Material and Methods

Patients, treatments, and operative procedure

Fertile female, post-menopausal female, and male patients (altogether 42), scheduled for elective cholecystectomy because of uncomplicated gallstone disease, were enrolled in the study. For inclusion, the patients had to have a total plasma cholesterol > 3.0 mmol/L, normal or slightly elevated plasma triglycerides (<3.6 mmol/L), and also to be non-obese (BMI<30). Evidence of hepatic, renal, metabolic or endocrine dysfunction and history of concomitant medication interfering with the metabolism of statins were criteria for exclusion, as was compliance to treatment of < 80%. Five patients did not complete the study because of low compliance and thus 12 males, 12 fertile females, and 13 post-menopausal females were evaluated (Table 1). Each of the 3 patient groups was randomized to three treatment arms: placebo, fluvastatin 20 mg/day (Low-ChSI) or atorvastatin 80 mg/day (High-ChSI) for 4 weeks prior to surgery. Fasting blood samples were drawn at randomisation and on the day before surgery. The last dose was administered the day before operation. Surgery was performed under general anesthesia between 09.00 h and 11.00 h after an overnight fast. The patients were all operated with a laparoscopic technique. A biopsy was obtained from the left lobe of the liver and the specimen was immediately frozen in liquid nitrogen. All patients had cholesterol gallstones, as judged from postoperative visual inspection. Except for safety parameters, all analyses were performed after the last patients had completed the study. Informed consent was obtained from all patients prior to inclusion into the study, which was approved by the Human Ethics Committee of Karolinska Institutet and by the Swedish Medical Product Agency.
Chemical analysis of plasma

Plasma analyses of total cholesterol, triglycerides, and apolipoproteins B and AI were performed by certified routine assays. Size-fractionation of lipoproteins was performed on 10 µl of individual plasma samples using a Superose 6 PC 3.2/30 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as described \(^1\). The respective lipoprotein fraction lipid concentrations were calculated after integration of the individual chromatograms. SDS/PAGE separation of apolipoproteins in the VLDL fraction was performed using pooled plasma after ultracentrifugation at \(d<1.006\ \text{g/mL}\) as previously described. \(^2\) The bands corresponding to apolipoproteins B and E were quantitated by densitometry.

Unesterified lathosterol, an indirect marker of HMG-CoA reductase activity in the liver \(^3\) and whole body cholesterol synthesis \(^4\), was determined by isotope dilution-mass spectrometry after the addition of deuterium-labeled internal standard \(^5\). 7\(\alpha\)-hydroxy-4-cholesten-3-one (C4), an intermediate in bile acid formation that directly correlates to bile acid synthesis, was analysed by HPLC in pooled serum samples as described \(^6\). The plant sterols sitosterol and campesterol were analysed by gas chromatography-mass spectrometry (GC/MS) using D\(_5\)-campesterol and D\(_5\)-sitosterol as internal standards \(^7\).

Preparation of hepatic membranes, ligand blot assay of LDL receptors and Western blot assays.

Liver plasma membranes were prepared from pools of liver as described previously \(^8\). For ligand blot assay of LDL receptors, polyacrylamide gels (10% with SDS) were loaded with membrane protein prepared from pooled samples of liver. Filters were incubated with \(^{125}\text{I}\)-labeled rabbit \(\beta\)-migrating very low density lipoproteins (\(\beta\)-VLDL), as described \(^8\). The LDL-receptor expression (120 kDa band) was determined using a Fuji Bio-imaging analyzer (BAS
CLA-I receptors were determined on liver membranes by Western Blot analysis. Reduced samples were separated on 3-8% Tris-acetate gels (NuPAGE Invitrogen, Carlsbad, CA). Proteins were then transferred onto nitrocellulose filters. CLA-I protein was detected with rabbit polyclonal antibodies (1:3000) against mouse SR-B1 (Novus Biologicals, Inc, Littleton, Co) as described elsewhere. ABCG8 was detected with rabbit polyclonal antibody to human ABCG8 following manufacturer’s instruction (Novus Biologicals, Inc, Littleton, Co). Western blots were quantified by Image Gauge software, (Fuji Photo Film Co, Japan).

**RNA preparation and mRNA determination**

Total RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA). One µg total RNA was transcribed into cDNA using random hexamer priming and Omniscript™ (Qiagen, Valencia, CA). Quantification of specific mRNAs was performed by SYBR® Green real-time PCR using an ABI PRISM 7000 thermocycler (PE Applied Biosystems, Foster City, CA). In order to prevent amplification of genomic DNA, primer sets were designed whenever possible to cross an exon-exon boundary (Supplementary Table 1). Data are expressed in arbitrary units and normalized by correction for the signal obtained in the same cDNA preparation for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Degraded RNA samples and samples showing >2 cycle difference for GAPDH from average were not used in the final computations.

**Isolation of microsomes, enzymatic analyses, and ACAT2 protein determination**

Isolation of hepatic microsomes and determination of total ACAT enzymatic activity were performed as described previously, except that preincubation was with a cholesterol-saturated solution of β-hydroxypropyl cyclodextrin for 30 min before addition of $^{14}$[C]-oleoyl-
CoA. In order to separately identify ACAT1 and ACAT2 activities, pyripyropene A, a specific ACAT2 inhibitor\textsuperscript{11}, was included in the preincubation and reaction mixture at a concentration of 5 µM. For ACAT2 protein analysis by Western blot, 10 or 20 µg of microsomal protein was analyzed. Separation and blotting were as described earlier\textsuperscript{12}. Immunoaffinity purified monkey ACAT2 antibody, prepared as previously described\textsuperscript{13}, was applied to the nitrocellulose at a concentration of 1 µg/ml in the blocking solution. Western blots were quantified by Image Gauge software (Fuji Photo Film Co, Japan).

**Analysis of biliary lipids and bile acid composition**

Biliary cholesterol, total bile acids and phospholipids in gallbladder bile were measured as previously described\textsuperscript{14}. The saturation index was calculated using Carey’s critical tables\textsuperscript{15}. For bile acid composition, bile was hydrolyzed in 1M potassium hydroxide at 110°C for 12 hrs. The deconjugated bile acids were thereafter extracted with diethyl ether after acidification to pH 1 by 8M HCl, preparation of trimethylsilyl ethers and analyzed by gas-liquid chromatograph (Agilent Technologies, Böblingen, Germany) using a HPI column (Agilent Technologies).

**Statistics**

Data are presented as means ± SEM. The significance of differences between groups was tested by one-way ANOVA followed by post-hoc comparisons of group means according to the LSD or the Dunnett methods (Statistica software, Stat Soft, Tulsa OK). Correlations were calculated with the Spearman rank order test.
References


