Hepatic cholesterol metabolism in cholesterol gallstone disease

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Abstract Hepatic cholesterol metabolism was examined in 27 Swedish patients with cholesterol gallstone disease and in 13 patients free of gallstones operated for roentgenographically suspect polyps in the gallbladder. All 40 patients underwent cholecystectomy, and a liver biopsy and gallbladder bile were obtained at surgery. The cholesterol saturation of gallbladder bile was significantly higher in patients with gallstones compared to the gallstone-free controls (131 ± 13 vs. 75 ± 5%, $P < 0.001$). Microsomal 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, governing cholesterol synthesis, did not differ between gallstone and gallstone-free patients (104 ± 11 vs. and 109 ± 22 pmol/min per mg protein, respectively). The activity of cholesterol 7 α-hydroxylase, catalyzing the catabolism of cholesterol to bile acids, was not significantly decreased in gallstone patients (6.2 ± 1.1 vs. 8.0 ± 2.0 pmol/min per mg protein). The capacity to esterify cholesterol, judged by the activity of acyl coenzyme A:cholesterol acyltransferase (ACAT), was similar in gallstone and gallstone-free patients (5.4 ± 0.4 vs. 6.7 ± 1.1 pmol/min per mg protein). In the presence of exogenous cholesterol, ACAT activity increased by more than fourfold in both groups. No correlation was found between the saturation of gallbladder bile and any of the mentioned enzyme activities in gallstone patients. It is concluded that distinct abnormalities in cholesterol metabolizing enzymes are not of major importance for development of gallstones in Swedish patients with cholesterol gallstone disease. The results support the contention that the etiology of cholesterol gallstones is multifactorial. —**Reihnér, E., B. Angelin, I. Björkhem, and K. Einarsson.** Hepatic cholesterol metabolism in cholesterol gallstone disease. *J. Lipid Res.* 1991. **32:** 469–475.

Supplementary key words 3-hydroxy-3-methylglutaryl coenzyme A reductase • cholesterol 7α-hydroxylase • acyl coenzyme A:cholesterol acyltransferase • bile • biliary lipids

Cholesterol gallstone disease is common in the Swedish adult population (1, 2). It has been reported that a population of Swedish patients with gallstones had almost 50% higher cholesterol secretion rate than gallstone-free controls, whereas the rate of bile acid secretion was normal (3). However there is still considerable uncertainty regarding the mechanisms and regulation of cholesterol secretion into the bile.

Several possible explanations for the development of supersaturated bile in cholesterol gallstone disease have been presented (4, 5). First, hepatic cholesterol synthesis might be increased. However, there is controversy as to whether HMG-CoA reductase, the rate-limiting enzyme for cholesterol biosynthesis, is increased (6–9) or unchanged (10, 11) in gallstone disease. Another possibility is an inappropriate down-regulation of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis (12). At present, there is some evidence for a decrease in the hepatocyte activity of cholesterol 7α-hydroxylase in cholesterol gallstone patients (6–8, 10), but the validity of the assay systems used may be questioned (12, 13). Hypersecretion of biliary free cholesterol in gallstone patients might also be due to a reduced esterification of cholesterol in the hepatocyte (14). The intracellular esterification of cholesterol is catalyzed by the microsomal enzyme acyl-CoA:cholesterol acyltransferase (ACAT) (15).

To the best of our knowledge, no previous studies exist where the activities of the three above-mentioned rate-limiting enzymes have been assayed simultaneously in the same subjects. In the present study these regulating enzymes of hepatic cholesterol metabolism have been assayed in subjects with and without gallstone disease. Attempts have also been made to correlate the results with bile composition. Despite highly significant increases in cholesterol concentration and saturation of gallbladder bile in patients with gallstones, the mean values in hepatic enzyme activities did not differ from those of the controls.

MATERIALS AND METHODS

Materials

[3-^{14}C]HMG-CoA (sp act 52 mCi/mmol), and oleoyl coenzyme A (sp act 53.5 mCi/mmol) were obtained from

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ACAT, acyl CoA:cholesterol acyltransferase.

DuPont Company Biotechnology Systems, Wilmington, DE. Radiolabeled HMG-CoA was diluted to a specific activity of 5.5 mCi/mmol. DL-[2-^{3}H]mevalonic acid lactone (sp act 125 mCi/mmol) was obtained from Radiochemical Center, Amersham, England and [1,2,6,7-^{3}H]cholesteryl oleate (sp act 82.7 mCi/mmol) was from New England Nuclear Corp., Boston, MA. Unlabeled HMG-CoA, mevalonic acid lactone, cholesteryl oleate, human serum albumin (free of fatty acids), EDTA, NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, dithiothreitol (DTT), bovine serum albumin, and Triton WR-1339 were purchased from Sigma Chemical Co., St. Louis, MO. Deuterium-labeled 7α-hydroxycholesterol was synthesized as described previously (16). [$^2H_7$]cholesterol was obtained from Applied Sciences Laboratories Inc., State College, PA.

Subjects

Studies were carried out in 27 patients (22 females and 5 males) with cholesterol gallstone disease and 13 controls (10 females and 3 males) without gallstones (**Table 1**). Indication for cholecystectomy in the latter group was roentgenographic suspicion of polyp or adenomyoma in the gallbladder (5 patients), or normal oral cholecystogram and ultrasonogram but a history of biliary colic inducible by cholecystokinin. However, no polyps or other macroscopic abnormalities were found in the removed gallbladders. Microscopic examination revealed only very mild chronic inflammation of the gallbladder in 4 patients. Subjects with cholesterolosis of the gallbladder mucosa were not included in the study. No patients showed evidence of gastrointestinal, endocrine, hepatic, or lipid disorders. Age or relative body weight did not differ significantly between the groups of patients. Informed consent was obtained from each patient before the operation, and the study was approved by the Ethical Committee at Huddinge University Hospital.

Experimental procedure

The patients were admitted to the hospital the day before operation and were given the regular hospital diet.

TABLE 1. Clinical data of gallstone patients and gallstone free controls

Subjects	Sex M/F[a]	Age	Relative Body Weight[b]	Serum Cholesterol	Serum Triglycerides
		yr	*%*	*mmol/l*	*mmol/l*
Gallstones	5/22	46 (22-73)	95 (64-119)	4.9 (3.6-6.9)	1.1 (0.3-2.3)
Controls	3/10	43 (17-74)	97 (73-122)	5.2 (3.6-7.0)	1.3 (0.6-2.1)

To convert mmol/l to mg/dl, multiply cholesterol by 38.7 and triglycerides by 88.5. Values given are means with range in parentheses.
[a]M, male; F, female.
[b]Calculated as (weight(kg)/height (cm) - 100) × 100.

To prevent any possible diurnal variation in enzyme activity, cholecystectomy was performed between 8 and 9 AM after a 12-h fast. Standardized anesthesia was given during operation (17). A wedge biopsy of 2-4 g wet weight was taken from the left lobe of the liver after opening the abdomen. A small portion was sent for histological examination. Liver morphology was normal except slight fatty infiltration in seven patients with gallstones and in one patient without gallstones. However, laboratory tests regarding liver function and plasma lipids were completely normal also in these subjects. The rest of the biopsy was immediately put into ice-cold homogenizing buffer and transported to the laboratory within 10 min.

The cystic duct was clamped and bile from the gallbladder was obtained by aspiration. Cholecystectomy was then performed without complications.

Examination for rhomboid monohydrate cholesterol crystals was made in fresh gallbladder bile by polarizing light microscopy.

Preparation of liver microsomes

One aliquot of the liver biopsy (approximatively 0.5-1 g) was weighed and placed into nine volumes (v/w) of ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM EDTA, 10 mM DTT, and 50 mM NaCl. In the ACAT assay only 1 mM EDTA was added and DTT was excluded. The liver specimens were homogenized and the microsomal fraction was prepared by ultracentrifugation as described previously (13, 17, 18). The microsomal content of protein was determined by the method of Lowry et al. (19).

Assay of microsomal HMG-CoA reductase activity

Microsomal fractions (30-95 μg of protein) were preincubated for 15 min at 37°C, and the HMG-CoA reductase assay was then initiated by the addition of 90 nmol of [3-^{14}C]HMG-CoA (0.5 μCi). The incubation was for 15 min and was stopped by the addition of 6 M HCl. Tritium-labeled mevalonic acid (0.01 μCi) was added as internal standard. After lactonization the mevalonic acid lactone formed was isolated by thin-layer chromatography and counted in a liquid scintillation spectrometer. Correction for losses was made by the internal standard. Details about the method have been published previously (17).

Assay of microsomal cholesterol 7α-hydroxylase activity

The activity of cholesterol 7α-hydroxylase was assayed as described (13). Microsomal fractions (0.25-1.1 mg protein) were incubated for 15 min at 37°C. After the reaction was stopped, deuterium-labeled 7α-hydroxycholesterol was added as internal standard. The amount of 7α-hydroxycholesterol formed was determined by combined gas-liquid chromatography-mass spectrometry and was expressed as pmol/min per mg protein.

Assay of microsomal ACAT activity

The assay of ACAT activity was performed in two different ways, as previously described (18). In the first assay system, microsomal fractions (0.1–0.3 mg protein) were preincubated for 5 min at 37°C. In the second assay system, microsomes were preincubated with 50 μmol of exogenous cholesterol (dissolved in 0.6 mg Triton WR-1339) for 20 min. The reaction was started by the addition of 25 nmol (1.45 μCi) of [^{14}C]oleoyl coenzyme A. The assay was stopped after 6 min, and [^{3}H]cholesteryl oleate (0.01 μCi) was added as internal standard. The cholesteryl oleate formed was isolated by thin-layer chromatography and the radioactivity was counted in a liquid scintillation spectrometer.

Analysis of hepatic cholesterol

To 20 μl of homogenized liver suspension or microsomes were added 2.0 μg [$^{2}H_{7}$]cholesterol and chloroform–methanol 2:1 (v/v). The chloroform phase was then evaporated and the residue was either hydrolyzed with 0.5 M KOH, extracted with hexane, and converted into trimethylsilyl ether, or directly converted into trimethylsilyl ether before analysis by gas–liquid chromatography–mass spectrometry (20).

Analysis of biliary lipids

Gallbladder bile was extracted with chloroform–methanol 2:1 (v/v), and the chloroform phase was analyzed with respect to cholesterol (21) and phospholipids (22). Total bile acid concentration was determined by an enzymatic method (23) in a separate portion of bile. The relative concentrations of cholesterol, bile acids, and phospholipids were expressed as molar percentage of total biliary lipids. The cholesterol saturation of bile (%) was calculated according to Carey (24). Bile from nonfunctioning gallbladders was not analyzed.

Statistical analysis

Data are given as the mean ± SEM. The statistical significance of differences was evaluated with the Mann-Whitney U-test. Correlations were tested by calculating Spearman's rank order correlation coefficient, r_s.

RESULTS

Gallbladder bile from all control subjects except one was unsaturated (mean 75 ± 5%) and contained no cholesterol monohydrate crystals. In the group of gallstone patients cholesterol crystals were observed in gallbladder biles from 20 of the patients (75%). The cholesterol saturation reached a mean value of 131 ± 13% and was significantly higher than in controls ($P < 0.001$). This difference in saturation of bile could be explained by a significantly higher proportion of cholesterol in the gallbladder bile from patients with gallstones compared to gallstone-free subjects (**Table 2**). Although the amount of bile acids seemed to be lower in bile from gallstone patients, the difference did not reach statistical significance. The two groups of patients also differed with respect to total lipid concentration: a lower mean value was obtained in gallstone disease. The molar ratio of cholesterol to bile acids was elevated in gallstone disease (0.11 ± 0.01 vs. 0.08 ± 0.01, $P < 0.01$) as was the ratio of cholesterol to phospholipids (0.36 ± 0.03 vs. 0.25 ± 0.02, $P < 0.005$).

It has been suggested that the pathogenesis of supersaturated bile is related to both increased hepatic cholesterol synthesis and decreased bile acid formation. However, in our study we could not find any statistical difference in hepatic HMG-CoA reductase activity between gallstone patients and the control group, 104 ± 11 and 109 ± 22 pmol/min per mg protein respectively (**Fig. 1**). In 14 gallstone patients and 4 gallstone-free subjects, liver microsomes were also prepared in sodium fluoride which prevents activation of inactive enzyme during preparation (17). However, the relative proportion of HMG-CoA reductase initially present in the active form was similar in gallstone patients and in controls (32% vs. 35%).

The activity of the microsomal enzyme cholesterol 7α-hydroxylase is believed to regulate the biosynthesis of bile acids. As can be seen in **Fig. 2**, there was no statistically significant difference between the two groups of patients, although the mean enzyme activity was somewhat lower in gallstone patients, 6.2 ± 1.1 versus 8.0 ± 2.0 pmol/min per mg protein in the controls. When the ratio of cholesterol 7α-hydroxylase activity to HMG-CoA reductase activity was calculated for each individual, a tendency towards a lower ratio was seen in gallstone disease (0.07 ± 0.01 vs. 0.09 ± 0.01), but the difference was not statistically significant. Thus, our results did not show any clear evidence for a reduced absolute or relative bile acid synthesis as measured by cholesterol 7α-hydroxylase activity in gallstone patients.

TABLE 2. Biliary lipid composition of gallbladder bile

Lipids	Controls (n = 12)	Gallstones (n = 19)
Cholesterol (molar%)	5.4 ± 0.5	7.8 ± 0.6[a]
Bile acids (molar%)	72.7 ± 1.2	70.2 ± 1.1
Phospholipids (molar%)	21.7 ± 0.8	21.9 ± 0.8
Total lipids (g/dl)	10.7 ± 1.2	5.8 ± 0.9[a]
Cholesterol saturation (%)	75 ± 5	131 ± 13[b]

Values given are means ± SEM.
[a] $P < 0.005$ versus control.
[b] $P < 0.001$ versus control.

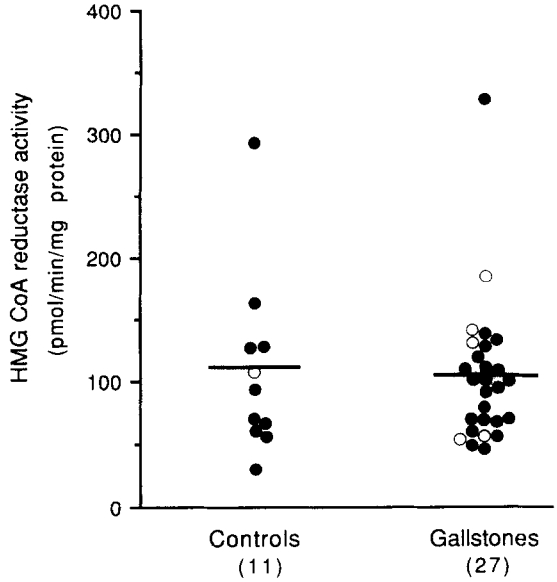

Fig. 1. HMG-CoA reductase activity in liver microsomes from patients with cholesterol gallstones and gallstone-free controls. Horizontal bars indicate means within each group; numbers of patients studied within parentheses; open symbols, males; closed symbols, females.

Another possible mechanism for the development of supersaturated bile could be a decreased ACAT activity. However, the ACAT activity was not significantly lower in our gallstone patients (5.4 ± 0.4 pmol/min per mg protein) than in the control group (6.7 ± 1.1 pmol/min per mg protein; **Fig. 3**). When ACAT was assayed with exogenous cholesterol, a nearly fivefold increase in ACAT activity was seen both in the control group and in the gallstone patients (33.2 ± 5.4 and 27.8 ± 3.7 pmol/min per mg protein, respectively). A negative correlation was obtained between ACAT activity and HMG-CoA reductase activity among gallstone patients ($r_S = -0.74$, $P < 0.002$).

Hepatic cholesterol concentrations were determined in a portion of the liver biopsy specimens used for enzyme analysis. Patients with gallstone disease were not different from subjects without gallstones with respect to the levels of free and esterified cholesterol in whole liver homogenates or in the microsomal fractions (**Table 3**).

DISCUSSION

In some previous studies cholesterol gallstone patients have been reported to display higher than normal levels of HMG-CoA reductase activity in the liver (6–9). Based on this, it has been suggested that the biliary cholesterol output may be determined by the rate of hepatic cholesterol synthesis. However, some objections to the selection of the controls in these studies could be made. Half of the control subjects in the first two studies (6, 7) had either chronic duodenal ulcer disease or Hodgkin's disease, conditions that could have interfered with their preoperative nutritional status. In the two latter studies (8, 9), the group of patients with gallstones had significantly higher relative body weight than the corresponding control group. It has previously been shown (25) that obesity is associated with elevated HMG-CoA reductase activity.

In an attempt to get an ideal control population, we have used subjects with suspect gallbladder polyps and no crystals in gallbladder bile. Despite the fact that they had the same operation performed as the patients with gallstones, their disease was not related to the formation of cholesterol gallstones or to any other hepatobiliary disease. In agreement with some previous studies (10, 11, 26), we found that the gallstone patients and their controls presented similar levels of hepatic HMG-CoA reductase activity. This result is further supported by the previously described lack of a general correlation between rate of hepatic cholesterol synthesis and biliary output or relative amount of cholesterol in bile (11, 27).

The molar fraction of cholesterol in bile is relatively high at low bile acid secretion rates (28). A negative relationship between cholesterol saturation and bile acid synthesis was recently observed in gallstone-free subjects (29). Thus, the possibility must be considered that bile acid synthesis in itself may exert a net regulatory effect on the quantity of cholesterol transported into bile. Shaffer and Small (30), and Reuben et al. (31) reported that pa-

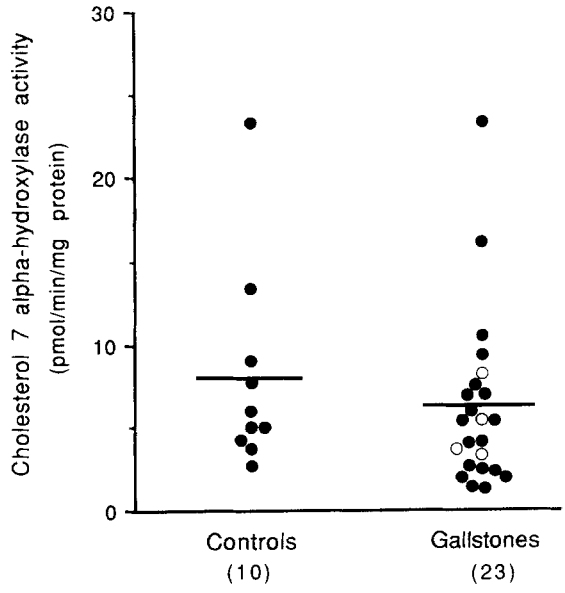

Fig. 2. Cholesterol 7α-hydroxylase activity in liver microsomes from patients with cholesterol gallstones and gallstone-free controls. Horizontal bars indicate means within each group; numbers of patients studied within parentheses; open symbols males; closed symbols, females.

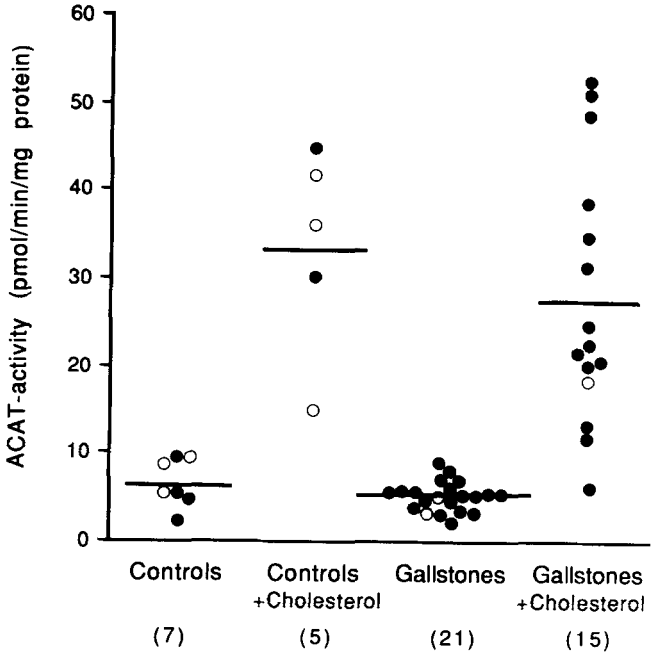

Fig. 3. ACAT activity in liver microsomes from patients with cholesterol gallstones and gallstone-free controls. The enzyme activity was assayed in the absence and presence of exogenous cholesterol (see Methods). Numbers of patients studied within parentheses; open symbols, males; closed symbols, females.

tients with cholesterol gallstones had a significantly reduced bile acid secretion. Nilsell et al. (3) could not confirm this finding although bile acid synthesis measured by isotope dilution tended to be subnormal in gallstone patients. Vlahcevic et al. (32) have reported a significantly lower production of cholic acid in patients with gallstones. In accordance with that finding the hepatic cholesterol 7α-hydroxylase activity was later found to be significantly lower in subjects having gallstones (6–8, 10).

The divergent finding in the present study of an equal enzyme activity among gallstone patients and gallstone-free subjects may possibly be explained by our selection of patients and/or controls or by our use of a different, and probably more precise, mass spectrometric assay for determination of cholesterol 7α-hydroxylase. In previous studies the assay of the cholesterol 7α-hydroxylase was based on conversion of exogenously added radiolabeled cholesterol. In view of the very low conversion of this cholesterol (in general less than 0.1%) and the difficulties of getting the added cholesterol equilibrated with the endogenous cholesterol, it is difficult to get reproducible results. The best assays of cholesterol 7α-hydroxylase activity are considered to be those based on the measurement of the actual mass of 7α-hydroxycholesterol formed from endogenous substrate (12). We have previously shown that the cholesterol 7α-hydroxylase in human liver microsomes is highly saturated with endogenous substrate (33).

Our data do not exclude the possiblity that certain individuals with gallstones might have an increased HMG-CoA reductase activity and/or a decreased 7α-hydroxylase activity, and there was, in fact, a tendency towards a lower ratio between 7α-hydroxylase activity and HMG-CoA reductase activity in our gallstone patients.

There is some evidence in man that only 20–30% of the biliary cholesterol input is derived from newly synthesized hepatic cholesterol (34). In view of that, changes in the kinetics of lipoprotein cholesterol uptake and distribution in the hepatocyte may well result in an increased amount of biliary cholesterol even if there is a normal hepatic cholesterogenesis (26). It has been suggested that hepatic cholesterol esterification may be the major intrahepatic determinant of the size of the biliary cholesterol precursor pool (35). In the rat, biliary cholesterol output is correlated to ACAT activity in a reciprocal manner (35). Thus, it was considered of importance to measure the ability to esterify free cholesterol in gallstone patients. However, the ACAT activity was not lower in our gallstone patients as compared to the control group. Nor was there a difference between the gallstone patients and controls in the ability to esterify exogenous cholesterol, which has been reported by Smith et al. (14). However, the patients with gallstone disease in that study were significantly older and overweight compared to the controls, which mainly consisted of cadaver kidney donors.

An interesting finding in the present study was the inverse correlation between ACAT and HMG-CoA reduc-

TABLE 3. Cholesterol content of homogenates and microsomal fractions of liver biopsies from patients with gallstones and controls

	Homogenates		Microsomes	
Cholesterol	Controls (n = 7)	Gallstones (n = 18)	Controls (n = 6)	Gallstones (n = 24)
	nmol/mg protein			
Total cholesterol	43.6 ± 4.6	41.8 ± 2.5	89.9 ± 6.5	97.3 ± 9.1
Free cholesterol	33.6 ± 3.6	33.5 ± 2.2	72.0 ± 3.6	80.6 ± 6.7
Esterified cholesterol	10.1 ± 2.9	8.3 ± 0.8	17.8 ± 5.0	16.4 ± 3.4

Values given are means ± SEM.

tase activities in the gallstone patients. To the best of our knowledge, this relation, which is known from experiments in rats, has not been reported in man previously (15). The balance between free and esterified cholesterol might be of importance in regulating hepatic cholesterol homeostasis. Unlike some previous authors (6, 10, 11) we did not find an elevated hepatic cholesterol concentration in patients with gallstone disease. Moreover, the proportion of esterified cholesterol was similar in the two groups of patients, which is in agreement with the reports of Nervi et al. (26) and Maton et al. (9).

In conclusion, we found no evidence of disturbances in the activities of HMG-CoA reductase, cholesterol 7α-hydroxylase, or ACAT in Swedish patients with cholesterol gallstones compared to controls. By virtue of the results in the present investigation, we believe that increased biliary cholesterol saturation and gallstone formation are not the results of a defect in a single regulatory mechanism. Rather the etiology of cholesterol gallstones is multifactorial. We cannot exclude, however, that there might be subgroups among gallstone patients displaying an increased activity of HMG-CoA reductase activity, and/or decreased activities of cholesterol 7α-hydroxylase or ACAT. Future studies should be directed at characterizing possible disturbances of hepatic lipoprotein cholesterol uptake and intracellular cholesterol channeling in patients with cholesterol gallstones.

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