SUPPLEMENTAL MATERIAL

Data S1.

SUPPLEMENTAL METHODS

Quantification of Ceramides

A two-dimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantification of C24:0, C22:0, and C16:0 ceramides was developed as a modification of a previously reported assay validated according to FDA guidance for bioanalytical method validation.[1, 2] During the course of this work, a similar assay was published by Kauhanen et al.[3]

Standard curves and quality control (QC) samples. Because of the endogenous presence of C16:0, C22:0, and C24:0 in human plasma, 5% bovine serum albumin (BSA) aqueous solution was used to prepare the calibration standards. Calibration curves were prepared by spiking the C16:0, C22:0, and C24:0 working solution into 5% BSA solution, and preparing serial dilutions that yielded eight calibration standards (0.01/0.04/0.1, 0.02/0.08/0.2, 0.05/0.2/0.5, 0.1/0.4/1, 0.2/0.8/2, 0.5/2/5, 1/4/10, and 2/8/20 μg/mL of C16:0/C22:0/C24:0 ceramides). 5% BSA solution served as blank. The standard curves prepared in 5% BSA solution were parallel to those prepared in human plasma, suggesting that the responsiveness of these ceramides in different matrices were the same and a calibration curve prepared in surrogate matrix was suitable for analysis of human plasma samples.

The pooled human plasma was analyzed to establish the mean concentration of endogenous C16:0, C22:0, and C24:0 ceramides. Low (LQC), middle (MQC), high (HQC), and dilution (DQC) quality control samples (endogenous level + 0/0/0 μg/mL, endogenous level + 0.75/3/7.5 μg/mL, endogenous level + 1.5/6/15 μg/mL, and endogenous level + 3/12/30 μg/mL) were prepared. The ceramides in the DQC samples were higher than the highest standard (2/8/20 μg/mL of C16:0/C22:0/C24:0 ceramides). The DQC sample was diluted 1:4 with 5% BSA solution, prior to extraction.

Sample preparation. Standards, QCs, blank or study samples (50 μL) were aliquoted into a 96-well (2 mL/well) plate. To each well 400 μL of internal standards/protein precipitation solution

(0.025/0.025/0.0625 μg/mL of d5-C16:0, d4-C22:0, and d4-C24:0 ceramides in isopropanol-chloroform (9:1) was added and 400 μL of isopropanol-chloroform (9:1) was used for a blank. The plate was vortexed for 3 min, centrifuged for 10 min at 3000 g, and 250 μL of supernatant transferred to clean 96 wells (1 mL/well) plate with a Tomtec Quadra 96 (Tomtec, Hamden, CT) for LC-MS/MS assay.

LC-MS/MS analysis. LC–MS/MS analysis was conducted on a Shimadzu (Columbia, MD) Prominence HPLC system coupled with an Applied Biosystems/MDS Sciex (Ontario, Canada) 4000QTRAP mass spectrometer using multiple reaction monitoring (MRM). The HPLC system consists of Prominence HPLC system with a CBM-20A system controller, 4 LC-20AD pumps, a SIL-20ACHT autosampler, a DGU-20A5R degasser, and a rack changer.

The chromatography was performed using an Atlantis HILIC silica column (3 × 50 mm, 3 μm; Waters, Milford, MA) as the first dimension at ambient temperature and Xselect HSS C18 (4.6 x 50 mm, 3.5 μm; Waters, Milford, MA) as the second dimension at ambient temperature. The compartments of the autosampler and rack changer were set at 4°C. For the first dimension LC, mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) were operated with a gradient elution as follows: 0–1.0 min 95% B, 1.0–1.2 min 95–50% B, 1.2–2.4 min 50% B, 2.4–2.5 min 50–95% B, and 2.5–5.0 min 95% B at a flow rate of 0.6 mL/min. The solvent gradient for second dimension LC using 0.1% formic acid in water (phase C) and 0.1% formic acid in isopropanol-acetonitrile (1:2) (phase D) at a flow rate of 1 mL/min was as follows: 0–0.9 min 95% D, 0.9–3.0 min 95–100% D, 3.0–4.5 min 100% D, 4.5–4.6 min 100–95% D, and 4.6–5.0 min 95% D. Valve 1 was kept at the A position during 0–0.5 min and 0.9–5.0 min, and at the B position during 0.5–0.9 min. Valve 2 was kept at the A position during 0–2.0 min and 3.7–5.0 min, and at the B position during 2.0–3.7 min. The injection volume was 5 μL. The ESI source temperature was 400 °C. The ESI spray voltage was 5500 V. For all the ceramides and their internal standards, the declustering potential, entrance potential, and the collision cell

exit potential were 66 V, 10 V, and 10 V, respectively. The collision and curtain gas were set at medium and 15, respectively. Both desolvation gas and nebulizing gas were set at 45 L/min. The collision energies for all the MRM transitions including *m/z* 538.5 to 264.3 (quantifier for C16:0), *m/z* 538.5 to 282.3 (qualifier for C16:0), *m/z* 622.6 to 264.3 (quantifier for C22:0), *m/z* 622.6 to 282.3 (qualifier for C22:0), *m/z* 650.6 to 264.3 (quantifier for C24:0), *m/z* 650.6 to 282.3 (qualifier for C24:0), *m/z* 543.5 to 264.3 (d5-C16:0), m/z 626.6 to 264.3 (d4-C22:0) and *m/z* 654.6 to 264.3 (d4-C24:0) were set at 40 eV. The dwell time was set at 50 ms for each mass transition. Data were acquired and analyzed by Analyst software (version 1.5.2). Calibration curves were constructed by plotting the corresponding peak area ratios of analyte/internal standard versus the corresponding analyte concentrations using weighted (1/x2) least-squares regression analysis.

Analysis of clinical samples. Samples analyzed consisted of calibration standards in duplicate, a blank, a blank with internal standards, QC samples (LQC, MQC and HQC), and unknown clinical samples. The total number of QC samples was at least 5% of that of unknown clinical samples. The standard curve covered the expected unknown sample concentration range, and samples that exceeded the highest standard could be diluted and re-assayed. In the dilution sample re-assay, a diluted QC in triplicate is also included in the analytical run. The LC-MS/MS run acceptance criteria included: 1) a minimum of six standards within ±15%, except for the lowest standard for which ±20% of the nominal value was accepted; 2) at least 67% of the QC samples within 15% of their respective nominal values; and 3) not all replicates at the same level of QC outside ±15% of the nominal value.[2] The analysis for FHS and SHIP samples was performed in 16 and 7 batches, respectively. All batches met acceptance criteria.

Analysis of SHIP Samples

Because exact dates of cardiovascular events are not available in SHIP, when modeling the association between ceramides and CHD or HF, we chose to use the Poisson model as opposed to Cox proportional hazards regression. In general, parametric models are more flexible to handle interval-censored data problems.[4, 5] In the present analysis, we chose the mid-point between a participant's SHIP-1 and SHIP-2 date as his/her event date among those participants with an event. This approach acknowledges the interval censoring, but assumes that events happened in the middle of the interval.[6-9] Furthermore, we utilized a Poisson model with an offset equal to the log follow-up time to model the event rates. This approach is equivalent to a parametric exponential survival (i.e. constant hazard) model and can therefore be used in time-to-event analysis.[5]

SUPPLEMENTAL REFERENCES:

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Table S1. C16:0, C22:0 and C24:0 Ceramides and C24:0/C16:0 and C22:0/C16:0 Ceramide Ratios in Healthy Volunteers.

Measure	Time 1 range	Time 1 mean	Time 2 range	Time 2 mean	Mean % change*	95% CI	*P*Value†
C16:0 ceramide	0.105 - 0.270	0.151	0.0612 - 0.187	0.129	-12.6	(-20.1, -5.0)	0.002
C22:0 ceramide	0.291 - 0.979	0.547	0.156 - 0.988	0.502	-8.8	(-16.6, -1.0)	0.028
C24:0 ceramide	0.879 - 3.50	2.04	0.735 - 3.08	1.82	-9.8	(-17.6, -2.0)	0.017
C24:0/C16:0 ceramide ratio	6.43 - 25.0	13.6	8.88 - 22.3	14.0	6.4	(-4.2, 17.0)	0.23
C22:0/C16:0 ceramide ratio	1.63 - 4.99	3.59	2.05 - 7.16	3.80	6.6	(-1.9, 15.2)	0.12

Ceramides were quantified in fasting plasma obtained in blood draws at times 1 and 2 (2 weeks apart) from 24 human volunteers who were free of diabetes, hypertension, obstructive coronary heart disease and smoking. Ceramide values are reported in μg/ml; ceramide ratios have no units.

The difference and percent change were calculated per subject and then aggregated and summarized by the mean difference and mean percent change, respectively. [Percent change = 100 (Time 2– Time 1)/Time 1 (calculated per patient)]

† Test to determine if percent change differs from 0%.

Table S2. Clinical Correlates of Plasma C22:0/C16:0 Ceramide Ratios in FHS and SHIP.

	FHS		SHIP	
Variable	βEstimate	*P*Value	βEstimate	*P*Value
Age	-0.012	<0.0001	-0.006	<0.001
Male	0.049	0.13	-0.025	0.28
Body Mass Index	0.015	<0.0001	0.019	<0.001
Systolic Blood Pressure	0.002	0.06	0.002	0.003
Antihypertensive Medication	-0.12	0.0006	-0.025	0.38
Smoking Status	-0.128	0.0195	-0.024	0.35
Diabetes Status	0.18	0.0002	0.055	0.10
Total/HDL Cholesterol	0.073	0.0003	0.055	<0.001
Triglycerides	0.002	<0.0001	0.0003	0.007
Lipid Lowering Medication	0.028	0.42	0.054	0.13
Prevalent CVD	-0.136	0.0028	-0.050	0.07

Multiple linear regression models were used, where the ceramide ratio served as the dependent variable and clinical correlates served as independent variables. βEstimates represent the increase in ceramide levels for a unit increase in continuous variables and for presence vs. absence of dichotomous variables.

FHS = Framingham Heart Study, SHIP = Study of Health in Pomerania

Table S3. Clinical Correlates of Individual Plasma Ceramide Species in FHS and SHIP.

	C16:0 Ceramide			
	FHS		SHIP	
Variable	*β*Estimate	*P*Value	*β*Estimate	*P*Value
Age	0.0006	< 0.0001	0.001	< 0.001
Male	-0.015	< 0.0001	-0.018	< 0.001
Body Mass Index	-0.001	< 0.0001	-0.0009	< 0.001
Systolic Blood Pressure	0.00005	0.23	0.0001	0.003
Antihypertensive Medication	-0.004	0.005	-0.008	< 0.001
Smoking Status	0.007	0.002	0.009	< 0.001
Diabetes Status	-0.004	0.0346	-0.008	0.002
Total/HDL Cholesterol	0.008	< 0.0001	0.009	< 0.001
Triglyceride	0.0001	< 0.0001	0.00003	< 0.001
Lipid Lowering Medication	-0.02	< 0.0001	-0.013	< 0.001
Prevalent CVD	-0.002	0.31	0.0002	0.91
	C22:0 Ceramide			
	FHS		SHIP	
Variable	*β*Estimate	*P*Value	*β*Estimate	*P*Value
Age	0.00009	0.80	0.002	< 0.001
Male	-0.051	< 0.0001	-0.064	< 0.001
Body Mass Index	-0.002	0.0047	0.001	0.048
Systolic Blood Pressure	0.0005	0.0055	0.0009	< 0.001
Antihypertensive Medication	-0.036	< 0.0001	-0.028	< 0.001
Smoking Status	0.002	0.88	0.022	0.002
Diabetes Status	0.007	0.41	-0.011	0.24
Total/HDL Cholesterol	0.042	< 0.0001	0.041	< 0.001

Triglyceride	0.001	< 0.0001	0.0002	< 0.001
Lipid Lowering Medication	-0.07	< 0.0001	-0.027	0.005
Prevalent CVD	-0.028	0.0012	-0.008	0.30

	C24:0 Ceramide			
	FHS		SHIP	
Variable	*β*Estimate	*P*Value	*β*Estimate	*P*Value
Age	-0.007	< 0.0001	0.005	< 0.001
Male	-0.086	0.0002	-0.102	< 0.001
Body Mass Index	-0.016	< 0.0001	-0.012	< 0.001
Systolic Blood Pressure	0.003	< 0.0001	0.004	< 0.001
Antihypertensive Medication	-0.137	< 0.0001	-0.141	< 0.001
Smoking Status	-0.018	0.64	0.053	0.037
Diabetes Status	-0.036	0.29	-0.098	0.003
Total/HDL Cholesterol	0.072	< 0.0001	0.121	< 0.001
Triglyceride	0.003	< 0.0001	0.0004	< 0.001
Lipid Lowering Medication	-0.253	< 0.0001	-0.081	0.018
Prevalent CVD	-0.132	< 0.0001	-0.049	0.07

Multiple linear regression models were used, where ceramides served as dependent variables and clinical correlates served as independent variables; beta estimates represent the increase in ceramide levels for a unit increase in continuous variables and for presence vs. absence of dichotomous variables.

FHS = Framingham Heart Study, SHIP = Study of Health in Pomerania

Table S4. Incremental Effect of Incorporating hsCRP and ntBNP on Model Discrimination.

Predictors in Model	FHS Samples				
	Incident CHD c-statistic	Incident HF c-statistic	All-Cause Mortality* c-statistic	CVD Mortality* c-statistic	Non-CVD Mortality* c-statistic
	n=2327	n=2533	n=2624	n=2624	n=2624
Standard Risk Factors (SRF) †	0.703	0.840	0.756	0.834	0.743
SRF + C24:0/C16:0 ratio	0.714	0.844	0.776‡	0.832	0.768‡
SRF + hsCRP	0.710	0.841	0.761‡	0.835	0.748‡
Predictors in Model	**SHIP Samples**				
	Incident CHD c-statistic	Incident HF c-statistic	All-Cause Mortality* c-statistic	CVD Mortality* c-statistic	Non-CVD Mortality* c-statistic
	n=1555	n=1643	n=2635	n=2635	n=2635
Standard Risk Factors (SRF) †	0.7159	0.6739	0.8593	0.9132	0.8483
SRF + C24:0/C16:0 ratio	0.7234	0.6764	0.8661‡	0.9176	0.8554
SRF + hsCRP	0.7172	0.6775	0.8606	0.9132	0.8503
SRF + ntBNP	0.7160	0.6758	0.8633‡	0.9192‡	0.8503

* Prevalent CVD added to standard risk factors

† Standard risk factors: age, sex, BMI, SBP, antihypertensive medication, current smoking status, diabetes, total/HDL cholesterol, triglycerides, and lipid-lowering medication.

‡ Confidence interval for change in c-statistic excludes 0

FHS= Framingham Heart Study

SHIP=Study of Health in Pomerania

Figure S1. Generation of FHS Samples.

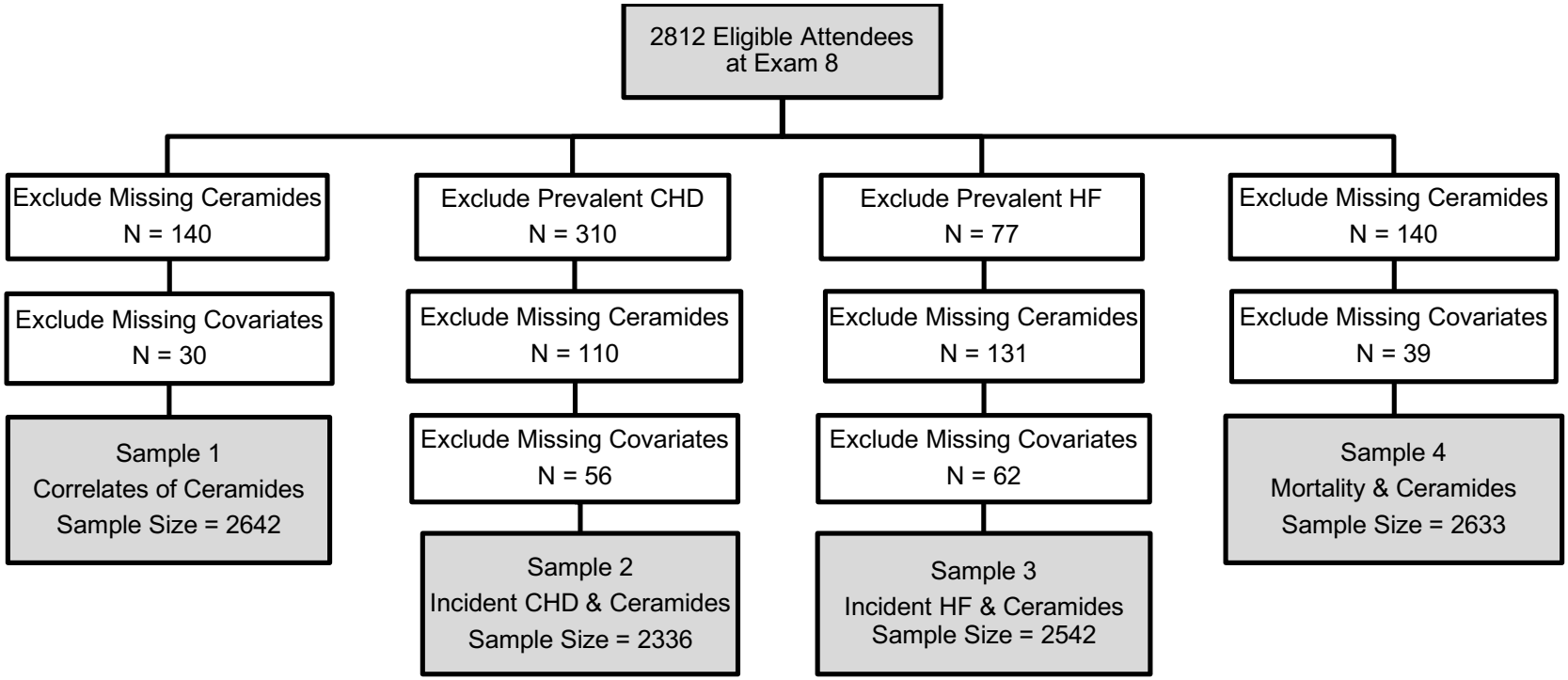


Figure S1. Generation of FHS Samples. From 2,812 participants in the Offspring Cohort who attended their 8th examination cycle, 4 participant samples were created based on availability of plasma samples and covariate data. For Sample 1, individuals were excluded if they were missing ceramide values or covariates. For Samples 2 and 3, individuals with prevalent coronary heart disease (CHD, sample 2) and heart failure (HF, sample 3) were excluded. For Sample 4, individuals with missing follow-up time were excluded.

Figure S2. Generation of SHIP Samples

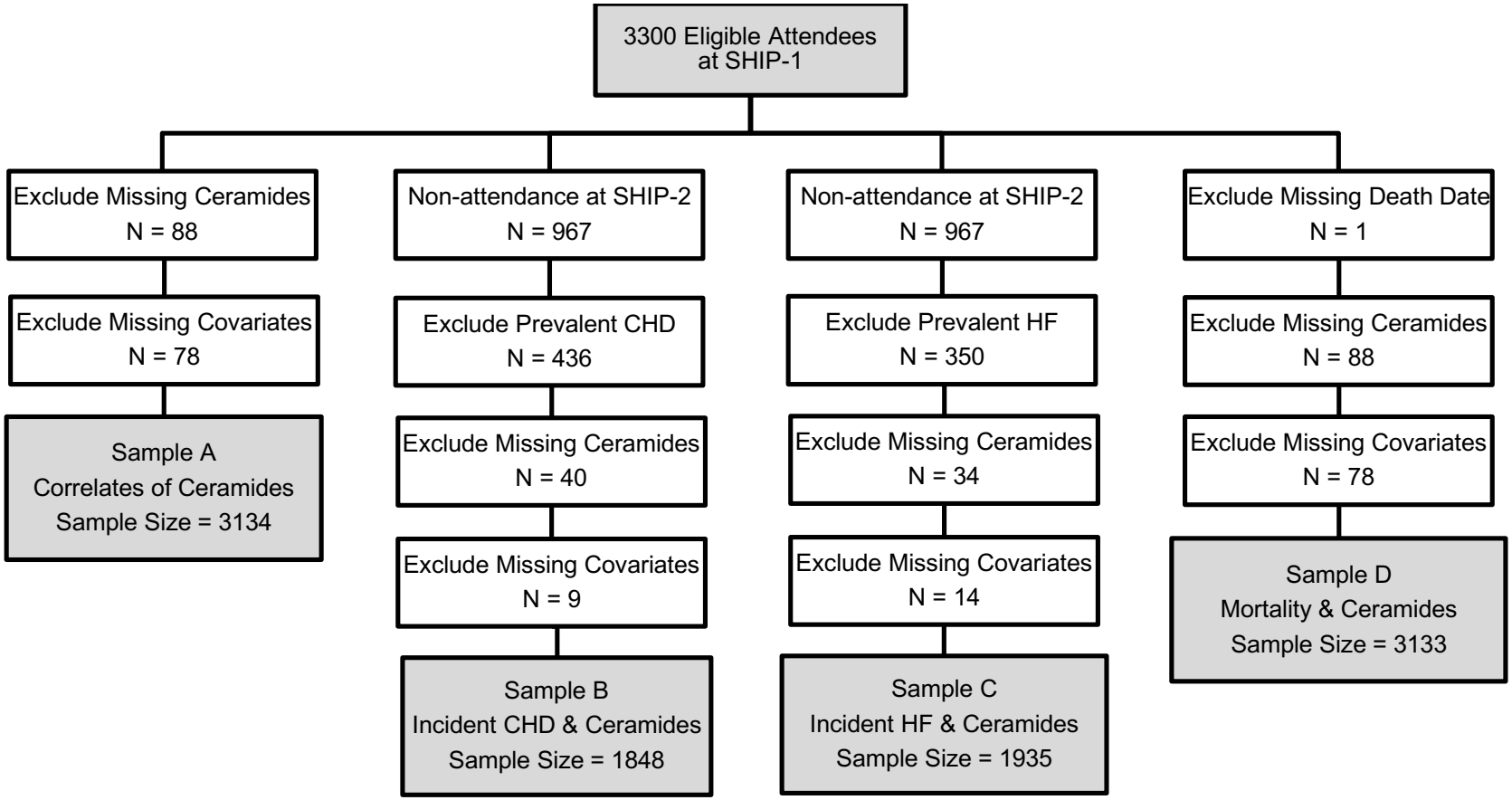


Figure S2. Generation of SHIP Samples. From 3,300 participants who attended SHIP-1, 4 participant samples were created based on availability of plasma samples and covariate data. For Sample A, individuals were excluded if they were missing ceramide values or covariate data. Samples B and C included those who had ceramide and covariate data and also attended SHIP-2 when CHD and HF were assessed (Sample B excluded those with CHD at SHIP-1 and Sample C excluded those with HF at SHIP-1. Samples B and C excluded individuals with uncertain event status at SHIP-2). Sample D excluded those with missing ceramides or covariates or with unknown death date.

Figure S3. Distributions of Plasma Ceramides in FHS and SHIP.

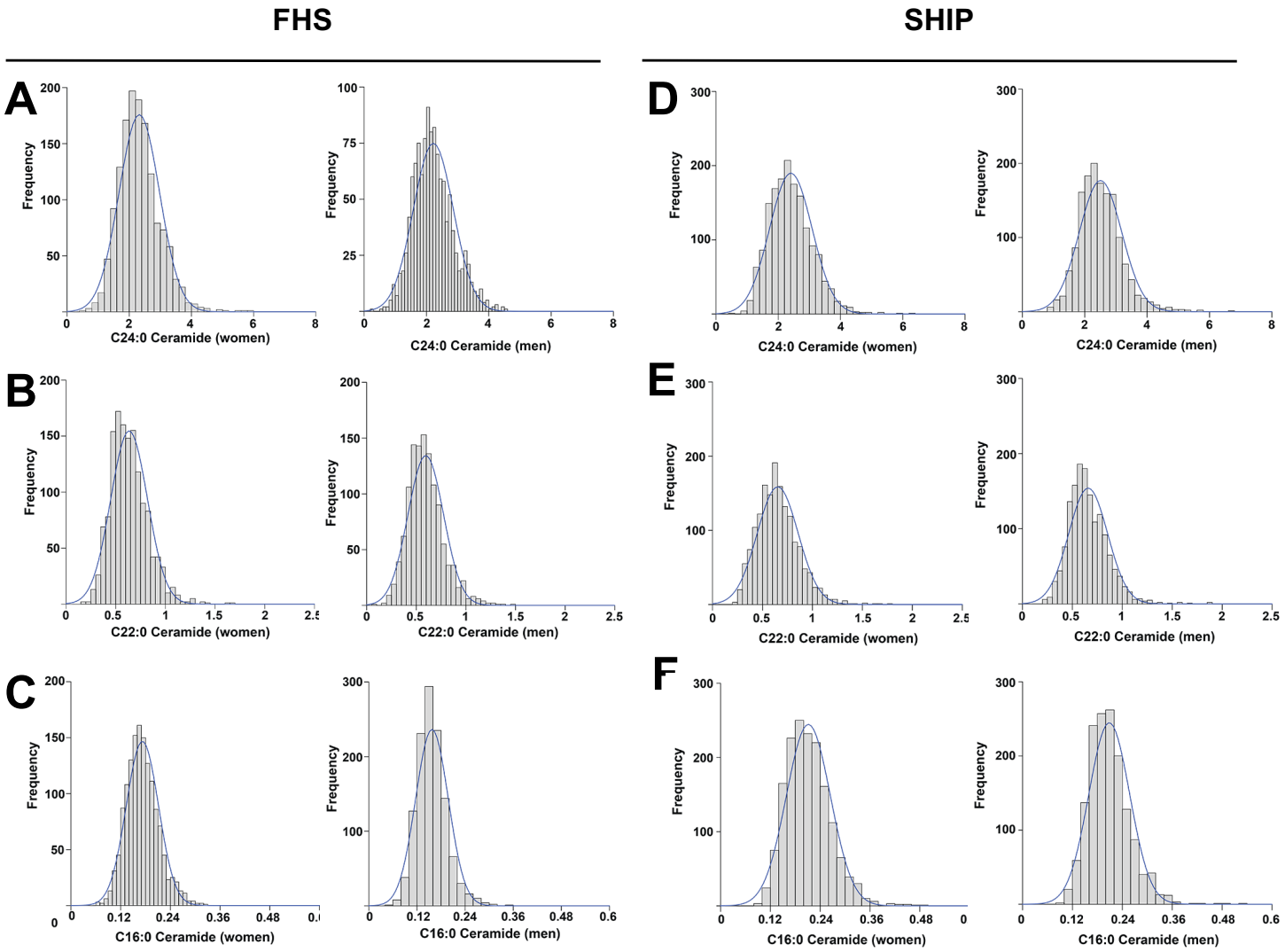
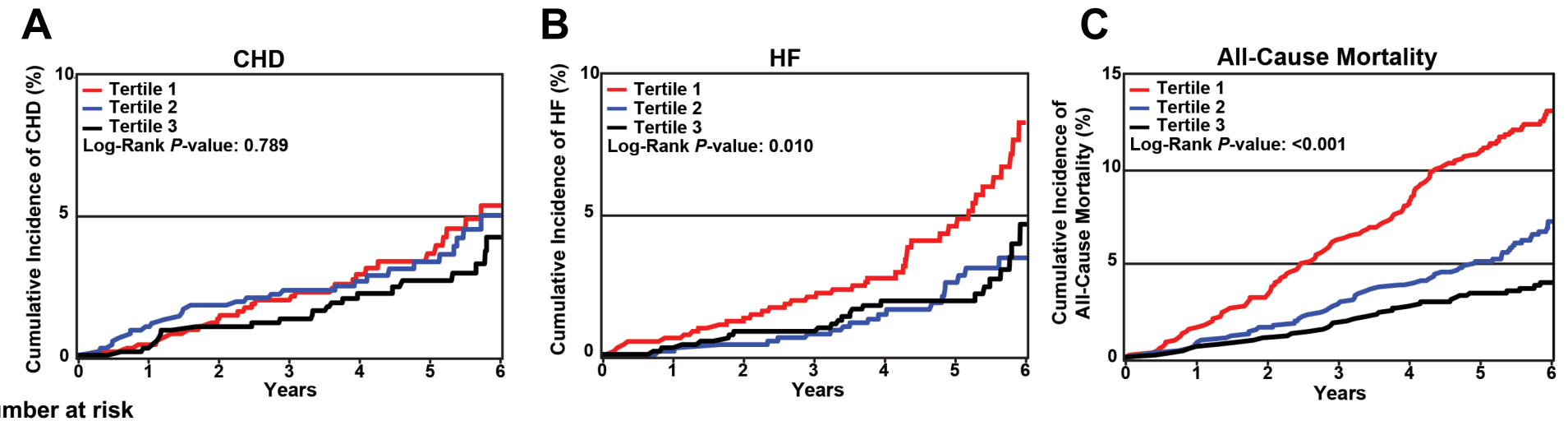


Figure S3. Distributions of Plasma Ceramides in FHS and SHIP. Plots display distribution of values for C24:0 (A, D), C22:0 (B, E), and C16:0 (C, F) ceramides in women and men FHS participants at examination 8 (A, B, C) and in SHIP participants at SHIP-1 examination (D, E, F).

Figure S4. Cumulative Incidence of Coronary Heart Disease, Heart Failure, and All-Cause Mortality in FHS by Tertiles of Plasma C22:0/C16:0 Ceramide Ratio.



Number at risk

Tertile 1	778	766	737	691	484	335	114	847	832	804	747	525	358	121	876	863	846	820	798	770	462
Tertile 2	780	770	749	694	495	364	101	846	840	818	755	529	384	98	880	874	866	852	842	818	491
Tertile 3	778	772	750	693	500	391	111	849	842	819	755	541	412	108	877	872	868	861	852	836	531

Figure S4. Cumulative Incidence of Coronary Heart Disease, Heart Failure, and All-Cause Mortality in FHS by Tertiles of Plasma C22:0/C16:0 Ceramide Ratio. Cumulative incidence of coronary heart disease (CHD, A), heart failure (HF, B), and all-cause mortality (C) are reported for tertiles of C22:0/C16:0 ceramide ratio. Tertile 1 includes participants with ceramide levels ≤ the 33rd percentile [1.0, 3.4]; tertile 2 includes participants with ceramide levels between the 33rd and 66th percentile [3.4, 4.1]; tertile 3 includes participants with ceramide levels ≥ the 66th percentile [4.1, 10.5].

Figure S5.Risk of Coronary Heart Disease, Heart Failure, and All-Cause mortality by C22:0/C16:0 Ceramide Ratio.

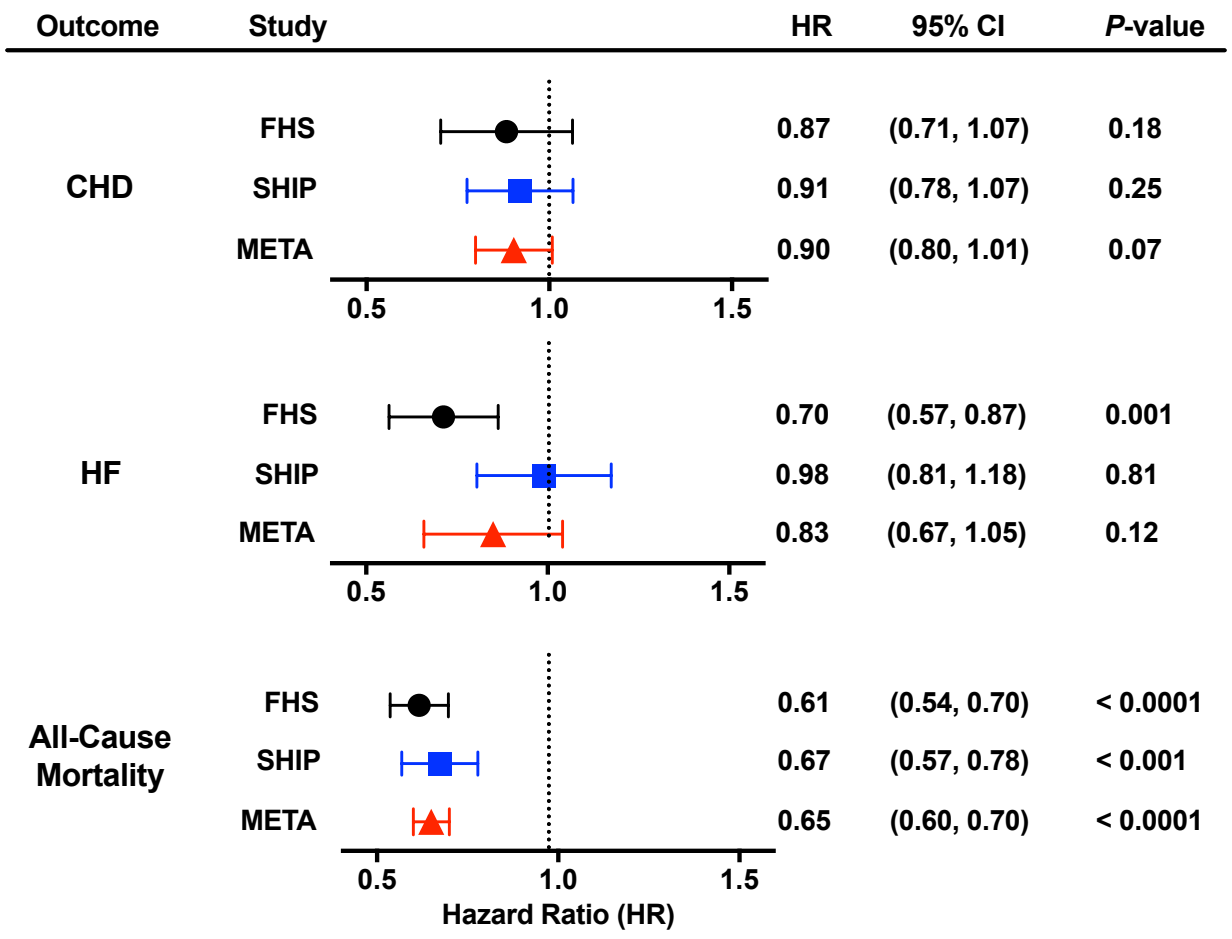


Figure S5. Risk of Coronary Heart Disease, Heart Failure, and All-Cause mortality by C22:0/C16:0 Ceramide Ratio. Hazard ratios (HR) for coronary heart disease (CHD), heart failure (HF), and all-cause mortality are reported with 95% confidence intervals (CI) for a 0.7-unit increase in C22:0/C16:0 ceramide ratio (average of standard deviations between FHS and SHIP), adjusting for all other variables in the model. Data is shown from analysis of subjects in FHS, SHIP and the combined meta-analysis. $I^2 = 0$ for CHD; $I^2 = 0.81$ for HF; $I^2 = 0.13$ for all-cause mortality.

Figure S6.Risk of Coronary Heart Disease, Heart Failure, and All-Cause Mortality by C24:0 Ceramide Level.

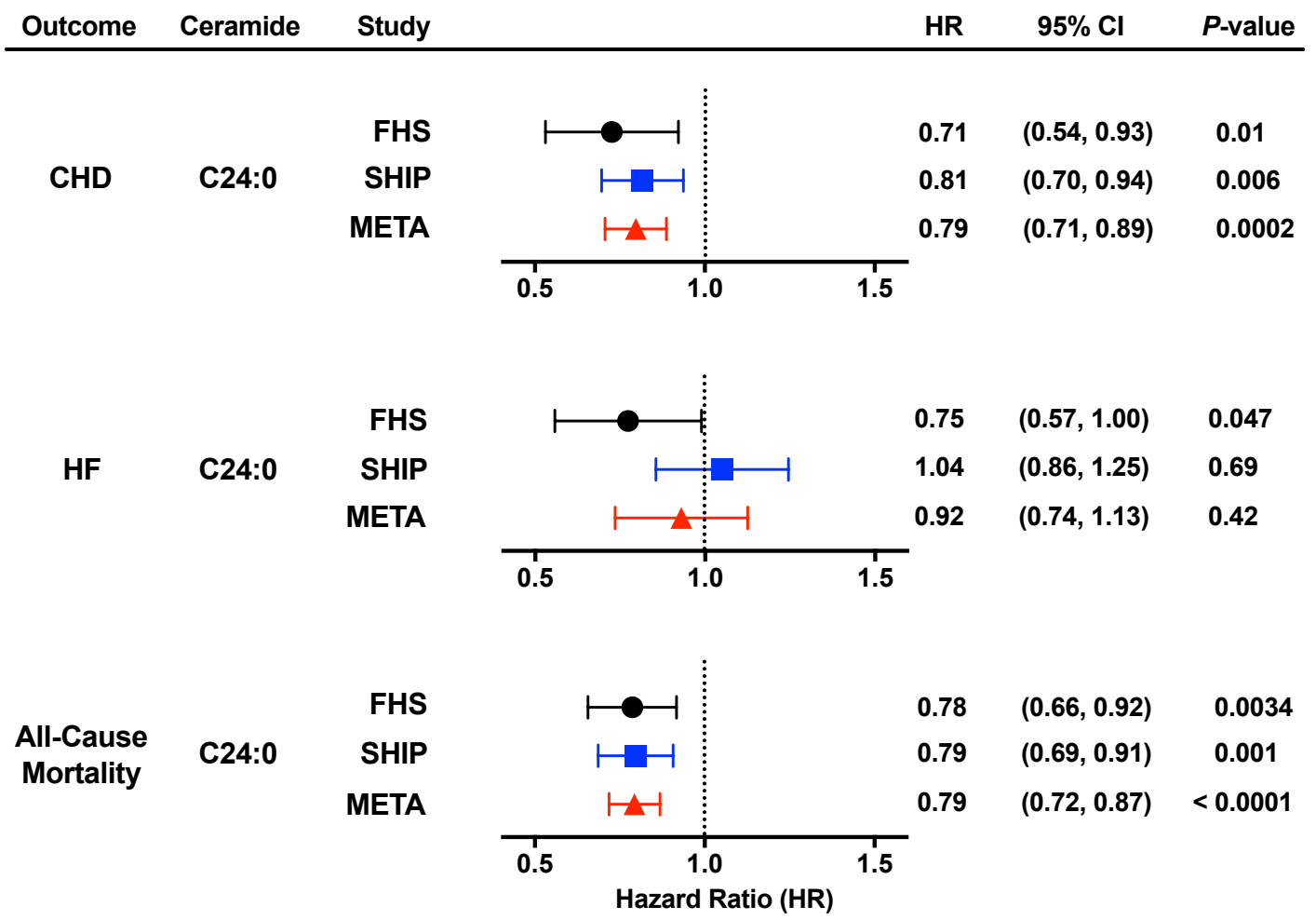


Figure S6. Risk of Coronary Heart Disease, Heart Failure, and All-Cause Mortality by C24:0 Ceramide Level. Hazard ratios (HR) for coronary heart disease (CHD), heart failure (HF), and all-cause mortality are reported with 95% confidence intervals (CI) for a 0.65 μg/ml increase in C24:0 ceramide level (average of standard deviations between FHS and SHIP), adjusting for all other variables in the model. Data is shown from analysis of subjects in FHS, SHIP and the combined meta-analysis. $I^2 < 0.0001$ for CHD; $I^2 = 0.71$ for HF; $I^2 = 0$ for all-cause mortality.

Figure S7. Risk of Coronary Heart Disease, Heart Failure, and All-Cause Mortality by C16:0 Ceramide Level.

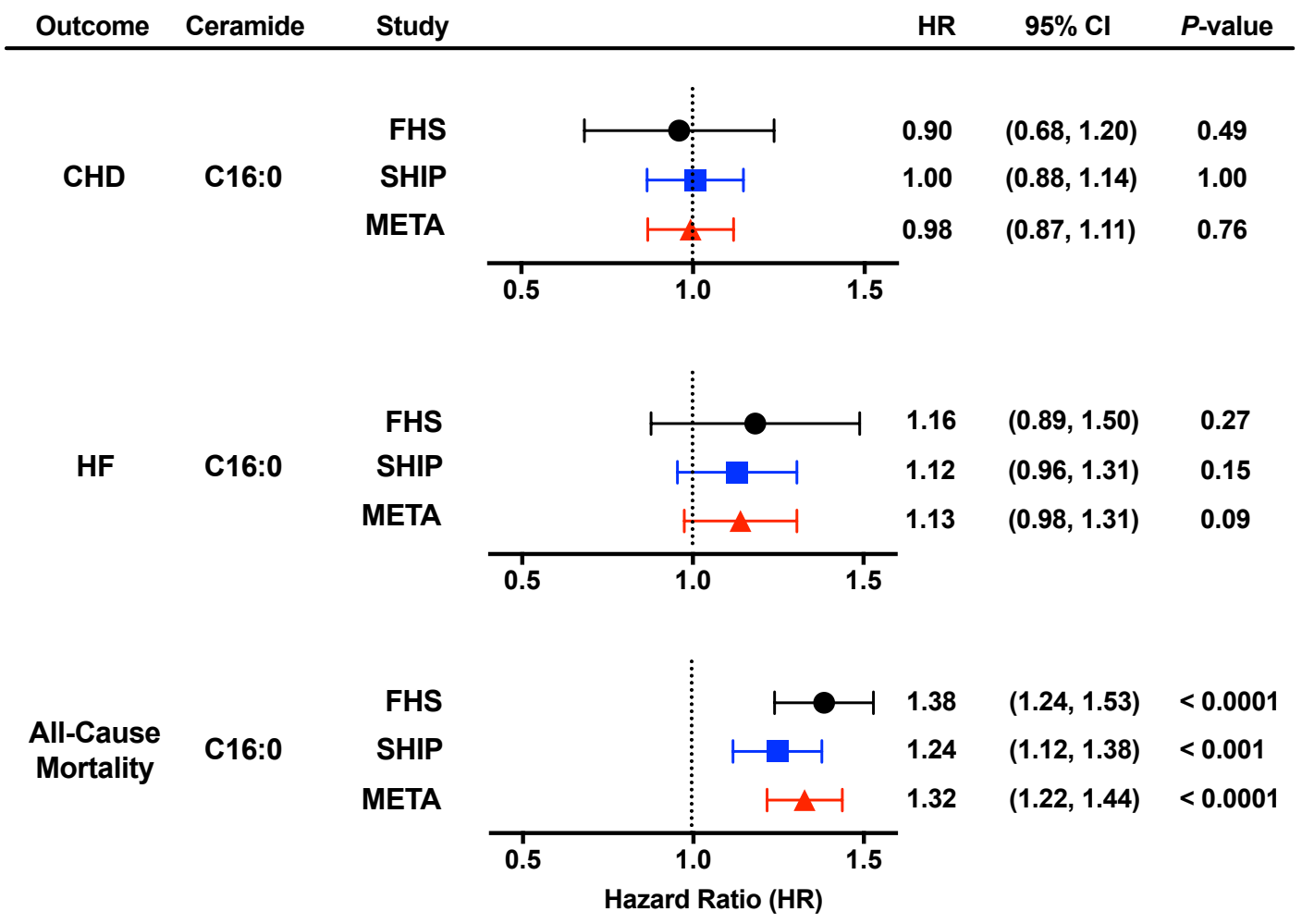


Figure S7. Risk of Coronary Heart Disease, Heart Failure, and All-Cause Mortality by C16:0 Ceramide Level. Hazard ratios for coronary heart disease (CHD), heart failure (HF), and all-cause mortality are reported with 95% confidence intervals (CI) for a 0.045 μg/ml increase in C16:0 ceramide level (average of standard deviations between FHS and SHIP), adjusting for all other variables in the model. Data is shown from analysis of subjects in FHS, SHIP and the combined meta-analysis. $I^2 = 0$ for CHD and for HF. $I^2 = 0.26$ for all-cause mortality.