

Ezetimibe in Combination With Simvastatin Reduces Remnant Cholesterol Without Affecting Biliary Lipid Concentrations in Gallstone Patients

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Background—In randomized trials (SHARP [Study of Heart and Renal Protection], IMPROVE-IT [Improved Reduction of Outcomes: Vytorin Efficacy International Trial]), combination of statin and ezetimibe resulted in additional reduction of cardiovascular events. The reduction was greater in patients with type 2 diabetes mellitus (T2DM), where elevated remnant cholesterol and high cardiovascular disease risk is characteristic. To evaluate possible causes behind these results, 40 patients eligible for cholecystectomy, randomized to simvastatin, ezetimibe, combined treatment (simvastatin+ezetimibe), or placebo treatment during 4 weeks before surgery, were studied.

Methods and Results—Fasting blood samples were taken before treatment start and at the end (just before surgery). Bile samples and liver biopsies were collected during surgery. Hepatic gene expression levels were assessed with qPCR. Lipoprotein, apolipoprotein levels, and content of cholesterol, cholesteryl ester, and triglycerides were measured after lipoprotein fractionation. Lipoprotein subclasses were analyzed by nuclear magnetic resonance. Apolipoprotein affinity for human arterial proteoglycans (PG) was measured. Biomarkers of cholesterol biosynthesis and intestinal absorption and bile lipid composition were analyzed using mass spectrometry. Combined treatment caused a statistically significant decrease in plasma remnant particles and apolipoprotein B (ApoB)/lipoprotein content of cholesterol, cholesteryl esters, and triglycerides. All treatments reduced ApoB-lipoprotein PG binding. Simvastatin and combined treatment modified the composition of lipoproteins. Changes in biomarkers of cholesterol synthesis and absorption and bile acid synthesis were as expected. No adverse events were found.

Conclusions—Combined treatment caused atheroprotective changes on ApoB-lipoproteins, remnant particles, bile components, and in ApoB-lipoprotein affinity for arterial PG. These effects might explain the decrease of cardiovascular events seen in the SHARP and IMPROVE-IT trials.

Clinical Trial Registration—URL: www.clinicaltrialsregister.eu. Unique identifier: 2006-004839-30). (*J Am Heart Assoc.* 2018;7:e009876. DOI: 10.1161/JAHA.118.009876.)

Key Words: ApoB-lipoproteins • bile • proteoglycan binding • statin therapy • T2DM

Statins are the main treatment for primary and secondary prevention of cardiovascular disease (CVD). Efforts to reach guideline goal levels for low-density lipoprotein cholesterol (LDL-C), when statins are not sufficient or poorly

tolerated, have led to studies where lipid-lowering drugs have been used in combination with statins.¹ One such drug is ezetimibe, an inhibitor of cholesterol absorption that blocks the intestinal Niemann-Pick C1-like 1 (NPC1L1) protein

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Accompanying Data S1 and Tables S1 through S4 are available at <https://www.ahajournals.org/doi/suppl/10.1161/JAHA.118.009876>

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Clinical Perspective

What Is New?

- This study demonstrated that combined treatment of simvastatin and ezetimibe caused a significant decrease of plasma cholesterol, cholesteryl esters, and triglycerides in remnant particles without changing the lipid composition of bile.
- Simvastatin, ezetimibe, and combined treatment reduced the binding of apolipoprotein B (ApoB)-containing lipoprotein to human arterial proteoglycans.
- The expected increase in biliary cholesterol following treatment with ezetimibe alone did not occur in patients with gallstone disease, yet we found evidence that ezetimibe was able to block hepatic Niemann-Pick C1-like 1 (NPC1L1) in humans.
- Addition of ezetimibe to simvastatin did not affect the hepatic mRNA levels of the sterol regulatory element binding transcription factor 2 (*SREBF2*) system.

What Are the Clinical Implications?

- Combined therapy of simvastatin and ezetimibe seems to be an optimal treatment for lipid disorders characterized by elevated remnant cholesterol (ie, type 2 diabetes mellitus) to reduce cardiovascular events.
- Addition of ezetimibe to simvastatin does not increase biliary cholesterol, and this should not lead to an increased risk for cholesterol gallstone disease.

transporter.² Consistent with previous studies,³ the IMPROVE-IT (Improved Reduction of Outcomes: Vytorin Efficacy International Trial) trial recently reported that adding ezetimibe to simvastatin therapy after acute coronary syndrome resulted in additional reduction of plasma LDL-C levels by $\approx 22\%$.⁴ This resulted in a 2% absolute risk reduction of death from CVD, major coronary events (nonfatal myocardial infarction, unstable angina, or coronary revascularization), or nonfatal stroke.⁴ This benefit was more pronounced in the subgroup of patients with type 2 diabetes mellitus (T2DM), with 5.5% absolute risk reduction.⁵ No additional adverse muscle, gallbladder, or hepatic effects were reported in the IMPROVE-IT trial. Similar results were observed in the SHARP (Study of Heart and Renal Protection), where the safety and efficacy of plasma LDL-C reduction in patients with chronic kidney disease were assessed. Treatment with simvastatin and ezetimibe in combination resulted in an average plasma LDL-C lowering of 0.85 mmol/L and a 17% reduction in major atherosclerotic events (nonfatal myocardial infarction, coronary death, non-hemorrhagic stroke, or arterial revascularization), without any excess risk of persistent elevation of hepatic transaminases, hepatitis, gallstone disease, or pancreatitis compared with placebo.⁶ In this trial, 22% of the patients, equally distributed in the treatment and placebo group, had T2DM.⁷

Remnant cholesterol in plasma consists of free cholesterol and cholesteryl esters (CEs) carried in triglyceride-rich lipoproteins, chylomicron remnants, very low-density lipoproteins (VLDLs) and intermediate-density lipoproteins (IDLs). All these particles contain apolipoprotein B (ApoB) as their main apolipoprotein. Remnant cholesterol can be estimated by subtracting LDL-C and high-density lipoprotein (HDL) cholesterol from total plasma cholesterol.⁸ The European Atherosclerosis Society recommends that nonfasting lipid profiling, including calculation of remnant cholesterol, can be used routinely in clinics given that both fasting and nonfasting conditions have been shown to be good predictors for CVD and for the evaluation of treatment response. Nonfasting remnant cholesterol ≥ 0.9 mmol/L and fasting remnant cholesterol ≥ 0.8 mmol/L can be considered as abnormal.⁹

Both elevated LDL-C and remnant cholesterol increase the risk for CVD. In contrast to LDL-C, elevated remnant cholesterol is also associated with a systemic low-grade inflammation.⁸ The residual CVD risk observed after lowering of LDL-C may partly be related to remnant particles that can be retained in the arterial intima by the interaction of the apoB-100 with extracellular intima proteoglycans.^{10,11} Therefore, the ability of a lipid-lowering therapeutic regimen to reduce circulating levels of all ApoB particles that can deposit cholesterol and CEs in the arteries (ie, remnants, IDL, and LDL) could be just as important as its ability to lower LDL-C.^{10,11}

Three enzymes are responsible for cholesterol esterification. Lecithin-cholesterol acyltransferase (LCAT) esterifies cholesterol only in the plasma, whereas acyl-coenzyme A: cholesterol acyltransferase (ACAT) 1 and 2 both acts intracellularly.¹² Intestinal and hepatic ACAT2 synthesizes the CEs used for assembly and secretion of nascent chylomicrons and VLDL, respectively.^{12,13} CEs synthesized by LCAT is transferred to VLDL.¹⁴ Atorvastatin has been shown to reduce the expression and activity of hepatic ACAT2, thereby lowering CEs in VLDL.¹⁵ Ezetimibe inhibits intestinal NPC1L1 and reduces the amount of cholesterol that can be esterified by intestinal ACAT2 and secreted with chylomicrons.¹⁶ Large and small dense LDLs (sdLDLs) are remnants of secreted VLDL, and their CEs are produced by hepatic ACAT2 and plasma LCAT.^{12,17} Concentration of sdLDL can be used to predict the risk of developing CVD in patients with T2DM.^{18,19} In these patients, the combination of rosuvastatin and ezetimibe reduced sdLDL-C more efficiently than doubling the rosuvastatin dose.²⁰ sdLDL particles are more prone to oxidation,²¹ glycation,²² and have a higher binding affinity to arterial proteoglycans (PG).²³ This last property is associated with cholesterol deposition in the intima and CVD and can be reduced by lipid-lowering treatments.^{24–26} Plasma ApoB (ApoB-100)-containing lipoprotein binding to human intima

PG causes lipoprotein deposition in the arterial wall. This is one of the initial steps in intimal deposition of cholesterol and consequent atherogenesis and is a central concept in the current “response to retention hypothesis” of atherosclerosis.^{11,23–31}

In contrast to mice, hepatic NPC1L1 is highly expressed in humans.³² The role and effects of ezetimibe on human hepatic NPC1L1 remain uncertain given that most of the available data are based on either in vitro models³³ or mouse models overexpressing human NPC1L1.^{34,35} The results from recent studies performed so far in humans are not conclusive because of very small sample size in addition to limitations of duodenal bile sampling methods.^{36,37}

In the present study, we evaluated whether ezetimibe in association with simvastatin has the ability to decrease cholesterol and CEs in remnant particles and LDL, and whether this translates into a reduced PG binding. We assessed the effects of ezetimibe on biliary lipid composition to gain more insight into the function of hepatic NPC1L1. In addition, the effects on mRNA levels of genes involved in the hepatic cholesterol metabolism were analyzed.

Methods

The data, analytical methods, and study materials will be made available on request to other researchers for the purposes of reproducing the results or replicating the procedure. All data are electronically stored at the division of Clinical Chemistry, Department of Laboratory Medicine, Karolinska Institutet and can be available to other researchers by contacting the coordinator (Osman Ahmed) or the principle investigators (Paolo Parini or Mats Eriksson) of the Stockholm study.

Patients, Treatments, and Specimen Collection

Forty patients (14 males, 13 fertile, and 13 postmenopausal females) with uncomplicated cholesterol gallstone disease, eligible for elective cholecystectomy at the Department of Surgery, Danderyd Hospital, Danderyd, Sweden, were enrolled in this study. The study was approved by the Stockholm Region Ethical Board (2006/1204-31/1), the Swedish Medical Agency, and registered at the EU clinical trial register (EudraCT number: 2006-004839-30). All patients gave their written informed consent to participate in this study.

Inclusion Criteria

Patients with gallstone disease, eligible to cholecystectomy, were aged between 25 and 80 years.

Exclusion Criteria

Patients with obesity (body mass index, >30), known familial phytosterolemia, active liver disease, or hepatic dysfunction (aspartate aminotransferase [S-AST] or alanine aminotransferase [S-ALT] ≥ 3 times the upper limit of reference), elevated creatinine phosphokinase (S-CPK), partial ileal bypass, metabolic diseases (ie, T2DM), inflammatory bowel disease, or other serious disease were not enrolled. Patients with known hypersensitivity to 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, ezetimibe, or treatment with drugs known to be associated with rhabdomyolysis in combination with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors were excluded.

Study Procedure

After a first screening-visit, patients qualifying for inclusion were randomized into 4 different treatment groups: simvastatin 80 mg daily (S), ezetimibe 10 mg daily (E), simvastatin 80 mg and ezetimibe 10 mg daily (S+E), or placebo (P). Treatment started 4 weeks (25–30 days) before the surgery and ended at the end-of study visit, preceding the surgical intervention. Investigators were aware of the treatment received, because this was a randomized single-blind study.

Fasting blood samples were collected at the first screening visit and at the end-of study visit. Liver biopsies and bile were collected after an overnight fast during the surgical intervention. All samples were rapidly frozen and stored at -70°C until analysis.

Statistical Analysis

Sample-size determination

The primary end points of the study were the relative reduction of LDL-C and the relative changes in hepatic gene expression. No data on the variation of gene expression in humans in a Swedish population were available. Therefore, we used LDL-C as the variable on which sample size and power for reaching significance in a 2-sided test at the 5% level (F test; 1-way ANOVA; fully randomized design) were measured. Mean LDL-C concentration in our Stockholm reference group was estimated to be 3.68 mmol/L, with an SD equal to 1.21. Considering an LDL-C lowering effect of 15%, 30%, and 50% by treatment with ezetimibe, simvastatin, and ezetimibe plus simvastatin, respectively (effect-size “ f ”: 0.6355), the calculated power was 0.8 if there were 10 patients in each treatment group for the study.

All data are presented as mean \pm SEM, unless otherwise stated. When appropriate, data were log transformed before parametric analysis when homoscedasticity was not present (unequal variance of different groups). Significance was tested by multiway ANOVA. Post hoc comparisons were done

according to the Fisher's least significant difference test. Outlier analysis was performed for all variables and rejection was done if outliers were present.

All statistical analyses were performed using Statistica (version 12.0; StatSoft, Tulsa, OK; Data S1).

Results

Effects on Total Body Cholesterol Synthesis and Intestinal Cholesterol Absorption

In this study, patients were randomized to treatment with placebo, simvastatin 80 mg/day, ezetimibe 10 mg/day, or combined simvastatin 80 mg/day and ezetimibe 10 mg/day (Table 1). Cholesterol synthesis (assessed by the total plasma lathosterol to cholesterol ratio) was reduced by simvastatin (−56%; $P<0.001$) and combined treatment (−29%; $P<0.001$), but increased by ezetimibe as a monotherapy (40%; $P<0.01$; Figure 1). As expected, simvastatin increased intestinal cholesterol absorption, assessed by the plasma campesterol to cholesterol ratio (21%; $P<0.05$), whereas ezetimibe reduced it as a monotherapy (−52%; $P<0.001$) and in combination with simvastatin (−41%; $P<0.01$; Figure 1).

Effects on Lipoproteins and Lipoprotein Subclasses

In order to determine in more detail the lipoprotein composition, plasma samples were subjected to analysis by nuclear magnetic resonance. Remnant cholesterol and LDL-C were reduced by both simvastatin (−51% and −52%; $P<0.001$) and ezetimibe (−18%; $P<0.001$ and −14%; $P<0.05$). Combined treatment with simvastatin and ezetimibe led to an even greater reduction of both remnant cholesterol (−65%; $P<0.001$) and LDL-C (−64%; $P<0.001$; Figure 2A). Simvastatin, ezetimibe, and the combined treatment also led to a reduction of remnant CEs (−53%, −20%, and −68%, respectively; $P<0.001$; Figure 2B). Ezetimibe added to simvastatin treatment showed an additional reduction (−15%; $P<0.01$) of remnant CEs compared with simvastatin alone (Figure 2B).

Simvastatin and combined treatment with ezetimibe showed reduction (−37%; $P<0.01$ and −50%; $P<0.001$) of remnant triglyceride (Figure 2C). Similarly, simvastatin and combined treatment with ezetimibe reduced LDL-triglyceride (−35% and −42%, respectively; $P<0.001$) and HDL-triglyceride (−23%; $P<0.01$ and −32%; $P<0.001$; Figure 2C). Ezetimibe in monotherapy had no significant effect on triglyceride levels. The lipid contribution in lipoprotein fractions is given in Table S1.

Lipoprotein fractions were divided into subclasses by nuclear magnetic resonance. The contribution of the

different subclasses to their respective fraction (ie, remnant, LDL, and HDL) baseline is presented in Table S2. Detailed analysis of the 7 different non-LDL, non-HDL lipoprotein subclasses (diameter size range down to 28.6 nm) showed that simvastatin alone or in combination with ezetimibe reduced particle number ($P<0.001$), triglyceride ($P<0.001$), and CE ($P<0.001$) in all subclasses (Figure 3). Ezetimibe treatment alone reduced the particle number in 3 subclasses (XXL-VLDL/chylomicron, XS-VLDL, and IDL; $P<0.05$), triglyceride in 1 subclass (XXL-VLDL/chylomicron; $P<0.05$), and CEs in all subclasses except XL-VLDL ($P<0.05$; Figure 3).

Analyses of LDL subclasses following treatment with simvastatin alone or in combination with ezetimibe showed reduced particle number ($P<0.001$), triglyceride ($P<0.001$), and CEs ($P<0.001$) in all 3 LDL subclasses (diameter size range, 18.7–25.5 nm; Figure 4A through 4C). Ezetimibe as monotherapy reduced particle number ($P<0.05$), triglyceride ($P<0.01$) in S-LDL (diameter size, 18.7 nm), and CEs ($P<0.05$) in L-LDL particles (diameter size, 25.5 nm; Figure 4A and 4C).

Evaluation of the effects on the 4 different HDL subclasses (diameter size range, 8.7–14.3 nm) showed that the different treatments only affected the larger HDL subclasses (L-HDL and XL-HDL; Figure 4D and 4E). Combined treatment reduced the particle number ($P<0.05$) in the XL-HDL subclass (diameter size, 14.3 nm). Triglycerides were reduced by simvastatin ($P<0.001$), ezetimibe ($P<0.05$), and by combined treatment ($P<0.001$), with a clear additive effect when ezetimibe was combined with simvastatin ($P<0.05$) in the XL-HDL subclass. Simvastatin and combined treatment reduced triglyceride ($P<0.05$) and increased CEs ($P<0.05$) in the L-HDL subclass (diameter size, 12.1 nm; Figure 4D and 4E).

Effects on the Major Apolipoproteins

ApoB levels were reduced by simvastatin (−38%; $P<0.001$), ezetimibe (−13%; $P<0.01$), and combined treatment (−48%; $P<0.001$). Ezetimibe had an additive effect in combination with simvastatin compared with simvastatin treatment alone ($P<0.05$; Figure 5A). No significant differences in plasma ApoA1 levels were observed. Thus, the ApoB to ApoA1 ratio was reduced following simvastatin, ezetimibe, or combined treatment (−34%; $P<0.001$, −10%; $P<0.05$ and −45%; $P<0.001$, respectively; Figure 5A). Ezetimibe had an additive effect on the ApoB to ApoA1 ratio in combination with simvastatin compared with simvastatin alone ($P<0.01$; Figure 5A). Similar decreases in ApoE levels were observed in the simvastatin (−24%; $P<0.01$) and combined treatment groups (−24%; $P<0.01$), whereas ezetimibe alone had no significant effect (Figure 5B). In addition, no significant effects were observed in ApoCIII levels (Figure 5B).

Table 1. Baseline Clinical Characteristics and Plasma Lipid Levels

	Placebo (n=10)	Simvastatin (n=10)	Ezetimibe (n=10)	Simvastatin+Ezetimibe (n=10)	P Value
Sex (M/FF/PF)	4/3/3	3/4/3	4/3/3	3/3/4	NS
Age, y	57.7±3.40	57.4±6.20	54.1±5.70	59.4±4.60	NS
BMI	25.8±0.70	26.4±0.80	25.5±0.80	25.2±0.90	NS
Plasma total cholesterol, mmol/L	5.80±0.40	5.80±0.30	5.50±0.40	5.50±0.30	NS
Plasma triglycerides, mmol/L	1.10±0.20	1.70±0.60	1.10±0.10	1.00±0.60	NS
Remnant cholesterol (non-LDL, non-HDL), mmol/L	0.90±0.20	1.10±0.30	0.80±0.10	1.00±0.10	NS
LDL cholesterol, mmol/L	3.10±0.30	3.20±0.20	3.20±0.20	3.00±0.20	NS
HDL cholesterol, mmol/L	1.80±0.20	1.50±0.10	1.60±0.10	1.50±0.20	NS
Apolipoprotein B, g/L	0.87±0.70	0.98±0.12	0.78±0.04	0.84±0.05	NS
Apolipoprotein A1, g/L	1.60±0.05	1.57±0.03	1.41±0.04	1.41±0.03	<0.05
Apolipoprotein E, g/L	0.05±0.00	0.05±0.00	0.05±0.00	0.04±0.00	<0.05
Apolipoprotein CIII, g/L	0.95±0.04	0.73±0.15	0.69±0.12	0.95±0.09	NS
Lathosterol/cholesterol, mg/mmol cholesterol	430±30.00	433±62.00	559±43.00	473±24.00	<0.05
Campesterol/cholesterol, mg/mmol cholesterol	568±74.00	697±108.0	511±58.00	489±39.00	NS

Data show mean±SEM. Lipoprotein profiling was determined after separation by size-exclusion chromatography. Multiway ANOVA followed by LSD test. BMI indicates body mass index; FF, fertile female; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LSD, least significant difference; M, male; NS, not significant; PF, postmenopausal female.

Effects on Plasma Binding to Human Aortic PG

Simvastatin, ezetimibe, and the combined treatment reduced plasma binding to arterial PG (−53%; $P<0.001$, −17%; $P<0.01$, and −57%; $P<0.001$, respectively) compared with placebo (Figure 6A). When plasma binding was divided by total cholesterol, simvastatin and combined treatment showed (−40%; $P<0.001$ and −28%; $P<0.01$) reduction, respectively, whereas the effects by ezetimibe were lost (Figure 6B). After correction for ApoB content, binding to human arterial PG was reduced by simvastatin (−27%; $P<0.01$), by ezetimibe (−11%; $P<0.05$), and by the combined treatment (−21%; $P<0.01$; Figure 6C).

When the percentage change from baseline in plasma PG binding was correlated with the percentage change from baseline in each lipoprotein subclass (Table S3), it was clear that the reduction in particle number and lipid composition (CEs and triglyceride) of all ApoB-lipoprotein contributed to the reduction of PG binding and especially the reduction of the remnant CEs.

Effects on Biliary Lipid Composition and on Bile Acids Synthesis

The effect of intestinal cholesterol absorption and/or cholesterol synthesis inhibition on biliary lipid composition in gallbladder bile after an overnight fast was assessed. As expected,¹⁵ simvastatin treatment reduced the absolute

biliary cholesterol concentration (−51%; $P<0.01$) when compared with placebo (Table 2). This reduction was still present when the % molar concentration of biliary cholesterol was calculated (−21%; $P<0.05$; Figure 7A). In contrast, ezetimibe alone had no significant effect on the absolute biliary cholesterol or on the % molar biliary cholesterol concentrations (Table 2 and Figure 7A). Addition of ezetimibe to simvastatin treatment increased the absolute concentration of all biliary lipids when compared with simvastatin alone ($P<0.05$ for all; Table 2). The new ratios between bile acids, phospholipids, and cholesterol translated to a loss of significant reduction in the % molar biliary cholesterol concentration when compared with placebo (Figure 7A). Hence, only significant reduction of cholesterol saturation index was observed following treatment with simvastatin (−15%; $P<0.05$; Figure 7D).

Simvastatin, ezetimibe, and combined therapy reduced the absolute concentration of campesterol in bile compared with placebo (−43%, −51%, and −54% respectively; $P<0.01$; Table 2). No significant effects were observed on the composition of individual bile acids or on bile acid synthesis (assayed by C4 to cholesterol ratio; Table 2).

Effects on Genes Involved in Hepatic Cholesterol Metabolism

The mRNA levels of key genes in hepatic cholesterol metabolism were analyzed to investigate the effect of

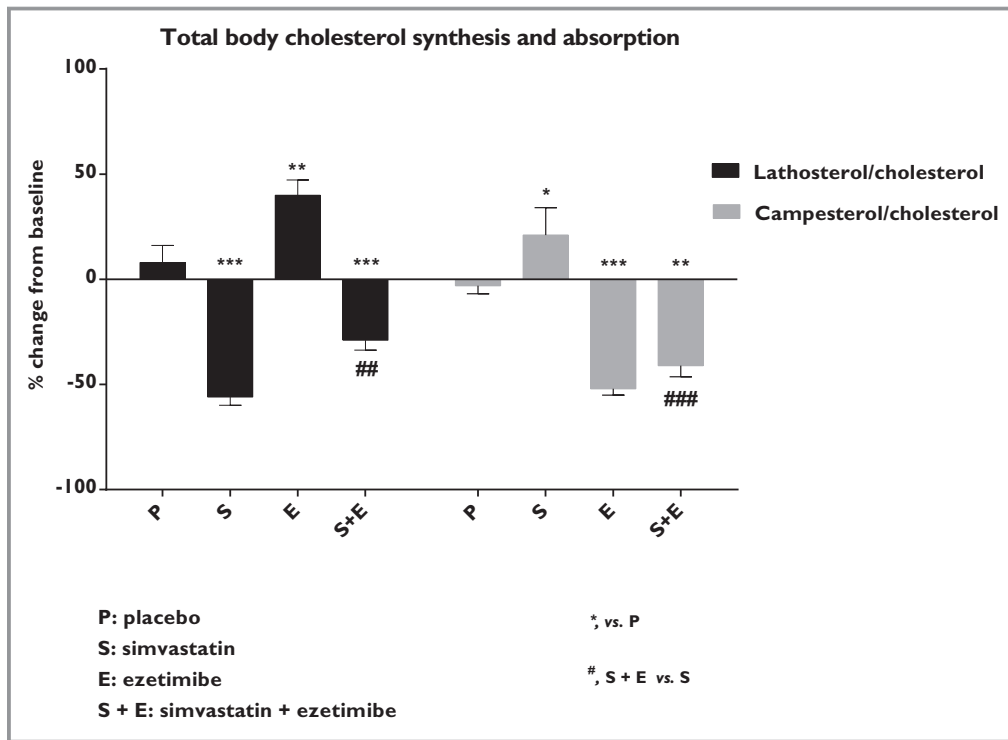


Figure 1. Effects on total body cholesterol synthesis and intestinal cholesterol absorption. The plasma lathosterol/cholesterol ratio is an indirect measurement of total body cholesterol synthesis whereas the plasma campesterol/cholesterol ratio reflects intestinal cholesterol absorption. Data show the % change from baseline and are expressed as mean±SEM. Baseline values are given in Table 1. Multiway ANOVA followed by LSD test. *vs placebo, $P<0.05$; **vs placebo, $P<0.01$; ***vs placebo, $P<0.001$; #simvastatin+ezetimibe vs simvastatin, $P<0.05$; ##simvastatin+ezetimibe vs simvastatin, $P<0.01$; and ###simvastatin+ezetimibe vs simvastatin, $P<0.001$ ($n=8-10$ /treatment group). LSD indicates least significant difference.

intestinal cholesterol absorption and/or cholesterol synthesis inhibition. Simvastatin and combined treatment significantly increased hepatic mRNA expression of sterol regulatory element binding transcription factor 2 (*SREBF2*) by 2-fold, 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*; 3.5- and 4-fold, respectively), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGCS1*; 5.5- and 6.5-fold, respectively), low-density lipoprotein receptor (*LDLR*; 3.5- and 2-fold, respectively), and proprotein convertase subtilisin/kexin type 9 (*PCSK9*; 3.5- and 2-fold, respectively; Figure 8A and Table 3). Simvastatin increased hepatic expression of *NPC1L1* 2-fold and microsomal triglyceride transfer protein (*MTP*) 2-fold. Ezetimibe added to simvastatin treatment caused 2-fold reduction in hepatic expression of *MTP* and cholesteryl ester transfer protein (*CETP*), compared with simvastatin monotherapy (Figure 8B and Table 3).

Safety Parameters

No significant between-group differences were observed in the percentage change from baseline of S-ALT, S-CPK, or

gamma-glutamyltransferase (Table S4). No adverse events were reported during or after the treatment period.

Discussion

In this randomized, single-blind, placebo-controlled study, we found previously unreported effects on plasma lipoprotein particle size, composition, apolipoprotein content, and binding to human arterial proteoglycan following simvastatin, ezetimibe, or combined treatment. In addition, we identified differences in hepatic gene expression of some key genes regulating cholesterol metabolism in the liver and different circulating lipoproteins.

In contrast to mice, humans express *NPC1L1* in the liver and its role for hepatic cholesterol and lipoprotein metabolisms has not been fully elucidated. Using a human transgenic mouse model, Temel et al³⁴ suggested that hepatic *NPC1L1* is responsible for biliary cholesterol reuptake and is a target for ezetimibe. Our study confirms that this hypothesis is also true in humans. We found that inhibition of cholesterol synthesis with simvastatin leads to a compensatory reduction of cholesterol secretion into bile

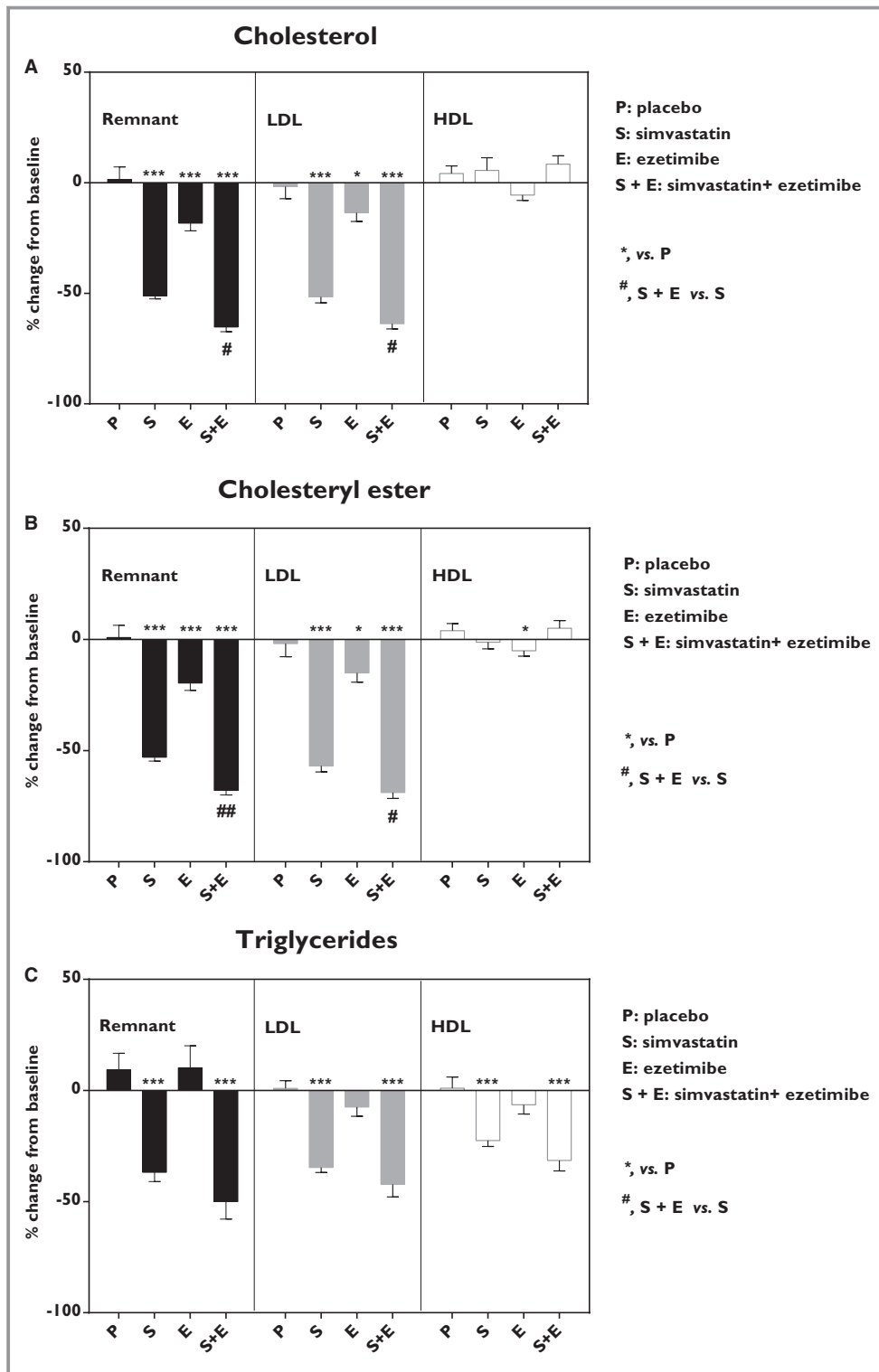


Figure 2. Effects on plasma lipoproteins. **A**, Cholesterol, **(B)** cholesteryl ester, and **(C)** triglycerides. Remnant particles, that is, non-high-density lipoproteins (non-HDL) and non-low-density lipoproteins (non-LDL; black), LDL particles (gray) and HDL particles (white). Data show the % change from baseline and are expressed as mean±SEM. Fasted plasma lipoproteins were subjected to analysis by nuclear magnetic resonance. Lipid contribution in lipoprotein fractions are given in Table S1. Multiway ANOVA followed by LSD test. *vs placebo, $P<0.05$; ***vs placebo, $P<0.001$; #simvastatin+ezetimibe vs simvastatin, $P<0.05$; and ##simvastatin+ezetimibe vs simvastatin, $P<0.01$ ($n=8-10$ /treatment group). LSD indicates least significant difference.

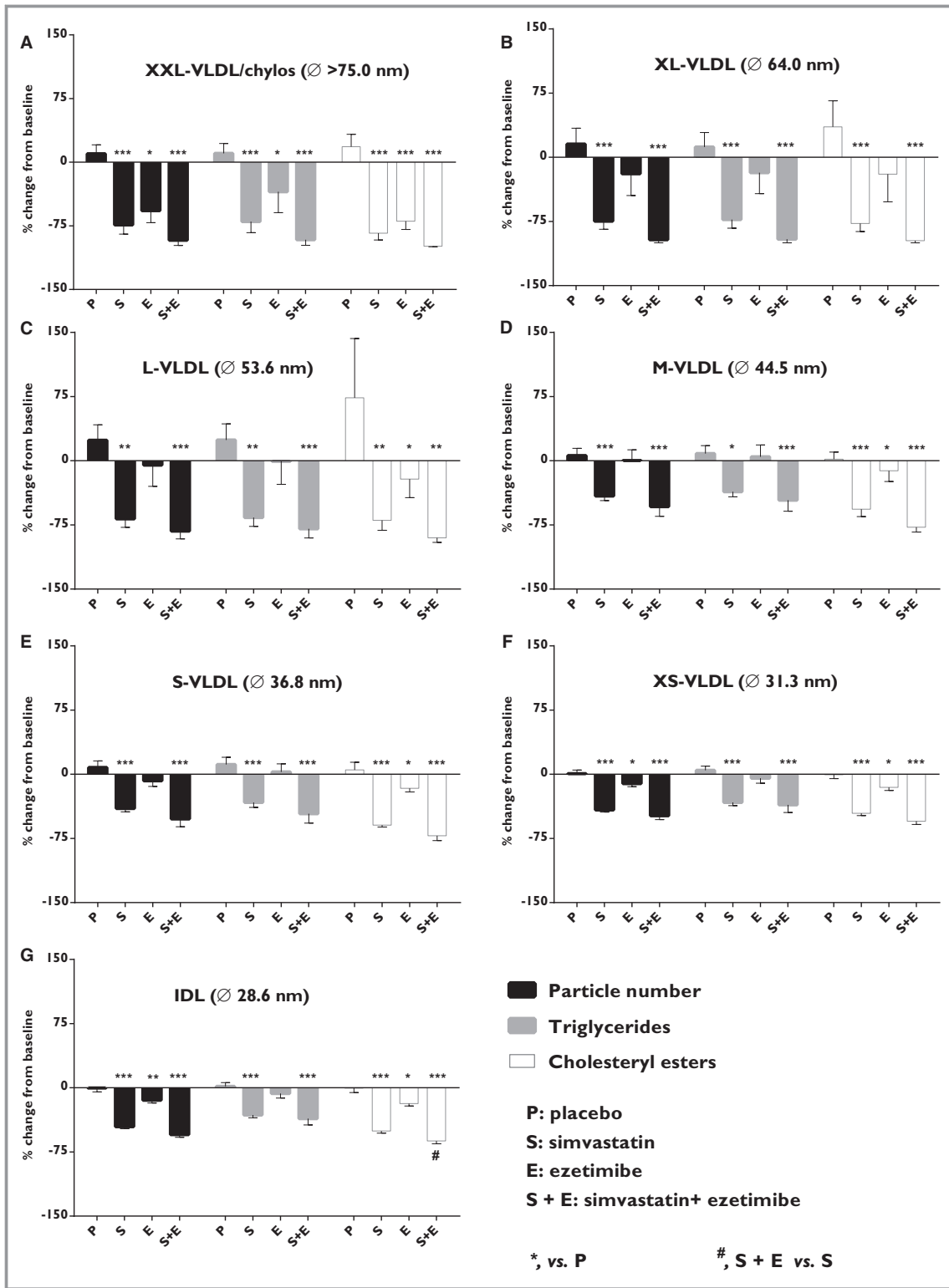


Figure 3. Effects on subclasses of non-LDL, non-HDL particles. Particle numbers (black), cholesteryl esters (white), and triglycerides (gray) were quantified in (A) extra-large very-low-density lipoproteins (XXL-VLDL)/chylomicrons, (B) extra-large-VLDL (XL-VLDL), (C) large-VLDL (L-VLDL), (D) medium-VLDL (M-VLDL), (E) small-VLDL (S-VLDL), (F) extra-small-VLDL (XS-VLDL), and (G) intermediate-density lipoprotein (IDL). Data show the % change from baseline and are expressed as mean±SEM. Fasted plasma lipoproteins were subjected to analysis by nuclear magnetic resonance. Multiway ANOVA followed by LSD test. *vs placebo, $P<0.05$; **vs placebo, $P<0.01$; ***vs placebo, $P<0.001$; and #simvastatin+ezetimibe vs simvastatin, $P<0.05$ ($n=8-10$ /treatment group). LSD indicates least significant difference.

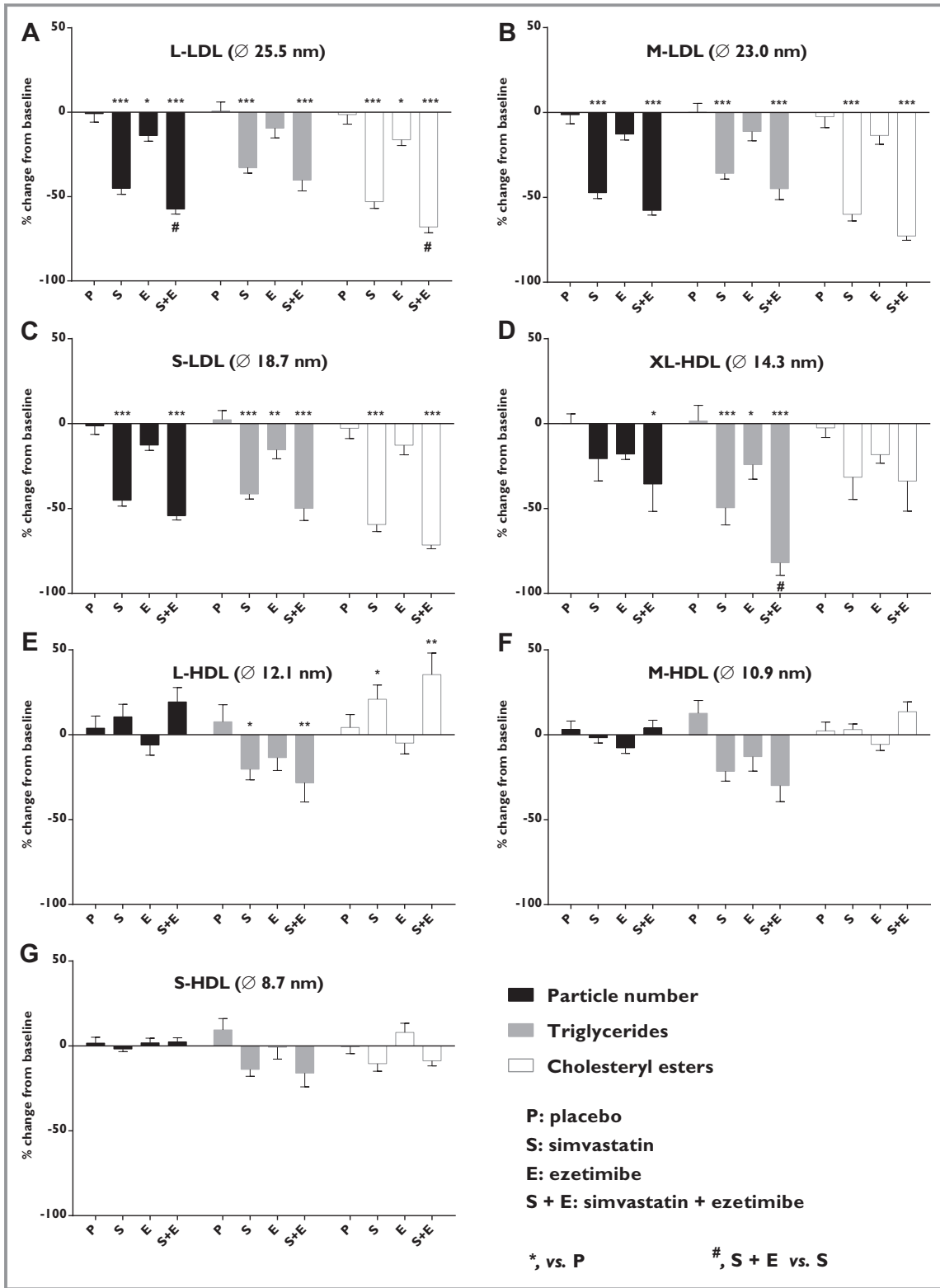


Figure 4. Effects on subclasses of LDL and HDL particles. Particle numbers (black), cholesteryl esters (white) and triglycerides (gray) were quantified in (A) large low-density lipoproteins (L-LDL), (B) medium-LDL (M-LDL), (C) small-LDL (S-LDL), (D) extra-large high-density lipoproteins (XL-HDL), (E) large-HDL (L-HDL), (F) medium-HDL (M-HDL), and (G) small-HDL (S-HDL). Data show the % change from baseline and are expressed as mean±SEM. Fasted plasma lipoproteins were subjected to analysis by nuclear magnetic resonance. Multiway ANOVA followed by LSD test. *vs placebo, $P<0.05$; **vs placebo, $P<0.01$; ***vs placebo, $P<0.001$; and #simvastatin+ezetimibe vs simvastatin, $P<0.05$ ($n=8-10$ /treatment group). LSD indicates least significant difference.

and to a consequent decrease in biliary cholesterol saturation index. Inhibition of cholesterol synthesis activates the *SREBF2* pathway, which also control hepatic *NPC1L1* expression in humans.^{38,39} Thus, the decreased biliary cholesterol that follows inhibition of cholesterol synthesis seems to be secondary to the concomitant 2-fold increase in hepatic *NPC1L1* expression and to a decrease of the intracellular cholesterol pool available for biliary secretion. The observation that ezetimibe in monotherapy did not affect biliary cholesterol in humans argues against the hypothesis that hepatic *NPC1L1* is also a target of this drug,

given that Temel et al³⁴ observed an increase in biliary cholesterol after treating transgenic mice with ezetimibe. This discrepancy may have different explanations. In gallstone disease a low hepatic expression of *NPC1L1* has been reported,⁴⁰ and this may reduce the magnitude of the effect of ezetimibe on hepatic *NPC1L1*. Alternatively, inhibition of intestinal cholesterol absorption by ezetimibe may lead to a decrease in the hepatic cholesterol pool available to biliary secretion counterbalancing the expected increase in biliary cholesterol that ezetimibe would have induced. When ezetimibe was added to statin treatment, a new condition

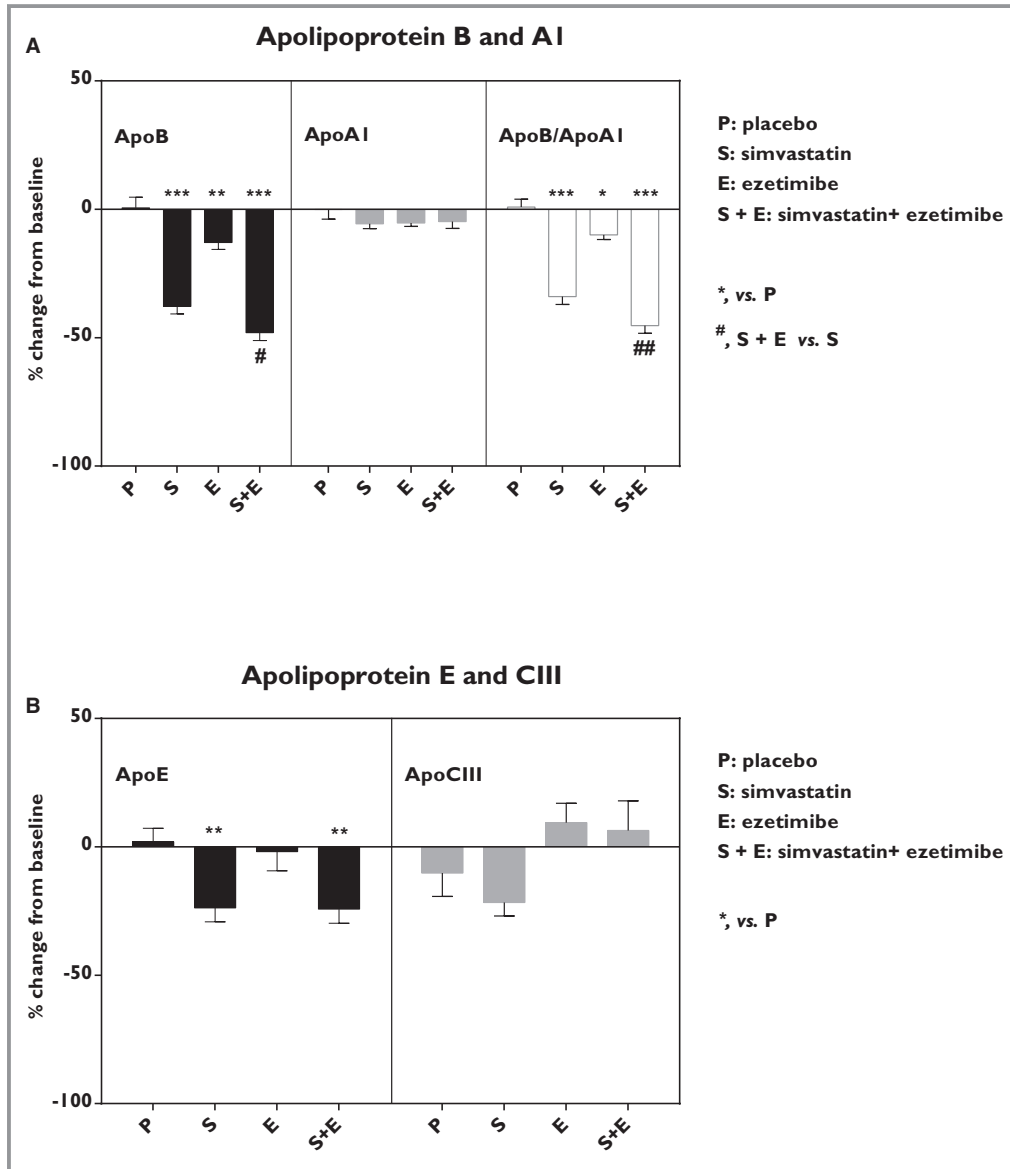


Figure 5. Effects on apolipoproteins. Levels of: **(A)** apolipoprotein B (ApoB) and apolipoprotein A1 (ApoA1) and the calculated ApoB/ApoA1 ratio, and **(B)** apolipoprotein E (ApoE) and apolipoprotein CIII (ApoCIII). Data show the % change from the baseline and are expressed as mean±SEM. Multiway ANOVA followed by LSD test. *vs placebo, $P<0.05$; **vs placebo, $P<0.01$; ***vs placebo, $P<0.001$; #simvastatin+ezetimibe vs simvastatin, $P<0.05$; and ##simvastatin+ezetimibe vs simvastatin, $P<0.01$ (n=8–10/treatment group). LSD indicates least significant difference.

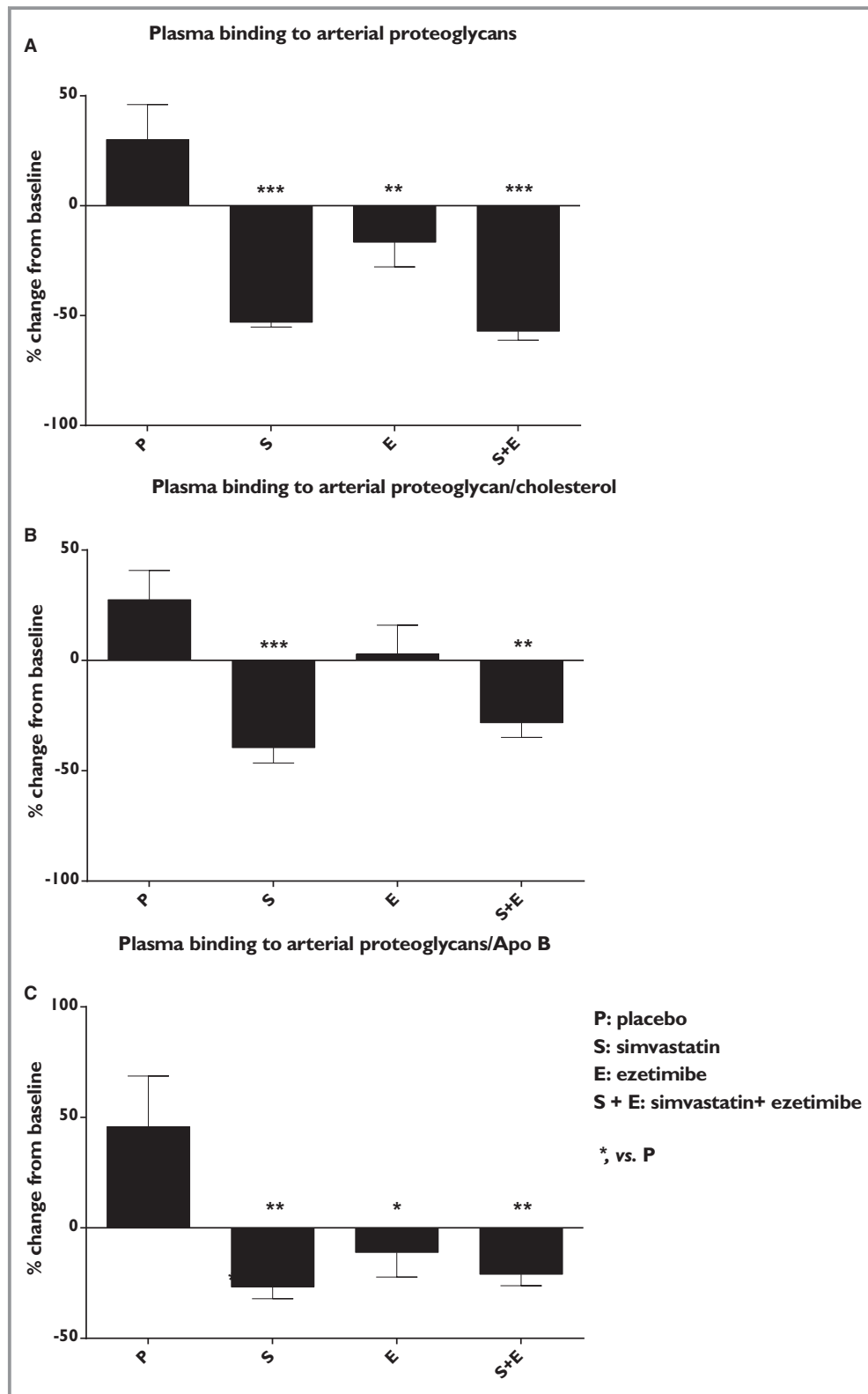


Figure 6. Effects on plasma binding to the human aortic PG. **(A)**, plasma binding to the human arterial proteoglycans (PG), **(B)** plasma binding to the arterial PG/total cholesterol ratio, and **(C)** plasma binding to the arterial PG/apolipoprotein B (ApoB) ratio. Data show the % change from baseline and are expressed as mean±SEM. Multiway ANOVA followed by LSD test. *vs placebo, $P<0.05$; **vs placebo, $P<0.01$; and ***vs placebo, $P<0.001$ ($n=8-10$ /treatment group). LSD indicates least significant difference.

Table 2. Bile Composition, Plasma Plant Sterols and 7 α -Hydroxy-4-Cholesten-3-One (C4)

	Placebo (n=10)	Simvastatin (n=10)	Ezetimibe (n=10)	Simvastatin+Ezetimibe (n=10)
Biliary cholesterol, mmol/L	17.7 \pm 2.57	8.60 \pm 1.13**	15.6 \pm 1.37	16.5 \pm 2.35 [#]
Biliary bile acids, mmol/L	110 \pm 9.80	74.8 \pm 8.30	97.1 \pm 12.8	139 \pm 33.9 [#]
Biliary phospholipids, mmol/L	52.2 \pm 7.00	30.5 \pm 4.20	43.2 \pm 6.70	65.5 \pm 11.9 [#]
Total biliary lipid, mmol/L	179 \pm 18.60	114 \pm 13.10	156 \pm 20.30	213 \pm 49.00 [#]
Cholesterol, % molar	9.10 \pm 0.52	7.20 \pm 0.20*	10.4 \pm 0.71	7.60 \pm 0.48
Bile acid, % molar	61.7 \pm 1.49	66.2 \pm 1.59	62.4 \pm 0.96	64.7 \pm 0.82
Phospholipids, % molar	28.5 \pm 1.20	26.2 \pm 1.31	27.2 \pm 1.19	27.8 \pm 0.71
Saturation index, %	123 \pm 8.60	104 \pm 4.70*	138 \pm 12.5	94.0 \pm 8.70
Cholic acid, %	24.0 \pm 1.49	21.5 \pm 1.53	21.5 \pm 2.16	22.7 \pm 1.42
Chenodeoxycholic acid, %	41.4 \pm 1.56	48.1 \pm 4.48	47.5 \pm 3.80	43.1 \pm 2.18
Deoxycholic acid, %	34.5 \pm 2.95	24.8 \pm 6.98	25.2 \pm 5.15	33.7 \pm 4.53
Ursodeoxycholic acid, %	1.50 \pm 0.28	1.92 \pm 0.39	1.45 \pm 0.27	1.46 \pm 0.31
Lithocholic acid, %	0.87 \pm 0.31	0.62 \pm 0.11	0.50 \pm 0.10	0.63 \pm 0.13
Bile campesterol, μ g/mL	29.7 \pm 3.60	16.8 \pm 3.9**	14.5 \pm 2.7**	13.7 \pm 1.78**
Plasma lathosterol/cholesterol, % variation from baseline	8.16 \pm 8.09	-56 \pm 3.1***	40 \pm 7.21**	-29 \pm 4.6***,##
Plasma campesterol/cholesterol, % variation from baseline	-3.5 \pm 3.90	21.2 \pm 13.1*	-52 \pm 3.1***	-41 \pm 5.3***,###
Plasma C4/cholesterol, ng/mmol/L	26.8 \pm 18.4	21.2 \pm 17.9	13 \pm 17.6	14 \pm 10.3

Data show mean \pm SEM. Multiway ANOVA followed by LSD test. LSD indicates least significant difference.

*vs placebo, $P<0.05$; **vs placebo, $P<0.01$; ***vs placebo, $P<0.001$; #simvastatin + ezetimibe vs simvastatin, $P<0.05$; ##simvastatin + ezetimibe vs simvastatin, $P<0.01$; and ###simvastatin+ezetimibe vs simvastatin, $P<0.001$.

was created as depicted by the analysis of the plasma lathosterol to cholesterol ratio: The liver cannot synthesize cholesterol in response to lower input of cholesterol from the intestine. In this new condition, biliary cholesterol levels were similar to placebo, suggesting that hepatic NPC1L1 in humans is likely a target for ezetimibe. If not, significantly lower levels of biliary cholesterol should have been observed. Our conclusions are in line with the observations that: (1) In pigs,⁴¹ ezetimibe added to simvastatin increased hepatic NPC1L1 expression following *SREBF2* induction; (2) statin therapy has been associated with a reduced risk of gallstone formation in an epidemiological study⁴²; and (3) genetic variation of NPC1L1 leading to lower activity are associated with an increased risk of gallstone disease.⁴³

Large genetic studies have recently recognized elevated remnant cholesterol, defined as non-LDL-C, non-HDL-C, as a causal risk factor for CVD and low-grade inflammation, whereas elevated LDL-C is associated causally only with CVD.⁸ Thus, the residual risk of CVD after LDL-C lowering can partly be attributed to remnant cholesterol.¹⁰ In the present study, all treatments, and especially combined therapy, were associated with decreased plasma CEs in almost all 7 subclasses of remnant particles. ACAT2 determines the content of atherogenic CEs secreted into nascent VLDL and chylomicrons¹² whereas LCAT adds CEs to VLDL in plasma.¹⁴ High-dose statin

treatment decreases the activity of hepatic ACAT2¹⁵ and CETP⁴⁴ whereas increasing the activity of LCAT⁴⁴⁻⁴⁶ in humans. As a net effect, plasma VLDL cholesterol level is decreased. Ezetimibe seems to decrease CETP and LCAT activity in plasma,⁴⁷ whereas it blocks the NPC1L1-mediated cholesterol availability to intestinal ACAT2. Consequently, ezetimibe reduces the chylomicron, CE, as shown in mice.¹⁶ In this study, ezetimibe reduced CE in almost all remnant lipoprotein subclasses, but not in all LDL subclasses. Thus, the additional reduction of CE in remnant particles by combined treatment compared with simvastatin monotherapy support the complementary and synergistic role of ezetimibe in combination with statin. In line with these findings, it has been shown that the addition of ezetimibe to ongoing statin therapy decreases remnant cholesterol to a greater extent than just doubling the statin dose.⁴⁸

The primary quantitative dyslipidemia in T2DM is characterized by high plasma triglyceride and low HDL-cholesterol levels while qualitatively by an increase in L-VLDL and sdLDL fractions.⁴⁹ The combined treatment showed consistent significant effects in all remnant lipoprotein subclasses both qualitatively (CE and triglyceride) and quantitatively (particle numbers). These findings suggest that ezetimibe in combination with simvastatin is more effective than simvastatin monotherapy in T2DM patients, who are characterized by high

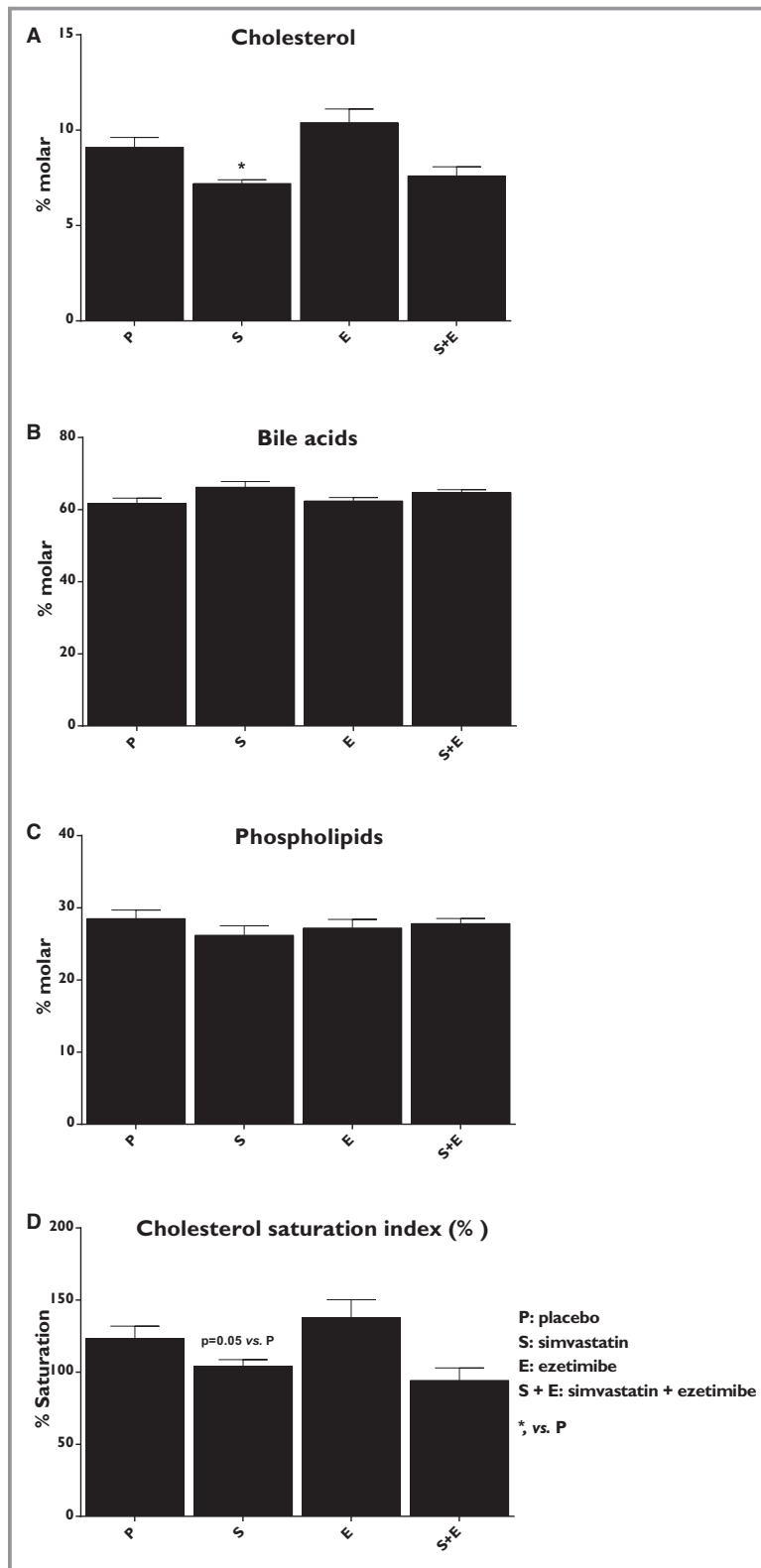


Figure 7. Effects on biliary lipid composition. **(A)**, Percentage molar cholesterol, **(B)** percentage molar bile acids, **(C)** percentage molar phospholipids, and **(D)** cholesterol saturation index. Data show the % change from the baseline and are expressed as mean±SEM. Multiway ANOVA followed by LSD test. *vs placebo, $P<0.05$ (n=8–10/treatment group). LSD indicates least significant difference.

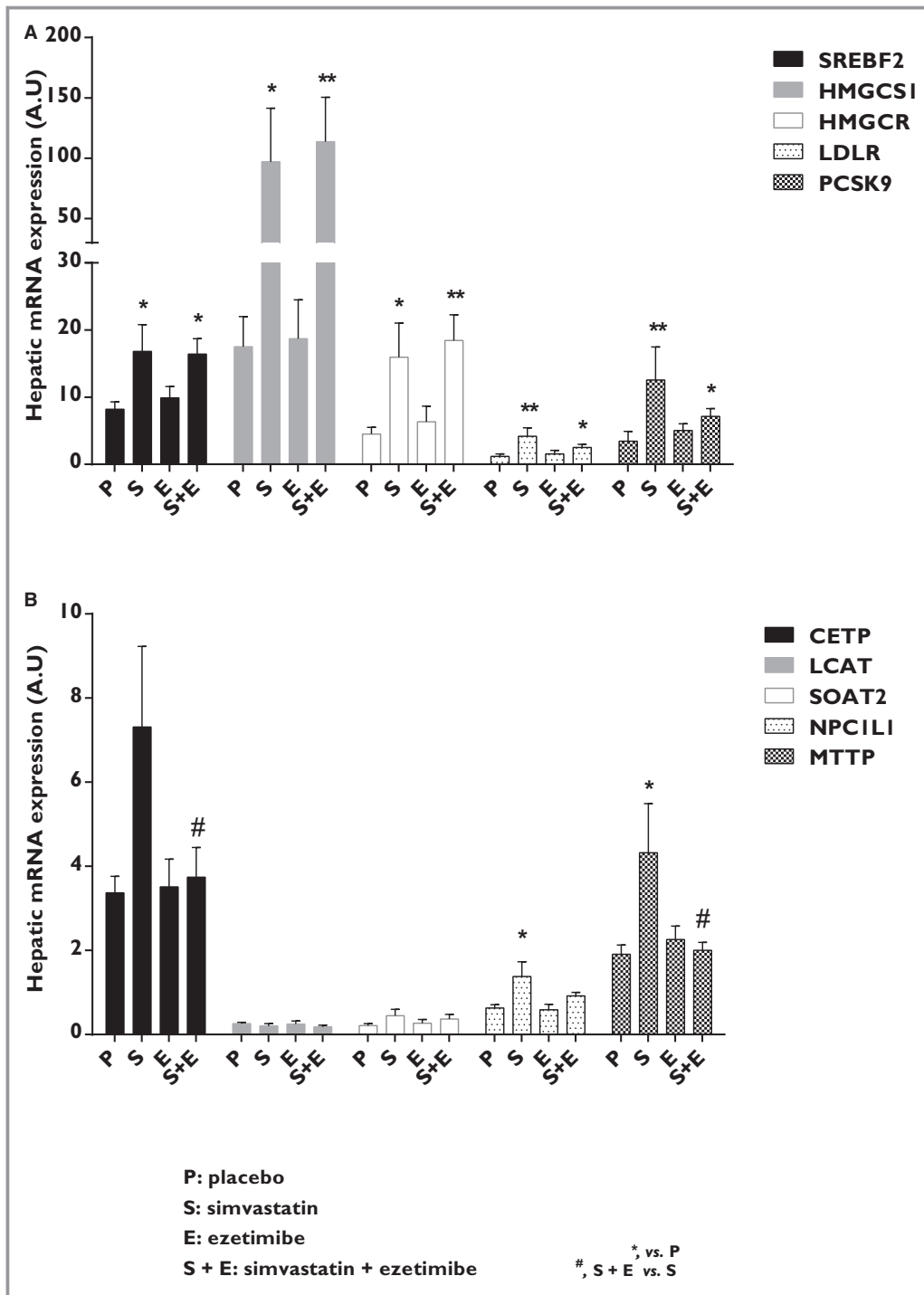


Figure 8. Effects on genes involved in hepatic cholesterol metabolism. **A** and **B**, Key genes regulating hepatic cholesterol metabolism. *CETP* indicates cholesteryl ester transfer protein; *HMGCR*, 3-hydroxy-3-methylglutaryl-CoA reductase; *HMGCS1*, 3-hydroxy-3-methylglutaryl-CoA synthase 1; *LCAT*, lecithin-cholesterol acyltransferase; *LDLR*, low-density lipoprotein receptor; LSD, least significant difference; *MTTP*, microsomal triglyceride transfer protein; *NPC1L1*, NPC1 like intracellular cholesterol transporter 1; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *SOAT2*, sterol O-acyltransferase 2; *SREBF2*, sterol regulatory element binding transcription factor 2. Data are expressed as mean±SEM. Primers sequences are given in Table 3. Multiway ANOVA followed by LSD test. *vs placebo, $P<0.05$; **vs placebo, $P<0.01$; and #simvastatin+ezetimibe vs simvastatin, $P<0.05$ (n=5–9/treatment group). A.U. indicates arbitrary units; LSD, least significant difference.

Table 3. Primer Sequences for Real-Time PCR

Gene	Primer Sequence (5' to 3')	Final Conc.
<i>HuSREBF2</i>	Fwd CAG CTG CAC ATC ACA GGG AA	700 nmol/L
	Rev GTA CAT CGG AAC AGG CGG AT	700 nmol/L
<i>HuHMGCS1</i>	Fwd GGC ACA GCT GCT GTC TTC AAT	200 nmol/L
	Rev ACC AGG GCA TAC CGT CCA T	200 nmol/L
<i>HuHMGCR</i>	Fwd ATA GGA GGC TAC AAC GCC CAT	200 nmol/L
	Rev TTC TGT GCT GCA TCC TGT CC	200 nmol/L
<i>HuLDLR</i>	Fwd CAG ATA TCA TCA ACG AAG C	700 nmol/L
	Rev CCT CTC ACA CCA GTT CAC TCC	700 nmol/L
<i>HuPCSK9</i>	Fwd CCA AGA TCC TGC ATG TCT TCC	200 nmol/L
	Rev AAC TTC AAG GCC AGC TCC AG	200 nmol/L
<i>Hu CETP</i>	Fwd GAG ACG AGT TCA AGG CAG TGC	200 nmol/L
	Rev TCC TGG TTG GTG TTG AAG CC	200 nmol/L
<i>HuLCAT</i>	Fwd TCC AAC GCC CCT GGT GTC CA	100 nmol/L
	Rev TCT CGT CCC GCA CGT AGC CA	100 nmol/L
<i>HuSOAT2</i>	Fwd GAG ACT TAC CCT AGG ACG CCC T	200 nmol/L
	Rev AGT TCT TGG CCA CAT AAT TCC AC	200 nmol/L
<i>HuNPC1L1</i>	Fwd CTT CAG ATG GCC AGG TTT TAG C	200 nmol/L
	Rev TGT AAT CCT GTG AGT TTT TCA GGG	200 nmol/L
<i>HuMTTP</i>	Fwd TTC ACG GTA GCC AGG TGG TT	100 nmol/L
	Rev GCG ATT AAG GCT TCC AGT CCT	100 nmol/L
<i>HuGAPDH</i>	Fwd TGA CAA CTT TGG TAT CGT GGA AGG	200 nmol/L
	Rev AGG CAG GGA TGA TGT TCT GGA GAG	200 nmol/L

All primer pairs were based on human sequences. *CETP* indicates cholesteryl ester transfer protein; Conc., concentration; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *HMGCR*, 3-hydroxy-3-methylglutaryl-CoA reductase; *HMGCS1*, 3-hydroxy-3-methylglutaryl-CoA synthase 1; *LCAT*, lecithin-cholesterol acyltransferase; *LDLR*, low-density lipoprotein receptor; *MTTP*, microsomal triglyceride transfer protein; *NPC1L1*, NPC1 like intracellular cholesterol transporter 1; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *SOAT2*, sterol O-acyltransferase 2; *SREBF2*, sterol regulatory element binding transcription factor 2.

levels of triglyceride-rich remnant particles. Hence, our study may, in part, explain why the combination therapy of simvastatin and ezetimibe was more efficient in reducing CVD events in patients with T2DM in the IMPROVE-IT⁵ and SHARP⁶ trials.

LDL particles from patients with coronary heart disease have a significantly higher affinity for arterial PG compared with healthy individuals.^{24,25} This affinity is reduced by statin treatment in patients with moderate hypercholesterolemia.²⁶ Here, we evaluated the effect of simvastatin and ezetimibe alone or in combination on the binding of lipoproteins in plasma to human arterial PG. The solid-phase analysis used mainly LDL particles and the triglyceride-rich VLDL and IDL particles, which have a lower affinity for arterial PG than LDL particles. Simvastatin and ezetimibe monotherapy treatments reduced the amount of plasma ApoB-lipoprotein cholesterol bound to PG compared with placebo, where simvastatin was more effective. However, addition of ezetimibe did not have any significant synergistic effect compared with simvastatin

alone. It has been shown that large LDL particles have a lower affinity for PG than sdLDL particles.^{11,25} Simvastatin reduced CE in all LDL subclasses whereas ezetimibe only reduced CE in L-LDL particles, with no effects on smaller and most atherogenic sdLDL subclasses. This might explain the greater effect of simvastatin and the lack of synergistic effect of ezetimibe on arterial PG binding. To assess further, whether the reduction of lipoprotein binding to PG was attributed to the lowering effect of the cholesterol and particle number, we corrected the PG binding for total cholesterol and ApoB, respectively. In contrast to ezetimibe, simvastatin was still able to reduce the binding to arterial PG. Thus, simvastatin seems to modify the characteristics of the particles and not only their number and cholesterol load.

Simvastatin monotherapy increased the hepatic mRNA levels for genes under the control of *SREBF2*, as expected. For the majority of these genes, addition of ezetimibe in combination with simvastatin treatment did not enhance this effect, suggesting that inhibition of cholesterol synthesis is

per se sufficient to fully activate the *SREBF2* pathway. Adding ezetimibe to simvastatin treatment blunted the induction of hepatic expression of *MTPP*. This effect may, in part, explain why the combined therapy modifies the lipid content of remnant particles. Differently from our previous study with atorvastatin,¹⁵ we did not observe a decrease in *Soat2* mRNA levels. Unfortunately, the size of the biopsies did not allow for measurement of ACAT2 activity, which is a limitation of this study.

In conclusion, the expected increase in biliary cholesterol following treatment with ezetimibe monotherapy does not occur in gallstone-diseased patients, yet we found evidence that ezetimibe is able to block hepatic NPC1L1 in humans. In addition, our results suggest that ezetimibe and simvastatin reduces atherogenic CE in remnant cholesterol particles. Moreover, the dramatic reduction of remnant cholesterol and the apparent atheroprotective changes in ApoB-lipoprotein plasma levels, their composition, size distribution, and possible reduction of affinity for human arterial proteoglycans may all explain why ezetimibe combined with simvastatin was effective in reducing cardiovascular events in the IMPROVE-IT and SHARP trials. Therefore, it seems reasonable to treat the lipid disorder in those conditions characterized by increased levels of remnant cholesterol, that is, patients with T2DM with a combination of simvastatin and ezetimibe.

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Disclosures

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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Chemical analysis

Blood and bile samples

All blood samples were collected in fasting state. C-reactive protein (CRP), creatine kinase (CK), ApoE and safety assessment analyses (e.g. AST and ALT) in addition to biliary total bile acids were performed by certified routine assays at the Karolinska University Laboratory, Stockholm, Sweden.

For lipoprotein profiling the total and free cholesterol, and TG content in the different lipoprotein fractions corresponding to VLDL, LDL and HDL were determined after separation by size-exclusion chromatography¹. Detailed analysis of lipoprotein subclasses as well as ApoA1 and ApoB were performed with Nuclear Magnetic Resonance spectroscopy (NMR) at Nightingale Health Ltd (formerly known as Brainshake) Laboratory, Vantaa, Finland².

ApoCIII was analyzed using an enzyme-linked immunosorbent assay (Abcam, Cambridge, USA) following the manufacturer's instructions. Plasma and biliary lathosterol and campesterol were determined by isotope dilution-mass spectrometry after addition of deuterium labeled internal standards^{3, 4}. The campesterol/cholesterol and the lathosterol/cholesterol ratios were calculated as biomarkers for intestinal cholesterol absorption and total body cholesterol synthesis, respectively⁵⁻⁸. The intermediate in bile acid

synthesis 7 α -hydroxy-4-cholesten-3-one (C4) was analyzed in plasma samples by LC-MS/MS as described⁹.

Biliary lipids were extracted using the Bligh-Dyer method¹⁰. Cholesterol and phospholipid concentrations were determined by enzymatic methods with commercially available kits (Roche Diagnostics, Mannheim, Germany). Cholesterol saturation index (CSI) was calculated according to the critical tables described by Carey¹¹. The individual bile acids were analyzed using GC-MS as previously described¹². Briefly, bile was diluted with water, ethanol, KOH and hydrolyzed overnight after addition of deuterium labeled bile acids as internal standards. After acidification with HCl to pH 1 the deconjugated bile acids were extracted with diethyl ether and trimethylsilylated prior injection into GC-MS (Agilent Technologies).

PG binding

In this study we used a solid phase binding procedure that has been found reproducible for evaluation of affinity of isolated, or in plasma, apoB-lipoproteins for human arterial proteoglycans or purified proteoglycans preparations^{13, 14}. Human aortic PG were isolated from the intima-media of human aortas essentially by the method of Hurt-Camejo *et al*^{15 16}. Glycosaminoglycans were quantified by the method of Bartold and Page¹⁷, and the amounts of PG are expressed in terms of their glycosaminoglycan content. The wells of polystyrene 96-well plates (Thermo Fisher Scientific) were coated with 100 μ L of the PGs (25 μ g/mL in PBS) by incubation at 4°C overnight. Wells were blocked with 1% BSA in PBS for 1 h at 37°C. Wells without PG served as controls. One microliter of plasma was added to the wells in a buffer containing 140 mM NaCl, 2 mM MgCl₂, 5 mM CaCl₂ and 10 mM MES, pH 5.5 and incubated for 1 h at 37°C. The wells were washed with 10 mM MES-50 mM NaCl, pH 5.5, and the amount of bound cholesterol was determined using the Amplex Red cholesterol kit (Molecular Probes).

RNA extraction, cDNA synthesis and real-time RT-PCR

Total RNA was extracted and transcribed into cDNA using Omniscript (Qiagen, Sollentuna, Sweden). The mRNA expression levels were then quantified and using specific primers (Table 3), arbitrary units were calculated by linearization of the CT values and normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Table S1. Lipid contribution in lipoprotein fractions used in Figure 2

Data show mean \pm SEM. Plasma lipoproteins were subjected to analysis by nuclear magnetic resonance.

LDL: low density lipoprotein, HDL: high density lipoprotein, Remnant: non-HDL and non-LDL, P: placebo; S: simvastatin, E: ezetimibe, S+E: simvastatin plus ezetimibe.

Pre-	Cholesterol				Cholesteryl ester				Triglycerides			
	P	S	E	S+E	P	S	E	S+E	P	S	E	S+E
Remnant (mmol/L)	1.4 \pm 0.2	1.5 \pm 0.2	1.2 \pm 0.1	1.4 \pm 0.1	0.9 \pm 0.1	1.0 \pm 0.1	0.8 \pm 0.1	0.9 \pm 0.1	0.9 \pm 0.1	1.2 \pm 0.1	0.9 \pm 0.1	0.9 \pm 0.1
LDL (mmol/L)	1.7 \pm 0.2	1.8 \pm 0.1	1.5 \pm 0.1	1.6 \pm 0.1	1.2 \pm 0.1	1.3 \pm 0.1	1.1 \pm 0.1	1.2 \pm 0.1	0.17 \pm 0	0.24 \pm 0	0.17 \pm 0	0.18 \pm 0.0
HDL (mmol/L)	1.6 \pm 0.1	1.4 \pm 0.1	1.4 \pm 0.1	1.3 \pm 0.1	1.2 \pm 0.1	1.1 \pm 0.0	1.1 \pm 0.1	1.0 \pm 0.0	0.14 \pm 0	0.16 \pm 0	0.13 \pm 0	0.14 \pm 0
Post-												
Remnant (mmol/L)	1.4 \pm 0.1	0.8 \pm 0.1	1.1 \pm 0.1	0.5 \pm 0.1	0.9 \pm 0.1	0.5 \pm 0.1	0.7 \pm 0.1	0.3 \pm 0.0	1.0 \pm 0.1	0.7 \pm 0.1	0.8 \pm 0.1	0.5 \pm 0.1
LDL (mmol/L)	1.7 \pm 0.2	0.9 \pm 0.1	1.4 \pm 0.1	0.6 \pm 0.1	1.3 \pm 0.1	0.6 \pm 0.1	1.0 \pm 0.1	0.3 \pm 0.1	0.18 \pm 0	0.14 \pm 0	0.15 \pm 0	0.10 \pm 0.0
HDL (mmol/L)	1.6 \pm 0.1	1.4 \pm 0.1	1.4 \pm 0.1	1.4 \pm 0.1	1.3 \pm 0.1	1.1 \pm 0.1	1.1 \pm 0.1	1.1 \pm 0.0	0.15 \pm 0.0	0.12 \pm 0.0	0.12 \pm 0.0	0.10 \pm 0.0

Table S2. Contribution of different subclasses for their respective fraction baseline.

	Percentage contribution of remnant subclasses (Non-LDL; Non-HDL) to overall remnant fraction						
	XXL-VLDL	XL-VLDL	L-VLDL	M-VLDL	S-VLDL	XS-VLDL	IDL
Particle number	0.1%	0.3%	2.0%	8.0%	14%	20%	55%
Triglyceride	2.0%	3.0%	14%	30%	25%	13%	14%
Cholesteryl ester	0.3%	0.6%	3.0%	9.0%	14%	18%	55%
	Percentage contribution of LDL subclasses to overall LDL fraction						
	L-LDL		M-LDL		S-LDL		
Particle number	36%		29%		34%		
Triglyceride	56%		27%		17%		
Cholesteryl ester	52%		30%		18%		
	Percentage contribution of HDL subclasses to overall HDL fraction						
	XL-HDL	L-HDL	M-HDL	S-HDL			
Particle number	5%	13%	30%	58%			
Triglyceride	10%	22%	32%	36%			
Cholesteryl ester	12%	24%	32%	32%			

Baseline contribution of lipoprotein subclasses for their respective fraction.

XXL-VLDL, extra extra large very low density lipoproteins; XL-VLDL, extra large-VLDL; L-VLDL, large-VLDL; M-VLDL, medium-VLDL; S-VLDL, small-VLDL; XS-VLDL, extra small-VLDL; IDL, intermediate density lipoprotein; L-LDL, large low density lipoprotein; M-LDL, medium-LDL; S-LDL, small-LDL; XL-HDL, extra-large high density lipoprotein; L-HDL, large-HDL; M-HDL, medium-HDL; S-HDL, small-HDL.

Table S3. Correlation of percentage changes of proteoglycan (PG) binding and lipoprotein particle number and composition.

	Percentage change correlation of PG binding with remnant (Non-LDL; Non-HDL) lipoprotein subclasses						
	XXL-VLDL	XL-VLDL	L-VLDL	M-VLDL	S-VLDL	XS-VLDL	IDL
Particle number (r ² ; p value)	0.52; <0.001	0.58; <0.001	0.42; <0.001	0.45; <0.001	0.51; <0.001	0.55; <0.001	0.60; <0.001
Triglyceride (r ² ; p value)	0.51; <0.001	0.55; <0.001	0.38; <0.001	0.39; <0.001	0.42; <0.001	0.43; <0.001	0.34; <0.001
Cholesteryl ester (r ² ; p value)	0.62; <0.001	0.61; <0.001	0.23; <0.01	0.59; <0.001	0.65; <0.001	0.59; <0.001	0.54; <0.001
	Percentage change correlation of PG binding with LDL lipoprotein subclasses						
	L-LDL		M-LDL		S-LDL		
Particle number (r ² ; p value)	0.49; <0.001		0.49; <0.001		0.50; <0.001		
Triglyceride (r ² ; p value)	0.32; <0.001		0.35; <0.001		0.48; <0.001		
Cholesteryl ester (r ² ; p value)	0.52; <0.001		0.49; <0.001		0.46; <0.001		
	Percentage change correlation of PG binding with HDL lipoprotein subclasses						
	XL-HDL	L-HDL		M-HDL	S-HDL		
Particle number (r ² ; p value)	0.06; NS	0.06; NS [#]		0.01; NS	0.17; <0.05 [#]		
Triglyceride (r ² ; p value)	0.33; <0.001	0.13; <0.05		0.40; <0.05	0.15; <0.05		
Cholesteryl ester (r ² ; p value)	0.06; NS	0.11; <0.05 [#]		0.04; NS [#]	0.16; <0.05		

Pearson correlation between percentage changes of PG binding and lipoprotein subclasses particle number and composition. [#] Inverse correlation

XXL-VLDL, extra extra large very low density lipoproteins; XL-VLDL, extra large-VLDL; L-VLDL, large-VLDL; M-VLDL, medium-VLDL; S-VLDL, small-VLDL; XS-VLDL, extra small-VLDL; IDL, intermediate density lipoprotein; L-LDL, large low density lipoprotein; M-LDL, medium-LDL; S-LDL, small-LDL; XL-HDL, extra-large high density lipoprotein; L-HDL, large-HDL; M-HDL, medium-HDL; S-HDL, small-HDL

Table S4. Safety assessment analyses.

	Placebo	Simvastatin	Ezetimibe	Simvastatin + Ezetimibe	P value
Alanine aminotransferase (ALT); % variation from baseline	1.68±7.26	-2.81±11.90	-6.43±11.2	21.74±16.0	NS
Gamma-glutamyltransferase (GT); % variation from baseline	5.46±13.7	-21.0±11.2	0.84±8.38	8.88±19.7	NS
Creatine Phosphokinase (CK), % variation from baseline	17.5± 14.7	22.5± 14.8	27.6± 17.5	-0.7± 12.6	NS

Data show mean ± SEM. Multi-Way ANOVA followed by LSD-test. NS: not significant.

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