

Ezetimibe ameliorates cholecystosteatosis

Abhishek Mathur, MD, Julia J. Walker, MS, Hayder H. Al-Azzawi, MD, Debao Lu, MD, Deborah A. Swartz-Basile, PhD, Attila Nakeeb, MD, and Henry A. Pitt, MD, Indianapolis, Ind

Background. Cholecystosteatosis is the accumulation of gallbladder wall fats leading to decreased gallbladder emptying. Ezetimibe inhibits intestinal fat absorption and prevents murine gallstone formation. However, the influence of ezetimibe on gallbladder emptying and cholecystosteatosis has not been studied. Therefore, we tested the hypothesis that ezetimibe would improve gallbladder motility by preventing the buildup of fats in the gallbladder wall.

Methods. Forty lean female mice were fed either a control diet or a lithogenic diet for 6 weeks. Half of the mice on each diet received ezetimibe. At 11 weeks of age, all mice were fasted overnight and underwent gallbladder ultrasonography to determine ejection fraction. One week later, the mice were fasted and underwent cholecystectomy. Bile was examined for cholesterol crystals. The gallbladders were snap-frozen for lipid analysis.

Results. The lithogenic diet significantly ($P < 0.05$) increased serum cholesterol, biliary crystals, gallbladder wall cholesterol and cholesterol/phospholipid ratio, and decreased gallbladder ejection fraction. All of these abnormalities were reversed ($P < 0.05$) by the addition of ezetimibe to the diet.

Conclusions. These data suggest that ezetimibe lowers serum cholesterol, prevents biliary crystals, and normalizes gallbladder wall fat and function. We conclude that ezetimibe ameliorates cholecystosteatosis and may be an effective agent for gallstone prevention. (Surgery 2007;142:228-33.)

From the Department of Surgery, Indiana University School of Medicine, Indianapolis

INTRODUCTION

Fatty infiltration of an organ, under conditions of oxidative stress, has the potential to precipitate an inflammatory cascade resulting in organ dysfunction.^{1,2} Obesity leads to steatosis of various organs including the heart, kidneys, and liver. Hepatic steatosis has been termed nonalcoholic fatty liver disease that may lead to nonalcoholic steatohepatitis.¹ We have demonstrated recently that congenitally obese mice and lean mice fed a high-fat diet have increased gallbladder wall fats and decreased gallbladder emptying.³ We also have shown that cholecystosteatosis, the accumulation of fats in the gallbladder wall leading to decreased emptying, occurs in humans.⁴ In addition, high-fat diets rich in

cholesterol have been documented to produce gallstones in animal models.⁵ Moreover, the type of dietary fat plays a role with saturated fats enhancing and unsaturated fats protecting against gallstone formation in both animal models and human studies.⁶⁻⁸

Ezetimibe is a drug that inhibits the absorption of both dietary and biliary cholesterol in the small intestine.⁹ Ezetimibe has been approved for use in humans to lower serum cholesterol. Ezetimibe is revolutionary because it is the only drug available that acts directly on enterocyte-mediated cholesterol absorption, via the Niemann-Pick C1 Like 1 (NPC1L1) protein.^{10,11} Moreover, ezetimibe ameliorates hepatic steatosis and cholesterol gallstone formation in animal models¹⁰⁻¹³; however, the influence of ezetimibe on in vivo gallbladder emptying and on gallbladder wall fat accumulation, cholecystosteatosis, has not been studied. Therefore, we tested the hypothesis that ezetimibe would improve gallbladder motility by preventing the buildup of fats in the gallbladder wall.

MATERIALS AND METHODS

Animals and diets. Forty 6-week-old lean C5BL/6J female mice were obtained from Jackson Laboratory (Bar Harbor, Me). At 7 weeks of age, 20 mice were fed a 25% fat control (CONT) diet with corn and soybean oil, and the remaining 20 were fed a

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Reprint requests: Henry A. Pitt, MD, Department of Surgery, Indiana University School of Medicine, 535 Barnhill Drive, RT 130D, Indianapolis, IN 46202. E-mail: hapitt@iupui.edu.

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lithogenic (LITH) diet with 23% butter fat, 1.5% cholesterol, and 0.5% cholic acid (Dyets Inc.; Bethlehem, Penn.) for 6 weeks. Half of the mice on each diet received ezetimibe (EZT) (0.004%) in their diet, which resulted in four study groups. Both the animals and the food were weighed weekly to determine growth, dietary intake, and ezetimibe dose in mg/kg/day. All protocols for these animal studies were approved by the Indiana University Institutional Animal Care and Use Committee.

Gallbladder ultrasonography. At 11 weeks of age, all mice were fasted overnight and underwent ultrasonography of the gallbladder under inhalation isoflurane anesthesia. Gallbladder volume before (resting volume) and 45 min after an intraperitoneal injection of 1 nmol/kg cholecystokinin (residual volume) were determined using the Visual Sonic Vivo 660TM High Resolution Imaging System. Gallbladder ejection fraction was determined from the resting and residual gallbladder volumes.

Serum, bile, and tissue collection. At 12 weeks of age, after an overnight fast with water allowed ad libitum, all mice were anesthetized with an intraperitoneal injection of xylazine (15 mg/kg) and ketamine (50 mg/kg). The animals were weighed and then underwent cholecystectomy. Bile was aspirated, and gallbladders were snap-frozen in liquid nitrogen. Whole blood was aspirated from the heart, and serum was isolated.

Serum analysis. Whole blood was spun at 15,000 rpm for 5 min to separate serum. Serum was pooled to give five pools in each group. Serum glucose was determined by the quantitative colorimetric method using the Glucose Liquicolor Kit (StanBio; Boerne, Tex.). Serum insulin was determined by ELISA, using the Ultra Sensitive Rat Insulin Kit (Crystal Chem.; Downers Grove, Ill.). HOMA Index, a measure of insulin resistance (fasting serum glucose X fasting serum insulin/22.5) was then calculated. Cholesterol and triglycerides were determined by using an enzymatic colorimetric method for their quantitative determination. The kits for the same were obtained from Wako Chem. USA, Richmond, Va., and Stanbio, Boerne, Tex., respectively. Serum leptin was determined by ELISA, using the Mouse Leptin ELISA Kit (Linco Research, St. Charles, Mo.).

Gallbladder wall lipid analysis. Gallbladders were pooled two/pool to give five pools in each group. Gallbladder wall lipids were analyzed by thin layer and gas chromatography by the Mouse Metabolic Phenotyping Center at Vanderbilt University Medical Center, as described previously by Goldblatt et al.³

Table I. Gallbladder volumes on ultrasound

Diet	EZT	Resting volume (μL)	Residual volume (μL)
CONT	No	16 \pm 3	7 \pm 1
CONT	Yes	18 \pm 3	9 \pm 2
LITH	No	33 \pm 3*	21 \pm 2*
LITH	Yes	43 \pm 4*†	18 \pm 5*

CONT, Control diet; LITH, lithogenic diet; EZT, ezetimibe.

* $P < 0.05$ vs CONT.

† $P < 0.05$ vs LITH-NO EZT.

Crystal analysis. Bile was spun at 15,000 rpm for 10 min and the pellet examined under polarized light microscopy. Both birefringent liquid “maltese cross” and solid cholesterol monohydrate crystals were counted in 10 high-power fields by an investigator who was blinded as to group.

Statistical analysis. Statistical analyses were performed using Sigma Stat Statistical Software (Jandel Corp., San Rafael, Calif.). All data are expressed as mean \pm SEM. All data were tested for statistical significance by 2-way ANOVA and Tukey test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Animal weights, dietary intake, and ezetimibe dose. No significant differences in weight existed among the four groups of mice at 7 weeks (initial, average weight = 14.4 \pm 0.2 g) and 12 weeks (final, average weight = 16.6 \pm 0.2 g). Average dietary intake per mouse was significantly increased in the lithogenic versus the control diet (17.5 \pm 0.8 vs 15.3 \pm 0.4 g, $P < 0.001$). The addition of ezetimibe to the diet did not affect dietary intake. Because of the difference in dietary intake, the ezetimibe dose was greater in the mice on the lithogenic diet (6.6 vs 5.2 mg/kg/day, $P < 0.01$).

Gallbladder ultrasonography. Gallbladder resting and residual volumes are shown in Table I, and gallbladder ejection fraction is depicted in Fig 1. The LITH diet increased resting volumes (33 \pm 3 vs 16 \pm 3 μL), residual volumes (21 \pm 2 vs 7 \pm 1 μL), and decreased ejection fraction (35 \pm 3 vs 60 \pm 5%) versus the CONT diet. Ezetimibe supplementation to the CONT diet did not alter any of these parameters. Ezetimibe supplementation to the LITH diet restored ejection fraction to normal levels but increased paradoxically ($P < 0.05$) resting and residual volumes.

Serum data. Serum cholesterol is presented in Fig 2, A. As expected, serum cholesterol was increased in mice on the LITH diet ($P < 0.05$). Ezetimibe administration decreased ($P < 0.01$) serum cholesterol concentration on the LITH diet,

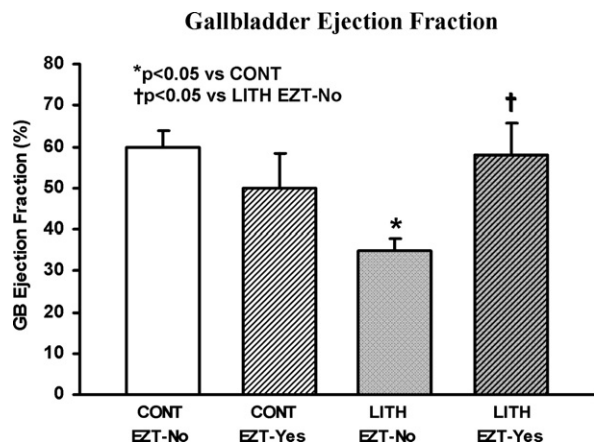


Fig 1. Gallbladder in vivo ejection fractions in all four groups.

but had no effect on the CONT diet. No differences were seen among the four groups in serum triglycerides (37 ± 2 mg/dL), glucose (76 ± 5 mg/dL), insulin (9 ± 0.2 μ IU/L), HOMA (2 ± 0.1), or leptin (1 ± 0.1 ng/mL).

Gallbladder wall lipids. Total cholesterol, cholesterol esters, and cholesterol/phospholipid ratio: Gallbladder (GB) total cholesterol (XOL) is shown in Fig 2, B. GB XOL esters are presented in Table II, and cholesterol/phospholipids (XOL/PL) ratio in Fig 3. GB total XOL, XOL esters, and XOL/PL ratio increased ($P < 0.05$) on the LITH diet. Ezetimibe administration decreased ($P < 0.05$) these tissue increases on the LITH diet, but had no effect on the CONT diet.

Triglycerides and phospholipids: Serum concentrations, GB triglycerides (TG), and phospholipids (PL) are shown in Table II. No changes in gallbladder TG were seen on the LITH diet. Ezetimibe administration had no effect on TG levels on the LITH diet. Ezetimibe decreased ($P < 0.01$) serum TG levels on the CONT diet in the GB. Phospholipids significantly ($P < 0.01$) decreased on the LITH diet. Ezetimibe supplementation to the CONT diet decreased ($P < 0.01$) PL in the GB. Ezetimibe supplementation to the LITH diet decreased ($P < 0.01$) PL in the gallbladder.

Free fatty acids: GB free-fatty acids (FFA) are shown in Table II. No changes in gallbladder FFA occurred on the LITH diet. In the GB ezetimibe supplementation did not alter FFA levels on either diet.

In vitro biliary crystal observation: In vitro crystal results are presented in Fig 2, C. For liquid crystals, the crystal mass/high-power fields were increased ($P < 0.01$) on the LITH diet vs other groups. Ezetimibe administration decreased ($P < 0.01$)

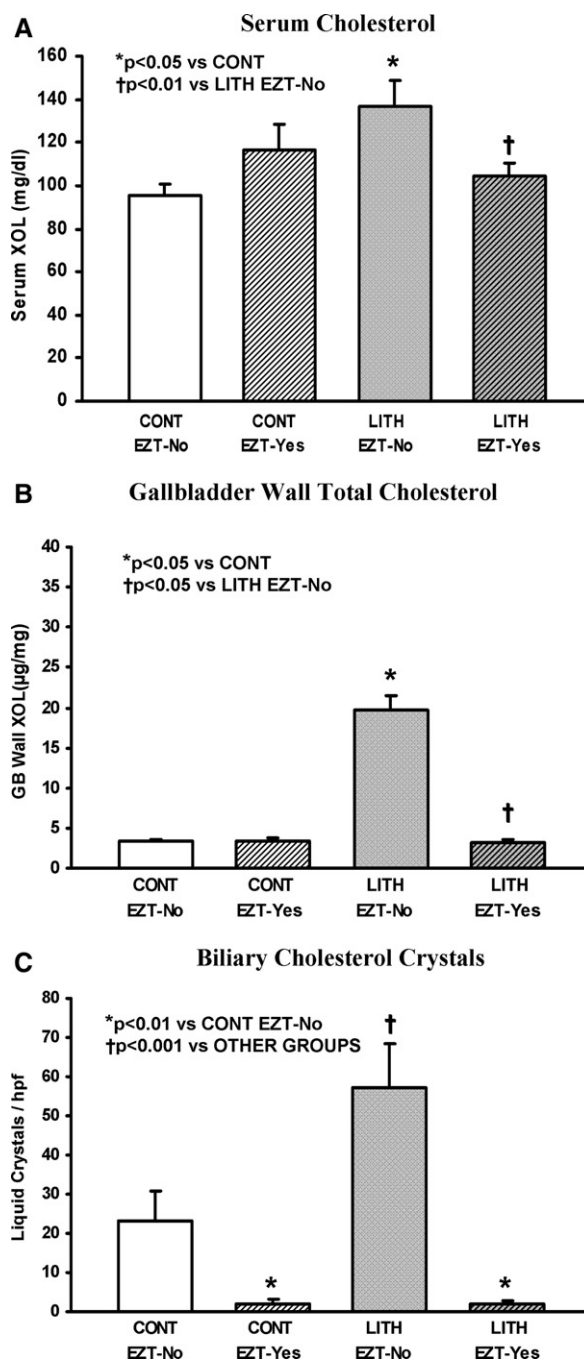


Fig 2. (A) Serum cholesterol (XOL) in milligram per deciliter in all four groups. (B) Gallbladder wall cholesterol (XOL) in microgram per milligram in all four groups. (C) Biliary liquid cholesterol (XOL) crystals per high-power field (hpf) in all four groups.

crystal mass in mice on both diets. No solid crystals were seen in any of the four groups.

DISCUSSION

In this study forty 6-week-old lean C5BL/6J female mice were fed either a 25% fat control diet

Table II. Gallbladder lipids

Diet	EZT	Cholesterol esters ($\mu\text{g}/\text{mg}$)	Triglycerides ($\mu\text{g}/\text{mg}$)	Phospholipids ($\mu\text{g}/\text{mg}$)	Free fatty acids ($\mu\text{g}/\text{mg}$)
CONT	No	0 \pm 0	58 \pm 8	30 \pm 1	2 \pm 0
CONT	Yes	0 \pm 0	29 \pm 7*	18 \pm 1*	1 \pm 0
LITH	No	24 \pm 4*	47 \pm 5	19 \pm 1*	2 \pm 0
LITH	Yes	1 \pm 0 Δ	31 \pm 3 Δ	11 \pm 1 $\dagger\Delta$	1 \pm 0

CONT, Control diet; LITH, lithogenic diet; EZT, ezetimibe.

* $P < 0.01$ vs CONT-EZT No.

$\dagger P < 0.001$ vs CONT-EZT Yes.

$\Delta P < 0.001$ vs LITH-EZT No.

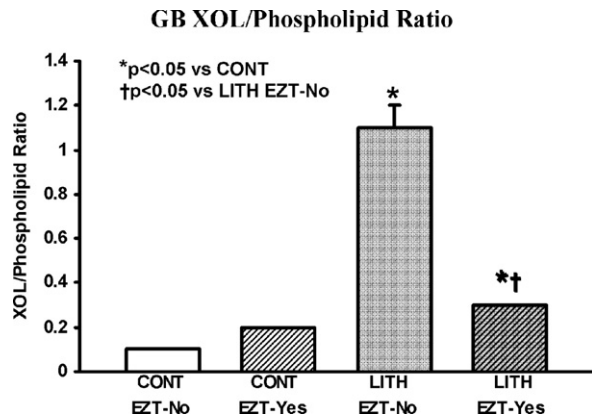


Fig 3. Gallbladder wall total cholesterol/phospholipid (XOL/PL) ratio in all four groups.

with corn and soybean oil ($n = 20$) or a lithogenic diet with 23% butter fat, 1.5% cholesterol, and 0.5% cholic acid ($n = 20$) for 6 weeks. Half of the mice on each diet received ezetimibe (6 mg/kg/day) in their diet. The lithogenic diet increased serum cholesterol, but not triglycerides. Additionally, the butter fat/cholesterol diet increased gallbladder wall total cholesterol, cholesterol esters, and cholesterol/phospholipid ratio and increased concomitantly resting and residual gallbladder volumes while decreasing gallbladder ejection fraction. In the bile, the lithogenic diet increased cholesterol crystal formation. Ezetimibe supplementation to the lithogenic diet decreased the increases in serum and gallbladder wall cholesterol and improved simultaneously gallbladder ejection fraction; however, ezetimibe also increased the resting gallbladder volume in the animals on the lithogenic diet. In contrast, ezetimibe ameliorated biliary cholesterol crystal formation on both diets.

In 1934, Patey et al first showed that a high-fat diet elevated the incidence of cholesterol gallstones.¹⁴ Since then, additional animal studies have further advanced our understanding of the effects of dietary fats on gallstone formation.^{5,6} Saturated

fats have been implicated in enhanced gallstone formation, whereas unsaturated fats have been shown to be protective.⁶ Epidemiologic studies have further bolstered these conclusions. Ortega et al showed that women with gallstones had increased dietary intake of total fat, saturated fat, cholesterol, and monounsaturated fats.⁷ In addition, a recent epidemiologic study of the role of dietary fats in gallstone formation comes from Tsai et al.⁸ In a population based cohort study of 45,756 men over 14 years, they showed that a high intake of poly- and mono-unsaturated fats is associated with a reduced risk of gallstones.

Obesity leads to fat infiltration of various organs, including the heart, kidney, and liver.^{1,2} In the liver, fat infiltration is termed nonalcoholic fatty liver disease, which may lead to nonalcoholic steatohepatitis.¹ In addition, a high-fat diet has been shown to cause hepatic steatosis and is used as an animal model for nonalcoholic fatty liver disease.¹⁵ Previous work from our laboratory by Goldblatt et al showed that obese leptin-deficient mice have increased infiltration of gallbladder wall with fat compared with their lean counterparts.³ Feeding a high-fat diet to lean mice also caused steatosis of the gallbladder, highlighting the role of diet in the development of cholecystosteatosis. Both congenital and diet-induced cholecystosteatosis were associated with decreased in vitro gallbladder emptying.^{3,16} Moreover, we have documented the phenomenon of cholecystosteatosis in humans by demonstrating increased fatty infiltration in the gallbladder of patients with acalculous and calculous cholecystitis compared with nondiseased controls.⁴

Cholesterol milieu in the serum and tissues is maintained through a closely knit mechanism of de novo synthesis, dietary absorption, biliary clearance, and excretion. Ezetimibe is an azetidione that is a novel drug targeting the absorption of both dietary and biliary cholesterol in the intestine.⁹ The drug and its glucuronidated form are absorbed in

the small intestine and recirculated via hepatobiliary excretion. In a classic case of the chicken coming before the egg, the identification of ezetimibe led to the discovery of its molecular target—enterocyte-expressed NPC1L1 protein. Interestingly, NPC1L1 mRNA also is expressed in the liver and gallbladder.^{10,11} Although the use of ezetimibe is established in humans in ameliorating hypercholesterolemia, the expression pattern of its molecular target suggest that it may also have effects in the liver and gallbladder. Furthering this theory, Davies et al showed that NPC1L1 knockout mice were resistant to diet-induced hypercholesterolemia, fatty liver, and gallbladder stasis.¹⁰

Decreased gallbladder motility plays a key role in the development of cholesterol gallstones. Excessive incorporation of cholesterol in the membranes of gallbladder smooth muscle has been documented in patients with gallstones and decreased gallbladder contractility.¹⁷ Additionally, increased gallbladder muscle cholesterol and cholesterol/phospholipid ratio decrease membrane fluidity and CCK receptor binding.¹⁸ Moreover, elimination of the excess cholesterol from the gallbladder smooth muscles normalized membrane fluidity and muscle contractility.¹⁷ In this and a previous study,³ we demonstrated a similar increase in gallbladder wall cholesterol and cholesterol/phospholipid ratio when mice were fed a lithogenic diet. Ezetimibe supplementation to this diet prevented the accumulation of cholesterol in the gallbladder wall and restored the impaired gallbladder ejection fraction. Ezetimibe, in addition to inhibiting intestinal NPC1L1, could potentially also reduce incorporation of cholesterol in the gallbladder by acting on gallbladder NPC1L1 receptors.

Resting gallbladder volumes are affected by a plethora of factors including hepatic bile flow, gallbladder absorption, fasting gallbladder tone, and cystic duct, or sphincter of Oddi resistance. We have shown that the lithogenic diet resulted in increased resting gallbladder volumes. Gallbladder wall inflammation is an important regulator of gallbladder absorption and secretion.¹⁹ Visceral fat infiltration results in initiation of an inflammatory cascade,¹ and Rege et al showed that gallbladder inflammation is an early phenomenon in gallstone formation.²⁰ In addition, gallbladder inflammation increases mucin production which may play an important role in cholesterol crystal formation.²¹ Moreover, cholesterol crystals, in turn, can induce the inflammatory process in the gallbladder²² and increase cystic duct resistance.²³ Thus the net effect of diminished gallbladder absorption and

increased cystic duct resistance is elevated gallbladder volume.

Paradoxically, gallbladder volumes increased on the lithogenic diet with ezetimibe. In contrast, Wang et al noted that ezetimibe at a dose of 1 mg/kg/day normalized resting gallbladder volumes and biliary cholesterol saturation index (CSI) and prevented completely gallstone formation in lean mice on a high-cholesterol diet.¹² In addition, Miquel et al have shown that administering ezetimibe at a dose of 5-6 mg/kg/day in lean mice on a lithogenic diet containing 15% fat, 1.25% cholesterol, and 0.5% cholic acid for 2, 4, or 8 weeks increased bile flow, decreased CSI, and prevented completely the formation of both biliary cholesterol crystals and gallstones.¹³ In the present study, mice on the lithogenic diet received a higher dose of ezetimibe than the mice on the control diet (6.6 mg/kg/day vs 5.2 mg/kg/day). This difference could have caused an increase in bile flow and resulted in an increased gallbladder volume; however, the increased gallbladder volume did not affect adversely biliary crystal formation because ezetimibe increased gallbladder motility that circumvented any deleterious effects of an increased resting gallbladder volume.

A previous study from our laboratory showed that lean mice formed cholesterol crystals when fed a lithogenic diet containing butter fat, cholesterol, and cholic acid.²⁴ In the present study, lean mice developed liquid cholesterol crystals on both the soybean/corn oil (primarily unsaturated) and the butter fat/cholesterol (primarily saturated) diet, but they formed more crystals on the lithogenic diet supplemented with cholesterol and cholic acid. These findings concur with literature citing enhanced gallstone formation with increased dietary saturated fats. In addition, Swartz-Basile et al have shown that biliary cholesterol saturation index is increased in lean mice fed a diet supplemented with cholesterol and cholic acid.²⁴ Ezetimibe treatment resulted in a dramatic decrease in liquid crystals of greater than 90% on both diets. These findings confirmed those by Wang et al¹² and Miquel et al,¹³ showing a similar decrease in cholesterol crystals when mice were treated with ezetimibe. Formation of cholesterol crystals involves a close interplay among CSI of bile, gallbladder motility, and pronucleating biliary glycoproteins. Miquel et al¹³ demonstrated that ezetimibe decreased biliary CSI in mice fed a high-cholesterol diet. We have shown that ezetimibe ameliorates gallbladder dysmotility. Further studies will be required to determine the effects of ezetimibe on biliary glycoproteins.

In conclusion, a 23% butter fat/1.5% cholesterol diet increases serum, bile, and gallbladder wall cholesterol and decreases gallbladder emptying. Ezetimibe lowers serum cholesterol, prevents biliary crystals, and normalized gallbladder wall fat and function. Therefore, we conclude that ezetimibe ameliorates cholecystosteatosis, and may be an effective agent for treatment of biliary dyskinesia and prevention of gallstones. Whether ezetimibe will be capable of eliminating symptoms and stones in patients with established cholelithiasis also needs to be studied.

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