

High-Dose Simvastatin Exhibits Enhanced Lipid-Lowering Effects Relative to Simvastatin/Ezetimibe Combination Therapy

Stuart G. Snowden, PhD*; Dmitry Grapov, PhD*; Magnus Settergren, MD, PhD;
Fabio Luiz D'Alexandri, PhD; Jesper Z. Haeggström, MD, PhD; Oliver Fiehn, PhD;
Tuulia Hyötyläinen, PhD; Theresa L. Pedersen, MS; John W. Newman, PhD; Matej Orešič, PhD;
John Pernow, MD, PhD; Craig E. Wheelock, PhD

Abstract—Statins are the frontline in cholesterol reduction therapies; however, their use in combination with agents that possess complimentary mechanisms of action may achieve further reductions in low-density lipoprotein cholesterol. Thirty-nine patients were treated with either 80 mg simvastatin (n=20) or 10 mg simvastatin plus 10 mg ezetimibe (n=19) for 6 weeks. Dosing was designed to produce comparable low-density lipoprotein cholesterol reductions, while enabling assessment of potential simvastatin-associated pleiotropic effects. Baseline and post-treatment plasma were analyzed for lipid mediators (eg, eicosanoids and endocannabinoids) and structural lipids by liquid chromatography tandem mass spectrometry. After statistical analysis and orthogonal projections to latent structures multivariate modeling, no changes were observed in lipid mediator levels, whereas global structural lipids were reduced in response to both monotherapy ($R^2Y=0.74$; $Q^2=0.66$; cross-validated ANOVA $P=7.0\times 10^{-8}$) and combination therapy ($R^2Y=0.67$; $Q^2=0.54$; cross-validated ANOVA $P=2.6\times 10^{-5}$). Orthogonal projections to latent structures modeling identified a subset of 12 lipids that classified the 2 treatment groups after 6 weeks ($R^2Y=0.65$; $Q^2=0.61$; cross-validated ANOVA $P=5.4\times 10^{-8}$). Decreases in the lipid species phosphatidylcholine (15:0/18:2) and hexosyl-ceramide (d18:1/24:0) were the strongest discriminators of low-density lipoprotein cholesterol reductions for both treatment groups ($q<0.00005$), whereas phosphatidylethanolamine (36:3e) contributed most to distinguishing treatment groups ($q=0.017$). Shifts in lipid composition were similar for high-dose simvastatin and simvastatin/ezetimibe combination therapy, but the magnitude of the reduction was linked to simvastatin dosage. Simvastatin therapy did not affect circulating levels of lipid mediators, suggesting that pleiotropic effects are not associated with eicosanoid production. Only high-dose simvastatin reduced the relative proportion of sphingomyelin and ceramide to phosphatidylcholine ($q=0.008$), suggesting a pleiotropic effect previously associated with a reduced risk of cardiovascular disease. (*Circ Cardiovasc Genet.* 2014;7:955-964.)

Hypercholesterolemia plays a central role in the pathology and exacerbation of numerous diseases, with reduction and management of cholesterol levels advised for multiple diseases, including cardiovascular disease^{1,2} and diabetes mellitus.^{3,4} Reducing cholesterol levels is a common therapeutic goal, with treatment guidelines describing reduction of low-density lipoprotein cholesterol (LDL-C) as a primary target and marker for the efficacy of clinical intervention.⁵ Multiple strategies exist for reducing both LDL-C and total cholesterol (TC) levels that generally start with lifestyle changes (eg, reduced dietary cholesterol intake, increased physical activity, smoking cessation).⁶ However,

if these steps are insufficient to achieve the targeted reduction in LDL-C and TC, pharmaceutical intervention can be useful. Statins represent the front line in cholesterol reduction drug therapies and efficacy in reducing the incidence of cardiovascular events.^{7,8} Statins lower cholesterol levels by reducing production in the liver via the inhibition of hydroxymethylglutaryl-coenzyme A reductase, which is involved in the rate-limiting conversion of hydroxymethylglutaryl-coenzyme A to mevalonic acid in cholesterol biosynthesis.⁹

In some instances, statin intervention alone is insufficient to achieve the targeted cholesterol reduction and supplementary

From the Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institutet, Stockholm, Sweden (S.G.S., F.L.D., J.Z.H., C.E.W.); NIH West Coast Metabolomics Center (D.G., O.F.) and Department of Nutrition (J.W.N.), University of California, Davis; Obesity and Metabolism Research Unit, USDA-ARS Western Human Nutrition Research Center, Davis, CA (D.G., T.L.P., J.W.N.); Department of Medicine, Unit of Cardiology, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden (M.S., J.P.); and Department of Bio- and Chemical Processes, VTT Technical Research Centre of Finland, Espoo, Finland (T.H., M.O.).

Current address for Drs Hyötyläinen and Orešič is Steno Diabetes Center A/S, Gentofte, Denmark.

*Drs Snowden and Grapov contributed equally to this work.

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Correspondence to Craig Wheelock, PhD, Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institutet, SE-17177, Stockholm, Sweden. E-mail craig.wheelock@ki.se

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strategies are required. To more effectively reduce blood cholesterol levels, statins can be combined with other lipid-lowering therapies such as cholesterol absorption inhibitors. A common approach is to combine statin therapy with ezetimibe treatment, which inhibits the absorption of cholesterol in the intestine by binding to Niemann-Pick C1 like 1 proteins on enterocytes, decreasing delivery of cholesterol to the liver.¹⁰ Ezetimibe treatment alone has been shown to significantly reduce LDL-C and TC in hypercholesterolemia patients.¹¹ However, the combination of ezetimibe and simvastatin results in a greater reduction in LDL-C than is produced by equivalent statin dose alone.^{12–14}

It has been suggested that the beneficial therapeutic effects of statins are 2-fold, first in their ability to reduce absolute levels of LDL-C and TC, and second their so-called pleiotropic effects (ie, therapeutic effects unrelated to lipid lowering),¹⁵ such as improving endothelial function.¹⁶ In the present study, patients with dysglycemia and coronary artery disease were given either high-dose simvastatin monotherapy or a combination of ezetimibe and low-dose simvastatin treatment to assess the pleiotropic effects of statin therapy. The study was designed to produce equivalent reductions in TC levels with both treatment regimes, to enable the pleiotropic effects to be examined independently of the level of cholesterol reduction. One advantage of combination therapy lies in an overall reduction of the required dosage of the individual drugs, minimizing the risk of side effects while maintaining therapeutic efficacy.¹⁷ However, combination therapies can also result in unique side effects not observed with the individual therapeutics.¹⁷ Accordingly, we compared the effect of high-dose simvastatin monotherapy and combined simvastatin/ezetimibe treatment on the levels of lipid mediators and structural lipids in circulating plasma.

Methods

Study Design

Samples were obtained from a double-blind randomized study of 39 patients with dysglycaemia and coronary artery disease randomized to 2 treatments, an 80 mg simvastatin monotherapy (n=20) and a combined 10 mg simvastatin plus 10 mg ezetimibe (n=19). The study was designed to achieve comparable reductions in LDL-C in both treatment groups. Because the combination of ezetimibe with low-dose simvastatin is known to reduce LDL-C by ≈50%, an 80 mg simvastatin monotherapy dose was required to achieve comparative LDL-C reductions in the 2 groups¹⁸ although the dose of 80 mg simvastatin is no longer recommended in the new guidelines on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk.¹⁹ Because ezetimibe as monotherapy is a weaker cholesterol-lowering compound than simvastatin, it was necessary to design the study with 1 group given low-dose simvastatin together with ezetimibe and a high dose of monotherapy simvastatin to achieve similar reductions in cholesterol, but with different doses of the statin. Treatments were taken daily in the evening for 6 weeks. Blood was sampled in the morning after a 12-hour fast at baseline and at the end of the treatment period. All drugs, with the exception of aspirin, clopidogrel, and glucose-lowering therapies, were withheld on the morning of collection, with treatment compliance monitored using pill counts (compliance was 100%). There was no significant sex imbalance or differences in medications between the 2 cohorts. Table 1 provides an overview of baseline group characteristics, none of which were significantly different between the 2 groups. However, for a detailed description of the cohorts, the reader is directed to the original publication by Settergren et al.²⁰

Table 1. Baseline Characteristics of Cohorts

Characteristic	Monotherapy (n=20)	Combination Therapy (n=19)
Age, y	70 (62–74)	74 (66–77)
Female/male, n (%)	5 (25)/15 (75)	8 (42)/11 (58)
Body mass index, kg/m ²	28 (25–31)	28 (26–29)
Smokers, n (%)	4 (20)	4 (21)
Type 2 diabetes mellitus/ impaired glucose tolerance, n (%)	17 (85)/3 (15)	19 (100)/0 (0)
Aspirin, n (%)	20 (100)	16 (84)
Clopidogrel, n (%)	3 (15)	2 (10)
β-Blockers, n (%)	18 (90)	15 (79)
Calcium channel blockers, n (%)	8 (40)	6 (31)
Angiotensin-converting enzyme inhibitors, n (%)	9 (45)	10 (52)
Statins, n (%)	0 (0)	0 (0)

Data are presented as median and quartiles. There were no significant differences between the 2 groups at the $P<0.05$ level based on the Mann-Whitney U test. The sex composition was not significantly different between the 2 cohorts based on Fisher exact test $P<0.05$.

Fasting blood samples were collected in EDTA tubes by puncture of a cubital vein. The samples were centrifuged at +4°C and 300g for 15 minutes. Plasma was removed and stored frozen at –80°C until analysis. All patients gave their written informed consent. The study protocol was approved by the ethics committee of the Karolinska University Hospital and conducted according to the Declaration of Helsinki.

Lipid Mediator Analysis

Oxylipins and endocannabinoids were analyzed as previously described.²¹ Briefly, a 250 μL aliquot of plasma was spiked with antioxidants, deuterated standards, and extracted using solid phase extraction cartridges (Waters Oasis HLB 3cc; 60 mg). Before analysis, dried solid-phase extraction eluents were reconstituted in 1:1 MeOH/acetonitrile containing 100 nmol/L 1-cyclohexyl-3-dodecanoic acid urea (Sigma-Aldrich) and filtered by centrifugation using 0.1 μm Durapore PVDF (Millipore). Compounds were separated using a reverse phase gradient with a 2.1×150 mm, 1.7 μm Acquity BEH column on a Waters Acquity Ultra Performance LC, with ionization in negative mode by electrospray ionization. Data were acquired in multireaction monitoring mode with an ABI 4000QTRAP triple quadrupole mass spectrometer. See the Data Supplement for a detailed description.

Structural Lipid Analysis

A mixture of lipid standards (20 μL) was added to plasma (10 μL), which was extracted using 2:1 chloroform/methanol followed by collection of the bottom phase (60 μL) and the addition of isotopically labeled standards.²² Lipids were separated using a 2.1×100 mm, 1.7 μm Acquity BEH column on an Acquity Ultra Performance LC coupled to Waters Q-ToF Premier mass spectrometer. Lipid profiling was done in electrospray ionization in positive mode, and the data were collected at a mass range of m/z 300 to 1200 with scan duration of 0.2 second. MZmine2 and an in-house spectral library were used for peak alignment, integration, identification, and normalization. Relative lipid concentrations (μmol/L) were calculated based on a ratio of peak heights (normalization) to corresponding standards followed by multiplication by the standards' concentration. Analytic variance and data quality were calculated based on a set of control samples (n=10), which were randomized within the study design and used to estimate the median (11%) and range (3–28%) of the relative

standard deviation of individual lipid measurements. See the Data Supplement for a detailed description.

Statistical Analysis and Multivariate Modeling

Individual lipid species that were not observed in $\leq 75\%$ of a given sample class or were structurally unidentified ($n=490$) were removed from all data analyses. Analyses were conducted separately for oxylipins, endocannabinoids, and structural lipids using the R language for statistical computing (version 3.0.1) and SIMCA-P 13 (Umetrics, Umeå, Sweden).

Power calculations were performed to estimate the minimum observable difference for changes in lipids from baseline to 6 weeks and between mono- and combination therapy at 80% power. The minimum detectable difference was calculated based on an effect size of 0.92 using the sum of the analytic and biological variances of each lipid.

Fisher exact test was used to confirm equal proportions of male and female patients among the mono- and combination therapy cohorts ($P=0.32$). Statistical comparisons between baseline and 6-week treatment were evaluated on logarithm (base 10)-transformed values using paired t tests, the significance levels (ie, P values) of which were adjusted for multiple hypotheses testing according to Benjamini and Hochberg at $q=0.05$,²³ and the adjusted P values are provided as P_{adj} . The false discovery rate (FDR) was also directly estimated according to the methods of Storey²⁴ and provided as q -values.

Orthogonal projections to latent structures–discriminant analysis (OPLS-DA) was conducted on logarithmic (base 10)-transformed, mean-centered and pareto-scaled data. Model performance was reported as cumulative correlation coefficient ($R^2Y[cum]$), 7-fold cross-validated fit to the training data ($Q^2[cum]$), and model significance was estimated using cross-validated ANOVA (CV-ANOVA). Feature selection was performed using variable importance in projection and $p(\text{corr})$ according to Wheelock and Wheelock.²⁵ See the Data Supplement for a detailed description.

Partial Correlation Network Analysis

Analysis of partial correlations was used to investigate direct empirical relationships between OPLS-DA selected lipids and clinical parameters. To identify pleiotropic effects between the 2 treatments,²¹ the coefficients of partial correlation, associated P values, and FDR-adjusted P values²³ were separately estimated for the mono- and combination therapy cohorts. Calculated networks were rendered in Cytoscape.²⁶ See the Data Supplement for a detailed description.

Results

Both mono- and combination therapy produced significant reductions in TC, LDL-C, and triglycerides (TGs; Table I in the Data Supplement). C-reactive protein was reduced ($P_{adj}=0.0004$) in response to the combination, but not monotherapy. However, the fold change in C-reactive protein at 6 weeks relative from baseline was not significantly different between the 2 treatments (Table I in the Data Supplement).

Eighty-one oxylipins representing 3 metabolic pathways, cyclooxygenase, lipoxygenase, and cytochrome P450, were screened. Of these, 35 were measured above the limit of detection, ranging in concentration between 30 pmol/L and 87 nmol/L (Table I in the Data Supplement). The observed oxylipins were predominantly cytochrome P450-, 12- and 15-lipoxygenase-derived products of linoleic and arachidonic acid. Thirty-three endocannabinoids were screened, 12 of which were present above the limit of detection, ranging in concentration from 40 pmol/L to 10.8 nmol/L (Table I in the Data Supplement). Statistical comparisons of both oxylipin and endocannabinoid levels between baseline and 6 weeks did not identify any significantly changed species after mono- or combination therapies (Table I in the Data Supplement). This observation was supported by OPLS-DA modeling, which did not produce informative models from either the oxylipin or endocannabinoid lipid measurements (Figure I and Table II in the Data Supplement).

A total of 800 lipid features were measured for the structural lipids, of which 310 were structurally identified and categorized into 7 distinct classes: cholesterol ester (CE; $n=8$), sphingomyelin and ceramide (SM|Cer; $n=36$), lysophosphatidylcholine (lysoPC; $n=18$), lysophosphatidylethanolamine (lysoPE; $n=3$), phosphatidylcholine (PC; $n=78$), phosphatidylethanolamine (PE; $n=44$), and TG ($n=123$).

Structural lipids displayed robust reductions in response to both mono- and combination therapy. Adjusting for FDR, monotherapy led to significant changes in 213 of the structural lipids (69%), compared with 159 species for the combined treatment (51%; Table 2; Figures II and III in the Data

Table 2. Comparison of Changes in Lipid Classes After Mono- and Combination Therapy

Lipid Class (No)*	Monotherapy			Combination			Comparison	
	% Change†	P_{adj} ‡	q Value§	% Change	P_{adj}	q Value	P_{adj}	q Value
SM+Cer (36)	-21±2	<0.0001	<0.0001	-15±2	<0.0001	<0.0001	0.2044	0.0188
(SM Cer)/(SM Cer+PC)	-8±2	0.0329	0.0080	-3±2	0.5382	0.1665	0.2044	0.0188
PC (78)	-14±3	0.0003	<0.0001	-13±2	<0.0001	<0.0001	0.7716	0.0709
PE (44)	-8±3	0.0051	0.0013	-3±4	0.0245	0.0079	0.3444	0.0314
LysoPC (18)	10±6	0.0279	0.0068	-4±5	0.0022	0.0005	0.2044	0.0188
LysoPE (3)	12±5	0.8429	0.0259	-7±5	0.0045	0.0012	0.0728	0.0067
CE (8)	-19±7	<0.0001	<0.0001	-16±4	0.0022	0.0006	0.7716	0.0709
TG (123)	-14±5	0.0036	0.0009	-5±5	0.0045	0.0013	0.3444	0.0316

CE indicates cholesterol ester; Cer, ceramide; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; SM, sphingomyelin; TG, triglyceride; SM|Cer, SM and Cer combined.

*Data are shown in a graphical format in Figure III in the Data Supplement.

†Mean percent change±SE comparing the sum of all lipids per lipid class at 6 wk relative to baseline.

‡Paired t test comparing 6 wk to baseline, adjusted for multiple hypotheses testing according to Benjamini and Hochberg at $q=0.05$.²³

§Direct estimate of false discovery rate according to the methods of Storey.²⁴

|| t Test contrasting changes in lipids from baseline to 6 wk in monotherapy compared with the combination therapy, adjusted for multiple hypotheses testing according to Benjamini and Hochberg at $q=0.05$.²³

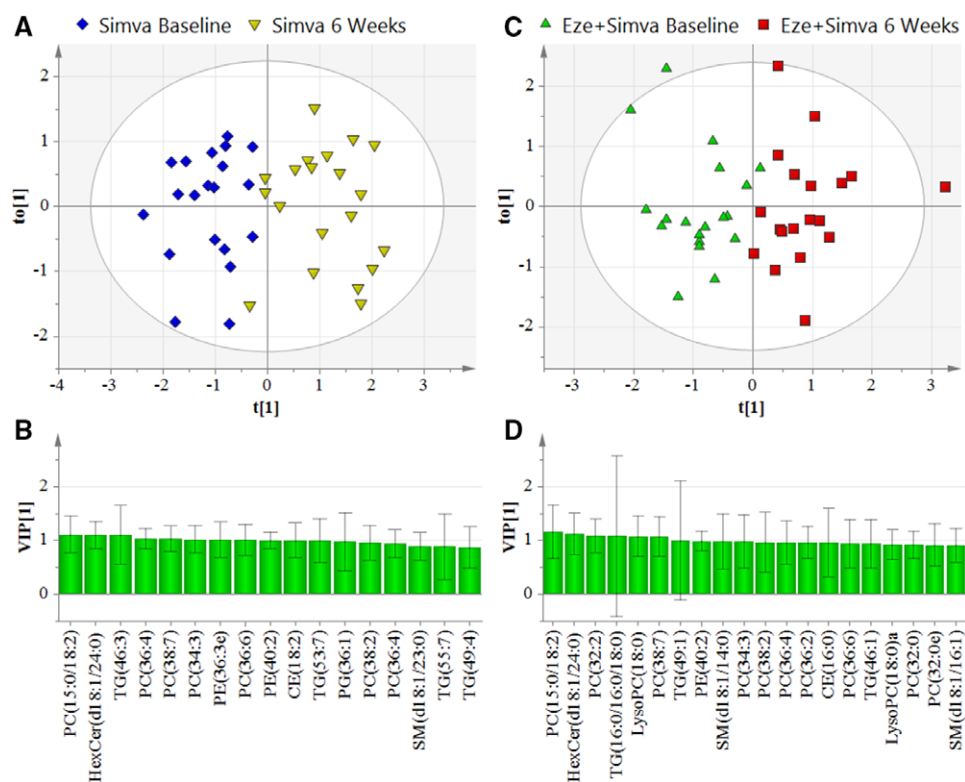


Figure 1. Orthogonal projections to latent structures–discriminant analysis modeling of structural lipid data after 3 iterative rounds of variable selection. **A**, Scores plot of simvastatin monotherapy at baseline vs 6 wk ($R^2Y=0.74$; $Q^2=0.66$; 1 plus 1 components; cross-validated ANOVA [CV-ANOVA] $P=7.0 \times 10^{-9}$). **B**, Top 10 lipid species from the variable importance in projection (VIP) plot of simvastatin monotherapy at baseline vs 6 wk. **C**, Scores plot of combination therapy at baseline vs 6 wk ($R^2Y=0.67$; $Q^2=0.54$; 1 plus 1 components; cross-validated ANOVA $P=2.6 \times 10^{-9}$). **D**, Top 10 lipid species from the VIP plot of combination therapy at baseline vs 6 wk. Eze indicates ezetimibe; and Simva, simvastatin. The orthogonal projections to latent structures–discriminant analysis models before variable selection are shown in Figure II in the Data Supplement. CE indicates cholesterol ester; HexCer, hexosyl-ceramide; LysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; and TG, triglyceride.

Supplement). The 2 treatments shared 40% of the observed changes in common. SM|Cer displayed the largest decreases relative to baseline after monotherapy ($-21 \pm 2\%$; $P_{\text{adj}} < 0.0001$) and the second largest decrease of all lipid classes after the combined treatment ($-15 \pm 2\%$; $P_{\text{adj}} < 0.0001$). Only the monotherapy led to significant reduction in the ratio between SM|Cer and PC, SM|Cer/(SM|Cer+PC), ($-8 \pm 2\%$; $P_{\text{adj}} = 0.033$). Furthermore, PC, PE, CE, and TG lipid classes were all significantly decreased after both mono- and combination therapy, but monotherapy led to greater absolute decreases. As a class, lysoPE lipids were unchanged after monotherapy, but reduced after the combined treatment ($-7 \pm 5\%$; $P_{\text{adj}} = 0.0045$). Similarly, lysoPC lipids showed differential regulation between the 2 treatments and were increased after monotherapy ($10 \pm 6\%$; $P_{\text{adj}} = 0.028$), but decreased ($-4 \pm 5\%$; $P_{\text{adj}} = 0.0022$) after the combined treatment.

The data were further interrogated using OPLS-DA multivariate classification modeling, which was used to assess the homogeneity of the treatment cohorts at baseline (Table II in the Data Supplement). Models comparing changes in oxylipins and endocannabinoids could not identify significant compositional differences between baseline and 6-week treatment for either the mono- or combination therapy cohort (Figure I in the Data Supplement), further supporting the results of the univariate statistical analyses of these species. Significant differences were observed in treatment models for structural

lipids after both mono- ($R^2Y=0.958$; $Q^2=0.703$; CV-ANOVA $P=2.0 \times 10^{-4}$; Figure IID in the Data Supplement) and combination therapies ($R^2Y=0.732$; $Q^2=0.503$; CV-ANOVA $P=8.9 \times 10^{-5}$; Figure IIC in the Data Supplement). An OPLS model comparing the ratio of the change in lipid species level for each treatment between baseline and 6 weeks gave a significant model; however, the model possessed overall low predictive power ($R^2Y=0.827$; $Q^2=0.298$; CV-ANOVA $P=0.01$).

OPLS-DA-based feature selection was used to generate curated models that identified the lipid species with the greatest ability to distinguish pre- versus post-treatment after mono- and combination therapies (Figure 1). The final models were highly significant for both the monotherapy ($R^2Y=0.74$; $Q^2=0.66$; CV-ANOVA $P=7.0 \times 10^{-8}$) and combination therapy ($R^2Y=0.67$; $Q^2=0.54$; CV-ANOVA $P=2.6 \times 10^{-5}$). The top 10 contributing lipids from each curated model were selected for treatment comparison (Table 3), and their structures were confirmed using MS/MS (Figure III in the Data Supplement). PC (15:0/18:2) and hexosyl-ceramide (HexCer; d18:1/24:0) were the 2 top-ranked predictors for both treatments. These 2 lipids were both significantly decreased by 50% after monotherapy and 40% and 30%, respectively, after the combined treatment (Table I in the Data Supplement). None of the top 10 predictors in Table 3 increased for either treatment. Although multiple lipids increased significantly after either monotherapy or the combined treatment; only lysoPC (20:4) and PE (36:6e)

Table 3. The 10 Variables With the Strongest Contribution to the OPLS-DA Models Comparing Treatment at Baseline Versus After 6 Weeks

OPLS VIP Rank*	Simvastatin Baseline vs 6 wk†			OPLS VIP Rank	Combined Baseline vs 6 wk‡		
	Lipid Species§	Fold Changell	P_{adj} ¶		Lipid Species	Fold Change	P_{adj}
1	PC(15:0/18:2)#	0.5	3.3×10^{-6}	1	PC(15:0/18:2)#	0.6	2.1×10^{-5}
2	HexCer(d18:1/24:0)#	0.5	3.3×10^{-6}	2	HexCer(d18:1/24:0)#	0.7	1.1×10^{-3}
3	TG(46:3)	0.4	3.9×10^{-4}	3	PC(32:2)	0.6	3.3×10^{-4}
4	PC(36:4)	0.6	3.5×10^{-5}	4	TG(16:0/16:0/18:0)	0.6	9.6×10^{-4}
5	PC(38:7)#	0.8	3.1×10^{-3}	5	LysoPC(18:0)	0.7	3.4×10^{-4}
6	PC(34:3)#	0.6	5.6×10^{-5}	6	PC(38:7)#	0.8	4.2×10^{-3}
7	PE(p16:0/18:2)	0.5	4.2×10^{-6}	7	TG(49:1)	0.6	6.9×10^{-4}
8	PC(36:6)	0.6	3.5×10^{-5}	8	PE(40:2)#	0.7	1.5×10^{-5}
9	PE(40:2)#	0.6	3.0×10^{-5}	9	SM(d18:1/14:0)	0.7	1.8×10^{-7}
10	CE(18:2)	0.6	3.1×10^{-5}	10	PC(34:3)#	0.7	2.0×10^{-4}

CE indicates cholesterol ester; HexCer, hexosyl-ceramide; LysoPC, lysophosphatidylcholine; OPLS-DA, orthogonal projections to latent structures–discriminant analysis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TG, triglyceride; and VIP, variable importance in projection.

*Variable ranking from the VIP plot from the OPLS-DA models shown in Figure 1.

†See Figure 1C for details of simvastatin OPLS model.

‡See Figure 1D for details of combined treatment OPLS model.

§These species were confirmed by MS/MS experiments. See Figure IV in the Data Supplement for the MS/MS spectra of PC(15:0/18:2) and HexCer(d18:1/24:0).

llFold change of means relative to 6 wk (see Table I in the Data Supplement).

¶ P values adjusted for multiple hypotheses testing according to Benjamini and Hochberg at $q=0.05^{23}$ (see Table I in the Data Supplement).

#Variables in common between the 2 models.

significantly increased in response to both treatments (Table I and Figure V in the Data Supplement). Contribution plots were generated for both treatment models, which showed similar shifts in overall lipid composition, with the exception of some lysoPC and lysoPE (Figure 2). The OPLS model of the ratio of the change in lipid species level between baseline and 6 weeks was curated via 3 rounds of feature selection to give a highly significant model for classifying treatment group (Figure 3A; $R^2Y=0.651$; $Q^2=0.605$; CV-ANOVA $P=5.4 \times 10^{-8}$). This model was driven by a subset of 12 lipids, of which PE

(36:3e) and SM (d18:0/24:0) were the strongest contributors to the overall model (Figure 3B). This finding was supported by the univariate analysis, in which PE (36:3e) was reported to differ significantly between treatment groups ($q=0.017$).

Lipid and clinical parameter partial correlation networks were developed to integrate the statistical and multivariate analysis results (Figure 4; Figure IV in the Data Supplement). Partial correlations are commonly used to decouple direct from indirect variable associations and, in the case of highly intracorrelated lipids, offer a unique approach for generating

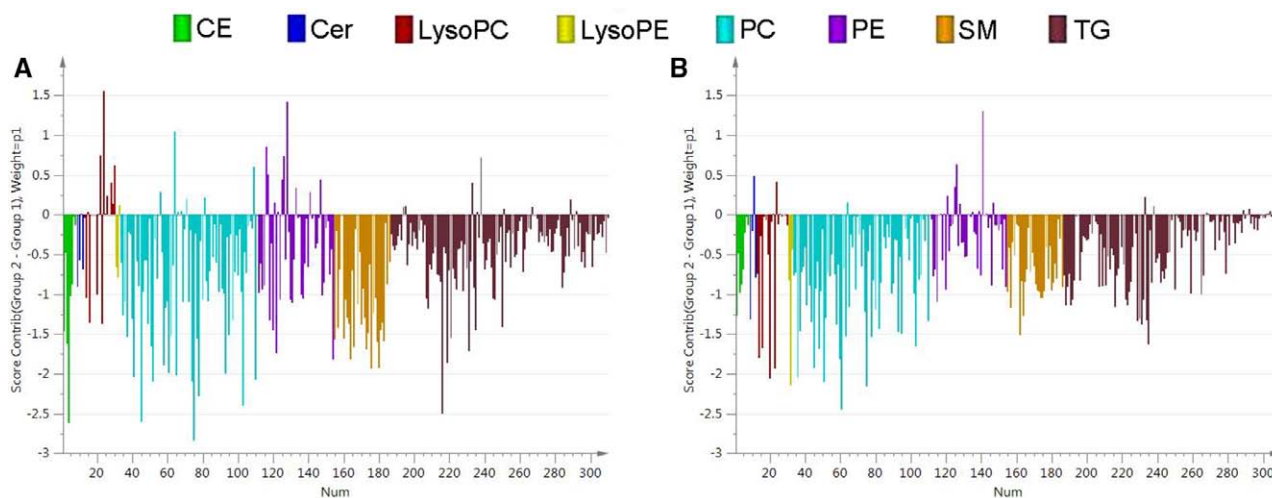


Figure 2. Contribution plots showing the influence of individual structural lipid species in the orthogonal projections to latent structures–discriminant analysis models. **A**, Simvastatin monotherapy baseline vs 6 wk ($R^2Y=0.99$; $Q^2=0.73$; cross-validated ANOVA $P=2.0 \times 10^{-4}$; see Figure ID in the Data Supplement). **B**, Combination therapy baseline vs 6 wk ($R^2Y=0.76$; $Q^2=0.50$; CV-ANOVA $P=8.9 \times 10^{-5}$; see Figure IC in the Data Supplement). CE indicates cholesterol ester; Cer, ceramide; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; SM, sphingomyelin; and TG, triglyceride.

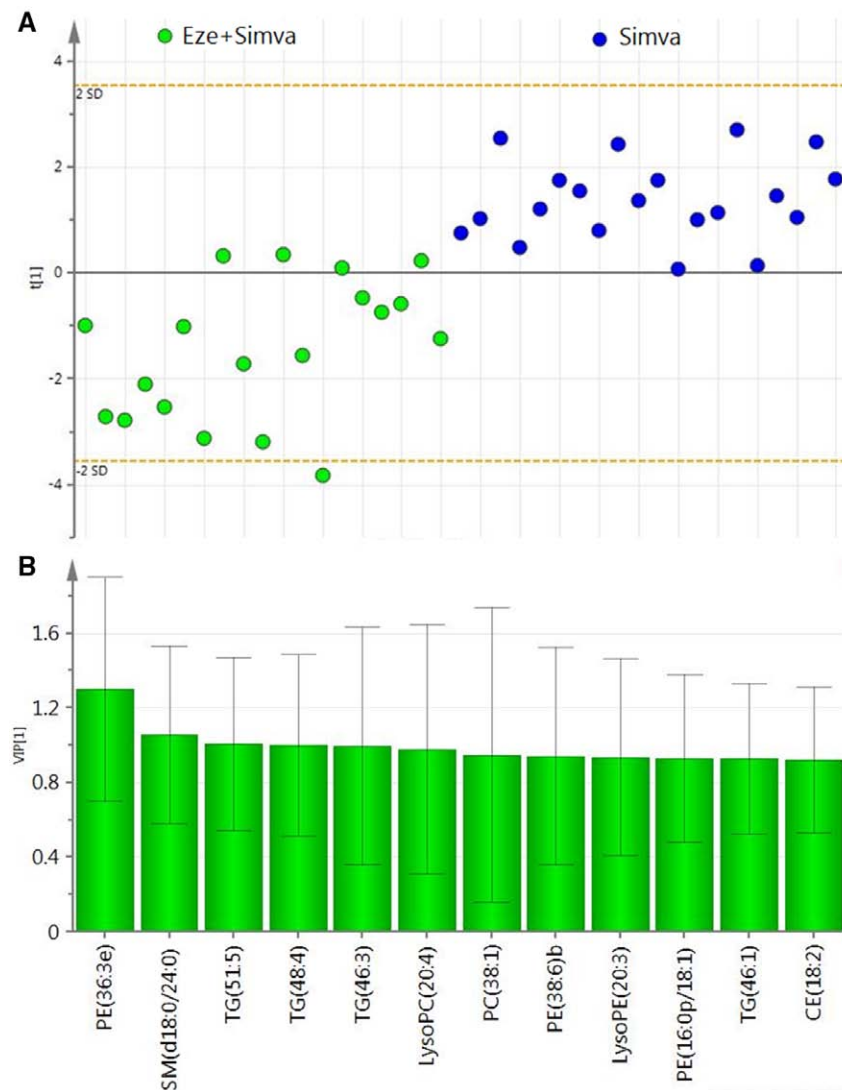


Figure 3. Orthogonal projections to latent structures–discriminant analysis modeling of the ratio of structural lipid levels at baseline vs 6 wk after 3 iterative rounds of variable selection. **A**, Scores plot of simvastatin monotherapy vs combination therapy ($R^2Y=0.65$; $Q^2=0.61$; 1 plus 0 components; cross-validated ANOVA $P=5.4\times 10^{-8}$). **B**, Variable importance in projection (VIP) plot of the 12 lipid species driving the model. CE indicates cholesterol ester; Eze, ezetimibe; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; Simva, simvastatin; SM, sphingomyelin; and TG, triglyceride.

simplified and often more informative dependency visualizations. Associations were calculated between the top lipid predictors of treatment effects after both therapies (Figure 1B and 1D) and clinical parameters (Table I in the Data Supplement). The relationships were adjusted for FDR and calculated separately for monotherapy and combination treatment to aid in the identification of nuanced changes because of potentially differing pleiotropic effects.²¹ In the case of the combination treatment, the decreases in LDL-C and TC were linked with the reduction in the ceramide species HexCer (d18:1/24:0), which was the second best predictor of the treatment effect after either therapy (Table 3). Similarly, clinically measured TG was significantly reduced after both treatments, but this change was differentially related to specific TG predictors (Figure 4) unique to each treatment model (Figure 1). However, the changes in individual TG lipids could all be linked to reductions in PC (38:7) after either treatment. For the monotherapy, the significant increase in lysoPC (20:4) correlated positively with HDL-C, and the decrease in SM (d18:1/23:0) was inversely related to plasma creatinine levels. PC (15:0/18:2), the top predictor of treatment after either therapy, was significantly reduced after both treatments, and

this decrease was positively correlated with similar decreases in PC (33:2) and PE (40:2) after both treatments. However, only after monotherapy was the decrease in PC (15:0/18:2) also indirectly linked to reductions in CE (18:2) and HexCer (d18:1/24:0; Figure 4). The related changes in differing lipid classes may arise because of these species sharing the same acyl chain, linoleic acid (18:2), which is true for PC (15:0/18:2) and CE (18:2) and likely for PC (33:2) and PE (40:2), but this would require confirmation via MS/MS experiments. PE (36:3e), the strongest discriminator of the treatment groups, correlated positively with HDL-C after both treatments; however, the magnitude of the correlation and level of decrease were greater for monotherapy.

A limitation of the current study is the relatively small number of individuals. Power analyses were conducted to estimate the minimum observable differences for changes in structural lipids from baseline to 6 weeks, between mono- and combination therapy. Based on the analytic (Table III in the Data Supplement) and biological variance in lipid measurements, the current study is well powered (80%) to detect changes in SM|Cer, LysoPC, LysoPE, and PC between the mono- and combination cohorts at changes in mean lipid levels from 27%

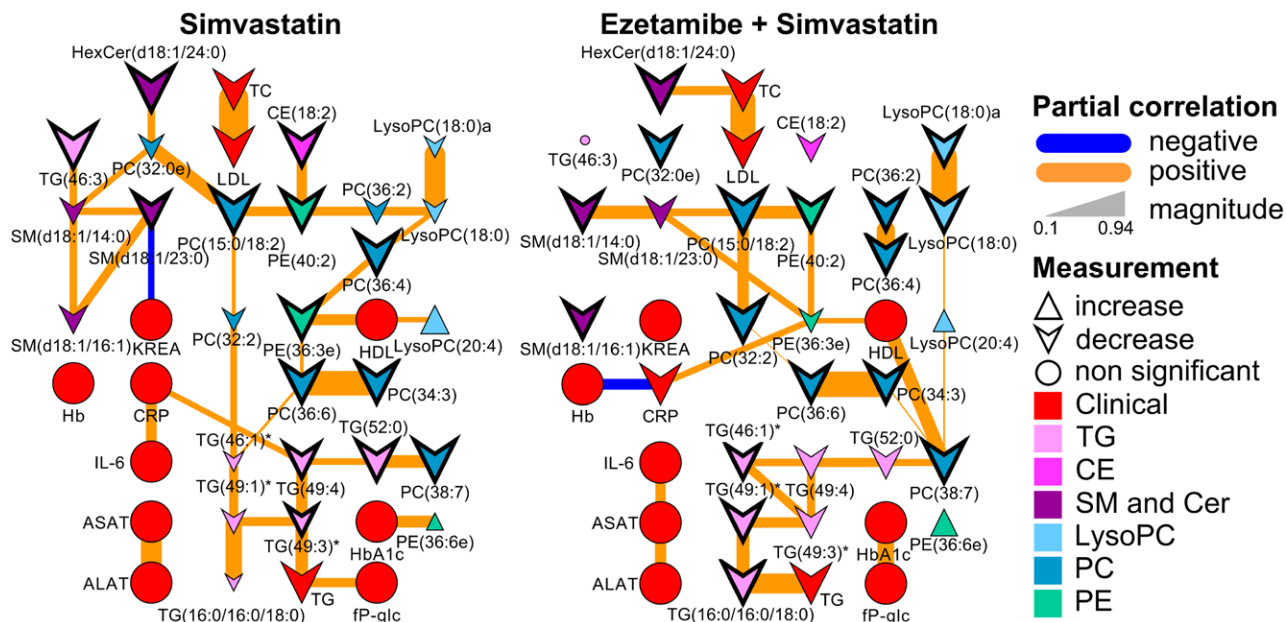


Figure 4. Partial correlation network displaying conditionally independent relationships between selected changed lipids listed in Table 3 and clinical parameters. Simvastatin (A) and simvastatin and ezetimibe treatment (B). Vertices, representing measured parameters, are connected based on significant partial correlations ($P_{adj} \leq 0.05$). Vertex size displays the parameter’s importance in the orthogonal projections to latent structures–discriminant analysis model (variable importance in projection [VIP] value, with clinical parameters size artificially set to the maximum VIP value). Vertex shape is used to encode the direction (triangle, increase; VEE, decrease) and statistical significance of the parameters change (circle; $P_{adj} > 0.05$). Vertices are colored according to variable type or lipid biochemical class, and treatment model–specific variables are highlighted with thick black borders. ALAT indicates alanine aminotransferase; ASAT, aspartate aminotransferase; CE, cholesterol ester; Cer, ceramide; Eze, ezetimibe; fP-glc, fasting glucose; Hb, hemoglobin; HexCer, hexosylceramide; KREA, blood creatinine; LysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; Simva, simvastatin; SM, sphingomyelin; and TG, triglyceride.

to 37% (Table IV in the Data Supplement). Consequently, the probability of a β (type II) error was $\leq 20\%$, and the probability of α (type I) errors was controlled via standard FDR approaches as described above. Accordingly, the chances of committing a type I or type II error in this study for 4 of the lipid classes were within the traditionally set limits for statistical acceptance. However, compared with the aforementioned lipids, there may exist a bias toward lack of detection of changes in PE, CE, and TG classes of lipids because of their increased analytic and biological variability (52–65%; Table IV in the Data Supplement).

Discussion

There is a sizeable body of literature examining the effects of both statins,^{2,27} and combined statin/ezetimibe treatment^{12–14} on LDL-C and TC levels. However, relatively few studies have examined the effects these treatments exert on global lipid composition, with most studies to date focusing on the effect of statins.^{28,29} The current study is the first to perform a comprehensive analysis of lipid species, with >900 measured lipid variables including lipid mediators (eg, endocannabinoids and oxylipins).

The general effects on oxylipin and endocannabinoid levels were minor, suggesting that neither treatment significantly impacts the metabolism of these species in circulating plasma. It has been suggested that eicosanoid production could play a role in the pleiotropic effects of statins.³⁰ In animal models, high-dose statins have been shown to modulate eicosanoid

production, for example, via the inhibition of leukotriene synthesis by activation of protein kinase A, which subsequently phosphorylates 5-lipoxygenase.³¹ In the current investigation, the 5-lipoxygenase product 5-hydroxyeicosatetraenoic acid was reduced after both treatments, but this change failed to reach significance. Accordingly, data from the current study suggest that high-dose simvastatin does not affect circulating levels of eicosanoids. However, it should be noted that only the free acid forms of these species were measured and it is possible that shifts occurred in the esterified pools, which are generally in greater abundance.^{32,33} For example, it was shown in Zucker rats that >90% of the whole plasma oxylipins were esterified to lipoproteins on a class-specific basis. These esterified oxylipins were substrates for lipoprotein lipase activity, whose distributions changed within the context of obesity-associated dyslipidemia.^{32,33} Accordingly, future studies should focus on the esterified species to comprehensively examine oxylipin dynamics in response to lipid reduction therapy.

Both the monotherapy and the combination therapy produced similar shifts in the composition of structural lipids, suggesting that simvastatin is the predominant driver for the observed changes. This view is supported by the greater reductions for the majority of measured lipid classes, and particularly SM|Cer, PC, and the ratio between the 2, in response to the monotherapy using a higher simvastatin dose. Alternatively, lysoPC and lysoPE species displayed greater reductions in response to the combined treatment, suggesting that ezetimibe has subtle effects on lysophosphatidyl

lipid metabolism. Although the implications of this shift are unclear, it is of interest that the observed increase in lysoPC (20:4) after monotherapy positively correlated with HDL (Figure 4).

The observed shifts in several lipid classes, CE, PC, PE, and TG, are in line with those previously reported for simvastatin therapy.^{29,34,35} Reduction in the abundance of esterified cholesterol is expected²⁹; however, the observed decrease in the levels of multiple phospholipid species is noteworthy. It has been postulated that simvastatin can directly decrease phospholipid synthesis *in vitro*³⁶; however, the correlation ($r^2=0.479$) between the observed reduction in CEs and PCs in patients receiving simvastatin was not particularly strong.²⁹ The current investigation suggests that the dominant CE species in plasma, CE (18:2), was significantly decreased after both treatments (Table I in the Data Supplement), and this reduction was positively correlated with a decrease in PC (38:2; Figure VI in the Data Supplement), which was a top 10 variable of importance in projection predictor for both treatments. Otherwise comparable reductions in phospholipid levels should have been observed in the current study, where both treatment groups exhibited comparable reductions in LDL-C. However, patients receiving the higher-dose statin exhibited larger overall reductions in phospholipid levels. This apparent effect of simvastatin on phospholipid metabolism provides evidence for 1 potential pleiotropic effect mechanism.

Another potential mechanism for pleiotropic effects was observed in an analysis of the relative levels of SM and PC, the SM/(SM+PC) ratio, of which both increases^{37,38} and decreases³⁹ have been reported to be associated with increased cardiovascular risk. The SM/PC ratio has been suggested as diagnostic marker for increased lipoprotein modifications in hyperlipidemic patients.⁴⁰ The combined ratio, SM|Cer/(SM|Cer+PC), was significantly reduced only after higher-dose statin monotherapy ($P=0.03$; Table 2). Changes in SM were most closely related to PE, and Cer to lysoPC (Figure VI in the Data Supplement). A trend in reduced plasma SM levels has been previously reported for patients undergoing statin treatment.³⁸ These results show a clear difference in the effect of monotherapy versus combination therapy on circulating levels of the ratio SM|Cer/(SM|Cer+PC). Accordingly, these data can be informative for other studies examining the potential relationship between these lipids and protective effects against coronary artery disease. It is not appropriate to extrapolate these findings within the context of the current study without information on future disease incidence in these patients. These results do indicate that further attempts to assess the role of SMs in disease incidence should control for potentially confounding effects of cholesterol reduction therapy.

Multivariate analysis of both treatments identified the lipid species PC (15:0/18:2) as the strongest discriminating variable for both treatment groups between baseline and post-treatment. There are currently no published reports of this lipid species; however, PCs are major components of cellular membranes playing critical roles in their structure and function. The pentadecanoic acid moiety is derived from dairy products and milk fat, whereas the linoleic acid

moiety is derived from seed oils. PCs interact with cholesterol, both in cell membranes as well as in plasma, which can affect the fluidity of the plasma membrane, with the nature of the interaction determined by the acyl chain length of the phospholipid.⁴¹ In the case of the monotherapy, the decrease in PC (15:0/18:2) was indirectly positively associated with a decrease in CE (18:2) and HexCer (d18:1/24:0; Figure 3). After both treatments, changes in PC (15:0/18:2) were positively correlated with decreases in PC (32:2) and PE (40:2). It is likely that all of these lipids contain linoleic acid (18:2), which may explain their related decrease after simvastatin therapy.

Both treatments led to a significant increase in lysoPC (20:4), which in the case of the monotherapy also displayed a positive association with HDL (Figure 3). LysoPC (20:4) has previously been reported to increase in response to simvastatin treatment.²⁹ It has also been shown to discriminate patient response to both high- and low-dose atorvastatin²⁸; however, Bergheanu et al²⁸ did not report how treatment affected the abundance of this lipid. Strauss et al⁴² showed that Wistar (CrI:WI[Han]) rats exposed to 28 days of treatment with both atorvastatin (70 mg/kg body weight) and pravastatin (200 mg/kg body weight) showed significant reductions in lysoPC (20:4). These findings are inconsistent with results from the current study and those of Kaddurah-Daouk et al.²⁹ However, the reductions in lysoPC (20:4) observed in Strauss et al⁴² were in rats, using different statins at higher doses. These findings are relevant within the context of the relationship between lysoPC and cardiovascular risk.⁴³ LysoPC is generated by phospholipase A₂ (PLA₂)–mediated hydrolysis of lipids and plays an important role in atherosclerosis as well as both acute and chronic inflammation.⁴⁴ Selective inhibition of lipoprotein-associated PLA₂ has been shown to reduce atherosclerotic lesion lysoPC content leading to a reduction in the development of advanced coronary atherosclerosis.⁴⁵ Lipoprotein-associated PLA₂ in carotid artery plaques is a predictor of future cardiac events,⁴⁶ and the associations between lipoprotein-associated PLA₂ and lysoPCs (as well as lyso-phosphatidic acid) in human plaques suggest that lysoPCs play a key role in plaque inflammation and vulnerability.⁴⁷ This body of literature highlights the need to specify the fatty acid content of lysoPC, under the pretext that not all lysoPCs necessarily evidence similar behavior as demonstrated herein. Accordingly, although lysoPC (20:4) seems to be a noteworthy lipid in the response to statin treatment, additional information is needed to describe its biological function. This is also the case for PE (36:6e), which is the only other species to increase after both treatments.

Our findings show that simvastatin monotherapy and simvastatin/ezetimibe combination therapy produce similar overall shifts in lipid levels; however, several treatment-specific effects were observed. Although the findings in the current study are of interest, there are limitations that restrict data interpretation. OPLS modeling provided significant classification of the treatment groups (CV-ANOVA $P=5.4\times 10^{-8}$; Figure 3); however, no adjusted P values reached significance on a lipid class–based comparison (Table 2) and only 2 lipid species had q values <0.05 (Table I in the Data Supplement).

Accordingly, a study with increased power is necessary to more fully examine the specificity of lipid reduction therapy on shifts in individual lipid species. In addition, to more fully determine the relative effects of simvastatin monotherapy versus simvastatin/ezetimibe combination therapy, additional work should include a group receiving ezetimibe monotherapy. This design would enable a more direct comparison of relative lipid-lowering efficacy of the different treatments. Furthermore, it remains unclear as to whether the observed effects are a general feature of statins or are specific for simvastatin. It should also be stressed that the current study focused solely on lipid metabolism. Verschuren et al⁴⁸ reported that in a transgenic mouse model a combined rosuvastatin/ezetimibe treatment enriched 16 biological processes not involved in lipid metabolism, none of which were affected by the individual drugs. These results should also be tempered with the knowledge that effects on lipid composition are statin-specific.²⁸ For example, Bergheanu et al²⁸ investigated the differential effects of rosuvastatin and atorvastatin on lipid composition. Both statins reduced the plasma levels of SMs; however, atorvastatin reduced the levels of PCs in plasma, whereas rosuvastatin increased PC levels. This differential response further supports the hypothesis that simvastatin directly affects phospholipid metabolism, rather than being a secondary effect of cholesterol reduction. It also raises the point that pleiotropic effects may be statin-specific, and it is not appropriate to discuss general statin-based effects. In addition, effects of combination therapy are most likely statin-dependent, with different statins interacting differently with ezetimibe. Several studies have looked at the effect of statin therapy on wider metabolism.^{42,49,50} Trupp et al⁴⁹ reported that simvastatin produced significant shifts in a range of metabolites, including several essential amino acids specifically those that are transported by cysteine and arginine transporters (cysteine, ornithine, arginine, and lysine). This would suggest that further statin-based studies should focus on a wider swathe of metabolic processes than lipids to more fully understand the metabolic effects of statin administration. Lastly, although the free acid forms of the lipid mediators were not significantly shifted after either treatment, there is evidence that structural lipids contain an abundance of esterified eicosanoid species.^{32,33} There is a subsequent need for the evaluation of the effect of statin therapy on structural lipid-bound eicosanoids and other lipid mediators to fully investigate potential pleiotropic effects of these treatments on the lipid mediator pool.

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Disclosures

None.

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KEY WORDS: eicosanoids ■ ezetimibe ■ hydroxymethylglutaryl-CoA reductase inhibitors ■ lipids ■ mass spectrometry ■ simvastatin

High-Dose Simvastatin Exhibits Enhanced Lipid-Lowering Effects Relative to Simvastatin/Ezetimibe Combination Therapy

Stuart G. Snowden, Dmitry Grapov, Magnus Settergren, Fabio Luiz D'Alexandri, Jesper Z. Haeggström, Oliver Fiehn, Tuulia Hyötyläinen, Theresa L. Pedersen, John W. Newman, Matej Oresic, John Pernow and Craig E. Wheelock

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