Aging per se is an independent risk factor for cholesterol gallstone formation in gallstone susceptible mice

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Abstract Cholesterol gallstones occur rarely in childhood and adolescence and increase linearly with age in both genders. To explore whether aging per se increases cholesterol saturation of bile and gallstone prevalence, and to investigate age-related changes in hepatic and biliary lipid metabolism, we studied gallstone-susceptible C57L mice and resistant AKR mice of both genders fed an lithogenic diet containing 1% cholesterol, 0.5% cholic acid, and 15% butter fat starting at (young adult) 8, (older adult) 36, and (aged) 50-weeks-of-age. After the 8-week feeding period, gallstone prevalence, gallbladder size, biliary lipid secretion rate, and HMG-CoA reductase activity were significantly greater but cholesterol 7α-hydroxylase activity was lower in C57L mice of both genders compared with AKR mice. Increasing age augmented biliary secretion and intestinal absorption of cholesterol, reduced hepatic synthesis and biliary secretion of bile salts, and decreased gallbladder contractility, all of which increased susceptibility to cholesterol cholelithiasis in C57L mice. We conclude that aging per se is an independent risk factor for cholesterol gallstone formation. Because aging increases significantly biliary cholesterol hypersecretion and gallstone prevalence in C57L mice carrying Lith genes, it is highly likely that Longevity (aging) genes can enhance lithogenesis of Lith (gallstone) genes.—Wang, D. Q-H. Aging per se is an independent risk factor for cholesterol gallstone formation in gallstone susceptible mice. J. Lipid Res. 2002. 43: 1950–1959.

Supplementary key words bile salt • bile flow • phospholipid • gallbladder size • mucin gel • HMG-CoA reductase • cholesterol 7α-hydroxylase

Cholesterol gallstone disease is influenced by a complex interaction of genetic and environmental factors (1) that determine the hypersecretion of biliary cholesterol into hepatic bile (2) and subsequently the precipitation of cholesterol crystals in gallbladder bile (3). Epidemiological and clinical investigations (4–8) have shown that cholesterol gallstones occur infrequently in childhood and adolescence, and the prevalence of cholesterol gallstone disease increases linearly with age in both genders and approaches 50% at age 70 in females. Furthermore, elderly individuals are at high risk for developing complications of gallstones and mortality from surgery is often unacceptably high in patients older than 65 (9–11). Although cholesterol saturation of bile is significantly higher in elderly Swedes (12) and Chilean women (13) and age correlates positively with increased hepatic cholesterol secretion rate (12), it has not been established how aging per se influences hepatic and biliary cholesterol metabolism. Furthermore, numerous studies on age-related hepatic cholesterol and bile salt metabolism (14–20), biliary cholesterol secretion and composition (12, 13, 21, 22), gallbladder contraction function (23–26), and intestinal cholesterol absorption (27, 28) have been performed in humans and in different animal species; however, effects of aging on these parameters varied tremendously, being either normal, diminished, or elevated compared to young subjects. In particular, little is known whether aging per se influences cholesterol gallstone formation. In this study, using a unique genetically gallstone-susceptible C57L mouse model with Lith genes (2, 3, 29) compared to resistant AKR mice, we investigated age-related hepatic cholesterol and bile salt metabolism, biliary lipid secretion rate, gallbladder bile lipid composition, gallbladder motility function, and intestinal cholesterol absorption, as well as gallstone prevalence rate. Our results showed that aging per se increases cholesterol gallstone formation through enhancing biliary secretion and intestinal absorption of cholesterol, decreasing hepatic synthesis and secretion of bile salts, and reducing gallbladder contractility in C57L mice.

MATERIALS AND METHODS

Chemois

Medium-chain triglyceride was purchased from Mead Johnson (Evansville, IN), and Intralipid (20%, w/v) was from Pharmacia

Abbreviations: CCK-8, sulfated cholecystokinin octapeptide; CSI, cholesterol saturation index; HMG-CoA, hydroxy-3-methylglutaryl coenzyme A.

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terol (access to water and normal mouse chow containing trace choles-
strate is homozygous for resistant cellular Care and Use Committees of Harvard University.
were executed according to accepted criteria for the care and ex-
amine at 8 weeks of feeding the lithogenic diet. All experiments
form gallstones at 4-weeks of feeding, 80% at 8-weeks (3), and
over, we have observed that when feeding the lithogenic diet
made on the right or left side of the neck and the jugular
vein was cannulated. Then, the incision was closed tightly with
silk sutures. Following this procedure, a cholecystectomy was
performed and gallbladder volume was measured by weighing
(see above), which was considered as control groups. Additional
groups of mice were injected intravenously through the jugular
vein with exactly 17 nmol/kg body weight of sulfated CCK oc-
tapeptide (CCK-8) dissolved in 100 μl of phosphate buffered sa-
line (PBS) solution, or 100 μl of only PBS solution. This pro-
cedure was carried out over 1 min to prevent cardiac arrest. In the
mouse, CCK-8 is a full agonist at both subtypes of CCK-A and B
receptors (33). Exactly 15 min after the injection, a cholecystec-
tomy was made and gallbladder size was determined by weighing.
Gallbladder contractile function was determined by comparing
gallbladder volumes of the CCK-8 groups with those of the con-
rol and the PBS groups.

Measurement of intestinal cholesterol absorption
Cholesterol absorption was determined by the plasma dual
isotope ratio method (32, 34) in chow-fed C57L and AKR mice
(n = 10 per group) of both genders. In brief, exactly 2.5 μg of
[14C]cholesterol in 100 μl of Intralipid was injected intravenously
(iv) into a jugular vein. Immediately, the animal was given by ga-
vage an intragastric (ig) dose of 1 μg of [14C]cholesterol in 150 μl
of medium-chain triglyceride. After dosing, mice were re-
turned to individual cages with wire mesh bottoms, where they
were free to eat chow for an additional 3 days. Mice were then
anesthetized and bled from the heart into heparinized micro-
tubes. Exactly 100 μl of plasma aliquots and the original dosing
mixture, respectively, were mixed with 10 ml of EcoLite (ICN
Biomedicals, Costa Mesa, CA). The vials were counted in a liquid
scintillation spectrometer (Beckman Instruments, San Ramon,
CA). The ratio of the two radiolabels in plasma was used for cal-
culating the percent of cholesterol absorption:

% Cholesterol absorption =

(Percent of IG dose [14C]cholesterol per ml plasma)
(Percent of IV dose [14C]cholesterol per ml plasma) × 100

Measurement of small intestinal transit time
Intestinal transit time was measured in three age groups of
chow-fed C57L and AKR mice (n = 5 per group) according to a
previously published method (34). Nonabsorbable [3H]sitost-
tanol is used as a reference marker (34). In brief, 2 μCi of
[3H]sitostanol in 100 μl of medium-chain triglyceride were in-
stilled into the small intestine via a duodenal catheter. Exactly 30
min after instilling, mice were anesthetized with pentobarbital.
The abdomen was opened quickly, and the stomach, small and
large intestines, and cecum were removed carefully. The small in-

Animals and diets
Inbred C57L/J and AKR/J mice of both genders, 6-weeks-old,
were purchased from The Jackson Laboratory, Bar Harbor, ME.
C57L strain is homozygous for susceptible Lthb alleles and AKR
strain is homozygous for resistant Lthb alleles (2, 3, 29). All ani-
mals were maintained in a temperature-controlled room (22 ±
1°C) with a 12-h day cycle (6 AM–6 PM), and were provided free
access to water and normal mouse chow containing trace chole-
sterol (≤0.02%). (The Mouse Diet 1401, St. Louis, MO). In gen-
eral, total life expectancy of C57L mice is ~80 weeks, and AKR
mice ~60 weeks (30, 31). Based upon measurements of physical
growth and sexual maturation, the different age groups of mice
were considered to be in the following stages of maturation: 8-weeks, young adult; 36-weeks, older adult; and 50-weeks, aged
(30, 31). To explore whether aging per se increases cholesterol
saturation of bile and gallstone prevalence in C57L and AKR
mice of both genders, we studied three groups of mice at differ-
et ages, i.e., 8-, 36-, and 50-weeks-old (n = 20 per group). More-
over, we have observed that when feeding the lithogenic diet
(diet 32) containing 1% cholesterol, 0.5% cholic acid, and 15% but-
ter fat starts at the age of 8-weeks-old, 27% of male C57L mice
form gallstones at 4-weeks of feeding, 80% at 8-weeks (3), and
100% at 52-weeks (32), suggesting that time consuming the litho-
genic diet is a risk factor. Therefore, all animals were fed 8-weeks
of the lithogenic diet starting at 8-, 36-, and 50-weeks-of-age, re-
spectively. Also, the biliary and gallstone phenotypes were exam-
ined at 8 weeks of feeding the lithogenic diet. All experiments
were executed according to accepted criteria for the care and ex-
perimental use of laboratory animals and euthanasia was consist-
tent with recommendations of the American Veterinary Medical
Association. All protocols were approved by the Institutional Ani-
imal Care and Use Committees of Harvard University.

Collection of gallbladder bile and gallstones and
microscopic studies
After the 8-week lithogenic diet feeding, non-fasted animals
were weighed and anesthetized with an intraperitoneal injection
of 35 mg/kg pentobarbital. After cholecystectomy was per-
formed, gallbladder volume was measured by weighing the whole
and calculating gallbladder weight (including stones) with gallbladder volume. Five microtomes of fresh gall-
bladder bile were examined for mucin gel, solid and liquid crys-
tals, and gallstones, which were defined according to previously
established criteria (3). After pooled gallbladder bile were ultra-
centrifuged at 100,000 g for 30 min at 37°C and filtered through
a preheated (37°C) Swinnex-GS filter (0.22 μm) assembly (Milli-
pore Products Division, Bedford, MA), samples were frozen and
stored at −20°C for further lipid analyses (see below).

Cannulation of the common bile duct and collection of
hepatic bile
Additional groups of mice (n = 5 per group) of different ages,
which were fed the lithogenic diet for 8 weeks, were used for bil-
ary lipid secretion studies (2). In brief, after cholecystectomy
was performed, the common bile duct was cannulated and he-
patic bile was collected by gravity. The first hour collection of he-
patic bile was used to study biliary lipid outputs. To determine
the circulating bile salt pool sizes, 8 h biliary “washout” studies
were performed according to previous method (2). After fresh
hepatic bile were examined by polarizing light microscopy and
their volumes were determined by weighing, all samples were fro-
zen and stored at −20°C for further lipid analyses (see below).
During surgery and hepatic bile collection, mouse body tempera-
ture was maintained at 37 ± 0.5°C with a heating lamp and mon-
itored with a thermometer.

Galbladder contraction study
To explore whether gallbladder emptying in response to exog-
eneously administered CCK changed with age, we studied three
different age groups of C57L and AKR mice (n = 5 per group) at
8-weeks of feeding the lithogenic diet. In brief, non-fasted mice
were anesthetized lightly with pentobarbital. An 0.4 cm incision
was made on the right or left side of the neck and the jugular
vein was cannulated. Then, the incision was closed tightly with
3-0 silk sutures. Following this procedure, a cholecystectomy was
performed and gallbladder volume was measured by weighing
(see above), which was considered as control groups. Additional
groups of mice were injected intravenously through the jugular
vein with exactly 17 nmol/kg body weight of sulfated CCK oc-
tapeptide (CCK-8) dissolved in 100 μl of phosphate buffered sa-
line (PBS) solution, or 100 μl of only PBS solution. This pro-
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mouse, CCK-8 is a full agonist at both subtypes of CCK-A and B
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[3H]sitostanol in 100 μl of medium-chain triglyceride were in-
stilled into the small intestine via a duodenal catheter. Exactly 30
min after instilling, mice were anesthetized with pentobarbital.
The abdomen was opened quickly, and the stomach, small and
large intestines, and cecum were removed carefully. The small in-
testine was cut into 20 segments equally with a scalpel blade. The individual segments were mixed with 10 ml of CHCl₂CH₂OH (2:1, v/v), and stored at 4°C for 48 h. One milliliter of aliquots was transferred into counting vials and the solvent was then evaporated under nitrogen. Seven milliliters of Cytoscint (ICN Biomedicals, Costa Mesa, CA) were added and the radioactivity was determined by liquid scintillation spectrometry. Samples of stomach, cecum, and large intestine were also analyzed, but none ever showed appreciable radioactivity. Intestinal transit time was evaluated by a geometric center method (34). The geometric center of the distribution of radioactivity within the small intestine was the center of gravity for the distribution of the reference marker and was calculated using the following equation:

\[
\text{Geometric center} = \sum (\text{fraction of } [\text{H}]\text{sitostanol per segment} \times \text{segment number})
\]

**(Eq. 2)**

**Determination of activities of hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase**

Liver samples were collected from non-fasted mice (n = 5 per group) after the 8-week lithogenic diet feeding. To minimize diurnal variations of hepatic enzyme activities, all procedures (35) were performed between 9:00 and 10:00 AM. Microsomal activities of HMG-CoA reductase were determined by measuring the conversion rate of \(^{14}\text{C}\)HMG-CoA to \(^{14}\text{C}\)mevalonic acid using a radiochemical assay (36). Products were quantified by liquid scintillation counting with \(^{3}\text{H}\)mevalonolactone as internal standard. Hepatic activities of cholesterol 7α-hydroxylase were determined by the HPLC-based assay system of Hylemon et al. (37). Protein concentration was determined by the assay of Bradford (38).

**Lipid analyses**

Total and individual bile salt concentrations were measured by HPLC according to the method of Rossi, Converse and Hofmann (39). Biliary phospholipids were determined as inorganic phosphorus by the method of Bartlett (40). Bile cholesterol as well as cholesterol content in the lithogenic diet, gallstones, and liver were determined by HPLC (2). Cholesterol saturation indexes (CSI) in gallbladder and hepatic biles were calculated from the critical tables (41). Hydrophobicity indexes of hepatic bile were calculated according to Heuman’s method (42).

**Statistical methods**

All data are expressed as means ± SD. Statistically significant differences among groups of mice were assessed by Student’s t-test or Chi-square tests. Analyses were performed with a SuperANOVA software (Abacus Concepts, Berkeley, CA). Statistical significance was defined as a two-tailed probability of less than 0.05.

**RESULTS**

**Influence of aging on gallstone prevalence and lipid compositions of gallbladder biles**

As expected for healthy rodents, mice of each strain displayed progressive weight gain from 28–35 g to 32–37 g from 8- to 50-weeks-of-age. AKR mice were 3–18% heavier than C57L mice, and males were 3–22% heavier than females in both strains. All these strains of mice ate similar amounts of the lithogenic diet (4.0–4.3 g/day), and displayed average dietary cholesterol intake of \(~40–43\) mg/day. The fecal outputs (1.2–1.4 g/day dry weight) and the weight (1.5–1.7 g) and length (39–42 cm) of the small intestine were very similar in all mice of different ages. All animals appeared healthy with the exception of 10 male (50%) and 13 female (65%) aged AKR mice. These developed evidence of listlessness, lethargy, anorexia, sleepiness, and weight loss, dying eventually between 45 and 57 weeks. At autopsy, no special causes of death relevant to the hepato-biliary system were found. These aged AKR mice were not used for the present study.

**Figure 1** demonstrates that at week 8 of feeding the lithogenic diet, gallstone prevalence rates in C57L mice of both genders were significantly (P < 0.001) greater compared with AKR mice of the same ages. Of note is that in young adult groups, gallstone prevalence rates were 2-fold higher (P < 0.01) in C57L males than females, but no gender differences were found in AKR mice. Moreover, gallstone prevalence rates were slightly but not significantly higher with increasing age in C57L mice of both genders; however this did not occur in AKR mice. With an increase in age, the gender difference in gallstone prevalence for C57L mice became smaller. The sterols extracted from the stones of each mouse strain contained only cholesterol, which constituted >99% of stone weight. In general, the size of gallstones was markedly greater in C57L mice than in AKR mice of various ages (Fig. 2). On average, the size of gallstones ranged from 0.28 to 0.38 mm in AKR mice with no gender or age differences. In contrast, the size of gallstones was 0.54 ± 0.28 mm in C57L males and was significantly (P < 0.01) bigger than females (0.31 ± 0.13 mm). Also, gallstone diameters in aged C57L mice of both genders became bigger compared with their young adults, but did not reach significant differences. Furthermore, Fig. 3 shows that the number of gallstones in C57L mice of both genders fell between 7 and 9, whereas in AKR mice the corresponding values were 4 and 6. Of note is that gallbladders of AKR mice exhibited much lower gallstone numbers than in C57L mice, and 75–90% of AKR mice were gallstone free.

**Table 1** lists biliary lipid compositions of pooled gallbladder biles. After the 8-week lithogenic diet feeding, all mice had supersaturated gallbladder biles (CSI = 1.30–1.77) in three different age groups of AKR and C57L mice. In general, the CSI values became higher gradually with increasing age. However, total lipid concentrations of gallbladder biles decreased (Table 1) and showed a reverse correlation with the age, suggesting that gallbladder concentration function is diminished with advancing age. Moreover, the pooled gallbladder biles of C57L mice displayed markedly higher CSI values but lower total lipid concentrations compared with AKR mice.

**Effect of aging on gallbladder volumes and emptying**

**Figure 4** shows that after the 8 weeks of lithogenic diet feeding, gallbladder sizes in young adult C57L mice were

\[\text{~40–43 mg/day.}\]
Wang

Aging increases cholesterol gallstone formation

25 ± 8 µl (males) and 21 ± 6 µl (females), being significantly ($P < 0.001$) greater than those in AKR mice (10 ± 4 µl in males and 9 ± 4 µl in females). Furthermore, the size of gallbladders in C57L mice of both genders was significantly ($P < 0.001$) increased with advancing age from 21–25 µl in the young adult, to 57–50 µl in the older adult, to 90–95 µl in the aged, but this did not occur in AKR mice. Figure 5 shows that after the 8-week feeding, the CCK-8 treatment induced significantly ($P < 0.05$) gallbladder emptying in three different age groups of male and female AKR mice. However, CCK-8-mediated gallbladder emptying was diminished in C57L mice of both genders and of various ages. Furthermore, the PBS administration did not influence gallbladder size in the two strains of mice.

**Figure 5.** Gallstone-susceptible C57L mice and resistant AKR mice of both genders (n = 20 per group) were fed 8 weeks with the lithogenic diet containing 1% cholesterol, 0.5% cholic acid, and 15% butter fat starting at young adult (8-), older adult (36-), and 50-weeks-of-age (aged). After the 8-week feeding, gallstone prevalence rates were significantly ($P < 0.01$) greater in male C57L mice than in females, and was independent of gender in AKR mice. Also, prevalence was significantly ($P < 0.001$) higher in C57L mice than in AKR mice, and increased slightly ($P = NS$) with age in both strains.

**Aging-related effects on bile flow and biliary lipid secretion rates**

We observed changes in bile flow rates (Table 2) for the first hour following interruption of the enterohepatic circulation in different age groups of AKR and C57L mice fed the lithogenic diet for 8 weeks. Bile flow rates in C57L mice (range 97–117 µl/min/kg) of both genders were 2-fold higher than those in AKR mice (range 41–49 µl/min/kg).
min/kg) of the same gender and of the same ages, and increased significantly (P < 0.05) with age in both strains. However, we did not find any gender differences in bile flow for C57L and AKR mice of various ages.

Table 2 also shows biliary cholesterol, phospholipid, and bile salt secretion rates during the first hour of biliary washout in mice fed the lithogenic diet for 8 weeks. In general, outputs of all three biliary lipids were significantly (P < 0.05) higher in C57L mice compared with AKR mice of the same ages. The most marked changes were in cholesterol and phospholipid outputs in C57L mice (cholesterol = 38.6–38.8 μmol/h/kg and phospholipid = 68.2–68.9 μmol/h/kg) of both genders at 50-weeks-of-age, being significantly (P < 0.05) higher compared to mice at 8-weeks-of-age (cholesterol = 29.4–29.7 μmol/h/kg and phospholipid = 52.6–53.5 μmol/h/kg). However, biliary bile salt outputs decreased significantly (P < 0.05) from 192–211 μmol/h/kg at 8-weeks-of-age to 145–157 μmol/h/kg at 50-weeks-of-age. Biliary cholesterol and phospholipid outputs increased and bile salt outputs decreased slightly in AKR mice of both genders with age; however, they did not reach statistically significant differences. The pool sizes were significantly (P < 0.05) larger in C57L mice (2.6–3.6 μmol) than in AKR mice (2.1–2.2 μmol), and there were no gender differences in the circulating bile salt pool sizes (Table 2) in both mouse strains. Also, the circulating bile salt pool sizes decreased significantly (P < 0.05) with age in C57L mice of both genders, but this did not occur in AKR mice. Compared with AKR mice, the total bile salt pool sizes, i.e., the circulating bile salt pool size plus the bile salt pool in the gallbladder, were 1.6–2.7-fold higher in C57L mice, and it is likely due to the enlarged gallbladder size with increasing age in the latter mouse strain. Moreover, because aged C57L mice displayed larger gallbladder size than their young adults, there were age differences in the total bile salt pool sizes in C57L mice, with the aged (11.0–11.4 μmol) being ~2-fold higher than in the young adult (5.8–6.4 μmol). In contrast, aged AKR mice (4.1–4.3 μmol) displayed essentially similar total bile salt pool sizes compared with their young adults (3.6–4.0 μmol).

After the 8-week lithogenic diet feeding, young adult C57L mice of both genders displayed that the predominant bile salts were taurocholate (57–64%), taurodeoxycholate (12–23%), and taurochenodeoxycholate (11–14%). Present in smaller concentrations were tauro-β-muricholate (6–8%), tauroursodeoxycholate (1–2%) and tauro-α-muricholate (1%). Furthermore, young adult AKR mice of both genders showed that the major bile salts were taurocholate (60–67%) and tauro-β-muricholate.

TABLE 1. Biliary lipid compositions of pooled gallbladder biles in inbred mice of different ages

<table>
<thead>
<tr>
<th>Age</th>
<th>Males</th>
<th></th>
<th></th>
<th></th>
<th>Females</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ch</td>
<td>PL</td>
<td>BS</td>
<td>PL/(PL+BS)</td>
<td>[TL]</td>
<td>CSI</td>
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<tr>
<td></td>
<td>mole%</td>
<td>g/dl</td>
<td>mole%</td>
<td>g/dl</td>
<td></td>
<td>mole%</td>
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<tr>
<td>AKR Mice</td>
<td></td>
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</tr>
<tr>
<td>8-weeks</td>
<td>9.40</td>
<td>19.71</td>
<td>70.88</td>
<td>0.22</td>
<td>14.08</td>
<td>1.31</td>
</tr>
<tr>
<td>36-weeks</td>
<td>9.53</td>
<td>19.33</td>
<td>71.14</td>
<td>0.21</td>
<td>13.20</td>
<td>1.35</td>
</tr>
<tr>
<td>50-weeks</td>
<td>10.17</td>
<td>20.29</td>
<td>69.55</td>
<td>0.23</td>
<td>11.71</td>
<td>1.43</td>
</tr>
<tr>
<td>C57L mice</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>8-weeks</td>
<td>9.58</td>
<td>19.11</td>
<td>71.32</td>
<td>0.21</td>
<td>8.87</td>
<td>1.48</td>
</tr>
<tr>
<td>36-weeks</td>
<td>9.89</td>
<td>18.21</td>
<td>71.90</td>
<td>0.20</td>
<td>7.38</td>
<td>1.63</td>
</tr>
<tr>
<td>50-weeks</td>
<td>12.41</td>
<td>18.95</td>
<td>68.64</td>
<td>0.22</td>
<td>7.35</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Ch, cholesterol; PL, phospholipid; BS, bile salt; [TL], total lipid concentration; CSI, cholesterol saturation index.

Table values were determined from the pooled gallbladder biles (n = 20 per group).

Based upon measurements of physical growth and sexual maturation, the different age groups of mice were considered to be in the following stages of maturation: 8-weeks, young adult; 36-weeks, older adult; and 50-weeks, aged (30, 31).

These values represent the mean CSIs of the pooled gallbladder biles calculated from the critical tables (41).
The small intestine was essentially similar between young adult groups of C57L mice and AKR mice of both genders, with peaks between segments 8 and 15. The geometric center of the distribution profile of radioisotope in the small intestine was 11.0 ± 1.0 in AKR mice and 11.1 ± 0.8 in C57L mice. Furthermore, small intestinal transit times in older adult (geometric center = 11.7 ± 1.2) and aged (geometric center = 12.1 ± 1.1) C57L mice were slightly slower compared to their young adults, but did not reach statistically significant differences. In contrast, in AKR mice of various ages, there were no age differences in small intestinal transit times, ranging from 11.0 (young adult) to 11.3 (old adult) to 11.5 (aged) for the geometric centers.

### Aging-related changes in hepatic cholesterol and bile salt synthesis

Table 3 shows that under the chow conditions, the activities of hepatic HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, were similar between AKR and C57L mice of both genders at 8-weeks-of-age. Increasing age reduced significantly \((P < 0.01)\) the activity of HMG-CoA reductase in male and female AKR mice, however, such alterations did not occur in C57L mice. Furthermore, the 8-week feeding of the lithogenic diet decreased significantly \((P < 0.01)\) this enzymatic activity in AKR mice of various ages (14 to 25 pmol/min/mg), but C57L mice still displayed high HMG-CoA reductase activities (54 to 62 pmol/min/mg).

Table 3 also demonstrates that in a chow-fed state, the activities of hepatic cholesterol 7α-hydroxylase were similar in the two strains of mice, and decreased significantly \((P < 0.05)\) with advancing age. Moreover, there were no significant gender differences in the activities of cholesterol 7α-hydroxylase in AKR and C57L mice of various ages. At week-8 of feeding the lithogenic diet, although the activities of cholesterol 7α-hydroxylase were decreased significantly \((P < 0.05)\) in both strains of mice, this enzymatic activity was significantly \((P < 0.05)\) greater in three age groups of AKR mice (4 to 10 pmol/min/mg) compared with C57L mice (2 to 4 pmol/min/mg).

### DISCUSSION

With improved life expectancy, the population older than the age of 65 years, which was 35 million in the United States in 2000, is expected to reach 78 million by 2050. More impressive is that cholesterol gallstone disease is more common in people over the age of 50, and 50% of females at age 70–75 and of males at age 80–85 has gallstones (7, 8). Why does the prevalence of gallstones increase with aging? To answer this question, we investigated gallstone prevalence, hepatic and biliary lipid metabolism, gallbladder contraction function, and intestinal cholesterol absorption in three different age groups (i.e., 8, 36-, and 50-week-old) of gallstone-susceptible C57L mice and resistant AKR mice of both genders fed the lithogenic diet for 8 weeks. The most important find-
Based upon measurements of physical growth and sexual maturation, the different age groups of mice were considered to be in the following stages of maturation: 8-weeks, young adult; 36-weeks, older adult; and 50-weeks, aged (30, 31).

Parameters of biliary output are shown in Table 2. In the present study, we found that aging per se increased biliary cholesterol hypersecretion and intestinal cholesterol absorption, and diminished hepatic bile salt synthesis and secretion, and induced an enlarged gallbladder with impaired contractility function, all of which increase susceptibility to cholesterol gallstone formation in C57L mice.

Our study showed that after the 8-week lithogenic diet feeding, secretion rates of biliary cholesterol and phospholipid were increased but secretion rates and circulating pool sizes of biliary bile salts (Table 2) were diminished with the progress of age in C57L mice. This supports the concept (2, 43) that in gallstone-susceptible mice, Lith genes induce both relative and absolute biliary cholesterol hypersecretion disproportionately to phospholipid and bile salt outputs thereby inducing lithogenic bile formation. Furthermore, gallstone prevalence rates favored males 2 to 1 (Fig. 1) in C57L mice of various ages, and were slightly but not significantly higher in aged C57L mice of both genders compared to their young adults. However, there were no age or gender differences in AKR mice. As C57L mice carry more than two Lith genes (29), it is highly probable that Longevity (aging) genes (44–47) influence Lith (gallstone) genes to augment cholelithogenesis. Our findings were in agreement with the results of Einarsson et al. (12) in Scandinavian subjects of both genders, but were different from those of Valdivieso et al. (13) in Chilean women. The latter research group observed that aging does not modify hepatic synthesis, biliary pool size, or turnover of bile salts, although there is an increment in the lithogenic index of gallbladder bile with increasing age. Nevertheless, although there may be ethnic differences in these human studies (12, 13), the available evidence (12, 13) indicated that increased biliary cholesterol secretion with age is a major risk factor in the pathogenesis of cholesterol gallstone formation.

Additionally, aged C57L mice showed enlarged gallbladders with poor emptying (Figs. 4 and 5), which may contribute to cholesterol gallstone formation by causing gallbladder stasis (43). We also found that exogenous CCK can induce significant gallbladder emptying in AKR mice of various ages. However, C57L mice showed enlarged gallbladders (Fig. 4) and a significant decrease in gallbladder stasis (43). We also found that exogenous CCK can induce significant gallbladder emptying in AKR mice of various ages. However, C57L mice showed enlarged gallbladders (Fig. 4) and a significant decrease in gallbladder stasis (43). We also found that exogenous CCK can induce significant gallbladder emptying in AKR mice of various ages. However, C57L mice showed enlarged gallbladders (Fig. 4) and a significant decrease in gallbladder stasis (43). We also found that exogenous CCK can induce significant gallbladder emptying in AKR mice of various ages. However, C57L mice showed enlarged gallbladders (Fig. 4) and a significant decrease in gallbladder stasis (43). We also found that exogenous CCK can induce significant gallbladder emptying in AKR mice of various ages. However, C57L mice showed enlarged gallbladders (Fig. 4) and a significant decrease in gallbladder stasis (43). We also found that exogenous CCK can induce significant gallbladder emptying in AKR mice of various ages. However, C57L mice showed enlarged gallbladders (Fig. 4) and a significant decrease in gallbladder stasis (43). We also found that exogenous CCK can induce significant gallbladder emptying in AKR mice of various ages. However, C57L mice showed enlarged gallbladders (Fig. 4) and a significant decrease in gallbladder stasis (43).
Aging increases cholesterol gallstone formation

Wang

Both genders and (P < 0.001) percent cholesterol absorption increased significantly (P < 0.001) in aged AKR mice compared with AKR mice of the same age and of the same gender.

Based upon measurements of physical growth and sexual maturation, the different age groups of mice were considered to be in the following stages of maturation: 8-weeks, young adult; 36-weeks, older adult; and 50-weeks, aged (30, 31).

To determine cholesterol absorption in chow-fed male (A) and female (bottom panel) AKR and C57L mice of various ages (n = 10 per group) was determined by the plasma dual isotope ratio method (32, 34). Three age groups of male and female C57L mice displayed significantly higher (P < 0.001) percent cholesterol absorption compared with AKR mice. Percentage of cholesterol absorption increased significantly (P < 0.001) in aged C57L mice of both genders and (P < 0.05) in aged AKR mice compared with their young adults, respectively.

Fig. 6. Percent cholesterol absorption in chow-fed male (A) and female (bottom panel) AKR and C57L mice of various ages (n = 10 per group) was determined by the plasma dual isotope ratio method (32, 34).

Three age groups of male and female C57L mice displayed significantly higher (P < 0.001) percent cholesterol absorption compared with AKR mice. Percentage of cholesterol absorption increased significantly (P < 0.001) in aged C57L mice of both genders and (P < 0.05) in aged AKR mice compared with their young adults, respectively.

Table 3. Activities of hepatic lipid regulatory enzymes in inbred mice of different ages

<table>
<thead>
<tr>
<th>Age</th>
<th>Males Hmgr</th>
<th>Males C7h</th>
<th>Females Hmgr</th>
<th>Females C7h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-weeks</td>
<td>61 ± 12 *</td>
<td>18 ± 3</td>
<td>55 ± 11 *</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>36-weeks</td>
<td>36 ± 10 *</td>
<td>12 ± 3 *</td>
<td>35 ± 8 *</td>
<td>10 ± 4 *</td>
</tr>
<tr>
<td>50-weeks</td>
<td>29 ± 9 *</td>
<td>9 ± 2 *</td>
<td>27 ± 8 *</td>
<td>7 ± 2 *</td>
</tr>
<tr>
<td>Lithogenic diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-weeks</td>
<td>25 ± 5 *</td>
<td>10 ± 2</td>
<td>27 ± 7</td>
<td>8 ± 3 *</td>
</tr>
<tr>
<td>36-weeks</td>
<td>19 ± 5</td>
<td>8 ± 2</td>
<td>20 ± 6</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>50-weeks</td>
<td>13 ± 4</td>
<td>6 ± 2</td>
<td>15 ± 5 *</td>
<td>4 ± 2 *</td>
</tr>
</tbody>
</table>

Hmgr, hydroxy-3-methylglutaryl coenzyme A reductase; C7h, cholesterol 7α-hydroxylase.

*Values were expressed in pmol/min/mg microsomal protein (n = 5 per group).

* P < 0.01, compared with mice at 8-weeks-of-age on the same diet.

* P < 0.05, compared with mice at 8-weeks-of-age on the same diet.

* P < 0.001, compared with chow-fed mice of the same age and of the same gender.

* P < 0.01, compared with chow-fed mice of the same age and of the same gender.

* P < 0.05, compared with chow-fed mice of the same age and of the same gender.

* P < 0.0001, compared with AKR mice of the same age and of the same gender on the same diet.

* P < 0.001, compared with AKR mice of the same age and of the same gender on the same diet.

* P < 0.01, compared with AKR mice of the same age and of the same gender on the same diet.

* P < 0.05, compared with AKR mice of the same age and of the same gender on the same diet.

Wang Aging increases cholesterol gallstone formation 1957
patic lipid regulatory enzymes in humans (12–15) and in animals (16–20) have been intensively performed. However, there is no consensus how aging per se influences these enzymatic activities during cholesterol gallstone formation. We found that in a chow-fed state, the activity of HMG-CoA reductase decreased by 47–49% from 8- to 50-weeks-of-age in AKR mice of both genders, but remained unchanged with increasing age in C57L mice. Further, the high dietary cholesterol reduced the activities of hepatic HMG-CoA reductase in AKR mice. The HMG-CoA reductase activity remained unchanged in C57L mice in the face of advancing age, increased intestinal cholesterol absorption, and the lithogenic diet feeding, suggesting that high cholesterol synthesis in the liver could contribute, in part, to biliary cholesterol hypersecretion. One explanation would be a mutation or influenced by Lith genes (32, 35). Furthermore, the activity of hepatic cholesterol 7α-hydroxylase gradually decreased from 8- to 50-weeks-of-age by 44–50% in male and female AKR mice and by 43–58% in C57L mice. Our data are consistent with the results of Choi et al. (59), who reported a significant decrease in the activity of cholesterol 7α-hydroxylase in the rat from 5- to 32-weeks-of-age. Under the lithogenic diet conditions, three age groups of C57L mice displayed significantly (P < 0.05) lower activities of cholesterol 7α-hydroxylase compared with AKR mice of the same ages. Therefore, it suggests that markedly lower cholesterol 7α-hydroxylase activities, which reflects a decreased capacity to metabolize cholesterol to bile salts, may contribute to the age-related decrease in biliary bile salt secretion in C57L mice (Table 2).

In summary, our study shows that gallstone prevalence rates increase with age as a consequence of enhanced biliary secretion and intestinal absorption of cholesterol, declined hepatic synthesis and secretion of bile salts, and decreased gallbladder contractility in gallstone-susceptible C57L mice. We conclude that aging per se is an independent risk factor for cholesterol gallstone formation. Furthermore, cholesterol gallstones are a multifactorial disease, which involves the interaction of multiple Lith genes as inferred from the mouse studies (2, 3, 29), with environmental conditions such as aging and the lithogenic diet. This work therefore provides a basic framework for further investigating molecular mechanisms of how Longevity (aging) genes influence Lith (gallstone) genes to enhance cholelithogenesis. Because the author is greatly indebted to Dr. Martin C. Carey (Gastroenterology Division, Brigham and Women’s Hospital, Boston, MA) for his support and encouragement of this study as well as for helpful discussions. The author also thanks Helen H-F. Wang and Drs. Akira Ohashi, Victor M. Morales, and Li-Ping Duan (Beth Israel Deaconess Medical Center) for excellent technical assistance. D.Q-H.W. is a recipient of a New Scholar Award from the Ellison Medical Foundation (1999–2003). This work was supported in part by a grant from the Ellison Medical Foundation and a Grant DK54012 from the National Institutes of Health.

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