SUPPLEMENTARY DATA

Supplementary Table 1. Association of sphingolipid species with plasma fasting insulin and change in insulin

Species	Outcome: Baseline insulin Estimate* (95% CI), p	Outcome: Follow-up insulin Estimate* (95% CI), p	Outcome: Change in insulin Estimate (95% CI), p
Cer-16:0	1.13 (1.07-1.20), $1.9x10^{-5}$	1.10 (1.01-1.21), 0.03	1.05 (0.95-1.16), 0.33
Cer-18:0	1.11 (1.06-1.15), $1.0x10^{-6}$	1.12 (1.06-1.19), 0.0001	1.08 (1.01-1.15), 0.03
Cer-20:0	1.12 (1.07-1.17), $1.5x10^{-7}$	1.10 (1.03-1.18), 0.003	1.06 (0.99-1.14), 0.09
Cer-22:0	1.13 (1.08-1.19), $3.9x10^{-7}$	1.12 (1.04-1.20), 0.004	1.06 (0.97-1.14), 0.20
Cer-24:0	1.05 (0.99-1.11), 0.08	1.09 (1.01-1.19), 0.04	1.07 (0.97-1.17), 0.16
SM-14:0	1.00 (0.96-1.05), 0.91	1.00 (0.93-1.07), 0.94	0.98 (0.91-1.06), 0.69
SM-16:0	0.89 (0.81-0.97), 0.009	0.96 (0.83-1.10), 0.53	0.99 (0.85-1.15), 0.91
SM-18:0	0.95 (0.89-1.02), 0.19	1.04 (0.93-1.15), 0.52	1.05 (0.94-1.18), 0.38
SM-20:0	0.95 (0.89-1.02), 0.17	1.02 (0.92-1.13), 0.73	1.03 (0.92-1.15), 0.64
SM-22:0	1.00 (0.93-1.07), 0.97	1.05 (0.94-1.16), 0.39	1.03 (0.92-1.16), 0.56
SM-24:0	0.96 (0.90-1.02), 0.22	1.00 (0.91-1.11), 0.92	1.01 (0.91-1.13), 0.79
GluCer-16:0	0.96 (0.90-1.02), 0.16	0.96 (0.88-1.05), 0.39	0.97 (0.88-1.07), 0.50
GluCer-22:0	0.93 (0.87-0.98), 0.013	0.97 (0.88-1.06), 0.46	0.98 (0.90-1.08), 0.71
GluCer-24:0	0.92 (0.86-0.97), 0.0023	0.95 (0.87-1.03), 0.24	0.97 (0.89-1.07), 0.54
LacCer-16:0	0.87 (0.82-0.94), 0.0001	0.93 (0.84-1.03), 0.16	0.98 (0.88-1.10), 0.71

*Fold change in insulin geometric mean with 2 fold higher sphingolipid

Supplementary Table 2. Association of sphingolipid species with HOMAIR and change in HOMAIR

Species	Baseline HOMAIR Estimate* (95% CI), p	Follow-up HOMAIR Estimate* (95% CI), p	Change in HOMAIR Estimate (95% CI), p
Cer-16:0	1.15 (1.08-1.22), $4.7x10^{-6}$	1.11 (1.01-1.22), 0.03	1.05 (0.95-1.17), 0.32
Cer-18:0	1.12 (1.08-1.17), $6.3x10^{-8}$	1.13 (1.06-1.20), $2.0x10^{-4}$	1.08 (1.01-1.16), 0.03
Cer-20:0	1.13 (1.08-1.18), $1.3x10^{-7}$	1.11 (1.04-1.19), 0.002	1.07 (0.99-1.15), 0.07
Cer-22:0	1.15 (1.09-1.21), $1.0x10^{-7}$	1.13 (1.04-1.22), 0.004	1.06 (0.97-1.16), 0.17
Cer-24:0	1.06 (1.00-1.13), 0.04	1.11 (1.01-1.21), 0.03	1.08 (0.98-1.19), 0.13
SM-14:0	1.01 (0.96-1.06), 0.79	1.00 (0.92-1.07), 0.92	0.98 (0.90-1.06), 0.62
SM-16:0	0.87 (0.79-0.96), 0.006	0.95 (0.82-1.10), 0.53	0.99 (0.84-1.16), 0.87
SM-18:0	0.96 (0.89-1.03), 0.24	1.03 (0.92-1.16), 0.59	1.05 (0.92-1.18), 0.48
SM-20:0	0.95 (0.89-1.02), 0.19	1.01 (0.90-1.13), 0.86	1.02 (0.90-1.15), 0.79
SM-22:0	1.00 (0.93-1.08), 0.91	1.04 (0.93-1.17), 0.45	1.03 (0.91-1.16), 0.68
SM-24:0	0.97 (0.90-1.03), 0.31	1.01 (0.91-1.12), 0.90	1.01 (0.90-1.13), 0.86
GluCer-16:0	0.95 (0.89-1.02), 0.15	0.96 (0.87-1.06), 0.43	0.97 (0.87-1.07), 0.51
GluCer-22:0	0.93 (0.87-0.98), 0.01	0.96 (0.88-1.06), 0.44	0.98 (0.88-1.08), 0.63
GluCer-24:0	0.92 (0.86-0.97), 0.004	0.95 (0.87-1.05), 0.31	0.97 (0.88-1.07), 0.59
LacCer-16:0	0.86 (0.80-0.92), $3.7x10^{-5}$	0.92 (0.82-1.02), 0.12	0.97 (0.86-1.09), 0.59

*Fold change in HOMAIR geometric mean with 2 fold higher sphingolipid

SUPPLEMENTARY DATA

Supplementary Table 3. Association of sphingolipid species with HOMAB and change in HOMAB

Species	Baseline HOMAB Estimate* (95% CI), p	Follow-up HOMAB Estimate* (95% CI), p	Change in HOMAB Estimate* (95% CI), p
Cer-16:0	1.04 (0.99-1.11), 0.13	1.06 (0.98-1.16), 0.14	1.04 (0.95-1.14), 0.35
Cer-18:0	1.04 (1.00-1.09), 0.03	1.09 (1.04-1.15), 0.001	1.06 (1.00-1.13), 0.045
Cer-20:0	1.06 (1.02-1.11), 0.003	1.06 (1.00-1.13), 0.04	1.04 (0.97-1.10), 0.28
Cer-22:0	1.07 (1.02-1.12), 0.004	1.09 (1.02-1.17), 0.02	1.04 (0.97-1.12), 0.26
Cer-24:0	1.00 (0.94-1.05), 0.86	1.05 (0.97-1.13), 0.20	1.04 (0.96-1.13), 0.34
SM-14:0	0.97 (0.93-1.01), 0.19	0.97 (0.91-1.04), 0.37	0.97 (0.91-1.04), 0.45
SM-16:0	0.89 (0.82-0.98), 0.01	0.93 (0.82-1.05), 0.25	0.98 (0.86-1.13), 0.80
SM-18:0	0.93 (0.87-0.99), 0.03	1.03 (0.94-1.14), 0.52	1.06 (0.96-1.18), 0.26
SM-20:0	0.94 (0.88-1.00), 0.05	1.02 (0.93-1.13), 0.64	1.04 (0.94-1.15), 0.48
SM-22:0	0.96 (0.90-1.03), 0.30	1.04 (0.94-1.14), 0.44	1.05 (0.94-1.16), 0.36
SM-24:0	0.93 (0.87-0.99), 0.02	0.98 (0.90-1.08), 0.69	1.02 (0.92-1.12), 0.76
GluCer-16:0	0.95 (0.89-1.00), 0.07	0.94 (0.87-1.03), 0.17	0.97 (0.89-1.06), 0.48
GluCer-22:0	0.91 (0.86-0.97), 0.002	0.97 (0.90-1.05), 0.47	1.01 (0.92-1.10), 0.89
GluCer-24:0	0.90 (0.85-0.95), 0.0002	0.92 (0.85-1.00), 0.04	0.96 (0.88-1.04), 0.33
LacCer-16:0	0.91 (0.85-0.98), 0.007	0.96 (0.88-1.06), 0.44	1.01 (0.91-1.12), 0.87

*Fold change in HOMAB geometric mean with 2 fold higher sphingolipid

Supplementary Table 4. Sphingolipids and baseline insulin – Additional adjustments for LDL and HDL

	Main Model Estimate* (95% CI), p	Adjustment for LDL Estimate* (95% CI), p	Adjustment for HDL Estimate* (95% CI), p
Cer-16:0	1.13 (1.07-1.20), $1.9x10^{-5}$	1.16 (1.10-1.24), $9.9x10^{-7}$	1.12 (1.06-1.19), $5.3x10^{-5}$
Cer-18:0	1.11 (1.06-1.15), $1.0x10^{-6}$	1.12 (1.08-1.17), $3.7x10^{-8}$	1.09 (1.04-1.13), $3.3x10^{-5}$
Cer-20:0	1.12 (1.07-1.17), $1.5x10^{-7}$	1.13 (1.09-1.18), $1.4x10^{-8}$	1.10 (1.06-1.15), $3.9x10^{-6}$
Cer-22:0	1.13 (1.08-1.19), $3.9x10^{-7}$	1.17 (1.11-1.23), $3.3x10^{-9}$	1.11 (1.05-1.16), $3.8x10^{-5}$
Cer-24:0	1.05 (0.99-1.11), 0.08	1.07 (1.01-1.14), 0.02	1.05 (0.99-1.11), 0.07
SM-14:0	1.00 (0.96-1.05), 0.91	1.02 (0.97-1.07), 0.50	1.06 (1.01-1.10), 0.02
SM-16:0	0.89 (0.81-0.97), 0.009	0.88 (0.79-0.97), 0.01	0.97 (0.88-1.06), 0.45
SM-18:0	0.95 (0.89-1.02), 0.19	0.96 (0.89-1.04), 0.37	0.98 (0.91-1.05), 0.58
SM-20:0	0.95 (0.89-1.02), 0.17	0.96 (0.89-1.04), 0.36	0.99 (0.92-1.06), 0.74
SM-22:0	1.00 (0.93-1.07), 0.97	1.03 (0.95-1.12), 0.49	1.02 (0.95-1.09), 0.61
SM-24:0	0.96 (0.90-1.02), 0.22	0.97 (0.90-1.05), 0.44	1.01 (0.94-1.07), 0.86
GluCer-16	0.96 (0.90-1.02), 0.16	0.96 (0.90-1.03), 0.27	0.98 (0.92-1.04), 0.49
GluCer-22	0.93 (0.87-0.98), 0.013	0.93 (0.87-0.99), 0.03	0.94 (0.89-0.997), 0.04
GluCer-24	0.92 (0.86-0.97), 0.0023	0.92 (0.86-0.97), 0.005	0.95 (0.90-1.01), 0.11
LacCer-16	0.87 (0.82-0.94), 0.0001	0.87 (0.81-0.94), 0.0002	0.89 (0.83-0.95), 0.0005

Fold change in baseline insulin geometric mean with 2 fold higher sphingolipid (95% CI)

SUPPLEMENTARY DATA

Supplementary Materials. Details of sphingolipid laboratory measurement

Sample preparation

Lipids were extracted from plasma using an organic protein precipitation with internal standard included in the precipitating solvent. The precipitation solvent was made by combining 12.85 mL of methyl tert-butyl ether (MTBE), 10.28 mL methanol, and 2.57 mL isopropanol. The solvent was spiked with 20 µL internal standard (Ceramide/Sphingoid Internal Standard Mixture I, 25 µM, Avanti Polar Lipids, LM-6002), which yielded a final concentration of 19.4 nM. After thawing, each sample was mixed by vortexing (10 s) and then centrifuged for 8 min at 3,000*g* (Accuspin Micro 17, Fisher). For each sample, quality control material, and single point calibrator, 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 multi-tube vortex (VWR) for 5 min at speed 10. Subsequently, in a fume hood, a 10µm 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 flowed through 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 analyzed using liquid chromatography-tandem mass spectrometry.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

A volume of 5 µL was injected using an autosampler (samples were cooled at 8 °C) were resolved using reversed phase chromatography on an Acquity BEH 300 C4 1.7µ 2.1x50 analytical column (Waters, Cat.No 186004495) equipped with a VanGuard BEH 300 C4 1.7µ guard column (Waters, Cat. No. 186004623). The columns were held at 50 °C. Mobile phases were: Optima water/0.2% formic acid (Buffer A) and 60% acetonitrile/40% isopropanol/0.2% formic acid (Buffer B). The gradient profile (400 µL/min) was:

Time (min)	%B	Valve
0.00	49%	Waste
1.50	49%	Waste
3.10		Instrument
11.50	79%	Instrument
11.55	95%	Instrument
12.00		Waste
15.00	95%	Waste
15.55	49%	Waste
19.90	49%	Waste
20.00		Stop

The transitions monitored and mass spectrometer settings for each analyte were:

SUPPLEMENTARY DATA

Analyte	Precursor	Fragment	DP	CE	Dwell (ms)
CerC12 (IS)	482.5	264.2	60	35	20
CerC25 (IS)	664.7	264.2	65	45	20
GluCerC12(IS)	644.5	264.2	39	45	20
LacCerC12(IS)	806.6	264.2	32	53	20
SM12 (IS)	647.5	184.1	75	35	20
Cer C14	510.5	264.2	30	35	20
Cer C16	538.5	264.2	60	40	20
Cer C18	566.6	264.2	30	38	20
Cer C20	594.6	264.2	59	37	20
Cer C22	622.6	264.2	58	42	20
Cer C24 18:1_24	650.6	264.2	60	43	20
Cer C24 18:2_24	648.6	264.2	60	43	20
GluCer 18:1_16:0	700.6	264.2	39	45	20
GluCer 18:1_18:0	728.6	264.2	44	57	20
GluCer 18:1_20:0	756.6	264.2	30	53	20
GluCer 18:1_22:0	784.7	264.2	47	55	20
GluCer 18:1_24:0	812.7	264.2	44	50	20
LacCer 18:1_16:0	862.62	264.2	32	53	20
LacCer 18:1_18:0	890.65	264.2	35	63	20
LacCer 18:1_20:0	918.68	264.2	30	63	20
LacCer 18:1_22:0	946.71	264.2	40	60	20
LacCer 18:1_24:0	974.74	264.2	48	63	20
SM14	675.5	184.1	55	45	20
SM16	703.6	184.1	55	39	20
SM18	731.6	184.1	45	42	20
SM20	759.6	184.1	50	48	20
SM22	787.7	184.1	60	48	20
SM24	815.7	184.1	45	45	20

DP: Declustering potential; CE: Collision energy; IS: Internal standard
Mode: Positive Ion Turbo Spray
Source temp: 600 C
Curtain gas: 25
Source: 3000 V
GS1: 30
GS2: 45

Data reduction

Chromatographic peak areas were quantified using SkyLine software, written by the MacCoss laboratory at the University of Washington (PMID: 20147306). Each peak area for each endogenous sphingolipid was divided by the sum of the peak area of the five internal standards (CerC12, CerC25, GluCerC12, LacCerC12, and 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.

Precision

The purpose of data normalization to the single point calibrator was to minimize variability of the assay over time. We determined the coefficient of variation (standard deviation divided by the mean) for a quality control sample that was run (precipitated and analyzed by LC-MS/MS) in duplicate in each batch. One replicate was injected onto the LC-MS/MS instrument near the beginning of the run and one replicate towards the end, in a random position in the each batch. The quality control sample was an independent pool of EDTA-anticoagulated plasma. The variability for each of the analytes across the project is shown below:

SUPPLEMENTARY DATA

Analyte	%CV	Analyte	%CV
Cer C14	44.2%	**LacCer 18:1_16:0**	14.4%
Cer C16	18.4%	**LacCer 18:1_18:0**	42.6%
Cer C18	21.2%	**LacCer 18:1_20:0**	47.4%
Cer C20	19.5%	**LacCer 18:1_22:0**	28.3%
Cer C22	13.9%	**LacCer 18:1_24:0**	31.0%
Cer C24 18:1_24	15.5%	**SM14**	18.2%
Cer C24 18:2_24	12.1%	**SM16**	11.5%
GluCer 18:1_16:0	13.4%	**SM18**	12.2%
GluCer 18:1_18:0	24.5%	**SM20**	12.6%
GluCer 18:1_20:0	24.7%	**SM22**	12.4%
GluCer 18:1_22:0	13.9%	**SM24**	13.3%
GluCer 18:1_24:0	16.0%		

Concentrations of lipids in the single point calibrator, linearity, and parallelism

The approximate concentration of each lipid was approximated by using the internal standards (IS) for each class according the following equation:

PAR.SPC * concentration_of_IS

As another approach to determine the approximate concentration of certain lipids in the single point calibrator, we also performed spiking experiments with synthetic lipids (Avanti Polar Lipids). The single point calibrator was spiked with a mixture of each analyte (final concentration in μM): Cer C16 (2.9), Cer C20 (1.6), Cer C22 (7.2), Cer C24 18:1_24:0 (20.9), LacCer 18:1_24:0 (2.3), SM16 (126.1), and SM24 (108.7). The concentration of each lipid was then calculated from the following equation:

[Final_Spiked_Concentration / (PAR.Spiked_SPC – PAR.Unspiked_SPC)] * PAR.Unspiked_SPC

To determine the linearity of the response (peak area ratio), the spiked single point calibrator (from the above experiment to determine the approximate concentration) was diluted with MSG3000 (Golden West Biologicals), which is a stripped pooled human serum sample. The linearity was assessed using Pearson correlation coefficient.

To assess matrix effects, we performed a parallelism experiment. A spiked single point calibrator was diluted with the same single point calibrator plasma pool. In addition, spiked MSG3000 was diluted with either MSG3000 or phosphate buffered saline. There were 5 points in total (spiked sample at 100%, diluent at 100% and 3 admixtures of these two samples for each dilution series, at 25:75, 50:50, and 75:25). The slope for each dilution series (peak area ratio vs. spiked concentration) was determined for each of the 3 dilution series. The consistency of the signal vs. concentration between the matrices was determined as the %CV of the 3 slopes.

SUPPLEMENTARY DATA

Analyte	IS (for concentration)	SPC PA (ave)	SCP IS PA (ave)	Conc (uM, based on IS)	Conc (uM, based on spike)	Linearity	CV slope
Cer C14	CerC12	588.4	22436.6	0.010		0.9878	
Cer C16	CerC12	13754.1	22436.6	0.226	0.294	0.9899	5.7%
Cer C18	CerC12	6233.3	22436.6	0.103		0.9633	
Cer C20	CerC25	3521.1	29636.3	0.044	0.075	0.9868	10.0%
Cer C22	CerC25	20153.4	29636.3	0.251	0.496	0.9932	11.1%
Cer C24 18:1_24	CerC25	56259.7	29636.3	0.701	2.726	0.9864	9.8%
Cer C24 18:2_24	CerC25	25354.1	29636.3	0.316		0.9899	
GluCer 18:1_16:0	GluCer12	10600.9	34525.6	0.113		0.9934	
GluCer 18:1_18:0	GluCer12	1182.6	34525.6	0.013		0.9511	
GluCer 18:1_20:0	GluCer12	1137.3	34525.6	0.012		0.9942	
GluCer 18:1_22:0	GluCer12	9410.7	34525.6	0.101		0.9860	
GluCer 18:1_24:0	GluCer12	9779.1	34525.6	0.105		0.9918	
LacCer 18:1_16:0	LacCer12	22179.6	19442.6	0.421		0.9937	
LacCer 18:1_18:0	LacCer12	530.1	19442.6	0.010		0.9871	
LacCer 18:1_20:0	LacCer12	204.0	19442.6	0.004		0.9870	
LacCer 18:1_22:0	LacCer12	668.7	19442.6	0.013		0.9981	
LacCer 18:1_24:0	LacCer12	552.6	19442.6	0.010	0.108	0.9882	7.3%
SM14	SM12	853982.9	81862.8	3.853		0.9978	
SM16	SM12	3457964.3	81862.8	15.602	117.239	0.9533	15.2%
SM18	SM12	892676.2	81862.8	4.028		0.9912	
SM20	SM12	381473.2	81862.8	1.721		0.9935	
SM22	SM12	567439.8	81862.8	2.560		0.9942	
SM24	SM12	245442.1	81862.8	1.107	13.874	0.9735	10.0%