Lovastatin Inhibits Gallstone Formation in the Cholesterol-fed Prairie Dog

KIMBERLY D. SAUNDERS, M.D., JOE A. CATES, M.D., MOHAMMAD Z. ABEDIN, PH.D., SHEILA REGE, M.D., SARKIS F. FESTEKDJIAN, B.A., WARREN HOWARD, B.A., and JOEL J. ROSLYN, M.D.

The efficacy of lovastatin, an inhibitor of hepatic cholesterol synthesis in the prevention of cholesterol gallstone formation, was evaluated in the prairie dog model. Two groups of animals were maintained on either nonlithogenic or 1.2% cholesterol-enriched chow for 21 days. Seven of the animals in each group received lovastatin, and the remaining six received only distilled water. All of the cholesterol-fed/water-treated animals had crystals and 83% had gallstones, but none of the cholesterol-fed/lovastatin-treated animals had gallstones and only three had microscopic crystals. These data indicate that lovastatin inhibits cholesterol gallstone formation in a diet-induced model of gallstone disease.

From the Research and Surgical Services, Sepulveda Veterans Administration Medical Center, Sepulveda, and the Department of Surgery, University of California at Los Angeles School of Medicine, Los Angeles, California

IN 1968, ADMIRAND AND Small[1] demonstrated that the hepatic secretion of cholesterol-saturated bile is a prerequisite to the formation of cholesterol gallstones. The clinical significance of this observation has been expanded by the finding that pharmacologic agents that reduce biliary cholesterol saturation are effective in the dissolution of existing gallstones in select patients.[2–4] Furthermore, with the advent of extracorporeal shock wave lithotripsy treatment for gallstones, these drugs have become increasingly important.[5–7] The efficacy of agents such as ursodeoxycholic acid in dissolving gallstones and fragments ultimately depends on their ability to reduce biliary cholesterol levels, probably *via* multiple mechanisms.[8]

Lovastatin, an inhibitor of hepatic cholesterol synthesis, has been proved to be effective in the treatment of hypercholesterolemia.[9] The drug is orally absorbed, extracted on first pass through the liver, where it exerts its primary effect, and is eliminated almost exclusively *via* biliary excretion. Lovastatin acts by competitively inhibiting HMG-CoA reductase,[8] the rate-limiting enzyme in cholesterol biosynthesis. In clinical trials using both healthy volunteers and patients with hypercholesterolemia, lovastatin administration (80 mg/day, in divided doses) resulted in significant reductions in serum total cholesterol, low-density lipid (LDL) cholesterol, very low-density lipid cholesterol, and triglycerides.[10]

Unlike the previously available cholesterol-lowering agent clofibrate, which increases biliary cholesterol,[11] cholesterol saturation index (CSI), and the incidence of clinical cholelithiasis,[12] HMG-CoA reductase inhibitors have been shown to reduce significantly both biliary cholesterol levels and CSIs in hypercholesterolemic patients[13] and healthy volunteers.[14] Recently Logan and Duane[14,15] demonstrated that combination therapy using lovastatin plus ursodeoxycholic acid resulted in significantly greater reductions in biliary CSI than did ursodeoxycholic acid therapy alone. These preliminary observations suggest that lovastatin may be a useful agent in the treatment of cholesterol gallstones.

The purpose of the present study, therefore, was to evaluate the efficacy of lovastatin in preventing the formation of diet-induced cholesterol gallstones. The prairie dog model was selected for study because its biliary lipid composition has been shown to be similar to that of humans.[16–19] Furthermore cholesterol crystals and gallstones are predictably induced in this model in a time-dependent manner by a diet enriched in cholesterol.[16,17]

Supported by the Veterans Administration.

Dr. Saunders was supported by Claude E. Welch Research Fellowship, Department of Surgery, Massachusetts General Hospital.

Address reprint requests to Joel J. Roslyn, M.D., Division of General Surgery, UCLA School of Medicine, Los Angeles, CA 90024.

Accepted for publication September 27, 1991.

Materials and Methods

Experimental Design

Adult male prairie dogs (*Cynomys ludovicianus*, trapped in the wild and obtained from Otto Marten Locke of New Braunfels, TX) weighing approximately 1 kg were individually caged in a thermoregulated (23 C) room. Use of the animals followed prior approval by the Veterans Administration Institutional Animal Care and Use Committee. Two groups of 13 animals each were maintained for 21 days on either nonlithogenic control chow (NL) (Purina Laboratory Chow, Ralston-Purina, St. Louis, MO) or 1.2% cholesterol-enriched chow (XOL) (Teklad, Sprague-Dawley, Madison, WI). Both diets contain ample essential fatty acids, minerals, and fiber. Twice a day, following mild sedation (ketamine, 25 mg/kg body weight), seven animals in each group received a 5-mL bolus through an orogastric tube of lovastatin (mevinolin, provided by Merck, Sharp & Dohme, West Point, PA) (1.6 mg/mL dH_2O), while the remaining six received only distilled water.

After a 16-hour fast (water *ad libitum*), each animal was anesthetized with ketamine (100 mg/kg body weight, intramuscularly) and xylazine (1.5 mg/kg body weight, intramuscularly). *Via* a midline laparotomy incision, the cystic duct was exposed, clamped proximally and distally, and divided. A meticulous cholecystectomy then was performed. The distal common bile duct was ligated and a 25-cm silastic catheter (inner diameter, 0.02 inches/outer diameter, 0.037 inches) was placed into the common bile duct. Hepatic bile was collected under mineral oil over ice on an hourly basis for a total of 3 hours. At the termination of the experiment, blood was obtained *via* direct cardiac puncture and serum was prepared. Serum total cholesterol, high-density lipoprotein (HDL), triglycerides, total protein, and albumin were determined using the MONARCH system (I.L. Fisher, Boston, MA).

Analytic Methods

Gallbladder and hepatic bile samples were examined both grossly and microscopically for the presence of gallstones and cholesterol crystals. Fresh bile was centrifuged to remove suspended gallstones and sludge, and the supernatant was aliquoted and subsequently analyzed for levels of cholesterol,[20] phospholipids,[21] total bile acids,[22] and calcium[23,24] using methods that have long been in use in this laboratory. A CSI was calculated using Carey's critical tables.[25]

Statistical Analysis

Statistical comparisons between the dietary groups were made using analysis of variance followed by Tukey's multiple comparison tests. Fisher's exact test was used to compare the incidence of crystals and gallstones among the treatment groups.

Results

Nutritional Parameters

The effects of cholesterol feeding and lovastatin administration on nutritional parameters are shown in Table 1. All animals tolerated the diets and interventions well. Their coats of fur remained healthy and there was no significant diarrhea or other apparent side effects. Serum total protein and albumin were similar among all treatment groups. While body weights decreased slightly ($\leq$ 1%) during the 21 days of the experiment, the trend was uniform throughout all groups. It has been previously noted in this laboratory (unpublished data) that dietary intake of these gregarious animals is slightly higher when they are housed in small groups; hence the necessity of maintaining the prairie dogs in individual cages probably accounts for the minimal decrease in body weight.

Gallstone Formation

Figure 1 summarizes the effects of lovastatin administration on the formation of diet-induced cholesterol gallstones. As expected neither the NL diet group that received water (NL/W) nor the group that received lovastatin developed either gallstones or cholesterol crystals. All of the cholesterol-fed/water animals (XOL/W) had crystals, and 83% (5 of 6 dogs) had gallstones ($p < 0.005$ *versus* NL/W). In contrast none of the cholesterol-fed/lovastatin-treated animals (XOL/LS) had gallstones ($p < 0.005$ *versus* XOL/W), and only three had microscopic crystals ($p < 0.05$ *versus* XOL/W).

Serum Cholesterol and Lipoproteins

The effects of cholesterol feeding and lovastatin administration on serum cholesterol and lipoprotein levels are shown in Table 2. Cholesterol-fed animals receiving water (XOL/W) had significant elevations in serum total cholesterol ($p < 0.005$), LDL ($p < 0.005$), and HDL ($p < 0.001$) as compared to NL diet-fed animals receiving

TABLE 1. *Nutritional Parameters*

Group	n	% Weight Change	T. Prot* (g/dL)	Albumin* (g/dL)
NL/W	6	1.0 ± 0.2	5.7 ± 0.8	2.4 ± 0.4
NL/LS	7	0.9 ± 0.1	5.7 ± 0.7	2.7 ± 0.4
XOL/W	6	0.9 ± 0.1	6.6 ± 0.7	2.9 ± 0.7
XOL/LS	7	1.0 ± 0.2	6.3 ± 1.0	2.7 ± 0.2

NL, normal diet; W, water-treated (5 mL *via* orogastric tube, twice a day); XOL, 1.2% cholesterol-enriched diet; LS, lovastatin-treated (5 mL of a 1.6 mg/mL solution via orogastric tube, twice a day).

* Serum levels; results are means ± SD.

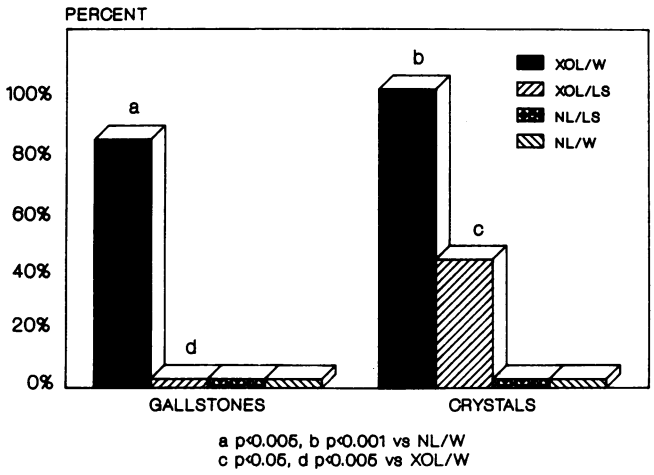

FIG. 1. Effect of lovastatin administration on the formation of cholesterol gallstones in prairie dogs. XOL/W = animals fed 1.2% cholesterol-enriched chow and treated with 5 mL water *via* orogastric tube twice a day (positive controls, n = 6); NL/LS = animals fed normal laboratory chow and treated with 8 mg lovastatin in 5 mL water *via* orogastric tube twice a day (negative controls, n = 7); XOL/LS = animals fed 1.2% cholesterol-enriched chow and treated with 8 mg lovastatin in 5 mL water *via* orogastric tube twice a day (experimental group, n = 7). Results represent percent of animals in each group found to have gallstones or cholesterol crystals in their gallbladder bile after 21 days of treatment.

water (NL/W). Cholesterol-fed/lovastatin-treated animals (XOL/LS) demonstrated a 20% to 30% reduction in total cholesterol, LDL, and HDL as compared to cholesterol-fed/water-treated animals (XOL/W). Similarly lovastatin-treated animals on the NL diet (NL/LS) had levels of serum total cholesterol and LDL that were approximately 25% lower than those seen in NL/W animals. While these differences were not significant at the 5% probability level, they were similar in magnitude to reductions in serum lipids seen in human studies with lovastatin.[6]

For further analysis cholesterol-fed/lovastatin-treated animals (XOL/LS) were divided into two groups based on the presence or absence of crystals in their gallbladder bile. When these two groups were compared with each other, significant reductions were seen in total XOL ($p < 0.05$), and LDL ($p < 0.005$) in XOL/LS animals with clear bile, as compared to XOL/LS animals with crystal-containing bile.

Biliary Lipid Composition

Due to the severe extent of gallstone disease in the XOL/W group, available gallbladder bile following centrifugation and gallstone removal was inadequate or unusable for subsequent biochemical analysis. The high prevalence of gallstones and crystals among these animals, however, suggests that gallbladder bile in this group was almost certainly supersaturated with cholesterol. Gallbladder biochemistry for the remaining treatment groups is presented in Table 3. Because the necessary positive control group data were unavailable, no statistical comparisons could be made among treatment groups. Subdivision of the lovastatin-treated animals based on crystal formation, however, revealed that crystal-containing animals had significant elevations in gallbladder bile XOL, phospholipids (PPL), Ca^{2+}, and CSI, as compared to animals with clear bile. Interestingly values for XOL/LS animals with clear bile were remarkably similar to both groups of animals maintained on the NL diets (NL/LS and NL/W).

The effects of lovastatin and cholesterol feeding on hepatic bile composition are shown in Figure 2. Hepatic bile XOL ($p < 0.001$) and PPL ($p < 0.01$) were significantly elevated in XOL/W animals as compared to the NL/W control group. Although hepatic bile CSIs also tended to be higher in the XOL/W animals (1.00 ± 0.34) as compared to the NL/W controls (0.70 ± 0.13), these differences did not achieve statistical significance at the 5% probability level. Lovastatin-treated cholesterol-fed animals (XOL/LS) had significantly reduced hepatic bile XOL levels ($p < 0.05$) than did cholesterol-fed animals that received water (XOL/W). Once again, while hepatic bile CSIs from the XOL/LS group also tended to be lower (0.78 ± 29) than among XOL/W animals (1.00 ± 0.34), these differences were not significant. There were no significant differences between XOL/W and XOL/LS animals with respect to hepatic PPL, total bile acids (TBA), or calcium levels (latter not shown). Administration of lovastatin to animals maintained on control NL chow induced no significant alteration in hepatic bile composition.

Lovastatin-treated cholesterol-fed animals with clear bile had significantly reduced hepatic XOL ($p < 0.001$) and PPL ($p < 0.025$) as compared to those animals that formed crystals (Fig. 3). In fact XOL and PPL levels in the clear bile group closely approximated those found in the NL diet control group. Similarly hepatic bile CSIs from XOL/LS animals with clear bile (0.66 ± 0.14) were very similar to those from the NL control group (0.70

TABLE 2. *Serum Lipid Profile*

Group	XOL (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
NL/W	144 ± 57	77 ± 13	36 ± 16
NL/LS	110 ± 30	79 ± 24	27 ± 19
XOL/W	325 ± 71*	139 ± 9†	168 ± 70*
XOL/LS	260 ± 87‡	114 ± 34	130 ± 65‡
Crys	336 ± 11§	128 ± 29	194 ± 30‖
Clear	202 ± 70	103 ± 38	81 ± 23

NL, normal diet; W, water-treated (5 mL *via* orogastric tube, twice a day); XOL, 1.2% cholesterol-enriched diet; LS, lovastatin-treated (5 mL of a 1.6 mg/mL solution *via* orogastric tube, twice a day); crys, subgroup with crystal-containing bile; clear, subgroup with clear bile; results are means ± SD.

* $p < 0.005$. † $p < 0.001$ *vs.* NL/W; ‡ $p < 0.02$ *vs.* NL/LS; § $p < 0.05$; ‖ $p < 0.005$ *vs.* XOL/LS clear.

TABLE 3. *Gallbladder Bile Composition*

Group	XOL (μmol/mL)	PPL (μmol/mL)	TBA (μmol/mL)	Calcium (mg/dL)	CSI
NL/W	6.0 ± 0.9	19.3 ± 5.7	136 ± 15	20.3 ± 4.6	0.72 ± 0.14
NL/LS	5.6 ± 2.2	21.7 ± 5.8	123 ± 34	20.6 ± 5.6	0.69 ± 0.15
XOL/LS	9.8 ± 5.4	32.8 ± 9.9	149 ± 51	27.4 ± 6.6	0.81 ± 0.17
Crys	13.9 ± 6.2*	41.2 ± 8.3*	185 ± 58	33.8 ± 2.0†	0.89 ± 0.23*
Clear	6.6 ± 1.3	26.4 ± 4.9	121 ± 25	22.5 ± 3.5	0.75 ± 0.08

NL, normal diet; W, water-treated (5 mL *via* orogastric tube, twice a day); XOL = 1.2% cholesterol-enriched diet; LS, lovastatin-treated (5 mL of a 1.6 mg/mL solution via orogastric tube, twice a day); crys, subgroup with crystal-containing bile; clear, subgroup with clear bile; results are mean ± standard deviation.

* $p < 0.05$, † $p < 0.01$ *vs.* XOL/LS clear.

± 0.13), whereas CSIs among XOL/LS crystal-containing animals tended to be higher (0.95 ± 0.40) ($p = 0.06$).

Discussion

These data represent the first report of which we are aware documenting the effects of lovastatin on gallstone formation. Using a diet-induced model of cholesterol gallstone disease, we demonstrated that oral administration of lovastatin, an inhibitor of cholesterol biosynthesis, altered biliary lipid composition and inhibited cholesterol gallstone formation. Prairie dogs maintained on a XOL diet for 3 weeks had increased biliary concentrations of cholesterol, phospholipids, and calcium, and furthermore 83% developed cholesterol gallstones. The addition of lovastatin to this potent lithogenic regimen markedly reduced the incidence of cholesterol gallstones in this model. Furthermore no apparent side effects were associated with use of this drug. These findings suggest that lovastatin might also be useful in the dissolution of existing cholesterol gallstones, although this hypothesis remains to be tested.

Many studies have demonstrated that the prairie dog is an animal model uniquely suited to the study of cholesterol gallstone disease.[16-19] Both the biliary lipid composition and the bile acid profile in the prairie dog are remarkably similar to those found in humans. Furthermore, to the extent that the pathogenesis of cholesterol gallstones is understood, the process appears to share similar features in humans and prairie dogs.

In the present study, 21 days of cholesterol feeding resulted in crystal formation in all placebo (XOL/W) animals and gallstones in five of six dogs (83%). In contrast only three of seven (42%) lovastatin-treated, cholesterol-fed animals (XOL/LS) had evidence of crystals, and none had gallstones. Interestingly, among the three XOL/LS animals that did have crystals, serum total cholesterol and

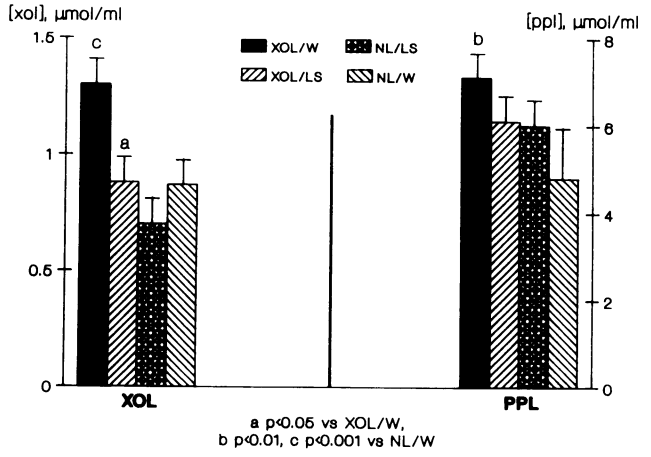

FIG. 2. Effect of lovastatin administration on hepatic biliary lipid composition in cholesterol-fed prairie dogs. XOL = cholesterol; PPL = phospholipids; TBA = total bile acids; XOL/W = animals fed 1.2% cholesterol-enriched chow and treated with 5 mL water *via* orogastric tube twice a day (positive controls, n = 6); NL/W = animals fed normal laboratory chow and treated with 5 mL water *via* orogastric tube twice a day (negative controls, n = 6); XOL/LS = animals fed 1.2% cholesterol-enriched chow and treated with 8 mg lovastatin in 5 mL water *via* orogastric tube twice a day (experimental group, n = 7). Animals were treated for a total of 21 days. Results represent mean ± SEM.

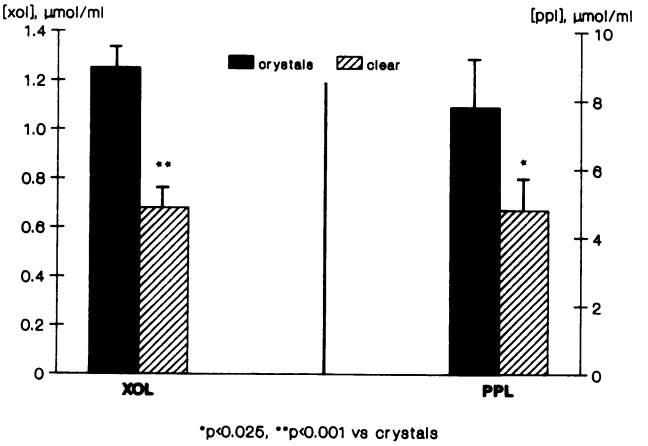

FIG. 3. Comparison of hepatic biliary lipids in cholesterol-fed/lovastatin-treated prairie dogs with clear *versus* crystal-containing gallbladder bile. XOL = cholesterol; PPL = phospholipids; All animals were fed 1.2% cholesterol-enriched chow and treated with 8 mg lovastatin in 5 mL water *via* orogastric tube twice a day for a total of 21 days. Animals were divided into two groups based on the presence (n = 3) or absence (n = 4) of cholesterol crystals in their gallbladder bile. Results represent mean ± SEM.

virtually all gallbladder and hepatic bile parameters measured were typical of untreated cholesterol-fed animals. Furthermore serum and biliary profiles among the four XOL/LS animals with clear bile were similar to those from control animals fed NL chow. These biochemical data, taken together with the gross observations, are consistent with a 'partial response' to lovastatin for the three animals with crystals, and a 'complete response' for the remaining four animals with clear bile. The present study cannot exclude the possibility, however, that lovastatin was effective simply in delaying gallstone formation in this model. Nonetheless this seems improbable given the virtual normalization of biliary biochemistry by lovastatin among animals with clear bile. Rather it seems more likely that the incomplete inhibition of crystal formation seen among lovastatin-treated animals may be due to animal-to-animal variations in gastrointestinal absorption or metabolism of administered drug. Serum lovastatin (mevinolin) levels, which would be most helpful in sorting out these variations, are not available at this time.

The effects of lovastatin administration on serum lipid profile seen in the present study approximate the reductions in serum lipids reported in human studies (using 40 mg, twice a day).[10] Lovastatin administration (8 mg/kg, twice a day) to cholesterol-fed prairie dogs resulted in a 20% to 30% reduction in serum total cholesterol, LDL, HDL, and triglycerides as compared to animals that received no drug. Similarly lovastatin-treated animals fed the control NL diet also showed a 25% decrease in serum total cholesterol and LDL compared to nonlovastatin-treated controls. Because fur coat appearance, body weight, and serum total protein and albumin were all similar among the lovastatin- and nonlovastatin-treated groups, observed differences in serum lipid profile presumably reflect a specific effect of the drug rather than a nonspecific perturbation in dietary intake.

It is impossible to define precisely the mechanism by which lovastatin exerts its inhibitory effect on gallstone formation based on the findings in the present experiment. We would speculate, however, that the observed inhibition of cholesterol crystal and gallstone formation resulted from a reduction in hepatic synthesis of cholesterol. Lovastatin directly inhibits the activity of HMG-CoA reductase,[8] the rate-limiting enzyme in cholesterol biosynthesis (Fig. 4). Furthermore studies by Stone and colleagues[26] using an *in vivo* rat model demonstrated inhibition of both hepatic cholesterol synthesis and secretion after lovastatin infusion, with no effect on bile flow, bile salt output, or phospholipid output. In the present study, lovastatin significantly reduced hepatic biliary cholesterol concentration without altering phospholipid, total bile acid, or calcium levels. These findings are consistent with, but do not prove, the hypothesis that inhibition of gallstone formation by lovastatin may be due to reduction in cholesterol secretion by the liver. Additional studies, specifically designed to examine the effects of lovastatin on cholesterol synthesis and secretion, are necessary to clarify this mechanism further.

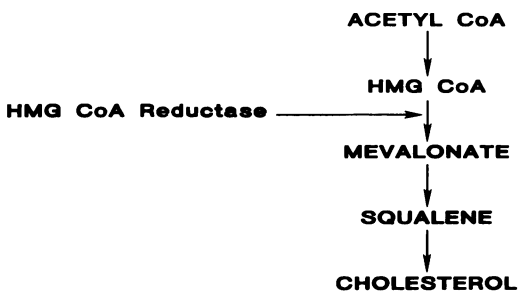

FIG. 4. Simplified version of the cholesterol biosynthetic pathway. HMG-CoA reductase, which converts HMG-CoA to mevalonate, is the rate-limiting enzyme in this sequence.

Data from our animal studies indicate that lovastatin, which suppresses cholesterol biosynthesis, inhibits cholesterol gallstone formation in a diet-induced model of gallstone disease. Studies are currently underway to define further the mechanism by which gallstone inhibition is achieved and to explore the possible role of lovastatin in gallstone dissolution.

References

1. Admirand WH, Small DM. The physicochemical basis of cholesterol gallstone formation in man. J Clin Invest 1968; 47:1043.
2. Roda E, Bazzoli F, Labate AMM, et al. Ursodeoxycholic acid vs. chenodeoxycholic acid as cholesterol gallstone-dissolving agents: a comparative randomized study. Hepatology 1982; 2:804–810.
3. Schoenfield LJ, Lachin JM, the Steering Committee, and the National Gallstone Study Group. Chenodiol (chenodeoxycholic acid) for dissolution of gallstones: The National Cooperative Gallstone Study. Ann Intern Med 1981; 95:257–282.
4. Salen G, Colalillo A, Verga D, et al. Effect of high and low doses of ursodeoxycholic acid on gallstone dissolution in humans. Gastroenterology 1980; 78:1412–1418.
5. Hofmann AF. Rationale of bile acid therapy after biliary lithotripsy. *In* Ferrucci JT, Delius M, Burhenne HJ, eds. Biliary Lithotripsy. Chicago: Year Book Medical Publishers, 1989, pp 151–157.
6. Sackmann M, Delius M, Sauerbruch T, et al. Shock-wave lithotripsy of gallbladder stones—The first 175 patients. N Engl J Med. 1988; 318:393–397.
7. Heberer, G, Paumgartner G, Sauerbruch T, et al. A retrospective analysis of three year's experience of an interdisciplinary approach to gallstone disease including shock-waves. Ann Surg 1988; 208: 274–277.
8. Angelin B, Ewerth S, Einarsson K. Ursodeoxycholic acid treatment in cholesterol gallstone disease: effects on hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, biliary lipid composition, and plasma lipid levels. J Lipid Res 1983; 24:461–468.
9. Havel RJ, Hunninghake DB, Illingworth DR, et al. A multicenter study of lovastatin (mevinolin) in the therapy of heterozygous familial hypercholesterolemia. Ann Intern Med 1987; 107:609–615.
10. Krukemyer JJ, Talbert RL. Lovastatin: A new cholesterol lowering agent. Pharmacotherapy 1987; 7:198–210.
11. Pertsemlidis DE, Panveliwaila D, Ahrens EH Jr. Effects of clofibrate and an estrogen-progestin combination on fasting biliary lipids

and cholic acid kinetics in man. Gastroenterology 1974; 66:565–573.
12. The Coronary Drug Project Research Group. Coronary Drug Project. Clofibrate and niacin in coronary heart disease. JAMA 1975; 231:360–381.
13. Duane WC, Hunninghake DB, Freeman ML, et al. Simvastatin, a competitive inhibitor of HMG-CoA reductase, lowers cholesterol saturation index of gallbladder bile. Hepatology 1989; 8(5):1147–1150.
14. Logan GM, Duane WC. Lovastatin aded to ursodeoxycholic acid further reduces biliary cholesterol saturation. Gastroenterology 1990; 98:1572–1576.
15. Logan GM, Duane WC. Lovastatin in combination with ursodeoxycholic acid lowers cholesterol saturation index of gallbladder bile more than either agent alone. Gastroentrology 1989; 96: A623(Abstr).
16. Brenneman DE, Connor WE, Forker EL, et al. The formation of abnormal bile and cholesterol gallstones from dietary cholesterol in the prairie dog. J Clin Invest 1972; 51:1495–1502.
17. DenBesten L, Safaie-Shirazi S, Connor WE, et al. Early changes in bile composition and gallstone formation induced by a high cholesterol diet in prairie dogs. Gastroenterology 1974; 66:1036–1045.
18. Gurll N, DenBesten L. Animal models of human cholesterol gallstone disease: a review. Lab Anim Sci 1978; 28:428–432.
19. Holzbach RT. Animal models of cholesterol gallstone disease. Hepatolotgy 1984; 4:1915–1985.
20. Roschlau F, Bernt E, Gruber W. Cholesterol and esterified cholesterol. *In* Bergmeyer HU, ed. Methods of Enzymatic Analysis, Vol. 4. Academic Press, New York, 1974, p 1890.
21. Dryer Rl, Tammes AR, Routh R. The determination of phosphorus and phosphatase with N-phenyl-p-phenylene diamine. J Biol Chem 1957; 225:177–183.
22. Iwata T, Yamasaki K. Enzymatic determination and TLC of bile acids in blood. J Biochem (Tokyo) 1964; 56:424–431.
23. Andregg C, Flaschka J, Sallman R, Schwarzenback GM. Metallindikatoren VII. Ein auf erdalkaliionen arsprechendes phtalein und seine analytische verwendung. Helv Chem Acta 1954; 37:113.
24. Connerty H, Briggs A. Determination of serum calcium by means of orthocresolphthalein complex one. Am J Clin Pathol 1966; 45:290.
25. Carey MC. Critical tables for calculating the cholesterol saturation index of native bile. J Lipid Res 1978; 19:945–955.
26. Stone BG, Erikson SK, Craig WY, Cooper AD. Regulation of rat biliary cholesterol secretion by agents that alter intrahepatic cholesterol metabolism. J Clin Invest 1985; 76:1773–1781.