Cholesterol gallstone formation in overweight mice establishes that obesity per se is not linked directly to cholelithiasis risk

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Abstract **The relationship between obesity and cholesterol cholelithiasis is not well understood at physiologic or genetic levels. To clarify whether obesity per se leads to increased prevalence of cholelithiasis, we examined cholesterol gallstone susceptibility in three polygenic (KK/H1J, NON/LtJ, NOD/LtJ) and five monogenic [carboxypeptidase E (*Cpe^{fat}*), agouti yellow (*A^{y}*), tubby (*tub*), leptin (*Lep^{ob}*), leptin receptor (*Lepr^{db}*)] murine models of obesity during ingestion of a lithogenic diet containing dairy fat, cholesterol, and cholic acid. At 8 weeks on the diet, one strain of polygenic obese mice was resistant whereas the others revealed low or intermediate prevalence rates of cholelithiasis. Monogenic obese mice showed distinct patterns with either high or low gallstone prevalence rates depending upon the mutation. Dysfunction of the leptin axis, as evidenced by the *Lep^{ob}* and the *Lepr^{db}* mutations, markedly reduced gallstone formation in a genetically susceptible background strain, indicating that in mice with this genetic background, physiologic leptin homeostasis is a requisite for cholesterol cholelithogenesis. In contrast, the *Cpe^{fat}* mutation enhanced the prevalence of cholelithiasis markedly when compared with the background strain. Since CPE converts many prohormones to hormones, a deficiency of biologically active cholecystokinin is a likely contributor to enhanced susceptibility to cholelithiasis through compromising gallbladder contractility and small intestinal motility. Because some murine models of obesity increased, whereas others decreased cholesterol gallstone susceptibility, we establish that cholesterol cholelithiasis in mice is not simply a secondary consequence of obesity per se. Rather, specific genes and distinct pathophysiological pathways are responsible for the shared susceptibility to both of these common diseases.—** Bouchard, G., D. Johnson, T. Carver, B. Paigen, and M. C. Carey. **Cholesterol gallstone formation in overweight mice establishes that obesity per se is not linked directly to cholelithiasis risk. *J. Lipid Res.* 2002.** 43: **1105–1113.**

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It is estimated that as many as half of North American adults are overweight or obese. Most likely, this epidemic has been caused by lifestyle changes in food consumption and exercise as well as a common genetic predisposition to obesity (1). It is believed that more than 200 genes and quantitative trait loci are linked to the regulation of body weight in humans, which clearly reflects the multiplicity of biochemical and pathophysiological pathways involved (2).

Often paralleling prevalence of obesity, cholesterol gallstones (ChGS) affect 20% to 60% of adults in the Americas and Europe and are caused by both genetic and environmental factors (3–5). Furthermore, because obese individuals are more likely than normal weight individuals to acquire cholelithiasis (6, 7), obesity is generally recognized as a major ChGS risk factor (3-7). Nonetheless, close association clinically and pathophysiologically between the two diseases is often inconsistent (3, 8, 9). For example, two important indicators of cholelithiasis risk, the cholesterol saturation index (CSI) of gallbladder bile and the speed and completeness of gallbladder emptying, display considerable intersubject variation among obese patients (10–15).

The purpose of this investigation was to determine in a cohort of murine models of obesity whether obesity per se leads to cholelithiasis or whether cross-susceptibility to both diseases is caused by specific genes that underlie obesity. Therefore, we studied ChGS prevalence rates in eight

Abbreviations: *A^{y}*, agouti yellow; B6, C57BL/6J inbred mouse strain; BKS, C57BLKS/J inbred mouse strain; CCK, cholecystokinin; CCKAR, cholecystokinin A receptor; ChGS, cholesterol gallstones; CPE, carboxypeptidase E; CSI, cholesterol saturation index; LEP, leptin; LEPR, leptin receptor; tub, tubby.

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murine models of obesity fed a lithogenic diet for 8 weeks to determine the cholalithogenic consequences. The strains surveyed included the polygenic obesity models [KK/H1J (KK), NON/LtJ (NON), NOD/LtJ (NOD)] and the monogenic obesity models, namely leptin (*Lep*^{ob}), leptin receptor (*Lepr*^{db}), carboxypeptidase E (*Cpe*^{fat}), tubby (*tub*), and a mutation of the agouti locus named "yellow" (*A*^{y}). For the monogenic models, the background strains included mice with either high or low ChGS susceptibilities. Thereby, the wild-type strains allowed a determination of the phenotypic effects of each obesity mutation compared with mice with normal alleles, but displayed distinct ChGS prevalence rates. Our hypothesis was that, if all obese mice developed heightened ChGS frequency compared with the background strains, we could conclude that gallstones were a secondary consequence of obesity per se. However, if only some murine strains revealed higher gallstone prevalence rates, then we would suppose that only certain genes cause obesity as well as gallstones. Finally, if certain obesity mutations decreased ChGS prevalence rates compared with the background strains, then we could think it likely that mutations in some homeostatic circuits actually counteract metabolic pathways leading to ChGS. Since our obesity models revealed that the development of gallstones was mutation specific, with certain obesity mutations preventing whereas others promoted ChGS formation, we conclude that only certain pathophysiologic pathways leading to obesity result in cross-susceptibility to both diseases.

EXPERIMENTAL PROCEDURES

Mice and diet

Colonies of male and female mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were acclimatized for at least 2 weeks. Animals were provided free access to Rodent Laboratory Chow (Purina Mills, Richmond, VA) and acidified water (adjusted with HCl to a pH of 2.8–3.2) to retard microbial growth. Mice were housed in a temperature (22–23°C) controlled room with alternating 14:10 h light-dark cycles of regular diurnal periodicity. When 10 weeks old, the mice were weighed and fed a lithogenic diet containing 15% butter fat, 1% cholesterol, and 0.5% cholic acid for 8 weeks (16). All animal protocols were approved by the Institutional Animal Care and Use Committees of The Jackson Laboratory and of Harvard University (Cambridge, MA).

We studied KK, NOD, and the NON strains of polygenic obesity and *A*^{y}, *tub*, *Lep*^{ob}, *Lepr*^{db}, and *Cpe*^{fat} models of monogenic obesity (**Table 1**). All mutant obesity models were homozygous, except for A^{y} mice, which are heterozygous since homozygous mice with this mutation are not viable. The *A*^{y} and *Lep*^{ob} mutants were obtained as congenics on a C57BL/6J (B6) background; that is, the original mutation was introgressed into the new background strain (B6) by multiple backcrossings. The *tub* mutant was studied as an isogenic B6 strain; that is, the mutation was maintained in the strain wherein the original spontaneous mutation occurred. The *Lepr*^{db} mutation was available as a congenic strain on both B6 and C57BLKS/J (BKS) backgrounds (Table 1). The *Cpe*^{fat} mutation was bred solely as a congenic strain on the BKS background. Controls for all monogenic obesity strains are therefore the wild-type B6 mouse, which carries *Lith* susceptibility alleles (3), and/or the BKS strain, which at the initiation of this study was of unknown status with respect to *Lith* alleles. Because all three polygenic obesity models are unique inbred strains, they cannot be matched with suitable controls, since mice with identical genetic backgrounds and normal alleles at all obesity loci are not available.

Tissue samples

After 8 weeks on the lithogenic diet, we re-weighed the mice and fasted them for 4 h, allowing free access to acidified water. Blood was collected from the retroorbital plexus into a capillary tube containing disodium EDTA (final concentration ~4.5 mM). Tubes were kept on ice for 1 to 3 hours prior to centrifugation at 5,000 *g* to separate plasma. Mice were sacrificed by cervical dislocation and the abdominal cavity was opened rapidly to allow ligation of the cystic duct and excision of the gallbladder (17, 18). Thereafter, the liver of each mouse was removed intact, weighed, and snap-frozen in liquid N_2.

Gallbladder volumes were determined by gravimetry, assuming a tissue plus bile density of 1 g/ml. We then placed fresh bile on an ethanol-washed slide and, using a polarizing microscope and a low power objective lens, counted sandy stones and true

TABLE 1. Murine models of monogenic obesity

Gene[a]	Protein Encoded	Chr	Original Genetic Background	Present Genetic Background
A^{y}	agouti signaling protein	2	unknown (mouse "fanciers" stocks)	C57BL/6J
tub	tubby (insulin signaling protein)	7	C57BL/6J	C57BL/6J
Lep^{ob}	leptin	6	multiple recessive stock	C57BL/6J
Lepr^{db}	leptin receptor	4	C57BLKS/J	C57BL/6J
Lepr^{db}	leptin receptor	4	C57BLKS/J	C57BLKS/J
Cpe^{fat}	carboxypeptidase E	8	HRS/J	C57BLKS/J

From left to right, the columns describe the common symbols employed for the five obesity mutations, the protein normally encoded by the obesity allele, the chromosomal (Chr) localization of the gene, the original genetic background in which the spontaneous mutation occurred, and the present genetic background wherein the mutation is maintained.

[a] The official names of the strains (and their abbreviations) are B6.Cg-*A*^{y} (*A*^{y}) , C57BL/6J-*tub*^{tub}/+ (*tub*), B6.V-*Lep*^{ob} (*Lep*^{ob}), B6.Cg-*m* +/+ *Lepr*^{db} and BKS.Cg-m +/+ *Lepr*^{db} (*Lepr*^{db}), and BKS.HRS-*Cpe*^{fat}/J (*Cpe*^{fat}), respectively. It is clear from the two right-hand columns that some strains are maintained on an isogenic background (in the strain where the original mutation occurred); however, most mutations have been introgressed into the present backgrounds (congenics) by multiple backcrossings. All mutant mice were homozygous, except for the heterozygous *A*^{y} which is not viable in the homozygous state.

Data are collated from the Jackson Laboratory Mouse Genome Informatics page (www.informatics.jax.org).

gallstones, defined and identified as described earlier (18). In a subset of each cohort of mice, we recorded the presence of cholesterol monohydrate crystals to ensure that 8 weeks on the lithogenic diet was sufficient for solid phase separation. In the remaining mice of each group, we aspirated the gallbladders and pooled the bile samples. We then froze biles rapidly at −20°C for subsequent lipid assays.

Biochemical analysis

After brief centrifugation of gallbladder bile (3,500 rpm for 5 min) to sediment cholesterol monohydrate crystals, total bile salt concentrations were determined using the 3α-hydroxysteroid dehydrogenase assay (17). After perchloric acid digestion, the levels of biliary phospholipids (mostly lecithin) were determined by inorganic phosphorus assay (18). We quantified biliary cholesterol using HPLC (17). The CSIs of the gallbladder biles were calculated from critical tables as described (18).

After thawing, we homogenized each liver, extracted the lipids (19), and determined the amounts of hepatic cholesterol by a cholesterol oxidase/esterase method (Cholesterol 20 kit, Number 352-20, Sigma Chemical Co., St. Louis, MO); the results are expressed per gram of wet tissue. To assay the amounts of unesterified hepatic cholesterol, we employed HPLC (17, 20). We then calculated the proportions of cholesteryl esters in the liver by measuring the difference between unesterified and total cholesterol concentrations.

Total and HDL-cholesterol (HDL-C) levels were assayed in fresh mouse blood after preparing plasma. We employed a Beckman CX5 Delta Chemistry Analyzer with the manufacturer's reagents and procedures (Beckman-Coulter, Fullerton, CA; product number 467825). Prior to HDL-C measurements, apolipoprotein B-containing lipoproteins were precipitated with polyethylene glycol 6000 (21).

Statistical analyses

We principally employed an SPSS statistical software package (SPSS, Inc., Chicago, IL). ANOVA was performed on data for each gender comparing obesity mutants and their respective background controls. In addition, ANOVA was performed between a single polygenic obesity model and each of the other polygenic obesity strains, followed by a Duncan analysis, where applicable. Significant differences in ChGS prevalence rates were established by Chi-square tests. For all statistical analyses, we set significance levels at a $P \leq 0.05$.

RESULTS

Characteristics of murine obesity models

It has been established (22–25) that each monogenic obesity mutation studied here leads to a unique phenotype principally with different times of onset of weight gain. Consistent with this, the *Lep*^{ob} and *Lepr*^{db} mutant mice exhibit rapid onsets of severe obesity, whereas the other models gain excess weight appreciably slower. **Figure 1A, B** plots body weights (grams) of female and male control mice containing the wild-type alleles, and of each monogenic obesity mutation prior to (chow) and during lithogenic diet feeding for 8 weeks. Compared with wild-type controls, essentially all monogenic mutant mice are distinguished by significant increases in body weight both on chow as well as on the lithogenic diet (Fig. 1A, B). Weight gain over control values varies markedly with a specific mutation, with progressive increases in the rank

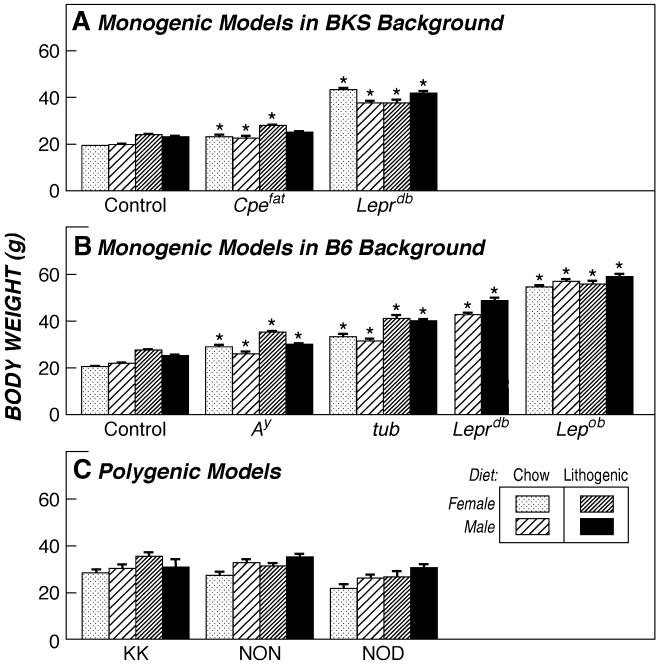

Fig. 1. Body weights (mean ± SEM) in female and male obese mice together with the control background strains, while ingesting chow or a lithogenic diet for 8 weeks. A: Control and monogenic obese mice with the C57BLKS/J inbred mouse strain (BKS) genetic background. B: Control and monogenic obese mice with the C57BL/6J inbred mouse strain (B6) genetic background. C: Mice with polygenic obesity; because these are polygenically obese inbred mice, suitable controls with normal alleles are unavailable. N values for initial body weight varied from 10 to 34 and for final body weight varied from 7 to 34 according to gender and strain designation. In the top and middle panels, asterisks indicate significant differences from the corresponding sets of controls, i.e., background strains, which are plotted to the left of panels A and B.

order: *Cpe*^{fat} < *A*^{y} < *tub* < *Lepr*^{db} < *Lep*^{ob}. However, there are no pronounced differences between genders and there is relative insensitivity to the lithogenic diet, except for *tub* mice (Fig. 1A). Polygenic obesity models (Fig. 1C) display no significant differences from each other, nor exhibit a diet or gender effect. However, compared with the monogenic obesity models, there is a tendency for a gender-dependent variation in obesity level prior to the lithogenic diet, with males showing higher body weight than females.

Cholesterol cholelithiasis in obese mice

Table 2 lists results documenting the number of mice examined for ChGS prevalence rates and stone counts according to mouse gender and group. For purposes of illustration, **Fig. 2** displays the influence of monogenic and polygenic obesity and gender on ChGS prevalence rates at 8 weeks on the lithogenic diet. In all cases, both monogenic obesity models in females and their controls display low to moderate, i.e., under 30%, ChGS prevalence rates. In the case of males, four of the five monogenic obesity models, especially the *Lepr*^{db} and *Lep*^{ob} mice, display gallstone prevalence rates that are zero or otherwise signifi-

TABLE 2. Influence of monogenic and polygenic models of murine obesity on cholesterol gallstone formation[a]

	N[b]	ChGS Prevalence[c]	Mean Number of Sandy GS[d]	Mean Number of True GS[e]
		%		
Monogenic obesity				
Female				
BKS control	19	5	0.0 ± 0.0	0.2 ± 0.2
Cpe^{fat}	17	29	1.5 ± 1.5[f]	1.9 ± 1.5
Lep^{db}	19	6	0.0 ± 0.0	0.0 ± 0.0
B6 control	20	10	0.2 ± 0.2	0.0 ± 0.0
A^{y}	18	11	0.2 ± 0.2	0.0 ± 0.0
tub	23	26	0.4 ± 0.3	1.4 ± 1.4
Lep^{ob}	17	0	0.0 ± 0.0	0.0 ± 0.0[f]
Male				
BKS control	13	15	0.4 ± 0.2	0.0 ± 0.0
Cpe^{fat}	18	78[f]	5.9 ± 3.6[f]	12.4 ± 5.6[f]
Lep^{db}	15	0	0.0 ± 0.0	0.0 ± 0.0
B6 control	16	69	2.2 ± 1.5	2.5 ± 1.3
A^{y}	34	24[f]	5.3 ± 2.9	3.7 ± 3.0
tub	26	31[f]	0.0 ± 0.0[f]	1.1 ± 1.1
Lepr^{db}	16	0[f]	0.0 ± 0.0[f]	0.0 ± 0.0[f]
Lep^{ob}	21	10[f]	0.3 ± 0.2[f]	0.0 ± 0.0[f]
Polygenic obesity				
Female				
KK	8	0	0.0 ± 0.0	0.0 ± 0.0
NON	9	0	0.0 ± 0.0	0.0 ± 0.0
NOD	8	33[g]	3.8 ± 2.0[g]	0.0 ± 0.0
Male				
KK	9	0	0.0 ± 0.0	0.0 ± 0.0
NON	10	10	0.4 ± 0.4	0.0 ± 0.0
NOD	6	38[g]	5.5 ± 4.9[g]	5.0 ± 5.0[g]

[a] Analyses were carried out after 8 weeks of consuming a lithogenic diet as described in Experimental Procedures.

[b] Number of mice employed per group.

[c] Percent of mice with cholesterol gallstones out of the total number tested (6–34, varying for each gender) within each group.

[d] Early, soft stones, partially birefringent, detected in individual mice from a group for each gender (including resistant mice).

[e] Hard stones, completely non-birefringent (see ref 18), detected in individual mice from a group for each gender (including resistant mice).

[f] Significant difference from the same sex background strains (control mice).

[g] Significant difference from other polygenic obesity models.

cantly lower than in the B6 background controls [0% in *Lepr^{db}*, 10% in *Lep^{ob}* vs. 69% in the B6 controls (Fig. 2, Table 2)]. In contrast, 78% (14 of 18) of male *Cpe^{fat}* mutant mice acquire ChGS, appreciably more frequently than controls (15%) with the BKS background (Table 2, Fig. 2). Polygenic obesity models (Table 2, Fig. 2) are either totally resistant to ChGS on the lithogenic diet (KK strain) or reveal a slight or low gender-sensitive susceptibility to cholelithiasis (NON), show modest prevalence but gender-indifferent susceptibility (NOD strain). In all groups, frequencies of cholesterol monohydrate crystals are similar to the ChGS prevalence rates (data not displayed).

Table 2 also summarizes the mean number of sandy and true gallstones found in each mouse group as functions of gender at 8 weeks on the lithogenic diet. Paralleling ChGS prevalence rates, females exhibit fewer sandy and true stones than males, with the exception of mice carrying the *tub* mutation, where no gender difference was observed. Moreover, mean stone number per gallbladder decreases in the *Lep^{ob}* or *Lepr^{db}* congenics compared with the B6 background strain, whereas a marked increase in stone number is observed in the *Cpe^{fat}* strain, correlating positively with its ChGS prevalence rate (Fig. 2).

Gallbladder volumes and biliary lipids

Figure 3 illustrates mean gallbladder volumes (μl) of controls and mutant mice as functions of each gender and strain on the lithogenic diet. It is clear that these values display large variations between mutant strains as well as gender of mice, with values ranging from 7 μl to 59 μl. Compared with their respective background controls (BKS and B6), gallbladder volumes are not influenced appreciably by the presence of the *A^{y}* and *tub* mutations; however, the *Cpe^{fat}* and particularly the *Lep^{ob}* and *Lepr^{db}* mutations result in considerably larger but not statistically significant gallbladder volumes.

Table 3 summarizes the biliary lipid compositions and cholesterol saturation indexes (CSI) of gallbladder biles of obese mice and their controls at 8 weeks on the lithogenic diet. With the exception of *Cpe^{fat}* males, the monogenic obesity mutations result in slight elevations of total lipid concentrations. A similar trend occurs with bile salt and phospholipid concentrations, but not with biliary cholesterol concentrations, which vary with gender and the obesity mutation. Molar percentages of biliary cholesterol decrease uniformly in the monogenic mutants, with the exception of the *Cpe^{fat}* male. In this model, total and individual biliary lipid concentrations are decreased. This is especially true in the case of bile salts, whereas cholesterol is proportionately less affected. In all monogenic obesity models, the CSI values are diminished, with the exception of the *Cpe^{fat}* males, where its value becomes markedly elevated (Table 3), consistent with the high ChGS prevalence rates (Fig. 2).

The polygenic obese mice reveal total and individual biliary lipid concentrations that are similar in magnitude to those observed with the monogenic obesity strains (Table 3). The exception is both males and females of the KK strain, where marked elevations in biliary phospholipid levels contribute to the very low CSI values. Within the polygenic obesity groups, the CSI value was most elevated in NOD males, consistent with their higher ChGS prevalence rates (Table 2), and is caused by a low phospholipid to total biliary lipid ratio (Table 3).

Hepatic and plasma cholesterol concentrations

Table 4 summarizes the effects of murine obesity on hepatic cholesterol levels compared with the corresponding background strains of mice at 8 weeks on the lithogenic diet. The *Lep^{ob}* and *Lepr^{db}* mutations induce the highest elevations of hepatic total and free cholesterol levels, whereas the *tub* mutation produces a net decrease in both. There are no significant effects of any obesity mutation on hepatic free to esterified cholesterol ratio, nor any correlation between unesterified cholesterol levels in liver and biliary cholesterol concentrations ($P = 0.19$, $r^2 = 0.10$).

Because low plasma HDL-C concentration has been linked epidemiologically to ChGS disease in humans and in mice (7, 26–31), we quantified the effects of obesity mutations on plasma total, HDL, and non-HDL choles-

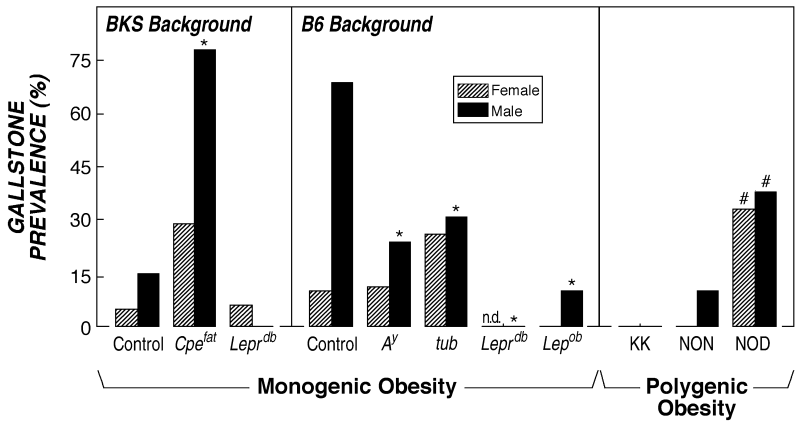

Fig. 2. Effects of murine obesity on cholesterol gallstone prevalence rates in female and male mice after 8 weeks of lithogenic diet feeding. Prevalence rates represent the percent of mice with cholesterol gallstones out of the total number tested within each group (see Table 2 for N values). Due to insufficient availability of female *Lepr*^{db} mice, ChGS prevalence was not determined (n.d.) in the B6 background. A data line at the origin above a designated strain indicates that prevalence rate was zero. Asterisk indicates significant difference from the background strains (control mice), whereas a pound sign denotes significant difference from all other polygenic obesity models. No SEM values are given since these experiments represent studies on single large cohorts.

terol levels (Table 4). The values display that none of the obesity mutations results in a significant decline in HDL-C concentrations. The *Lep*^{ob} or *Lepr*^{db} mutations that prevent ChGS formation are associated with marked elevations of plasma total cholesterol without significant influence on the distribution of cholesterol within lipoprotein fractions. The *Cpe*^{fat} mutation, which induces high susceptibility to ChGS, is also linked to an elevated plasma cholesterol level compared with the BKS control. However, this occurs through an increase in the non-HDL-C fraction accompanied by a reciprocal decline in percent HDL-C. Mice with polygenic obesity also show significant differences from each other, but total hepatic and plasma cholesterol values lie within the ranges observed in the monogenic obesity models (Table 4).

DISCUSSION

As a new approach to understanding a major ChGS risk factor in humans, we focused on genetic obesity in mice and tested its influence on ChGS prevalence rates using polygenic as well as monogenic murine models. Two polygenic obesity models (KK, NON) revealed either zero or low susceptibilities to cholelithiasis whereas one (NOD) achieved a frequency (33–38%; Fig. 2) typical of age-adjusted gallstone prevalence rates in Western humans (3–5). The monogenic obesity models differed considerably from each other (Fig. 2, Table 2), and only male mice with the *Cpe*^{fat} mutation demonstrated markedly elevated ChGS prevalence rates (Fig. 2) as well as number of stones per gallbladder (Table 2). Compared with the background strains, essentially all other monogenic obesity models, particularly the *Lep*^{ob} and *Lepr*^{db} mutations, either induced no change in ChGS prevalence, especially in females, or induced marked decreases in cholelithiasis susceptibility in the case of males. Therefore, this study establishes that the lithogenic consequences of murine monogenic obesity, as well as polygenic obesity, on ChGS formation are not uniform but exhibit a considerable influence of gender and are not directly related to obesity per se. We believe that these findings underscore the importance of the underlying genetic and therefore pathophysiologic causes of obesity in comprehending its impact on ChGS prevalence rates.

A vast body of epidemiologic information in humans suggests that plasma cholesterol levels are correlated inversely with ChGS risk, which may be related to the common occurrence of obesity as a confounding factor in ChGS formation (reviewed in 3). Many different studies of plasma lipids in various ChGS-prone population groups have produced results that are not uniform (7, 26–31). There are studies that identified a strong correlation between low plasma HDL-C and increased risk of ChGS, but

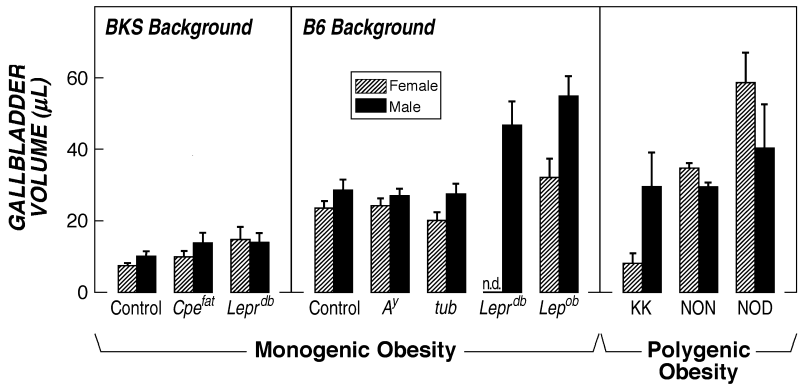

Fig. 3. Gallbladder volumes (µl) in female and male mice with monogenic and polygenic obesity and control background strains at 8 weeks of lithogenic diet feeding. Gallbladder volumes were quantified by differences in weight of the filled and emptied gallbladder as described in Experimental Procedures. Results represent mean ± SEM of N that varied from 5 to 31 mice per group (see Table 2, for details). Due to an insufficient availability, gallbladder volumes were not determined (n.d.) for female *Lepr*^{db} in the B6 background strain. Compared with the respective background strains, no significant differences in gallbladder volumes were found.

TABLE 3. Influence of murine obesity mutations on absolute and relative biliary lipid compositions of gallbladder bile obtained from pooled samples[a]

		Concentration			Moles			
	TL	BS	PL	Ch	BS	PL	Ch	CSI
	g/dl		*mM*			%		
Monogenic obesity								
Female								
BKS control	10.5	153	30	17	77	15	8	1.50
Cpe^{fat}	14.8	210	47	21	76	17	7	1.17
B6 control	11.9	169	36	20	75	16	9	1.46
A^{y}	14.6	208	46	20	76	17	7	1.14
tub	14.2	217	38	16	80	14	6	1.05
Lep^{ob}	13.7	200	43	15	77	17	6	0.93
Male								
BKS control	10.1	146	30	16	76	16	8	1.45
Cpe^{fat}	4.1	50	16	10	66	21	13	2.13
B6 control	11.9	179	32	15	79	14	7	1.26
A^{y}	13.3	197	39	15	78	16	6	1.02
tub	15.1	222	46	17	78	16	6	0.96
Lepr^{db}	17.0	253	53	12	79	17	4	0.79
Lep^{ob}	13.0	191	41	12	78	17	5	0.59
Polygenic obesity								
Female								
KK	17.1	237	68	15	74	21	5	0.60
NON	16.3	250	43	16	81	14	5	0.91
NOD	17.5	266	49	14	81	15	4	0.71
Male								
KK	12.2	152	62	15	69	27	7	0.80
NON	16.0	240	45	17	79	15	6	0.94
NOD	14.5	226	32	16	82	12	6	1.17

TL, total lipid concentration; BS, bile salt; PL, phospholipids; Ch cholesterol; and CSI, cholesterol saturation index.

[a] Analyses carried out at 8 weeks on the lithogenic diet. Results were obtained from pooled biles according to strain and gender as described in Experimental Procedures employing the following numbers of gallbladder biles (listed for females followed by males): BKS (N = 14 and 10), *Cpe^{fat}* (N = 8 and 7), B6 (N = 10 and 10), *A^{y}* (N = 13 and 10), *tub* (N = 13 and 12), *Lepr^{db}* (N = 14, male only), *Lep^{ob}* (N = 10 and 13), and all polygenic models (N = 5 and 5).

other work suggested a strong statistical link between low plasma LDL-C levels and ChGS. In the present work we found, unexpectedly, that the obesity mutation *Cpe^{fat}* increased ChGS prevalence rates markedly, especially in males (Fig. 2). Moreover, this observation is associated with a decrease in the ratio of HDL to total cholesterol in plasma (Table 4). Nonetheless, considering the data on plasma lipids as a whole, we failed to observe any statistically significant correlation between plasma cholesterol, its sub-fractions, or percent HDL and ChGS prevalence rates. In these murine obesity models, biochemical analysis of gallbladder bile not unexpectedly revealed that ChGS prevalence rates correlated positively with biliary CSI ($P = 0.01$, $r^2 = 0.33$), and negatively with total hepatic cholesterol concentrations ($P = 0.05$, $r^2 = 0.19$); but unanticipated was the positive correlation with low biliary phospholipid levels ($P = 0.01$, $r^2 = 0.33$). The latter finding clearly showed that in these obese mice low phospholipid concentration was the variable with the strongest predictive value for ChGS formation. Most importantly, reduced levels of biliary phospholipids lead to heightened CSI and risk of ChGS by shifting relative lipid compositions of bile to lower phospholipid mole fractions (18). Of considerable relevance is that mutations in the canalicular phosphatidylcholine transmembrane translocator, ABCB4 (also known and MDR2 in mice and MDR3 in humans), appear to underlie the molecular basis of intrahepatic cholesterol calculi and "unusual" gallstones in both humans and mice (32–35). Moreover, although the extremely high prevalence rates of cholelithiasis in Chileans are undoubtedly genetically based (3), there has also been a substantiated association with consumption of certain Andean legumes in this population, which lower biliary phospholipid levels and thereby heighten cholesterol supersaturation of bile (36). Further understanding of the influence of different genetic varieties of obesity on biliary phospholipid secretion in humans may provide an understanding as to why obesity per se does not invariably lead to heightened risk of ChGS formation.

The dual susceptibility of mice with the *Cpe^{fat}* mutation to both obesity and ChGS (Fig. 2) establishes *Cpe^{fat}* as a particularly novel "*Lith*" gene. Mice with the *Cpe^{fat}* mutation lack functional carboxypeptidase E and therefore cannot hydrolyze many prohormones efficiently to their bioactive form, including procholecystokinin to cholecystokinin (CCK) (37–39). We have shown elsewhere that inactivation of CCK's hormonal axis, by targeted mutation of the murine cholecystokinin A receptor (*Cckar*) gene, results in heightened susceptibility to ChGS in mice with an otherwise gallstone-resistant genetic background (40). Nonetheless, mice with the *Cpe^{fat}* mutation are not totally deficient in hormonally-active CCK because some hydrolysis of pro-CCK occurs by other, less specific, intracellular carboxypeptidases (37–39). Despite such ancillary mechanisms, the "rescue" hydrolases fail to produce a normal post-prandial elevation of circulating levels of bioactive CCK in response to a meal (37). Not only does circulating CCK promote gallbladder contractility and relaxation of Oddi's sphincter, but physiological CCK levels are a powerful stimulant of small intestinal motility (41). In humans, intestinal cholesterol absorption is known to be appreciably augmented by hypomotility of the small intestine (42). Moreover, gallbladder hypomotility results in prolonged stagnation of bile (43), which is an important risk factor for ChGS, by facilitating phase separation of cholesterol liquid and solid crystals from supersaturated gallbladder bile (3). Most likely, therefore, a key contributing factor to ChGS susceptibility in the *Cpe^{fat}* mutant mouse is its inability to elevate circulating CCK levels postprandially (37).

The present study clearly established that *A^{y}*, *tub*, *Lep^{ob}*, and *Lepr^{db}* obesity mutations, particularly in males, counteracted the high susceptibility to ChGS in the B6 genetic background (Table 2, Fig. 2), which carries susceptible *Lith* alleles (44). In these obesity models, the chromosomal locations of obesity mutations are distinct from *Lith* genes, since their map positions do not overlap with currently identified *Lith* loci (44, 45). Therefore, our results suggest that obesity mutations may act epistatically on the *Lith* genes to abrogate their lithogenic actions. It is important to emphasize that none of these obesity mutations encode proteins that are specifically expressed in the liver or

TABLE 4. Influences of murine obesity mutations on hepatic and plasma cholesterol concentrations[a]

	Hepatic Concentration[b]			Plasma Concentration[c]			
	Total	Free	Free to Esterified Ratio	Total	HDL	Non-HDL	% in HDL
	mg/g liver			*mg/dl*			
Monogenic obesity							
Female							
BKS control	42 ± 3	3.8 ± 0.3	0.10 ± 0.01	174 ± 8	64 ± 3	109 ± 6	37 ± 1
Cpe^{fat}	40 ± 4	5.3 ± 0.5	0.17 ± 0.01	342 ± 30[d]	66 ± 5	275 ± 31[d]	21 ± 2[d]
Lepr^{db}	41 ± 3	5.4 ± 0.5	0.17 ± 0.02	389 ± 22[d]	94 ± 11[d]	294 ± 31[d]	28 ± 4
B6 control	42 ± 4	4.8 ± 0.3	0.13 ± 0.01	177 ± 5	56 ± 4	125 ± 7	29 ± 3
A^{y}	48 ± 4	5.3 ± 0.3	0.12 ± 0.02	203 ± 3	64 ± 4	144 ± 14	30 ± 2
tub	31 ± 2	4.5 ± 0.8	0.19 ± 0.04	196 ± 6	65 ± 4	130 ± 7	34 ± 2
Lep^{ob}	55 ± 5	6.9 ± 0.5[d]	0.14 ± 0.01	377 ± 26[d]	63 ± 4	314 ± 28[d]	18 ± 2
Male							
BKS control	28 ± 4	4.4 ± 0.3	0.18 ± 0.02	194 ± 15	61 ± 2	131 ± 16	34 ± 2
Cpe^{fat}	30 ± 2	3.9 ± 0.2	0.16 ± 0.01	274 ± 15[d]	64 ± 4	208 ± 15[d]	24 ± 2[d]
Lepr^{db}	40 ± 3	6.0 ± 0.5	0.22 ± 0.02	271 ± 10[d]	108 ± 12[d]	162 ± 17[d]	41 ± 5
B6 control	41 ± 3	5.9 ± 0.3	0.15 ± 0.00	275 ± 12	61 ± 2	213 ± 12	23 ± 1
A^{y}	31 ± 3	5.4 ± 0.2	0.22 ± 0.04	236 ± 6	69 ± 4	166 ± 8	29 ± 2
tub	24 ± 2[d]	4.5 ± 0.6	0.24 ± 0.03	200 ± 5[d]	75 ± 3	123 ± 5[d]	38 ± 1[d]
Lepr^{db}	56 ± 3	9.1 ± 0.6[d]	0.19 ± 0.01	415 ± 29[d]	81 ± 3	333 ± 32[d]	20 ± 22
Lep^{ob}	55 ± 6	6.5 ± 0.1	0.19 ± 0.03	396 ± 21[d]	73 ± 6	324 ± 26[d]	20 ± 2
Polygenic obesity							
Female							
KK	49 ± 2	5.1 ± 0.4	0.12 ± 0.01	296 ± 13	69 ± 5	229 ± 17	23 ± 3
NON	32 ± 3[e]	6.1 ± 0.6	0.31 ± 0.04[f]	201 ± 4[e]	79 ± 3	122 ± 6[e]	39 ± 2
NOD	46 ± 6	6.1 ± 0.5	0.23 ± 0.06	311 ± 28	99 ± 6[e]	211 ± 28	33 ± 2
Male							
KK	45 ± 5	5.8 ± 0.6	0.20 ± 0.02	189 ± 10[e]	106 ± 6	83 ± 9	53 ± 2
NON	41 ± 2	5.6 ± 0.3	0.16 ± 0.02	261 ± 9	134 ± 8	123 ± 12	53 ± 4
NOD	26 ± 3[e]	5.4 ± 0.2	0.30 ± 0.03[g]	303 ± 9	133 ± 9	170 ± 9[e]	44 ± 3

[a] Analyses are as described in Experimental Procedures carried out at 8 weeks of consuming a lithogenic diet.
[b] Assays of total and free hepatic cholesterol concentrations were performed respectively, on the first 10 and 6 mice available, per gender and group as described in Experimental Procedures.
[c] Plasma values were obtained from 4 to 24 mice according to gender and strain (see Experimental Procedures for details).
[d] Significantly different compared to the background controls.
[e] Significantly different compared to the other polygenic models.
[f] Significantly different from the KK strain.
[g] Significantly different from the NON strain.

gallbladder; however, they appear to display major influences on signaling/hormonal pathways outside the hepatobiliary system (46–48). The strongest gallstone protective effect was demonstrated by the *Lep*^{ob} and *Lepr*^{db} obesity mutations (Fig. 2), where ChGS formation was almost entirely abrogated and CSI levels halved, despite the highly susceptible ChGS proclivity of the background strain (Fig. 2, Table 2). The *Lepr*^{db} mutation also appeared to reduce the susceptibility to cholesterol cholelithiasis on the gallstone-resistant BKS background, especially in males (Table 2). Taken together, these observations reinforce the concept that absence of an intact leptin hormonal axis in *Lep*^{ob} and *Lepr*^{db} mice inhibits the development of ChGS by decreasing biliary cholesterol concentrations and CSI values, most likely secondary to a diminution in hepatic cholesterol secretion (Tables 2 and 3). This contrasts with the actions of these mutations in elevating both total hepatic and plasma cholesterol levels (Table 4). At least in the basal state, HDL-C is a major source of biliary cholesterol, and non-HDL-C is a source of de novo bile salt synthesis (3, 49). Furthermore, our data do not support the notion that when cholesterol pools are augmented in the liver, they become major determinants of biliary cholesterol secretion (3). Nonetheless, we show that an intact leptin axis is crucial for normal biliary lipid coupling and cholesterol hypersecretion in obese mice ingesting a lithogenic diet. Recent observations (50) in leptin-deficient rats support this concept since biliary cholesterol secretion is uncoupled negatively from secretion rates of the solubilizing lipids, i.e., bile salts plus phospholipids. However, acute infusions of leptin have been shown to counteract this effect (50).

From these mouse studies, we can infer a number of pathophysiological correlations that might be extrapolated cautiously to ChGS disease in humans. Paralleling clinical and epidemiological reports (10, 11, 14, 15, 51), we have shown that murine obesity has variable consequences on biliary lipid composition, especially lithogenicity of gallbladder bile and ChGS risk, all of which are dependent on the underlying genetic basis of obesity. These models of murine obesity exhibit multiple hormonal and metabolic abnormalities, including those affecting insulin, glucagons, and cortisol regulation, just as occurs in obese humans (22–25, 52). Although these dysregulations in hormonal homeostasis could contribute, in part, to obesity and gallstones in humans (53–55), our study ex-

tends these concepts to highlight preponderant roles of two additional hormones in these diseases, carboxypeptidase E and leptin, at least in obese mice. Disruption of the effects of the former strongly promotes ChGS formation, and disruption of the latter markedly inhibits ChGS formation. Monogenic etiologies of ChGS and obesity in mice are likely to relate to ChGS susceptibility in some obese humans. For example, polymorphisms of the *CCKAR* gene have been reported in patients with ChGS (56), and mutations in the *CCKAR* gene promoter were shown to be linked to abnormally high proportions of body fat (57); moreover, aberrant splicing of the *CCKAR* receptor gene was discovered in one obese patient with cholesterol cholelithiasis (10). Nonetheless, no focused human studies are yet available on the extent of the contributions of the CPE-CCK pathway to cross-susceptibility for both obesity and ChGS. Although uncommon, mutations and polymorphisms in the *LEP* and/or *LEPR* genes are correlated with human obesity and percent body fat (58). Along the same lines, our data suggest that polymorphisms in the *LEP* and *LEPR* genes might exert both positive and negative influences on biliary lipid secretion and CSI values. For example, we speculate that if these genes were hypofunctional in humans, they would increase resistance to ChGS and, in contrast, if they were upregulated, they could possibly promote ChGS formation.

In summary, this systematic study of ChGS in monogenic and polygenic obese mice helps clarify why the consequences of obesity on biliary lipids, gallbladder function, and CSI have been so variable in humans (3, 8–15). Based on our findings, we propose that in determining ChGS risk, obesity and *Lith* genes frequently act independently, sometimes in common, and occasionally epistatically, and that the presence of multiple alleles may modify the phenotypes. It is highly probable that obesity and ChGS formation in humans share several pathophysiologic and genomic pathways, as well as several independent biochemical and biophysical pathways (2, 3, 7–13, 52, 59, 60). We propose that further studies of the complex relationships that underlie the molecular basis of obesity and ChGS and the environmental triggers that lead to their genetic expression may provide clues to a fundamental understanding of the genetic and pathophysiological relationships between these two remarkably common human diseases.

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