

## Pathophysiological preconditions promoting mixed “black” pigment plus cholesterol gallstones in a $\Delta F508$ mouse model of cystic fibrosis

Folke Freudenberg,<sup>1,2</sup> Monika R. Leonard,<sup>2</sup> Shou-An Liu,<sup>2</sup> Jonathan N. Glickman,<sup>3</sup> and Martin C. Carey<sup>1,2</sup>

<sup>1</sup>Department of Medicine, Harvard Medical School and Harvard Digestive Diseases Center; <sup>2</sup>Department of Medicine, Gastroenterology Division, Brigham and Women’s Hospital, and <sup>3</sup>Pathology Department, Harvard Medical School and Brigham and Women’s Hospital, Boston, Massachusetts

Submitted 19 August 2009; accepted in final form 24 April 2010

**Freudenberg F, Leonard MR, Liu SA, Glickman JN, Carey MC.** Pathophysiological preconditions promoting mixed “black” pigment plus cholesterol gallstones in a  $\Delta F508$  mouse model of cystic fibrosis. *Am J Physiol Gastrointest Liver Physiol* 299: G205–G214, 2010. First published April 29, 2010; doi:10.1152/ajpgi.00341.2009.—Gallstones are frequent in patients with cystic fibrosis (CF). These stones are generally “black” pigment (i.e., Ca bilirubinate) with an appreciable cholesterol admixture. The pathophysiology and molecular mechanisms for this “mixed” gallstone in CF are unknown. Here we investigate in a CF mouse model with no overt liver or gallbladder disease whether pathophysiological changes in the physical chemistry of gallbladder bile might predict the occurrence of “mixed” cholelithiasis. Employing a  $\Delta F508$  mouse model with documented increased fecal bile acid loss and induced enterohepatic cycling of bilirubin (*Am J Physiol Gastrointest Liver Physiol* 294: G1411–G1420, 2008), we assessed gallbladder bile chemistry, morphology, and microscopy in CF and wild-type mice, with focus on the concentrations and compositions of the common biliary lipids, bilirubins,  $\text{Ca}^{2+}$ , and pH. Our results demonstrate that gallbladder bile of CF mice contains significantly higher levels of all bilirubin conjugates and unconjugated bilirubin with lower gallbladder bile pH values. Significant elevations in Ca bilirubinate ion products in bile of CF mice increase the likelihood of supersaturating bile and forming black pigment gallstones. The risk of potential pigment cholelithogenesis is coupled with higher cholesterol saturations and bile salt hydrophobicity indexes, consistent with a proclivity to cholesterol phase separation during pigment gallstone formation. This is an initial step toward unraveling the molecular basis of CF gallstone disease and constitutes a framework for investigating animal models of CF with more severe biliary disease, as well as the human disease.

cystic fibrosis transmembrane conductance regulator; hepatobiliary disease; enterohepatic cycling; bilirubin; gallbladder

CYSTIC FIBROSIS (CF) is a common inherited disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, with >1,500 examples documented to date (23, 54, 57). Dysfunction of *CFTR*, an apical plasma membrane  $\text{Cl}^-$  channel regulated by cAMP, impairs  $\text{Cl}^-/\text{HCO}_3^-$  exchange via anion exchanger-2 on apical plasma membranes of specific parenchymal cells, most notably lung, pancreas, and small intestine. In the hepatobiliary system, *CFTR* is located on the apical plasma membranes of large cholangiocytes and cholecystocytes, but not hepatocytes (18, 20, 35). Approximately 10–25% of pediatric CF patients (16, 28, 50) and 30–72% of adult patients with CF (42, 43) display hepatobiliary abnor-

malities, most notably “biliary sludge” and gallstones (1, 43). Earlier authoritative publications (19, 58, 67) assumed that cholesterol stones form uniquely in CF because of the frequent bile salt malabsorption in the disease (58, 67), analogous to regional ileitis (19). However, in CF the gallstones are known to be insoluble with ursodeoxycholic acid therapy (21), and microscopy of CF gallbladder biles aspirated by duodenal drainage shows an absence of cholesterol crystals (1). In contrast to most common “black” pigment gallstones, the stones in CF are radiolucent, suggesting little, if any, crystalline calcium carbonate or calcium phosphate and/or high cholesterol content (1). The pathophysiological basis for the molecular pathogenesis of these stones is unknown.

The aim of this study was to investigate in a mouse model carrying the  $\Delta F508$  mutation (68) whether chemical changes that might presage the pathophysiology of such “mixed” gallstones occur in gallbladder bile. Not only is the  $\Delta F508$  mutation the most common *CFTR* gene mutation (~70%) in CF patients (57), but the pathophysiology of the intestinal tract and the liver in this mouse model has been well described (30, 68). Moreover, gross and direct light microscopy of the hematoxylin-and-eosin (H&E)-stained gallbladders of a small number of very young  $\Delta F508$  mice revealed histopathology that was indistinguishable from the gallbladders of wild-type (WT) mice (68). In studies of other CF mouse models, several gallbladders were reported to be distended with “black bile” (33), suggesting that a physical chemical change and precipitation had occurred. Patch-clamp studies on epithelial cells from  $\Delta F508$  CF mice reveal that the number of functional *CFTR* channels is ~1% of normal (29), which seems to be sufficient to prevent gross hepatobiliary disease in these mutants. Nonetheless, a high percentage of animals develop meconium ileus and growth retardation (30, 68).

Our prior publication (30) was a systematic evaluation of the secretory, i.e., production, rates of the common biliary lipids and lipopigments carried out without appreciable perturbation of the enterohepatic circulation. We showed (30) that hepatic bile of these CF mice was rendered more cytotoxic to cholangiocytes by events that occurred in the distal small and large intestines. Specifically, we demonstrated significantly reduced ileal pH levels, as well as increased fecal bile acid loss (30), leading to “hyperbilirubinemia” (increased secretion of conjugated bilirubins into bile) from induced enterohepatic cycling (EHC) of unconjugated bilirubin (UCB) (64). We also found significantly increased secretion rates of all common biliary lipids (30), especially cholesterol, in CF mice. We now propose that the same scenario sets in place biochemical and pathophysiological changes in bulk gallbladder bile that render

Address for reprint requests and other correspondence: M. C. Carey, Dept. of Medicine, Thorn Bldg., Rm. 1430, Brigham and Women’s Hospital, 75 Francis St., Boston, MA 02115 (e-mail: mccarey@rics.bwh.harvard.edu).

it potentially lithogenic, i.e., a “pre-stone” stage for Ca bilirubinate and cholesterol phase separation. We found significant increases in the lithogenic potential for biliary cholesterol and Ca bilirubinates that would allow these molecules to eventually precipitate from gallbladder bile if the observed levels were to become further elevated. This study forms a framework for understanding the pathophysiological and molecular mechanisms underlying gallstone disease in animal models with a more severe CF phenotype, as well as in the human disease.

## METHODS

**Animals.** Heterozygous breeding pairs of  $\Delta$ F508 mice (68) on a 75% C57BL/6-25% 129SvEv background were donated by Dr. Marie Egan (Yale University School of Medicine, New Haven, CT). Breeding pairs of G551D mice (25) were obtained from Dr. Gerald Pier (Channing Laboratory, Brigham and Women’s Hospital and Harvard Medical School). Of the two murine CF models [ $\Delta$ F508 (68) and G551D (25)] that were evaluated in preliminary studies, both displayed bile acid malabsorption (G551D >  $\Delta$ F508). However,  $\Delta$ F508 mice exhibited hepatic lipopigment secretory data consistent with EHC of bilirubin (30), whereas G551D mice did not (F. Freudenberg and M. C. Carey, unpublished observations). This was due to chronic diarrhea in the G551D, but not the  $\Delta$ F508, mice, thereby curtailing colonic residence times, and is analogous to our previous observations in *Asbt*-null mice (24, 31).  $\Delta$ F508 mice and WT controls were housed in the animal facility in the Thorn Research Building (Brigham and Women’s Hospital). To minimize the risk of potentially lethal meconium ileus, all animals were housed on corn cob bedding (The Andersons, Maumee, OH), and for the same reason, a polyethylene glycol-3350-electrolyte solution (GoLYTELY, Braintree Laboratories, Braintree, MA) was added to their drinking water. Mice were fed a diet that contained 11% fat and was replete in nutrients, vitamins, and minerals (Mouse Diet 5015, Labdiet, Richmond, IN). Housing illumination consisted of normal 12:12-h light-dark cycles employing conventional fluorescent lamps. All surgeries were performed at the same time of day on age-matched (unless otherwise noted, 3–14 mo of age) and sex-matched homozygous CF and WT mice that had been fasted for 4–8 h. Each mouse was genotyped as follows. Tails were clipped at 3 wk of age, and DNA was isolated using DNeasy kits (Qiagen, Valencia, CA). For PCR amplification, we used Amplitaq Gold Master Mix (Applied Biosystems, Foster City, CA) with the primer sequences GAG TGT TTT CTT GAT GAT GTG and ACC TCA ACC AGA AAA ACC AG. The amplified DNA was restricted using the enzyme *RsaI* (Applied Biosystems) and separated by agarose gel electrophoresis. Mice were weighed prior to surgery. Unless stated otherwise, all analyses were performed on individual bile samples. Humane protocols for induction of anesthesia, laparotomy, cholecystectomy, and mouse euthanasia were approved by the Harvard University Medical Area Standing Committee on Animals.

**Gallbladder bile volume, pH, and microscopy.** After induction of anesthesia (intraperitoneal injection of ketamine, xylazine, and atropine in 0.15 M NaCl), laparotomy was performed (30), and gallbladders were examined under direct and transmitted illumination (SZ40 Stereo Zoom Microscope, Olympus America, Chelmsford, MA). Cholecystectomy was then performed by standard technique for mice. Subsequently, the mouse was euthanized with an overdose of ketamine and xylazine, and bilateral thoracotomy was performed. After emptying gallbladder bile completely into tared 200- $\mu$ l collection tubes, we measured bile volumes gravimetrically by numerically equating weight and volume. Immediately thereafter, pH values were measured on individual biles by microelectrode (Thermo Electron, Beverly, MA). Gallbladder histopathology of prepared sections stained with H&E was assessed as described previously (30). Gallbladder bile was examined by direct and polarizing light microscopy for insoluble mucin gel, phase-separated amorphous and crystalline

precipitates, and pigment and cholesterol gallstones. In addition, intact gallbladders of nonfasted mice (4 female and 2 male CF, 5 female and 3 male WT) were fixed in 20-fold volumes of formalin solution, dehydrated serially with ethanol and H<sub>2</sub>O, embedded, and sectioned along the longitudinal axis to expose maximum luminal volumes and mucosa. For each gallbladder, separate slides were stained with Alcian blue to detect acidic mucins and with periodic acid-Schiff (PAS) to detect neutral mucins. All sections were coded and read blindly by three of the investigators in conjunction with an experienced rodent pathologist. We analyzed and scored stained sections as follows: 0, no mucin on apical columnar cells or within the gallbladder lumen; 1, a few wisps of mucin within the lumen and a uniformly thin layer of mucin adherent to the apical columnar cells; 2, a mucin layer of variable thickness adherent to apical columnar cells, small aggregates of mucin within the gallbladder lumen; 3, a mucin layer of variable thickness on epithelial cells, moderate amounts of mucin within the gallbladder lumen; 4, a few agglomerated mucin spherules within the gallbladder lumen, moderate-to-large amount of mucin within the lumen; and 5, many stained globules or mucin spherules within the gallbladder lumen and/or nonagglomerated mucin filling the gallbladder lumen.

**Quantitation of Ca<sup>2+</sup> activities.** Immediately after measurement of gallbladder bile pH values, we quantified Ca<sup>2+</sup> activities. To adjust for the ionic strength of bile and to prepare standard solutions, Na<sup>+</sup> activities were measured in a preliminary experiment (Clinical Chemistry Core Laboratory of Children’s Hospital, Boston, MA) using a Roche/Hitachi cobas c 6000 analyzer (Roche Diagnostics, Indianapolis, IN) with a calibrated ion-selective electrode. Employing pooled samples ( $n = 4$ ; necessitated by the volumetric requirement of the analyzer) of mouse gallbladder bile diluted fivefold (to protect the analyzer’s electrode from bile salt detergency), we determined a range of 190–205 mM Na<sup>+</sup> in murine gallbladder bile. Accordingly, we adjusted all CaCl<sub>2</sub> standards to an ionic strength of 200 mM NaCl. Semilogarithmic calibration curves were generated from five stock solutions ranging from 10<sup>-1</sup> to 10<sup>-5</sup> M CaCl<sub>2</sub> prepared by serial dilution of a 0.1 M CaCl<sub>2</sub> standard solution (Microelectrodes). Ca<sup>2+</sup> activities were measured in millivolts using a pH meter (Orion model 720A, Thermo Fisher Scientific, Waltham, MA) and an ion-specific microelectrode (MI-600, Microelectrodes, Bedford, NH) by introduction of the Ca<sup>2+</sup> and reference microelectrodes into individual gallbladder bile samples. Between measurements, Ca<sup>2+</sup> and reference electrodes were rinsed with deionized, ultrafiltered H<sub>2</sub>O (Fisher Scientific) and stored in 0.1 M CaCl<sub>2</sub> solution. Bile sample readings were interpolated on the standard curve to obtain millimolar Ca<sup>2+</sup> concentrations. Ion products were calculated as the product of molar concentrations of Ca<sup>2+</sup> and unconjugated bilirubin (UCB) or monoconjugated bilirubin.

**Bilirubin molecular species.** To separate and quantify bilirubin molecular species in bile, we injected a 10- $\mu$ l aliquot of individual bile samples onto a reverse-phase HPLC precolumn-and-column assembly (59) within 5 min of bile collection. Prior to HPLC, samples were maintained in darkness to preserve bile pigments from actinic degradation. Concentrations of the two bilirubin monoglucuronoside (BMG) isomers in bile, the principal biliary conjugates in the mouse; bilirubin diglucuronosides (BDG); all other minor bilirubin mono- and diconjugates (BMX and BDX); and UCB are reported in micromolar concentrations.

**Common biliary lipids in gallbladder bile.** Molecular species of individual bile salts in gallbladder bile were separated and quantified by HPLC (56). Concentrations of total bile salts were assayed by the 3 $\alpha$ -hydroxysteroid dehydrogenase method (62), biliary phospholipids were determined as inorganic phosphorus (2), and biliary cholesterol was extracted (40) prior to HPLC assay (63). Bile salt hydrophobicity indexes were quantified using the method of Heuman (36). The major biliary lipids are reported as millimolar concentrations. We calculated cholesterol saturation indexes (CSIs) of gallbladder bile samples using critical tables (11), with the initial assumption that all bile salts present

are Na taurocholate. Then “urso-correction” factors (12) were employed to adjust the initial CSI values on the basis of the percent total muricholate plus ursodeoxycholate conjugates in each bile sample.

**Statistics.** Values for most measurements are means  $\pm$  SE. For these comparisons between mutant  $\Delta$ F508 and WT groups, statistical significance was assessed using an unpaired, two-tailed Student’s *t*-test, corrected with Welch’s correction factor in cases of unequal variance. However, data for the Ca bilirubinate ion products show skewness and kurtosis. To evaluate these nonparametric data, we used the Mann-Whitney test (also known as Wilcoxon’s rank sum test) to report medians and Mann-Whitney *U* and *P* values. For all statistics, *P* < 0.05 is considered significant.

## RESULTS

**General.** As we described earlier (30), not only does breeding these CF mice yield fewer-than-expected offspring on the basis of Mendelian predictions, but in the pre- and postweaning periods a higher proportion of  $\Delta$ F508 CF offspring die spontaneously, usually from unavoidable intestinal obstruction. This occurs despite corn cob bedding and GoLYTELY in the drinking water. There is also a significant weight difference in age-matched, mature mice (Fig. 1A): CF mice (*n* = 21) are  $\sim$ 34% lighter than WT mice (*n* = 20, *P* < 0.0001).

**Gallbladder volumes, microscopy, and mucin scores.** Gallbladders are markedly larger in CF than WT mice, whether expressed as absolute volumes (Fig. 1B; CF 23% greater than WT, *P* = 0.04) or normalized to body weight (Fig. 1C; CF 79% larger than WT, *P* < 0.0001, *n* = 21 and 20, respectively). All biles are bright yellow and translucent. By direct light microscopy, neither yellow precipitates of phase-separated Ca bilirubinates nor black gall-sand or black gallstones are observed in the gallbladders or found in gallbladder biles of CF (*n* = 7) or WT (*n* = 5) mice. Under direct and polarized light microscopy, we sometimes noted one or two narrow, birefringent crystalline objects of unknown chemistry per high-power field in CF and WT gallbladder biles. There are no phase-separated liquid crystals, cholesterol monohydrate crystals, inorganic Ca carbonate/Ca phosphate crystals, or amor-

phous bilirubinate precipitates in the gallbladders and bile samples of CF and WT mice. By direct light microscopy, gallbladders of CF mice invariably contain a mild-to-moderate amount of insoluble mucin gel. Some gallbladders of WT mice are completely isotropic, although moderate amounts of mucin gel are found in the gallbladders of two 8- and 9-mo-old WT mice. We further assessed epithelial-adherent and luminal gallbladder mucins semiquantitatively by Alcian blue and PAS stains in 3- to 18-mo-old male and female CF and WT mice (*n* = 6 and 8, respectively). PAS scores confirm those of sections stained with Alcian blue, being identical in all but one case. Blinded mucin scores (see METHODS) range from 0 to 4 for WT mice and from 1 to 4 for CF mice, and mean scores for both genotypes are essentially identical (CF mucin score = 2.4, WT mucin score = 2.5); no sex differences are found. Although a trend toward increasing scores with age is observed in CF mice, this is not the case with WT mice.

**Gallbladder morphology.** The gross appearances of gallbladders from CF (6–19 mo old) and WT (4–9 mo old) mice, despite marked differences in size (Fig. 1B), are identical; in particular, no yellow or black staining of the CF gallbladders is noted, and, by transillumination, no phase-separated precipitates are observed. The H&E-stained gallbladder tissues were read by two independent pathologists with different backgrounds who were blinded as to their origin. After examination of 12 CF and 13 WT mouse gallbladders, no significant histopathological difference could be documented to distinguish gallbladders of CF from WT mice. Specifically, one gastrointestinal histopathologist, a specialist in human tissues, scored the histology as 0 to 2+ inflammation, with no difference between CF and WT genotypes. A dedicated murine pathologist scored WT and CF sections as within normal limits for the laboratory mouse.

**Gallbladder bile pH values.** As displayed in Fig. 1D, pH values of gallbladder bile are significantly (*P* = 0.004) less alkaline in  $\Delta$ F508 mutant CF ( $7.32 \pm 0.04$ , *n* = 21) than WT ( $7.53 \pm 0.06$ , *n* = 20) mice.

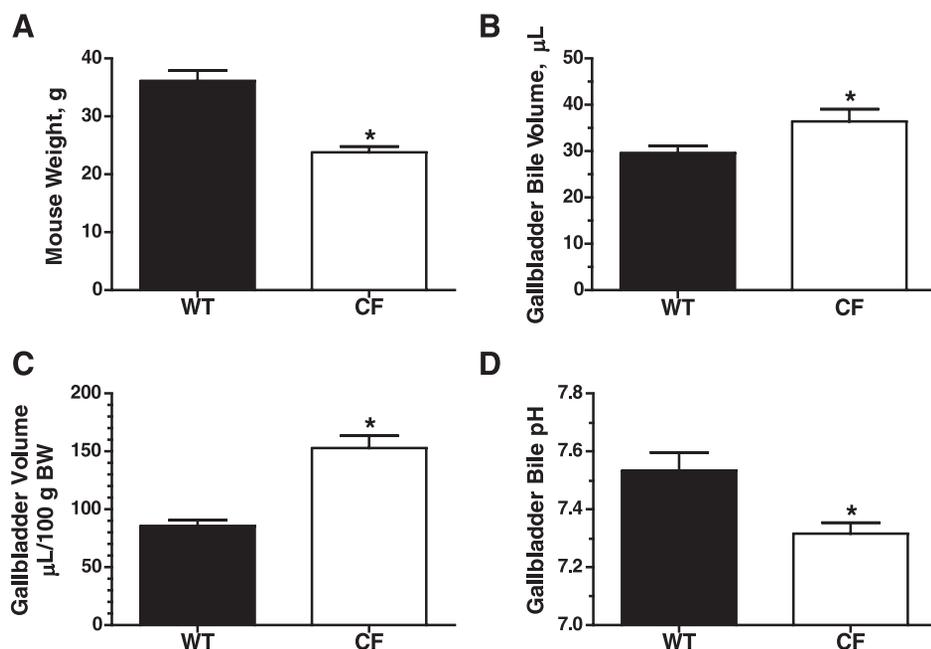


Fig. 1. Mouse weight, gallbladder volume, and bulk pH of  $\Delta$ F508 [cystic fibrosis (CF)] and control (WT) mice (*n* = 21 and 20, respectively). \*Statistically significant differences between  $\Delta$ F508 CF and WT mice. A: mouse weights at time of cholecystectomy are significantly lower in CF than WT mice ( $23.8 \pm 0.9$  vs.  $36.1 \pm 1.8$  g, *P* < 0.0001). B: gallbladder bile volumes are significantly (*P* = 0.04) larger in CF than WT mice ( $36.4 \pm 2.7$  vs.  $29.6 \pm 1.6$   $\mu$ L). C: normalized gallbladder bile volumes are markedly different in the same direction ( $153.2 \pm 10.4$  and  $85.4 \pm 5.3$   $\mu$ L/100 g body wt for CF and WT mice, respectively, *P* < 0.0001). D: individual gallbladder bile pH values measured by microelectrode immediately following bile expression from gallbladders. The pH values are significantly (*P* = 0.004) lower in CF than in WT mice ( $7.32 \pm 0.04$  vs.  $7.53 \pm 0.06$ ).

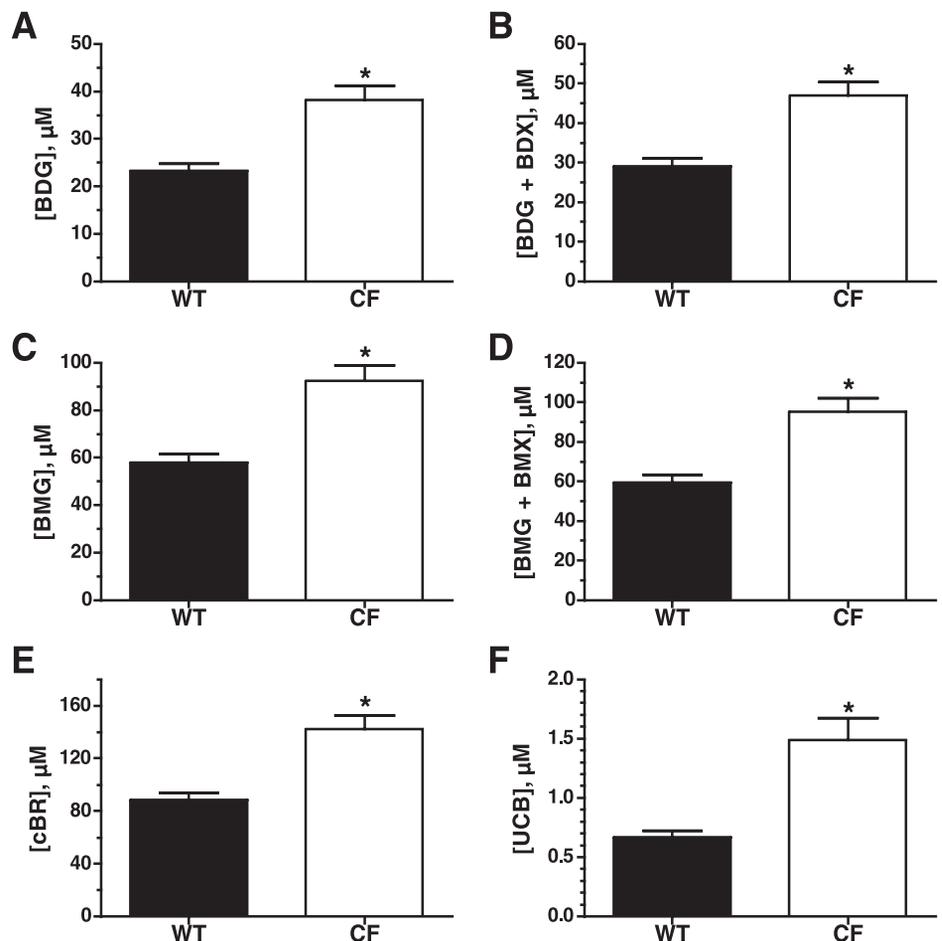
**Concentrations of bilirubin molecular species in gallbladder bile.** Figure 2 shows significantly higher concentrations of all bilirubin molecular species, including UCB, in gallbladder bile of CF mice. The differences between CF ( $n = 21$ ) and WT ( $n = 20$ ) mice are marked and highly significant ( $P < 0.0001$ ) for all conjugates and at the  $P = 0.0002$  level for UCB concentrations:  $38.2 \pm 3.0$  and  $23.3 \pm 1.6 \mu\text{M}$  BDG for CF and WT, respectively (Fig. 2A);  $47.0 \pm 3.5$  and  $29.1 \pm 1.9 \mu\text{M}$  BDG + BDX for CF and WT, respectively (Fig. 2B);  $92.3 \pm 6.6$  and  $58.0 \pm 3.7 \mu\text{M}$  BMG for CF and WT, respectively (Fig. 2C);  $95.4 \pm 6.8$  and  $59.5 \pm 3.7 \mu\text{M}$  BMG + BMX for CF and WT, respectively (Fig. 2D);  $142.3 \pm 10.1$  and  $88.6 \pm 5.6 \mu\text{M}$  total of conjugated bilirubin species for CF and WT, respectively (Fig. 2E); and  $1.5 \pm 0.2$  and  $0.7 \pm 0.1 \mu\text{M}$  UCB for CF and WT, respectively (Fig. 2F). Within each mouse genotype, we found sex differences in the concentrations of individual bilirubin species, with higher levels in female ( $n = 7/\text{group}$ ) than male ( $n = 6/\text{group}$ ) mice; these differences are significant in WT mice. However, all molecular species of bilirubins are significantly elevated in CF females compared with WT females and in CF males compared with WT males (comparisons not shown).

**Common biliary lipids in gallbladder bile.** Absolute concentrations of bile salts, phospholipids, and cholesterol in individual gallbladder biles of CF and WT mice ( $n = 10$  and  $8$ , respectively, for both bile salts and phospholipids;  $n = 6$  and  $7$  for cholesterol) plus their respective total lipid concentrations

( $n = 6$  and  $7$ ) are delineated in Fig. 3, A–D. Bile salt concentrations are decreased significantly ( $P = 0.02$ ) in CF compared with WT mice ( $132.3 \pm 5.7$  vs.  $155.1 \pm 6.5$  mM; Fig. 3A). Although slightly depressed in CF mice, no significant differences in biliary phospholipid levels are noted between CF and WT mice ( $18.6 \pm 1.3$  and  $20.7 \pm 1.9$  mM, respectively,  $P = 0.38$ ; Fig. 3B). Cholesterol concentrations are appreciably greater in biles of CF than WT mice (Fig. 3C), but the mean value marginally misses statistical significance ( $3.23 \pm 0.56$  vs.  $1.77 \pm 0.18$  mM, respectively,  $P = 0.056$ ). However, molar percentages of cholesterol are doubled in CF compared with WT mice ( $2.06 \pm 0.34$  vs.  $0.99 \pm 0.14$  mol%,  $P = 0.01$ ). Total lipid concentrations were calculated for the CF and WT samples for which sufficient bile was available to measure all three common biliary lipids in the same sample, with values of  $8.8 \pm 0.5$  and  $10.3 \pm 0.5$  g/dl for CF and WT mice, respectively. Comparison of the means barely misses statistical significance ( $P = 0.057$ ; Fig. 3D).

**Cholesterol saturation indexes.** Figure 3E shows the CSI values (uncorrected for percent muricholates and ursodeoxycholates in murine bile). CSI values are significantly higher ( $P = 0.003$ ) in gallbladder biles of CF ( $0.45 \pm 0.05$ ,  $n = 6$ ) than WT ( $0.23 \pm 0.04$ ,  $n = 7$ ) mice. When appropriate urso-correction factors (12) tailored to the total percent muricholate and ursodeoxycholate conjugates in individual bile samples are applied, these values remain statistically significant, increasing to  $0.56 \pm 0.05$  for CF mice and  $0.32 \pm 0.05$  for WT mice ( $P = 0.01$ ; Fig. 3F).

Fig. 2. Concentrations of bilirubins in individual (10- $\mu\text{l}$  aliquots) gallbladder biles of WT ( $n = 20$ ) and CF ( $n = 21$ ) mice measured by HPLC immediately following cholecystectomy. \*Statistically significant differences between groups. A: bilirubin diglucuronoside (BDG) concentrations ([BDG]). B: concentrations of bilirubin diconjugates (BDG + BDX), which include pure and mixed conjugates with xylose and glucose (BDX). C: bilirubin monoglucuronoside concentrations ([BMG]). D: concentrations of bilirubin monoconjugates, including conjugates with xylose and glucose ([BMG + BMX]). E: concentrations of total conjugated bilirubins ([cBR]) in gallbladder bile. F: unconjugated bilirubin concentrations ([UCB]). In the case of the conjugates (A–E) and UCB (F), concentrations are significantly higher ( $P < 0.0001$  and  $0.0002$ , respectively) in CF than WT mice. As inferred visually from these plots (means  $\pm$  SE), all bilirubin levels in gallbladder bile of CF mice are 1.5- to  $>2$ -fold greater than in WT mice.



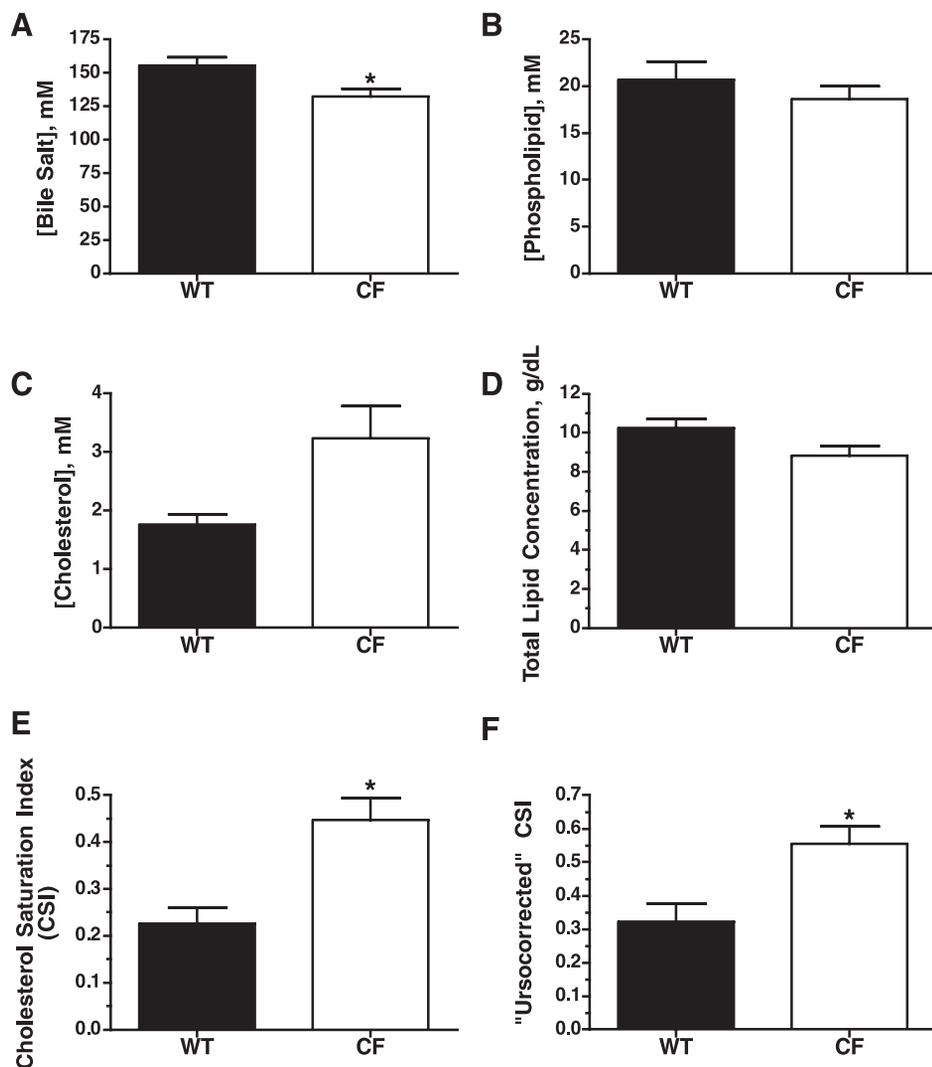


Fig. 3. Concentrations of common biliary lipids in gallbladder biles of WT ( $n = 7$  or  $8$ ) and CF mice ( $n = 6$  or  $10$ ). \*Statistically significant differences between CF and WT mice. *A*: bile salts are decreased significantly ( $P = 0.02$ ) in gallbladder bile of CF mice compared with WT mice. *B*: no significant differences in total phospholipid concentrations are noted between WT and CF mice. *C*: cholesterol concentrations are markedly increased in CF compared with WT gallbladders, but the values miss statistical significance at the  $P = 0.056$  level. *D*: total biliary lipid concentrations in gallbladder bile, despite the marked trend, are not statistically different in CF mice compared with WT mice ( $P = 0.057$ ). *E*: cholesterol saturation index (CSI) of murine biles based on biliary taurocholate only. These estimates in CF biles are approximately double those in WT biles ( $P = 0.003$ ). *F*: with the "urso-corrected" values [i.e., corrected individually for percent muricholates + percent ursodeoxycholate in each bile (12)], CSI values remain significantly elevated in CF compared with WT biles ( $P = 0.01$ ).

**Bile salt molecular species and hydrophobicity indexes.** Figure 4, A–G, shows the bile salt molecular species, calculated as percentage of total bile salt concentration, in gallbladder biles of CF and WT mice ( $n = 17$  per group). Significantly lower proportions of tauro- $\beta$ -muricholate ( $P = 0.01$ ) are present in CF than WT mice ( $34.2 \pm 1.6$  vs.  $42.3 \pm 2.5\%$  bile salt; Fig. 4B). Similarly, tauroursodeoxycholate is decreased in CF compared with WT mice ( $3.5 \pm 0.4$  vs.  $5.1 \pm 0.4$ ,  $P = 0.006$ ; Fig. 4D). In contrast, the percent taurocholate is significantly ( $P = 0.02$ ) greater in CF than WT mice ( $51.1 \pm 2.4$  vs.  $42.3 \pm 2.7\%$ ). We found no significant differences between  $\Delta$ F508 CF and WT mice in terms of percent bile salt concentration for tauro- $\alpha$ -muricholate, taurochenodeoxycholate, or taurodeoxycholate. As shown in Fig. 4H, hydrophobicity indexes are significantly increased (i.e., less negative and more hydrophobic) in CF compared with WT mice ( $-0.35 \pm 0.02$  and  $-0.41 \pm 0.02$ , respectively,  $n = 17$  per group,  $P < 0.05$ ).

**Ionized Ca and ion products for Ca bilirubinates.**  $\text{Ca}^{2+}$  levels in gallbladder bile samples of CF and WT mice ( $n = 13$  for each group, 3–11 mo old) are  $0.42 \pm 0.04$  and  $0.45 \pm 0.06$  mM, respectively (data not shown). Clearly,  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]$ ) did not differ between groups, as was also the case when

males and females were compared:  $0.40 \pm 0.03$  and  $0.56 \pm 0.09$  mM for CF and WT males, respectively ( $n = 6$  per group,  $P = 0.15$ ), and  $0.44 \pm 0.07$  and  $0.37 \pm 0.06$  mM for CF and WT females, respectively ( $n = 7$  per group,  $P = 0.45$ ). Differences in  $[\text{Ca}^{2+}]$  between sexes for each genotype are at the  $P = 0.62$  and  $0.10$  levels for CF and WT mice, respectively.

Figure 5 displays scatter plots of the negative logarithms (base 10) of the ion products of  $\text{Ca}^{2+}$  activities and the bilirubin molecular species that theoretically are cation-sensitive and can form insoluble salts with  $\text{Ca}^{2+}$ . As anticipated (Fig. 2), each ion product is significantly increased in CF compared with WT mice ( $n = 13$  per group). Nontransformed median ion products for  $[\text{Ca}^{2+}] \times [\text{HUCB}^-]^2$ , the monoanionic, protonated form of UCB, are  $9.0 \times 10^{-16}$  and  $1.4 \times 10^{-16}$  for CF and WT mice, respectively (Mann-Whitney  $U = 33$ ,  $P = 0.009$ ; Fig. 5A). Corresponding values for  $[\text{Ca}^{2+}] \times [\text{UCB}^{2-}]$  (dianionic form of UCB) ion products are  $5.6 \times 10^{-10}$  and  $2.2 \times 10^{-10}$  for CF and WT mice, respectively ( $U = 38$ ,  $P = 0.02$ ; Fig. 5B). For  $[\text{Ca}^{2+}] \times ([\text{BMG}^-] + [\text{BMX}^-])^2$  (anionic form of BMG and anionic form of BMG conjugated with glucose or xylose, respectively), the corresponding CF and WT values are  $3.2 \times 10^{-12}$  and  $1.4 \times 10^{-12}$  ( $U = 18$ ,  $P = 0.0007$ ; Fig. 5C).

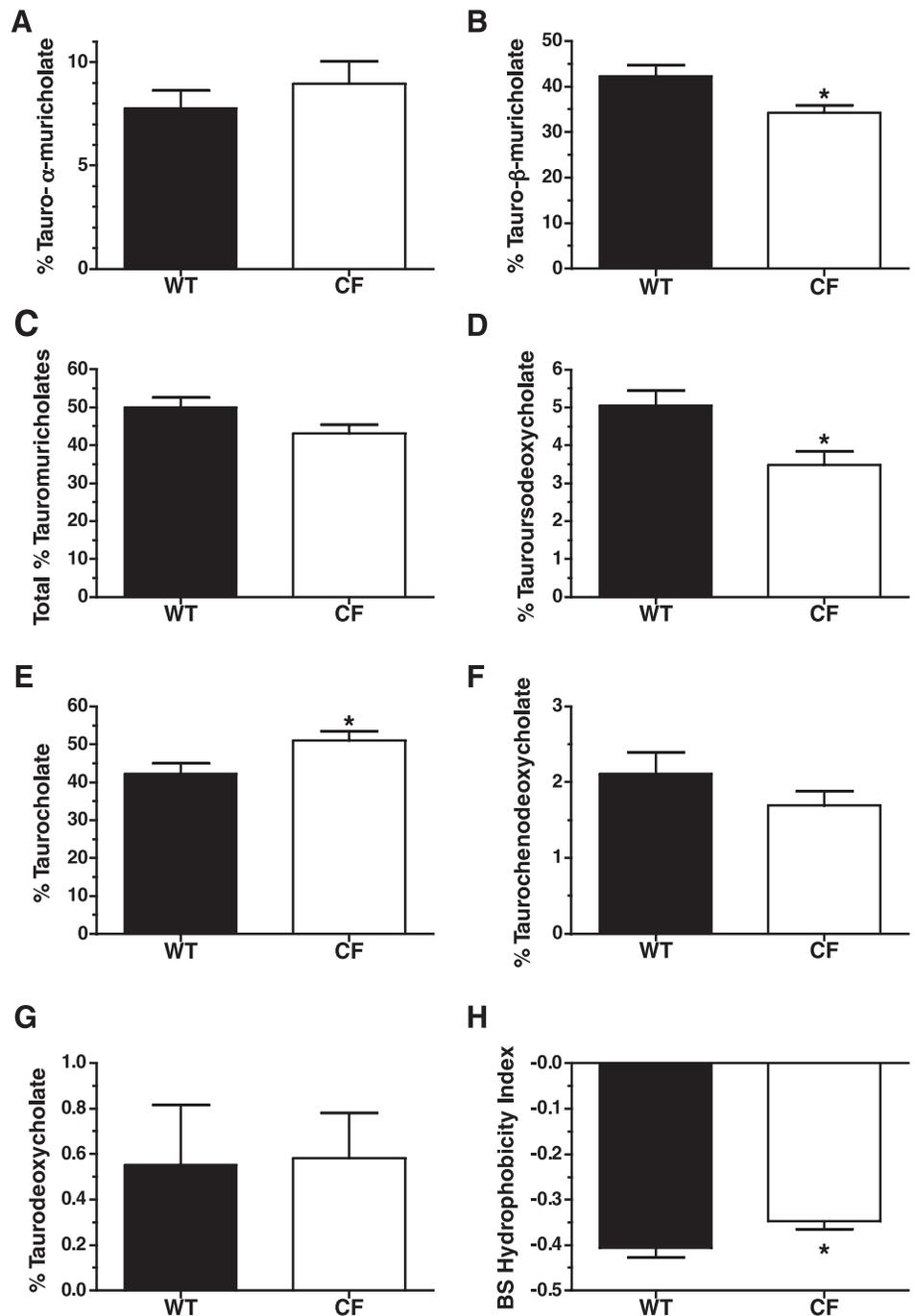


Fig. 4. Bile salt molecular species, as a percentage of total moles of bile salts, and “hydrophobicity indexes” in gallbladder bile samples. \*Statistically significant differences between groups. A–G: individual bile salt molecular species for WT and CF mice ( $n = 17$  per group). Percent tauro- $\beta$ -muricholate (B) and percent tauroursodeoxycholate (D) are significantly lower (both  $P \leq 0.01$ ) in gallbladder bile of CF than WT mice, whereas percent taurocholate (E) is significantly higher ( $P = 0.02$ ) in  $\Delta$ F508 CF than WT mice. Percent tauro- $\alpha$ -muricholate (A), total tauromuricholates (C), taurochenodeoxycholate (F), and taurodeoxycholate (G) are not different between genotypes. H: bile salt hydrophobicity indexes (36) are significantly more hydrophobic (i.e., less negative;  $P < 0.05$ ) in gallbladder biles of CF than WT mice.

## DISCUSSION

In the present work, our aim was to obtain the putative molecular “fingerprints” underlying gallstone disease in CF, employing this mouse model of the commonest human mutation ( $\Delta$ F508) with documented bile acid malabsorption. We were interested in the integrated biliary lipid and lipopigment compositions of gallbladder bile and how they represent secretory data (30) averaged over a short ( $6 \pm 2$  h) fasting period with obligatory gallbladder modifications. Although in humans the  $\Delta$ F508 mutation is known to be associated with a relatively high incidence of liver disease (3, 28, 43) and gallstones (1, 27, 60), despite 99% CFTR ablation (29), the  $\Delta$ F508 mouse

acquires neither clinically significant liver disease (30) nor gallstones (68). In preliminary studies, we tested the same hypothesis in another CF mouse model (G551D) that also exhibits no hepatobiliary disease (25). Despite highly significant bile acid malabsorption in G551D CF compared with WT mice, we did not observe hyperbilirubinemia but did document higher biliary cholesterol secretory rates. We determined that the lack of induced EHC of UCB occurred because these animals suffer from chronic diarrhea analogous to the *Asbt* (*Slc10a2*)-null mouse (24), where insufficient colonic residence time curtails UCB reabsorption (F. Freudenberg and M. C. Carey, unpublished observations; 31). Accordingly, the

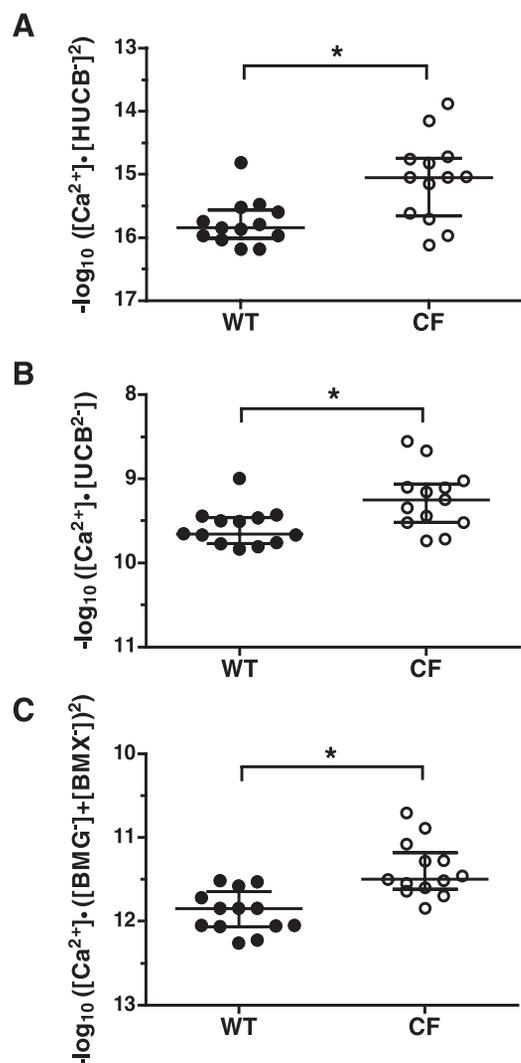


Fig. 5. Ca bilirubinate ion products, calculated by multiplying activities of  $\text{Ca}^{2+}$  and the appropriate bilirubin anion(s) in gallbladder bile. [Although dimensionless, activities are derived from molar concentrations (48)]. Since ion product data showed both skewness and kurtosis, the Mann-Whitney test was used to calculate statistical significance. Because of very low values and broad range of data in each group, ion products are displayed for individual gallbladder bile samples ( $n = 13$  per group) as negative logarithms (base 10), together with medians and interquartile ranges. y-Axes are reversed to depict graphically that nontransformed median ion product values are greater for CF than WT mice. \*Statistically significant differences between CF and WT mice. A: ion products for  $[\text{Ca}^{2+}] \times [\text{HUCB}^-]^2$ , the monoanionic, protonated form of unconjugated bilirubin (UCB) at neutral pH, are  $9.0 \times 10^{-16}$  and  $1.4 \times 10^{-16}$  for CF and WT mice, respectively ( $P = 0.009$ ). B: ion products for  $[\text{Ca}^{2+}] \times [\text{UCB}^{2-}]$ , the dianionic form of unconjugated bilirubin, are  $5.6 \times 10^{-10}$  and  $2.2 \times 10^{-10}$  for CF and WT mice, respectively ( $P = 0.02$ ). C: corresponding ion products for  $[\text{Ca}^{2+}] \times ([\text{BMG}^-] + [\text{BMX}^-])^2$  are  $3.2 \times 10^{-12}$  and  $1.4 \times 10^{-12}$  for CF and WT mice, respectively ( $P = 0.0007$ ).  $\text{BMG}^-$  is the anionic form of bilirubin monoglucuronosides;  $\text{BMX}^-$  is the anionic form of bilirubin monoconjugates conjugated with glucose or xylose.

$\Delta F508$  mouse model was chosen for these systematic studies, since it exhibits well-formed stools and biliary lipid chemistry unperturbed by subclinical or overt hepatobiliary disease, as well as elevations of cholesterol and lipopigment levels in bile that should presage the mixed pigment and cholesterol gallstone findings in CF humans with the same mutation (1). Although no mouse model expresses the holophenotype ob-

served in CF patients (27), fecal bile acid loss is increased significantly in the  $\Delta F508$  homozygous mice ( $\sim 2$ -fold) compared with WT controls (5, 30), a feature seen in the human disease, where  $\sim 30\%$  of CF patients exhibit bile salt malabsorption (49, 67). We documented earlier (6, 7) that when increased spillage of bile acids into the colon occurs and provided colonic residence time is sufficient (31), EHC of UCB is induced and leads to hyperbilirubinemia (30, 64); moreover, upregulated bile acid synthesis leads to increased hepatic availability of cholesterol and its hypersecretion into bile. While these factors contribute to cytotoxicity of bile to cholangiocytes (30), we now propose that the same scenario sets in place the physical-chemical preconditions for mixed black pigment plus cholesterol gallstones in the gallbladder.

We found that CF gallbladder bile is indeed richer in all conjugated and unconjugated bilirubins by a factor of  $\sim 2$  (Fig. 2). This is in line with the evidence that secretion rates of all bilirubin molecular species are elevated in hepatic biles of the same model (30). Moreover, we anticipated that the *Cfr* mutation on cholecystocytes, in addition to cholangiocytes, would lead to an appreciable decrease in gallbladder pH values, as shown in Fig. 1D. It is reasonable to speculate that enzymatic deconjugation of bilirubin conjugates by biliary endogenous  $\beta$ -glucuronidase, with its pH optimum of  $\sim 5$ , is active at pH  $\sim 7$  (37–39) and would be augmented by nonenzymatic hydrolysis in the setting of significantly lower pH in CF gallbladder bile. Indeed, gallbladder bile UCB was significantly elevated (Fig. 2F), but UCB also forms more easily from BMG, the principal murine conjugate, than from BDG (9). One can dismiss direct secretion of UCB into bile in this setting (26) since UCB has no affinity for the canalicular transporter MRP2 (ABCC2) (32). It is generally believed that UCB, which at neutral biliary pH is in the monoanionic ( $\text{HUCB}^-$ ) form (9), would precipitate as insoluble calcium hydrogen bilirubinate salts. However, all Ca monobilirubinate ion products are elevated in CF compared with WT mice (Fig. 5). It has been demonstrated that, in the black pigment gallstones of the hemolytic *nb/nb* mouse model (61), Ca salts of bilirubin monoconjugates also separate from solution in addition to  $\text{Ca}(\text{HUCB})_2$ . The experimental solubility products (dimensionless) for  $\text{Ca}(\text{HUCB})_2$  and  $\text{CaUCB}$  in 50 mM Na taurocholate are reported to be  $6.5 \times 10^{-13}$  and  $1.8 \times 10^{-8}$ , respectively (9). Using these values as approximate estimates for murine gallbladder bile [since muricholate and ursodeoxycholate solubilization of Ca bilirubinate is minimal (M. D. Berman and M. C. Carey, unpublished observations) and biliary phosphatidylcholine decreases UCB solubility (14)], we find that the ion products (Fig. 5) are unsaturated in murine bile by  $\sim 2$  orders of magnitude. Because microscopic examination of a sizable cohort of  $\Delta F508$  gallbladder biles of all ages did not reveal any Ca bilirubinate precipitates, it is likely that, in this study, CF mouse gallbladder biles are unsaturated with these Ca bilirubinate salts (Fig. 5). Furthermore, we did not observe any evidence of  $\text{CaCO}_3$  crystals when gallbladder biles of  $\Delta F508$  and WT mice were examined microscopically. Moore and Vérine (46, 47) demonstrated that the formation constants of  $\text{Ca}(\text{HCO}_3)_2$  and  $\text{CaCO}_3$  are also promoted by gallbladder acidification of hepatic bile; hence, in CF it is likely that these salts could contribute to black pigment gallstone formation (9).

In the face of small differences in absolute bile salt and phospholipid concentrations between CF and WT mice, gallbladder bile of CF mice was more enriched in cholesterol (Fig. 3). Even as the cholesterol results typified hepatic bile (30), the gallbladder compositions of bile salts and phospholipids showed a reverse trend compared with their increased secretion rates in hepatic bile of CF compared with WT mice. The 2.8-fold increase in bile salt secretion in CF compared with WT mice that we found in our previous work (30) may be accounted for, in part, by hepatic bile flow that was nearly doubled in CF compared with WT mice (30). It is likely that CFTR-ablated function at the level of the cholecystocytes is also partly responsible for the decrease in bile salt concentration found in gallbladders of CF compared with WT mice. Gallbladder phospholipid concentrations were decreased in CF compared with WT mice by  $-2.1$  mM, a fact that is not surprising given the gallbladder's selective absorption of biliary phosphatidylcholine in addition to cholesterol (22). The markedly higher cholesterol levels (Fig. 3C) and CSI values (Fig. 3E), which were nearly twofold greater in CF than WT mice, are only slightly diminished when the values were "urso-corrected" (see METHODS; Fig. 3F). It would be feasible to present these mice, whose genetic background (see METHODS) contains multiple *Lith* genes (41, 44), with powerful lithogenic challenges, such as a cholesterol lithogenic diet (52) and bacterial  $\beta$ -glucuronidase per os, to induce EHC of UCB from the upper small intestine. However, we did not believe it was within the scope of the present work to perform these chronic epidemiological experiments. Nonetheless, on the basis of analogy (13), if a percent elevation in CSI occurred in CF humans similar to that found in the  $\Delta$ F508 CF mice (Fig. 3E), the CSI values, normally falling  $\sim 0.8$ – $1.2$  in human bile, would increase to  $1.4$ – $1.6$ .

The bile salt pool is significantly more hydrophobic in CF than WT mice (Fig. 4H). We reasoned earlier (6, 30) that the increased hydrophobicity is secondary to increased spillage of bile salts into the colon with augmented fecal bile acid loss. Although, in theory, the necessity for GoLYTELY in the drinking water to prevent small intestinal obstruction could have contributed to bile acid loss, our WT controls were hydrated in an identical manner and displayed approximately half the bile acid wastage as did the CF mice. Interestingly, the high levels of secondary bile salts from colonic anaerobic catabolism of primary bile salts are not fully "corrected" hepatically in CF mice. The significant shift in hydrophobicity index (Fig. 4H), which in gallbladder bile is accompanied by an increase in UCB levels (Fig. 2F), should render bile more cytotoxic to cholecystocytes (8, 10, 15, 51). Paralleling the significant taurocholate elevation in  $\Delta$ F508 mice (Fig. 4E), studies in CF patients show that cholate conjugates constitute a higher percentage of total bile salts, with chenodeoxycholate conjugates, from which muricholate conjugates are derived in the mouse, constituting a lower percentage (4, 60). Both changes likely reflect upregulated bile salt synthesis via the classic pathway because of increased fecal bile acid loss (5, 30).

Gallbladders are also significantly enlarged in  $\Delta$ F508 mice compared with WT mice (Fig. 1, B and C), and, in mouse models of CF in contrast to humans with CF, distended gallbladders are the rule (33, 34). Although not tested here, larger gallbladders in CF mice might be a surrogate index of decreased motility, but in our CF mice, they are not a result of mucin gel accumulation (see RESULTS) (65, 66). It is, however,

possible that cholecystocytic (and smooth muscle cell) absorption of elevated levels of cholesterol (17) and UCB (53) from gallbladder bile plus T cell activation (45) may compromise gallbladder motility by ablating smooth muscle function. In CF humans, the common  $\Delta$ F508 mutation causes more severe disease, with gallstones forming early within a highly viscous gallbladder mucin gel and the ensuing chronic cholecystitis leading to fibrosis and scarring (27).

This systematic study of gallbladder bile chemistry and early pathophysiology in a large cohort of  $\Delta$ F508 CF and WT mice is likely to presage the earliest events in human gallbladder disease secondary to CF. These mice exhibit neither gross liver nor biliary disease yet display bile acid malabsorption, which is the only pathophysiological abnormality they share with CF humans. Nonetheless, the preponderance of the evidence based on the current data and earlier work (30) suggests that mild bile acid malabsorption is sufficient to perturb bile pigment and bile lipid chemistry at the level of the gallbladder and likely provides the mechanistic setting that promotes mixed stone formation in humans with CF disease (1, 21). Our studies will obviously need to be confirmed noninvasively in humans and extended to animal models of severe CF that acquire gallstones (55). If these findings are translatable to humans with higher CSI values from the beginning, it could explain the high cholesterol admixture in the black pigment stones of CF patients found by Angelico et al. (1). It is hoped that this work will lead to therapeutic targets and, perhaps, new options for preventing and treating this common hepatobiliary complication in CF patients.

#### ACKNOWLEDGMENTS

We are grateful to Bian B. Yu for excellent technical assistance and meticulous animal care. We are indebted to Roderick Bronson (Rodent Pathology Core at Harvard University Medical School) for reviewing gallbladder tissue sections. We thank Elisa Ferreira (Yale University School of Medicine, New Haven, CT) for guidance with mouse husbandry.

Present addresses: F. Freudenberg, Division of Pediatric Gastroenterology and Hepatology, Dr. von Haunersches Kinderspital, Ludwig Maximilians University, Lindwurmstrasse 4, 80337 Munich, Germany; J. N. Glickman, GI Pathology Services, Caris Diagnostics, Newton, MA.

#### GRANTS

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-073687 and DK-036588 to M. C. Carey and Deutsche Forschungsgemeinschaft Grant FR 1733/1-1 to F. Freudenberg.

#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### REFERENCES

1. Angelico M, Gandin C, Canuzzi P, Bertasi S, Cantafora A, De Santis A, Quattrucci S, Antonelli M. Gallstones in cystic fibrosis: a critical reappraisal. *Hepatology* 14: 768–775, 1991.
2. Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem* 234: 466–468, 1959.
3. Bartlett JR, Friedman KJ, Ling SC, Pace RG, Bell SC, Bourke B, Castaldo G, Castellani C, Cipolli M, Colombo C, Colombo JL, Debray D, Fernandez A, Lacaille F, Macek M Jr, Rowland M, Salvatore F, Taylor CJ, Wainwright C, Wilschanski M, Zemkova D, Hannah WB, Phillips MJ, Corey M, Zielenski J, Dorfman R, Wang Y, Zou F, Silverman LM, Drumm ML, Wright FA, Lange EM, Durie PR, Knowles MR. Genetic modifiers of liver disease in cystic fibrosis. *JAMA* 302: 1076–1083, 2009.

4. **Becker M, Staab D, Leiss O, von Bergmann K.** Biliary lipid composition in patients with cystic fibrosis. *J Pediatr Gastroenterol Nutr* 8: 308–312, 1989.
5. **Bijvelds MJ, Bronsveld I, Havinga R, Sinaasappel M, de Jonge HR, Verkade HJ.** Fat absorption in cystic fibrosis mice is impeded by defective lipolysis and post-lipolytic events. *Am J Physiol Gastrointest Liver Physiol* 288: G646–G653, 2005.
6. **Brink MA, Méndez-Sánchez N, Carey MC.** Bilirubin cycles enterohepatically after ileal resection in the rat. *Gastroenterology* 110: 1945–1957, 1996.
7. **Brink MA, Slors JF, Keulemans YC, Mok KS, De Waart DR, Carey MC, Groen AK, Tytgat GN.** Enterohepatic cycling of bilirubin: a putative mechanism for pigment gallstone formation in ileal Crohn's disease. *Gastroenterology* 116: 1420–1427, 1999.
8. **Brito MA, Silva RF, Brites D.** Bilirubin induces loss of membrane lipids and exposure of phosphatidylserine in human erythrocytes. *Cell Biol Toxicol* 18: 181–192, 2002.
9. **Cahalane MJ, Neubrand MW, Carey MC.** Physical-chemical pathogenesis of pigment gallstones. *Semin Liver Dis* 8: 317–328, 1988.
10. **Calligaris S, Cekic D, Roca-Burgos L, Gerin F, Mazzone G, Ostrow JD, Tiribelli C.** Multidrug resistance associated protein 1 protects against bilirubin-induced cytotoxicity. *FEBS Lett* 580: 1355–1359, 2006.
11. **Carey MC.** Critical tables for calculating the cholesterol saturation of native bile. *J Lipid Res* 19: 945–955, 1978.
12. **Carey MC, Ko G.** The importance of total lipid concentration in determining cholesterol solubility in bile and the development of critical tables for calculating “percent cholesterol saturation” with a correction factor for ursodeoxycholate-rich bile. In: *Biological Effects of Bile Acids*, edited by Paumgartner G, Stiehl A, Gerok W. Lancaster, UK: MTP, 1979, p. 299–308.
13. **Carey MC, Small DM.** The physical chemistry of cholesterol solubility in bile. Relationship to gallstone formation and dissolution in man. *J Clin Invest* 61: 998–1026, 1978.
14. **Carey MC, Spivak W.** Physical chemistry of bile pigments and porphyrins with particular reference to bile. In: *Bile Pigments and Jaundice. Molecular, Metabolic, and Medical Aspects*, edited by Ostrow JD. New York: Dekker, 1986, p. 81–132.
15. **Cesaratto L, Calligaris SD, Vascotto C, Deganuto M, Bellarosa C, Quadrifoglio F, Ostrow JD, Tiribelli C, Tell G.** Bilirubin-induced cell toxicity involves PTEN activation through an APE1/Ref-1-dependent pathway. *J Mol Med* 85: 1099–1112, 2007.
16. **Chaudry G, Navarro OM, Levine DS, Oudjhane K.** Abdominal manifestations of cystic fibrosis in children. *Pediatr Radiol* 36: 233–240, 2006.
17. **Chen Q, Amaral J, Biancani P, Behar J.** Excess membrane cholesterol alters human gallbladder muscle contractility and membrane fluidity. *Gastroenterology* 116: 678–685, 1999.
18. **Chinet T, Fouassier L, Dray-Charier N, Imam-Ghali M, Morel H, Mergey M, Dousset B, Parc R, Paul A, Housset C.** Regulation of electrogenic anion secretion in normal and cystic fibrosis gallbladder mucosa. *Hepatology* 29: 5–13, 1999.
19. **Cohen S, Kaplan M, Gottlieb L, Patterson J.** Liver disease and gallstones in regional enteritis. *Gastroenterology* 60: 237–245, 1971.
20. **Cohn JA, Strong TV, Picciotto MR, Nairn AC, Collins FS, Fitz JG.** Localization of the cystic fibrosis transmembrane conductance regulator in human bile duct epithelial cells. *Gastroenterology* 105: 1857–1864, 1993.
21. **Colombo C, Bertolini E, Assaisso ML, Bettinardi N, Giunta A, Podda M.** Failure of ursodeoxycholic acid to dissolve radiolucent gallstones in patients with cystic fibrosis. *Acta Paediatr* 82: 562–565, 1993.
22. **Corradini SG, Elisei W, Giovannelli L, Ripani C, Della Guardia P, Corsi A, Cantafora A, Capocaccia L, Ziparo V, Stipa V, Chirletti P, Caronna R, Lomanto D, Attili AF.** Impaired human gallbladder lipid absorption in cholesterol gallstone disease and its effect on cholesterol solubility in bile. *Gastroenterology* 118: 912–920, 2000.
23. **Couzin-Frankel J.** Genetics. The promise of a cure: 20 years and counting. *Science* 324: 1504–1507, 2009.
24. **Dawson PA, Haywood J, Craddock AL, Wilson M, Tietjen M, Kluckman K, Maeda N, Parks JS.** Targeted deletion of the ileal bile acid transporter eliminates enterohepatic cycling of bile acids in mice. *J Biol Chem* 278: 33920–33927, 2003.
25. **Delaney SJ, Alton EW, Smith SN, Lunn DP, Farley R, Lovelock PK, Thomson SA, Hume DA, Lamb D, Porteous DJ, Dorin JR, Wainwright BJ.** Cystic fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations. *EMBO J* 15: 955–963, 1996.
26. **Erlinger S.** Review article: new insights into the mechanisms of hepatic transport and bile secretion. *J Gastroenterol Hepatol* 11: 575–579, 1996.
27. **Feranchak AP.** Hepatobiliary complications of cystic fibrosis. *Curr Gastroenterol Rep* 6: 231–239, 2004.
28. **Feranchak AP, Sokol RJ.** Cholangiocyte biology and cystic fibrosis liver disease. *Semin Liver Dis* 21: 471–488, 2001.
29. **French PJ, van Doorninck JH, Peters RH, Verbeek E, Ameen NA, Marino CR, de Jonge HR, Bijman J, Scholte BJ.** A  $\Delta$ F508 mutation in mouse cystic fibrosis transmembrane conductance regulator results in a temperature-sensitive processing defect in vivo. *J Clin Invest* 98: 1304–1312, 1996.
30. **Freudenberg F, Broderick AL, Yu BB, Leonard MR, Glickman JN, Carey MC.** Pathophysiological basis of liver disease in cystic fibrosis employing a  $\Delta$ F508 mouse model. *Am J Physiol Gastrointest Liver Physiol* 294: G1411–G1420, 2008.
31. **Freudenberg F, Yu BB, Leonard MR, Dawson PA, Carey MC.** No evidence for enterohepatic cycling of bilirubin in the *Slc10a2* (*Asbt/Ibat*)<sup>-/-</sup> mouse (Abstract). *Gastroenterology* 130: M1823, 2006.
32. **Geier A, Wagner M, Dietrich CG, Trauner M.** Principles of hepatic organic anion transporter regulation during cholestasis, inflammation and liver regeneration. *Biochim Biophys Acta* 1773: 283–308, 2007.
33. **Grubb BR, Boucher RC.** Pathophysiology of gene-targeted mouse models for cystic fibrosis. *Physiol Rev* 79: S193–S214, 1999.
34. **Guilbault C, Saeed Z, Downey GP, Radzich D.** Cystic fibrosis mouse models. *Am J Respir Cell Mol Biol* 36: 1–7, 2007.
35. **Hayden UL, Carey HV.** Cellular localization of cystic fibrosis transmembrane regulator protein in piglet and mouse intestine. *Cell Tissue Res* 283: 209–213, 1996.
36. **Heuman DM.** Quantitative estimation of the hydrophilic-hydrophobic balance of mixed bile salt solutions. *J Lipid Res* 30: 719–730, 1989.
37. **Ho KJ, Hsu SC, Chen JS, Ho LH.** Human biliary  $\beta$ -glucuronidase: correlation of its activity with deconjugation of bilirubin in the bile. *Eur J Clin Invest* 16: 361–367, 1986.
38. **Ho YC, Ho KJ.** Human  $\beta$ -glucuronidase. Measurement of its activity in gallbladder bile devoid of intrinsic interference. *Dig Dis Sci* 33: 435–442, 1988.
39. **Ho YC, Ho LH, Ho KJ.** Human hepatic  $\beta$ -glucuronidase: an enzyme kinetic study. *Enzyme (Basel)* 33: 9–17, 1985.
40. **Jeske DJ, Dietsch JM.** Regulation of rates of cholesterol synthesis in vivo in the liver and carcass of the rat measured using [<sup>3</sup>H]water. *J Lipid Res* 21: 364–376, 1980.
41. **Khanuja B, Cheah YC, Hunt M, Nishina PM, Wang DQ, Chen HW, Billheimer JT, Carey MC, Paigen B.** *Lith1*, a major gene affecting cholesterol gallstone formation among inbred strains of mice. *Proc Natl Acad Sci USA* 92: 7729–7733, 1995.
42. **Kopelman H.** Cystic fibrosis. 6. Gastrointestinal and nutritional aspects. *Thorax* 46: 261–267, 1991.
43. **Lindblad A, Glaumann H, Strandvik B.** Natural history of liver disease in cystic fibrosis. *Hepatology* 30: 1151–1158, 1999.
44. **Lyons MA, Wittenburg H.** Cholesterol gallstone susceptibility loci: a mouse map, candidate gene evaluation, and guide to human *LITH* genes. *Gastroenterology* 131: 1943–1970, 2006.
45. **Maurer KJ, Rao VP, Ge Z, Rogers AB, Oura TJ, Carey MC, Fox JG.** T-cell function is critical for murine cholesterol gallstone formation. *Gastroenterology* 133: 1304–1315, 2007.
46. **Moore EW, Vérine HJ.** Pancreatic calcification: formation constants of  $\text{CaHCO}_3^+$  and  $\text{CaCO}_3^0$  complexes determined with  $\text{Ca}^{2+}$  electrode. *Am J Physiol Gastrointest Liver Physiol* 241: G182–G190, 1981.
47. **Moore EW, Vérine HJ.** Pathogenesis of pancreatic and biliary  $\text{CaCO}_3$  lithiasis: the solubility product ( $K^{\text{sp}}$ ) of calcite determined with the  $\text{Ca}^{++}$  electrode. *J Lab Clin Med* 106: 611–618, 1985.
48. **Nelson DL, Cox MM.** Water. In: *Lehninger Principles of Biochemistry* (4th ed.), edited by Lehninger AL. New York: Freeman, 2004, p. 47–74.
49. **O'Brien S, Mulcahy H, Fenlon H, O'Broin A, Casey M, Burke A, FitzGerald MX, Hegarty JE.** Intestinal bile acid malabsorption in cystic fibrosis. *Gut* 34: 1137–1141, 1993.
50. **Oppenheimer EH, Esterly JR.** Hepatic changes in young infants with cystic fibrosis: possible relation to focal biliary cirrhosis. *J Pediatr* 86: 683–689, 1975.
51. **Ostrow JD, Pascolo L, Brites D, Tiribelli C.** Molecular basis of bilirubin-induced neurotoxicity. *Trends Mol Med* 10: 65–70, 2004.
52. **Paigen B, Carey MC.** Gallstones. In: *The Genetic Basis of Common Diseases* (2nd ed.), edited by King RA, Rotter JJ, Motulsky AG. London: Oxford University Press, 2002, p. 298–335.

53. **Portincasa P, Di Ciaula A, Baldassarre G, Palmieri V, Gentile A, Cimmino A, Palasciano G.** Gallbladder motor function in gallstone patients: sonographic and *in vitro* studies on the role of gallstones, smooth muscle function and gallbladder wall inflammation. *J Hepatol* 21: 430–440, 1994.
54. **Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, Drumm ML, Iannuzzi MC, Collins FS, Tsui LC.** Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245: 1066–1073, 1989.
55. **Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB Jr, Zabner J, Prather RS, Welsh MJ.** Disruption of the *CFTR* gene produces a model of cystic fibrosis in newborn pigs. *Science* 321: 1837–1841, 2008.
56. **Rossi SS, Converse JL, Hofmann AF.** High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids. *J Lipid Res* 28: 589–595, 1987.
57. **Rowe SM, Miller S, Sorscher EJ.** Cystic fibrosis. *N Engl J Med* 352: 1992–2001, 2005.
58. **Roy CC, Weber AM, Morin CL, Combes JC, Nussle D, Megevand A, Lasalle R.** Abnormal biliary lipid composition in cystic fibrosis. Effect of pancreatic enzymes. *N Engl J Med* 297: 1301–1305, 1977.
59. **Spivak W, Yuey W.** Application of a rapid and efficient h.p.l.c. method to measure bilirubin and its conjugates from native bile and in model bile systems. Potential use as a tool for kinetic reactions and as an aid in diagnosis of hepatobiliary disease. *Biochem J* 234: 101–109, 1986.
60. **Strandvik B, Einarsson K, Lindblad A, Angelin B.** Bile acid kinetics and biliary lipid composition in cystic fibrosis. *J Hepatol* 25: 43–48, 1996.
61. **Trotman BW, Bernstein SE, Bove KE, Wirt GD.** Studies on the pathogenesis of pigment gallstones in hemolytic anemia: description and characteristics of a mouse model. *J Clin Invest* 65: 1301–1308, 1980.
62. **Turley SD, Dietschy JM.** Re-evaluation of the  $3\alpha$ -hydroxysteroid dehydrogenase assay for total bile acids in bile. *J Lipid Res* 19: 924–928, 1978.
63. **Vercaemst R, Union A, Rosseneu M.** Separation and quantitation of free cholesterol and cholesteryl esters in a macrophage cell line by high-performance liquid chromatography. *J Chromatogr* 494: 43–52, 1989.
64. **Vítek L, Carey MC.** Enterohepatic cycling of bilirubin as a cause of 'black' pigment gallstones in adult life. *Eur J Clin Invest* 33: 799–810, 2003.
65. **Wang DQ, Afdhal NH.** Genetic analysis of cholesterol gallstone formation: searching for *Lith* (gallstone) genes. *Curr Gastroenterol Rep* 6: 140–150, 2004.
66. **Wang HH, Afdhal NH, Gendler SJ, Wang DQ.** Evidence that gallbladder epithelial mucin enhances cholesterol cholelithogenesis in MUC1 transgenic mice. *Gastroenterology* 131: 210–222, 2006.
67. **Weber AM, Roy CC, Morin CL, Lasalle R.** Malabsorption of bile acids in children with cystic fibrosis. *N Engl J Med* 289: 1001–1005, 1973.
68. **Zeiber BG, Eichwald E, Zabner J, Smith JJ, Puga AP, McCray PB Jr, Capecchi MR, Welsh MJ, Thomas KR.** A mouse model for the  $\Delta$ F508 allele of cystic fibrosis. *J Clin Invest* 96: 2051–2064, 1995.

