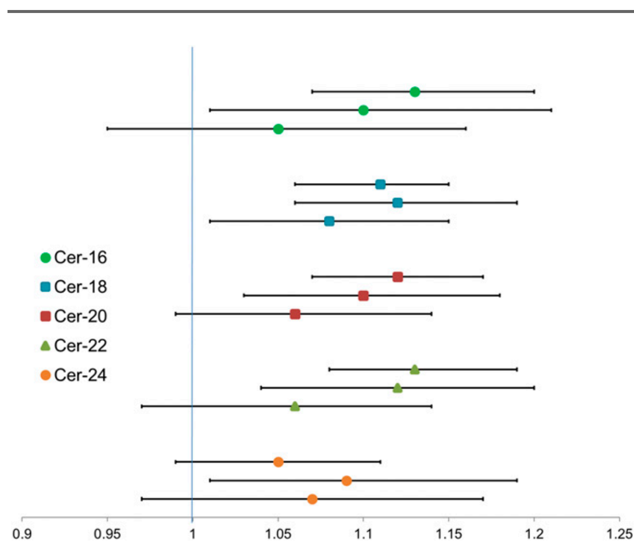

Figure 2—Association of plasma ceramides with plasma fasting insulin and change in plasma fasting insulin. Shown is the ratio of insulin geometric means associated with twofold higher ceramide. Within each ceramide, top estimate represents the association with insulin at baseline, middle estimate the association with insulin at follow-up, and bottom estimate the association with change in insulin between baseline and follow-up.

Table 5—Ratio of geometric mean ratios (95% CI) of baseline and follow-up insulin, HOMA-IR, and HOMA-B, associated with doubling of sphingomyelin species at different BMIs

	BMI (kg/m^2)						
	20	25	30	35	40	45	*P* for interaction
Baseline insulin							
SM-18	0.80 (0.69–0.91)	0.88 (0.81–0.96)	0.96 (0.89–1.03)	1.03 (0.95–1.12)	1.10 (0.98–1.23)	1.16 (1.00–1.34)	0.0027
SM-20	0.80 (0.70–0.91)	0.89 (0.82–0.96)	0.97 (0.90–1.04)	1.04 (0.95–1.13)	1.11 (0.99–1.24)	1.17 (1.02–1.35)	0.0011
SM-22	0.82 (0.72–0.93)	0.92 (0.85–1.00)	1.01 (0.94–1.08)	1.09 (1.00–1.19)	1.17 (1.05–1.31)	1.25 (1.08–1.44)	0.0005
SM-24	0.76 (0.67–0.86)	0.87 (0.80–0.94)	0.96 (0.90–1.03)	1.06 (0.98–1.14)	1.14 (1.03–1.27)	1.22 (1.07–1.39)	2.7×10^{-5}
Follow-up insulin							
SM-18	0.85 (0.69–1.05)	0.96 (0.84–1.09)	1.05 (0.94–1.17)	1.14 (0.99–1.31)	1.23 (1.02–1.47)	1.30 (1.03–1.64)	0.028
SM-20	0.79 (0.65–0.96)	0.92 (0.81–1.04)	1.05 (0.94–1.17)	1.17 (1.02–1.35)	1.29 (1.07–1.55)	1.41 (1.12–1.77)	0.0020
SM-22	0.80 (0.65–0.98)	0.94 (0.83–1.07)	1.08 (0.97–1.20)	1.21 (1.05–1.39)	1.34 (1.11–1.61)	1.46 (1.15–1.84)	0.0019
SM-24	0.76 (0.63–0.93)	0.90 (0.79–1.01)	1.02 (0.92–1.13)	1.14 (1.01–1.30)	1.26 (1.06–1.49)	1.37 (1.10–1.70)	0.0014
Baseline HOMA-IR							
SM-18	0.79 (0.69–0.92)	0.88 (0.81–0.97)	0.96 (0.89–1.04)	1.04 (0.94–1.14)	1.10 (0.98–1.25)	1.17 (1.00–1.36)	0.0039
SM-20	0.78 (0.68–0.89)	0.88 (0.81–0.96)	0.97 (0.90–1.04)	1.05 (0.96–1.15)	1.13 (1.00–1.27)	1.20 (1.03–1.40)	0.0006
SM-22	0.81 (0.71–0.93)	0.92 (0.84–1.00)	1.02 (0.95–1.09)	1.11 (1.01–1.21)	1.19 (1.06–1.34)	1.27 (1.09–1.48)	0.0005
SM-24	0.76 (0.66–0.87)	0.87 (0.80–0.95)	0.97 (0.91–1.04)	1.06 (0.98–1.16)	1.15 (1.03–1.29)	1.24 (1.08–1.42)	5.2×10^{-5}
Follow-up HOMA-IR							
SM-18	0.86 (0.69–1.07)	0.96 (0.83–1.10)	1.05 (0.93–1.18)	1.13 (0.98–1.31)	1.21 (0.99–1.47)	1.28 (1.00–1.64)	0.052
SM-20	0.78 (0.63–0.95)	0.91 (0.80–1.04)	1.04 (0.93–1.17)	1.17 (1.01–1.35)	1.29 (1.06–1.56)	1.40 (1.10–1.79)	0.0030
SM-22	0.80 (0.64–0.99)	0.94 (0.82–1.07)	1.07 (0.96–1.20)	1.20 (1.04–1.40)	1.33 (1.09–1.62)	1.45 (1.13–1.86)	0.0036
SM-24	0.77 (0.62–0.94)	0.90 (0.79–1.02)	1.02 (0.92–1.14)	1.14 (1.00–1.31)	1.26 (1.05–1.51)	1.37 (1.09–1.72)	0.0029
Baseline HOMA-B							
SM-18	0.77 (0.68–0.88)	0.86 (0.79–0.93)	0.93 (0.87–1.00)	1.00 (0.92–1.09)	1.07 (0.95–1.19)	1.13 (0.98–1.30)	0.0020
SM-20	0.84 (0.74–0.95)	0.90 (0.83–0.97)	0.95 (0.88–1.01)	0.99 (0.91–1.08)	1.03 (0.92–1.15)	1.07 (0.93–1.23)	0.033
SM-22	0.82 (0.72–0.93)	0.90 (0.83–0.98)	0.98 (0.91–1.04)	1.04 (0.96–1.14)	1.11 (0.99–1.24)	1.17 (1.02–1.34)	0.0022
SM-24	0.75 (0.66–0.84)	0.84 (0.78–0.91)	0.93 (0.87–0.99)	1.01 (0.94–1.09)	1.09 (0.99–1.21)	1.16 (1.02–1.32)	5.3×10^{-5}
Follow-up HOMA-B							
SM-18	0.80 (0.67–0.97)	0.93 (0.83–1.05)	1.05 (0.95–1.16)	1.17 (1.03–1.32)	1.27 (1.08–1.51)	1.38 (1.11–1.70)	0.0026
SM-20	0.81 (0.68–0.97)	0.94 (0.84–1.05)	1.05 (0.95–1.16)	1.16 (1.02–1.31)	1.26 (1.07–1.49)	1.35 (1.10–1.67)	0.0029
SM-22	0.80 (0.66–0.96)	0.94 (0.84–1.05)	1.07 (0.97–1.18)	1.19 (1.05–1.35)	1.31 (1.11–1.56)	1.43 (1.16–1.77)	0.0009
SM-24	0.73 (0.61–0.88)	0.87 (0.78–0.97)	1.00 (0.91–1.09)	1.12 (1.00–1.26)	1.24 (1.06–1.45)	1.36 (1.12–1.65)	0.0002

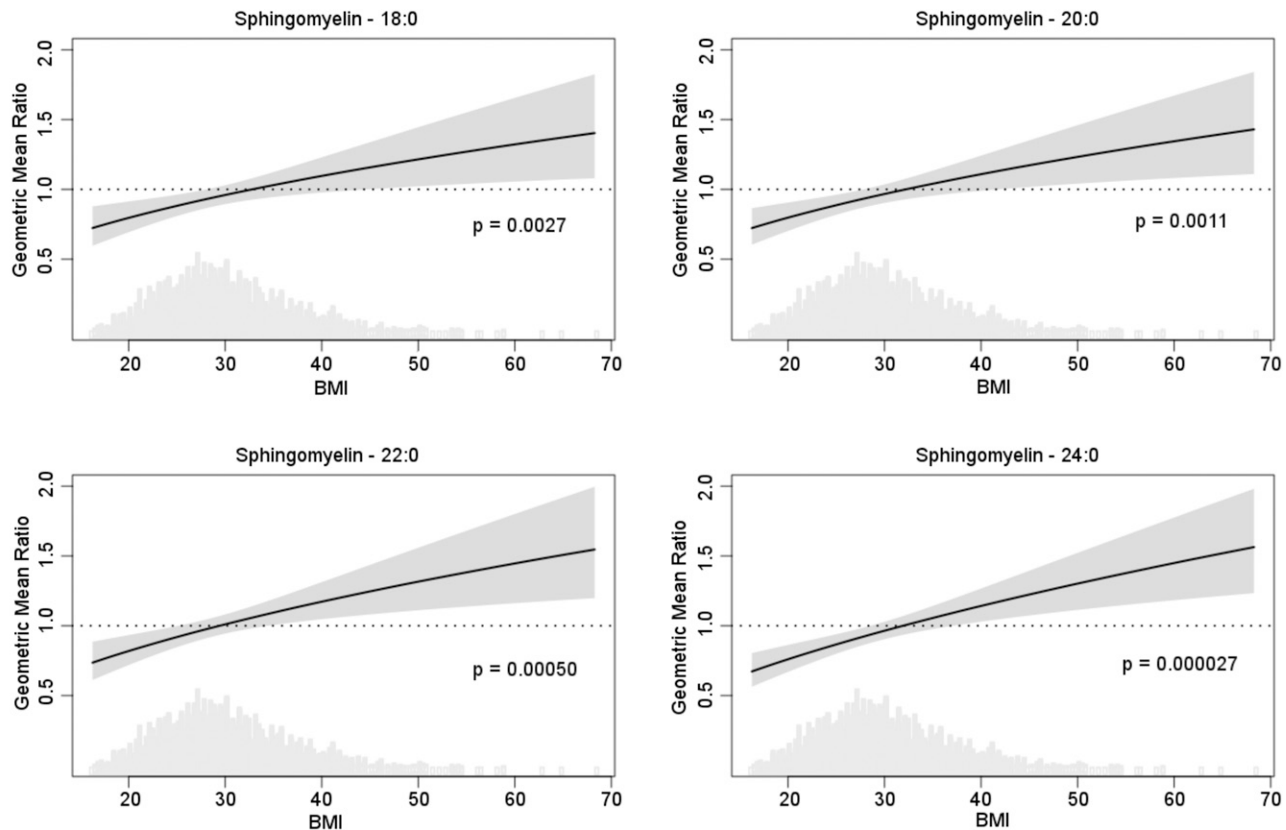

Figure 3—Geometric mean ratio of insulin levels associated with twofold difference in sphingomyelin species as a function of BMI (kg/m^2). Each plot shows the ratio of baseline insulin geometric means associated with twofold higher sphingomyelin species as a function of BMI (solid line) and 95% CI (shaded area). A geometric mean ratio of 1.0 indicates no association. Values less than 1 indicate an association with lower insulin, and values above 1 indicate an association with higher insulin. The density plot shows the distribution of BMI values in the cohort.

Finland, circulating Cer-16 and Cer-18 were associated with risk of incident major cardiovascular events (28). Our study complements these by suggesting that ceramides may associate early with insulin resistance, prior to its progression to diabetes and its complications.

The fatty acid 16:0 appears unique in its ability to promote endogenous ceramide synthesis and insulin resistance in experimental systems such as myotubes in culture (8,29). In the circulation, 16:0 is the most abundant saturated fatty acid, and total circulating 16:0 is associated with diabetes risk (30–33). In contrast, circulating concentrations of the fatty acids 20:0, 22:0, and 24:0 are associated with lower diabetes risk (11,30). However, we found similar associations of ceramides with different saturated fatty acids with insulin and HOMA-IR, suggesting similar potential biological activities of different ceramides with saturated fatty acids on insulin resistance.

Although sphingomyelins did not show significant associations with any of the outcomes, we provide strong evidence that BMI modifies the associations of the sphingomyelin species SM-18, SM-20, SM-22, and SM-24 with fasting insulin, HOMA-IR, and HOMA-B in both cross-sectional and prospective analyses. Likely as a result of these interactions, overall associations of sphingomyelins were not detected.

Basic studies paint a complex picture of sphingomyelin and insulin sensitivity. In β-cells in culture, increasing sphingomyelin by inhibiting its hydrolysis protects against palmitate-induced lipotoxicity (34), and inhibiting sphingomyelin synthase in myotubes, also resulting in a decrease in sphingomyelin, impairs insulin signaling (35). In contrast, the lowering of sphingomyelin by inhibition of sphingomyelin synthase in knockout mice prevents high-fat–induced obesity and increases insulin sensitivity (36,37). Whether adipogenesis or another aspect of the animal's metabolism influences the consequences of decreased sphingomyelin is not known.

Studies of sphingomyelins and insulin in humans are limited to cross-sectional studies. Among metabolomics studies that measured sphingomyelins, findings for species with saturated fatty acids ranged from positive to negative associations to no associations with HOMA-IR (38–41). The largest such study measured 47 sphingomyelins among 1,100 young adults; none of the sphingomyelin species, including saturated sphingomyelins, were associated with HOMA-IR (38), mirroring our findings of no main associations. In a smaller study, patients with insulin resistance and those with diabetes had lower SM-16 (39), whereas two studies that included obese individuals observed positive associations of sphingomyelins with saturated fatty acids

with HOMA-IR (40,41). Our findings of interactions with BMI may explain these apparently contradictory findings.

Plasma phospholipid levels of 20:0, 22:0, and 24:0 are associated with lower risk of incident diabetes (11,30), lower triglyceride levels, and a better insulin sensitivity score (11). Furthermore, we reported modification of the associations between plasma phospholipid 22:0 and 24:0 levels and diabetes risk by BMI: the association of these fatty acids with lower risk of diabetes was most pronounced among participants with normal BMI (11). The fatty acids 20:0, 22:0, and 24:0 are primarily found in sphingolipids (42), and sphingomyelins are the primary sphingolipids in phospholipids. Our findings that sphingomyelin species with 20:0, 22:0, and 24:0 are associated with lower levels of insulin among those with normal BMI raise the possibility that these sphingomyelins were the lipids associated with lower risk of diabetes in the previous study. Further studies are needed to investigate if circulating sphingomyelin species with 20:0, 22:0, and 24:0 are in fact associated with lower diabetes risk.

Higher levels of LacCer-16 and GluCer-24 were associated with lower fasting insulin, HOMA-IR, and HOMA-B, although these associations were only observed when examined cross-sectionally. Glucosyl ceramide and lactosyl ceramide are the precursors of larger glycosphingolipids such as gangliosides (43). Inhibition of glycosylceramide synthase, which affects the whole pathway, enhances insulin sensitivity in rodents (44). This effect appears to be due to gangliosides interfering with insulin action in adipocytes but not in myotubes, where gangliosides actually enhance insulin sensitivity (45). Biological properties of glucosyl and lactosyl ceramides and their metabolites in humans need to be pursued.

Study strengths include its large size; the study setting of a family-based, well-phenotyped cohort, with recorded data on many potential confounders; and the longitudinal study design in additional to cross-sectional analyses. Study limitations include its observational nature, which precludes assessment of causality. We were not able to investigate all the ceramide species containing saturated fatty acids, such as lactosyl and glycosyl ceramides, due to measurement error with the laboratory assay; however, we measured most underivatized ceramides and sphingomyelins with saturated fatty acids with good precision. The study included a single ethnicity; however, the relatively young cohort, at high risk of diabetes, is an ideal setting for the discovery of early risk factors for diabetes. We cannot eliminate the possibility that drift in the laboratory assay for insulin between baseline and follow-up influenced our measurements of change in insulin over time. However, we had no evidence of drift and it would be unlikely any drift would be differential by sphingolipid level.

Like many others (46,47), we used HOMA-IR and insulin as proxies for insulin resistance because only these measures were available in the SHFS population. It is not surprising that associations with HOMA-IR were very similar to those with fasting insulin, suggesting that values of HOMA-IR are largely driven by insulin levels in this relatively young population with an excess of obesity (48–50). Further, these basal estimates of insulin resistance, calculated only using fasting insulin and glucose values, may differ from estimates based on dynamic measurements of insulin and glucose responses or those derived from clamp experiments with uncertainties regarding contributions of peripheral, hepatic, or whole-body insulin resistance to these measures.

In summary, we have shown that higher plasma levels of ceramides with saturated fatty acids are prospectively associated with higher fasting insulin and HOMA-IR; and, in contrast, higher sphingomyelins with saturated fatty acids are associated with lower fasting insulin, HOMA-IR, and HOMA-B in those with normal BMI. Similar associations were seen for species within the same class, with different saturated fatty acids. The study suggests the lowering of circulating ceramides with saturated fatty acids might be a target in prediabetes, and targeting an increase in circulating sphingomyelins should take into account a person's BMI.

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