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

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Abstract

Statins are the frontline in cholesterol reduction therapies; however use in combination with agents that possess complimentary mechanisms of action may achieve further reductions in LDL-C. Thirty-nine patients were treated with either 80mg simvastatin (n=20) or 10mg simvastatin plus 10mg ezetimibe (n=19) for 6 weeks. Dosing was designed to produce comparable LDL-C reductions, while enabling assessment of potential simvastatin-associated pleiotropic effects. Baseline and post-treatment plasma were analyzed for lipid mediators (*e.g.*, eicosanoids, endocannabinoids) and structural lipids by liquid chromatography tandem mass spectrometry. Following statistical analysis and orthogonal projections to latent structures (OPLS) multivariate modeling, no changes were observed in lipid mediator levels, while global structural lipids were reduced in response to both mono- (R^2Y=0.74, Q^2=0.66, CV-ANOVA $p=7.0\times10^{-8}$) and combination therapy (R^2Y=0.67, Q^2=0.54, CV-ANOVA $p=2.6\times10^{-5}$). OPLS modeling identified a subset of 12 lipids that classified the two treatment groups after 6 weeks (R^2Y=0.65, Q^2=0.61, CV-ANOVA $p=5.4\times10^{-8}$). Decreases in the lipid species PC(15:0/18:2) and HexCer(d18:1/24:0) were the strongest discriminators of LDL-C reductions for both treatment groups ($q<0.00005$), while PE(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

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of sphingomyelin and ceramide to phosphatidylcholine (q=0.008), suggesting a pleiotropic effect previously associated with a reduced risk of cardiovascular disease.

Keywords

statin therapy; cholesterol-lowering drugs; eicosanoids; lipids; mass spectrometry

Introduction

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[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 (*e.g.*, 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-CoA reductase (HMGR), which is involved in the rate-limiting conversion of HMG-CoA to mevalonic acid in cholesterol biosynthesis[9].

In some instances statin intervention alone is insufficient to achieve the targeted cholesterol reduction and supplementary 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 Niemanh-Pick C1 like 1 (NPC1L1) 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 twofold, firstly in their ability to reduce absolute levels of LDL-C and TC, and secondly their so-called pleiotropic effects (*i.e.*, therapeutic effects unrelated to lipid lowering)[15], such as improving endothelial function[16]. In the present study, patients with dysglycaemia and coronary heart disease (CAD) 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 upon 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 CAD randomized to two treatments, an 80mg simvastatin monotherapy (n=20) and a combined 10mg simvastatin plus 10mg ezetimibe (n=19). The study was designed to achieve comparable reductions in LDL-C in both treatment groups. Since the combination of ezetimibe with low dose simvastatin is known to reduce LDL-C by ~50%, an 80mg simvastatin monotherapy dose was required to achieve comparative LDL-C reductions in the two groups[18] although the dose of 80mg simvastatin is no longer recommended in the new guidelines on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk[19]. Because ezetemibe as monotherapy is a weaker cholesterol-lowering compound than simvastatin, it was necessary to design the study with one group given low dose simvastatin together with ezetemibe 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 following a 12 h 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 gender imbalance or differences in medications between the two cohorts. Table 1 provides an overview of baseline group characteristics, none of which were significantly different between the two groups. However, for a detailed description of the cohorts the reader is directed to the original publication by Settergren et al. 2008[20].

Fasting blood samples were collected in EDTA tubes by puncture of a cubital vein. The samples were centrifuged at +4°C and 300*g* for 15 min. 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 (ECs) 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 (SPE) cartridges (Waters Oasis HLB 3cc, 60 mg). Prior to analysis, dried SPE eluents were reconstituted in 1:1 MeOH/acetonitrile containing 100nM 1-cyclohexyl-3-dodecanoic acid urea (CUDA; Sigma-Aldrich), and filtered by centrifugation using 0.1 μm Durapore PVDF (Millapore). Compounds were separated using a reverse phase gradient with a 2.1 × 150mm, 1.7μm Acquity BEH column on a Waters

Acquity UPLC, with ionization in negative mode by electrospray ionization (ESI). Data were acquired in multi-reaction monitoring mode (MRM) with an ABI 4000QTRAP triple quadrupole mass spectrometer. See supplemental methods for a detailed description.

Structural lipid analysis

A mixture of lipid standards (20 μl) were 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 × 100mm, 1.7μm Acquity BEH column on an Acquity Ultra Performance LC (UPLC) coupled to Waters Q-Tof Premier mass spectrometer. Lipid profiling was done in ESI in positive mode, and the data were collected at a mass range of *m/z* 300-1200 with scan duration of 0.2 sec. MZmine2 and an in-house spectral library were used for peak alignment, integration, identification and normalization. Relative lipid concentrations (μM) were calculated based on a ratio of peak heights (normalization) to corresponding standards followed by multiplication by the standards' concentration. Analytical 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 supplemental methods for a detailed description.

Statistical Analysis and Multivariate Modeling

Individual lipid species that were not observed in at least 75% of a given sample class or were structurally unidentified (n=490) were removed from all data analyses. Analyses were conducted separately for oxylipins, ECs and structural lipids using the R language for statistical computing (v3.0.1) 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 analytical and biological variances of each lipid.

Fisher's 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 weeks treatment were evaluated on logarithm (base 10) transformed values using paired t-Tests, the significance levels (*i.e.*, 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 Storey[24] 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]), seven-fold cross validated fit to the training data (Q^2[cum]), and model significance was estimated using cross validated analysis of variance (CV-ANOVA). Feature selection was performed using VIP (variable importance in projection) and p(corr) according to Wheelock ÅM and Wheelock CE[25]. See supplemental methods 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 two treatments[21], the coefficients of partial correlation, associated p-values and FDR adjusted p-values[23] were separately estimated for the mono- and combination therapy cohorts. Calculated networks were rendered in Cytoscape[26]. See supplemental methods for a detailed description.

Results

Both mono- and combination therapy produced significant reductions in TC, LDL-C and triglycerides (Table S1). C-reactive protein (CRP) was reduced (p_{adj}=0.0004) in response to the combination-, but not monotherapy. However, the fold change in CRP at 6 weeks relative from baseline was not significantly different between the two treatments (Table S1).

Eighty-one oxylipins, representing three metabolic pathways, cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) were screened. Of these, 35 were measured above the limit of detection (LOD), ranging in concentration between 30 pM and 87 nM (Table S1). The observed oxylipins were predominantly CYP450-, 12-and 15-LOX-dervied products of linoleic and arachidonic acid. Thirty-three ECs were screened, 12 of which were present above the LOD, ranging in concentration from 40 pM to 10.8 nM (Table S1). Statistical comparisons of both oxylipin and EC levels between baseline and 6 weeks did not identify any significantly changed species after mono- or combination therapies (Table S1). This observation was supported by OPLS-DA modeling, which did not produce informative models from either the oxylipin or EC lipid measurements (Figure S1 and Table S2).

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 (SM) and ceramide (Cer) (SM|Cer n=36), lysophosphatidylcholine (lysoPC, n=18), lysophosphatidylethanolamine (lysoPE, n=3), phosphatidylcholine (PC, n=78), phosphatidylethanolamine (PE, n=44), and triglyceride (TG, n=123).

Structural lipids displayed robust reductions in response to both mono- and combination therapy. Adjusting for FDR, monotherapy lead to significant changes in 213 of the structural lipids (69%), compared to 159 species for the combined treatment (51%) (Table 2, Figure S2 and S3). The two treatments shared 40% of the observed changes in common. SM and Cer (SM|Cer) displayed the largest decreases relative to baseline following monotherapy (-21±2%, p_{adj}<0.0001) and the second largest decrease of all lipid classes following the combined treatment (-15±2%, p_{adj}<0.0001). Only the monotherapy led to significant reduction in the ratio between SM|Cer and PC, SM|Cer/(SM|Cer+PC), (-8±2%, p_{adj}=0.033). Furthermore PC, PE, CE and TG lipid classes were all significantly decreased following both mono- and combination therapy, but monotherapy lead to greater absolute decreases. As a class, lysoPE lipids were unchanged after monotherapy, but reduced following the combined treatment (-7±5%, p_{adj}=0.0045). Similarly, lysoPC lipids showed deferential

regulation between the two treatments, and were increased after monotherapy (10±6%, p_{adj}=0.028), but decreased (-4±5%, p_{adj}=0.0022) following 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 S2). Models comparing changes in oxylipins and ECs could not identify significant compositional differences between baseline and six weeks treatment for either the mono- or combination therapy cohort (Figure S1), 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 $p=2.0\times10^{-4}$; Figure S2D) and combination therapies (R^2Y=0.732, Q^2=0.503, CV-ANOVA $p=8.9\times10^{-5}$; Figure S2C). 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- *vs.* 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 $p=7.0\times10^{-8}$) and combination therapy (R^2Y=0.67, Q^2=0.54, CV-ANOVA $p=2.6\times10^{-5}$). 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 S3). PC(15:0/18:2) and HexCer(d18:1/24:0) were the two top ranked predictors for both treatments. These two lipids were both significantly decreased by 50% following monotherapy and 40% and 30%, respectively after the combined treatment (Table S1). None of the top 10 predictors in Table 3 increased for either treatment. While multiple lipids increased significantly following either monotherapy or the combined treatment; only lysoPC(20:4) and PE(36:6e) significantly increased in response to both treatments (Table S1, Figure S5). 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 $p=5.4\times10^{-8}$). 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 (Figures 4 and S6). Partial correlations are commonly used to decouple direct from indirect variable associations, and in the case of highly intra-correlated lipids, offer a unique approach for generating simplified and often more informative dependency visualizations. Associations were calculated between the top lipid predictors of treatment effects following both therapies (Figure 1B and 1D) and clinical parameters (Table S1). The relationships were adjusted for FDR, and calculated separately for monotherapy and combination treatment to aid in the identification of nuanced changes

due to 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 following either therapy (Table 3). Similarly, clinically measured TG was significantly reduced following both treatments, but this change was deferentially 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) following 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 (KREA) levels. PC(15:0/18:2), the top predictor of treatment following either therapy, was significantly reduced following both treatments, and this decrease was positively correlated with similar decreases in PC(33:2) and PE(40:2) following 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 due to these species sharing the same acyl chain, linoleic acid (18:2), which is true for PC(15:0/18:2) and CE18:2 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 following 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 analytical (Table S3) 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 27-37% (Table S4). Consequently, the probability of a beta (Type II) error was ≤20% and the probability of alpha (Type I) errors were 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 to the aforementioned lipids, there may exist a bias towards lack of detection of changes in PE, CE and TG classes of lipids due to their increased analytical and biological variability (52-65%; Table S4).

Discussion

There is a sizeable body of literature examining the effects of both statins[2, 27], and combined statin/ezetimibe treatment[12-14] upon LDL-C and TC levels. However, relatively few studies have examined the effects these treatments exert upon 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 (*e.g.*, ECs and oxylipins).

The general effects upon oxylipin and EC 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-LOX[31]. In the current investigation, the 5-LOX product 5-HETE was reduced following 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 two, 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 upon lysophosphatidyl lipid metabolism. While the implications of this shift are unclear, it is of interest that the observed increase in lysoPC(20:4) following 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 expected[29]; 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 following both treatments (Tables S1) and this reduction was positively correlated with a decrease in PC(38:2) (Figure S6), which was a top ten VIP 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 one 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 decreases[39] 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 following the higher dose statin monotherapy (p=0.03, Table 2). Changes in SM were most closely related to PE, and Cer to lysoPC (Figure S6). 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 *vs.* combination therapy upon 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 CAD. 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, while 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). Following 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 following simvastatin therapy.

Both treatments led to a significant increases 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 atorvastatin[28]; however, Bergheanu *et al*. 2008 did not report how treatment affected the abundance of this lipid. Strauss *et al*. 2012 showed that Wistar (Crl:WI[Han]) rats exposed to 28 days of treatment with both atorvastatin (70mg/kg bw) and pravastatin (200mg/kg bw) showed significant reductions in lysoPC(20:4)[42]. These findings are inconsistent with results from the current study and those of Kaddurah-Daouk *et al*. 2010[29]. However the reductions in lysoPC(20:4) observed in Strauss *et al.* 2012 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 (PLA_2)-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 PLA_2 (Lp-PLA_2) has been shown to reduce atherosclerotic lesion lysoPC content leading to a reduction in the development of advanced coronary atherosclerosis[45]. Lp-PLA_2 in carotid artery plaques is a predictor of future cardiac events[46], and the associations between Lp-PLA_2 and lysoPCs (as well as lysophosphatidic acid; LPA) 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, while lysoPC(20:4) appears 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 following both treatments.

Our findings show that simvastatin monotherapy and simvastatin/ezetimibe combination therapy produce similar overall shifts in lipid levels; however, a number of treatment-specific effects were observed. While 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 $p=5.4\times10^{-8}$; 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 less than 0.05 (Table S1). Accordingly, a study with increased power is necessary to more fully examine the specificity of lipid-reduction therapy upon shifts in individual lipid species. In addition, in order to more fully determine the relative effects of simvastatin monotherapy *vs.* simvastatin/ezetimimibe combination therapy, additional work should include a group receiving ezetimimib 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 al*. 2012 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[48]. These results should also be tempered with the knowledge that effects on lipid composition are statin-specific[28]. For example Bergheanu *et al*. 2008 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, whilst rosuvastatin increased PC levels[28]. 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. A number of studies have looked at the effect of statin therapy on wider metabolism[42, 49, 50]. Trupp *et al*. 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)[49]. This would suggest that further statin-based studies should focus on a wider swathe of metabolic processes than lipids in order to more fully understand the metabolic effects of statin administration. Lastly, while the free acid forms of the lipid mediators were not significantly shifted following 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 in order to fully investigate potential pleiotropic effects of these treatments on the lipid mediator pool.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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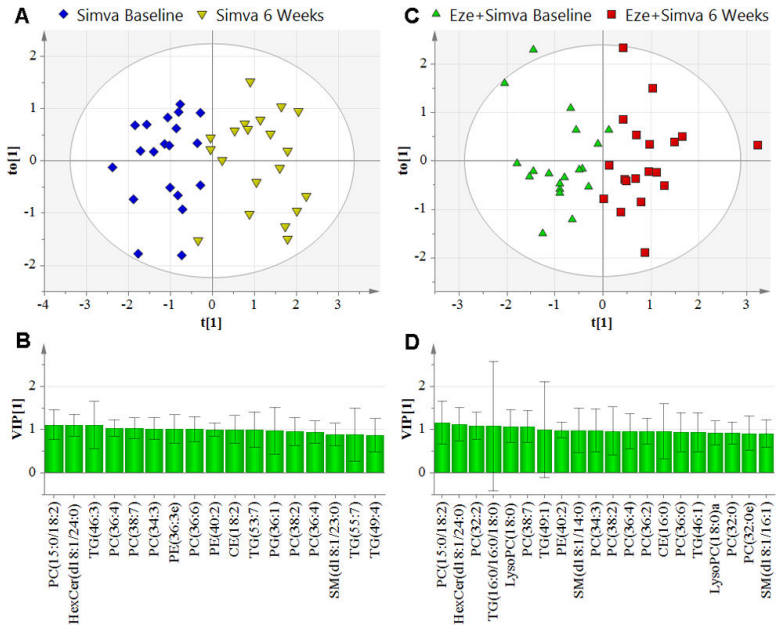

Figure 1.

OPLS-DA treatment-specific modeling of structural lipid data following 3 iterative rounds of variable selection. **A**) Scores plot of simvastatin monotherapy at baseline *vs.* 6 weeks (R^2Y=0.74, Q^2=0.66, 1+1 components, CV-ANOVA p=7.0×10^{-8}); **B**) Top 10 lipid species from the Variable Importance in Projection (VIP) plot of simvastatin monotherapy at baseline *vs.* 6 weeks; **C**) Scores plot of combination therapy at baseline *vs.* 6 weeks (R^2Y=0.67, Q^2=0.54, 1+1 components, CV-ANOVA p=2.6×10^{-5}); **D**) Top 10 lipid species from the Variable Importance in Projection (VIP) plot of combination therapy at baseline *vs.* 6 weeks. Eze=ezetimibe, Simva=simvastatin. The OPLS-DA models prior to variable selection are shown in Figure S2.

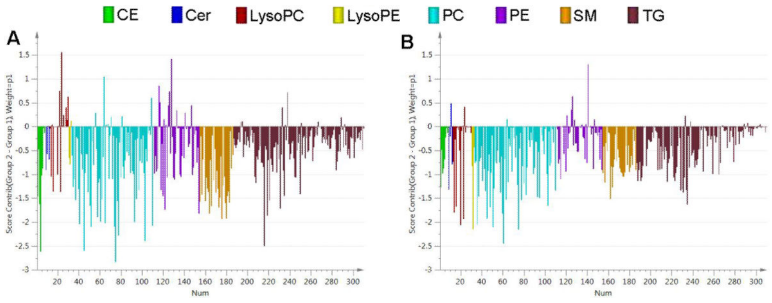

Figure 2.

Contribution plots showing the influence of individual structural lipid species in the OPLS-DA models. **A)** Simvastatin monotherapy baseline *vs*. 6 weeks (R^2Y=0.99, Q^2=0.73, CVANOVA p=2.0×10^{-4}; see Figure S1D); **B)** Combination therapy baseline *vs.* 6 weeks (R^2Y=0.76, Q^2=0.50, CV-ANNOVA p=8.9×10^{-5}; see Figure S1C). Abbreviations: CE=Cholesterol Esters, Cer=Ceramides, LysoPC=Lysophosphatidylcholines, LysoPE=Lysophosphatidylethanolamine, PE=Phosphatidylethanolamine, SM=Sphingomyelins, TG=Triglycerides.

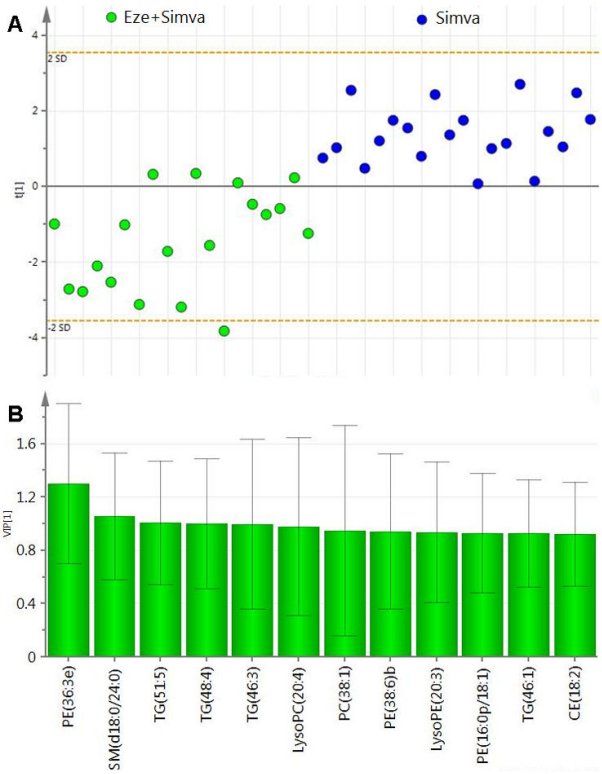

Figure 3.

OPLS-DA modeling of the ratio of structural lipid levels at baseline *vs.* 6 weeks following 3 iterative rounds of variable selection. **A**) Scores plot of simvastatin monotherapy *vs.* combination therapy (R^2Y=0.65, Q^2=0.61, 1+0 components, CV-ANOVA p=5.4×10^{-8}); **B**) Variable Importance in Projection (VIP) plot of the 12 lipid species driving the model. Eze=ezetimibe, Simva=simvastatin.

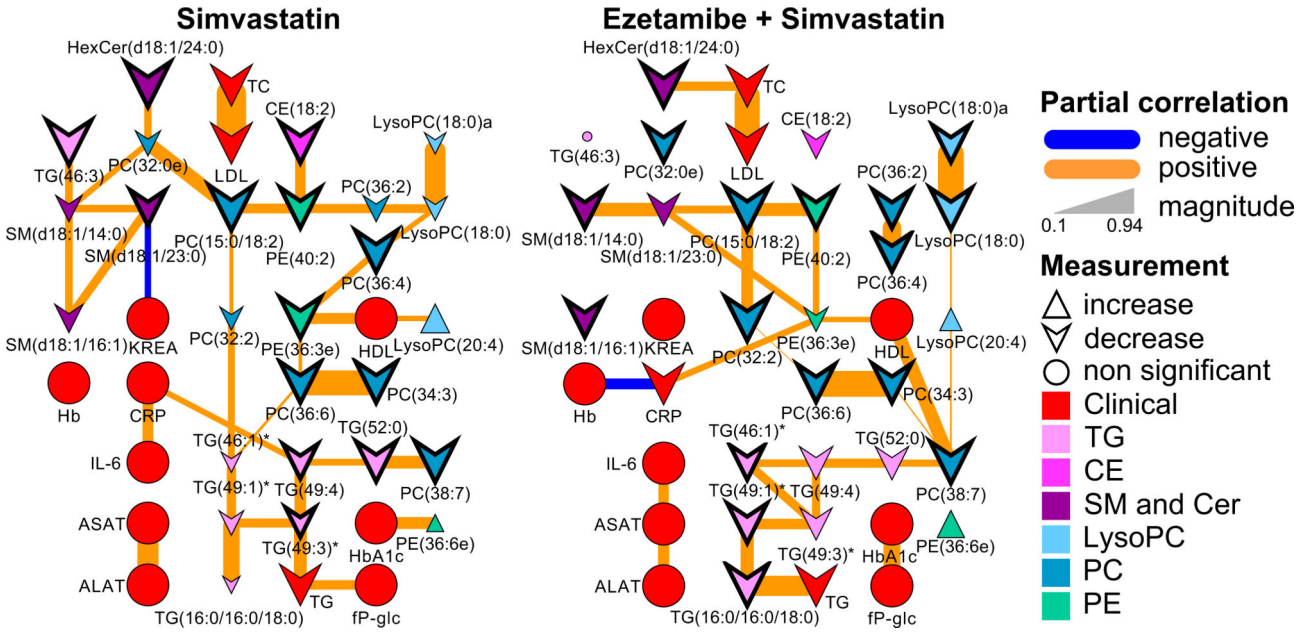

Figure 4.

Partial correlation network displaying conditionally independent relationships between selected changed lipids listed in Table 3 and clinical parameters. **A)** Simvastatin and **B)** Simvastatin and Ezetimibe treatment. 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 OPLS-DA model (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. Abbreviations: fP-glc=fasting glucose, ASAT=aspartate aminotransferase, ALAT=alanine aminotransferase, KREA=blood creatinine, Hb=hemoglobin.

Table 1

Baseline characteristics of cohorts*

Characteristic	Monotherapy (n=20)	Combination Therapy (n=19)
Age (years)	70 (62-74)	74 (66-77)
Female/male, n (%)	5 (25)/15 (75)	8 (42)/11 (58)
Body mass index (kg/m2)	28 (25-31)	28 (26-29)
Smokers, n (%)	4 (20)	4 (21)
Type 2 diabetes/impaired glucose tolerance, n (%)	17 (85)/3 (15)	19 (100)/0 (0)
Aspirin n (%)	20 (100)	16 (84)
Clopidogrel n (%)	3 (15)	2 (10)
Beta-blockers n (%)	18 (90)	15 (79)
Calcium channel-blockers n (%)	8 (40)	6 (31)
ACE-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 two groups at the $p<0.05$ level based on the Mann-Whitney U test. The gender composition was not significantly different between the two cohorts based on Fisher's exact test $p<0.05$.

Table 2

Comparison of changes in lipid classes following mono- and combination therapy.

	Monotherapy			Combination			Comparison//	
Lipid Class (N°)*	**% change†**	**p_{adj}-value‡**	**q-value§**	**% change**	**p_{adj}-value**	**q-value**	**p_{adj}-value**	**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

*Data are shown in graphical format in Figure S3. (N°) denotes the number of lipids per class. Abbreviations: CE=Cholesterol Esters, Cer=Ceramides, LysoPC=Lysophosphatidylcholines, LysoPE=Lysophosphatidylethanolamine, PE=Phosphatidylethanolamine, SM=Sphingomyelins, TG=Triglycerides, SM|Cer=Sphingomyelins and Ceramides combined.

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

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

§Direct estimate of FDR according to the methods of Storey [24].

//t-Test contrasting changes in lipids from baseline to 6 weeks in monotherapy compared to the combination therapy, adjusted for multiple hypotheses testing according to Benjamini and Hochberg at q=0.05 [23].

Table 3

The 10 variables with the strongest contribution to the OPLS-DA models comparing treatment at baseline *vs.* after 6 weeks

Simvastatin Baseline *vs.* 6 Weeks[*]				Combined Baseline *vs.* 6 Weeks[†]			
OPLS VIP Rank[‡]	Lipid Species[§]	FC[//]	p_{adj}[#]	OPLS VIP Rank	Lipid Species	FC	p_{adj}
1	*PC(I5:0/18:2)*	0.5	3.3×10^{-6}	1	*PC(I5:0/18:2)*	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}

– variables in common between the two models are italicized.

[*] See Figure 1C for details of simvastatin OPLS model.

[†] See Figure 1D for details of combined treatment OPLS model.

[‡] Variable ranking from the Variable Importance in Projection (VIP) plot from the OPLS-DA models shown in Figure 1.

[§] These species were confirmed by MS/MS experiments (see Figure S4 for the MS/MS spectra of PC(15:0/18:2) and HexCer(d18:1/24:0)). Abbreviations: CE=Cholesterol Esters, HexCer=hexosyl-ceramide, LysoPC=Lysophosphatidylcholine, PC=Phosphatidylcholine, PE=Phosphatidylethanolamine, SM=Sphingomyelins, TG=Triglycerides.

[//] Fold change of means relative to six weeks (see Table S1).

[#] p-values adjusted for multiple hypotheses testing according to Benjamini and Hochberg at q=0.05 [23] (see Table S1).