Circulating Sphingolipids and Incident Diabetes: the Strong Heart Study

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Abbreviations: AI, American Indian; SHS, Strong Heart Study; SHFS, Strong Heart Family Study; BMI, body mass index; CI, confidence interval; cer-ceramide; SM, sphingomyelin

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Abstract

Background: Ceramides and sphingomyelins have been implicated in several biological processes related to diabetes in cell and animal studies, but few prospective studies have assessed the associations of ceramides and sphingomyelins with diabetes in humans. **Objective:** We investigated the associations of 15 circulating ceramides and sphingomyelin species with development of diabetes in 2 studies. **Design:** This analysis included 2,337 participants from the Strong Heart Study and the Strong Heart Family Study, two large multi-tribal prospective studies of risk factors for cardiovascular disease among American Indians. Using logistic regression and parametric survival models within studies, and an inverse-variance weighted meta-analysis across studies, we examined the associations of 15 ceramides and sphingomyelin species with incident diabetes. **Results:** There were 446 cases of incident diabetes across the two studies. Higher circulating levels of ceramides containing stearic acid (Cer-18), arachidic acid (Cer-20), and behenic acid (Cer-22) were each associated with a higher risk of incident diabetes. The relative risk (RR) for incident diabetes per 2-fold higher levels of each ceramide species were1.42 (95% confidence interval, 1.17, 1,72) for Cer-18, 1.41 (95% CI, 1.17, 1.71) for Cer-20, and 1.56 (95% CI, 1.26, 1.92) for Cer-22, after adjustment for age, sex, site, education, smoking, physical activity, BMI, and waist circumference. Although the magnitude of the risk estimates for the association of Cer-24 with incident diabetes was similar to those for Cer-18, Cer-20, and Cer-22 (RR=1.44; 95% CI, 1.11, 1.87), this did not achieve statistical significance after correction for multiple testing (p=0.007). Ceramides carrying palmitic acid (Cer-16), sphingomyelins, glucosyl-ceramides, or a lactosyl ceramide were not associated with diabetes risk. **Conclusion**: Higher levels of circulating Cer-18, Cer-20, and Cer-22 were each associated with a higher risk of developing diabetes.

Introduction

Type 2 diabetes and related complications are leading causes of morbidity and mortality among American Indians (AIs) in the United States. AIs are 2.5 times more likely to have diagnosed diabetes than non-Hispanic whites of similar age (1). Although many risk factors for diabetes in AIs are well-known, including diet, physical activity, smoking, elevated triglycerides, obesity, and genetic factors (2-7), strategies to prevent diabetes have had varying levels of success over the past few decades, and diabetes remains a major health problem in AI communities (2, 8, 9). Identifying novel and potentially modifiable factors that influence diabetes may provide insights to inform future diabetes prevention efforts.

Sphingolipids are a class of lipids containing a backbone sphingoïd base (sphingosine) and one acylated fatty acid. Sphingosine N-acylated with a fatty acid forms ceramides, and moieties can attach to ceramides to form more complex sphingolipids; for sphingomyelins, phosphoryl choline is attached. Ceramides and sphingomyelins have been implicated in several biological processes related to diabetes in cell and animal studies, including high levels of impaired insulin signaling and glucose transport, insulin resistance, inflammation, oxidative stress, and apoptosis in beta-cells (10-17). In animal studies, inhibition of *de novo* sphingolipid synthesis improves insulin sensitivity and glucose homeostasis, major risk factors for diabetes, and reduces lipotoxic responses associated with metabolic disorders such as obesity (15, 16, 18-21). However, few prospective studies have assessed the associations of ceramides and sphingomyelins with diabetes-related phenotypes in humans. In the Strong Heart Family Study (SHFS), a family-based study of risk factors for cardio-metabolic diseases in American Indians, several plasma ceramide species were associated with higher fasting insulin levels among participants without

diabetes—suggesting a role of circulating ceramides in pre-diabetes (22). In untargeted analyses of 135 lipid species from several small case-control studies, ceramide and dihydroceramide species were associated with higher risk of incident diabetes (23).

The purpose of this analysis was to examine associations of 15 circulating ceramides and sphingomyelin species with incident diabetes among AIs who participated in the Strong Heart Study (SHS) and SHFS.

Methods

Study Design

We used plasma samples stored at -80 degrees Celsius from the SHS and SHFS cohorts to measure sphingolipids and to examine the associations of sphingolipids with incident diabetes. In the SHS, a case-control design was used to preserve existing stored blood specimens/optimize study resources. In the SHFS, a longitudinal design was used, and sphingolipids were measured on all participants using plasma samples at baseline. Analyses examined the associations of each sphingolipid with risk of incident diabetes in the SHS and SHFS separately, and then risk estimates from the two cohorts were combined in meta-analyses.

Setting

The SHS is a population-based longitudinal study of risk factors for cardiovascular diseases in 12 AI communities in Arizona, North and South Dakota, and Oklahoma. The cohort included 4,549 participants between the ages of 45-74 years who were seen at a baseline exam conducted in 1989-1991 (phase 1), and two follow-up exams conducted in 1993-1995 (Phase 2) and 1998-

1999 (Phase 3). As part of the Phase 3 exam, the SHS conducted a pilot study to better understand the genetic determinants of cardiovascular diseases in large families. As part of this pilot, 1st, 2nd, and 3rd degree relatives of original SHS participants from large families were recruited. This study, called the SHFS, was expanded in 2001-2003; in total, the SHFS comprised 2,780 participants between the ages of 14-93 years from 91 families. SHFS participants completed two examinations over an eleven-year period: 2001-2003 (Phase 4) and 2007-2009 (Phase 5). Subsequent to the study examinations, surveillance for major cardio-metabolic outcomes, including diabetes, continued for all SHS and SHFS participants through December 2017 using a single phone interview and medical record review (Phase 6). Details of the study designs, survey methods and laboratory techniques of the SHS and the SHFS have been reported previously (24, 25). The institutional review board from each Indian Health Service region and all communities approved the study, and written informed consent was obtained from all participants at each exam.

Study Sample

SHS: From participants without diabetes at the Phase 2 exam, we randomly selected a sample of 146 participants who developed diabetes within 15 years of the Phase 2 exam as cases. For each case, we randomly selected two age- and gender-matched controls who did not develop diabetes during the same follow-up time (n=289). Specifically, for those cases whose diabetes was identified at the Phase 3 exam, controls were chosen from those without diabetes at the Phase 3 exam. Later cases of diabetes were matched to controls who participated in Phase 6 and did not have a diabetes diagnosis in the 15 years after the Phase 2 exam. In total, there were 435 participants from the SHS in the analytic sample.

SHFS: Among the 2,713 participants with available sphingolipid measures at the Phase 4 exam, we excluded participants with prevalent diabetes (n=510) and those without follow-up (n=274). Additionally, participants missing data for measures of baseline glucose (n=6), waist circumference (n=14), and education (n=7) were excluded. In total, there were 1,902 participants from the SHFS in the analytic sample.

Data Collection

Each SHS and SHFS examination included a standardized personal interview, physical examination, and laboratory work-up. The data collection procedures were identical at all SHS and SHFS exams (24, 25), except for physical activity and diabetes ascertainment, as described below. Information regarding previous/current medical conditions, education, and smoking were collected at the personal interviews. Anthropometric measures were obtained with the participant wearing lightweight clothing and no shoes. Body mass index (BMI) was calculated as body weight divided by height-squared (kg/m^2). Waist circumference was measured at the umbilicus while the participant was in a supine position. In the SHS, participation in leisure-time and occupational physical activities was assessed using a questionnaire designed specifically for AIs (3, 26). In the SHFS, usual levels of ambulatory activity (i.e., average steps per day over one week) was estimated using Accusplit AE120 pedometers (Yamax, Japan) (4, 27). Both the physical activity questionnaire and pedometers have been shown to be reliable and valid in free-living conditions (28, 29).

Blood samples were collected after a 12-hour overnight fast and were stored at -80 degrees Celsius. Plasma glucose, total cholesterol, triglycerides, low density lipoprotein (LDL) and high density lipoprotein (HDL) were measured using enzymatic methods (30). In the SHS, each study participant who was not currently treated with insulin/oral hypoglycemic agents or did not have fasting glucose ≥ 225 mg/dL was given an oral glucose tolerance test (OGTT) (31). The SHFS did not perform OGTTs.

Measurement of Sphingolipids

Sphingomyelins and ceramides containing distinct saturated fatty acids 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), were measured using stored plasma samples from the SHS and the SHFS by sequential liquid chromatography and mass spectroscopy (LC/MS). Details of the laboratory methods and quality control procedures have been reported in detail previously (22). In total, 22 sphingolipid species were measured. For the current investigation, we restricted analyses to the 15 sphingolipid species with coefficient of variation less than or equal to 20%. This included 6 ceramide species (i.e., ceramide with 16:0 (Cer-16), ceramide with 18:0 (Cer-18), ceramide with 20:0 (Cer-20), ceramide with 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); 6 sphingomyelin (SM) species (i.e., SM-14, SM-16, SM-18, SM-20, SM-22, SM-24); 3 glucosyl-ceramides (GluCer) (i.e., GluCer-16, GluCer-22, and GluCer-24); and 1 lactosyl-ceramide (LacCer) (i.e., LacCer-16).

Diabetes Ascertainment

In the SHS, cases of diabetes were based on the 1999 World Health Organization (WHO) definition, including any participant taking insulin or oral anti-diabetic medications, participants with follow-up fasting plasma glucose (FPG) ≥ 126 mg/dl, 2-hour OGTT ≥ 200 mg/dL, or evidence of a physician-diagnosis of diabetes from annual medical record surveillance and review (32). In the SHFS, incident diabetes was defined using American Diabetes Association criteria, which included all WHO criteria except for OGTT. After Phase 5 and through December 2017, cases of diabetes were ascertained through phone interview and confirmed by documentation in medical records. Since type 1 diabetes is rare in AI populations, we assumed all new occurrences of diabetes in the SHS and the SHFS were type 2.

Statistical Analyses

SHS: Conditional logistic regression was used to evaluate the associations of plasma sphingolipids and incident diabetes, with 2 controls matched to each case based on age (± 5 years), sex, and site. There were 3 models fit to examine associations of sphingolipids with incident diabetes. Model 1 (a minimally-adjusted model) included a term for age to account for residual confounding within matched sets. Model 2 (primary model) additionally adjusted for *a priori* confounders, including education (years), smoking (never, former, current), physical activity (MET hours per week), BMI (log-transformed), and waist circumference. In model 3 (exploratory model), we additionally adjusted for low density lipoprotein (LDL) cholesterol to assess if LDL cholesterol levels mediate or confound the relationship of each sphingolipid of interest with incident diabetes. Observed associations were estimated by odds ratios associated

with two-folder higher sphingolipid levels—corresponding approximately to the difference between the 90th percentile and the 10th percentile of each sphingolipid species.

SHFS: Parametric survival models with a Weibull distribution were used to examine the associations of each sphingolipid with incident diabetes. These models were selected for the analysis to account for interval-censored incident diabetes. The SHFS comprised extended families, so robust standard error estimates that account for clustering of risk factors among family members were used.

Similar to analyses in the SHS, we fit 3 models to examine the associations of sphingolipid and incident diabetes in the SHFS. Model 1 (a minimally-adjusted model) adjusted for age, sex, and site. Model 2 (primary model) included further adjustments for education (years), smoking (never, former, current), physical activity (steps per day), BMI (log-transformed), and waist circumference. Model 3 (exploratory model) additionally included adjustment for LDL cholesterol since sphingolipids are associated with LDL. Results include estimated hazard ratios for diabetes associated with a 2-fold difference in each sphingolipid.

As associations of sphingolipid species with incident diabetes may differ by age, sex, or BMI, we examined potential effect modification of these factors with each sphingolipid species of interest on incident diabetes in exploratory analyses in both the SHS and the SHFS. To evaluate effect modification, a multiplicative term was created individually for sex, age, and BMI, with each sphingolipid, and included in the primary model (model 2). Wald tests were used to test the statistical significance of the interaction terms.

In the SHS and the SHFS analyses, multiple imputations were used (20 replicates) to address occasional missing values for physical activity (n=16 in the SHS and n=157 in the SHFS) using information on age, sex, site, BMI and waist circumference. Imputations were executed in the MICE package in R using the Fully Conditional Expectation method with predictive mean matching methods, as described previously (22, 33, 34).

Meta-Analysis: Results from each cohort were compiled and meta-analyses were performed using inverse-variance-weighted fixed effect methods in STATA 13.1 (Stata Corporation, College Station, TX). Heterogeneity between studies was assessed using the I^2 index derived from the Cochran Q statistic (35). A Bonferroni correction was used to adjust for multiple comparisons; the significance threshold of 0.0033 (0.05/15 sphingolipid species) was used.

Results

Baseline characteristics of SHS and SHFS participants are described in **Table 1**. The mean age across the cohorts ranged from 37 years (SHFS) to 57 years (SHS), and approximately 34% (SHS) and 39% (SHFS) of participants from each cohort were male. In both cohorts, participants reported an average of 12 years of education, mean BMI was 31 kg/m^2, and 37-38% of participants reported current smoking. Mean LDL cholesterol was 127 mg/dL in the SHS and 100 mg/dL in the SHFS, while mean HDL cholesterol was 43 mg/dl in the SHS and 52 mg/dl in the SHFS. In both the SHS and the SHFS, circulating levels of sphingolipid species were correlated, but the observed correlations were slightly lower in the SHS than in the SHFS (supplemental tables 1 and 2).

For this analysis, we had data available on 446 cases of incident diabetes (a subset of 146 cases from the SHS and all incident cases (n=300) from the SHFS). Higher circulating levels of Cer-18, Cer-20, and Cer-22 were each associated with a higher risk of incident diabetes. Pooling across studies, for 2-fold higher levels of each ceramide species, the relative risk for incident diabetes was 1.42 (95% confidence interval, 1.17, 1,72; p<0.001) for Cer-18, 1.41 (95% CI, 1.17, 1.71 p<0.001) for Cer-20, and 1.56 (95% CI, 1.26, 1.92 p<0.001) for Cer-22 after adjustment for age, sex, site, education, smoking, physical activity, BMI, and waist circumference (**Figure 1**). Although the magnitude of the risk estimate for the association of Cer-24 with incident diabetes was similar to those for Cer-18, Cer-20, and Cer-22 with incident diabetes, the relative risk for Cer-24 with incident diabetes did not achieve statistical significance at the pre-specified threshold for multiple testing (p=0.007). Adjusting for LDL cholesterol did not meaningfully change reported relative risks (Supplemental Figure 2).

Sphingomyelin species, glucosyl-ceramides and lactosyl ceramides were not associated with diabetes risk—although higher levels of circulating SM-22 showed marginal positive associations with diabetes risk before correction for multiple testing (**Figures 2-3**). Similar to the analyses for ceramides, additional adjustment for LDL cholesterol (Supplemental Figures 4 and 6) did not produce materially different results.

We observed little evidence of effect modification for each sphingolipid species with age (no p-interaction<0.05), sex (smallest p-interaction=0.02 for Cer-22), or BMI (smallest p-interaction=0.02 for Cer-24) on risk of incident diabetes after adjustment for multiple-

comparisons. Although we observed heterogeneity across studies for analyses of Cer-16, SM-14, SM-16, SM-20, SM-22, SM-24, GC-16, GC-22, and GC-24 with incident diabetes (i.e., I^2 index for each primary analyses >50%), tests for heterogeneity only reached statistical significance for SM-16 and SM-22 (p=0.02 and p=0.009, respectively).

Discussion

In this meta-analysis that included AIs from 12 communities throughout the United States, higher levels of circulating ceramides carrying fatty acids 18:0, 20:0, and 22:0 (i.e., cer-18, cer-20, and cer-22) were each associated with a higher risk of developing diabetes. On the other hand, circulating sphingomyelins, glucosyl-ceramides, or a lactosyl ceramide did not appear to be associated with diabetes risk. These findings suggest that the relationships of circulating sphingolipids with diabetes risk differ by sphingolipid species.

Several studies in animals and *in vitro* indicate that ceramides play a role in diabetes-related biomarkers, including insulin resistance and glucose homeostasis (15, 36). Obese, insulin-resistant rodents treated with myriocin, a drug that inhibits serine-palmitoyl transferese (i.e., a key enzyme in the metabolism of ceramides and sphingomyelins), had lowered levels of ceramides in liver, muscle, and circulation (18, 20), weight loss (20), improvements in insulin sensitivity and glucose tolerance (16, 19, 20) in liver and muscle, and were less likely to develop diabetes during a six-week follow-up (16). Findings from *in vitro* studies indicate that exposing cell cultures to 16:0 increased ceramides and inhibited Akt/protein Kinase B (Akt/PKB)—a key regulator in insulin-mediated glucose transport (15, 36). Similarly, the addition of ceramide analogs to cultured cells, muscles, adipocytes, and hepatocytes inhibited insulin-mediated

glucose uptake and activation Akt/PKB (37). Lowering ceramides by inhibiting enzymes needed for ceramide synthesis, including serine-palmitoyl transferese (i.e., enzyme that myriocin targets) negated the effects of 16:0 on impaired insulin-mediated glucose uptake and transport (10).

Only a handful of studies have assessed the associations of ceramides and diabetes-related biomarkers in humans. In two small studies (n<64), individuals with type 2 diabetes had higher circulating levels of Cer-18 and Cer-20, but not Cer-22 (38) or Cer-24 (39), than individuals without diabetes (38, 39). Results from two case-control studies (n=<300) indicate that higher levels of plasma dihydroceramide 18:0 (d(18:0)), a precursor to ceramides, is associated with a higher risk of developing diabetes (23). In a large study in Finland (n=8045) that assessed the associations of 4 ceramides (i.e., Cer-16, Cer-18, Cer-24, and Cer-24:1) with diabetes risk, higher levels of Cer-18, Cer-20, Cer-22, and Cer-24:1, and the ratios of Cer-18/Cer-16, Cer-18/Cer-24, and Cer-18/Cer-24:1, were each associated with a higher risk of incident diabetes (40). Although we did not include analyses of ceramide ratios with diabetes risk in our pre-specified analysis plan, in post-hoc meta-analyses, we also observed an association of Cer-18/Cer-16 with incident diabetes (RR=1.71 (95% CI, 1.17, 2.50) in the SHS and the SHFS, while the relative risk for Cer-18/Cer-24 with incident diabetes was of borderline significance (RR=1.87, 95% CI, 0.97, 3.58). The results of these studies complement the findings in this report, suggesting that ceramides are involved in processes related to both: (1) the development of diabetes and (2) the diagnosed diabetes phenotype.

Insulin resistance is an early marker of diabetes risk, and previous work in the SHFS indicated that higher levels of Cer-16, Cer-20, and Cer-22 are associated with higher fasting insulin levels

among AIs without diabetes. Specifically, a two-fold higher concentration of each ceramide was associated with 11%-16% higher fasting plasma insulin and homeostasis model assessment-insulin resistance (HOMA-IR) (22). On the other hand, in that analysis, higher levels of SM-18, SM-20, and SM-22 were each associated with lower fasting insulin levels, HOMA-IR, and HOMA-beta—but only among participants with normal BMI. We did not observe interaction of any of the sphingomyelins with BMI on risk of diabetes in the present analysis. This may be due to limited power to assess interaction of sphingomyelins with diabetes. Alternatively, this may suggest that the impact of BMI on the relations of sphingomyelins and metabolic dysfunction may occur early in the trajectory to diabetes.

Previous studies have consistently reported differential associations of plasma phospholipid saturated fatty acids of different chain length with diabetes risk (41-43). Particularly, circulating levels of 16:0 have been associated with a higher risk of diabetes while very-long-chain saturated fatty acids 20:0, 22:0, and 24:0 have been associated with a lower risk of diabetes. Because plasma phospholipids contain sphingolipids, especially sphingomyelins, it may seem reasonable to hypothesize that sphingolipids may be driving the observed associations of plasma phospholipid saturated fatty acids with diabetes risk. However, the findings reported herein do not support this hypothesis as irrespective of chain-length, sphingomyelins were not associated with a lower risk of diabetes. More studies are needed to better understand the interplay of sphingolipids, fatty acids, and diabetes risk.

This study has many strengths. The SHS and the SHFS are large multi-site prospective studies of risk factors for cardiovascular disease in an underserved population of AIs. The availability of

detailed data on demographic, behavioral, and health factors maximized our capacity to control for potential confounders. This study also has limitations. Although many sphingolipids were measured with good precision, it was not possible to examine the associations of all ceramide species carrying saturated fatty acids (e.g., lactosyl and glycosyl ceramides) due to measurement error associated with the laboratory assay. We adjusted for several demographic and behavioral factors that may be related to both circulating sphingolipids and diabetes risk, but residual confounding due to unmeasured or poorly measured factors is possible. Finally, circulating sphingolipid levels are related to diet, metabolism, and genetics, and the generalizability of these findings to other populations is unknown.

In conclusion, these findings suggest that higher levels of circulating ceramides carrying different saturated fatty acids (i.e., cer-18, cer-20, and cer-22) are associated with a higher risk of diabetes in two large cohorts of AI men and women over a wide age range. Replication of these findings in other studies is needed to better understand if lowering circulating ceramides may be a plausible target for diabetes prevention efforts.

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Table 1. Characteristics of the Strong Heart Study (SHS) and the Strong Heart Family Study (SHFS) participants at the time of circulating sphingolipids assessment [a]

	SHS		SHFS	
	Mean or %	**SD**	**Mean or %**	**SD**
Age (years)	57	7	37	16
Male (%)	34%		39%	
BMI (kg/m2)	31	6	31	7
Waist circumference (cm)	104	14	100	17
Education (years)	12	3	12	2
Smoking				
Never	29%		41%	
Ever	32%		21%	
Current	37%		38%	
Physical activity				
MET hours per week	92	84	--	--
Steps Per Day	--	--	6316	3987
Total Cholesterol (mg/dL)	199	38	181	36
HDL Cholesterol (mg/dL)	43	15	52	15
Triglycerides (mg/dL)	146	92	149	94
LDL Cholesterol (mg/dL)	127	34	100	30
Fasting Insulin (uU/mL)	-	-	16	16
Fasting Glucose (mg/dL)	-	-	94	10

[a] In the SHS, physical activity was assessed in 1989-1991 (i.e., 2-6 years before sphingolipid assessment). All other assessments in the SHS and the SHFS were done at the time of the sphingolipids assessment. Abbreviations: SHS, Strong Heart Study; SHFS, Strong Heart Family Study; BMI, body mass index; MET, metabolic equivalent task.; LDL, HDL, high density lipoprotein; LDL, low density lipoprotein

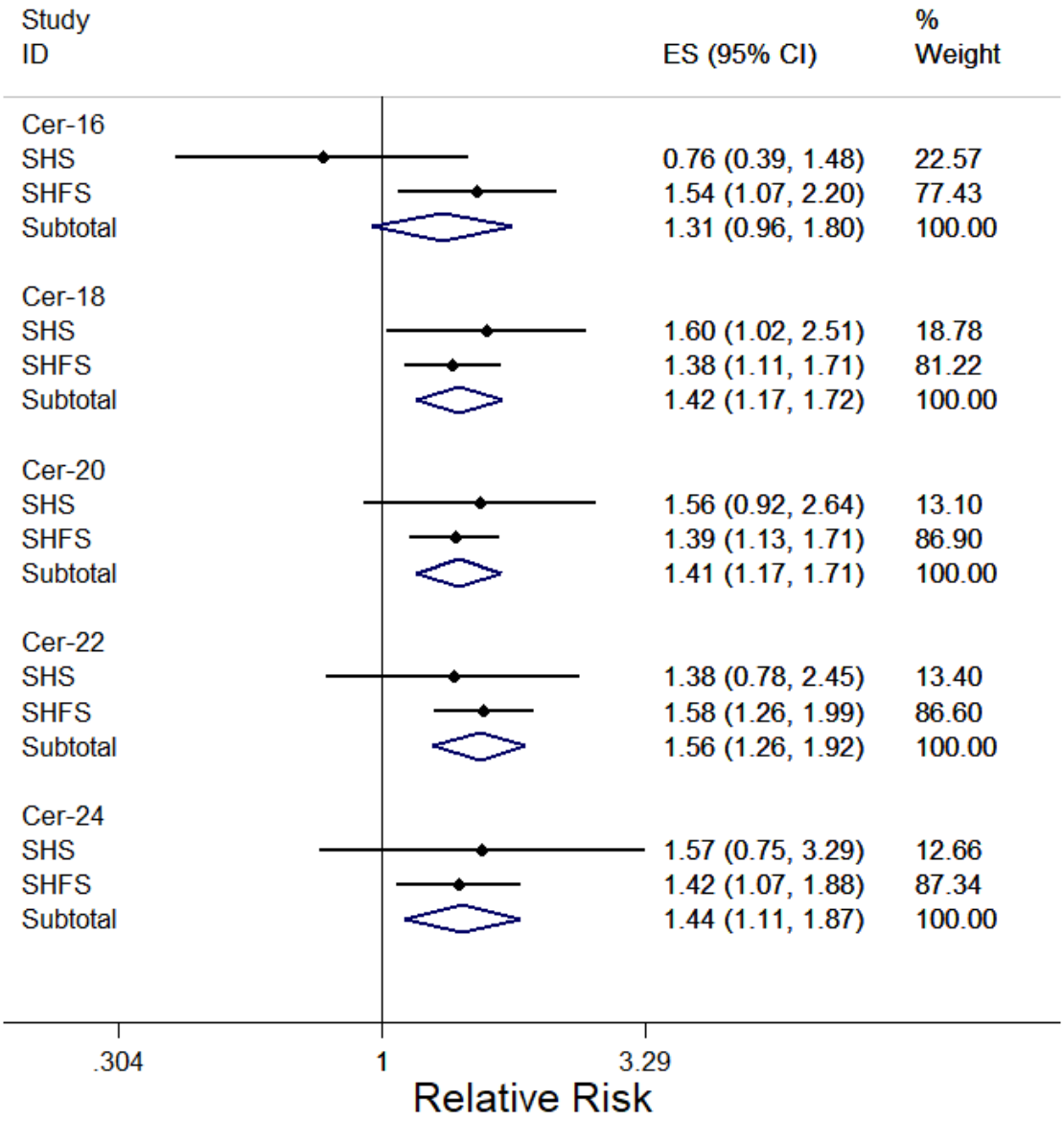

Figure 1. Forest plots of prospective associations of circulating ceramides with incident diabetes in the Strong Heart Study and the Strong Heart Family Study. Relative Risks and 95% Confidence Intervals per doubling of each ceramide species are represented by a filled circle and horizontal line for each cohort, and by a diamond for the overall pooled results. Cohort-specific associations were assessed in multivariable models adjusted for age, sex, site, education, physical activity, smoking, BMI and waist circumference.

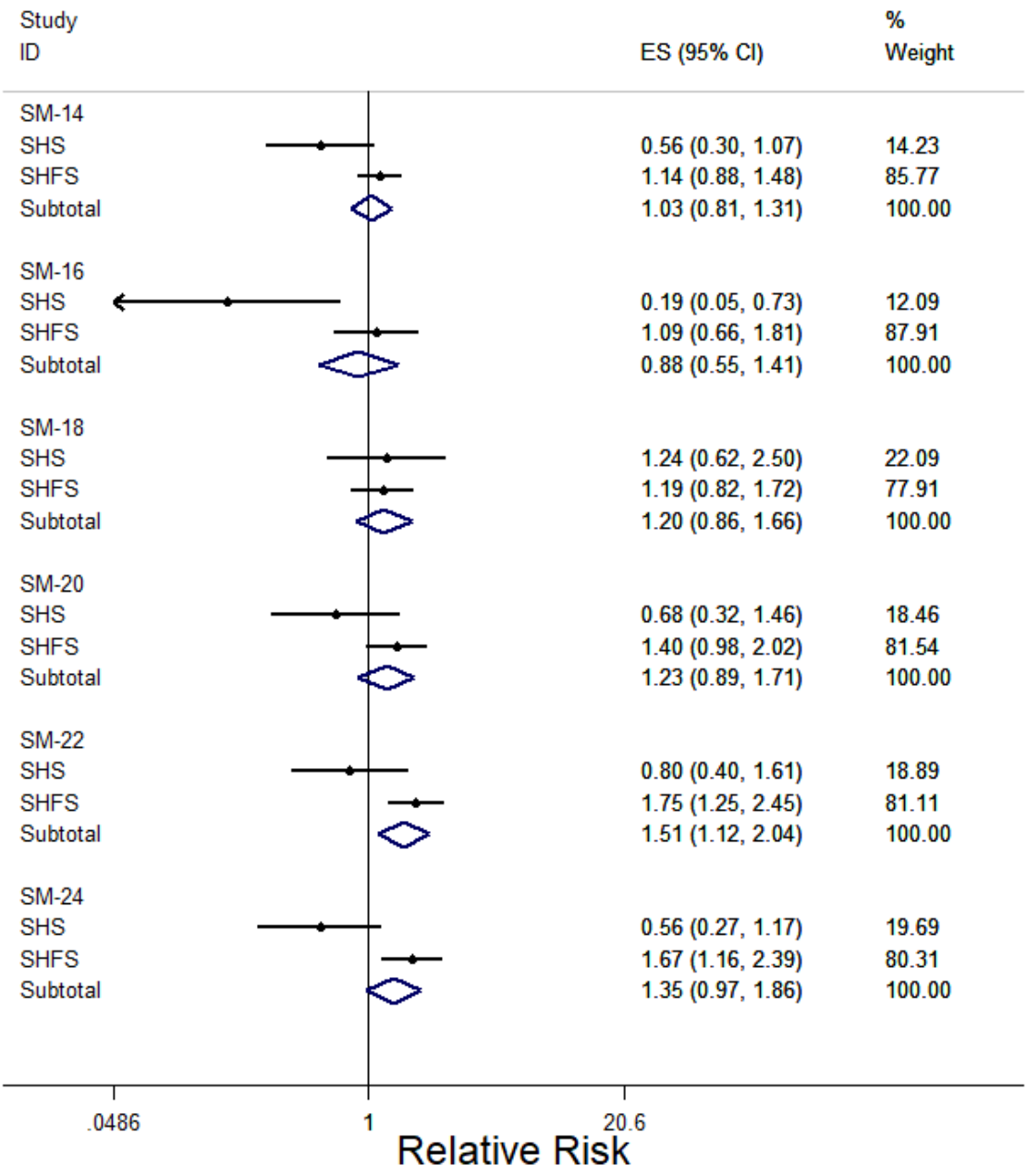

Figure 2. Forest plots of prospective associations of circulating sphingomyelin species with incident diabetes in the Strong Heart Study and the Strong Heart Family Study. Relative Risks and 95% Confidence Intervals per doubling of each sphingomyelin are represented by a filled circle and horizontal line for each cohort, and by a diamond for the overall pooled results. Cohort-specific associations were assessed in multivariable models adjusted for age, sex, site, education, physical activity, smoking, BMI and waist circumference.

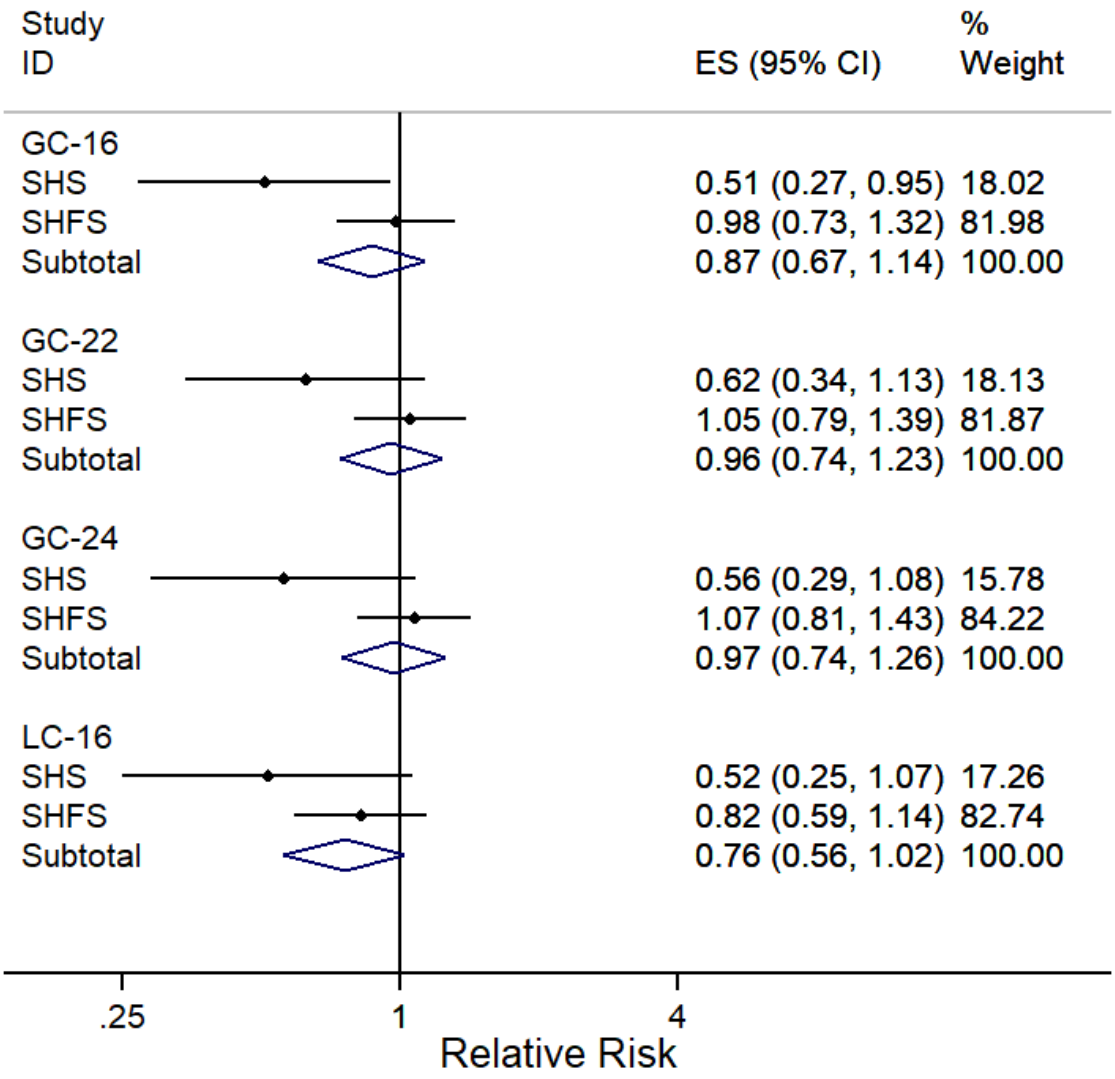

Figure 3. Forest plots of prospective associations of circulating glucosyl-ceramides and lactosyl ceramides with incident diabetes in the Strong Heart Study and the Strong Heart Family Study. Relative Risks and 95% Confidence Intervals per doubling of each glucosyl-ceramides species are represented by a filled circle and horizontal line for each cohort, and by a diamond for the overall pooled results. Cohort-specific associations were assessed in multivariable models adjusted for age, sex, site, education, physical activity, smoking, BMI and waist circumference.