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Chapter 5

Dietary sphingolipids lower plasma cholesterol and triacylglycerol and prevent liver steatosis in APOE*3Leiden mice

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Abstract

The prevalence of dyslipidemia and obesity resulting from excess energy intake and physical inactivity is increasing. The liver plays a pivotal role in systemic lipid homeostasis. Effective, natural dietary interventions that lower plasma lipids and promote liver health are needed. Our goal was to determine the effect of dietary sphingolipids on plasma lipids and liver steatosis. APOE*3Leiden mice were fed a Western-type diet, supplemented with different sphingolipids. Body cholesterol and triacylglycerol (TG) metabolism, as well as hepatic lipid levels and lipid-related gene expression, were determined. Dietary sphingolipids dose-dependently lowered both plasma cholesterol and TG in APOE*3Leiden mice. 1% phytosphingosine (PS) reduced plasma cholesterol and TG by 57% and 58%, respectively. PS (a) decreased the absorption of dietary cholesterol and free fatty acids by 50% and 40%, respectively, whereas intestinal TG lipolysis was not affected; (b) increased hepatic VLDL-TG production by 20%, whereas plasma lipolysis was not affected; and (c) increased the hepatic uptake of VLDL remnants by 60%. Hepatic mRNA concentrations indicated enhanced hepatic lipid synthesis and VLDL and LDL uptake. The net result of these changes was a strong decrease of plasma cholesterol and TG. The livers of 1%PS-fed mice were less pale, 22% lighter, and contained 61% less cholesteryl ester and 56% less TG than livers of control mice. Furthermore, markers of liver inflammation (SAA) and liver damage (ALAT) decreased by 74% and 79%, respectively, in PS-fed mice. We conclude that sphingolipids lower plasma cholesterol and TG and protect the liver from fat- and cholesterol-induced steatosis.

Introduction

In our modern Western society, excess energy intake and physical inactivity are the leading causes of the epidemic prevalence of obesity. Obesity in turn is associated with an increased prevalence of cardiovascular risk factors, such as dyslipidemia, insulin resistance, and hypertension (collectively called the metabolic syndrome¹). Obesity-related dyslipidemia is characterized by mildly elevated concentrations of very low density lipoprotein (VLDL)-triacylglycerol and low density lipoprotein (LDL)-cholesterol and decreased concentrations of high density lipoprotein (HDL)-cholesterol. The liver plays a central role in the maintenance of systemic lipid homeostasis, since it synthesizes and secretes VLDL and, thus, is involved in the redistribution of lipids, primarily triacylglycerol (TG), for storage and utilization by peripheral tissues. Lipid accumulation in the liver leads to the development of steatosis, a condition closely associated with insulin resistance².

The metabolic syndrome is usually treated by (a combination of) life-style and dietary changes. Currently used drugs target one aspect of the metabolic syndrome. For example hypercholesterolemia is treated with HMG-CoA reductase inhibitors (statins) and cholesterol absorption inhibitors (ezetimibe, phytosterols, and stanols), hypertriglyceride-

mia is treated with PPAR α agonists (fibrates), hypertension with β -blockers (atenolol, metoprolol, and propranolol), and insulin resistance with thiazolidinediones (pioglitazone and rosiglitazone) or metformin. Although recently developed compounds, such as the gli-tazars, target more than one aspect of the metabolic syndrome, treatment of multiple aspects of the metabolic syndrome with a single natural dietary compound could be an attractive alternative.

We recently observed in a pilot study that consumption of a Western-type diet supplemented with sphingolipids lowered both plasma cholesterol and TG in hyperlipidemic APOE*3Leiden mice. The APOE*3Leiden mouse has a lipoprotein profile that closely resembles the human profile. In these mice, plasma cholesterol can be titrated to various concentrations by varying the amount of cholesterol in the diet³. Moreover, in contrast with wild-type mice, LDL-receptor-deficient mice and ApoE-deficient mice, APOE*3Leiden mice, are highly sensitive to treatment with hypolipidemic drugs, such as statins^{4,5} and fibrates. We wondered, therefore, whether supplementation of the diet with sphingolipids could be used to treat the dyslipidemia characteristics of the metabolic syndrome.

Materials and methods

Sphingolipids

We used the 3 sphingolipids that represent the most abundant and simplest natural sphingolipid classes, the sphingoid bases (see **Figure 1**; I, II and III), which can be formed by enzymatic breakdown of complex sphingolipids in the intestine, and the 3 complex natural sphingolipids (**Figure 1**; IV, V and VI). Sphingomyelin (mainly N-palmitoyl-sphingosine-1-phosphocholine) from egg was obtained from Larodan Fine Chemicals (Stockholm, Sweden). Yeast-derived (semi)synthetic ceramide III (N-stearoyl-phytosphingosine), cerebroside (N-stearoyl-phytosphingosine-1-glucose) and phytosphingosine (PS) were from Cosmoferm BV (Delft, The Netherlands). Sphinganine and sphingosine were from Avanti Polar Lipids (Albaster, AL, USA).

Animals and diets

Female heterozygous APOE*3Leiden transgenic mice³ (6 months old) were fed a Western-type diet (Hope Farms, Woerden, the Netherlands) containing 15% cocoa butter, 0.25% cholesterol, 1% corn oil, 40.5% sucrose, 20% acid casein, 10% corn starch and 5.95% cellulose (all w/w) for 5 weeks. The mice were housed in clean-conventional animal rooms (relative humidity 50-60%, temperature ~ 21°C, light cycle 6.00 A.M. to 6.00 P.M.), and supplied with food and acidified tap water *ad libitum* during the experiment. Mice were housed in macrolon cages (3 mice per cage).

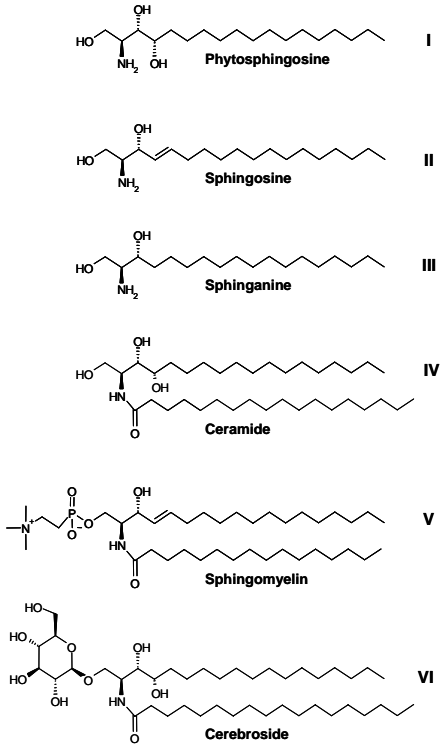


Figure 1. Structures of the sphingolipids used in this study

Body weight (20.9 ± 0.7 g) and food intake (2.5 ± 0.1 g/mouse/day) were monitored weekly. In the first experiment (**Figure 2**), the mice were randomized after these 5 weeks into 7 groups ($n = 6$ per group), based on plasma cholesterol, TG and body weight. Subsequently, the mice were fed the same diet with or without 0.1% (w/w) PS, sphingosine, sphinganine, cerebroside, ceramide III, or sphingomyelin for 3 weeks. Then, the sphingolipid dose was increased to 0.2% (w/w) for 3 weeks and finally to 0.4% (w/w) for 3 weeks. Tail-vein blood samples were obtained after a 4 h-fast, at randomization, and at 3, 6, and 9 weeks.

In all subsequent experiments, female APOE*3Leiden transgenic mice (6 months old) were fed the Western-type diet for 5 weeks and then randomized as described. Subsequently, the mice were fed the Western-type diet for 5 weeks or the same diet supplemented with 1.0 % (w/w) PS before being subjected to experimentation. All experiments were approved by the TNO Animal Care and Use Committee.

Plasma parameters

Tailblood samples were collected in EDTA-coated cups, or in paraoxon-coated capillaries to prevent lipolysis⁶. Plasma parameters were determined using commercial kits for total cholesterol (Roche Diagnostics, Mannheim, Germany), non-esterified free fatty acids (NEFA-C; Wako chemicals, Neuss, Germany), TG (Triglyceride GPO-Trinder; Sigma, St. Louis, MO, USA), β -hydroxybutyrate (β -HB; Sigma) and alanine aminotransferase (ALAT) (Reflotron GPT; Roche). Serum amyloid A (SAA) was measured by enzyme-linked immunoabsorbent assay (ELISA) (Biosource, Nivelles, Belgium) and fibrinogen by sandwich ELISA, as described⁷. For lipoprotein fractionation, groupwise-pooled plasma was size-fractionated by fast protein liquid chromatography (FPLC) on a Superose 6 column (Åkta; Amersham Pharmacia Biotech, Uppsala, Sweden). Fractions were assayed for total cholesterol and TG as described.

Intestinal cholesterol absorption

We assessed the cholesterol absorption using the fecal dual-isotope method described by Borgstrom⁸ and Wang and Carey⁹ in mice fed the diet with or without 1% PS (6 per group) for 5 weeks. The mice were housed individually. At 5.00 P.M. the mice received a dose of 200 μ l of olive oil, containing [¹⁴C]cholesterol (1 μ Ci/mouse; Amersham Biosciences, Little Chalfont, UK) and [³H]sitostanol (1 μ Ci/mouse; ARC, St. Louis, MO, USA) by gavage. Body weight and food intake were monitored, and feces were collected for 4 days. Feces were freeze-dried, homogenized, pooled per mouse over the 4-day period, and dissolved in ethanolic potassium (3 mol/l, 60% ethanol). Radioactivity was determined in the fecal samples to assess the amount of radiolabeled cholesterol and sitostanol. Sitostanol was used as the reference compound since it is known to be poorly absorbed (< 3%) in mice⁹. The formula used to calculate cholesterol absorption was: % cholesterol absorption = $\frac{([14\text{C}]/[3\text{H}] \text{ dosing mixture} - [14\text{C}]/[3\text{H}] \text{ feces})}{[14\text{C}]/[3\text{H}] \text{ dosing mixture}} \times 100$.

Determination of neutral sterols in feces

Two mice were housed per cage, and feces were collected in 2 subsequent 3-day periods from 3 cages per group. Feces were separated, freeze-dried and weighed. 10 mg of dried feces was used to extract neutral sterols by treatment with alkaline methanol (3 parts of methanol and 1 part of 1 N NaOH) and petroleum ether with the use of 5 α -cholestane as internal standard, as described previously¹⁰. Analysis of the sterol derivatives was performed by gas chromatography.

Intestinal TG and FFA absorption

Intestinal absorption of TG and FFA was determined in overnight-fasted mice (n = 6 per group) fed 1% PS-containing or control Western-type diet. The mice received an intragastric load of 200 μ l olive oil containing [³H]triolein (12 μ Ci/mouse) and [¹⁴C]oleic acid (3.3 μ Ci/mouse) (Amersham Biosciences) directly after the i.v. injection of Triton WR1339 (Tyloxapol, Sigma; 500 mg/kg in 100 μ l saline) to block lipoprotein lipase (LPL)-mediated TG hydrolysis¹¹. For PS-fed animals, 1% PS was added to the olive oil load, since overnight-fasting might negate any direct effect that PS may have on intestinal absorption. Blood samples were collected by tail-vein incision at 1, 2, 3, and 4 h after the intragastric load into pre-cooled paraoxon-coated capillaries, and plasma radioactivity and TG were determined.

Hepatic VLDL-TG production

The rate of hepatic VLDL-TG production, *de novo* apoB secretion, and VLDL composition were determined in overnight-fasted mice. Mice were anaesthetized with fluanisone/fentanyl/midazolam i.p., and injected i.v. with 0.1 ml PBS containing 100 μ Ci Tran³⁵S-label™ (ICN Biomedicals, Irvine, CA, USA) to measure *de novo* apoB synthesis. After 30 min, the animals received a Triton WR1339 injection (500 mg/kg body weight) to prevent systemic lipolysis of newly secreted hepatic VLDL-TG¹². Blood samples were drawn at 0, 15, 30, 60, and 90 min after Triton WR1339 injection, and plasma TG concentrations were determined. After 90 min, the animals were sacrificed and blood was collected by retro-orbital bleeding for isolation of VLDL.

VLDL composition

VLDL particles (d < 1.019) were separated from other lipoproteins in the plasma by density gradient ultra-centrifugation, as described previously¹³. The protein content of the VLDL fraction was determined by Lowry's assay¹⁴, and TG and total cholesterol were determined as described above. Phospholipids and free cholesterol were determined using standard commercial kits (Wako Chemicals, Neuss, Germany). The [³⁵S]-apoB content of VLDL was measured after selective precipitation of apoB with isopropanol^{15,16}.

***In vivo* clearance of VLDL-like TG-rich particles**

To determine whether 1% PS accelerates the clearance of TG-rich lipoproteins from plasma, we used radiolabeled emulsion particles as a tool. VLDL-like emulsion particles containing 200 μCi [^3H]triolein and 20 μCi [^{14}C]cholesteryl oleate were prepared and characterized as described^{15,17}. Fed mice ($n = 6$) were anaesthetized as described above and laparotomy was performed. Emulsion particles were injected into the vena cava inferior, at a dose of 300 μg TG per mouse. At 2, 5, 10, 20, and 30 min, blood samples (50 μl) were taken from the vena cava inferior and liver samples were tied off, excised, and weighed. [^3H] and [^{14}C]-activities were counted in 10 μl of serum and corrected for total serum volume (ml) calculated as $0.04706 \times \text{body weight (g)}$ ¹⁸. After the last liver and blood samples were taken, the remainder of the liver, heart, spleen, hind limb muscle, and gonadal, perirenal and intestinal white adipose tissues were harvested. Lipids were extracted overnight at 60°C in 500 μl of SolvableTM (Perkin Elmer, Wellesley, MA, USA), and radioactivity was counted¹⁷.

RNA isolation and RT-PCR

Livers from 4 h-fasted mice fed 1% PS-containing diet or control Western-type diet for 5 weeks were removed immediately after sacrifice, flushed with cold 0.9% NaCl and snap-frozen in liquid nitrogen. Total RNA was isolated as described by Chomczynski and Sacchi¹⁹ by use of RNA-BeeTM (Campro Scientific, Berlin, Germany). cDNA synthesis was performed according to Bloks et al.²⁰ Real-time quantitative PCR²¹ was performed using an Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands) 7700 Sequence detector. Primers were obtained from Invitrogen (Paisley, UK) and fluorogenic probes, labeled with 6-carboxyfluorescein (6-FAM), and 6-carboxytetramethylrhodamine (6-TAMRA), were made by Eurogentec (Seraing, Belgium). Primers and probes used were described earlier²²⁻²⁴. All expression data were subsequently standardized for hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA concentrations.

Liver lipid concentration

Liver samples taken from 4 h-fasted mice fed 1% PS-containing or control Western-type diet for 5 weeks were homogenized in phosphate-buffered saline (PBS) (10% wet wt/vol) and the protein content was measured by Lowry's assay¹⁴. The lipid content was determined by lipid extraction using the Bligh and Dyer method²⁵, followed by lipid separation using high performance thin layer chromatography (HPTLC) on silica gel plates as described²⁶ and analysis by TINA2.09 software²⁷ (Raytest Isotopen Meßgeräte, Straubenhardt, Germany).

Liver histology

Livers from 4 h-fasted mice fed 1% PS-containing or control Western-type diet for 5 weeks were fixed in 10% formalin and paraffin-embedded. Liver sections were stained with hematoxylin-phloxine-saffron (HPS) for morphological analysis.

Statistics

Differences in responses during the intervention period between the control group and the treatment groups were analyzed by two-way ANOVA. In case of a significant overall effect, this analysis was followed by comparison of all treatment groups with the control group (Dunnnett's test). When only 2 groups (control and treatment) were compared, Student's t-test, Welch's t-test or the Mann-Whitney U test was used. Time-course experiments were analyzed by two-way ANOVA. In all statistical tests performed, the null hypothesis was rejected at the 0.05 level of probability. All data are presented as mean \pm SD. Statistical analyses were performed using SPSS11.0 (SPSS, Chicago, IL).

Results

Sphingolipids lower plasma cholesterol and TG in APOE*3Leiden mice

For our initial experiment, to evaluate the effect of sphingolipids on plasma cholesterol and TG concentrations in APOE*3Leiden mice, we used 3 simple and 3 complex sphingolipids (**Figure 1**). The data were analyzed by two-way ANOVA. After demonstrating that the interaction between dose and treatment was significant ($P < 0.001$), we analyzed the data for each dose separately by ANOVA, followed by Dunnnett's test. With 0.1% of these sphingolipids in the diet, no significant effect on plasma cholesterol ($P = 0.978$) and TG ($P = 0.398$) was seen (**Figure 2**). At a dose of 0.2% (w/w), the sphingolipids (ceramide and cerebroside excepted) significantly decreased plasma cholesterol concentration by 20-40% ($P = 0.0096$; **Figure 2A**). At a dose of 0.4% sphingolipid, plasma cholesterol was decreased even more, and ceramide also had a significant cholesterol lowering effect ($P = 0.0009$; **Figure 2A**). The decrease in TG concentration was ~ 40% for all sphingolipids at dietary sphingolipid concentrations of 0.2% and 0.4%, a significant decrease for all compounds both at week 6 and at week 9; **Figure 2B**). No differences in food intake or body weight were observed throughout the experiment between the mice fed sphingolipids and the control animals (data not shown). Remarkably, the simplest sphingolipids, the sphingoid bases (**Figure 1**), had the same potent cholesterol- and TG-lowering effect as their complex sphingolipid derivatives.

To study the mechanisms underlying the cholesterol- and TG-lowering effects, we performed studies in mice fed the Western-type diet with or without 1% (w/w) PS for 5 weeks. This sphingolipid was chosen for all subsequent studies because it is one of the simplest in the sphingolipid class; it is the central structural element of ubiquitous sphingolipids of plants and yeasts that are part of our diet; and this lipid may be formed *in situ* in the intestine by enzymatic degradation of complex sphingolipids. No effects on body weight or food intake were ever observed (data not shown).

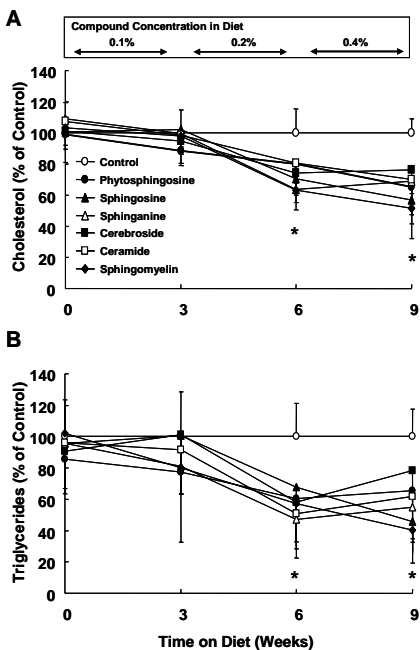


Figure 2. Plasma cholesterol (A) and triacylglycerol (B) concentrations in APOE*3Leiden mice after a 4h-fast

After a run-in period of 5 weeks with Western-type diet, female APOE*3Leiden mice were fed control Western-type diet, or this diet supplemented with increasing doses (0.1, 0.2 or 0.4% (w/w)) of the various sphingolipids (see also **Figure 1**) for periods of three weeks each ($n = 6$ per group). Baseline values for cholesterol and triacylglycerol were 15.0 ± 1.9 and 2.3 ± 0.5 mmol/l, respectively. Data shown are mean \pm SD of percentages relative to the control group. * $P < 0.05$ vs. control using ANOVA, followed by Dunnett's test

Table 1. Plasma parameters in 4 h-fasted APOE*3Leiden mice fed a control Western-type diet or a 1% PS-containing Western-type diet for 5 weeks

	Control	1% PS	%Δ
Total cholesterol (mmol/l)	14.0 ± 1.2	6.1 ± 1.1***	-57
Triacylglycerol (mmol/l)	2.80 ± 0.52	1.19 ± 0.39***	-58
Free fatty acids (mmol/l)	1.67 ± 0.31	1.06 ± 0.14***	-36
β-hydroxybutyrate (mmol/l)	0.40 ± 0.25	0.49 ± 0.39	NS

Values shown are mean ± SD of 6 mice per group. ***P=0.001 vs. control, using the Student t-test. NS not significant, PS phytosphingosine

As can be seen in **Table 1**, mice fed a 1% PS-containing diet showed, as expected, a strong and significant decrease in plasma cholesterol and TG. Plasma FFA concentration also significantly decreased, whereas plasma β-hydroxybutyrate (a liver-derived keton body) did not change significantly (**Table 1**). Groupwise-pooled plasma was used to determine lipoprotein profiles of these mice. The lipoprotein profiles showed that the decrease in cholesterol and TG was confined to the VLDL and IDL/LDL fractions, while HDL cholesterol did not change (**Figure 3**).

Phytosphingosine reduces intestinal cholesterol, TG, and FFA absorption

We assessed the effects of dietary sphingolipids on intestinal cholesterol absorption by measuring absorption with the fecal dual-isotope method, using [¹⁴C]cholesterol and [³H]sitostanol, in mice fed a 1% PS-containing or control Western-type diet for 5 weeks. No differences in body weight, food intake, and fecal output were observed between the two groups (data not shown). We observed that the intestinal cholesterol absorption in PS-fed mice was only one-half of that in the control diet-fed mice (**Figure 4A**). This reduction was also reflected by the fecal neutral sterol excretion, which was twice as high in the PS-fed mice as in control mice (**Figure 4B**). We next determined whether the observed decrease in plasma TG and FFA was also due to decreased intestinal absorption. Mice fed a control or 1% PS-containing Western-type diet for 5 weeks were used. After an overnight-fast, the mice were i.v. injected with Triton WR1339 to inhibit lipoprotein lipolysis, followed by an intragastric gavage of [³H]triolein and [¹⁴C]oleic acid in olive oil. In the group fed 1% PS, the olive oil contained, in addition to [³H]triolein and [¹⁴C]oleic acid, 1% PS. Serum TG, [³H]-activity, and [¹⁴C]-activity were assayed over a 4h-period. The time-dependent appearance of plasma [³H]-activity (a measure of intestinal TG uptake) is depicted for the two groups in **Figure 5A**. PS reduced the intestinal [³H]TG uptake by 33% after 4 h. The plasma appearance of [¹⁴C]oleate (a measure of intestinal FFA uptake) was 43% lower in PS-fed mice than in the control mice at 4 h after administration (**Figure 5B**).

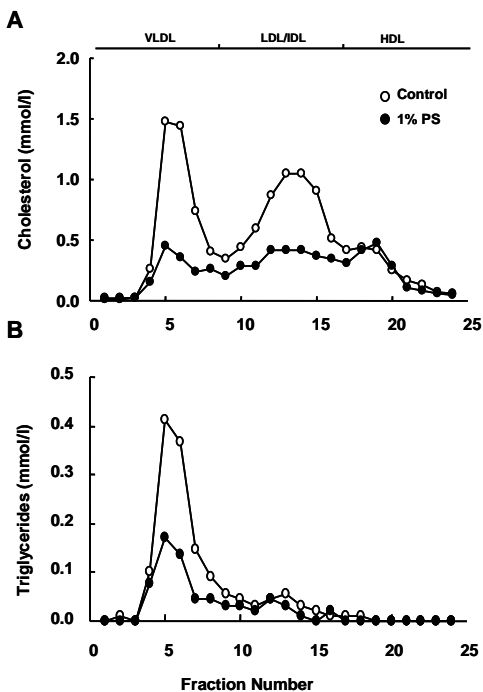


Figure 3. Cholesterol (A) and triacylglycerol (B) profiles after FPLC separation of plasma lipoproteins Pooled plasma of six 4 h-fasted APOE*3Leiden mice fed a control Western-type diet (open circles) or this diet supplemented with 1% (w/w) PS (closed circles) for 5 weeks was used. Cholesterol and triacylglycerol concentrations were determined in the individual fractions after separation by fast-liquid chromatography (FPLC). IDL intermediate density lipoprotein, HDL high density lipoprotein, LDL low density lipoprotein, PS phytosphingosine, VLDL very low density lipoprotein

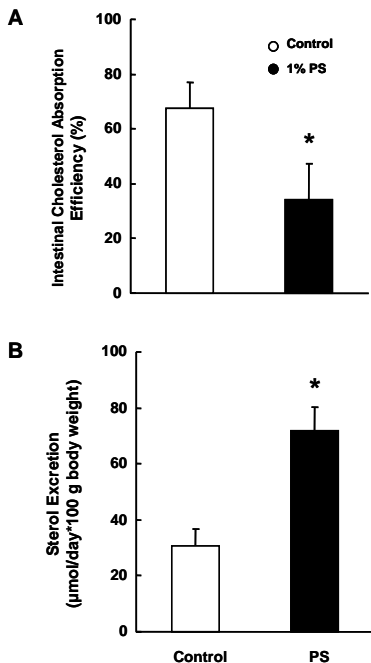


Figure 4. Intestinal cholesterol absorption (A) and neutral sterol excretion (B)

Mice ($n = 6$ per group) were fed 1% PS containing (closed bars) or control (open bars) Western-type diet for 5 weeks. The mice received a dose of 200 μl of olive oil, containing [^{14}C]cholesterol (1 $\mu\text{Ci}/\text{mouse}$) and [^3H]sitostanol (1 $\mu\text{Ci}/\text{mouse}$) by gavage. Feces were collected for 4 days. Radioactivity was determined in the fecal samples to assess the amount of radiolabeled cholesterol and sitostanol. Sitostanol was used as the reference compound (A). For neutral sterol excretion analysis two mice were housed per cage, and feces were collected in 2 subsequent 3-day periods from 3 cages per group. Feces were analyzed as described in the materials and methods section (B). Data shown are mean \pm SD. * $P < 0.05$ vs. control (Student's t-test)

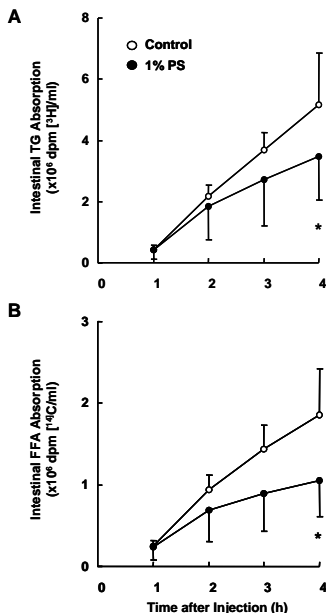


Figure 5. Intestinal TG (A) and FFA (B) absorption

After an overnight fast, mice ($n = 6$ per group) fed 1% PS-containing (closed circles) or control Western-type diet (open circles) for 5 weeks were injected with Triton WR1339 to block LPL-mediated TG hydrolysis and were given an intragastric load of 200 μ l olive oil containing [3 H]triolein (12 μ Ci/mouse) and [14 C]oleic acid (3.3 μ Ci/mouse). For PS-fed animals, 1% PS was added to the olive oil load, since overnight fasting might annihilate any direct effect that PS may have on intestinal absorption. Plasma samples were obtained to determine the appearance of TG derived [3 H]-FA (A) or [14 C]-FFA (B) in the blood. Data shown are mean \pm SD. After establishing significant interaction between time and treatment by two-way ANOVA (Figure A and B both $P < 0.05$), differences between control and PS-treated mice were calculated (* $P < 0.05$ vs. control). FFA free fatty acids, PS phytosphingosine, TG triglycerides

Phytosphingosine increases hepatic VLDL-TG production

Hepatic VLDL-TG production was studied in overnight-fasted mice by use of the Triton WR1339 method and by measuring plasma TG accumulation. In these experiments, plasma TG concentration increased faster in the mice fed 1% PS-containing diet than in mice fed the control Western-type diet. The VLDL-TG production rate, as determined from the slope of the curves, was 20% higher in PS-fed mice than in control mice (**Table 2**). Analysis of the composition of the VLDL particles (isolated by ultracentrifugation) revealed that the TG content as well as the phospholipid content increased by 66% and 17%, respectively, in VLDL particles derived from PS-fed mice (**Table 2**). Total cholesterol, in contrast, decreased by 51% in the VLDL particles from the PS-fed mice. The *de novo* total apoB production rate in newly synthesized VLDL particles did not differ between mice fed PS-containing diet and the control mice (**Table 2**). The data indicate that the number of VLDL particles secreted by the liver was not affected; however the VLDL particles from PS-fed mice contained less cholesterol, but more TG.

Table 2. Production rates of VLDL-TG and VLDL-apoB, and VLDL particle composition in APOE*3Leiden mice fed a control Western-type diet or the same diet supplemented with 1% PS for 5 weeks

	Control	1% PS	%Δ
Production rates of VLDL			
VLDL-TG production rate (μmol TG/h)	3.01 ± 0.44	3.63 ± 0.52***	+20
apoB production rate (x10 ⁴ dpm/ml/h)	12.80 ± 2.57	13.47 ± 2.45	NS
Composition of VLDL relative to protein			
Total cholesterol (μmol/mg VLDL protein)	24.39 ± 3.73	11.94 ± 2.16**	-51
Triacylglycerol (μmol/mg VLDL protein)	22.28 ± 2.37	36.93 ± 7.17**	+66
Phospholipid (μmol/mg VLDL protein)	6.47 ± 0.92	7.57 ± 1.13*	+17

Mice were fasted overnight and injected with ³⁵S label and Triton WR1339. Timed blood samples were taken to determine TG concentration. At 2 h, VLDL (d < 1.006g/ml) was isolated by ultracentrifugation, and TG, total cholesterol, phospholipid and protein content were determined. Values represent mean ± SD for 10 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control using Student's or Welch's t-test; NS, not significant. Apo apolipoprotein, PS phytosphingosine, TG triglycerides, VLDL very low density lipoprotein

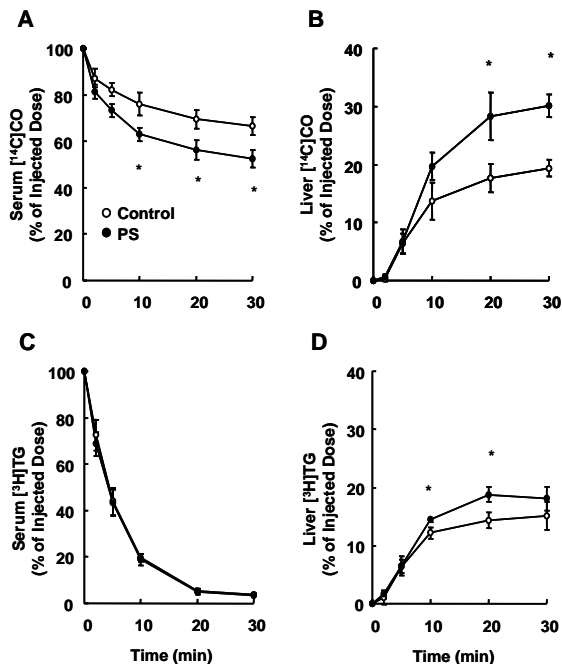


Figure 6. *In vivo* clearance of VLDL-like emulsion particles

After 5 weeks of 1% PS-containing (closed circles) or control (open circles) Western-type diet, fed mice ($n = 6$ per group) were injected into the vena cava inferior with VLDL-like emulsion particles, containing [³H]triolein (TG) and [¹⁴C]cholesteryl oleate (CO), at a dose of 300 μ g TG per mouse. Over time, serum (A,C) and liver (B,D) samples were taken to determine radioactivity. Data shown are mean \pm SD. After establishing significant interaction between time and treatment by two-way ANOVA (Figure A, B and D all $P < 0.05$), differences between control and PS-treated mice were calculated (* $P < 0.05$ vs. control). In Figure 6C, differences were not significant. PS phytosphingosine

Phytosphingosine increases liver-mediated clearance of plasma cholesterol but not plasma TG

Plasma cholesterol and TG concentrations are not only determined by their production rate but also by their clearance, *i.e.*, by their uptake, lipolysis, or both. VLDL-like particles containing [³H]triolein and [¹⁴C]cholesteryl oleate, which were previously shown to mimic the metabolic behavior of TG-rich lipoproteins^{17,28}, were used to determine the effects of a 1% PS-containing Western-type diet on plasma clearance. PS accelerated the plasma clearance of [¹⁴C]cholesteryl oleate ($t_{1/2}$ 39.5 ± 5.3 vs. 74.5 ± 9.9 min in PS-treated vs. control mice, respectively; $P < 0.05$; **Figure 6A**). The enhanced removal of cholesterol from the blood is corroborated by the increased liver uptake of [¹⁴C]cholesteryl oleate (+60% at 20 min; **Figure 6B**). Although lipoprotein lipase (LPL)-dependent serum clearance of [³H]TG was not affected ($t_{1/2}$ 4.7 ± 0.3 vs. 4.9 ± 0.3 min in PS-treated vs. control mice; **Figure 6C**), [³H]TG uptake in the liver 10 min and 20 min after injection increased significantly in the PS-fed mice (**Figure 6D**). No effects were observed on the uptake of [¹⁴C]cholesteryl oleate or [³H]TG-derived radioactivity by various peripheral muscle and adipose tissues (data not shown). Taken together, the results of this experiment indicate an increase in VLDL particle remnant uptake in the liver.

Hepatic mRNA concentrations indicate increased lipid synthesis

The hepatic expression of several genes was studied by using RT-PCR on liver samples of 4 h-fasted mice given the 1% PS-containing or the control Western-type diet for 5 weeks (**Table 3**). The mRNA concentrations of genes involved in FA and TG synthesis and secretion (*sreb1c*, *fas*, *mttp* and *dgat2*) increased. *Acc1*, *dgat1*, *aco* and *apob* transcription concentrations were unaltered. Furthermore, mRNA concentrations of cholesterol homeostasis genes, such as *sreb2*, *ldlr*, and *hmgcoAred*, also increased. In strong contrast, the two genes involved in bile salt formation studied decreased (*cyp7a1* and *lxrβ*), while *lxra* and *fxr* increased. *Ppara*, *ppary* and *abca1* expression did not change (**Table 3**). Overall, these changes suggest increased hepatic lipid and cholesterol synthesis and decreased bile formation, which indicates a shift in hepatic TG and cholesterol homeostasis compared with the control situation.

Phytosphingosine protects livers from steatosis

Increased remnant uptake and increased mRNA expression of genes involved in FA and TG synthesis suggested that intrahepatic TG and cholesterol concentrations increased in our setting. However, at autopsy of these mice, it was noted that the livers of the mice fed the 1% PS-containing Western-type diet were of normal size and had a dark-red appearance, whereas the livers of the control mice fed the Western-type diet without PS were enlarged and yellowish (**Figure 7A and B**). This indicates that the hepatic lipid content was lower in the PS-fed mice than in the control mice. Microscopical examination of HPS-

stained sections showed that, compared with the controls, the PS-fed mice had less lipid-filled vacuoles in the liver cells (**Figure 7C and D**). The livers of PS-fed mice weighed significantly less (22%) than those of the control mice (**Table 4**). Lipid analysis revealed that livers of PS-fed mice contained less TG (56%) than livers of control mice. Furthermore, liver cholesteryl esters were decreased by 61% as was free cholesterol by 11% in PS-fed mice. All these differences were significant (**Table 4**).

Table 3. Hepatic mRNA expression of genes involved in FA and TG and cholesterol homeostasis of APOE*3Leiden mice fed a control or a 1% PS-containing Western-type diet for 5 weeks, as determined by RT-PCR

mRNA of	Control	1% PS	Change
FA/TG homeostasis			
<i>srebp1c</i>	100 ± 7 %	194 ± 3 %**	↑
<i>fas</i>	100 ± 6 %	237 ± 33 %*	↑
<i>mtfp</i>	100 ± 4 %	167 ± 5 %**	↑
<i>dgat2</i>	100 ± 17%	185 ± 23 %*	↑
<i>acc1</i>	100 ± 6 %	138 ± 29 %	NS
<i>dgat1</i>	100 ± 25 %	112 ± 27 %	NS
<i>aco</i>	100 ± 12 %	56 ± 9 %	NS
<i>apob</i>	100 ± 15 %	105 ± 25 %	NS
Cholesterol homeostasis			
<i>srebp2</i>	100 ± 9 %	317 ± 33 %*	↑
<i>ldlr</i>	100 ± 7 %	209 ± 14 %**	↑
<i>hmgcoAred</i>	100 ± 7 %	444 ± 71 %**	↑
<i>cyp7a1</i>	100 ± 10 %	7 ± 2 %*	↓
<i>lxrβ</i>	100 ± 18 %	16 ± 8 %*	↓
<i>lxra</i>	100 ± 4 %	162 ± 13 %*	↑
<i>fxr</i>	100 ± 15 %	164 ± 58 %*	↑
<i>ppara</i>	100 ± 1 %	125 ± 10 %	NS
<i>pparγ</i>	100 ± 7 %	119 ± 10 %	NS
<i>abca1</i>	100 ± 12 %	79 ± 6 %	NS

Values represent mean ± SD of 5 mice per group. mRNA expression was normalized to HPRT. *P < 0.05, **P < 0.01 vs. control using the Mann-Whitney U test. *abca1* ATP-binding cassette transporter, *acc* acetyl-CoA carboxylase, *aco* acyl-coenzyme A oxidase, *apo* apolipoprotein, *cyp7a1* Cholesterol 7- α -hydroxylase, *dgat* acyl:diacylglycerol transferase, *fas* fatty acid synthase, *fxr* farnesoid X receptor, *hmgcoAred* 3-hydroxy-3-methyl glutaryl coenzyme A reductase, HPRT hypoxanthine guanine phosphoribosyl transferase, *ldlr* LDL receptor, *lxr* liver X receptor; *mtfp* microsomal TG transfer protein, NS not significant, *ppar* peroxisome proliferator-activated receptor, PS phytosphingosine, *srebp*, sterol regulatory binding protein

Phytosphingosine lowers plasma inflammatory markers

Hepatic steatosis is often associated with liver inflammation. We determined plasma ALAT concentration, as a measure for liver damage. The ALAT concentration decreased by 79% (P < 0.05) after PS-feeding. A 74% (P < 0.05) decrease in the concentration of the acute phase marker SAA was found in PS-fed mice. The fibrinogen concentration was 42% higher (P < 0.05) in PS-treated mice compared with controls (**Table 4**).

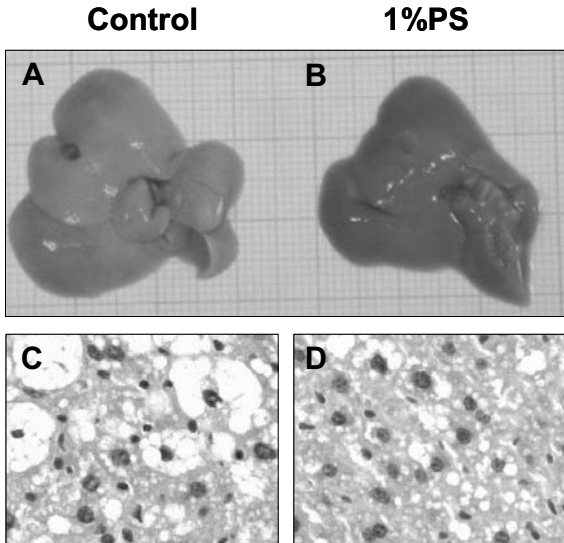


Figure 7. Liver analysis

Macroscopic appearance of a liver from a mouse fed the control Western-type diet for 5 weeks (A) and from a mouse fed the same diet with 1% PS (B). Haematoxylin-phloxine-saffron stained histological micrographs of paraffin-embedded livers from a mouse fed the Western-type diet for 5 weeks (C) and a mouse fed the same diet containing 1% PS (D). PS phytosphingosine.

Table 4. Liver lipid parameters and plasma inflammation markers determined in 4 h-fasted APOE*3Leiden mice after feeding a control or a 1% PS-containing Western-type diet for 5 weeks

	Control	1% PS	%Δ
Liver parameters			
Liver weight (g)	1.34 ± 0.12	1.05 ± 0.08***	-22
Triacylglycerol (μg/mg protein)	34.62 ± 2.23	15.28 ± 3.84***	-56
Free cholesterol (μg/mg protein)	7.81 ± 0.51	6.95 ± 0.78*	-11
Cholesteryl ester (μg/mg protein)	13.44 ± 0.79	5.20 ± 0.79***	-61
Plasma inflammation markers			
ALAT (U/l)	189 ± 49	41 ± 21***	-79
SAA (μg/ml)	11.2 ± 3.6	3.0 ± 0.3**	-74
Fibrinogen (mg/ml)	1.6 ± 0.5	2.3 ± 0.7***	+42

Values represent mean ± SD of 6 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control using Student's or Welch's t-test. ALAT alanine aminotransaminase, PS phytosphingosine, SAA serum amyloid A

Discussion

From this study we conclude that sphingolipids dose-dependently decrease plasma cholesterol and TG concentrations in APOE*3Leiden mice fed a Western-type diet. This cholesterol- and TG-lowering effect is mediated through inhibition of intestinal absorption of both cholesterol and TG and, eventually, leads to protection of the liver from fat and cholesterol-induced steatosis.

Since the intestinal absorption of FA and TG were inhibited to the same extent, we conclude that intestinal TG lipolysis *per se* is not inhibited by sphingolipids but that the absorption of FA is impaired. We do not know the exact mechanism underlying the PS-mediated inhibition of FA absorption. However, the FA-PS complex formation via an ionic interaction between the negatively charged carboxylic acid group of FA and the positively charged primary amine of PS might be an explanation. Although complex sphingolipids do not have such a primary amine group, in the intestine they can be lipolyzed to some extent into sphingoid bases and, thus, the same mechanism will, eventually, also hold for these complex molecules. However, it has been observed that some dietary sphingolipids are not fully digested and are partly excreted via the feces²⁹.

The intestinal FA absorption experiments (**Figure 5**) were performed applying an intragastric olive oil load. As compared with conditions of normal *ad libitum* food intake, intragastric olive oil loads represent extreme conditions of intestinal FA absorption. Thus, quantitative extrapolation of the observed inhibitory effect of PS on the FA absorption during a intragastric fat load to FA absorption under normal feeding conditions is hazardous. Under normal feeding conditions the effect of PS on the FA absorption is assumed to be more modest, and the mice may compensate with increased glucose utilization. The observation that a PS-containing diet does not significantly affect food intake or body weight in the long term is in line with this reasoning.

Intestinal absorption of cholesterol depends on bile salts, and is favored by the presence in the intestine of TG-derived FA that form mixed micelles with bile salts in which cholesterol is solubilized³⁰. Since we observed no effect of PS on intestinal TG hydrolysis (**Figure 5**), disturbance of the composition of these mixed micelles by sphingolipids, leading to hampered solubilization of cholesterol, seems unlikely. The formation of stable cholesterol and sphingomyelin (or sphingosine) complexes has been described^{29,30} and could, as such, be the cause of a reduced intestinal absorption of cholesterol. However, whether the formation of cholesterol-sphingolipid complexes also occurs in the intestine has been questioned, since the high affinity of cholesterol for sphingomyelin is lost in the presence of bile salts^{31,32}. We found that both simple and complex sphingolipids (**Figures 1 and 2**) decrease plasma cholesterol and TG concentrations in APOE*3Leiden mice. Because of the diversity in chemical structure between the various sphingolipid species, a wide range in physical and chemical properties may be expected and, thus, the inhibition of intestinal absorption of cholesterol and TG by PS is not likely to be explained by specific complex formation with bile salts or disturbance of bile salt micelles in the intestinal lumen. However, other yet unknown biochemical processes may be influenced by dietary sphingolipids or their metabolites. For example, the effect of the sphingolipid diet on the bile salt profile was not investigated, but changes in that profile (and in bile salt production) potentially may affect the intestinal lipid absorption and may thus influence plasma lipid concentrations. This hypothesis is sustained by the observation that genes involved in the bile salt synthesis are indeed affected by the sphingolipid diet (see **Table 3**).

The lipid-lowering effect of both cholesterol and TG of PS-feeding in APOE*3Leiden mice clearly differs from the effects of feeding stanol esters, which results in cholesterol-lowering only, while plasma TG remains unaffected⁵. Importantly, in contrast with the present dietary sphingolipid study, the expression of hepatic key genes involved in lipid metabolism (*i.e.*, *ldlr* and *cyp7a1*), were not affected in APOE3*Leiden mice fed with plant stanol esters³³. In addition, PS proved to be about twice as effective at decreasing plasma cholesterol in APOE*3Leiden mice as stanol esters. At a dose of 1% of plant stanol ester for 9 weeks, a 33% decrease in total plasma cholesterol was observed⁵, whereas PS-feeding at the same concentration reduced plasma cholesterol by 57%. Reciprocally, as did PS, dietary stanol esters decreased hepatic cholesteryl esters, free cholesterol, and TG concentrations in APOE*3Leiden mice³³.

Since blood samples were taken from animals that were fasted for 4 hours and are thus not expected to have intestine-derived chylomicrons in their plasma, the decreased concentration of plasma cholesterol and TG could not be directly ascribed to the PS-mediated inhibition of intestinal cholesterol and TG absorption. We reasoned that reduction in the absorption of dietary plus biliary cholesterol leads to a reduction in the liver cholesterol pool as presented in **Table 4**. A reduction in the cholesterol pool in the liver leads to a reduction in bile acid synthesis as reflected by a reduced expression in the liver of bile

acid synthesis genes such as *Ixrβ* and *cyp7a1*, concomitant with an increased expression of genes involved in hepatic cholesterol synthesis (*hmgcoA* reductase) and hepatic cholesterol uptake from plasma (*ldlr*) (**Table 3**). It can also be concluded from the results presented in **Table 3** that the expression of genes involved in lipid synthesis (*srebp1c*, *fas* and *dgat2*) and VLDL production (*mtt*) increased. Indeed, as presented in **Table 2**, measurement of VLDL-TG production *in vivo* revealed a 20% enhanced secretion of VLDL-TG by the liver, whereas the number of VLDL particles secreted into the circulation (apoB synthesis) remained constant.

The observations that these newly synthesized VLDL particles exhibit a 50% reduction in cholesterol content (**Table 2**) and a strongly increased hepatic uptake of their remnants (**Figure 6B**), the latter is in line with the increased expression of the hepatic *ldlr* (**Table 3**), offers an explanation for the cholesterol-lowering effect of dietary PS. In contrast, the increased hepatic secretion of VLDL-TG does not sustain the TG-lowering effect of PS. From **Figures 6B** and **D** it can be concluded that, after LPL-mediated lipolysis more than 80% of VLDL-derived TG is taken up by peripheral tissues for within 20 minutes; the remainder is taken up by the liver (**Figure 6D**). A difference in plasma clearance of VLDL-TG between PS- and control-fed mice could not be observed under the experimental conditions used (**Figure 6C**). However, as depicted in **Figure 6D** the hepatic uptake of VLDL-TG increased significantly in the PS-fed group, thereby sustaining the TG-lowering effect of PS-feeding. In PS-fed mice, the newly synthesized VLDL particles were larger and relatively enriched in TG compared with control fed mice (**Table 2**). Since the affinity of TG-rich emulsion particles¹⁷ and lipoproteins³⁴ for LPL is higher with increasing particle size, this change in lipid composition could also (partly) explain the TG-lowering effect of PS-feeding.

Although sphingolipids have been suggested to be PPAR α agonists³⁵, we found no changes in expression of PPAR α or in genes that are under PPAR α control, such as *aco* (**Table 3**). Furthermore, we did not observe enlarged livers in PS-treated mice, as would be expected with PPAR α agonists in rodents^{36,37}. In contrast, the compared with control mice, the livers of PS-fed mice weighed even significantly less, than those of the control mice, contained less lipid (cholesteryl esters and TG, **Table 4**), and contained smaller lipid-filled vacuoles in the parenchymal cells (**Figure 7D**). To maintain its lipid homeostasis, the liver compensates for the lower PS-mediated dietary and biliary cholesterol and TG supply from the intestine by increasing its endogenous cholesterol and FA synthesis, as is reflected in the increased hepatic mRNA concentrations of *fas* and *hmgcoAred*. However, hepatic lipid synthesis is well regulated by a feed-back mechanism. Consequently, on PS-feeding, hepatic cholesterol or TG synthesis increased but lipids do not accumulate in the liver (see **Table 4**).

In steatotic livers, lipid accumulation is accompanied by an increase in plasma ALAT and SAA, which are markers of liver damage and liver inflammation, respectively. In

PS-fed mice both markers decreased dramatically, pointing to a true hepato-protective effect of dietary PS under conditions of Western-type diet feeding. Liver steatosis, and elevated ALAT and SAA concentrations are associated with insulin resistance and the metabolic syndrome in mice and humans^{38,39}. Furthermore, inflammatory reactions are involved in the atherosclerotic process⁴⁰. Therefore, dietary sphingolipids should be considered as compounds for treating or ameliorating not only the lipid component of cardiovascular disease, but also the inflammatory processes involved in atherosclerosis and insulin resistance. We suggest that dietary sphingolipids hold great potential to treat multiple aspects of the metabolic syndrome, such as dyslipidemia, insulin resistance and cardiovascular diseases.

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