TITLE: Animal Models of Cystic Fibrosis: Gastrointestinal, Pancreatic and Hepatobiliary Disease and Pathophysiology

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RUNNING TITLE: Animal Models of Cystic Fibrosis Gastrointestinal Disease

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ABSTRACT
Multiple organ systems including the gastrointestinal tract, pancreas and hepatobiliary systems are affected by cystic fibrosis (CF). Many of these changes begin early in life and are difficult to study in young CF patients. Recent development of novel CF animal models has provided expanded opportunities in the field to better understand of CF pathogenesis and evaluate traditional to innovative therapeutics. In this review, manifestations of CF disease in gastrointestinal, pancreatic and hepatobiliary systems of humans and animal models will be discussed. We also compare the similarities and limitations of animal models, and discuss future directions for modeling CF.

Key Words: Cystic Fibrosis, Gastrointestinal Tract, Liver, Pancreas, CFTR
INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) (97, 101, 129). CFTR is an anion channel that transports chloride (Cl\(^-\)) and bicarbonate (HCO\(_3\)^-), and thereby regulates fluid secretion (22, 48, 108). While there are many theories about mechanisms of CF disease pathogenesis (18, 29, 92, 122), the fundamental lack of CFTR function produces luminal secretions with reduced pH and fluid volume (91). These changes in luminal environment are thought to biophysically and functionally alter mucus and proteinaceous secretions that obstruct and alter function in various organs (47, 60, 114, 122).

CF research has benefited from animal models and historically this has been limited to mice with multiple CFTR mutations, which have been summarized elsewhere (54, 130). CFTR-deficient mice have intestinal disease, but lack characteristic pancreatic and lung phenotypes commonly seen in humans (54, 94, 111). Even so, mouse models have been useful. Recently, researchers have developed mouse models with conditional, cell-specific CFTR expression and such novel approaches may be useful to further dissect CF pathogenesis (16, 58, 59). Because CF mice lacked a lung phenotype like humans; new model species were recently developed for study CF (Table 1). Targeted mutations in CFTR have been described in at least four mammalian species including the mouse (111), pig (100), ferret (121) and most recently the rat (124), with other species being considered.

CFTR-deficient animal models offer several advantages for study of CF pathogenesis and disease. First, study of early CF pathogenesis is limited in young CF infants and children due to appropriate ethical concerns. Animal models can be studied early in life, prior to significant remodeling and secondary features (e.g. inflammation, destruction) that can confound the interpretation. Second, animal models allow for controlled comparison of organ systems in the study of CF pathophysiology and in response to treatments. CF patients are often being administered multiple prophylactic and therapeutic treatments, all of which could confound organ specific interpretations. Third, comparison of the spectrum of CF disease between species can lead to a better understanding of CF disease pathogenesis and / or identify candidate modifier genes.
In this review, CF pathophysiology and disease of gastrointestinal, pancreatic and hepatobiliary systems are reviewed. For context, CF disease in humans is presented first followed by contemporary CF animal models.

**GASTROINTESTINAL**

**CFTR expression**

CFTR expression in the human gastrointestinal tract is defined by anatomic location and often relevant function (117). The stomach has low mRNA expression throughout the mucosa. Upon entering the small intestine, robust CFTR expression is found in the Brunner’s glands – consistent with the immediate requirement for bicarbonate-rich secretions to buffer acidic ingesta. The small intestine has a decreasing expression gradient from duodenum to ileum, and the colon has moderate levels of expression. In the small intestine and colon, the crypts have the highest CFTR mRNA expression with a decreased expression gradient towards cells extending into the lumen. In the small intestine, scattered high expressing cells have been described in the duodenum and jejunum, but the relevance of these is not clear. The high expression of CFTR in the crypts corresponds with the anatomic location of the mucoproteinaceous secretions obstructing and distending the crypts – a diagnostic feature of CF intestinal disease in humans and CF animal models (Figure 1) (15, 54, 79, 120).

**Intestinal obstruction**

Meconium ileus is an intestinal obstruction seen in ~15-20% of CF births and often detected in the first 48 hours following birth (Figure 2) (41, 86, 127). Typically, the obstruction is located in the distal ileum to proximal colon – being found on either side of the ileocecal junction. Immediately proximal to the obstruction, the bowel is distended by abnormally sticky and tenacious meconium, and distal to the obstruction the bowel is of small caliber (“microcolon”) with multifocal luminal casts (“cords” or “pellets”) comprised of mucous, proteinaceous and cellular debris (5). Abnormal intestinal secretions are believed to play an important role in pathogenesis of the intestinal obstruction (20, 96), but the exact mechanism is not well-defined. Interestingly, recent studies in the CF pig lung suggest abnormal mucus may not be able to detach properly.
from their site of origin (60). Speculatively, a similar mechanism in the intestine could possibly contribute to the development of intestinal obstruction.

In meconium ileus, approximately half of the cases are "complicated" by atresia, perforations, adhesions, pseudocyst formation or generalized peritonitis requiring surgical intervention (5, 96, 110), whereas the remainder are considered "uncomplicated" (81, 86). A recent review of fifty-nine cases of meconium ileus reported that Gastrograffin© (Bracco Diagnostics Inc., Anjou, Quebec Canada) enemas were successful in only about 9% (5 of 19 cases) and laparotomy was required for 91% (54/59) (41). Review of these fifty-four cases recorded three major surgical approaches including: stoma formation (72%), enterotomy with washout (13%), or resection and anastomosis (15%). All cases received N-acetyl cysteine until enteral feeding was resumed. With improved medical and surgical therapies, most patients with meconium ileus survive the neonatal obstruction.

Distal intestinal obstruction syndrome (DIOS), also called "meconium ileus-equivalent", is an intestinal obstruction that occurs at a rate of ~6.2 events per 1000 patient years and can be seen at any age after the newborn period. The obstruction is located on either side of the ileocecal junction and can generally appear similar to meconium ileus, but without meconium filling the bowel (61). Coincidence rates of meconium ileus and later DIOS presentations have been reported to be 44% (61), and another study has suggested that a history of meconium ileus is a significant risk factor for the necessity of surgical intervention for DIOS cases (118). Genetic analysis indicates that DIOS development is influenced by environmental factors and not modifier genes like meconium ileus (13, 61). Consensus diagnosis of DIOS in CF patients has recently been defined into two categories – impending and complete. Impending DIOS is characterized by the presence of a viscid fecal mass in the ileocolon along with abdominal pain/distension. Complete DIOS has, in addition to impending DIOS, evidence of complete obstruction such as bilious vomiting and/or fluid levels in abdominal radiographs (61, 127). In a retrospective study, DIOS medical therapy with Gastrograffin© (oral or enema administration) was reported to be successful in 71% of cases with the remainder of the cases requiring laparotomy (41).
Constipation is clinically less severe than DIOS and is reported to occur in 47% of patients, but this condition may be under-recognized in CF patients (126, 127). Diagnostic definitions of constipation include: 1) Abdominal pain and/or distension or 2) reduced frequency and/or increased consistency of bowel movements, and 3) improvement of the above symptoms with the use of laxatives. In CF animal models, there is a lack of diagnostic parameters and techniques to distinguish DIOS from constipation. Accordingly, discussion of intestinal obstructions that occur after the first week of life will be referred to as “DIOS/constipation” unless otherwise specified. For a summary of gastrointestinal manifestations in CF, refer to Table 2.

Mouse Model

In 1992, the first CF mouse model was described and intestinal disease was the major defect in the CF mouse phenotype (111). Intestinal obstruction in CF mice is highly variable in incidence and is dependent on the genetic background, CFTR mutation, and also dietary factors (54). CF mice can have up to a three-fold increased rate of mortality compared to control mice in the first few days of life that is attributed to intestinal obstruction (i.e. meconium ileus) (54, 94, 111). Following this, disease is generally subclinical until weaning when a significant wave of mortality occurs; this is again attributed to intestinal obstruction (i.e. DIOS/constipation). Dietary supplementation of a 6% polyethylene glycol and electrolyte solution significantly increased the survival rates post-weaning (53). A common pathologic finding in small intestinal crypts of laxative-treated and untreated CF mice is crypt filling and distension by Paneth cell cryptins, an alpha-defensin antimicrobial peptide (25).

One of the historic advantages of mouse models has been the ability to control genomic and tissue specific expression of target genes. Ectopic CFTR expression in intestinal epithelium of CF mice leads to mitigation of intestinal obstruction (88, 133). Furthermore, a recent conditional CFTR expression model demonstrated that intestinal epithelium was a significant regulator of intestinal obstruction, but not of weight loss even though CF mice lack pancreatic disease (59). As another example, genetic analysis has shown MSRA (methionine sulfoxide reductase A gene) is a modifier gene of meconium ileus in human infants. This premise was validated using CF mice with
wild type, heterozygous or mutated MSRA. Mice deficient in both CFTR and MSRA had reduced severity of intestinal obstruction suggesting that therapeutic modifications to reduce MSRA expression may be protective in CF (57). The variation of phenotypes seen in humans or in mice with the same CFTR mutation has raised questions about alternative anion channels that may modulate the CF mouse phenotype (54). A recent evaluation of candidate gene expression profiles in two strains of CF mice did not identify genes that might rescue CFTR mutation, but did identify one candidate gene product (mTTYH3) that was consistently down regulated in CF and was speculated to possible increase severity of CF disease (19).

Pig model
Genetically engineered CF pigs were first reported in 2008 (100). At birth, 100% of CF pigs with homozygous null or ΔF508 CFTR mutations had meconium ileus (87, 100). The obstruction was characterized proximally by dilated meconium filled bowel and distally by small caliber to atretic bowel containing mucus changes and multifocal mucocellular cords (“casts”, “pellets”) (79). The obstruction was often located in the distal small intestine or proximal spiral colon (79) and was unresponsive to medical therapy (laxatives, enemas, supportive care), but often complicated by small caliber bowel, atresia, perforation and / or peritonitis (79, 87, 113). Interestingly, the site of obstruction was dynamic and could sometimes transition distally after oral feeding as demonstrated by the accumulation of mucocellular cords in the colon, similar to that reported in humans (79, 110). Since CF pigs required surgery for meconium ileus and were of similar size to a small infant, the CF pig has become a useful model for pediatric surgeons to optimize surgical techniques (ileostomies, cecostomies) in the management and post-operative care of complicated meconium ileus cases (87, 113). Furthermore, improved knowledge and surgical skills gained from this spontaneous obstruction model can be applied as a learning tool for other types of intestinal obstruction in which animal models are sparse and often artificially induced (90). Recently, the generation of a CFTR<sup>−/−</sup> pig model with intestinal-specific CFTR expression under the intestinal fatty acid binding protein (iFABP) promotor alleviated the meconium ileus. Interestingly, only
~20% of wild type CFTR expression levels was required to correct meconium ileus obstruction and gives a target threshold for future gene therapy interventions (115).

In CF pigs, DIOS/constipation was prophylactically treated with daily administration of oral polyethylene glycol (113). In some cases, CF pigs had reduced stools and became anorexic, suggestive of postnatal intestinal obstruction. Supportive treatments with enemas and increased oral laxatives with fluids have been used to correct the situation. Surgical treatment was not pursued in refractory cases due to the requirement to evaluate respiratory disease in this model and to avoid potentially confounding lung disease with surgical complications.

Ferret model

A CFTR⁻ ferret model (CF ferret) first reported in 2010, had a 75% incidence of meconium ileus at birth (121). While CF ferrets lack a macroscopically distinct landmark for the ileocecal junction, the site of the meconium ileus in CF kits was usually located within the distal third of the length of the small and large intestine resulting in a 50% perforation rate. Meconium dilation and distal microcolon were seen similar to human infants. Interestingly, evaluation of breeding animals showed that the genetic background of the hob (paternal male) had a significant influence on the incidence of meconium ileus, suggesting the presence of genetic modifiers. Intestinal expression of CFTR using a FABP promotor has been also reported to alleviate intestinal obstruction in the CF ferret (121). DIOS/constipation are reportedly treated prophylactically in ferrets, but occasional DIOS/constipation episodes still occurred and were successfully managed medically (119).

Rat model

In 2014, a CFTR⁻ rat (CF rat) model was initially characterized (124). At birth, CF rats lack meconium ileus, but by two weeks of age they start to have reduced weight gain and begin to die of intestinal obstruction (DIOS/constipation) after weaning. By six weeks of age only ~30% of the CF rats survive. Use of GoLytely® (Braintree Laboratories, Inc.) significantly improves the survival to ~64%. Intestinal histology
showed cell sloughing and crypts dilated with a mucoid substance. While the CF rat has a DIOS/constipation phenotype, the pancreas and liver appear normal.

**Diverticulosis**

CF patients have a 7-14 fold increased risk of diverticulosis and these have been observed in the vermiform appendix and in the colon (11, 50). In the general population, diverticulosis/diverticulitis does not become apparent until the approximately 4th decade of life; however, in one study of CF patients, diverticula were detected in eight cases (14%, 8 of 57 cases) ranging between six and twenty two years of age (median, 14.5 years). Diverticula are proposed to form from chronic/repeated exposure to luminal distension and pressure causing herniation of the mucosa/submucosa through the tunica muscularis at the weakest muscular sites, i.e. at sites of vascular entry along the mesenteric border (50, 77). The total incidence in CF patients from autopsy studies is likely underestimated as diverticula can be difficult to discern during pathologic examination.

**Animal Models**

Diverticula, as a consequence of CF disease, have only been reported in CF pig models. This may be a reflection of the severity and/or chronicity of these intestinal obstructions. At birth, CF pigs frequently have diverticula along the mesenteric border, suggesting these formed in utero due to increased luminal pressures from the meconium ileus obstruction (79). Development of diverticula seems to be related to increased severity of meconium ileus in CF pigs and when meconium ileus was not present, such as in CF pig model with intestinal correction of CFTR expression, intestinal diverticula were absent (115).

**Atresia**

CF infants have increased risk (>200 times) for intestinal atresia and its mechanisms are poorly understood. Theories include intestinal ischemia from meconium ileus or malformations during fetal development (99). Volvulus, reported in
up to 50% of meconium ileus cases, is suggested as a possible contributing cause of intestinal ischemia during development (2).

Animal models

Atresias have only been reported in the CF pig model. Atretic bowel is associated with the presence of meconium ileus and when present is consistently found distal to the obstruction (79, 100). Atresia in CF pigs seems to be related to the severity of meconium ileus obstruction and in pigs with intestinal CFTR correction, atresias are lacking (115).

Gastroesophageal Reflux Disease

CF patients, including children, are prone to acidic gastroesophageal reflux disease (GERD) with incidence rates ranging as high as 35-80% (106, 132). A comparison of GERD patients suggested that those with CF did not have an increased frequency of reflux, but rather had decreased ability to buffer and restore esophageal pH following reflux events. This may be a result of hyperacidity of the gastric fluids (132), or from inadequate bicarbonate-rich secretions in saliva and/or esophageal glands (22, 56, 80). Additionally, the proximal small intestine has a diminished ability to buffer acidified gastric contents (49), likely a result of decreased bicarbonate-rich secretions from Brunner’s glands, pancreas and to a lesser extent the intestinal epithelium. CF patients are often placed on proton pump inhibitors or H2 antagonists, in part, to mitigate the GERD symptoms (21). The use of proton pump inhibitors to mitigate gastric pH has been recently questioned due to potential for adverse changes in CF lung disease (21, 37).

Animal Models

Clinical signs of GERD have not been defined in animal models of CF. Even so, CF pigs and ferrets typically follow a postnatal protocol that includes proton pump inhibitors or H2 blocker principally to mitigate their susceptibility to gastric ulceration (87, 113, 121), but also to minimize potential for GERD-related disease. In contrast, these therapies are not routinely used in studies involving CF mice and rats.
Motility

Alterations in intestinal motility can contribute to intestinal disease phenotype. Transit times in the CF intestine, especially that of the small intestine, is reported to be delayed using traditional hydrogen breath tests (8) and more recently using techniques such as a magnet-based motility tracking system (55) and wireless motility capsule (49). During end-stage lung disease, the esophagus and stomach of CF patients are also reported to have similar delayed transit (44). Histopathological examination of the intestine from CF patients at autopsy have reported prominent smooth muscle (i.e. tunica muscularis) in the intestinal wall (15, 134), which could be a marker of an adaptive change or even a primary smooth muscle phenotype.

Animal models

Of the CF animal models, only mice and pigs have reported alterations in intestinal motility or smooth muscle phenotypes. Ex vivo ileal smooth muscle from CF mice exhibited increased peak isometric forces following stimulation and also had increased relaxation following isoproterenol administration compared to non-CF controls. Smooth muscle mass in the wall of the ileum was also variably increased and many of these changes were specific to background strain, suggesting a role for modifier genes (98). CF pigs also have an intestinal smooth muscle phenotype with increased thickness of the tunica muscularis from duodenum to the colon (79). Since these changes are found at birth, bacterial regulation is not likely the cause, but could possibly contribute to the phenotype postnatally.

Dysbiosis

Intestinal dysbiosis is defined as a change in the microbiome and/or bacterial populations of the intestine. A recent study of CF patients found that changes in fecal bacterial populations were associated with both genotype and clinical disease severity. Homozygous ΔF508 mutations or CF patients with severe disease had increased growth of pathogenic bacterial populations (e.g. E. coli) and decreased presence of beneficial bacterial populations (e.g. Bifidobacterium spp) (104). Another recent study
suggested that probiotic supplementation can mitigate clinical gastrointestinal disease and significantly reduce pathogenic proteobacterial populations (36). Small intestinal bacterial overgrowth (SIBO, a form of dysbiosis) is also recognized in CF, but any link with intestinal inflammation is not yet clear (71, 72).

**Animal models**

Much of the modeling of CF dysbiosis has been performed in the mouse and more recently the ferret model. CF mice have reduced diversity of beneficial bacteria (e.g. Lactobacilliales members) and increased pathogenic bacteria (e.g. *Bacteroides fragilis*) that are associated with intestinal inflammation and immunomodulation. The increases in Enterobacteriaceae and Clostridiaceae in CF mice are speculated to have a negative regulation on growth of beneficial bacteria (75). Another study found that cryptins (*alpha*-defensins from Paneth cells) and bacterial numbers were both increased in CF mice, but even with increased activated cryptin protein levels, CF mice intestines had significantly reduced capacity to kill cryptin-sensitive bacteria (25). This may be due to the poor dissolution and entrapment of cryptins in the villus crypts (25). Additionally, deficient bicarbonate transport in CF mice (48) could produce an abnormally acidic microenvironment that could potentially dampen the efficacy of endogenous antimicrobials (1). In CF mice, intestinal microbiota can modulate intestinal motility as evidenced by treatment with either antibiotics or laxatives to flush out enteric bacteria - both can normalize intestinal motility (33). Additionally, the postnatal onset of the smooth muscle phenotype in mice directly correlated with the development of bacterial overgrowth. The influence of bacteria appears to be related to inflammation regulated through phospholipase A genes, which suggests a prostaglandin mediated effect (32).

In the ferret, fecal bacterial diversity appears to be genotype-independent between CF and non-CF pairs suggesting the environment plays an important role in the intestinal microbiome (119). Bacterial overgrowth is common in CF ferrets and characterized by a dilated, ingesta-filled small intestine. Microscopically, blunted villi lined by abundant bacteria were common along with lymphoplasmacytic inflammation. Aerobic and anaerobic titers were significantly higher from the small intestine of CF ferrets with several types of bacteria that distinguished CF from non-CF ferrets (119).
Rectal prolapse was historically seen in up to 20% of CF patients, with a preferential incidence in the first few years of life (5, 15). Predisposing factors may include weakened muscular tone of pelvic floor, numerous bulky stools and respiratory disease (e.g. increased intraabdominal pressure from persistent/recurrent cough). Conservative medical therapy aimed at mitigating the inciting cause is often successful, but surgical therapies may be required in refractory cases (109).

Animal models
Rectal prolapse is a common finding in CF ferrets with up to 30% affected (119), but is not described in other CF animal models. Rectal prolapse in CF animals occurs within a very narrow window at approximately one month of age. Proactive laxative and pancreatic enzyme therapy is now effective in treating the majority of the affected CF ferrets.

Cancer
CF patients have an overall low cancer burden compared to the general population (82). However, long-term studies have suggested that CF may predispose patients to specific cancers. For instance, CF patients have increased incidence of digestive tract cancers and this risk is further elevated following lung transplantation (76, 82). Increased incidence of testicular cancer and lymphoid leukemias are also reported in CF patients (76).

Mouse models and the more recent CF animal models (e.g. pig, ferret, rat) lack studies that validate a predisposition to cancer. The CF pig, ferret and rat models are relatively novel and thus aging studies to assess cancer incidence have yet to be reported. CF mice have intestinal disease characterized by a variety of histological changes including increased cell proliferation (Gallagher, 2001 #463), but whether these contribute to eventual cancer development in the model, remains unclear.
Future Directions

All of the species with targeted CFTR disruption demonstrate gastrointestinal phenotypes – most notably intestinal obstruction. Better treatments and prophylaxis for intestinal obstructions are an obvious target goal for the animal models and will require better understanding the molecular mechanisms initiating the obstruction. Furthermore, CF animal models will be useful towards understanding the intestinal microbiome and its effects on gastrointestinal function as well as overall health status (e.g. lung disease) of CF patients. Lastly, emerging technologies such as organoids may be useful for study of intestinal CF disease (66, 73, 78). These cultured cell systems are a hybrid of *in vivo* and *in vitro* models that produce small cellular aggregates with cellular differentiation, three-dimensional structure and physiologic properties that resemble specific organs or tissues. While organoid research is still early, it has potential for usefulness in study of CF pathogenesis as well as testing the efficacy and safety of novel therapeutics.

LIVER / GALLBLADDER

The incidence of CF liver disease (CFLD) is 5-10% and it results in 2.5% of the overall mortality in CF patients (28). The mean age of diagnosis of CFLD is ten years of age (65, 105). There is a spectrum of hepatobiliary diseases defined as CFLD including: focal biliary cirrhosis, multilobular biliary cirrhosis and portal hypertension, with the most common being focal biliary cirrhosis. The diagnostic criteria for CFLD are met if at least two of the following are present: hepatomegaly, abnormalities in liver function tests (AST and ALT over a 12 month period), ultrasonographic evidence of liver involvement or portal hypertension (34). Elevations in AST, ALT, and GGT are not predictive of liver disease and/or fibrosis, and up to 41% of children with CF will have abnormalities in liver transaminases by the time they reach 12 years of age (65). Hepatic steatosis is common in CF patients, likely associated with nutritional deficiencies, and has not proven to be associated with cirrhosis in CF patients (28).

The pathogenesis of CFLD is complex and not well defined. CFTR is expressed in cholangiocytes (bile duct cells) and not in other cells of the liver (27). Dysfunction of CFTR results in abnormal biliary sections and decreased bile flow, which leads to
thickening of the bile and formation of plugs within the bile ducts. Bile plugging/obstruction is present focally to multifocally and not in every duct. Duct obstruction results in up-regulation of chemokines (93), activation of hepatic stellate cells (68), and peribiliary fibrogenesis that is characteristic of CFLD. In a subset of patients, focal biliary cirrhosis progresses to multilobular biliary cirrhosis and portal hypertension.

Early diagnosis of CFLD is confounded by the focal nature of the disease. Ultrasound can detect changes associated with cirrhosis, but is not reliable in detecting early stages of fibrosis. A recent study demonstrated that fibrosis staging on liver biopsy predicted the development of clinically significant CFLD and advised that obtaining two biopsy cores samples for histopathological evaluation improved detection (69). Given that approximately 40% of CF patients exhibit liver function test abnormalities, but only a very small percentage develop cirrhosis and hypertension, suggest that other factors must be involved in the pathogenesis of CFLD. No specific CFTR mutation has been associated with the occurrence or severity of liver disease; however CF patients with the α1-antitrypsin (SERPINA1) Z allele are at increased risk of CFLD indicating that modifier genes likely play a role in pathogenesis (9).

There is no effective treatment for CFLD. The use of ursodeoxycholic acid (UDCA) is recommended in the best practice guidelines for the management of CFLD, but the effectiveness of the treatment has been controversial (23, 34, 83). Liver transplantation may be considered for CFLD patients, but there are no guidelines for the optimal timing for transplantation.

Neonatal cholestasis is an uncommon early manifestation of liver disease. Leeuwen, et al. reported that cholestasis occurred in 5.7% (23 of 401) infants and there was a higher incidence of cholestasis in infants with meconium ileus than those without meconium ileus. In this study, cholestasis resolved in all the children and was not associated with the later development of CFLD (67). Even without clinical liver disease or evidence of cholestasis at biopsy, liver function tests can be elevated in the majority of pediatric cases during the first 2-3 years of life (70).
CFTR is also expressed in gallbladder epithelial cells. Gallbladder abnormalities are found in 24-50% of CF patients and include microgallbladder and gallbladder dysfunction (45, 131). No treatment is required for microgallbladder.

Mouse models

The majority of CF mouse models do not have liver changes, but when present are often mild. As previously discussed, the severity of the intestinal manifestations often limits the survival of CF mice. In a long term study, CF mice demonstrated liver changes that progressed as the mice aged. At the culmination of the study at 12-24 months, CF mice developed focal biliary cirrhosis with some showing lobular cirrhosis very similar to CF patients (39). \( \text{CFTR}^{\Delta F508/\Delta F508} \) mice exhibit mild histopathologic liver disease characterized by mild and patchy bile ductular proliferation and very mild fibrosis (46).

Although CF mouse models have minimal liver disease, they have been utilized to investigate bile acid flow and bile acid metabolism. CF mice are reported to have enlarged gallbladders with emptying defects that can contribute to altered bile flow and shunting. Expression of fibroblastic growth factor-15, a key hormone regulating gallbladder filling, was decreased in the ileum (a normal site of expression), but overexpressed in the gallbladder – suggesting a possible mechanism contributing to CFLD (35). Additionally, increased hydrophobicity of the bile salt is thought to be more toxic to the hepatobiliary system thereby causing injury (10). \( \text{CFTR}^{\Delta F508/\Delta F508} \) mice have more hydrophobic bile acid and reduced bile salt-dependent bile flow possibly contributing to biliary epithelial injury (46). In a DSS induced colitis model, \( \text{CFTR}^- \) mice (C57BL/6J-\( \text{Cftr}^{\text{tm1Unc}} \)) had increased biliary epithelial cell damage in response to endotoxin administration as compared to controls and had an exaggerated innate immune response mediated by toll-like receptor 4 and NF-kB (42). This could suggest that targeting inflammatory processes may be a potential therapy for CFLD.

Given the controversial nature of UDCA treatment in CF patients, several CF mouse studies have investigated mechanisms of UDCA therapy. Chronic UDCA treatment in CF mice reduced the hydrophobicity of the bile salt pool and stimulated bile
flow independent of CFTR (14). These results support a potential beneficial effect of UDCA on bile flow and bile salt metabolism in cystic fibrosis conditions; however, beneficial effects in CF patients remain controversial.

Pig

Neonatal CF pigs have focal biliary cirrhosis with portal inflammation, biliary hyperplasia and mild fibrosis (79). With age, multilobular cirrhosis can develop (115). Microgallbladder was present in all CF pigs with diffuse epithelial mucinous change and cystic epithelial proliferation in some cases (79). Microgallbladders contained thick bile/mucus material plugging the lumen. Newborn CFTRΔF508/ΔF508 pigs had similar liver and gallbladder lesions as those described for CFTR+/− pigs (87). The pH of gallbladder bile was significantly lower in CF neonatal pigs (125), consistent with loss of bicarbonate transport.

Ferret

Neonatal ferrets demonstrate early abnormalities in liver enzymes without histologic evidence of cholestasis, similar to CF infants (11, 67). Treatment with UCDA resulted in normalization of these values. CF ferrets and controls had lymphocytic portal inflammation but no evidence of biliary proliferation or fibrosis (15). Gallbladder changes were not evident at birth, but as CF ferrets aged several animals had dark gallbladders on gross examination with thick bile present within the lumen. Histologically, the majority of older CF ferrets had gallbladder epithelium with mucinous change and cystic proliferation (15). However, unlike the CF pig, newborn CF ferret gallbladder bile pH was not significantly lower than non-CF control (43).

Future Directions

Future goals utilizing animal models will be directed at the prevention or reversal of liver changes, to mitigate the potential development of multilobular cirrhosis. Development of diagnostics for early detection of liver disease and understanding factors that result in increased susceptibility of CF patients would also be valuable.
The pancreas is one of the most common organs affected in CF. CFTR is highly expressed in ductal epithelial cells and functions to transport fluid and anions into the ductal lumen. The pancreas secretes large quantities of digestive enzymes along with alkaline fluid. Altered fluid and anion transport in CF results in reduced luminal fluid volume, decreased pH and hyperconcentration of macromolecules that thereby cause obstruction and damage of exocrine acini (Figure 3) (38, 103).

Pancreatic disease severity is highly dependent on genotype (85, 102) and these CFTR mutations can be organized into classes based on their functional biology (102). Pancreatic insufficient (PI) patients tend to have mutations that result in absent or nonfunctional CFTR at the cell surface (class I-III mutations) while individuals that are pancreatic sufficient (PS) have more mild CFTR mutation with partially functional CFTR on the cell surface (class IV-V mutations) (6). In contrast, CFTR mutations can produce disproportional decreases in anion transport leading to altered bicarbonate to chloride ratios ($\text{HCO}_3^-:\text{Cl}^-$) that can influence phenotype. For example, CFTR mutations producing a low ($\text{HCO}_3^-:\text{Cl}^-$) transport ratio were more likely patients with pancreatic insufficiency whereas higher ($\text{HCO}_3^-:\text{Cl}^-$) transport ratios were often associated with pancreatic sufficient patients and milder disease (24).

Pancreatic lesions begin in utero and progress from early plugging (obstruction) in acini and small ducts with eventual acini injury and destruction resulting in inflammation, fibrosis and fatty replacement (62). As acini are destroyed, duct proliferations ("tubular complexes") are sometimes observed and have been suggested to represent a reparative response (79, 116). Altered zymogen secretions form much of the early luminal obstruction material. In more severe or advance disease, secondary changes such as mucous cell metaplasia with its mucus secretions may further complicate luminal obstructions (123).

Serum immunoreactive trypsinogen (IRT) (64), a pancreatic enzyme precursor, is elevated in the blood of CF infants and utilized as a newborn screening tool (112). Evaluation of feces for pancreatic enzymes such as elastase-1 are also useful to diagnose pancreatic insufficiency (74). It is important to note that these immunological tests use antibodies that do not cross react with elastase found in porcine-derived
pancreatic replacement supplements. Additionally, fecal fat levels are used to classify CF patients as PS or PI. IRT levels and fecal fat levels are inversely correlated in CF patients (112). Transition from PS to PI increases in frequency throughout the first year of life with a significant impact on growth and nutrition (62, 128). Approximately 85% of CF patients are classified as PI and require lifelong pancreatic enzyme supplementation. PS patients have evidence of pancreatic damage, as demonstrated by high IRT during neonatal screening with reduced but sufficient exocrine pancreatic function for normal digestion (7, 40, 85). The estimated occurrence of pancreatitis among CF patients is 1.24% but PS patients are at increased risk (10.3%) (30). Interestingly, patients with idiopathic pancreatitis and no history of CF have a higher frequency of CFTR gene mutations (12, 107).

Mouse model

Of the CF mouse models, the major phenotype has been intestinal obstruction, but only subtle pancreatic disease is detectable. The lack of pancreatic disease may be due to several factors including: developmental/structural differences of the mouse pancreas, low CFTR expression in the rodent pancreas with higher expression of alternative anion channels, modifier genes and/or short life-span of CF mice (26, 39, 52, 63, 89).

There are many CF mouse strains (see (130) for a recent overview of CF mouse models) available for pancreas study, but examples of those which demonstrate pancreatic changes will be discussed here. It is important to note that in some studies, CF mice may have altered physiology (e.g. CCK secretion, fluid homeostasis, etc.) as a result of being maintained on a long-term liquid diet and to what extent that contributes to any observed pancreatic changes is not clear (17, 31). In one study, CF mice maintained on a liquid diet developed mild intraluminal obstruction that progresses with age, with a decrease in acinar cell volume by 15-24 months of age (39). CFTR\textsuperscript{tm1CAM} mice (15-50 days of age) demonstrated mild pancreatic pathology characterized by focal pancreatic duct plugging in 50% of the mice (94). In another study, CF mice maintained with polyethylene glycol 4000 in water had increased expression of Muc6 (51). This mucin is similarly localized to acini and luminal plugs in human CF pancreas
Additional studies may be useful to determine if Muc6 has a role in the pathogenesis of CF pancreas disease.

**Pig Model**

Pancreatic pathology in CF newborn pigs is similar to CF patients with plugging/obstruction and acinus destruction with mild inflammation. The newborn CF pig pancreas is characterized by small lobules separated by increased loose connective tissue, scattered inflammation, dilation of centroacinar spaces, duct proliferation and mucinous metaplasia (79, 100). Interestingly, duct proliferation and mucinous metaplasia were not present in fetal pancreas at ninety days gestation (~115 days term gestation is term) indicating that remodeling occurred in the weeks prior to birth (3). While inflammation is present in the CF pig pancreas at birth, there is a lack of systemic inflammation indicating a pancreas-specific response (4). Pancreatic enzyme replacement therapy is started shortly after birth in CF pigs (113). As animals age, acini are destroyed and are replaced by fibrosis and adipose tissue (79). The volume and pH of pancreatic fluid are lower in newborn CF pigs with higher protein concentration. Similarly, CF pigs have diminished response following the administration of secretin (125). Reduced volume and pH of pancreatic fluid are also features of human CF disease (36).

**Ferret model**

The CF ferret presents with mild pancreatic disease at birth characterized by multifocal acinar lumen dilation by zymogen material with few dilated ducts (11). Within the first month of life there is rapid progression of disease characterized by acinar loss, duct dilation, inflammation and fibrosis (126). This pancreatic destruction coincides with glycemic instability in CF ferrets and progressive glucose intolerance (84), which may highlight the CF ferret as a suitable model to study CF-related diabetes. The majority of CF ferrets have progressive pancreatic destruction that results in exocrine PI and necessitates enzyme replacement therapy. Interestingly, there is a small percentage (~15%) of CF ferrets that have a predominantly normal pancreas with normal fecal
elastase levels (15), indicating other genetic modifiers may play a role in pancreatic disease.

**Future Directions**

The current treatment for PI is pancreatic enzyme replacement therapy that can help prevent the most severe complications. Enzymes are not fully effective given the acidity of the duodenal fluid, therefore acid-resistant lipases are currently being developed. PS patients may benefit from therapies that target the basic defect, inflammation or other factors that play a role in the progression of the disease. Animal models will allow us to better evaluate the spectrum and time-course of pancreatic disease that would facilitate therapeutic development and testing.

**SUMMARY OBSERVATIONS**

CF research is fortunate to have a rich diversity of animal models for the study of CF disease. Inter-organ and inter-species comparison of gastrointestinal, pancreatic and hepatobiliary pathogenesis allows some fundamental observations. First, altered secretions are highly relevant features of disease in these organs and mucus is often the targeted culprit. However, altered proteinaceous secretions (and not just mucus) are clearly evident histopathologically in intestinal crypts and as common components of pancreatic obstructions. These under-reported observations suggest that the CF luminal microenvironment could have a profound influence on many secreted materials (proteins and mucus) – both in structure and function. Second, comparison between CF species may allow for better targeting of genetic or environmental modifiers of CF disease severity. For instance why is meconium ileus obstruction absent in early reports of the rat model, yet meconium ileus has variable penetrance in the other three models? Could outbred models such as the CF ferret or CF pig offer wider genetic diversity to help identify candidate modifier genes that could then be further tested in inbred models such as CF rat and mouse? In conclusion, CF researchers can take advantage of the similarities and differences in CF models to optimize CF disease modeling, advance the understanding of CF pathogenesis and validate therapeutic targets.
Acknowledgements

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**FIGURE LEGEND**

**Figure 1. CF colon (A & B) and small intestine (C & D).** Absence of CFTR in the crypts can result in multifocal plugging/obstruction (arrows). (A) CF infant, 4 months old. (B) CF pig, newborn. (C) CF ferret, 2.5 week old. (D) CF mouse, 1 month old, courtesy of Lynda Ostedgaard (University of Iowa). Hematoxylin and eosin (HE) stain.

**Figure 2. Meconium ileus in the newborn CF pig model.** (A) Meconium ileus obstruction in the distal small intestine. Proximally, the intestine is distended by sticky meconium (white asterisks). The site of obstruction (arrows) represents an abrupt transition from dilated meconium-filled bowel to small meconium-devoid bowel. Distal to the obstruction (black asterisks), the intestine is of small caliber (“microcolon”). (B) The meconium (asterisk) proximal to the obstruction (Figure 2A, white asterisks) can cause distension and thinning (arrows) of the intestinal mucosa and in severe cases cause hemorrhage and necrosis leading to perforation. (C) Microcolon distal to obstruction can have multifocal cords (“casts” or “pellets”, asterisk) composed of mucus, protein and cellular debris. (D) Proximal to the obstruction, diverticula can be detected (arrows). (E) Diverticula are mucosal herniations through the tunica muscularis (arrow). HE stain (C-E).

**Figure 3. Pancreas in health and CF disease.** (A) Schematic of healthy pancreas. (a) Acini have numerous zymogen granules (red circles) in the apical cytoplasm that upon stimulation are secreted into the duct system. (b) Duct cells secrete bicarbonate-rich fluids into the lumen (black arrows) to mix with the acinar secretions to form pancreatic fluid (red arrows). Note that mucous cells (blue) are normally restricted to the larger ducts. (B) Pathophysiology of CF disease in the pancreas. (a) Acini secrete zymogen material and ducts secrete reduced liquid (represented small black arrows) into a microenvironment that promotes obstructions (red masses). (b) Obstructions injure nearby acini and ducts leading to destruction, inflammation, fibrosis and adipose replacement of the organ. Note that during CF disease, mucous cells increase in number and secretions (blue color in lumen) contributing to obstructions. (C-D) CF pancreas, 2 month old infant. (C) Many pancreatic acini have been destroyed and
replaced by connective tissue. (D) Remnant acini and ducts are often distended and obstructed by altered zymogen secretions. (E) CF pancreas, 14 year old. With progressive destruction, adipose tissue (asterisks) replaces the pancreas. The remaining lobules are composed of scattered ducts, a few acini and multiple islets. Note: Continued disease progression can result in nearly complete destruction of exocrine tissue with replacement by adipose tissue leaving only scattered islets as the only evidence of pancreas. (F,G) Newborn CF pig pancreas. (F) The CF pig pancreas has small degenerate lobules separated by loose connective tissue (asterisks). (G) Acini and ducts are distended by altered zymogen secretions (arrows) and have evidence of inflammation (asterisks). (H) CF ferret, newborn. Pancreatic acini and ducts have obstructions (arrows) by altered zymogen secretions with little evidence of inflammation or remodeling.

### Table 1. CFTR mutations recently developed in novel CF animal models

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Pig</th>
<th>Ferret</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{CFTR^{ΔF508}/ΔF508}$</td>
<td>2008 (100)</td>
<td>2010 (121)</td>
<td>2014 (124)</td>
</tr>
<tr>
<td>$^{CFTR^{ΔF508}/ΔF508}$</td>
<td>2011 (87)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$^{CFTR^{−}; iFABP-CFTR}$</td>
<td>2013 (115)</td>
<td>2010 (121)</td>
<td>–</td>
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</tbody>
</table>

(“gut-corrected”)
Table 2. Gastrointestinal manifestation of CF disease in humans and animal models

<table>
<thead>
<tr>
<th>Manifestation</th>
<th>Human</th>
<th>Mouse</th>
<th>Pig</th>
<th>Ferret</th>
<th>Rat</th>
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</thead>
<tbody>
<tr>
<td>Meconium ileus obstruction</td>
<td>~15% incidence (41, 86, 127)</td>
<td>Up to 3+ fold increased mortality compared to controls in first few days of life (54, 94, 111)</td>
<td>100% incidence (87, 100)</td>
<td>75% incidence (121)</td>
<td>0%* (124)</td>
</tr>
<tr>
<td>DIOS obstruction / constipation</td>
<td>DIOS incidence: 6.2 events per 1000 patient years (61)</td>
<td>~0-100% mortality weeks after weaning, dependent on background (54, 94, 111)</td>
<td>Prophylaxis treatment with some “episodes” (113)</td>
<td>Prophylaxis treatment with some “episodes” (119, 121)</td>
<td>~70% mortality due to intestinal obstruction by 6 weeks of age (124)</td>
</tr>
<tr>
<td>Diverticula</td>
<td>7-14 fold increased risk (50)</td>
<td>NR</td>
<td>Incidence may be proportional to meconium ileus severity (79)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Atresia</td>
<td>CF infants have &gt;200 fold increased risk (99)</td>
<td>NR</td>
<td>Incidence may be proportional to meconium ileus severity (79)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>GERD</td>
<td>~35-80% incidence (106, 132)</td>
<td>NR</td>
<td>NR, but treated as prophylaxis (87, 113)</td>
<td>NR, but treated as prophylaxis (121)</td>
<td>NR</td>
</tr>
<tr>
<td>Motility</td>
<td>Delayed transit with prominent intestinal smooth muscle (15, 49, 55, 134)</td>
<td>Increased peak isometric force and increased relaxation in CF intestines with increased smooth muscle (98)</td>
<td>Increased size of tunica muscularis from duodenum to colon (79)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Dysbiosis</td>
<td>Genotype and disease severity influence dysbiosis; SIBO also reported (36, 71, 72, 104)</td>
<td>SIBO / dysbiosis reported, may alter motility and inflammation; defective antimicrobial peptides from crypt may</td>
<td>NR</td>
<td>Dysbiosis / SIBO influenced by environment and not genotype (119)</td>
<td>NR</td>
</tr>
<tr>
<td>Condition</td>
<td>Description</td>
<td>NR</td>
<td>NR</td>
<td>Up to 30% at about 1 month of age (119)</td>
<td>NR</td>
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<tr>
<td>Rectal prolapse</td>
<td>Historically up to 20%, especially in young children (5, 15)</td>
<td>NR</td>
<td>NR</td>
<td></td>
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</tr>
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</table>

* Based on preliminary phenotypic study

NR – Not reported
REFERENCES


