Online-Only Supplemental Material

Table S1. Stability of the established ultra-high performance liquid chromatography-tandem mass spectrometry method ($N = 5$)

	Positive mode					Negative mode			
No.	RT (min)	RSD (%)	*m/z*	RSD (%)	No.	RT (min)	RSD (%)	*m/z*	RSD (%)
1	3.68	0.0135	274.2645	0.0022	11	1.42	0.0122	187.0308	0.0023
2	4.62	0.0141	468.3036	0.0021	12	2.26	0.0452	367.1758	0.0018
3	5.14	0.0235	494.3237	0.0132	13	5.56	0.0032	564.3293	0.0056
4	5.44	0.0121	520.3373	0.0014	14	6.29	0.0214	540.3300	0.0011
5	5.80	0.0025	520.3392	0.0023	15	6.74	0.0325	540.3323	0.0003
6	6.60	0.0364	496.3378	0.0051	16	7.33	0.0045	566.3434	0.0005
7	7.16	0.0214	522.3544	0.0032	17	9.09	0.0112	568.3606	0.0023
8	8.42	0.0524	524.3700	0.0011	18	13.75	0.0423	745.5305	0.0074
9	8.92	0.0112	524.3707	0.0063	19	14.74	0.0021	826.5321	0.0014
10	14.86	0.0032	758.5818	0.0125	20	14.95	0.0325	802.5365	0.0012

RSD: relative standard deviation; RT: retention time.

Table S2. Repeatability of the established Ultra high-performance liquid chromatography-tandem mass spectrometry method ($N = 5$)

	Positive mode					Negative mode			
No.	RT (min)	RSD (%)	*m/z*	RSD (%)	No.	RT (min)	RSD (%)	*m/z*	RSD (%)
1	3.68	0.0032	274.2645	0.0035	11	1.42	0.0023	187.0308	0.0026
2	4.62	0.0563	468.3036	0.0102	12	2.26	0.0012	367.1758	0.0009
3	5.14	0.0127	494.3237	0.0143	13	5.56	0.0081	564.3293	0.0024
4	5.44	0.0036	520.3373	0.0057	14	6.29	0.0043	540.3300	0.0013
5	5.80	0.0124	520.3392	0.0101	15	6.74	0.0084	540.3323	0.0005
6	6.60	0.0135	496.3378	0.0111	16	7.33	0.0101	566.3434	0.0001
7	7.16	0.0058	522.3544	0.0421	17	9.09	0.0016	568.3606	0.0012
8	8.42	0.0103	524.3700	0.0215	18	13.75	0.0165	745.5305	0.0075
9	8.92	0.0012	524.3707	0.0037	19	14.74	0.0045	826.5321	0.0017
10	14.86	0.0017	758.5818	0.0321	20	14.95	0.0135	802.5365	0.0022

RSD: relative standard deviation; RT: retention time.

Table S3. CV-ANOVA test of the established orthogonal partial least squares discriminant analysis models

NGT_non-CVD vs. DM_CVD (detected in positive mode)	SS	DF	MS	F	*P* value	SD
Total corr.	293	293	1			1
Regression	291.431	4	72.8578	13422.9	< 0.0001	8.53568
Residual	1.56865	289	0.005428			0.073674
NGT_non-CVD vs. DM_non-CVD (detected in positive mode)	SS	DF	MS	F	*P* value	SD
Total corr.	336	336	1			1
Regression	335.991	4	83.9976	$2.94E^{+06}$	< 0.0001	9.16502
Residual	0.009478	332	$2.85E^{-05}$			0.005343
NGT_non-CVD vs. DM_CVD (detected in negative mode)	SS	DF	MS	F	*P* value	SD
Total corr.	197	197	1			1
Regression	196.423	12	16.3686	5249.55	< 0.0001	4.04581
Residual	0.576848	185	0.003118			0.05584
NGT_non-CVD vs. DM_non-CVD (detected in negative mode)	SS	DF	MS	F	*P* value	SD
Total corr.	229	229	1			1
Regression	228.323	12	19.0269	6096.56	< 0.0001	4.36198
Residual	0.67724	217	0.003121			0.055865

Total corr: total corrected; CV: coefficient of variation.; DM_CVD: diabetes mellitus with cardiovascular disease; NGT_non-CVD: normal glucose tolerance without cardiovascular disease; DM_non-CVD: diabetes mellitus without cardiovascular disease.

Table S4 Anti-diabetes and Cardiovascular medications in the discovery group's subjects with diabetes

	DM_non−CVD group (*N* = 60)	DM_CVD group (*N* = 60)
Antihyperglycemic treatment, N (%)	52 (86.7)	54 (90.0)
Oral antihyperglycemic drugs, *N* (%)	31 (51.6)	26 (45.6)
Metformin	10 (16.7)	6 (10.0)
Sulfonylurea	11 (18.)	5 (8.3)
Alpha glucosidase inhibitors	3 (5.0)	6 (10.0)
Thiazolidinedione	3 (5.0)	0 (0)
Glinides	1 (1.7)	2 (3.3)
Other	3 (5.0)	7 (11.7)
Insulin treatment, *N* (%)	35 (58.3)	45 (75.0)
Antihypertensive drugs, *N* (%)	26 (43.3)	41 (68.3)
Angiotensin -converting enzyme inhibitors /angiotensin receptor blockers	6 (10.0)	18 (30.0)
Beta -blockers	6 (10.0)	7 (11.7)
Diuretics	1 (1.7)	4 (6.7)
Calcium channel blockers	13 (21.7)	20 (33.3)
Alpha- blockers	0 (0)	0 (0)
Other	6 (10.0)	7 (11.7)
Hypolipidemic drugs, *N* (%)	5 (8.3)	15 (25)
Statins	2 (3.3)	7 (11.7)
Fibrates	1 (1.7)	1 (1.7)
other	3 (5.0)	7 (11.7)

DM_non-CVD: diabetes mellitus without cardiovascular disease; DM_CVD: diabetes mellitus with cardiovascular disease.

Table S5. Association between each metabolite and cardiovascular disease in patients with diabetes adjusted for conventional cardiovascular risk factors

	unadjusted				adjusted*				adjusted†			
Metabolite	OR	95% CI		*P* value	OR	95% CI		*P* value	OR	95% CI		*P* value
Dihydroxyacetone phosphate‡	1.04	1.02	1.07	0.002	1.05	1.02	1.08	0.002	1.04	1.02	1.08	0.0010
Unidentified metabolite 1‡	1.11	1.04	1.18	0.001	1.11	1.04	1.18	0.001	1.097	1.05	1.18	0.0005
Taurocyamine‡	0.87	0.83	0.92	< 0.0001	0.87	0.83	0.92	< 0.0001	0.88	0.83	0.92	< 0.0001
Lactosylceramide (d18:1/12:0)	1.05	1.02	1.07	< 0.0001	1.05	1.02	1.07	< 0.0001	1.04	1.03	1.06	< 0.0001
PC (18:1/18:1)‡	1.04	1.02	1.07	0.0001	1.04	1.02	1.07	0.0001	1.04	1.02	1.07	0.0002
palmitoyl sphingomyelin	5.40	2.49	11.72	< 0.0001	5.22	2.41	11.32	< 0.0001	4.80	2.36	11.08	< 0.0001
PC (20:0/18:2)§	1.05	1.03	1.08	< 0.0001	1.05	1.03	1.08	< 0.0001	1.005	1.003	1.007	< 0.0001
Diacylglycerol (22:1n9/0:0/22:6n3)‖	1.04	1.02	1.06	< 0.0001	1.04	1.03	1.06	< 0.0001	1.04	1.03	1.06	< 0.0001
Unidentified metabolite 2‖	1.23	1.11	1.36	< 0.0001	1.23	1.12	1.38	< 0.0001	1.19	1.12	1.32	< 0.0001
PC (24:1/14:0)	1.37	1.19	1.58	< 0.0001	1.37	1.19	1.58	< 0.0001	1.33	1.19	1.55	< 0.0001
Glucosylceramide (d18:1/18:0)	1.04	1.02	1.06	< 0.0001	1.04	1.02	1.06	< 0.0001	1.04	1.02	1.06	< 0.0001
Unidentified metabolite 3	1.02	1.01	1.03	< 0.0001	1.02	1.01	1.03	< 0.0001	1.02	1.01	1.03	< 0.0001
Diacylglycerol (18:4/24:1/0:0)	1.69	1.37	2.09	< 0.0001	1.68	1.36	2.08	< 0.0001	1.66	1.38	2.12	< 0.0001
SM (d18:1/24:1)	4.14	2.37	7.22	< 0.0001	4.15	2.36	7.30	< 0.0001	4.12	2.48	8.21	< 0.0001
1-stearoylglycero phosphoinositol	1.25	1.13	1.40	< 0.0001	1.25	1.12	1.40	< 0.0001	1.21	1.11	1.34	< 0.0001
Stearoylcarnitine	1.28	1.13	1.45	< 0.0001	1.26	1.11	1.43	0.0003	1.24	1.11	1.40	0.0003

*Adjusted for age and sex in baseline. †Adjusted for age, sex, smoking, BMI, systolic blood pressure, fasting plasma glucose, and total serum cholesterol. ‡Per 0.01-unit increase. §Per 0.1-unit increase. ‖Per 10-unit increase. CVD, cardiovascular disease PC: glycerophosphocholines; SM: sphingomyelins. Given the multiplicity of comparisons, *P* values less than 0.003 were considered statistically significant with Bonferroni's method.

Table S6. Association between all metabolites and cardiovascular disease in patients with diabetes adjusted for conventional cardiovascular risk factors (step wise anaysis)

Metabolites	adjusted*			
	OR	95% CI		*P* value
palmitoyl sphingomyelin	1.81	1.29	2.53	0.0006
Unidentified metabolite 2†	1.24	1.12	1.37	<0.0001

*Adjusted for age, sex, smoking, systolic blood pressure, BMI, fasting plasma glucose, and serum total cholesterol at baseline. †: Per 10-unit increase. Other metabolites did not enter the model at $p<0.05$ levels.

Table S7 Association of one-SD increase in palmitoyl sphingomyelin and cardiovascular disease in the validation dataset

Model (per 1 SD increase)	OR (95% CI)	*P* value
age (1-SD = 6.7 year)	1.29 (0.96, 1.73)	0.01
sex (male = 1)	2.05 (1.03, 4.10)	0.02
smoking (yes = 1)	1.35 (0.69, 2.65)	0.42
SBP (1-SD = 21.7 mmHg)	1.25 (0.94, 1.66)	0.13
TC (1-SD = 1.1 mmol/L)	1.02 (0.77, 1.37)	0.87
FPG (1-SD =3.0 mm/ml)	1.44 (1.10, 1.89)	0.008
PSM (1-SD = 6.0 μg/mL)	2.90 (2.11, 3.98)	< 0.0001

FPG: fasting plasma glucose; PSM: palmitoyl sphingomyelin; SBP: systolic blood pressure; TC: total cholesterol; SD: standard deviation.

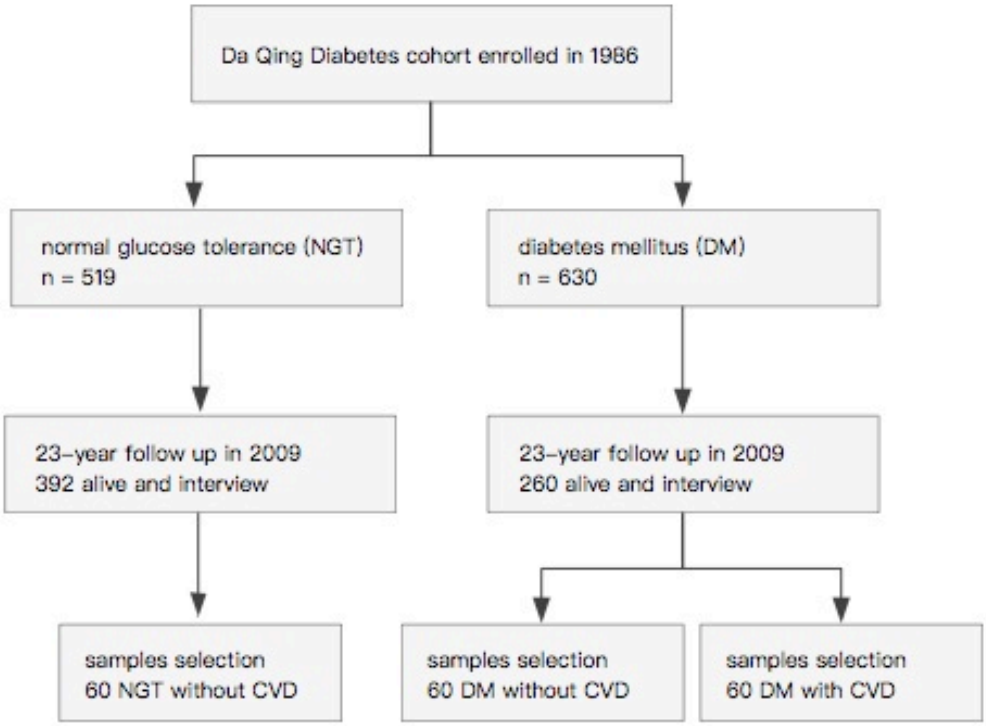


Fig. S1 Flow chart of discovery dataset and plasma metabolic profiles of patients using PCA analysis. In 1986, the Da Qing Diabetes Study was conducted among residents of Da Qing, China. Based on 75-g OGTT, participants were classified (by the 1985 World Health Organization criteria) into groups of DM or NGT. In 2009, a follow-up study was conducted. We selected 120 participants with type 2 diabetes with a 1:1 ratio of CVD and non-CVD, and 60 participants with NGT and without CVD. DM: diabetes mellitus; CVD: cardiovascular disease; NGT: normal glucose tolerance; OGTT: oral glucose tolerance test; PCA: principal component analysis.

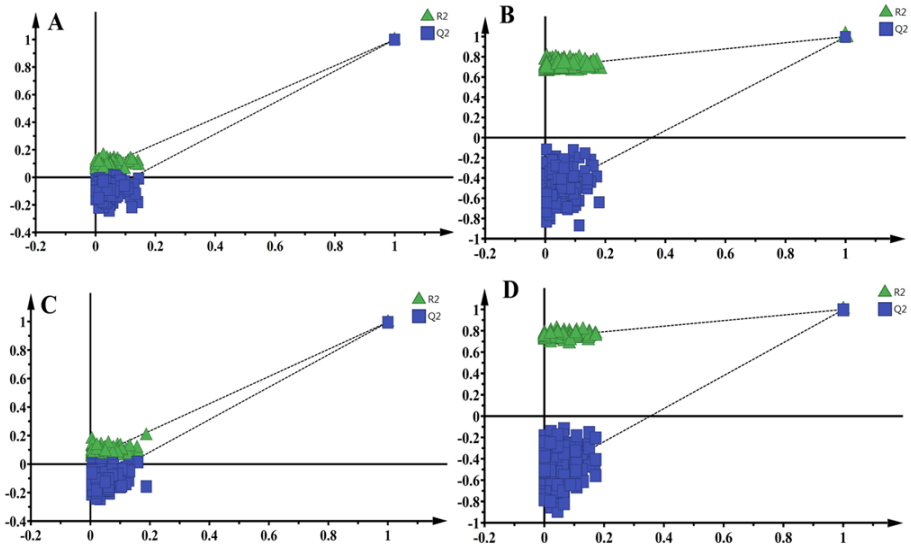


Fig. S2 Permutation plots (N = 200). Data from NGT_non-CVD vs. DM_CVD group (A) in positive mode R2 = (0.0, 0.0502), Q2 = (0.0, −0.142) and (B) negative mode R2 = (0.0, 0.698), Q2 = (0.0, −0.548); Data from NGT_non-CVD vs. DM_non-CVD group (C) in positive mode R2 = (0.0, 0.0416), Q2 = (0.0, −0.148) and (D) negative mode R2 = (0.0, 0.739), Q2 = (0.0, −0.548).
CVD: cardiovascular disease; DM: diabetes mellitus; NGT: normal glucose tolerance.

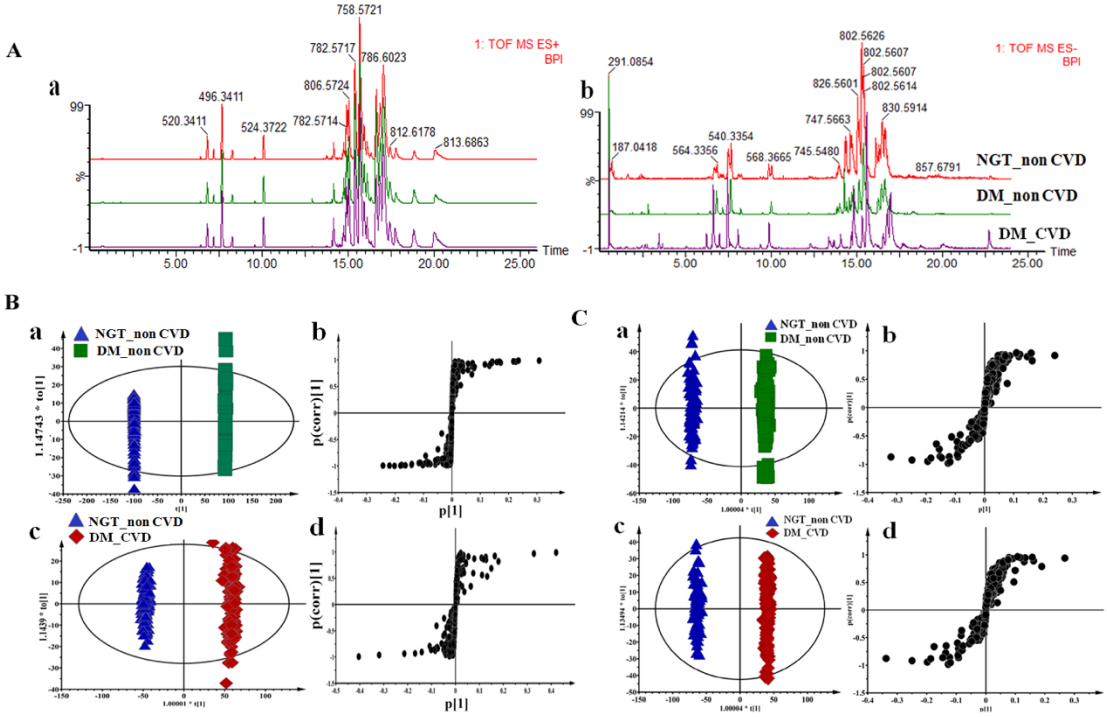


Fig. S3 The base peak intensity chromatograms (A) and orthogonal partial least squares discriminant analysis of plasma profiles (B, C) from all experimental groups: DM_CVD, DM_non-CVD and NGT_non-CVD. A(a) Positive mode; A(b) Negative mode. B. Positive mode. a: Score plot of DM_non-CVD vs. NGT_non-CVD (R2X = 0.868, R2Y = 1.0, Q2 = 1.0); b: S-plot of DM_non-CVD vs. NGT_non-CVD; c: Score plot of DM_CVD vs. NGT_non-CVD (R2X = 0.577, R2Y = 0.996, Q2 = 0.995); d: S-plot of NDD+CVD vs. NGT-CVD. C. Negative mode. a: Score plot of DM_non-CVD vs. NGT_non-CVD (R2X = 0.519, R2Y = 0.999, Q2 = 0.997); b: S-plot of DM_CVD vs. NGT_non-CVD; c: Score plot of DM_non-CVD vs. NGT_non-CVD (R2X = 0.503, R2Y = 0.999, Q2 = 0.997); d: S-plot of DM_non-CVD vs. NGT_non-CVD. DM_CVD: diabetes mellitus with cardiovascular disease; NGT_non-CVD: normal glucose tolerance without cardiovascular disease; DM_non-CVD: diabetes mellitus without cardiovascular disease.

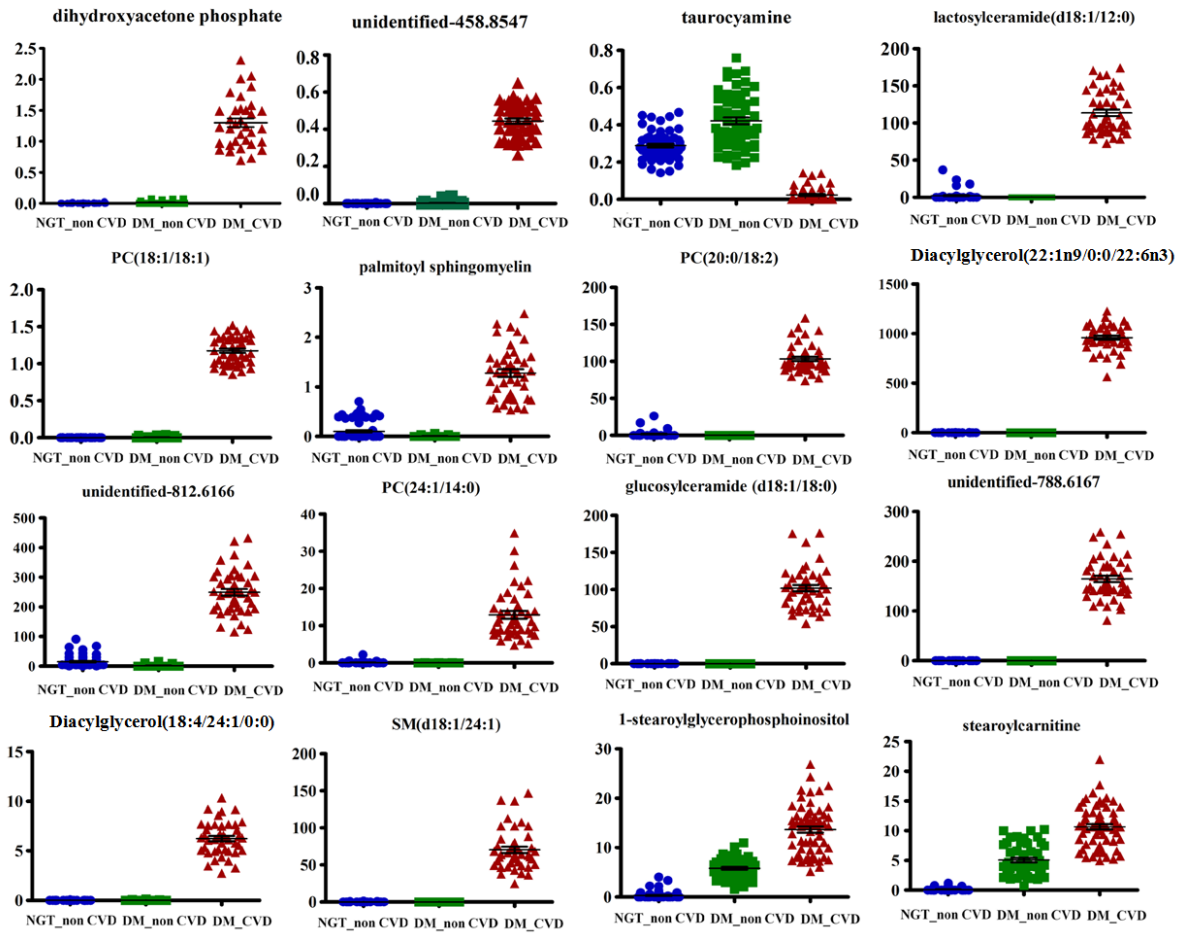


Fig. S4 Normalized peak intensities of the 16 metabolites in plasma samples from NGT_non-CVD, DM_non-CVD, and DM_CVD groups. DM_CVD: diabetes mellitus with cardiovascular disease; NGT_non-CVD: normal glucose tolerance without cardiovascular disease; DM_non-CVD: diabetes mellitus without cardiovascular disease; PC: glycerophosphocholines; SM: sphingomyelins; DAG: diacylglycerol; SD: standard deviation.

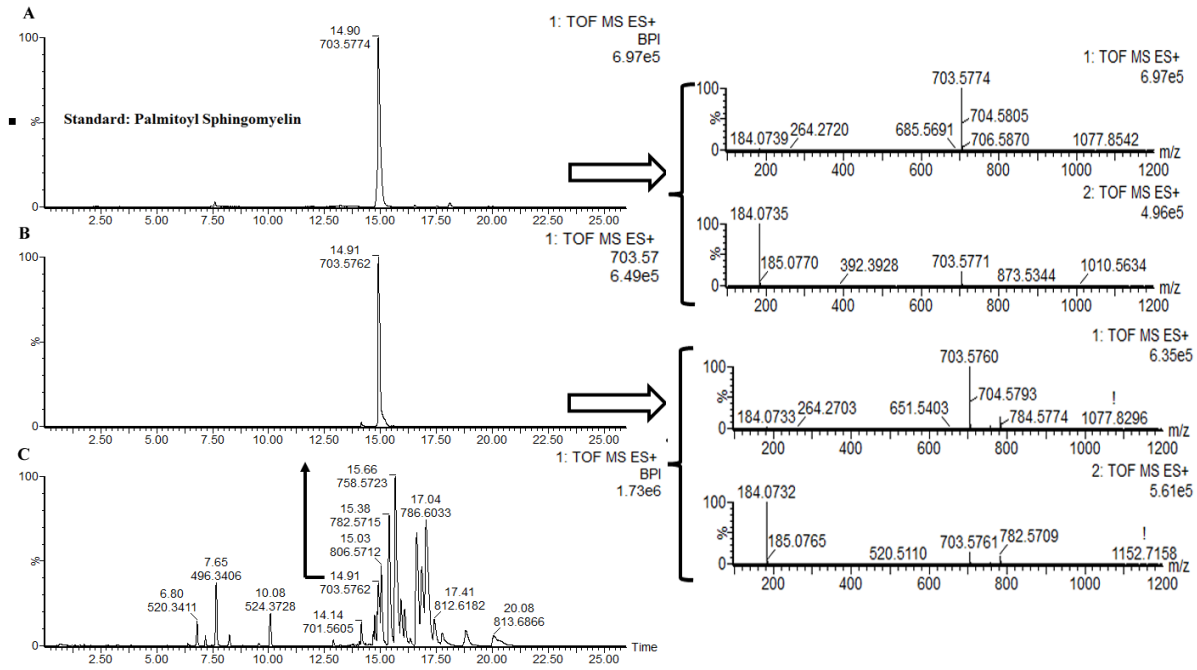


Fig. S5 Confirmation of the metabolite palmitoyl sphingomyelin using standard compounds. The base peak ion (BPI) chromatogram of (A) standard palmitoyl sphingomyelin and (C) plasma sample; (B)The extracted ion chromatogram of *m/z* 703.57 from the plasma sample. Measured MS and MS2 spectral fragmentation profiles (bottom) match the chemical standard (top).

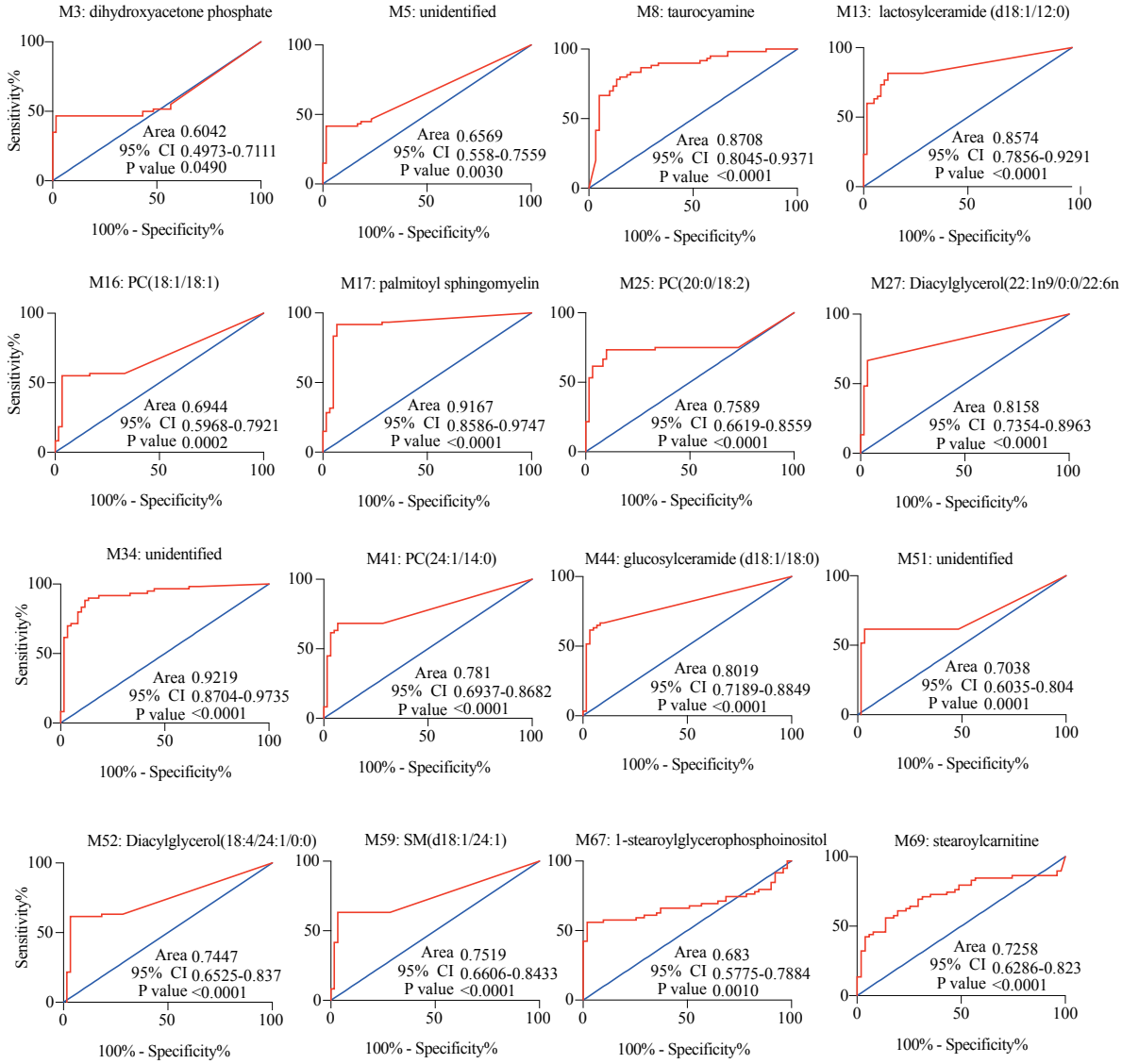


Fig. S6 Receiver operating characteristic curve of each metabolite for cardiovascular diseases in type 2 diabetes. PC: glycerophosphocholines; SM: sphingomyelins; DAG: diacylglycerol; ROC: receiver operating characteristic.

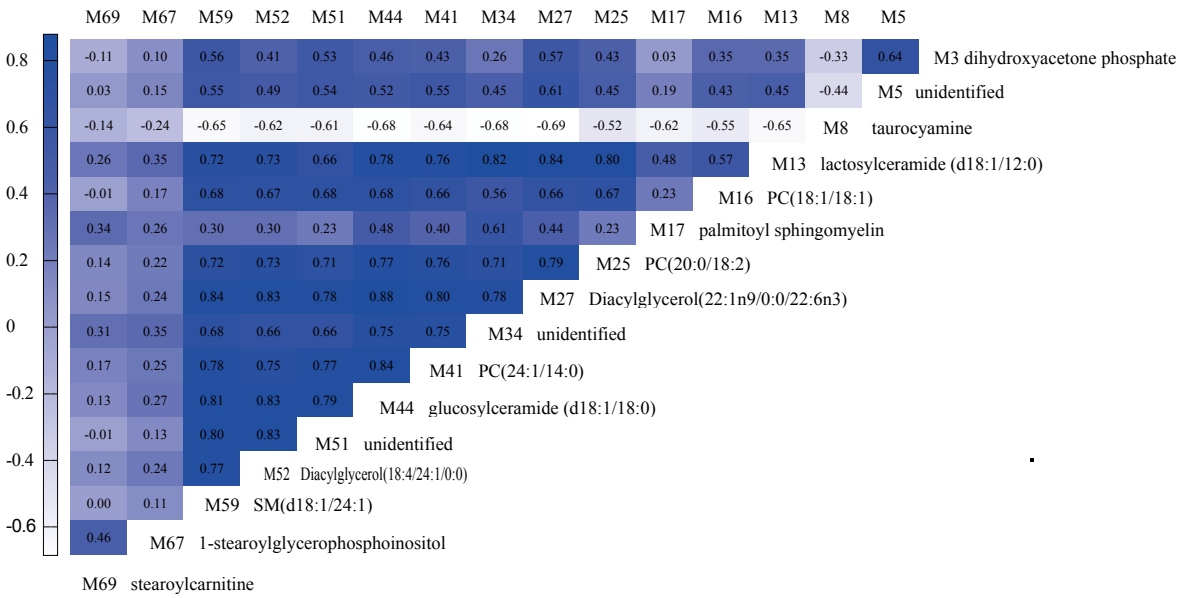
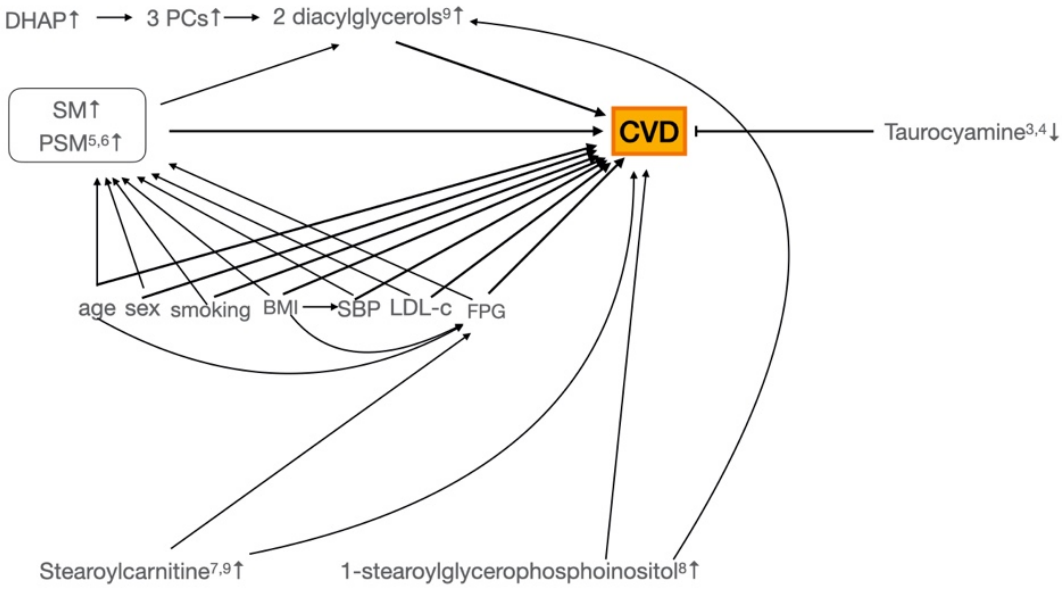


Fig. S7 Heat map showing the correlations between the 16 metabolites using Spearman's correlation coefficients analysis. Correlations are characterized according to direction (positive in blue, negative in white) and strength (intensity of color). Spearman's correlations with $P > 0.05$ are indicated with a gray background.



DHAP: Dihydroxyacetone phosphate;
PC: glycerophosphocholines;
PSM:palmitoyl sphingomyelin;
SM: sphingomyelin.
CVD: cardiovascular disease

1. KEGG SPHINGOLIPID SIGNALING PATHWAY – REFERENCE PATHWAY HTTPS://WWW.GENOME.JP/PATHWAY/MAP04071+C00550
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Fig. S8 Directed acyclic graphs.

Approaches

Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry Based Metabolomics Analysis Method Validation

Ten ions were extracted from the base peak intensity chromatogram and selected for method validation. The stability and reproducibility of ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) under the positive and negative ion modes were evaluated using six replicates of quality control (QC) samples in each mode. Relative standard deviations of retention time and *m/z* of the selected ions are listed in Tables S1 and S2, respectively.

Metabolomics Data Preprocess Parameters

The main parameters of the metabolomics data preprocess were set as follows: RT range 0.5–24 min; mass range 100–1200; extracted ion chromatogram window, 0.02 Da; automatically calculate peak width and peak-peak baseline noise; use raw data during deconvolution; marker intensity threshold (count), 300; mass tolerance, 0.02 Da; RT windows, 0.2 s; noise elimination level, 6; retain isotopic peaks.