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Differences in the Distribution of Ceramides and Sphingosine among Lipoprotein and Lipoprotein-Depleted Fractions in Patients with Type 2 Diabetes Mellitus

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Aim: In addition to the quantity and quality, the carriers, such as lipoproteins and albumin, can affect the physiological properties and clinical significance of lipids. This study aimed to elucidate the modulation of the levels of ceramides and sphingosine, which are considered as proatherosclerotic lipids, in lipoproteins and lipoprotein-depleted fractions in subjects with type 2 diabetes.

Methods: We separated the serum samples collected from healthy subjects ($n=22$) and subjects with type 2 diabetes ($n=39$) into Triglyceride (TG)-rich lipoproteins (TRL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and lipoprotein-depleted fractions *via* ultracentrifugation. Then, we measured the levels of six species of ceramides, sphingosine, and dihydrosphingosine via LC-MS/MS and statistically analyzed them to identify the sphingolipids in each fraction, which are associated with diabetes as well as cardiovascular and renal complications.

Results: In subjects with diabetes, the levels of sphingosine and dihydrosphingosine in the TRL, LDL, and lipoprotein-depleted fractions were higher, whereas those in the HDL were lower. In addition, the ceramide levels in HDL were lower, whereas those in lipoprotein-depleted fractions were higher. Furthermore, The levels of ceramides in lipoproteins, especially LDL, were negatively associated with the presence of cardiovascular diseases and stage 4 diabetic nephropathy.

Conclusions: The contents of ceramides and sphingosine in lipoproteins and lipoprotein-depleted fractions were differently modulated in diabetes and associated with cardiovascular diseases and diabetic nephropathy. The carrier might be an important factor for the biological properties and clinical significance of these sphingolipids.

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Key words: Ceramides, Sphingosine, Lipoproteins, Diabetes, Cardiovascular diseases

List of Abbreviations: ApoM, apolipoprotein M; Cer, ceramide; CVD, cardiovascular disease; dhSph, dihydrosphingosine; OPLS, orthogonal projection to latent structures; PL, phospholipids; ROC, receiver operating characteristic; S1P, sphingosine 1-phosphate; Sph, sphingosine; TC, total cholesterol; TRL, TG-rich lipoprotein

Introduction

Many factors, other than the levels alone, have been reported to determine the bioactivities and clinical significance of lipids. Among such factors, the qualities of lipids, such as the molecular species, have been well investigated. For example, saturated fatty acids can induce lipid toxicities, such as endoplasmic reticulum stress, oxidative stress, and inflammation[1]. Furthermore, $\omega 6$ fatty acids can induce inflammation, whereas $\omega 3$ fatty acids exhibit anti-inflammatory properties[2]. In fact, the ratio of $\omega 3$ fatty acids to $\omega 6$

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fatty acids has been reported to be associated with the risk of atherosclerosis development[3], and ω3 fatty acid reagents are reported to protect against cardiovascular diseases (CVDs)[4].

In addition to quantity and quality, as described above, the carrier proteins of lipids, such as lipoproteins and albumin, also seem to influence the physiological properties of lipids. Based on the results of clinical studies, it has become established that the distribution of cholesterol between high-density lipoprotein (HDL) and low-density lipoprotein (LDL) determines the clinical significance of cholesterol on the risk of atherosclerosis. In addition to the case of cholesterol, we and others have also demonstrated the potential importance of carrier proteins in determining the properties of bioactive lipids. For example, sphingosine-1-phosphate (S1P) is a potent bioactive lipid, which is mainly bound to HDL *via* apolipoprotein M (apoM) and albumin in the plasma[5-7]. S1P, in the apoM/HDL-bound form, exhibits beneficial activities, such as anti-apoptotic[8], anti-inflammatory[9], vaso-protective[10-13], insulin-stimulatory[14], and mitochondria-protective[15] activities, whereas S1P bound to albumin exerts harmful effects, such as the induction of PAI-1[16], promotion of mesangial cell proliferation[17], induction of vasoconstriction[18], and induction of hepatic fibrosis[19, 20]. Recently, we reported that the carrier proteins also influenced the physiological properties of other lipids; for example, albumin-associated lysophosphatidylinositol exerted proinflammatory effects on macrophages, whereas lipoprotein-associated lysophosphatidylinositol had only a minimal or no such proinflammatory effects[21].

Similar to S1P, ceramides and sphingosine are also classified as sphingolipids. Ceramides have been demonstrated to be associated with human diseases, including atherosclerosis[22, 23]. Furthermore, they have been reported to exhibit proapoptotic[24] and proinflammatory properties[25], which could promote atherosclerosis. In fact, numerous clinical studies have demonstrated the existence of positive associations between circulating ceramides and the risk of atherosclerotic disease development[26-32]. Sphingosine is closely associated with ceramides in the metabolic context; ceramides can be hydrolyzed to sphingosine, and sphingosine can be recycled into ceramides[33]. S1P is also produced from sphingosine by sphingosine kinases. Dihydrosphingosine, which is very similar to sphingosine in terms of structure, can be converted into ceramides through dihydroceramide. Dihydrosphingosine can also be phosphorylated into dihydrosphingosine 1-phosphate by sphingosine kinases, which is an agonist for S1P receptors[33]. At present, the association between sphingosine and atherosclerosis remains unknown; however, sphingosine has been reported to exhibit proapoptotic properties, like ceramides[34]. Moreover, ceramides and sphingosine have also been reported to be associated with type 2 diabetes mellitus and obesity, which are potent risk factors for the development of atherosclerotic diseases[35-37]. Therefore, ceramides and sphingosines, which are modulated in diabetes, might somehow contribute to atherosclerotic complications that are commonly observed in diabetes.

Although ceramides and sphingosine are found in lipoproteins[38, 39], most previously reported clinical studies have measured the overall levels of ceramides and/or sphingosine in the serum or plasma samples and not in lipoprotein fractions and/or lipoprotein-depleted fractions. Only a limited number of studies have investigated the modulation of lipoprotein-associated ceramides in atherosclerotic diseases and/or diabetes mellitus[40, 41]. To the best of our knowledge, studies that have comprehensively investigated the modulation of the distribution of ceramides and sphingosine in the TG-rich lipoprotein (TRL), LDL, HDL, and lipoprotein-depleted fractions of the blood samples are scarce. Considering the possible importance of the carrier proteins on the physiological properties and clinical significance of lipids, as described above, we were prompted to investigate the modulation of the distribution of ceramides and sphingosine among lipoproteins and lipoprotein-depleted fractions in subjects with atherosclerosis and diabetes mellitus.

Aim

Considering the background described above, the present study was conducted to elucidate the modulation of the distribution of ceramides and sphingosine among the serum lipoprotein and lipoprotein-depleted fractions in patients with type 2 diabetes and the association of such modulation with the presence of diabetic complications, such as CVD and diabetic nephropathy.

Methods

Subjects

Serum samples were collected from healthy volunteers without any specific history (n=22) and subjects with type 2 diabetes (n=39). Several of the samples were the same as those used in previous studies[21, 42]. In the present study, we classified subjects with diabetes into those who were receiving (DM+statin, n=21) or not receiving (DM, n=18) statins, since statins have been reported to influence the

ceramide levels, in addition to their effect on the serum cholesterol levels[43, 44]. Among the subjects with diabetes who were treated with statin, three were treated with 5 mg of atorvastatin, three with 10 mg of atorvastatin, one with 40 mg of atorvastatin, three with 1 mg of pitavastatin, four with 2.5 mg of rosuvastatin, one with 5 mg of pravastatin, and six with 10 mg of pravastatin. With regard to the duration, all the subjects had received statins for more than 1 year.

History of CVD was defined as history of coronary artery diseases diagnosed based on coronary angiography with more than 75% of stenosis of coronary artery or that of acute cerebral infarction diagnosed by MRI. The stage of diabetic nephropathy was categorized according to the 2014 classification for diabetic nephropathy published by the Research Group of Diabetic Nephropathy in Japan[45].

Ethics

The present study was conducted according to the Declaration of Helsinki. Written informed consent for the analysis of human blood samples and information on the subjects' medical records was obtained from them. This study was approved by the Institutional Research Ethics Committee of the Faculty of Medicine, The University of Tokyo (Approval number: 10266 and 11158).

Separation of Lipoproteins

Whole blood specimen was collected after overnight fasting and directly placed into a thrombin-based blood-collecting tube and left for 15 min at room temperature to allow blood clot formation. Subsequently, the serum was separated *via* centrifugation at 1500×g for 5 min.

We separated the lipoprotein fractions *via* ultracentrifugation. First, after adjusting the density of the solutions containing the serum samples to 1.019 by adding NaCl, we ultracentrifuged the samples for 24 h at 170,000×g using MLA-80 equipped with Optima MAX-XP (Beckman Coulter, Inc., Brea, CA). Then, we collected the upper layer as the TRL fraction. Next, after adjusting the density of the sample solutions to 1.063 by adding NaCl, we ultracentrifuged the samples for 24 h at 170,000×g and collected the upper layer as the LDL fraction. Finally, after adjusting the density of the sample solutions to 1.21 by adding NaBr, we ultracentrifuged the samples for 48 h at 170,000×g after and collected the upper layer as the HDL fraction and the lower layer as the lipoprotein-depleted fraction. The separated fractions were dialyzed against phosphate-buffered saline (PBS) for 48 h prior to measurement of the lipids.

Measurement of Ceramides and Sphingosine

We measured the ceramide and sphingosine levels using an LC8060 system, which consists of a quantum ultra-triple quadrupole mass spectrometer (Shimadzu, Japan). We measured the contents of sphingosine (Sph) and dihydrosphingosine (dhSph) and six ceramide species (Cer d18:1/16:0 [C16:0], Cer d18:1/18:0 [C18:0], Cer d18:1/18:1 [C18:1], Cer d18:1/20:0 [C20:0], Cer d18:1/22:0 [C22:0], and Cer d18:1/24:0 [C24:0]), as described previously[46, 47]. Briefly, the samples (10 µL) were mixed with 190 µL of methanol (Wako Pure Chemical Industries, Japan) acidified with 0.1% formic acid, including an internal standard. C17:1 sphingosine (Avanti Polar Lipids, Alabama), C17:1 dihydrosphingosine (Avanti Polar Lipids, Alabama), and Cer d18:1/17:0 (Avanti Polar Lipids, Alabama) at 1.0 ng/mL (final concentration) were used as internal standards. The samples were sonicated for 3 min in an ultrasonic bath and centrifuged at 16,400×g for 10 min at 4℃; then, the supernatants were collected. Then, the supernatants (1.0 µL) were injected, and LC separation was performed using the InertSustain Swift C8 PEEK column (150 mm, 2.1 mm I.D., 3-µm particle size; GL science, Japan) with a gradient elution of solvent A (0.3% formic acid) and solvent B (0.3% formic acid, 95% acetonitrile) at 400 µL/min. We described the detail conditions of the mass spectrometry in a previous paper[46]. The analyses were conducted in the MRM mode in the positive ion mode for Cer d18:1/16:0 (*m/z*, 538.65 >264.45), Cer d18:1/18:0 (*m/z*, 564.65 >264.45), Cer d18:1/18:1 (*m/z*, 566.70 >264.45), Cer d18:1/20:0 (*m/z*, 594.75 >264.45), Cer d18:1/22:0 (*m/z*, 622.75 >264.45), Cer d18:1/24:0 (*m/z*, 650.75 >264.45), Sph (*m/z*, 300.50 >282.40), dhSph (*m/z*, 302.50 >284.45), C17:1 sphingosine (*m/z*, 286.45 >268.40), C17:1 dihydrosphingosine (*m/z*, 88.45 >270.50), and Cer d18:1/17:0 (*m/z*, 552.70 >264.45). The data were analyzed using the Lab Solution software (Shimadzu, Japan). We calculated the levels of lipids using the area ratio to 1.0 ng/mL C17:1 sphingosine, C17:1 dihydrosphingosine, or Cer d18:1/17:0.

Measurement of the apoB or apoA-I Levels in Lipoprotein Fractions

The levels of apoB in the TRL and LDL fractions and the levels of apoA-I in the HDL fractions were measured using enzyme-Linked Immunosorbent Assay (ELISA) kits (3715-1HP and 3710-1HP, respectively; MabTech AB, Stockholm, Sweden).

Measurement of the total cholesterol levels and total phospholipid levels

The total cholesterol (TC) and total phospholipid (PL) levels were measured using enzymatic methods (439-17501 and 296-63801, respectively; Wako Pure Chemical Industries).

Statistical Analysis

The results were expressed in dot plots. The data were analyzed using SPSS (Chicago, IL) or SIMCA (MKS Umetrics). In the present study, we conducted statistical analyses of the sphingolipid contents adjusted to apoB (TRL and LDL) or apoA-I (HDL) and those to the total PL levels, as well as the absolute sphingolipid levels, to investigate the modulation of sphingolipids in individual lipoprotein particles and determine whether the modulation of sphingolipids in each fraction was specific to the sphingolipids, respectively. The levels of sphingolipids among healthy subjects, subjects with diabetes treated with statin, and subjects with diabetes not treated with statin were compared *via* analysis of covariance (ANCOVA) to adjust for age, followed by the Bonferroni test as a *post hoc* test **(Fig. 1–4, Supplemental Fig. 1–3)**, since an obvious difference in age was observed between healthy subjects and subjects with diabetes, and the levels of sphingolipids were reportedly influenced by age[48]. The differences between the two groups were evaluated using the Mann–Whitney *U* test for **Fig. 5**, and those among three groups in clinical characteristics were evaluated using the Kruskal–Wallis test, followed by the Steel–Dwass test as a *post hoc* test for **Supplemental Table 1**. Correlations between two parameters were evaluated using Spearman's correlation test. A receiver operating characteristic (ROC) curve analysis was conducted to investigate the sphingolipid contents in specific fractions contributing to type 2 diabetes, the use of statin, or history of CVD. The cutoff values were determined based on Youden's index. *P*-values of less than 0.05 were deemed to denote statistical significance in all the analyses. Statistical analysis by the orthogonal projections to latent structures (OPLS) was performed using SIMCA to explore the variables associated with CVD or stage 4 diabetic nephropathy, considering the contents of the sphingolipids, TC, and PL in the lipoprotein and lipoprotein-depleted fractions (Model 1), the ratio of sphingolipids to apoB or apoA-I (Model 2), or the ratio of the sphingolipid to PL contents (Model 3), together with age, sex, HbA1c value, history of statin use, and presence/absence of diabetic nephropathy and presence/absence of CVD as potential contributing variables. In the OPLS analyses for stage 4 diabetic nephropathy, we also considered serum albumin level as a potential contributing variable.

Results

Characteristics of the Subjects in the Present Study

The characteristics of the subjects enrolled in this study are described in **Supplemental Table 1**. In the TRL fractions, the TC content was relatively high in the DM group, whereas the PL content was relatively high in the DM + statin group, as compared with the healthy control group. When adjusted to the apoB levels in TRL, the TC and PL contents were significantly higher in the DM and DM + statin groups than in the control group. In the LDL fractions, the TC content was relatively low in the DM and DM + statin groups as compared with the healthy group, whereas no significant difference was observed in the PL content in the LDL fraction between the two groups. When adjusted to the apoB levels in LDL, the TC levels were higher in the DM group, and the PL content was higher in the DM and DM + statin groups than in the healthy group. In the HDL fraction, both the TC and PL contents were relatively low in the DM and DM + statin groups as compared with the healthy control group. When adjusted to the apoA-I levels in HDL, the TC and PL contents were significantly higher in the DM and DM + statin groups than in the control group. These results indicate that subjects with diabetes in the present study had high TRL and low HDL levels, a lipid profile characteristic of the metabolic syndrome, that the size of lipoproteins might be larger in subjects with diabetes than in the healthy subjects, and that statin treatment reduced the TC content in LDL in this cohort study.

The Contents of Sph and dhSph in the TRL Fraction were Higher in Subjects with Diabetes

Fig. 1 presents the contents of Sph, dhSph, and ceramides in the TRL fraction. The contents of Sph and dhSph were higher in the TRL fraction, whereas those of ceramides were not significantly different in the DM group as compared with the healthy control subjects **(Fig. 1A, C, E)**. In the DM + statin group, the contents of C18:0, C20:0, C22:0, and C24:0 ceramides, as well as those of Sph and dhSph, were significantly higher than in the healthy group. When the contents of sphingolipids in the TRL fraction were adjusted to the apoB level, the contents of Sph, dhSph, C18:0, and C20:0 ceramides were higher in the DM and DM + statin groups, whereas the content of C24:0 ceramide was higher in the DM + statin group **(Fig. 1B, D, F)**. When the contents of

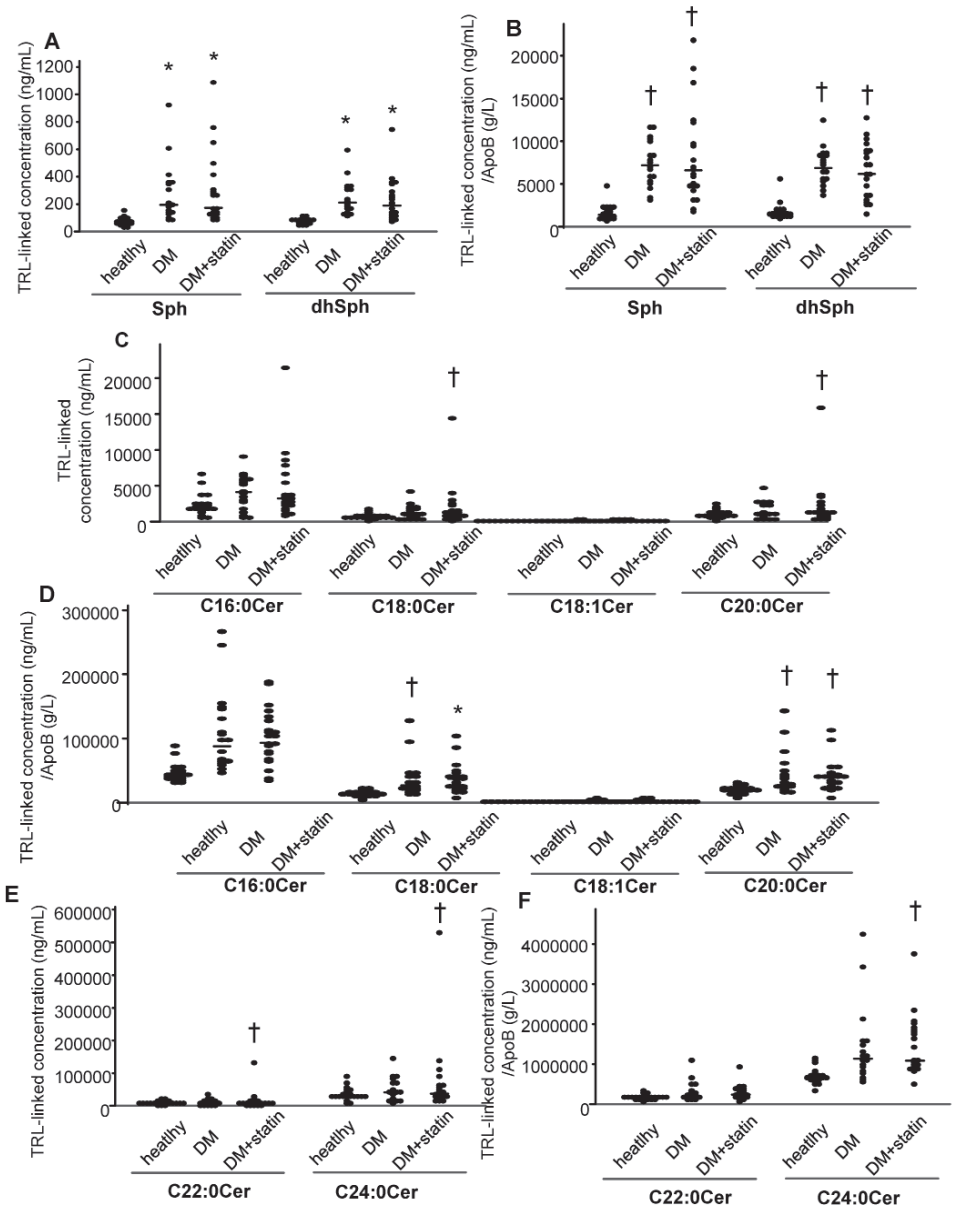

Fig. 1. Modulation of the sphingolipid contents in the Triglyceride (TG)-rich lipoprotein fraction in subjects with diabetes

Serum samples were collected from healthy volunteers ($n=22$) and subjects with type 2 diabetes not receiving treatment with statins (DM, $n=18$) or receiving statins (DM + statin, $n=21$). The samples were separated into lipoprotein and lipoprotein-depleted fractions via ultracentrifugation for the measurement in these fractions of sphingosine (Sph), dihydrosphingosine (dhSph), and the following ceramides: Cer d18:1/16:0 (C16:0 Cer), Cer d18:1/18:0 (C18:0 Cer), Cer d18:1/18:1 (C18:1 Cer), Cer d18:1/20:0 (C20:0 Cer), Cer d18:1/22:0 (C22:0 Cer), Cer d18:1/24:0 (C24:0 Cer). (A, C, E) Levels of sphingolipids in the TG-rich lipoprotein fraction (TRL). (B, D, F) Levels of sphingolipids after adjustment for the apoB content in the TRL fraction.

Differences were evaluated using the ANCOVA to adjust for age, followed by the Bonferroni test as a *post hoc* test. $^{*}P<0.01$ vs. healthy, $^{\dagger}P<0.05$ vs. healthy. The horizontal bars indicate the median of independent samples.

sphingolipids in the TRL fraction were adjusted to the total PL level, no significant difference was observed among the three groups **(Supplemental Fig. 1)**.

The Contents of Sph and Ceramides were Higher in the LDL Fraction in Subjects with Diabetes, which were not Observed in Subjects Treated with Statin

Fig. 2 presents the contents of Sph, dhSph, and ceramides in the LDL fractions. The contents of Sph, C16:0, and C24:0 ceramides in the LDL fraction were

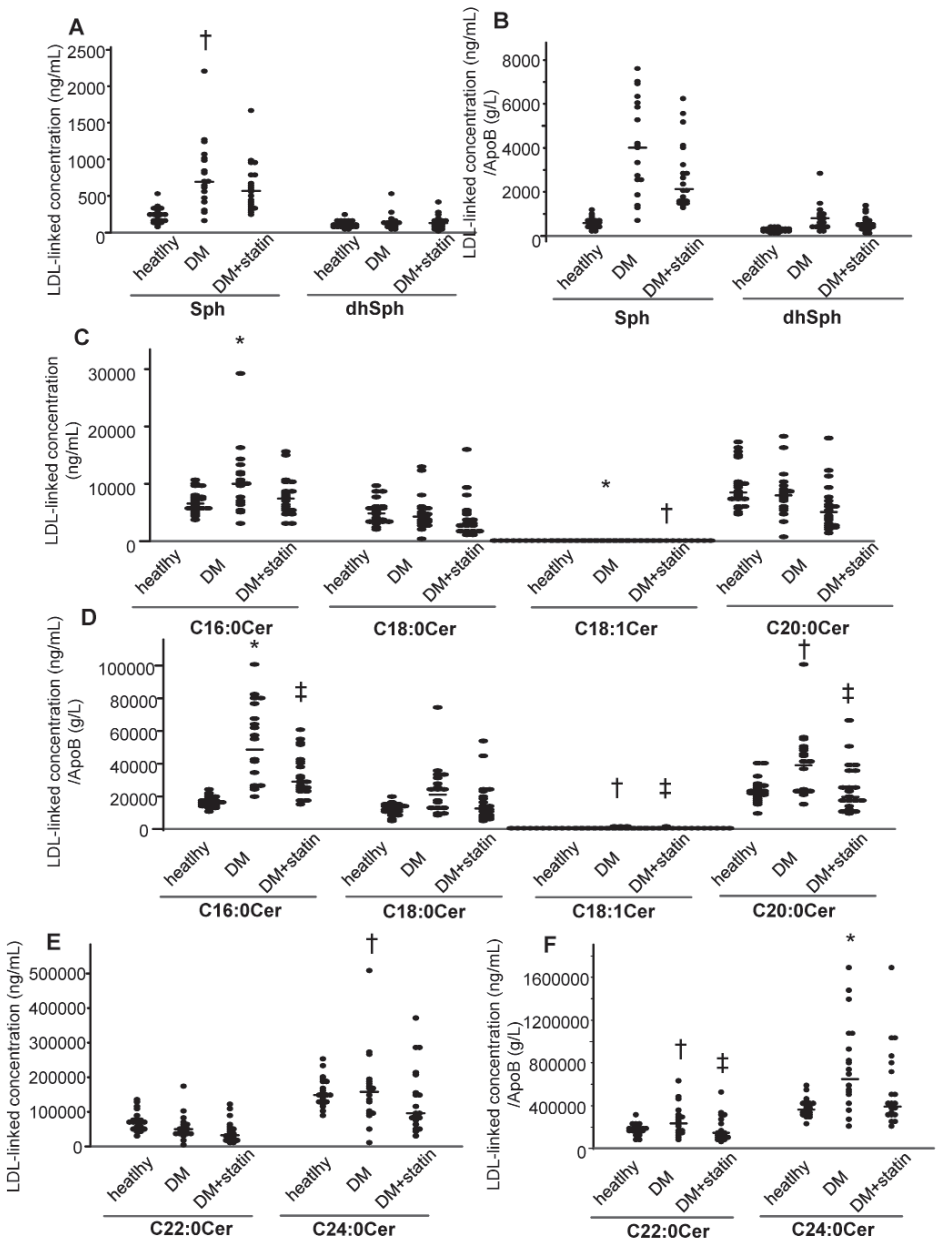

Fig. 2. Modulation of the sphingolipid contents in the LDL fraction in subjects with diabetes

The levels of sphingolipids in the LDL fraction, prepared and measured as described in Fig. 1, are presented. (A, C, E) Levels of the sphingolipids in the LDL fraction. (B, D, F) Levels of the sphingolipids after adjustment for the apoB content in the LDL fraction. Differences were evaluated using the ANCOVA to adjust for age, followed by the Bonferroni test as a *post hoc* test. $^{*}P<0.01$ vs. healthy, $^{\dagger}P<0.05$ vs. healthy, $^{\ddagger}P<0.05$ vs. DM. The horizontal bars represent the medians of independent samples.

higher in the DM group but not in the DM + statin group, whereas the content of C18:1 ceramide was higher in the DM and DM + statin groups **(Fig. 2A, C, E)**. When adjusted to the apoB level, the contents of C16:0, C18:1, C20:0, C22:0, and C24:0 ceramides were higher in the DM group than in the control group, whereas the contents of C16:0, C18:1, C20:0, and C22:0 ceramides were significantly lower in the DM + statin group than in the DM group **(Fig. 2B, D, F)**, which is consistent with a previous report[43, 44]. After the adjustment for the total PL content, the content of C20:0 ceramide was higher in the DM group than in the healthy group, whereas the contents of C16:0 and C20:0 ceramides were significantly lower in the DM + statin group than in the DM group **(Supplemental Fig. 2)**.

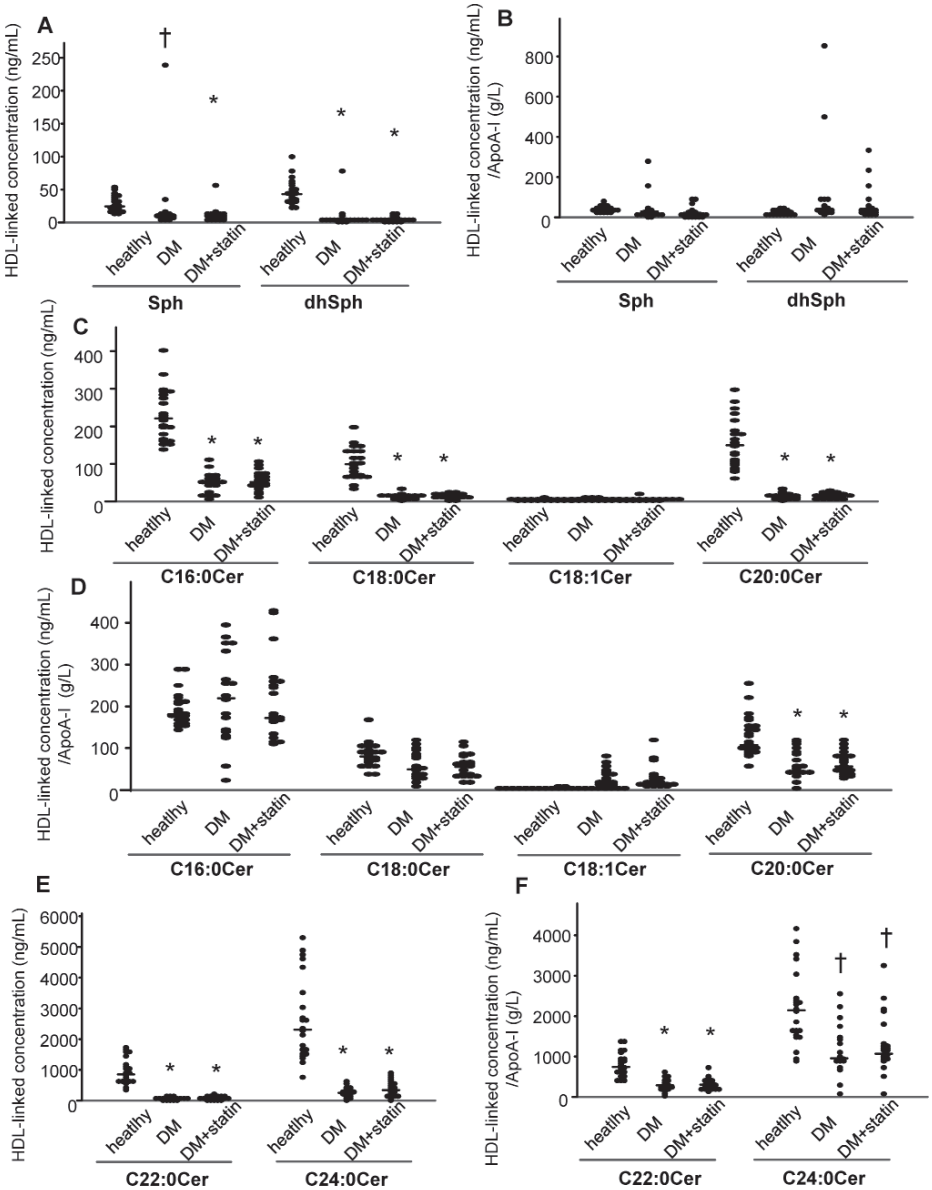

Fig. 3. Modulation of the sphingolipid contents in the HDL fraction in subjects with diabetes

The levels of sphingolipids in the HDL fraction, prepared and measured as described in Fig. 1, are presented. (A, C, E) Levels of sphingolipids in the HDL fraction. (B, D, F) Levels of sphingolipids after adjustment for the total phospholipid (PL) content in the HDL fraction. Differences were evaluated using the ANCOVA to adjust for age, followed by the Bonferroni test as a *post hoc* test. *$P < 0.01$ vs. healthy, †$P < 0.05$ vs. healthy. The horizontal bars represent the median of independent samples.

The Contents of Sph, dhSph, and Ceramides, Except for C18:1 Ceramide, in the HDL Fraction were Lower in Subjects with Diabetes

Fig. 3 presents the contents of Sph, dhSph, and ceramides in the HDL fraction. The levels of Sph, dhSph, and ceramides, except for C18:1 ceramide, were significantly reduced in the HDL fraction of subjects with diabetes **(Fig. 3A, C, E)**. When adjusted to the apoA-I level, the levels of C20:0, C22:0, and C24:0 remained significantly higher in the DM and DM + statin groups **(Fig. 3B, D, F)**. When adjusted to the total PL level, the levels of C16:0, C18:0, C20:0, C22:0, and C24:0 were significantly higher in the DM and DM + statin groups **(Supplemental Fig. 3)**. As for C18:1 ceramide, the HDL-linked C18:1 ceramide content was not different between the healthy subjects and subjects with diabetes and was, in fact, higher in the latter after adjustment for the total PL content **(Supplemental Fig. 3C)**. The use of statins had no effect on the levels of these

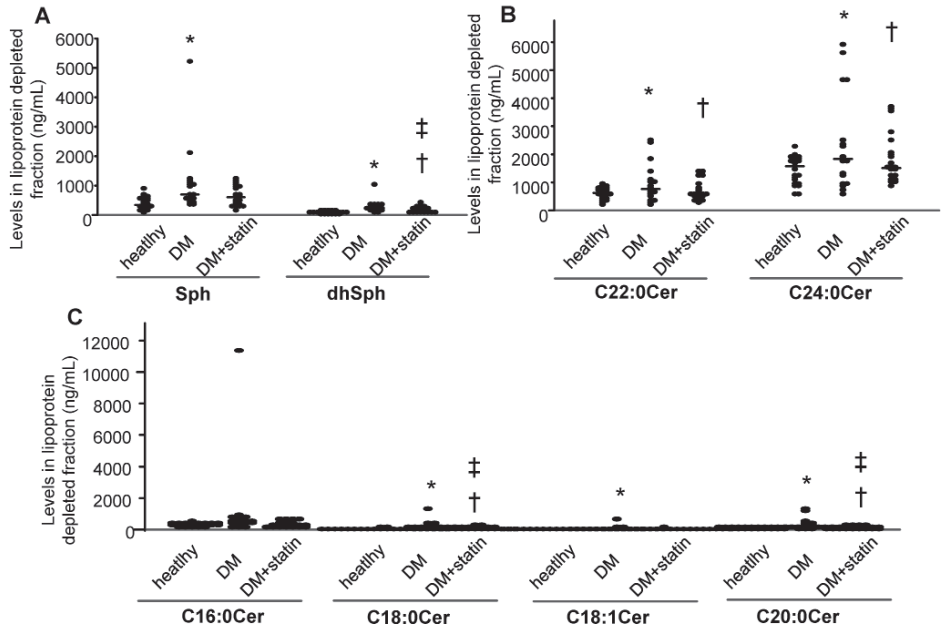

Fig. 4. Modulation of the sphingolipid contents in the lipoprotein-depleted fraction in subjects with diabetes

The levels of sphingolipids in the lipoprotein-depleted fractions, prepared and measured as described in Fig. 1, are presented. Differences were evaluated using the ANCOVA to adjust for age, followed by the Bonferroni test as a *post hoc* test. $^{*}P<0.01$ vs. healthy, $^{\dagger}P<0.05$ vs. healthy, $^{\ddagger}P<0.05$ vs. DM. The horizontal bars represent the medians of independent samples.

sphingolipids in the HDL fraction.

The Contents of Sph, dhSph, and Ceramides in the Lipoprotein-Depleted Fraction were Higher in Subjects with Diabetes, Especially those who were not Receiving Statins

Fig. 4 presents the levels of Sph, dhSph, and ceramides in the lipoprotein-depleted fractions. The levels of Sph, dhSph, C18:0, C18:1, C20:0, C22:0, and C24:0 ceramides in the lipoprotein-depleted fraction were significantly higher in the DM group. In the DM + statin group, the levels of dhSph, C18:0, C20:0, C22:0, and C24:0 ceramides were higher than in the healthy control group, whereas the levels of dhSph, C18:0, and C20:0 ceramides were lower than in the DM group.

The Contents of Sphingolipids in Lipoproteins and Lipoprotein-Depleted Fractions Significantly Discriminated Type 2 Diabetes and the Use of Statin in the Present Study

When we conducted the ROC curve analyses of the sphingolipid contents contributing to diabetes, many species of sphingolipids in specific fractions, especially HDL, were found to significantly discriminate type 2 diabetes **(Supplemental Table 2)**. When we investigated the sphingolipid contents contributing to the use of statin in subjects with diabetes, we observed that several ceramide species in LDL and lipoprotein-depleted fraction significantly contributed to the use of statin and that all the monitored ceramide species together with Sph adjusted to the apoB level in LDL contributed to the use of statin. After adjustment for the total PL contents, C16:0 Cer in TRL and LDL and C20:0 Cer in LDL were selected as significant discriminating contents for the use of statin **(Supplemental Table 3)**.

The Contents of Sphingolipids in the Lipoprotein-Depleted Fraction were Negatively Correlated with Urinary Total Protein Levels and Positively Correlated with the eGFR Levels in Subjects with Diabetes

We investigated the correlations between the levels of Sph, dhSph, and ceramides in each separated fraction and clinical parameters, such as HbA1c, urinary albumin levels, urinary total protein levels, and eGFR levels, in patients with diabetes. As presented in **Table 1A**, many contents of sphingolipids in lipoprotein-depleted fraction were negatively correlated with urinary total protein levels and positively correlated with eGFR levels. In other fractions, we observed a significant positive correlation only between the LDL-linked C18:1 ceramide content and eGFR and a significant negative correlation only between the HDL-linked Sph content and urinary

Table 1. Correlations of the sphingolipid contents in the separated fractions with clinical parameters

A. Absolute levels of sphingolipids

		HbA1c (%) $n=39$	Urinary albumin/Cr (mg/gCr) $n=35$	Urinary total protein/Cr (g/gCr), $n=29$	eGFR (ml/min/1.73m^2), $n=39$
TRL	TC	-0.059	-0.068	0.345	-0.132
	PL	-0.061	-0.093	0.337	-0.114
	Sph	-0.126	-0.137	0.135	0.194
	dhSph	-0.226	-0.090	0.189	0.185
	C16:0 Cer	0.050	-0.020	0.284	-0.083
	C18:0 Cer	-0.017	-0.230	0.172	0.053
	C18:1 Cer	-0.069	-0.167	0.211	0.010
	C20:0 Cer	-0.063	-0.197	0.136	0.085
	C22:0 Cer	0.055	-0.106	0.084	0.128
	C24:0 Cer	0.077	-0.133	0.150	0.053
LDL	TC	0.275	0.169	0.026	-0.069
	PL	0.141	0.070	-0.119	0.000
	Sph	0.299	0.362*	-0.025	-0.077
	dhSph	0.033	0.163	0.131	-0.202
	C16:0 Cer	0.228	0.001	-0.223	0.223
	C18:0 Cer	0.206	-0.061	-0.078	0.133
	C18:1 Cer	0.134	-0.221	-0.235	0.421†
	C20:0 Cer	0.179	-0.044	-0.078	0.133
	C22:0 Cer	0.237	-0.038	-0.129	0.178
	C24:0 Cer	0.240	0.000	-0.151	0.121
HDL	TC	0.284	0.263	-0.039	-0.111
	PL	0.273	0.159	-0.092	-0.130
	Sph	-0.070	-0.180	-0.389*	0.265
	dhSph	-0.097	-0.061	-0.174	0.171
	C16:0 Cer	0.161	0.071	-0.245	-0.015
	C18:0 Cer	0.060	-0.119	-0.145	-0.009
	C18:1 Cer	-0.003	-0.011	-0.114	0.223
	C20:0 Cer	0.099	-0.026	-0.085	-0.062
	C22:0 Cer	0.153	0.016	-0.204	-0.091
	C24:0 Cer	0.116	0.002	-0.245	-0.150
lipoprotein-depleted fraction	Sph	0.065	-0.125	-0.013	0.206
	dhSph	0.156	-0.259	-0.285	0.420†
	C16:0 Cer	0.145	-0.208	-0.351	0.395*
	C18:0 Cer	0.100	-0.257	-0.431*	0.380*
	C18:1 Cer	0.144	-0.280	-0.420*	0.394*
	C20:0 Cer	0.046	-0.219	-0.380*	0.420†
	C22:0 Cer	0.182	-0.114	-0.327	0.346*
	C24:0 Cer	0.253	-0.244	-0.412*	0.404*

B. Levels of the sphingolipids after adjustment for the apoB or apoA-I content

		HbA1c (%) $n=39$	Urinary albumin/Cr (mg/gCr) $n=35$	Urinary total protein/Cr (g/gCr), $n=29$	eGFR (ml/min/1.73m^2), $n=39$
TRL	Sph	-0.203	-0.047	-0.059	0.225
	dhSph	-0.383*	-0.028	-0.027	0.226
	C16:0 Cer	0.033	0.099	0.117	-0.023
	C18:0 Cer	0.018	-0.179	-0.059	0.183
	C18:1 Cer	-0.160	-0.165	-0.006	0.144
	C20:0 Cer	-0.043	-0.153	-0.151	0.228
	C22:0 Cer	0.030	-0.014	-0.063	0.200
	C24:0 Cer	0.123	-0.020	-0.066	0.188

(Cont. Table 1)

		HbA1c (%) $n=39$	Urinary albumin/Cr (mg/gCr) $n=35$	Urinary total protein/Cr (g/gCr), $n=29$	eGFR (ml/min/1.73m^2), $n=39$
LDL	Sph	0.151	0.151	-0.134	0.090
	dhSph	-0.067	0.016	-0.087	0.002
	C16:0 Cer	0.078	-0.094	-0.342	0.367*
	C18:0 Cer	0.161	-0.137	-0.173	0.238
	C18:1 Cer	0.031	-0.252	-0.318	0.456†
	C20:0 Cer	0.138	-0.091	-0.187	0.233
	C22:0 Cer	0.209	-0.075	-0.235	0.276
	C24:0 Cer	0.202	-0.069	-0.266	0.262
HDL	Sph	-0.306	-0.286	-0.296	0.268
	dhSph	-0.282	-0.226	-0.235	0.228
	C16:0 Cer	-0.276	-0.077	-0.071	0.175
	C18:0 Cer	-0.340*	-0.285	-0.137	0.218
	C18:1 Cer	-0.345*	-0.190	-0.070	0.292
	C20:0 Cer	-0.282	-0.279	-0.056	0.214
	C22:0 Cer	-0.091	-0.150	-0.127	0.092
	C24:0 Cer	-0.132	-0.201	-0.214	-0.042

C. Levels of the sphingolipids after adjustment for the total phospholipid content

		HbA1c (%) $n=39$	Urinary albumin/Cr (mg/gCr) $n=35$	Urinary total protein/Cr (g/gCr), $n=29$	eGFR (ml/min/1.73m^2), $n=39$
TRL	Sph	-0.105	0.018	-0.104	0.194
	dhSph	-0.097	0.078	-0.159	0.261
	C16:0 Cer	0.212	0.221	-0.131	0.152
	C18:0 Cer	0.138	-0.176	-0.281	0.310
	C18:1 Cer	-0.109	-0.105	-0.185	0.197
	C20:0 Cer	0.059	-0.051	-0.210	0.273
	C22:0 Cer	0.177	0.069	-0.180	0.214
	C24:0 Cer	0.286	0.024	-0.266	0.221
LDL	Sph	0.271	0.307	0.050	-0.029
	dhSph	-0.153	0.147	0.135	-0.116
	C16:0 Cer	0.155	0.012	-0.251	0.321*
	C18:0 Cer	0.256	-0.037	-0.053	0.184
	C18:1 Cer	0.060	-0.171	-0.227	0.367*
	C20:0 Cer	0.193	0.013	-0.070	0.200
	C22:0 Cer	0.284	0.065	-0.136	0.222
	C24:0 Cer	0.302	0.044	-0.117	0.159
HDL	Sph	-0.314	-0.292	-0.256	0.320*
	dhSph	-0.307	-0.208	-0.143	0.274
	C16:0 Cer	-.371*	-0.125	0.001	0.202
	C18:0 Cer	-.387*	-0.312	-0.073	0.220
	C18:1 Cer	-.372*	-0.218	0.065	0.238
	C20:0 Cer	-.369*	-0.275	0.046	0.186
	C22:0 Cer	-0.222	-0.159	-0.118	0.195
	C24:0 Cer	-.321*	-0.146	-0.121	-0.011

Correlations between two parameters were evaluated by Spearman's correlation test.
*$P<0.05$, †$P<0.01$.

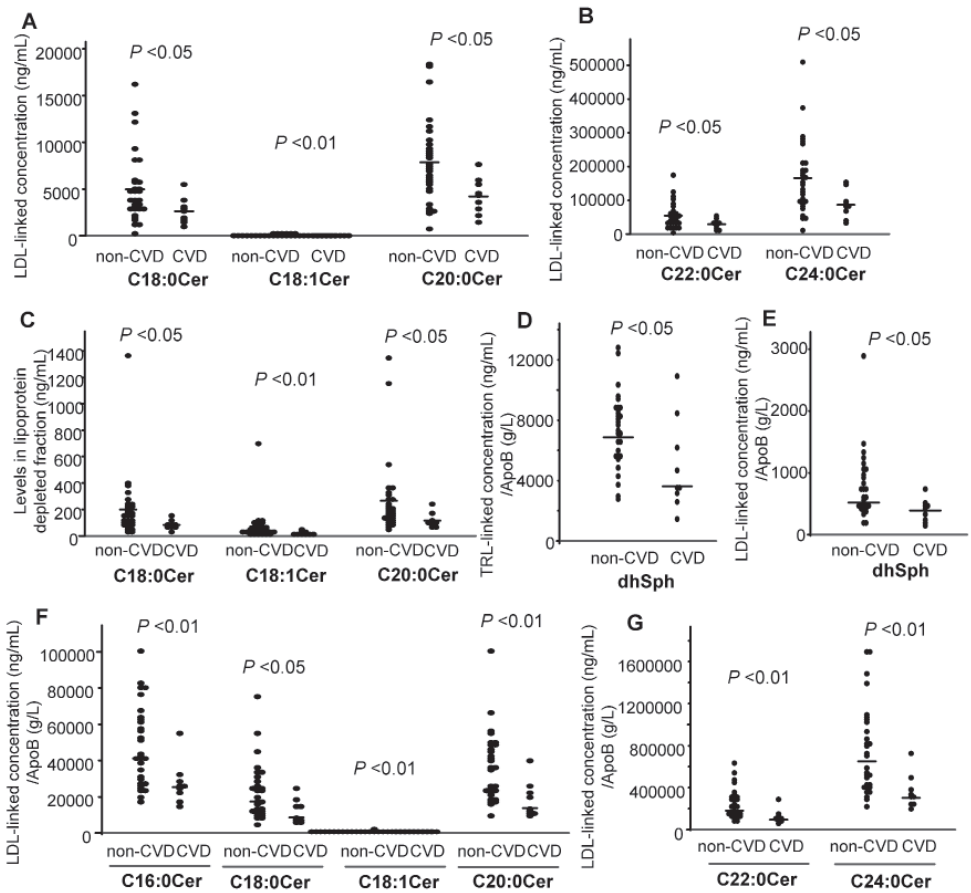

Fig. 5. Association of the levels of sphingolipids in the lipoprotein and lipoprotein-depleted fractions with cardiovascular disease

The levels of sphingolipids in the lipoprotein and lipoprotein-depleted fractions were compared between subjects with diabetes who have no cardiovascular disease (non-CVD, $n=30$) and subjects with diabetes who have cardiovascular disease (CVD, $n=9$).
Differences were evaluated using the Mann–Whitney U test. The horizontal bars represent the medians of independent samples.

total protein levels. When adjusted to the apoB or apoA-I levels, the TRL-linked dhSph content and HDL-linked C18:0 and C18:1 ceramides were negatively correlated with HbA1c, whereas the LDL-linked C16:0 and C18:1 ceramides were positively correlated with the eGFR levels **(Table 1B)**. After adjusting for the total PL content, the HDL-linked ceramide contents, except C22:0 ceramide, had significant negative correlations with HbA1c, whereas the LDL-linked C16:0 and C18:1 ceramide contents and the HDL-linked Sph content had significant positive correlations with eGFR **(Table 1C)**.

The Contents of Ceramides in the LDL and Lipoprotein-Depleted Fractions were Lower in Subjects with Diabetes who Have a History of Cardiovascular Disease

Next, we investigated the associations of the ceramide and sphingosine contents in the serum lipoprotein and lipoprotein-depleted fractions with the complications of diabetes mellitus, such as CVD and diabetic nephropathy. In this study, nine subjects had a history of CVD **(Supplemental Table 1)**. When we compared the contents of sphingolipids in each of the serum fractions between diabetes patients with and without CVD, we found that the contents of C18:0, C18:1, C20:0, C22:0, and C24:0 ceramides in the LDL fraction and those of C18:0, C18:1, and C20:0 ceramides in the lipoprotein-depleted fractions were lower in subjects with diabetes who have CVD as compared with subjects with diabetes who have no CVD **(Fig. 5A–C)**. After adjusting for the apoB level, the contents of dhSph in TRL and the contents of dhSph and all the monitored ceramide species were significantly lower in subjects with diabetes who have CVD **(Fig. 5E–G)**. After adjusting for the total PL content, the contents of Sph, dhSph, and C18:1 ceramide in the TRL fraction, of C20:0, C22:0 and C24:0 ceramides in the LDL fraction, and of Sph in the HDL fraction were significantly lower in subjects

with diabetes who have CVD than in subjects with diabetes who have no CVD **(Supplemental Fig. 4)**. When we conducted ROC curve analyses of the sphingolipid contents contributing to the history of CVD, several ceramide species in LDL and lipoprotein-depleted fraction significantly discriminated the history of CVD **(Supplemental Table 4A)**. After adjusting for the apoB level, the contents of dhSph in TRL and the contents of dhSph and all the monitored ceramide species significantly contributed to the history of CVD **(Supplemental Table 4B)**. After adjusting for the total PL content, Sph in TRL and LDL, dhSph in TRL, C18:1 Cer in TRL, and C20:0, C22:0, and C24:0 Cer in LDL significantly contributed to the history of CVD **(Supplemental Table 4C)**.

Considering that several potential confounding factors, such as the use of statins, could affect the contents of lipids in the lipoproteins, we conducted statistical analysis using the OPLS method, taking into consideration the potential confounding factors, to explore the variables that might contribute to the development of CVD. When we used the absolute contents of lipids in the analysis using OPLS (Model 1), the contents of C16:0, C18:0, C18:1, C20:0, C22:0, and C24:0 ceramides in the LDL fraction were selected as significant negative predictive variables for CVD, in addition to the absence of history of statin treatment **(Table 2A)**. The loading scatter plot and loading column plot for the OPLS are presented in **Supplemental Fig. 5**. When adjusted to the apoB or apoA-I contents (Model 2), the contents of Sph and all the monitored ceramides in the LDL fraction, together with the absence of statin use and stage 1 diabetic nephropathy, were selected as significant negative predictive variables for CVD **(Table 2B)**. The loading column plots for the OPLS are presented in **Supplemental Fig. 6**. When adjusted to the total PL contents (Model 3), the contents of Sph, dhSph, and C16:0, C18:1, C20:0, and C22:0 ceramides in the TRL fraction, of C20:0 ceramide in the LDL fraction, and of C16:0, C18:0, C18:1, C20:0, and C22:0 ceramides in the HDL fraction were selected as significant negative predictive variables for CVD **(Table 2C)**. The loading scatter plot and loading column plot for the OPLS are presented in **Supplemental Fig. 7**.

The Contents of Ceramides in the LDL Fraction might be Associated with Stage 4 Diabetic Nephropathy

With regard to diabetic nephropathy, no obvious associations were observed, except that the contents of C18:1 ceramide in the LDL fraction were significantly lower in subjects with diabetes who have stage 4 diabetic nephropathy than in those with stage 1 and 2 diabetic nephropathy. When we conducted statistical analysis using OPLS (Model 1), the contents of C18:0 and C20:0 ceramides in the LDL fraction, together with the absence of history of CVD and no statin use, were identified as significant negative predictive variables for stage 4 diabetic nephropathy **(Supplemental Table 5 and Supplemental Fig. 8)**, and after adjusting for the apoB or apoA-I levels or the total PL contents (Model 2 and Model 3), no significant predictive variables were selected.

Discussion

Ceramides and sphingosine are considered as proatherosclerotic sphingolipids, as elevated serum/plasma concentrations of sphingosine/ceramides have been reported be associated with an elevated risk of atherosclerotic diseases and diabetes mellitus[22, 23]. The carrier proteins emerge as important determinants of the physiological properties of lipids, in addition to the quantity and quality of lipids, as described in the Introduction section. While substantial amounts of ceramides and sphingosine are distributed in lipoproteins[38, 39], the associations of the distributions of ceramides and sphingosine in the lipoprotein and the lipoprotein-depleted fractions of patients with diabetes and atherosclerosis have not yet been comprehensively investigated. This study was conducted to investigate the modulation of the distribution of these lipids in each of the aforementioned serum fractions in subjects with diabetes and their associations with the complications of diabetes. The major modulations of these lipids were the increase in the Sph and dhSph levels in the TRL, LDL, and lipoprotein-depleted fractions, decrease in the Sph and dhSph levels in the HDL fraction, decrease in the ceramide levels in the HDL fraction, and increase in the ceramide levels in the lipoprotein-depleted fractions. In subjects with diabetes treated with statin, the contents of sphingolipids in the LDL and lipoprotein-depleted fractions decreased, as compared with subjects with diabetes who were not treated with statin **(Fig. 1–4)**. The major findings with regard to the associations of the contents of sphingolipids in each fraction with the risk of CVD and diabetic nephropathy were that the contents of ceramides in the LDL fraction were negatively associated with CVD and stage 4 diabetic nephropathy **(Fig. 5, Table 2, Supplemental Table 5)**. To date, studies have reported that the contents of ceramides in the HDL fraction were negatively associated with atherosclerosis[40] and type 1 diabetes

Table 2. OPLS analysis with variable importance in projection for cardiovascular disease

A. Model 1

Positive predicting variable		Negative predicting variable	
Variables	VIP	Variables	VIP
Statin use	0.2140 ± 0.2104	No statin use	-0.2140 ± 0.2104
		C18:1 Cer in LDL	-0.1574 ± 0.1303
		C20:0 Cer in LDL	-0.1452 ± 0.1049
		C18:0 Cer in LDL	-0.1256 ± 0.1130
		C16:0 Cer in LDL	-0.1192 ± 0.0896
		C22:0 Cer in LDL	-0.1024 ± 0.0924
		C24:0 Cer in LDL	-0.0992 ± 0.0905

B. Model 2

Positive predicting variable		Negative predicting variable	
Variables	VIP	Variables	VIP
Statin use	0.2011 ± 0.1505	C16:0 Cer in LDL	-0.2530 ± 0.0822
		C20:0 Cer in LDL	-0.2421 ± 0.1226
		C18:1 Cer in LDL	-0.2411 ± 0.0503
		C22:0 Cer in LDL	-0.2162 ± 0.1160
		C18:0 Cer in LDL	-0.2159 ± 0.1469
		C24:0 Cer in LDL	-0.2044 ± 0.1059
		No statin use	-0.2011 ± 0.1505
		Sph in LDL	-0.1839 ± 0.0733
		Stage1 diabetic nephropathy	-0.1545 ± 0.1218

C. Model 3

Positive predicting variable		Negative predicting variable	
Variables	VIP	Variables	VIP
Statin use	0.2373 ± 0.0747	No statin use	-0.2373 ± 0.0747
		Stage1 diabetic nephropathy	-0.2188 ± 0.0982
		C18:1 Cer in TRL	-0.2178 ± 0.1299
		dhSph in TRL	-0.1942 ± 0.1047
		Sph in TRL	-0.1909 ± 0.0677
		C22:0 Cer in HDL	-0.1593 ± 0.0807
		C20:0 Cer in LDL	-0.1445 ± 0.1210
		C20:0 Cer on TRL	-0.1309 ± 0.1062
		C20:0 Cer on HDL	-0.1306 ± 0.0590
		C18:0 Cer on HDL	-0.1181 ± 0.0794
		C16:0 Cer on TRL	-0.1104 ± 0.1014
		C16:0 Cer on HDL	-0.1067 ± 0.0894
		C22:0 Cer in TRL	-0.0922 ± 0.0905
		C18:1 Cer on HDL	-0.0805 ± 0.0647

Data are the means ± 2.40419 x SEM. VIP denotes means variable importance in projection, which was calculated in the OPLS analyses using SIMCA.

mellitus[41] and that those in the LDL fraction were positively associated with diabetes mellitus[49]; however, no studies have investigated the ceramide levels in lipoprotein-depleted fraction simultaneously.

In this study, the modulation of the contents of ceramides differed in terms of direction among the separated serum fractions; the contents of ceramides decreased, especially in the HDL fraction, whereas

those in the lipoprotein-depleted fraction increased. The results indicating that the HDL-linked ceramide levels were lower in subjects with type 2 diabetes were consistent with those of previous study on subjects with type 1 diabetes[41]. Furthermore, the results indicating that the ceramide contents in lipoprotein-depleted fraction were higher in type 2 diabetes seemed reasonable, since many clinical studies have demonstrated positive associations between the sphingolipids in plasma or serum and diabetes[36, 37, 50-52]. Although the carrier-dependent physiological properties of ceramides have remained largely unknown, considering that basic studies have demonstrated that ceramides impair insulin secretion[53] and worsen insulin resistance[54], it is possible that the ceramides in the lipoprotein-depleted fractions, not those in the lipoprotein fractions, especially the HDL fraction, are responsible for the proposed involvement of ceramides in the pathogenesis of diabetes.

Boon *et al.* demonstrated that the ceramide contents in LDL were higher in the subjects with diabetes[49], whereas a significant difference was not observed in LDL-linked ceramide levels. Moreover, in the present study, the levels of sphingolipids in various fractions were lower in subjects with diabetes who have history of CVD **(Fig. 5)**. These discrepancies might reflect the lipid profiles and/or the use of statin of the subjects in the present study, considering the influences of statin on the sphingolipid levels[43, 44]. In this study, the subjects with diabetes had rather lower LDL cholesterol levels than the control subjects, may be well controlled in terms of treatment with statin, and had extremely lower HDL cholesterol levels than the control subjects. In addition, although the TC and PL levels in LDL were not different between subjects with diabetes who have CVD and those without CVD, all subjects with CVD received statin, whereas 12 out of 30 subjects without CVD received statin. However, after adjustment to the apoA-I and total PL levels, the HDL-linked ceramide levels still exhibited substantially lower levels, suggesting that at least the HDL-linked sphingolipid levels would be certainly lower and the sphingolipids in lipoprotein-depleted fraction would be higher in subjects with type 2 diabetes.

In the present study, we observed that the sphingolipid levels in the LDL fraction were lower in subjects with diabetes who were treated with statin than in those not treated with statin **(Fig. 3)**, which is consistent with the findings of previous studies[43, 44]. Interestingly, treatment of statin also seemed to decrease the sphingolipid levels in the lipoprotein-depleted fraction **(Fig. 4)**. The mechanism for the effects of statin in the sphingolipids in the lipoprotein-depleted fraction remains unknown. At present, the possible interaction between the LDL fraction and the lipoprotein-depleted fraction as well as the possible pleiotropic effects of statin, such as anti-inflammation, might be involved. The modulation of sphingolipids in the lipoprotein-depleted fraction as well as in the LDL fraction might be a novel pleiotropic effect of statin.

When we adjusted the contents of sphingolipids in the lipoprotein fractions to the apoB or apoA-I levels in the same fractions, these parameters reflected the contents of sphingolipids in individual lipoprotein particles. After adjusting for apoB, we especially observed that the ceramide contents in the LDL and TRL particles are higher in subjects with diabetes and that treatment with statin decreases the ceramide contents in LDL particles. At present, the physiological meaning of this observation remains unknown; however, considering the harmful properties of sphingolipids in the fields of atherosclerosis, these findings may suggest a novel proatherosclerotic modulation of apoB-containing lipoproteins in diabetes. Although the TC levels in LDL were higher in the DM group than in the healthy control, we could not completely exclude the possibility that these findings cannot be applied to the general diabetic population, in which the size of LDL was estimated to be small by the TC levels adjusted to apoB in the LDL fractions.

With regard to the species of ceramide, we observed overall similar modulation in subjects with diabetes, except that the levels of C18:1 ceramide in HDL were not significantly modulated in the DM group and positively contribute to the discrimination of type 2 diabetes, whereas the levels of other ceramides in HDL were extremely lower in the DM group and negatively contribute to the discrimination of type 2 diabetes. To date, differences in biological properties have been reported for six kinds of ceramide synthases, and it has been proposed that such differences result from the distinct acyl chain lengths[55]. Furthermore, whether C18:0 Cer and C18:1 Cer possess similar biological properties remains unknown. The modulation of C18:1 Cer has not been monitored well in clinical studies[22]. The potential unique roles of C18:1 ceramide raised from the present study necessitate further investigation on the different biological properties between C18:1 ceramide and C18:0 ceramide.

The mechanisms underlying these sphingolipid modulations still need to be elucidated. With regard to the HDL-associated ceramides, the lower levels of HDL in subjects with diabetes could have certainly

affected the results; however, even after adjusting for the apoA-I and total PL contents, the ceramide contents were reduced in subjects with diabetes. At present, how ceramides are incorporated into HDL remains unknown. Since HDL takes up lipids from peripheral tissues by a reverse cholesterol transport system, HDL might also take up ceramides in addition to cholesterol, and the impaired HDL functions observed under the diabetic condition might reduce the capacity of HDL to induce efflux of ceramides from peripheral tissues. With regard to apoB-containing lipoproteins, MTP and apoB have been reported to play important roles in carrying ceramides[56]. As for the lipoprotein-depleted fraction, considering that the results were contrary to those of HDL, the results could not be attributed to the technical contamination of this fraction with HDL. In the lipoprotein-depleted fraction, the lipids might be carried on albumin[57] or in the form of exosomes. Considering that lipids can be nonspecifically bound to albumin and that exosomes are produced from the cell membrane, the elevated levels of ceramides in these fractions might reflect the increased cellular levels of ceramides. In fact, the contents of ceramides in the liver, muscle, and adipose tissue have been reported to be elevated in subjects with type 2 diabetes[58, 59].

With regard to Sph and dhSph, the contents of these sphingolipids were higher in the apoB-containing lipoproteins. These lipids have been reported promote overproduction of apoB-containing lipoproteins in the liver[60], which is consistent with the findings of the present study, whereas the mechanisms regulating HDL-linked Sph and dhSph remain unknown. Although dhSph is structurally akin to Sph, the two lipids play different roles in the metabolism of sphingolipids. Ceramides are hydrolyzed into Sph and Sph can be recycled to ceramides, whereas dhSph is concerted into ceramides through dihydroceramides[33]. In general, while Sph and dhSph appeared to be modulated similarly in each serum fraction, only the Sph contents in the LDL fractions were higher in subjects with diabetes **(Fig. 2A)**. Until now, no obvious differences in the physiological properties between Sph and dhSph have been demonstrated, and considering that Sph is converted into S1P and dhSph into dihydrosphingosine 1-phosphate and that they are differently bound to HDL[61], the different modulations of the two lipids in the LDL fraction could have some as-yet-unclear significance.

When we investigated the correlations between sphingolipids in specific fractions and clinical parameters, we especially found that the contents of sphingolipids in the lipoprotein-depleted fractions were negatively correlated with urinary total protein levels and positively correlated with eGFR **(Table 1A)**. After adjusting for the apoB or apoA-I contents, the dhSph content in TRL and the ceramides in HDL were negatively correlated with HbA1c, whereas the ceramides in LDL were positively correlated with the eGFR levels **(Table 1B)**. After adjusting for the total PL contents, especially the HDL-linked sphingolipids had negative correlations with HbA1c **(Table 1C)**. Of course, this is just a speculation; considering the harmful properties of ceramides and sphingosine in the pathogenesis of diabetes and CVD, the correlations with clinical parameters related to diabetic nephropathy might reflect the altered conditions in the LDL and the lipoprotein-depleted fractions, such as albumin and exosome, whereas the negative correlations with HbA1c might reflect the impaired ability of HDL in the efflux of sphingolipids. Essentially, the redox status of albumin affects the binding ability of lipids[62], and apoA-I has been reported to reduce the contents of sphingolipids in atherosclerotic lesions[63].

The finding that the contents of ceramides in the lipoprotein fractions, especially the LDL fraction **(Fig. 5)**, were negatively associated with CVD was unexpected, since the serum and plasma ceramide levels are known to be higher in patients with CVD[22, 23]. Although the use of statin could have affected the results, the association remained significant even when we considered the use of statins as a confounding variable in the statistical analysis factor **(Table 2)**. Considering that the contents of ceramides in the lipoprotein-depleted fraction did not exhibit any significant negative correlation with CVD, the involvement of ceramides in the pathogenesis of atherosclerosis might depend on the carrier proteins of ceramides: lipoprotein-associated ceramides might have antiatherosclerotic properties, whereas albumin- or exosome-associated ceramides might exhibit proatherosclerotic properties. The carrier-dependent bioactivities of ceramides need to be elucidated to address this question.

Many elegant clinical and basic studies have demonstrated that statins[64, 65], inhibitors of the renin-angiotensin system, such as angiotensin-converting enzyme inhibitor and angiotensin receptor blockers[66-68], have protective pleiotropic properties for cardiovascular dysfunction and/or diabetic nephropathy. However, at present, although the protective effects of antidyslipidemia reagents in atherosclerosis have been established, there remains an issue of cardiovascular residual risk[69]; moreover, the effects of antidyslipidemia reagents in diabetic nephropathy

were controversial, including the roles of statin in renal outcome[70, 71]. To overcome these issues, recent studies have proposed several promising targets. Sphingolipid is one of these promising therapeutic targets. Sphingolipids have been demonstrated to be involved in the pathogenesis of both atherosclerosis and kidney injuries. In addition to the roles of sphingolipids in the pathogenesis of atherosclerosis described in the Introduction section[24, 25], since ceramide and sphingosine exhibit proapoptosis, sphingolipids have been proposed to aggravate the apoptosis of mesangial cells, renal tubular epithelial cells, and microvascular endothelial cells in diabetic nephropathy[72]. The results of the present study indicating that the sphingolipid contents in LDL and HDL were negatively associated with history of CVD and stage 4 diabetic nephropathy seemed to contradict the proposed mechanisms at a glance. However, considering that sphingolipids exert proapoptotic effects intracellularly through the activation of PP2A[73] and that sphingolipids are ubiquitously produced in the body[74], the inverse association between the sphingolipid contents in lipoproteins and CVD or diabetic nephropathy might reflect the impaired removal system of sphingolipids from peripheral tissues and organs through lipoproteins. Essentially, HDL-associated sphingolipids were significantly lower in subjects with diabetes **(Fig. 3)**. The therapeutic strategy for the removal of sphingolipids from peripheral tissues and organs through enhanced reverse transport of sphingolipids by HDL might contribute to the suppression of cardiovascular residual risk and improve the outcome of diabetic nephropathy in the future.

Since this was only a cross-sectional study, we could not conclude that the modulation of the ceramide and sphingosine distribution on the carrier lipoproteins in subjects with diabetes was directly involved in the pathogenesis of diabetes or in the pathogenesis of diabetic complications. In addition, we could not infer that the sphingolipid contents in the LDL and lipoprotein-depleted fractions were influenced by the treatment with statin. More prospective and basic studies are needed to resolve this issue. Another limitation of the present study was that the number of subjects was rather small, and the control group characteristics did not match those of the DM group, especially in terms of the age of the subjects **(Supplemental Table 1)**. Essentially, plasma ceramide levels have been reported to be higher in elderly than younger subjects[48]. Moreover, although these data were not available for this study, diet[75], exercise[76], obesity[77], and blood pressure[78], which could have differed between the groups, have also been reported to affect the circulating ceramide levels. In the aspect of methodology, we could not deny the possibility that the some amounts of lipids could be transferred to other fractions, as we described in the previous reports[12, 61]. In any case, considering that the lipid profiles were largely different between subjects with diabetes and healthy subjects enrolled in the present study, that the differences in sphingolipid levels were significant even when we conducted the ANCOVA considering age as a potential confounding factor, and that this is the first study to comprehensively investigate the modulation and significance of the sphingolipids in the serum lipoprotein and lipoprotein-depleted fractions in subjects with diabetes, we believe that the present study lends support to the novel concept that the carrier protein is one of the important determinants of the biological properties and clinical significance of the sphingolipids.

Conclusion

In summary, the contents of ceramides and sphingosine in the serum lipoprotein and lipoprotein-depleted fractions were differently modulated in subjects with diabetes. The present results indicated the increased secretion of sphingosine from the liver in TRL and the increased contents of sphingosine and ceramides in LDL particles and the lipoprotein-depleted fraction, which might be characteristics of disturbed sphingolipid metabolism observed in diabetes. The impaired ability of HDL (and maybe LDL) to carry sphingolipids might accelerate the accumulation of sphingolipids in the cardiovascular system and kidneys, which might be involved in the pathogenesis of diabetic complications, such as CVD and nephropathy **(Fig. 6)**. These results indicate that the carrier proteins may be an important factor influencing the biological properties and clinical significances of sphingolipids.

Data Availability Statement

The datasets generated or analyzed in the current study will be made available upon reasonable request.

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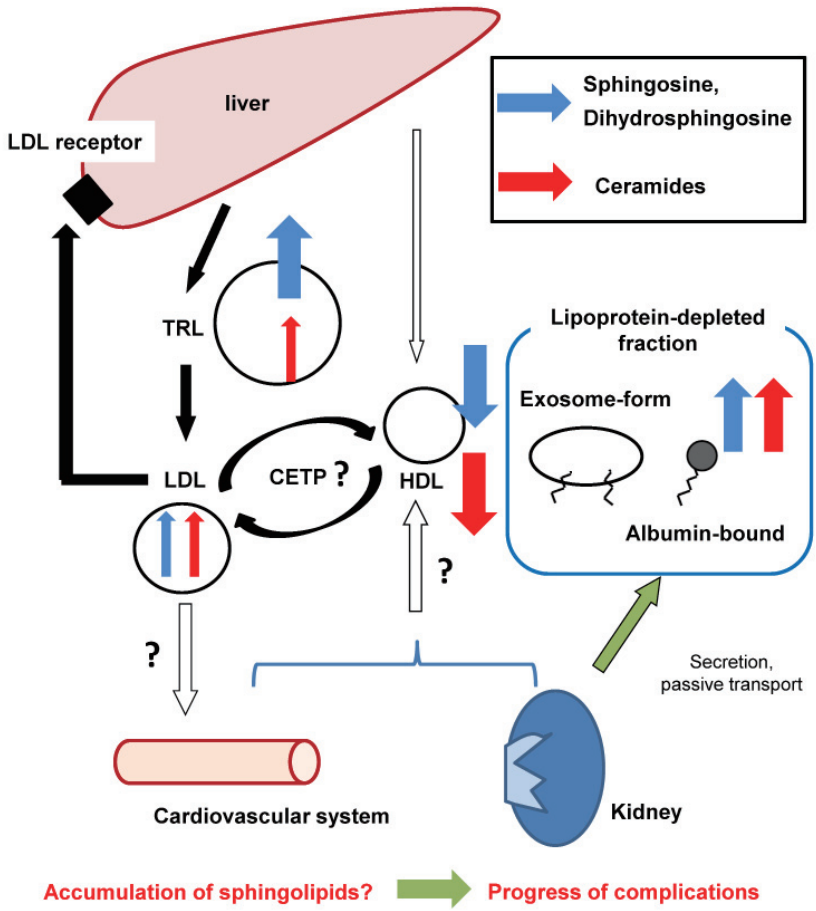

Fig. 6. Scheme of the results of the present study and their interpretation

This is a representative scheme of the present study. The red arrows indicate the modulation of sphingosine and dihydrosphingosine, and the blue ones represent that of ceramides in subjects with diabetes.

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Author Contributions

M. Kurano designed the research and analyzed the data; M. Kurano and E. Sakai performed the research; M. Kurano, K. Tsukamoto, and Y. Yatomi wrote the paper. All authors have read and approved the final manuscript.

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The authors have no competing financial interests to declare.

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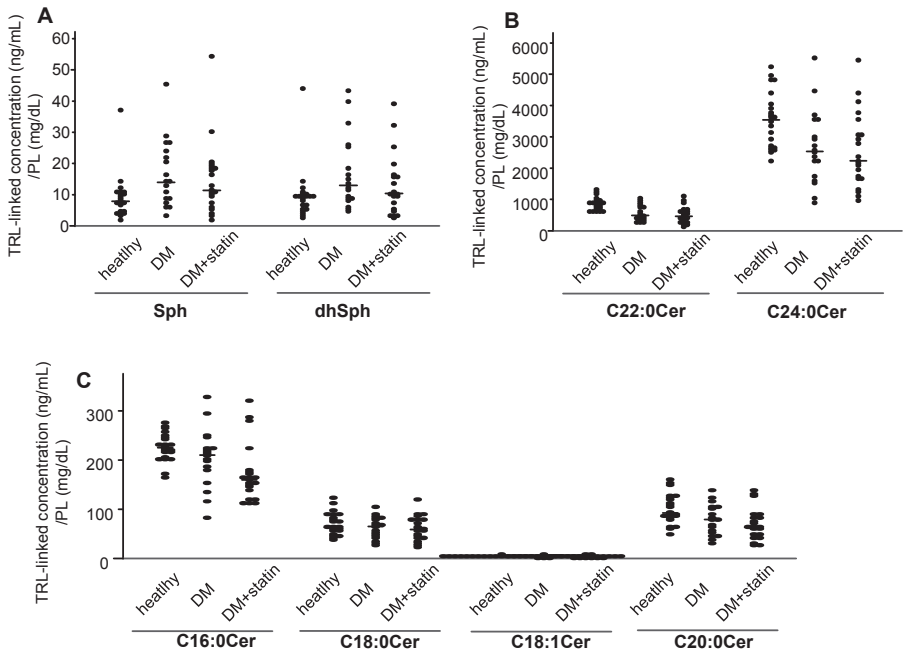
Supplemental Table 1. Characteristics of the subjects in the present study

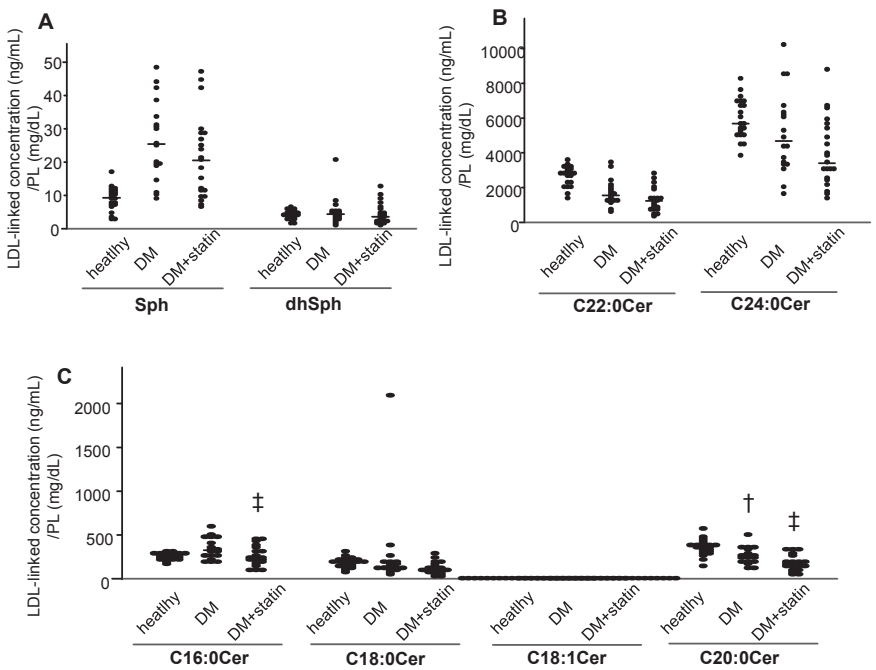
	Healthy	DM	DM + statin	Significance
N	$n=22$	$n=18$	$n=21$	
Age (year)	27.0 ± 2.6	73.5 ± 12.4	68.0 ± 10.0	$P<0.001$, *, †
Sex (male/female)	13/9	12/6	11/10	
Serum Total Cholesterol (mg/dL)	185.0 ± 35.7	172.0 ± 37.8	180.0 ± 40.9	
Serum Total Triglycerides (mg/dL)	79.5 ± 37.4	95.0 ± 94.2	141.0 ± 93.8	
Serum LDL cholesterol (mg/dL)	not assessed	107.0 ± 32.6	98.0 ± 33.5	
Serum HDL cholesterol (mg/dL)	not assessed	45.6 ± 18.2	55.6 ± 18.7	
TRL apoB (g/L)	0.046 ± 0.018	0.034 ± 0.022	0.030 ± 0.042	$P<0.001$, *, †
TRL Total Cholesterol (mg/dL)	7.2 ± 5.4	16.9 ± 11.1	13.4 ± 14.8	$P=0.028$, *
TRL Total Phospholipid (mg/dL)	9.4 ± 7.4	20.9 ± 19.2	23.2 ± 26.8	$P=0.013$, †
TRL Total Cholesterol (mg/dL)/ApoB (g/L)	147.6 ± 59.8	406.1 ± 226.8	458.9 ± 157.5	$P<0.001$, *, †
TRL Total Phospholipid (mg/dL)/ApoB (g/L)	185.9 ± 88.2	501.8 ± 398.8	653.0 ± 269.6	$P<0.001$, *, †
LDL apoB (g/L)	0.405 ± 0.107	0.182 ± 0.097	0.242 ± 0.089	$P<0.001$, *, †
LDL Total Cholesterol (mg/dL)	64.2 ± 21.0	41.6 ± 17.5	38.0 ± 32.5	$P=0.004$, *, †
LDL Total Phospholipid (mg/dL)	26.1 ± 7.5	29.9 ± 10.7	32.7 ± 18.5	
LDL Total Cholesterol (mg/dL)/ApoB (g/L)	159.9 ± 32.1	214.2 ± 60.4	172.4 ± 89.4	$P<0.001$, *
LDL Total Phospholipid (mg/dL)/ApoB (g/L)	63.5 ± 11.8	135.4 ± 47.1	132.2 ± 45.1	$P<0.001$, *, †
HDL apoA-I (g/L)	1.116 ± 0.368	0.278 ± 0.147	0.295 ± 0. 121	$P<0.001$, *, †
HDL Total Cholesterol (mg/dL)	31.3 ± 9.7	10.5 ± 7.7	15.4 ± 8.9	$P<0.001$, *, †
HDL Total Phospholipid (mg/dL)	41.8 ± 12.1	20.5 ± 12.4	28.2 ± 15.5	$P<0.001$, *, †
HDL Total Cholesterol (mg/dL)/ApoA-I (g/L)	27.85 ± 4.70	47.82 ± 11.24	53.08 ± 20.88	$P<0.001$, *, †
HDL Total Phospholipid (mg/dL)/ApoA-I (g/L)	39.23 ± 8.14	83.52 ± 19.34	100.97 ± 29.90	$P<0.001$, *, †
HbA1c	not assessed	7.25 ± 1.49	7.2 ± 1.44	
Urinary albumin/Cr (mg/gCr)	not assessed	44.8 ± 59.6	132.4 ± 276.3	
Urinary total protein/Cr (g/gCr)	not assessed	355.6 ± 815.3	1261.6 ± 2161.6	
eGFR (ml/min/1.73m^2)	not assessed	65.0 ± 23.9	51.5 ± 26.9	
Cardiovascular disease (%, n)	0%, $n=0$	0%, $n=0$	42.9%, $n=9$	
DM nephropathy stage 1 (%, n)		66.7%, $n=12$	38.1%, $n=8$	
DM nephropathy stage 2 (%, n)		22.2%, $n=4$	28.6%, $n=6$	
DM nephropathy stage 3 (%, n)		0 %, $n=0$	0 %, $n=0$	
DM nephropathy stage 4 (%, n)		11.1%, $n=2$	33.3%, $n=7$	

The differences were assessed using the Kruskal-Wallis test, followed by the Steel-Dwass test as a post-hoc test. Significance shows the *p*-values of the Kruskal-Wallis test. *$P<0.05$ between the Healthy and DM group and †$P<0.05$ between the Healthy and DM + statin group. For urine albumin/Cr and urine total protein/Cr, the data from 4 subjects (DM, $n=1$; DM + stain, $n=3$) and 10 subjects (DM, $n=5$; DM + stain, $n=5$) were missing.



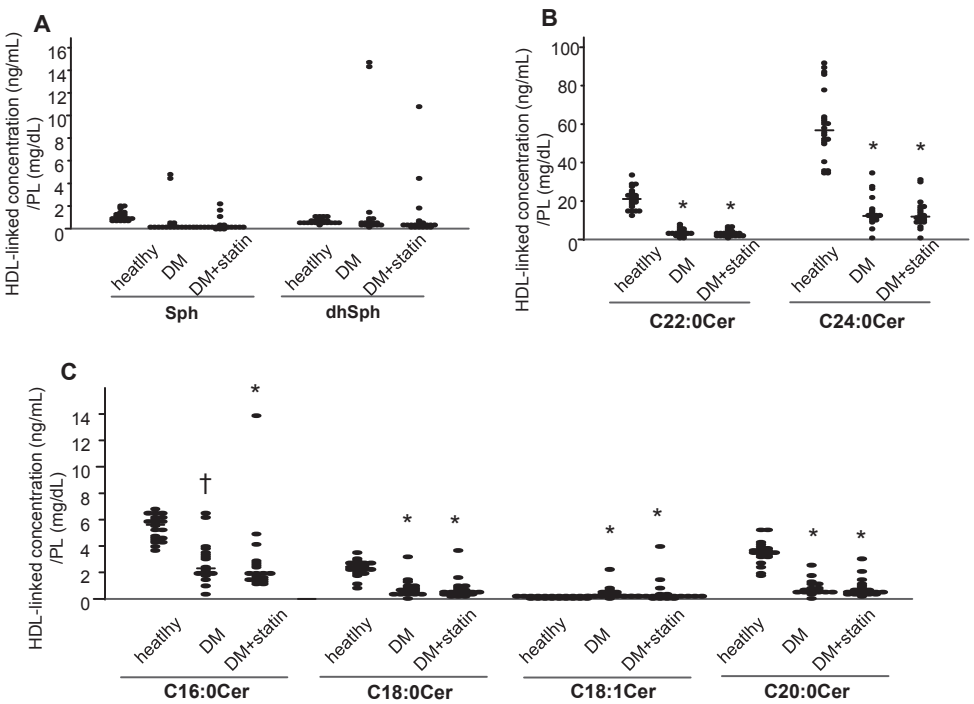
Supplemental Fig. 1. Modulation of the sphingolipid contents adjusted to the total PL level in the TG-rich lipoprotein fraction in diabetic subjects

The levels of sphingolipids adjusted to the total PL level in the TRL fraction, prepared and measured as described in Figure 1, are shown. Differences were evaluated using the ANCOVA to adjust for age, followed by the Bonferroni test as a post-hoc test. The horizontal bars represent the median of independent samples.



Supplemental Fig. 2. Modulation of the sphingolipid contents adjusted to the total PL level in the LDL fraction in diabetic subjects

The levels of sphingolipids adjusted to the total PL level in the LDL fraction, prepared and measured as described in Figure 1, are shown. Differences were evaluated using the ANCOVA to adjust for age, followed by the Bonferroni test as a post-hoc test. $^{\dagger}P<0.05$ vs. healthy, $^{\ddagger}P<0.05$ vs. DM. The horizontal bars represent the median of independent samples.



Supplemental Fig. 3. Modulation of the sphingolipid contents adjusted to the total PL level in the HDL fraction in diabetic subjects

The levels of sphingolipids adjusted to the total PL level in the HDL fraction, prepared and measured as described in Figure 1, are shown. Differences were evaluated using the ANCOVA to adjust for age, followed by the Bonferroni test as a post-hoc test. $^{*}P<0.01$ vs. healthy, $^{\dagger}P<0.05$ vs. healthy. The horizontal bars represent the median of independent samples.

Supplemental Table 2. ROC analyses of sphingolipids in lipoprotein and lipoprotein-depleted fractions contributing to type 2 diabetes

A. Sphingolipid contents (ng/mL)

		AUC	*p*-value	cut-off value	sensitivity	specificity	positive predictive value	negative predictive value
TRL	Sph	0.95	0.00	115.31	0.82	0.95	0.97	0.75
	dhSph	0.97	0.00	103.50	0.92	0.91	0.95	0.87
	C16:0 Cer	0.70	0.01	2648.95	0.67	0.82	0.87	0.58
	C18:0 Cer	0.69	0.01	991.39	0.56	0.86	0.88	0.53
	C18:1 Cer	0.70	0.01	41.19	0.72	0.68	0.80	0.58
LDL	Sph	0.92	0.00	384.32	0.77	0.95	0.97	0.70
	C18:0 Cer	0.65	0.05	3306.21	0.49	0.86	0.86	0.49
	C18:1 Cer	0.72	0.00	85.94	0.69	0.82	0.87	0.60
	C20:0 Cer	0.69	0.01	7311.13	0.59	0.73	0.79	0.50
	C22:0 Cer	0.77	0.00	40002	0.54	0.95	0.95	0.54
HDL	Sph	0.98	0.00	17.55	0.97	1.00	1.00	0.96
	dhSph	0.91	0.00	16.00	0.90	0.91	0.95	0.83
	C16:0 Cer	1.00	0.00	124.85	1.00	1.00	1.00	1.00
	C18:0 Cer	1.00	0.00	30.29	0.97	1.00	1.00	0.96
	C20:0 Cer	1.00	0.00	49.31	1.00	1.00	1.00	1.00
	C22:0 Cer	1.00	0.00	262.95	1.00	1.00	1.00	1.00
	C24:0 Cer	1.00	0.00	1045.03	1.00	0.95	0.98	1.00
lipoprotein-depleted fraction	Sph	0.76	0.00	686.92	0.49	0.95	0.95	0.51
	dhSph	0.89	0.00	104.03	0.92	0.77	0.88	0.85
	C18:0 Cer	0.77	0.00	111.20	0.54	1.00	1.00	0.55
	C18:1 Cer	0.87	0.00	12.28	1.00	0.59	0.81	1.00
	C20:0 Cer	0.71	0.01	162.47	0.49	0.95	0.95	0.51

B. Sphingolipid contents (ng/mL) adjusted to the apoB or apoA-I levels (g/L)

		AUC	*p*-value	cut-off value	sensitivity	specificity	positive predictive value	negative predictive value
TRL	Sph	0.97	0.00	2319.98	0.97	0.91	0.95	0.95
	dhSph	0.98	0.00	2752.91	0.95	0.95	0.97	0.91
	C16:0 Cer	0.92	0.00	57128.31	0.87	0.91	0.94	0.80
	C18:0 Cer	0.92	0.00	18502.13	0.85	0.86	0.92	0.76
	C18:1 Cer	0.97	0.00	1102.68	0.95	0.86	0.93	0.90
	C20:0 Cer	0.85	0.00	23462.11	0.79	0.82	0.89	0.69
	C22:0 Cer	0.76	0.00	192798.84	0.74	0.86	0.91	0.66
	C24:0 Cer	0.87	0.00	810717.06	0.87	0.86	0.92	0.79
LDL	Sph	0.86	0.00	398.18	0.77	0.95	0.97	0.70
	dhSph	0.99	0.00	1217.79	0.97	1.00	1.00	0.96
	C16:0 Cer	0.95	0.00	22565.25	0.90	0.95	0.97	0.84
	C18:0 Cer	0.66	0.05	15945.70	0.51	0.95	0.95	0.53
	C18:1 Cer	0.92	0.00	270.86	0.82	0.95	0.97	0.75
	C24:0 Cer	0.69	0.01	480433.30	0.56	0.91	0.92	0.54
HDL	Sph	0.74	0.00	24.29	0.82	0.64	0.80	0.67
	dhSph	0.81	0.00	23.79	0.74	0.91	0.94	0.67
	C18:0 Cer	0.75	0.00	65.20	0.67	0.77	0.84	0.57
	C18:1 Cer	0.97	0.00	6.75	0.97	1.00	1.00	0.96
	C20:0 Cer	0.90	0.00	82.19	0.74	0.95	0.97	0.68
	C22:0 Cer	0.95	0.00	391.67	0.79	0.95	0.97	0.72
	C24:0 Cer	0.81	0.00	1471.17	0.77	0.86	0.91	0.68

(Cont. Supplemental Table 2)

C. Sphingolipid contents (ng/mL) adjusted to the total PL levels (mg/dL)

		AUC	*p*-value	cut-off value	sensitivity	specificity	positive predictive value	negative predictive value
TRL	Sph	0.71	0.01	12.52	0.54	0.91	0.91	0.53
	dhSph	0.66	0.04	10.31	0.59	0.82	0.85	0.53
	C16:0 Cer	0.74	0.00	201.10	0.64	0.91	0.93	0.59
	C20:0 Cer	0.73	0.00	79.79	0.62	0.82	0.86	0.55
	C22:0 Cer	0.83	0.00	593.61	0.64	1.00	1.00	0.61
	C24:0 Cer	0.75	0.00	2525.36	0.54	0.95	0.95	0.54
LDL	Sph	0.87	0.00	13.52	0.72	0.95	0.97	0.66
	C18:0 Cer	0.76	0.00	145.74	0.67	0.82	0.87	0.58
	C18:1 Cer	0.70	0.01	3.94	0.44	1.00	1.00	0.50
	C20:0 Cer	0.87	0.00	288.86	0.79	0.86	0.91	0.70
	C22:0 Cer	0.89	0.00	1981.91	0.77	0.91	0.94	0.69
	C24:0 Cer	0.74	0.00	4451.95	0.56	0.95	0.96	0.55
HDL	Sph	0.89	0.00	0.63	0.87	1.00	1.00	0.81
	dhSph	0.70	0.01	0.42	0.59	0.95	0.96	0.57
	C16:0 Cer	0.92	0.00	3.62	0.82	1.00	1.00	0.76
	C18:0 Cer	0.94	0.00	1.69	0.95	0.91	0.95	0.91
	C18:1 Cer	0.79	0.00	0.17	0.62	1.00	1.00	0.59
	C20:0 Cer	0.99	0.00	1.77	0.92	1.00	1.00	0.88
	C22:0 Cer	1.00	0.00	10.60	1.00	1.00	1.00	1.00
	C24:0 Cer	1.00	0.00	32.71	0.97	1.00	1.00	0.96

ROC curve analyses were performed to investigate sphingolipid contents in lipoprotein and lipoprotein-depleted fractions contributing to type 2 diabetes. The cutoff values were determined based on Youden's index. The lipid contents with significant area under the curve (AUC) were described in the tables.

Supplemental Table 3. ROC analyses of sphingolipids in lipoprotein and lipoprotein-depleted fractions contributing to the use of statin

A. Sphingolipid contents (ng/mL)

		AUC	*p*-value	cut-off value	sensitivity	specificity	positive predictive value	negative predictive value
LDL	C20:0 Cer	0.70	0.03	5553.34	0.57	0.83	0.80	0.63
	Sph	0.69	0.04	120.29	0.38	1.00	1.00	0.58
	C16:0 Cer	0.70	0.03	357.13	0.62	0.78	0.76	0.64
	C18:1 Cer	0.69	0.04	40.45	0.81	0.61	0.71	0.73
lipoprotein-depleted fraction	C20:0 Cer	0.70	0.03	137.43	0.62	0.78	0.76	0.64

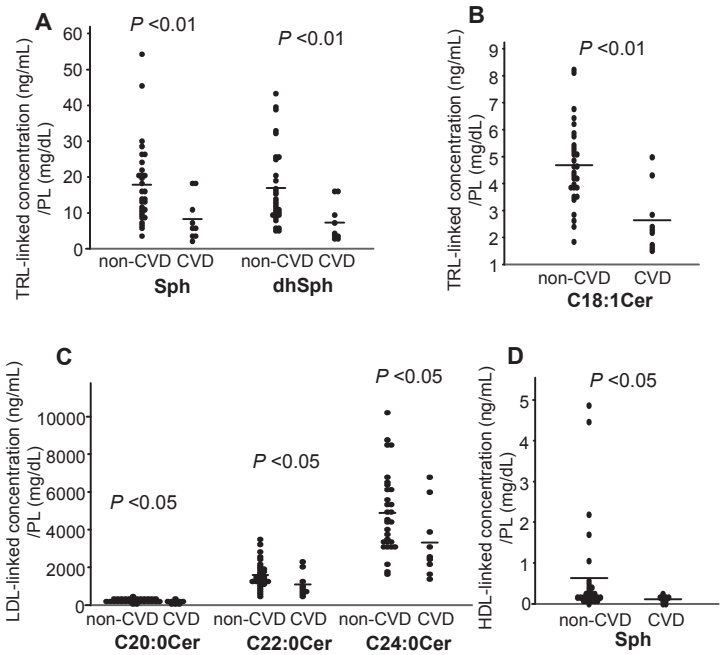
B. Sphingolipid contents (ng/mL) adjusted to the apoB or apoA-I levels (g/L)

		AUC	*p*-value	cut-off value	sensitivity	specificity	positive predictive value	negative predictive value
LDL	Sph	0.69	0.05	3301.79	0.76	0.61	0.70	0.69
	C16:0 Cer	0.75	0.01	55244.45	0.95	0.56	0.71	0.91
	C18:0 Cer	0.71	0.02	23495.69	0.81	0.56	0.68	0.71
	C18:1 Cer	0.72	0.02	687.25	0.86	0.50	0.67	0.75
	C20:0 Cer	0.76	0.01	20522.71	0.52	0.94	0.92	0.63
	C22:0 Cer	0.71	0.02	150044.43	0.67	0.78	0.78	0.67
	C24:0 Cer	0.70	0.03	423279.37	0.62	0.83	0.81	0.65

C. Sphingolipid contents (ng/mL) adjusted to the total PL levels (mg/dL)

		AUC	*p*-value	cut-off value	sensitivity	specificity	positive predictive value	negative predictive value
TRL	C16:0 Cer	0.70	0.03	178.93	0.81	0.78	0.81	0.78
LDL	C16:0 Cer	0.71	0.02	269.69	0.67	0.78	0.78	0.67
	C20:0 Cer	0.75	0.01	180.55	0.57	0.89	0.86	0.64

ROC curve analyses were performed to investigate sphingolipid contents in lipoprotein and lipoprotein-depleted fractions contributing to the use of statin in the diabetic subjects. The cutoff values were determined based on Youden's index. The lipid contents with significant area under the curve (AUC) were described in the tables.



Supplemental Fig. 4. Association of the levels of sphingolipids in the lipoprotein and lipoprotein-depleted fractions with cardiovascular disease

The levels of sphingolipids in the lipoprotein and lipoprotein-depleted fractions were compared between the diabetic subjects without cardiovascular disease (non-CVD, $n=30$) and diabetic subjects with cardiovascular disease (CVD, $n=9$).
Differences were evaluated using the Mann-Whitney U test. The horizontal bars represent the medians of independent samples.

Supplemental Table 4. ROC analyses of sphingolipids in lipoprotein and lipoprotein-depleted fractions contributing to the past history of CVD

A. Sphingolipid contents (ng/mL)

		AUC	*p*-value	cut-off value	sensitivity	specificity	positive predictive value	negative predictive value
LDL	C18:0 Cer	0.74	0.03	2784.27	0.67	0.77	0.46	0.88
	C18:1 Cer	0.79	0.01	69.72	0.67	0.93	0.75	0.90
	C20:0 Cer	0.78	0.01	6086.57	0.89	0.63	0.42	0.95
	C22:0 Cer	0.77	0.01	32326	0.78	0.80	0.54	0.92
	C24:0 Cer	0.77	0.01	96450	0.78	0.73	0.47	0.92
lipoprotein-depleted fraction	C18:0 Cer	0.76	0.02	81.81	0.78	0.77	0.50	0.92
	C18:1 Cer	0.83	0.00	30.55	0.89	0.73	0.50	0.96
	C20:0 Cer	0.76	0.02	118.04	0.78	0.80	0.54	0.92

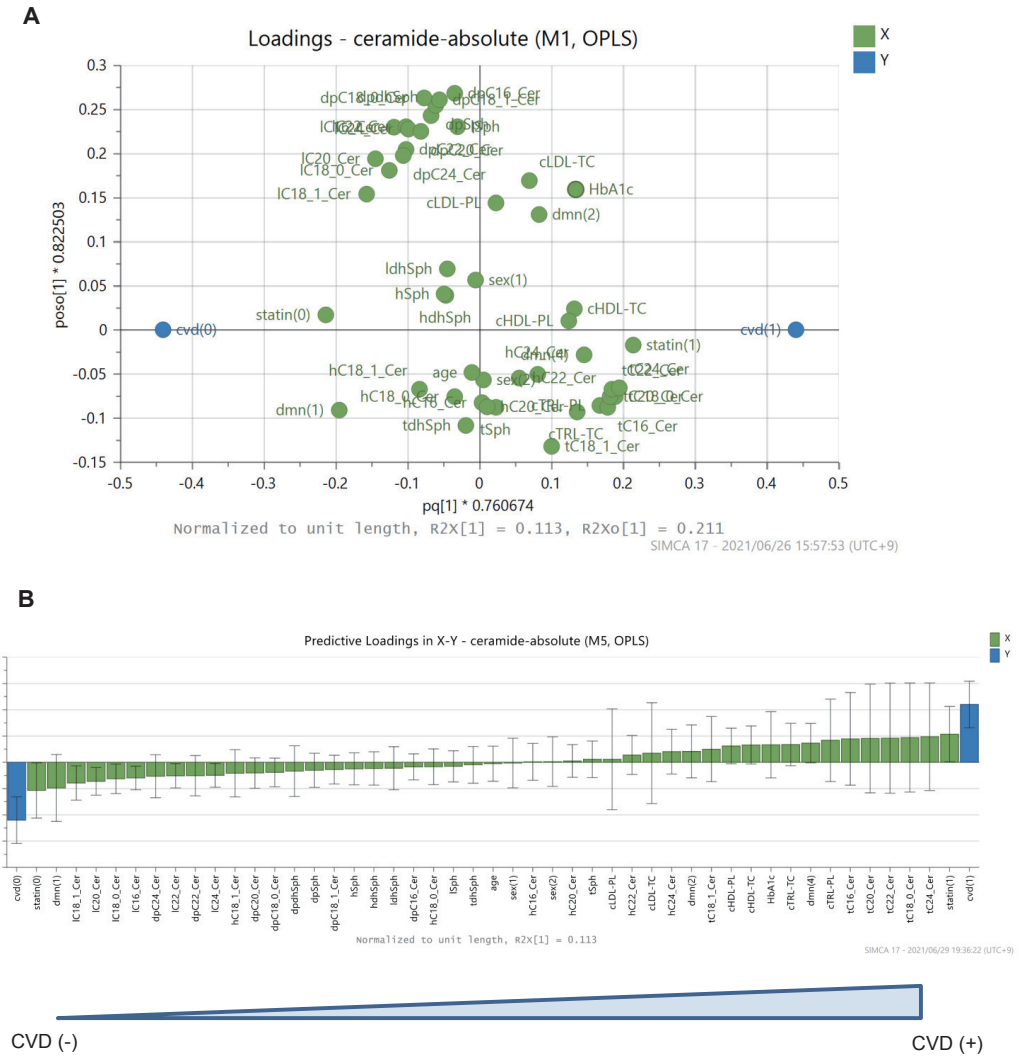
B. Sphingolipid contents (ng/mL) adjusted to the apoB or apoA-I levels (g/L)

		AUC	*p*-value	cut-off value	sensitivity	specificity	positive predictive value	negative predictive value
TRL	dhSph	0.74	0.03	4773.88	0.67	0.87	0.60	0.90
LDL	dhSph	0.76	0.02	507.23	0.89	0.57	0.38	0.94
	C16:0 Cer	0.80	0.01	33607.11	0.89	0.67	0.44	0.95
	C18:0 Cer	0.77	0.01	7957.20	0.56	0.97	0.83	0.88
	C18:1 Cer	0.79	0.01	424.78	0.89	0.70	0.47	0.95
	C20:0 Cer	0.82	0.00	14648.24	0.56	0.97	0.83	0.88
	C22:0 Cer	0.83	0.00	129389.24	0.78	0.83	0.58	0.93
	C24:0 Cer	0.84	0.00	388520.34	0.78	0.83	0.58	0.93

C. Sphingolipid contents (ng/mL) adjusted to the total PL levels (mg/dL)

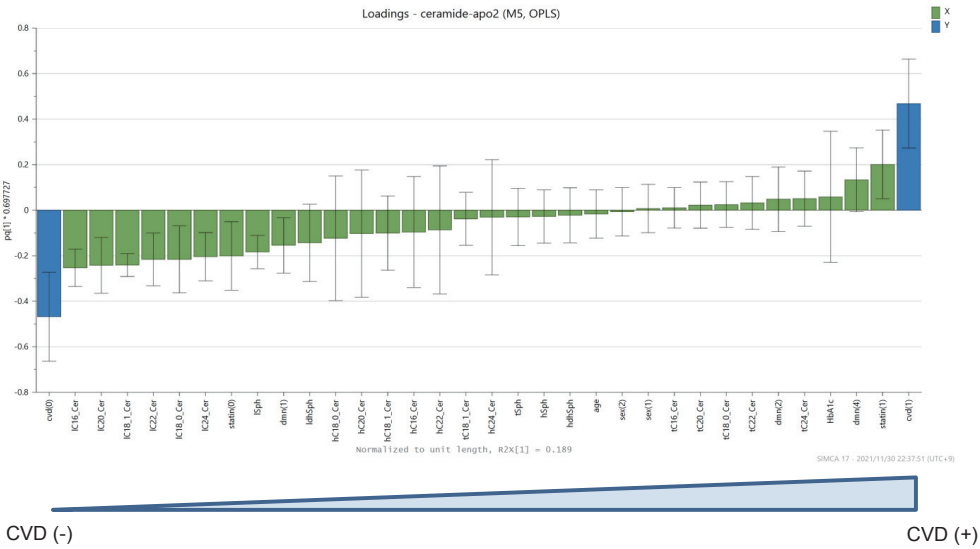
		AUC	*p*-value	cut-off value	sensitivity	specificity	positive predictive value	negative predictive value
TRL	Sph	0.81	0.01	7.93	0.67	0.87	0.60	0.90
	dhSph	0.81	0.00	7.52	0.67	0.87	0.60	0.90
	C18:1 Cer	0.86	0.00	2.83	0.78	0.90	0.70	0.93
LDL	C20:0 Cer	0.75	0.02	174.42	0.67	0.77	0.46	0.88
	C22:0 Cer	0.73	0.04	1024.88	0.67	0.83	0.55	0.89
	C24:0 Cer	0.73	0.04	2779.33	0.56	0.90	0.63	0.87
HDL	Sph	0.72	0.05	0.22	1.00	0.47	0.36	1.00

ROC curve analyses were performed to investigate sphingolipid contents in lipoprotein and lipoprotein-depleted fractions contributing to the past history of CVD in the diabetic subjects. The cutoff values were determined based on Youden's index. The lipid contents with significant area under the curve (AUC) were described in the tables.



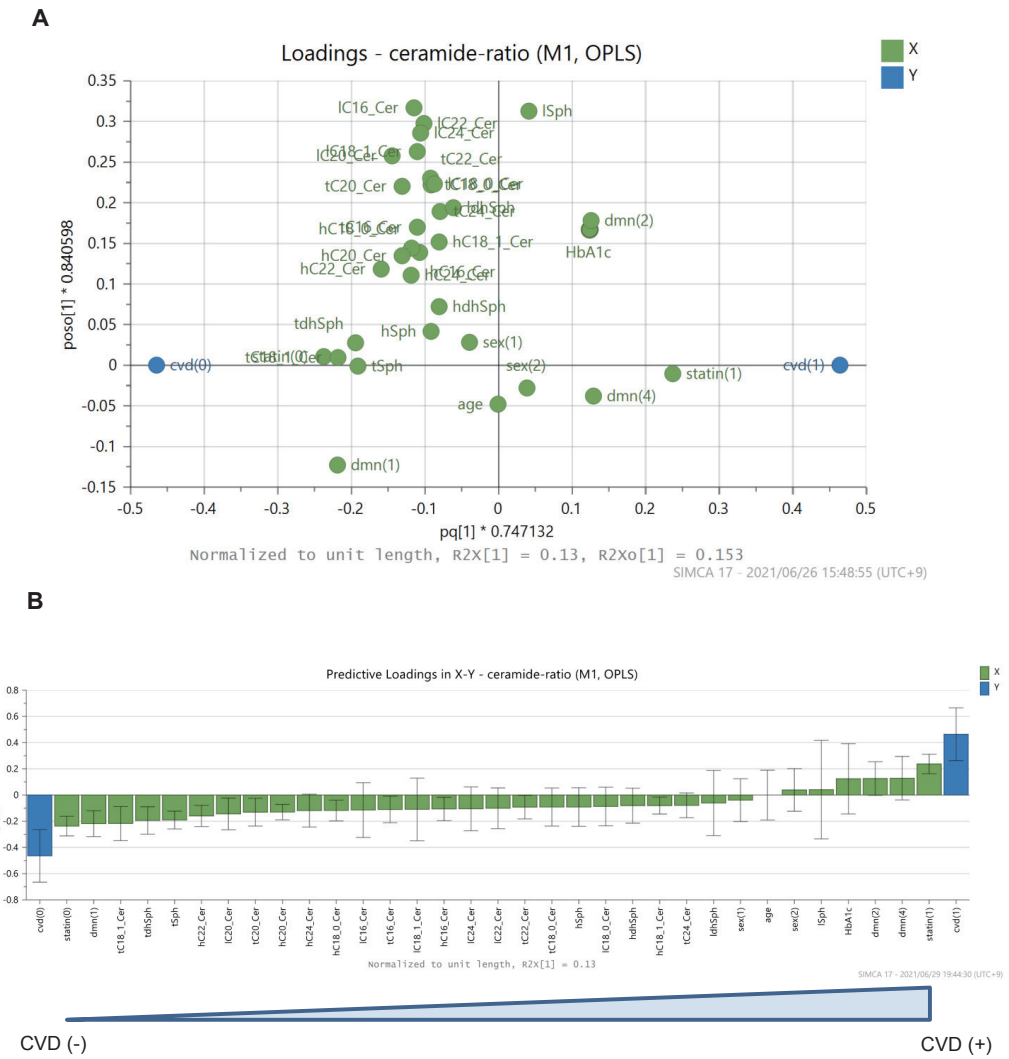
Supplemental Fig. 5. OPLS analysis for the absolute levels of sphingolipids in each serum fraction with variable importance in projection for cardiovascular diseases

An OPLS analysis was performed to investigate the absolute levels of sphingolipids in each fraction with variable importance in projection for cardiovascular diseases. The loading scatter plot (A) and loading column plot (B) for the OPLS are shown.



Supplemental Fig. 6. OPLS analysis for the levels of sphingolipids adjusted to apoB or apoA-I levels in each fraction with variable importance in projection for cardiovascular diseases

An OPLS analysis was performed to investigate the levels of sphingolipids adjusted to the total phospholipid levels in each fraction with variable importance in projection for cardiovascular diseases. The loading column plot for the OPLS are shown.



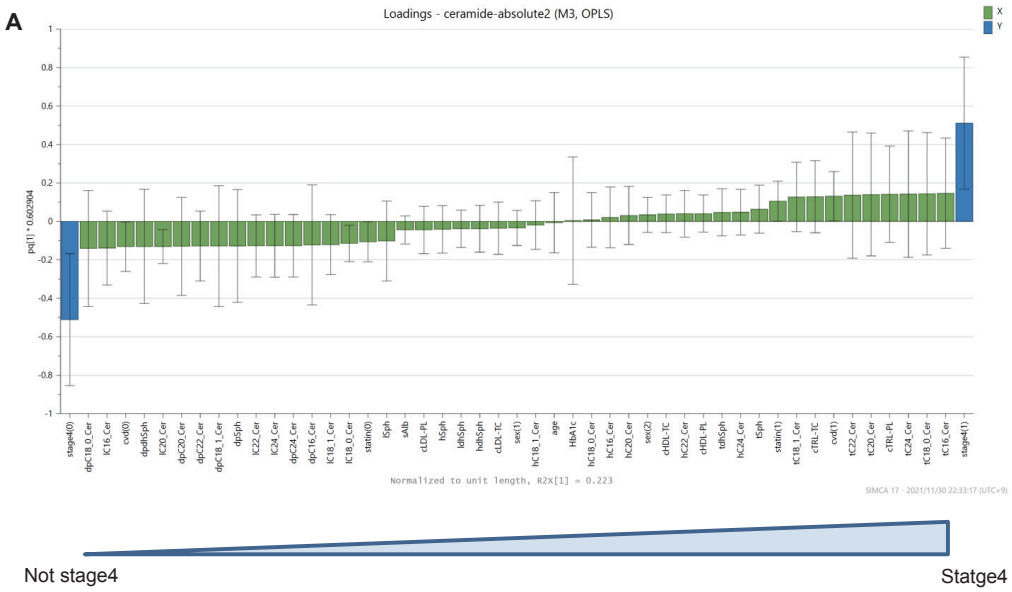
Supplemental Fig. 7. OPLS analysis for the levels of sphingolipids adjusted to total phospholipid levels in each fraction with variable importance in projection for cardiovascular diseases

An OPLS analysis was performed to investigate the levels of sphingolipids adjusted to the total phospholipid levels in each fraction with variable importance in projection for cardiovascular diseases. The loading scatter plot (A) and loading column plot (B) for the OPLS are shown.

Supplemental Table 5. OPLS analysis with variable importance in projection for stage 4 diabetic nephropathy (Model 1)

Positive predicting variable		Negative predicting variable	
Variables	VIP	Variables	VIP
Presence of CVD	0.1313 ± 0.1284	Absence of CVD	-0.1313 ± 0.1284
Statin use	0.1058 ± 0.1043	C20:0 Cer in LDL	-0.1302 ± 0.0883
		C18:0 Cer in LDL	-0.1150 ± 0.0946
		No statin use	-0.1158 ± 0.1043

Data are the means ± SEM. VIP denotes variable importance in projection, which was calculated in the OPLS analyses using SIMCA.



Supplemental Fig. 8. OPLS analysis for the levels of sphingolipids in each serum fraction with variable importance in projection for stage 4 diabetic nephropathy

An OPLS analysis was performed to investigate the absolute levels of sphingolipids in each fraction with variable importance in projection for stage 4 diabetic nephropathy. The loading column plots for the OPLS are shown.