Pathophysiologic preconditions promoting mixed “black” pigment plus cholesterol gallstones in a ΔF508 mouse model of cystic fibrosis

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Running head
Preconditions for gallstones in ΔF508 (CF) mice

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Abstract

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 ΔF508 mouse model with documented increased fecal bile acid loss and induced enterohepatic cycling of bilirubin (Freudenberg F et al. *Am J Physiol Gastrointest Liver Physiol*. 2008. 294:G1411-1420), we assessed gallbladder bile chemistry, morphology, and microscopy in CF and wild type mice, focusing on the concentrations and compositions of the common biliary lipids, bilirubins, 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 were present in biles of CF mice, increasing 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.
Keywords: CFTR, hepatobiliary disease, enterohepatic cycling, bilirubin, gallbladder
Introduction

Cystic fibrosis (CF) is a common inherited disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene with over 1,500 examples documented to date (23, 54, 57). Dysfunction of CFTR, an apical plasma membrane Cl⁻ channel regulated by cAMP, impairs Cl⁻/HCO₃⁻ exchange via anion exchanger-2 (AE2) 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 to 25% of pediatric CF patients (16, 28, 50) and 30 to 72% of adult patients with CF (42, 43) display hepatobiliary abnormalities, 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 ΔF508 mutation (68) whether chemical changes that might presage the pathophysiology of such “mixed”
gallstones occur in gallbladder bile. Not only is the ΔF508 mutation the most common CFTR gene mutation (about 70%) in CF patients (57), but the pathophysiology of the intestinal tract and the liver in this mouse model have 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 ΔF508 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 ΔF508 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 in this journal (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 “hyperbilirubinbilia” (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), but especially cholesterol, in CF mice. We now propose that the same scenario sets in place
biochemical and pathophysiological changes in bulk gallbladder bile that renders 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 both 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 more severe CF as well as in the human disease.
Methods

Animals. Heterozygous breeding pairs of ΔF508 mice (68) on a 75% C57BL/6:25% 129SvEv background were donated by Dr. Marie Egan, Yale University Medical School, New Haven, CT. Breeding pairs of G551D mice (25) were obtained from Dr. Gerald Pier of the Channing Laboratory in our institution. Of the two murine CF models [ΔF508 (68) and G551D (25)] that were evaluated in preliminary studies, both displayed bile acid malabsorption (G551D > ΔF508). However, ΔF508 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 mice but not in the ΔF508 mice, thereby curtailing colonic residence times, and is analogous to what we documented in Asbt null mice (24, 31). ΔF508 mice and WT controls were housed in the Thorn Research Building’s animal facility at Brigham and Women’s Hospital, Boston, MA. To minimize the risk of potentially lethal meconium ileus, all animals were housed on corncob bedding (The Andersons, Maumee, OH), and for the same reason GoLYTELY (Braintree Laboratories, Braintree, MA) was added to their drinking water. Mice were fed a diet containing 11% fat and replete in nutrients, vitamins, and minerals (Mouse Diet 5015, Labdiet, Richmond, IN). Housing illumination consisted of normal 12-h light:12-h dark cycles employing conventional fluorescent lamps. All surgeries were performed at the same time of day on age- (unless otherwise noted, mouse ages ranged from 3 - 14 mo) and sex-matched homozygous CF and WT mice that had been fasted for 4 - 8 h. Each mouse was genotyped as follows: After tail clipping at 3 wk of age, 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.** Following induction of anesthesia (i.p. injection of ketamine, xylazine and atropine in 0.15 M NaCl), laparotomy was performed (30) and gallbladders were examined under direct and transmitted illumination (Olympus SZ40 Stereo Zoom Microscope, Olympus America, Inc., Chelmsford, MA). Cholecystectomy was then performed by standard technique for mice. Subsequently, the mouse was euthanized employing an overdose of ketamine and xylazine followed by bilateral thoracotomy. After emptying gallbladder bile completely into tared 200-μL collection tubes, we measured bile volumes gravimetrically by numerically equating weight and volume. Immediately following, pH values were measured on individual biles by microelectrode (Thermo Electron Corp., 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 for pigment and cholesterol gallstones. In addition, intact gallbladders of non-fasted mice (n = 4 female plus 2 male CF and 5 female plus 3 male WT) were fixed in 20-fold volumes of formalin
solution, dehydrated serially with ethanol and H₂O, 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 (R. B., in Acknowledgements). We analyzed and scored stained sections as follows: 0, no mucin either on apical columnar cells or within gallbladder lumina; 1, few wisps of mucin within the lumina 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 gallbladder lumina; 3, a mucin layer of variable thickness on epithelial cells, moderate amounts of mucin within the gallbladder lumina; 4, few agglomerated mucin spherules within gallbladder lumina, moderate to large amount of mucin within lumina; and 5, many stained globules or mucin spherules within gallbladder lumina and/or non-agglomerated mucin filling gallbladder lumina.

Quantitation of ionized Ca²⁺ activities. Immediately following measurement of gallbladder bile pH values, we quantified Ca²⁺ ion activities. To adjust for the ionic strength of bile and to prepare standard solutions, Na⁺ ion 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 5-fold (to protect the analyzer’s electrode from bile salt detergency), we
determined a range of 190-205 mM Na⁺ in murine gallbladder bile. Accordingly, we adjusted all CaCl₂ standards to an ionic strength of 200 mM NaCl. Semi-logarithmic calibration curves were generated from five stock solutions ranging in concentration from 10⁻¹ to 10⁻⁵ M CaCl₂ prepared by serial dilution of a 0.1 M CaCl₂ standard solution (Microelectrodes, Inc.). Calcium ion activities were measured in mV using an Orion pH meter Model 720A (Thermo Fisher Scientific, Waltham, MA) and an ion-specific microelectrode (MI-600, Microelectrodes, Bedford, NH) by introducing both the calcium and reference microelectrodes into individual gallbladder bile samples. Between measurements, calcium and reference electrodes were rinsed with deionized, ultrafiltered water (Fisher Scientific) and stored in 0.1 M CaCl₂ solution. Bile sample readings were interpolated on the standard curve to obtain millimolar Ca²⁺ concentrations. Ion products were calculated as the product of molar concentrations of Ca²⁺ and unconjugated or monoconjugated bilirubins.

*Bilirubin molecular species.* To separate and quantify bilirubin molecular species in bile, we injected a 10-μl aliquot of individual bile samples onto a reverse phase HPLC pre-column 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, BDX); and unconjugated bilirubin (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 3α-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). Concentrations of the major biliary lipids are expressed as millimolar concentrations. We calculated cholesterol saturation indexes (CSIs) of gallbladder bile samples using critical tables (11), initially assuming that all bile salts present are sodium taurocholate. This was followed by employing “urso-correction” factors (12) 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 expressed as means ± SEM. For these comparisons between mutant ΔF508 and WT groups, statistical significance was assessed using an unpaired, 2-tailed Student’s t-test, corrected with Welch’s correction factor in cases of unequal variance. However, data for the Ca^{2+}-bilirubinate ion products show both skewness and kurtosis. To evaluate these non-parametric data, we used the Mann-Whitney test (also known as the Wilcoxon Rank Sum test) to report medians, Mann-Whitney U, and P values. For all statistics, P < 0.05 is considered significant.
Results

General. As described earlier in Freudenberg et al (30), not only does breeding these CF mice yield fewer than expected offspring based on Mendelian predictions, but in the pre- and post-weaning periods, a higher proportion of ΔF508 CF offspring die spontaneously, usually from unavoidable intestinal obstruction. This occurs despite corncob bedding and GoLYTELY in the drinking water. There is also a significant weight difference in age-matched, mature mice (Fig. 1A) in that CF mice \( n = 21 \) are \( \approx 34\% \) lighter than WT mice \( n = 20; \ P < 0.0001 \).

Gallbladder volumes, microscopy, and mucin scores. Fig. 1B and C display gallbladder sizes that are markedly larger in CF than in WT mice, whether expressed as absolute volumes (Fig. 1B; CF 23% greater than WT; \( P = 0.04 \)) or normalized to body weight (BW) (Fig. 1C; CF 79% larger than WT; \( P < 0.0001 \)) \( (n = 21 \text{ and } 20, \text{ respectively}) \). All biles were bright yellow and translucent. By direct light microscopy, neither yellow precipitates of phase-separated Ca bilirubinates nor “black” gall-sand or “black” gallstones were observed in the gallbladders or found in gallbladder biles of either CF \( n = 7 \) or WT \( n = 5 \) mice. Under direct and polarized light microscopy, we sometimes noted 1 or 2 narrow, birefringent crystalline objects of unknown chemistry per high power field in both CF and WT gallbladder biles. There was a total absence of phase-separated liquid crystals, cholesterol monohydrate crystals, inorganic calcium carbonate/calcium phosphate crystals, or amorphous bilirubinate precipitates in the gallbladders and bile samples of both CF and WT mice. By direct light microscopy, gallbladders of CF mice invariably contained a mild to moderate amount of insoluble
mucin gel. Some gallbladders of WT mice were completely isotropic, although moderate amounts of mucin gel were found in the gallbladders of two WT mice aged 8 and 9 mo. We further assessed epithelial-adherent and luminal gallbladder mucins semi-quantitatively by both Alcian Blue and PAS stains in male and female CF and WT mice (n = 6 and 8, respectively; mice of both genotypes aged 3 – 18 mo). PAS scores confirmed those of sections stained with Alcian Blue, being identical in all but one case. Blinded mucin scores (see Methods) ranged from 0 – 4 for WT mice and from 1 – 4 for CF mice, and mean scores for both genotypes were essentially identical (CF mucin score = 2.4; WT mucin score = 2.5); no gender differences were found. Although a trend toward increasing scores with age was observed in CF mice, this was not the case with WT mice.

Gallbladder morphology. The gross appearances of gallbladders from CF (6 – 19 mo) and WT (4 – 9 mo) mice, despite marked differences in size (Fig. 1B), were identical; in particular, no yellow or black staining of the CF gallbladders was noted and, by transillumination, no phase-separated precipitates were observed. The H&E-stained gallbladder tissues were read by two independent pathologists with different backgrounds, who were blinded as to their origin. After examining 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 read both 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 ΔF508 mutant CF mice ($7.32 \pm 0.04, n = 21$) compared with WT mice ($7.53 \pm 0.06, n = 20$).

**Concentrations of bilirubin molecular species in gallbladder bile.** Fig. 2A - F shows that gallbladder bile of CF mice contains significantly higher concentrations of all bilirubin molecular species, including UCB. 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. The μM concentrations for CF compared with WT mice, respectively, are: BDG, $38.2 \pm 3.0, 23.3 \pm 1.6$ (Fig. 2A); BDG + BDX, $47.0 \pm 3.5, 29.1 \pm 1.9$ (Fig. 2B); BMG, $92.3 \pm 6.6, 58.0 \pm 3.7$ (Fig. 2C); BMG + BMX, $95.4 \pm 6.8, 59.5 \pm 3.7$ (Fig. 2D); total of conjugated bilirubin species, $142.3 \pm 10.1, 88.6 \pm 5.6$ (Fig. 2E); and UCB, $1.5 \pm 0.2, 0.7 \pm 0.1$ (Fig. 2F). Within each mouse genotype, we found gender differences in the concentration of individual bilirubin species, with higher levels in female mice ($n = 7$/group) than in male mice ($n = 6$/group); these differences were significant in WT mice. However, all molecular species of bilirubins were 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.** Fig. 3A-D delineates the absolute concentrations of bile salts ($A$), phospholipids ($B$), and cholesterol ($C$) 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 (D) (n = 6 and 7). Bile salt concentrations are decreased significantly (P = 0.02) in CF compared with WT mice (132.3 ± 5.7, 155.1 ± 6.5 mM) (A). Although slightly depressed in CF mice, no significant differences in biliary phospholipid levels were noted between CF (18.6 ± 1.3 mM) and WT (20.7 ± 1.9 mM) mice (P = 0.38) (B). Cholesterol concentrations in biles of CF mice are appreciably greater than in biles of WT mice (C), but the mean value marginally misses statistical significance (3.23 ± 0.56 compared with 1.77 ± 0.18 mM, respectively; P = 0.056). However, molar percentages of cholesterol are doubled in CF compared with WT mice (2.06 ± 0.34 and 0.99 ± 0.14 mol %, respectively; 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 ± 0.5 g/dL for CF mice and 10.3 ± 0.5 g/dL for WT mice. Comparison of the means barely misses statistical significance; P = 0.057 (D).

Cholesterol saturation indexes (CSIs). Fig. 3E shows the CSI values (uncorrected for percent muricholates and ursodeoxycholates in murine bile): These are significantly higher (P = 0.003) in gallbladder biles of CF mice (0.45 ± 0.05; n = 6) compared with WT mice (0.23 ± 0.04; n = 7). 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 ± 0.05 for CF mice and 0.32 ± 0.05 for WT mice (P = 0.01) (Fig. 3F).
Bile salt molecular species and hydrophobicity indexes. Fig. 4A – G shows the bile salt molecular species, calculated as percent of total bile salt concentration, in gallbladder biles of CF and WT mice (n = 17 per group). Significantly lower proportions of tauro-ß-muricholate (P = 0.01) are present in CF (34.2 ± 1.6% BS) compared with WT mice (42.3 ± 2.5% BS) (Fig. 4B). Likewise, tauroursodeoxycholate is decreased in CF compared with WT mice (3.5 ± 0.4 and 5.1 ± 0.4, respectively; P = 0.006) (Fig. 4D). In contrast, the percent taurocholate is significantly (P = 0.02) greater in CF (51.1 ± 2.4%) than in WT mice (42.3 ± 2.7%). We found no significant differences between ΔF508 CF and WT mice in terms of percent bile salt concentration for tauro-α-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 ± 0.02 and −0.41 ± 0.02, respectively; n = 17 per group; P < 0.05).

Ionized calcium and ion products for calcium bilirubinates. The mean ± SEM levels of Ca\(^{2+}\) in gallbladder bile samples of CF and WT mice (n = 13 for each group; mice aged 3 – 11 mo) are 0.42 ± 0.04 and 0.45 ± 0.06 mM, respectively (data not shown). Clearly, [Ca\(^{2+}\)] did not differ between groups, as was also the case when males and females were compared (CF and WT males: 0.40 ± 0.03 and 0.56 ± 0.09 mM, respectively, n = 6 per group, P = 0.15; CF and WT females: 0.44 ± 0.07 and 0.37 ± 0.06 mM, n = 7 per group, P = 0.45.) Differences in [Ca\(^{2+}\)] between sexes for each genotype are at the P = 0.62 and 0.10 levels for CF and WT mice, respectively.
Fig. 5A-C displays scatter plots of the negative logarithms (base 10) of the ion products of Ca$^{2+}$ activities and the bilirubin molecular species that theoretically are cation-sensitive and can form insoluble salts with Ca$^{2+}$. As anticipated (Fig. 2), each ion product is significantly increased in CF mice compared with WT mice ($n = 13$ per group). Non-transformed median ion products for $[\text{Ca}^{2+}] \times [\text{HUCB}^{-}]^2$ 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 $[\text{Ca}^{2+}] \times [\text{UCB}^{2-}]$ ion products are $5.6 \times 10^{-10}$ and $2.2 \times 10^{-10}$ for CF and WT mice; $U = 38$; $P = 0.02$ (Fig. 5B). For $[\text{Ca}^{2+}] \times ([\text{BMG}^{-}] + [\text{BMX}^{-}])^2$ ion products, the corresponding CF and WT values are $3.2 \times 10^{-12}$ and $1.4 \times 10^{-12}$; $U = 18$; and $P = 0.0007$ (Fig. 5C).
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 (ΔF508) 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 fasting period (6 ± 2 h) with obligatory gallbladder modifications. Although in humans the ΔF508 mutation is known to be associated with a relatively high incidence of liver disease (3, 28, 43) and gallstones (1, 27, 60), yet despite 99% CFTR ablation (29), the ΔF508 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 hyperbilirubinbilia 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, and (31)]. Accordingly, the ΔF508 mouse model was chosen for these systematic studies since it exhibits well-formed stools and biliary lipid chemistry unperturbed by sub-clinical or overt hepatobiliary disease as well as displaying elevations of both 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 observed in CF patients (27), fecal bile acid loss is increased
significantly in the ΔF508 homozygous mice (approximately twofold) compared with
WT controls (5, 30), a feature seen in the human disease where approximately 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 “hyperbilirubinbilia” (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 \( \approx 2 \) (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 \( C f t r \) mutation on cholecystocytes in addition to
cholangiocytes would lead to an appreciable decrease in gallbladder pH values, as was
found (Fig. 1D). It is reasonable to speculate that enzymatic deconjugation of bilirubin
conjugates by biliary endogenous \( \beta \)-glucuronidase, with its pH optimum of about 5, is
active at pH \( \approx 7 \), (37-39) and would be augmented by non-enzymatic 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 (HUCB\(^{−}\)) form (9), would precipitate as insoluble calcium hydrogen bilirubinate salts. However, of note is that 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 Ca(HUCB)\(_2\). The experimental solubility products (dimensionless) for Ca(HUCB)\(_2\) and CaUCB in 50 mM Na taurocholate are reported to be 6.5 x 10\(^{-13}\) and 1.8 x 10\(^{-8}\), respectively (9). Using these values as approximate estimates for murine gallbladder bile [since muricholate and ursodeoxycholate solubilization of Ca bilirubinates 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 approximately two orders of magnitude. Because microscopic examination of a sizable cohort of ΔF508 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 CaCO\(_3\) crystals when gallbladder biles of ΔF508 and WT mice were examined microscopically. Moore and Vérine (46, 47) demonstrated that the formation constants of Ca(HCO\(_3\))\(_2\) and 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 to 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 levels of cholesterol (Fig. 3C) and CSIs (Fig. 3E) that were ≈2-fold greater in CF compared with 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 cholesterol lithogenic diet (52) and bacterial β-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, based on analogy (13), if a similar percent elevation in CSI occurred in CF humans as we found in the ΔF508 CF mice (Fig. 3E), the CSI values, normally falling around ≈0.8 – 1.2 in human bile, would increase to values in the range of 1.4 – 1.6.
The bile salt pool is significantly more hydrophobic in CF than in 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 ΔF508 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 ΔF508 mice compared with WT mice (Fig. 1B 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
ΔF508 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 ΔF508 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) suggest 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
observed in humans with CF disease (1, 21). Our studies will obviously need to be
confirmed non-invasively 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 ab initio, 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 open new options for preventing and treating this
common hepatobiliary complication in CF patients.
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Figure Legends

**Fig. 1.** Mouse weights (g), gallbladder volumes (μL), and bulk pH values of ΔF508 (CF) mice compared with control (WT) mice (n = 21 and 20, respectively). A: Mouse weights at time of cholecystectomy are significantly lower in CF mice (23.8 ± 0.9) compared with WT mice (36.1 ± 1.8; P < 0.0001). B: Gallbladder bile volumes of CF mice (36.4 ± 2.7) are significantly (P = 0.04) larger than in WT mice (29.6 ± 1.6). C: Normalized gallbladder bile volumes (μL/100g BW) are markedly different in the same direction with 153.2 ± 10.4 for CF mice, compared with 85.4 ± 5.3 for WT mice; P < 0.0001. D: Individual gallbladder bile pH values were measured by microelectrode immediately following bile expression from the gallbladders. The pH values are significantly (P = 0.004) lower in CF compared with WT mice (7.32 ± 0.04 vs. 7.53 ± 0.06).

**Fig. 2.** Concentrations of bilirubins in individual (10-μL aliquots) gallbladder biles of both WT (n = 20) and CF (n = 21) mice measured by HPLC immediately following cholecystectomy. A: Bilirubin diglucuronosides (BDG); B: Bilirubin diconjugates (BDG + BDX), which include pure and mixed conjugates with xylose and glucose (BDX); C: Bilirubin monoglucuronosides (BMG); D: Bilirubin monoconjugates including conjugates with xylose and glucose (BMG + BMX); E: Concentrations of total conjugated bilirubins (cBR) in gallbladder bile; and F: Unconjugated bilirubin (UCB). In the case of the conjugates and UCB, the concentrations are significantly higher (P < 0.0001 and 0.0002, respectively) in CF compared with WT mice. As inferred visually
from these plots (means ± SEM), all bilirubin levels in the gallbladder bile of CF mice are 1.5- to more than 2-fold greater than in WT mice.

**Fig. 3.** Concentrations (mM) of the common biliary lipids in gallbladder biles of WT ($n = 7$ or $8$) and CF mice ($n = 6$ or $10$). *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:* We found marked increases in cholesterol concentrations in CF gallbladders compared with WT, 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:* CSI values 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 plus percent ursodeoxycholate in each bile (12)], CSI values remain significantly elevated in CF compared with WT biles ($P = 0.01$).

**Fig. 4.** Bile salt molecular species, as a percentage of total moles bile salts, and “Hydrophobicity Indexes” in gallbladder bile samples. Panels *A – G* display the individual bile salt molecular species for WT and CF mice ($n = 17$ per group). Of note is that percent tauro-β-muricholate (*B*) and percent taoursodeoxycholate (*D*) are significantly lower in gallbladder bile of CF compared with WT mice, whereas percent taurocholate (*E*) is significantly higher in ΔF508 CF compared with WT mice. Percent tauro-α-muricholate (*A*), total tauomuricholates (*C*), taurochenodeoxycholate (*F*), and
taurodeoxycholate ($G$) are not different between genotypes. Bile salt hydrophobicity indexes (36), shown in panel $H$, are significantly more hydrophobic (i.e., less negative) in gallbladder biles of CF mice compared with WT mice.

**Fig. 5.** Ca$^{2+}$-bilirubinate ion products, calculated by multiplying activities of 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 the very low values and the 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. The y-axes have been reversed to depict graphically that the non-transformed median ion product values are greater for CF than for WT mice. 

$A$: Ion products for $[\text{Ca}^{2+} \times [\text{HUCB}^-]^2$, the monoanionic, protonated form of unconjugated bilirubin (UCB) present 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 $2.2 \times 10^{-10}$, 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 $1.4 \times 10^{-12}$; $P = 0.0007$. BMG$^-$ is the anionic form of bilirubin monoglucuronosides; BMX$^-$ is the anionic form of bilirubin monoconjugates conjugated with glucose or xylose.
A

\[-\log_{10}(\text{[Ca}^{2+}\text{]•([BMG] + [BMX] + [HUCB])})\]

\[\text{WT} \quad \text{CF}\]

B

\[-\log_{10}(\text{[Ca}^{2+}\text{]•([BMG] + [BMX] + [HUCB])})\]

\[\text{WT} \quad \text{CF}\]

C

\[-\log_{10}(\text{[Ca}^{2+}\text{]•([BMG] + [BMX] + [HUCB])})\]

\[\text{WT} \quad \text{CF}\]