ANIMAL MODELS

Gastrointestinal Pathology in Juvenile and Adult CFTR-Knockout Ferrets


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Cystic fibrosis (CF) is a multiorgan disease caused by loss of a functional cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel in many epithelia of the body. Here we report the pathology observed in the gastrointestinal organs of juvenile to adult CFTR-knockout ferrets. CF gastrointestinal manifestations included gastric ulceration, intestinal bacterial overgrowth with villous atrophy, and rectal prolapse. Metagenomic phylogenetic analysis of fecal microbiota by deep sequencing revealed considerable genotype-independent microbial diversity between animals, with the majority of taxa overlapping between CF and non-CF pairs. CF hepatic manifestations were variable, but included steatosis, necrosis, biliary hyperplasia, and biliary fibrosis. Gallbladder cystic mucosal hyperplasia was commonly found in 67% of CF animals. The majority of CF animals (85%) had pancreatic abnormalities, including extensive fibrosis, loss of exocrine pancreas, and islet disorganization. Interestingly, 2 of 13 CF animals retained predominantly normal pancreatic histology (84% to 94%) at time of death. Fecal elastase-1 levels from these CF animals were similar to non-CF controls, whereas all other CF animals evaluated were pancreatic insufficient (<2 μg elastase-1 per gram of feces). These findings suggest that genetic factors likely influence the extent of exocrine pancreas disease in CF ferrets and have implications for the etiology of pancreatic sufficiency in CF patients. In summary, these studies demonstrate that the CF ferret model develops gastrointestinal pathology similar to CF patients. (Am J Pathol 2014, 184: 1309–1322; http://dx.doi.org/10.1016/j.ajpath.2014.01.035)

Accepted for publication January 30, 2014.
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Cystic fibrosis (CF) is the most common life-threatening, autosomal recessive, genetic disorder among Caucasians, occurring in approximately 1 in 3500 births. Defects in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that disrupt function of this chloride channel cause abnormalities in electrolyte and fluid movement across many epithelia of the body, leading to viscous, poorly hydrated secretions. Although chronic bacterial infections in the lung are the most significant cause of mortality in CF, pathology in multiple other organs contributes to the progression of disease and overall health of CF patients. These organs include the intestine, pancreas, liver, and gallbladder for which clinical and/or histological disease is seen in CF patients at frequencies of 10% to 90%. In the current study, we evaluated gastrointestinal disease in juvenile and adult CFTR-knockout ferrets.

Mouse models of CF have been critical to our understanding of CFTR function in several organs, however, CF mice fail to develop spontaneous disease in the lung and pancreas, and have relatively minor disease of the liver and...
gallbladder. The recent creation of new CF pig and ferret models has provided the field with new tools to dissect CF disease pathophysiology and the factors that influence disease severity in CF patients. Comparisons between CF mouse, pig, and ferret models have clearly demonstrated that species-specific differences in organ physiology and CFTR biology influence the extent of pathology in major organs affected in CF. For example, all newborn CF models have intestinal pathology that manifests as meconium ileus at birth in the case of CF pigs (100% of animals) and CF ferrets (75% of animals), or as intestinal obstruction at weaning in the case of CF mice. By contrast, pancreatic phenotypes at birth are highly variable between species with disease being most severe in CF pigs, less severe in CF ferrets, and absent in CF mice. Similarly, CF pigs demonstrate histopathology in the gallbladder and liver at birth, whereas disease in these organs is relative minor in newborn CF ferrets and CF adult mice.

Elucidating the differences in the severity of CF gastrointestinal disease at birth and in disease progression between the various species may aid in dissecting genetic and environmental factors that influence gastrointestinal disease severity in CF patients. Here, we report the phenotype of gastrointestinal organs (pancreas, liver, gallbladder, stomach, and intestine) in older CF animals reared on antibiotics until 6 months of age, for the time at which they were euthanized due to severity of disease. We have also evaluated the extent of bacterial overgrowth in the CF intestine and the types of bacterial flora found in the intestine of non-CF and CF animals. Our findings demonstrate that juvenile and adult CF ferrets naturally acquire gastrointestinal disease at frequencies similar to that observed in CF patients. Of great interest, a small subset of CF animals was pancreatic sufficient from birth, implicating modifier genes that can compensate for the loss of CFTR in the exocrine pancreas. These studies suggest that the CF ferret model could be useful for testing therapies aimed at gastrointestinal organs and dissecting how genetic variation influences disease in CF.

Materials and Methods

Rearing of CF Ferrets

The previously described CFTR exon-10 disrupted ferret model was used for all studies. The colony of CFTR+/– ferrets was backcrossed from sable to both albino and cinnamon coat colors to increase genetic diversity. Heterozygous matings were performed at Marshall Farms (North Rose, NY) and pregnant jills were shipped to the University of Iowa at 21 to 28 days gestation. After birth, kits were rapidly genotyped as previously described. CF kits that passed meconium were paired with a non-CF animal, and generally all other kits were removed from the litter. CF and non-CF kits were reared as previously described. Each CF animal was paired with a non-CF sibling control and treated identically in terms of feeding, antibiotics, oral laxative, and pancreatic enzyme supplementation. This approach was used to control the variable clinical care of each CF animal (ie, antibiotics to control lung infection and oral laxatives to control gut obstruction). All kits were reared from birth on 20 mg/kg metronidazole s.c. (2× daily) and 4.0 mg/kg piperacillin-tazobactam s.c. (2× daily), with each dose in 100 μL of saline. If weight gain decreased in CF animals over a 12- to 18-hour period, both the non-CF control and CF animals were placed on 10 mg/kg enrofloxacin s.c. (2× daily). If weight gain decreased a second time, the non-CF control and CF animals were placed on 30 mg/kg cefazolin s.c. (2× daily). This antibiotic protocol was required because CF ferrets are highly susceptible to lung infections during the early neonatal period.

Porcine pancreatic enzymes (Viokase-V; Neogen Corporation, Lexington, KY) supplementation was initiated at approximately 20 to 30 days when kits were transitioned to artificial nipple feeding. When animals reached 21 weeks of age, the dose of antibiotics was reduced by 25% per week until the animals were free from antibiotics at 6 months of age.

Histology, Immunohistochemistry, and Morphometry

Standard histopathology analysis was performed on paraffin sections from formalin-fixed tissues. The tissues were collected at the time of euthanasia (clinical death) and immediately placed in 10% neutral buffered formalin for at least 72 hours. Tissues were then paraffin-embedded, sectioned (4 μm) and stained with H&E or periodic acid-Schiff. Histopathological examination was performed by a veterinary pathologist, and age-matched CFTR+/– and CFTR+/+;+/- controls were used. Formalin-fixed, paraffin-embedded pancreata were stained by immunohistochemistry for insulin and glucagon (both from MP Biomedicals, Santa Ana, CA) as previously described. Slides for morphometric assessment were scanned with an Aperio ScanScope CS (Buffalo Grove, IL) and images analyzed with Image-Pro Premier software version 9.0 (Media Cybernetics, Rockville, MD). Morphometry was performed to determine the percent normal pancreas in two pancreatic sufficient and two pancreatic insufficient CF animals. For the CF animals at approximately 250 days of age, four sections from different areas of the pancreas were evaluated because the entire pancreas could not fit onto one slide. For CF animals at 19 days of age, three sections of the entire pancreas (100 μm apart) were evaluated for morphometry because the entire pancreas fit onto one slide. Normal tissue was defined by an experienced veterinary pathologist as lacking inflammation and structural changes associated with CF pancreata.

Human CF and Non-CF Pancreatic Tissue

Formalin-fixed human pancreatic tissues were obtained from the National Disease Research Interchange (Philadelphia, PA). Tissue samples were collected from three non-CF
brain dead donors: patients with head trauma, traumatic injury, and traumatic brain injury. Six CF patient samples were evaluated: three diagnosed with CF-related diabetes and three that were not diagnosed with CF-related diabetes.

Fecal EL-1 Assays

Fecal elastase-1 (EL-1) assays were performed using a canine EL-1 ELISA kit (ScheBo Biotech AG, Giessen, Germany) according to the manufacturer’s instructions. Before ELISA, fecal material was first processed using an E1 Quick-Prep-Canine extraction kit (ScheBo Biotech AG) according to the manufacturer’s instructions. This fecal EL-1 assay detects ferret EL-1, but does not cross-react with porcine EL-1 in reconstitution assays performed with Viokase-V pancreatic enzyme (Neogen Corporation).

Bacteriology of Intestinal Samples

At the time of necropsy, a portion of the duodenum and ileum (containing fecal material) was homogenized in sterile saline and a portion was plated directly onto blood agar, MacConkey agar, colistin and naladixic acid agar, colistin and naladixic acid reducible agar, and chocolate agar in a four-quadrant streak pattern. Cultures were incubated aerobically and anaerobically for 24 to 48 hours at 35°C. The remaining tissue homogenate was mixed with glycerol to a final concentration of 10% and frozen in aliquots in liquid nitrogen. Assays for bacterial diversity were performed at the Iowa State University Veterinary Diagnostic Bacteriology Laboratory (Ames, Iowa) by an experienced bacteriologist (J.M.K.) who subcultured unique colony morphologies and performed preliminary classification using biochemical reactions, and final identification was performed using 16S sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. Quantification of the major culturable bacteria from the ileum was performed on frozen glycerol stock of tissue homogenates. Serial dilutions of the homogenate were performed on blood agar under aerobic and anaerobic conditions. The colony forming unit titers were calculated and normalized to milligram protein in the tissue homogenate.

Fecal Microbiota Analysis by Shotgun Sequencing

Fecal DNA Extraction

DNA was extracted using the repeated bead beating plus column (RBB+C) method as described by Yu and Morrison with modifications. Briefly, by using a sterile scalpel

Figure 1  Histopathology in the CF ferret pancreas. Sections of pancreata from four CF and one non-CF animals ranging from 3 to 8 months of age are shown. Pancreas sections from a pair of 8-month-old non-CF (A and B) and CF (C–F) ferrets (CF-1). A predominantly normal pancreas in both genotypes with structurally intact islets is shown in B and D. Focal lobular destruction of part of the pancreas (arrow, E) is noted in CF-1 with ductular enlargement and plugging with eosinophilic secretions (F). Boxed regions in A, C, and E are enlarged in B, D, and F, respectively. Photomicrographs of pancreatic sections representing the most common histological findings in three additional CF animals older than 3 months (G–L). There is significant fibrosis with ductular enlargement and plugging with eosinophilic secretions. Epithelial-like cellular clusters predominate in the CF pancreas (examples marked with arrowheads in G–L). Insets in A, C, G, I, and K are gross images of the pancreas (arrows). All sections were stained with H&E. Scale bars: 500 μm (A, C, E, and G); 200 μm (I); 100 μm (F and K); 75 μm (H); and 50 μm (B, D, J, and L).
blade, 250 mg of frozen fecal sample was transferred into a 2 mL sterile tube containing 1 mL of lysis buffer (500 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 8.0, 50 mmol/L EDTA, 4% sodium dodecyl sulfate, and 0.5 g of sterile DNA-free silica/zirconia beads [BioSpec, Bartlesville, OK]). Subsequent extraction was performed as described. The resulting DNA samples were stored at −80°C until sequenced.

Illumina Sequencing and Microbiota Analysis Using Metagenomic Phylogenetic Analysis
Well-established methods for ultra-high throughput sequencing were used to identify members of the bacterial community in whole fecal samples from three pairs of CF and non-CF ferrets, as described for human fecal samples. Briefly, sequencing libraries were made from DNA preparations using Illumina’s Nextera technology and were sequenced on the MiSeq platform. There were 15 to 20 million paired-end reads (150 nucleotides long) generated per sample, according to the manufacturer’s standards (Illumina Inc., San Diego, CA). Reads corresponding to ferret DNA (http://www.ncbi.nlm.nih.gov; GenBank accession number AEYP00000000.1) were filtered from each sample before analysis of the microbiome using the BMTagger program generated by the NIH Human Microbiome Project (http://www.mmnt.net/db/0/0/ftp.ncbi.nlm.nih.gov/pub/agarwala/bmtagger, last accessed December 19, 2013). Metagenomic Phylogenetic Analysis was used to profile the composition of microbial communities from metagenomic shotgun sequencing data. Metagenomic Phylogenetic Analysis relies on unique clade-specific marker genes identified from 3000 reference genomes and compares each metagenomic read from a sample to this marker catalog to identify high-confidence matches at the species or higher taxonomic levels. The total number of reads in each clade was normalized by the nucleotide length of its markers to provide the relative abundance of each taxonomic unit. This method provided the identities and relative abundances of bacterial taxa identified in each sample.

Figure 2  Islets are highly disorganized in the CF ferret and human pancreata. Sections of pancreata from non-CF and CF ferrets and humans are shown as marked. Each column of photomicrographs represents a different individual. Insulin immunostaining of pancreatic sections from non-CF ferret (A and E), CF ferret (B and F), non-CF human (C and G), and CF human (D and H). Glucagon immunostaining of pancreatic sections from non-CF ferret (I and M), CF ferret (J and N), non-CF human (K and O), and CF human (L and P). Photomicrographs in E–H and M–P emphasize islet structure. The non-CF patient in C and G died of head trauma. The CF patient in D and H died of respiratory failure and was not diagnosed with cystic fibrosis–related diabetes. CF ferret and human islets, identified by insulin and glucagon staining, were present within fibrotic regions of the pancreas and characterized by large clusters of endocrine cells with poorly defined islet boundaries (arrows in B, D, J, and L). By contrast, non-CF islets were smaller with well-defined boundaries and were more evenly dispersed throughout the pancreas (A, C, I, and K). For additional histological examples, see Supplemental Figure S2. Scale bars: 500 μm (A–D and I–L); 50 μm (E–H and M–P).
Study Approval

This study was performed according to protocols approved by the Institutional Animal Care and Use Committee (University of Iowa). Human pancreatic tissues were obtained from the National Human Tissue Resource Center with IRB approval (University of Iowa).

Results

Significant Pancreatic Remodeling Occurs in Most Juvenile and Adult CF Ferrets

Exocrine pancreatic insufficiency significantly impacts the health and nutrition of approximately 85% to 90% of CF patients.20,21 Both exocrine and endocrine pancreatic disease contribute to malnutrition and the development of diabetes in CF patients.21,22 We recently reported that juvenile CF ferrets develop CF-related diabetes with many of the phenotypes observed in CF patients.13 These studies reported a significant loss (approximately 50%) in islet mass of CF ferrets within the first month of age. In the current study, we evaluated the histopathology of the pancreas in older CF and non-CF animals (Figure 1). Eighty-five percent (11 of 13) of CF animals had significant loss of the exocrine pancreas with associated fibrosis, ductal proliferation, and plugging of intralobular ducts (Figure 1, G–L). Interestingly, two CF animals (CF-1 and CF-12) had pancreata that were largely histologically normal (Figure 1, A–D, and Supplemental Figure S1, A–G). However, each of these CF animals also had focal lobules with extensive loss of exocrine parenchyma and cystic dilation of ducts similar to that seen in other CF animals (Figure 1, E and F, and Supplemental Figure S1, H and I). These findings demonstrate that the ferret model retains the variability in pancreatic phenotypes also seen in CF patients,23 where 10% to 15% of CF patients retain partial or complete exocrine pancreas function.

Similar to the exocrine pancreas, there was significant remodeling of the endocrine pancreas in all CF animals, except CF-1 and CF-12 (compare Figure 1, A–D, to panels G–L). Islets in most CF animals were poorly organized (Figure 1, H, J, and L) in variably sized clusters surrounded by thick bands of fibrous tissue that replaced most of the acinar mass of the pancreas. Immunostaining for insulin and glucagon revealed that these cellular clusters in the CF pancreas were indeed disorganized islets composed of beta and alpha cells (Figure 2, A, B, E, F, I, J, M, and N, and Supplemental Figure S2, B, C, E, F, H, I, K, and L). When compared to human CF pancreas, similar disorganization of islet structure within fibrotic regions of the pancreas was observed (Figure 2, C, D, G, H, K, L, O, and P, and Supplemental Figure S2, A, D, G and J).

A Small Subset of CF Ferrets is Pancreatic Sufficient

Morphometric analysis demonstrated that CF-1 and CF-12 had 94% and 84% normal pancreatic histology, respectively

Figure 3

A small subset of CF ferrets are pancreatic sufficient. A: Morphometric analysis of pancreatic histopathology in four CF animals that were pancreatic sufficient (PS) or pancreatic insufficient (PI) based on fecal EL-1 levels. The mean percentage of normal pancreas (±SEM) is shown for the analysis of three to four nonoverlapping sections from each animal. B: Fecal EL-1 levels at various ages from CF-14 and its age-matched non-CF control. C: Indicated amount of Viokase-V pancreatic enzymes were spiked into 1 g of CF feces lacking EL-1 activity or non-CF feces and the samples were then evaluated for EL-1 activity. D and E: Fecal EL-1 levels at various ages from the indicated CF animals and their age-matched non-CF control. These CF animals (CF-1 and CF-12) demonstrated near-normal pancreatic histology. The arrowhead (B and D) marks the age at which both the CF and non-CF animal were placed on Viokase-V pancreatic enzymes (ie, including all time points past the arrowhead). F: Summary of fecal EL-1 levels (means ± SEM) for N independent CF and non-CF control animals for three age groups as indicated. Comparison of non-CF fecal EL-1 levels between various age brackets demonstrated a significant increase in 0 to 30 versus 31 to 80 days (P < 0.001), but not 31 to 80 versus 81 to 150 days (P = 0.448), time points by Student’s t-test. *P < 0.0001 between genotypes by Student’s t-test. Dagger indicates two time points for which fecal EL-1 levels dipped below 200 μg/g feces (level considered to be pancreatic sufficient in CF humans).
The finding that 15% of the CF ferrets analyzed had largely normal pancreatic histology raised the possibility that these CF animals were pancreatic sufficient from birth. To this end, we evaluated fecal elastase-1 (EL-1) levels in samples collected from CF animals with abnormal and largely normal pancreatic morphology at the time of death. All CF ferrets, with the exception of CF-1 and CF-12, had fecal EL-1 levels <2 μg EL-1/g feces at all time points evaluated (Figure 3, B and F), regardless of whether animals were fed oral porcine pancreatic enzymes (Viokase-V) (Figure 3B). This level of fecal EL-1 in CF animals was >1000-fold lower than non-CF controls. Reconstitution experiments spiking various amounts of Viokase-V into CF feces lacking immunoreactive EL-1, or non-CF feces containing significant immunoreactive EL-1, did not alter EL-1 ELISA results (Figure 3C). In contrast to most CF animals, the two CF animals with largely normal pancreatic morphology (CF-1 and CF-12) had levels of fecal EL-1 that did not differ from non-CF control littermates (Figure 3, D and E). These findings demonstrate that a small subset of CF ferrets is pancreatic sufficient. Although 2 of 13 CF animals evaluated in this study were pancreatic sufficient, the overall prevalence of this pancreatic sufficient phenotype in the CF colony is likely much less frequent. This conclusion is based on the finding that CF-1 and CF-12 had growth curves from birth that were similar to non-CF controls and the fact the frequency of this observation in our rearing experience is approximately 1% to 2%.

**Gallbladder Abnormalities Are More Common than Liver Abnormalities in Older CF Ferrets**

Gallbladder disease occurs in approximately 30% of CF patients. Although newborn CF ferrets demonstrated little gallbladder pathology,12 the majority of CF ferrets older than 1 month of age demonstrated significant mucus changes (78%) and mucosal proliferation (89%). In these cases (Figure 4, G–L), there was often thickening of the gallbladder wall due to mucosal proliferation with the formation of cystic structures that were variably sized and contain eosinophilic homogenous material confirmed to be mucus by periodic acid-Schiff staining. Grossly, the CF gallbladder was dark in appearance in cases where histopathology was identified (Figure 4, G and J). In one CF case

![Figure 4](https://example.com/figure4.png)
GI Pathology in Adult CF Ferrets

(CF-1), the animal with a predominantly normal pancreas, the gallbladder was grossly and histologically very similar to the non-CF control (Figure 4, A–F). However, even in this case there were fewer mucosal folds and evidence for multifocal cystic structures (Figure 4, E and F). Although no clear association between pancreatic sufficiency and gallbladder disease in CF patients has been noted, it is interesting that the older CF animal that appeared pancreatic sufficient also had a fairly normal gallbladder. There were no gallbladder abnormalities observed in CF animals that were less than 1-month-old (Table 1).

Liver abnormalities are not uncommon in CF patients and are characterized by biliary cirrhosis (25%), hepatic steatosis (30%), and clinical cirrhosis (5%).2,24,25 Although newborn CF ferrets demonstrated consistent abnormalities in liver enzymes and bile plugging at birth,12,13 histopathological abnormalities in older CF ferrets were highly variable. The majority of both non-CF (75%) and CF (67%) animals demonstrated minor-to-moderate portal lymphoid aggregates (Figure 5, A–D and F). Biliary ducts were generally unremarkable except for rare luminal cell debris and rare lymphocytes within the epithelium. There were 3 of 10 CF animals older than 1 month of age that demonstrated hepatic pathology not observed in non-CF controls (Table 1). These included multifocal mid-zonal necrosis (Figures 4G and 5E) in CF-4 and lipid vacuoles consistent with steatosis (Figures 5, G and H, and 4J) in CF-3 and CF-10. The pattern of hepatic necrosis in CF-4 is atypical of human CF liver disease. The cause of the necrosis is unknown, however, sepsis can induce hepatic midzonal necrosis and/or centrilobular necrosis due to hypovolemic shock.26–28 Oil red O stain confirmed hepatic steatosis in 20% of CF animals greater than 1 month of age (Figure 5, M and N). To assess whether fat accumulation was a consistent finding in CF animals, we measured the triglyceride content in six CF animals. These results confirmed a correlation between histological hepatic steatosis (Figure 5, M and N) and elevated hepatic triglycerides (Figure 5O). Two of three CF animals younger than 1 month of age demonstrated histological evidence of hepatic steatosis, biliary hyperplasia, biliary fibrosis, and/or bile duct plugging (Figure 5, I–L, and Table 1).

**Bacterial Overgrowth in the CF Intestine Was Accompanied with Villous Atrophy and Crypt Mucous Plugging**

Approximately 75% of newborn CF kits are born with meconium ileus,12 suggesting that the intestinal phenotype may be more severe than that observed in CF patients. Consistent abnormalities in the small intestine of CF ferrets older than 1 month included villous atrophy with blunting and fusion in 50% of animals (Figure 6 and Table 1). All CF animals had lymphoplasmacytic inflammation within the lamina propria. More severe villous atrophy was associated with marked lymphoplasmacytic inflammation that extended into the epithelium. Intestinal inflammation of varying degrees was also noted in some of the non-CF controls; however, villous atrophy was not a feature. Crypt dilatation with the accumulation of periodic acid-Schiff-positive mucin staining was common to all CF animals older than 1 month of age. The percentage of CF animals with any histological abnormalities is given. 2,24,25

### Table 1

**Summary of CF Animals Evaluated and Disease States**

<table>
<thead>
<tr>
<th>CF Ferret ID</th>
<th>Life span (days)</th>
<th>Antibiotics</th>
<th>Liver</th>
<th>Pancreas</th>
<th>Gallbladder</th>
<th>Intestine</th>
<th>Stomach</th>
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<tr>
<td>CF-1</td>
<td>257</td>
<td>6 months</td>
<td>None</td>
<td>94% normal, PS</td>
<td>DMF</td>
<td>CMP</td>
<td>None</td>
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<td>CF-2</td>
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<td>6 months</td>
<td>None</td>
<td>F, EXD, PI</td>
<td>MA, MP</td>
<td>VA, CMP</td>
<td>None</td>
</tr>
<tr>
<td>CF-3</td>
<td>120</td>
<td>OTD</td>
<td>FL</td>
<td>F, EXD, PI</td>
<td>MA, MP</td>
<td>VA, CMP, BA</td>
<td>None</td>
</tr>
<tr>
<td>CF-4</td>
<td>100</td>
<td>OTD</td>
<td>MFN</td>
<td>F, EXD, PI</td>
<td>MA, MP</td>
<td>VA, CMP, BA</td>
<td>ED</td>
</tr>
<tr>
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<td>OTD</td>
<td>None</td>
<td>F, EXD, PI</td>
<td>MA, MP</td>
<td>VA, CMP, BA</td>
<td>ED, UC, GD</td>
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<tr>
<td>CF-6</td>
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<td>6 months</td>
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<td>F, EXD, PI</td>
<td>MA, MP</td>
<td>CMP, BA</td>
<td>None</td>
</tr>
<tr>
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<td>35*</td>
<td>OTD</td>
<td>None</td>
<td>F, EXD, PI</td>
<td>NE</td>
<td>CMP</td>
<td>GD</td>
</tr>
<tr>
<td>CF-8</td>
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<td>OTD</td>
<td>None</td>
<td>F, EXD, PI</td>
<td>MP</td>
<td>VA, CMP</td>
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<td>CF-10</td>
<td>40</td>
<td>OTD</td>
<td>FL</td>
<td>F, EXD, PI</td>
<td>MA, MP</td>
<td>CMP</td>
<td>ED</td>
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<tr>
<td>CF-11</td>
<td>68</td>
<td>OTD</td>
<td>None</td>
<td>F, EXD, PI</td>
<td>MA, MP</td>
<td>CMP</td>
<td>GD</td>
</tr>
<tr>
<td>CF-12</td>
<td>19</td>
<td>OTD</td>
<td>FL, G</td>
<td>84% normal, PS</td>
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<td>None</td>
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<tr>
<td>CF-13</td>
<td>19</td>
<td>OTD</td>
<td>BH, BF, BDP</td>
<td>F, EXD, PI</td>
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<td>None</td>
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<tr>
<td>CF-14</td>
<td>&gt;200</td>
<td>6 months</td>
<td>None</td>
<td>PI</td>
<td>39%</td>
<td>85%</td>
<td>77%</td>
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</table>

*Animals were sacrificed due to a rectal prolapse.

This number reflects uniform histological abnormalities in the pancreas because there was a clear distinction between pancreatic insufficient and pancreatic sufficient animals in the extent of pancreatic fibrosis and exocrine destruction. CF animals (86%) were pancreatic insufficient based on fecal EL-1 levels and histological destruction of the exocrine pancreas.

BA, bacteria adherent to mucosal epithelium; BDP, bile duct plugging; BF, biliary fibrosis; BH, biliary hyperplasia; CF, cystic fibrosis; CMP, crypt mucous plugging; DMF, decreased mucosal folds; ED, interstitial edema; EXD, exocrine destruction; F, fibrosis; FL, fatty liver; G, glycogen; GD, fundic gland dilatation with mucus; MA, mucus accumulation; MFN, multifocal midzonal necrosis; MP, mucosal proliferation; NE, not evaluated as sample was not harvested; OTD, on antibiotics until death; PI, pancreatic insufficient; PS, pancreatic sufficient; UC, ulceration of gastric mucosa; VA, villus atrophy.
The lumen of the CF small intestine was often dilated by ingesta and contained significantly greater number of bacterial colonies, often adhered to the epithelial surface and within the ingesta, confirmed by colony forming unit titers of intestinal tissue and Gram stain (Figure 7, A and C). Both aerobic and anaerobic titers of bacteria cultured from the small intestine (at the level of the ileum) were significantly higher in CF animals (>10^3-fold) as compared to controls (Figure 7A). Additionally, several types of bacteria were cultured from the intestine that distinguished CF or non-CF animals (Figure 7B and Table 2). Importantly, all CF and non-CF pairs (Table 1) were reared identically in terms of antibiotic treatments.

Using an alternative approach to interrogate differences in the intestinal microbiota between animals, we performed deep sequencing and metagenomic phylogenetic analysis of fecal microbiota from the three oldest CF and non-CF pairs for which intestinal histopathology was obtained (Figure 7I and Supplemental Table S1). This culture-independent method demonstrated considerable diversity in microbiota between animals, regardless of CFTR genotype. In total, 66 bacterial taxa were identified from the six animals tested, including the majority of the bacteria observed by culture analysis of intestinal samples. Streptococcus was the most abundant genus observed in all animals (>50%), with the exception of CF-2 control (Figure 7I), consistent with this genus being one of the most commonly identified in the lungs of CF ferrets by culture. Escherichia coli, which has been shown to be significantly more abundant in feces from children with CF, was 2.1- and 7.5-fold higher in abundance in two of the CF animals as compared to their non-CF paired controls (Supplemental Table S1). On average, 69 ± 6.4% of taxa identified in the feces of a CF animal overlapped with its non-CF pair, with three to four major
taxa at higher abundance (>1%) being conserved between each pair (Supplemental Table S1). An ecological measure of association (Whittaker’s index of association), which accounts for species identity and relative abundance, demonstrated no significant clustering by CFTR genotype. However, when this analysis was performed using the presence or absence (not abundance) of specific bacteria, a nonsignificant sib-based clustering of the paired animals was observed. Overall, these findings suggest that the environment (ie, early co-habitation on the jill) more strongly influences the fecal microbiome in the ferret than the CFTR genotype.

At the level of the colon, colonic crypts were dilated with mucus and often contained bacterial colonies in CF animals. A mild increase in inflammatory cells within the lamina propria was also observed in CF animals. In one CF animal, the colon was white in color on gross examination. Histologically the abnormal appearance was due to lymphangiectasia with dilation of lymphatic vessels, rupture, and inflammation within the lamina propria and submucosa (Figure 8, A–F). Rectal prolapse was observed in several CF animals at approximately 1 month of age (Figure 8, G–I, and Table 1). Of the 13 animals reported in this study, two animals were sacrificed due to rectal prolapse, and two others showed signs of early stages of rectal prolapse, but were rescued by increased use of laxatives. Although rectal prolapse did not occur in all animals, all CF animals demonstrated constipation and diarrhea at different times in the rearing process. Constipation was treated with increased GoLYTELY (Braintree Laboratories Inc, Braintree, MA) administration and/or MiraLax (Merck Sharp & Dohme Corp, Whitehouse Station, NJ), whereas diarrhea was treated with probiotic. The use of probiotics typically resolved diarrhea within 3 to 4 days.

**Other Organ Abnormalities Are Seen in CF Animals**

Abnormalities in the stomach were also observed in 50% of CF animals older than 1 month of age (Figure 9 and Table 1). The most common change was submucosal edema (30% of animals) (Figure 9, B, F, and J) and gastric gland dilation by mucus (30% of animals) (Figure 9L). In one CF animal (CF-5), there was gross evidence of digested blood within the gastric contents indicative of loss of mucosal integrity that was histologically confirmed as mucosal ulceration with submucosal edema and inflammation (Figure 9, E–H). Two other CF animals (CF-4 and CF-10) exhibited moderate mucosal erosion with submucosal edema (Figure 9, I–L). The finding of compromised gastric integrity in some CF animals may be linked to CFTR function in the gastric glands. Alternatively, because all CF animals failed to thrive during end-stage disease, these findings may simply reflect the increased stress that the CF animals are under. In older CF male animals, the vas deferens was difficult to find and absent.
in most sections (Supplemental Figure S3). Infrequently, remnant islands of vas epithelium (dysplastic and lacking) could be detected. These changes are consistent with progressive loss or absence of the vas deferens.

**Discussion**

The goal of this study was to evaluate the progression of gastrointestinal disease in CF ferrets. Given the extreme susceptibility of newborn CF ferrets to lung infections, all CF animals were reared on antibiotics with the goal of removing them at 6 months of age (the age ferrets are considered to be sexually mature). Of the 14 CF animals studied, only four lived beyond the age of 6 months, despite continued antibiotic therapy. As observed in CF patients, variability in the severity of disease in these organs of CF patients has been thought to be due to multiple factors, including the type of CFTR mutation, modifier genes, and environment. Our results, demonstrating that disease variability exists on a CFTR null background, emphasize the importance of modifier genes and/or environmental factors in determining CF
phenotypes. Given the more controlled environment in which CF ferrets are reared (ie, housing, chow, etc.), as compared to humans, modifier genes likely play a more dominant role in the variability of organ disease severity.

In newborn CF ferrets, pancreatic disease is mild at the histological level and there is no obvious gallbladder disease.12,13 Unlike CF pigs, which have fairly uniform and extensive pancreatic and gallbladder disease at birth,9,11 we observed variability in the severity of disease in these organs of juvenile and adult CF ferrets. Similar to CF patients, for which 85% to 90% demonstrate exocrine pancreatic insufficiency,20,21 the majority of the exocrine pancreas was histologically destroyed in 85% of CF ferrets (11 of 13 animals). Of the CF ferrets older than 1 month of age, 78% had extensive gallbladder disease characterized by mucosal cystic proliferation with excessive mucus production. This percentage is much higher than the 15% to 30% of CF patients who demonstrate histological gallbladder disease at the time of autopsy.4,34,35

Intestinal complications in CF ferrets were quite diverse and included rectal prolapse, constipation, and diarrhea at frequencies similar to that observed in CF patients.36,37 Overgrowth of bacteria in the small intestine is a common finding in CF mice38 and humans,39 and this was also observed in CF ferrets. Whether the bacterial overgrowth was the cause or consequence of villous atrophy frequently observed in the CF ferrets remains unclear. However, these findings do suggest some level of impaired innate immunity in the CF ferret intestine, which could result from altered motility of ingesta and/or nutrients that are not absorbed. Metagenomic deep

Table 2  Culturable Bacteria Observed in the Gut of CF and Non-CF Animals

<table>
<thead>
<tr>
<th>CF ferret ID</th>
<th>Bacterial taxa cultured</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF-3</td>
<td>Enterococcus faecalis, E. faecium, E. gallinarum, Escherichia coli, Lactococcus garvieae, Citrobacter braakii, Clostridium perfringens, Proteus mirabilis, Pseudomonas putida, Staphylococcus pseudintermedius, Streptococcus alpha hemolytic</td>
</tr>
<tr>
<td>CF-3 Control</td>
<td>E. faecalis, E. avium, E. hirae, E. coli, Rothia dentocariosa, Veillonella ccrici</td>
</tr>
<tr>
<td>CF-4</td>
<td>E. faecalis, E. faecium, E. avium, E. coli, Lactococcus lactis, P. mirabilis, S. pseudintermedius, Arthrobacter pigmenti</td>
</tr>
<tr>
<td>CF-4 Control</td>
<td>E. faecalis, E. faecium, E. avium, E. hirae, E. coli, L. garvieae, L. lactis, Pasteurella canis, P. mirabilis, Streptococcus suis, Vagococcus fluvialis, V. luteae, V. ccrici</td>
</tr>
<tr>
<td>CF-5</td>
<td>E. faecalis, E. faecium, E. gallinarum, E. coli, Klebsiella pneumoniae, P. mirabilis, Streptococcus galloyticus, S. lutetiens, V. fluvialis</td>
</tr>
<tr>
<td>CF-5 Control</td>
<td>E. faecalis, E. faecium, C. perfringens, E. coli, L. garvieae, P. mirabilis, S. alpha hemolytic, Vagococcus luterae</td>
</tr>
<tr>
<td>CF-9</td>
<td>E. faecalis, E. faecium, E. avium, Lysinibacillus sphaericus, Acinetobacter genomospecies 3, Rothia nasimurum</td>
</tr>
<tr>
<td>CF-9 Control</td>
<td>E. faecalis, E. coli, Staphylococcus delphini, S. galloyticus</td>
</tr>
<tr>
<td>CF-10</td>
<td>E. faecalis, E. faecium, Staphylococcus hominis, Streptococcus pneumoniae, Penicillium spp.</td>
</tr>
<tr>
<td>CF-10 Control</td>
<td>E. hirae</td>
</tr>
<tr>
<td>CF-11</td>
<td>E. faecium, Staphylococcus epidermidis, S. xlyosus</td>
</tr>
<tr>
<td>CF-11 Control</td>
<td>E. faecalis, Gemella sanguinis, P. mirabilis, S. epidermidis, S. warneri</td>
</tr>
</tbody>
</table>

CG, cystic fibrosis.

Figure 8  Pathology of the colon and rectum in CF ferrets. Histological sections from the colon from a non-CF (A–C) and CF (D–F) ferret stained with H&E. Insets in A and D are gross images of the colon (arrow) demonstrating its white color in the CF animal. Boxed regions in B and E are enlarged in C and F, respectively, and show lymphangiectasia with dilation of lymphatic vessels in the CF animal (F). Rectal prolapse in a CF ferret at 28 days of age; gross image (G) and H&E-stained histology sections (H and I). Boxed region in H is enlarged in I. Scale bars: 1 mm (A and D); 500 μm (B, E, and H); and 100 μm (C, F, and I).
sequence analysis also demonstrated that *Streptococcus*, *Enterococcus*, and *Escherichia* were among the most abundant genera found in CF ferret feces. These bacteria are the predominant pathogens that colonize the lungs of CF ferrets, representing >80% of culturable bacteria in the lung of 9 of 10 CF animals tested.\(^{14}\) Thus, these findings reinforce the likely importance of fecal to oral transfer of pathogens before lung colonization, as suggested by a recent study in CF infants.\(^{40}\) This study demonstrated that the fecal microbiome significantly overlaps with the oropharyngeal microbiome in CF infants, with pathogens such as *Enterococcus*, and *Escherichia* (also observed in CF ferret feces and lungs) increasing in the stool before oropharyngeal colonization.\(^{46}\)

CF ferrets exhibited minor gastric pathology, predominantly gastric gland dilation with less frequent gastric mucosal changes (erosion and ulceration). It is presently unclear if pathological changes in the CF stomach are the direct result of loss of CFTR function or an indirect consequence of stress in the animals. However, it is interesting that gastric ulcers have been reported to occur in >50% of older CF pigs, requiring euthanasia before terminal end-stage lung disease.\(^{31}\) CF-specific liver disease was relatively minor in the ferret model when compared to controls; however, hepatic steatosis and biliary fibrosis were observed at frequencies similar to CF patients.\(^{32}\)

One of the more interesting findings in the CF ferret model was the phenotypic variability observed in the extent of pancreatic disease. Approximately 85% to 90% of CF patients demonstrate pancreatic exocrine insufficiency.\(^{20,21}\) Based on histological criteria, 2 of 12 CF animals had a predominantly normal exocrine pancreas and maintained fecal EL-1 levels throughout life that were similar to non-CF controls. In contrast, CF pig are born with the majority of their exocrine pancreas destroyed,\(^{11}\) whereas CF mice lack pancreatic disease.\(^{33}\) This finding is somewhat surprising, given that the CFTR genotype has been proposed to significantly influence pancreatic phenotypes in CF patients.\(^{33}\) Thus, one could interpret findings in the CF ferret model to suggest that either genetic modifiers and/or environmental factors play an unappreciated role in determining the extent of exocrine pancreatic damage in CF. However, because the two pancreatic sufficient CF ferrets (CF-1 and CF-12) had this phenotype from birth, genetic factors are likely more important in determining the pancreatic phenotypes than the environment. During the first year of life, fecal EL-1 levels in CF infants have been shown to dramatically fluctuate.\(^{44}\) In this later study, 4 of 82 infants had fecal EL-1 levels that rose from <200 µg/g at 2 months of age to >200 µg/g by 12 months of age.\(^{34}\) CF-1 demonstrated two time points in which fecal EL-1 dropped below 200 µg/g feces within the first 35 days of life, whereas CF-12 maintained fecal EL-1 levels above this threshold for defining pancreatic insufficiency. Very little is known about the early pathology in the neonatal human CF pancreas because only indirect measures of function can be used to interrogate pathology. In humans, the extent of pancreatic disease has been thought to be due predominantly to the severity of CFTR mutation. However, because the CF

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**Figure 9** Histological abnormalities observed in the gastric mucosa of CF ferrets. Gross images and histological images of the stomach from a non-CF (A–D) and two CF animals (E–L). Each row of images represents a single animal, depicting the gross image of the stomach (A, E, and I) and various magnification of the gastric mucosa (B–D, F–H, and J–L). In this CF animal (E–H), gastric pathology was noted grossly by the multifocal dark areas on the serosal surface of the stomach (E, inset). The stomach contents contained digested blood indicative of mucosal ulceration. Histologically, there was erosion and ulceration of the mucosa (arrow, F) with marked submucosal edema (bracketed, F) and inflammation (arrowheads, H). A different CF animal (I–L), with less severe mucosal erosion and submucosal edema and gastric gland dilation (asterisk in L). Interstitial edema and gastric gland dilation were the most common changes in CF animals. Scale bars: 200 µm (B, F, and J); 100 µm (C, G, and K); and 50 µm (D, H, and L).
ferrets studied were deficient in CFTR, our findings suggest that modifier genes are the most likely explanation for the pancreatic sufficiency from birth in CF-1 and CF-12. Such modifiers may include alternative chloride or bicarbonate channels that compensate for the lack of CFTR. If these factors could be identified and manipulated, they could be of significant value to CF patients.

One other finding about ferret fecal EL-1 levels is worth noting. There was a significant ($P < 0.001$) increase in fecal EL-1 levels between 1 to 2 months of age in wild-type control animals, and this increase also occurred in CF-1. This is the window in which ferrets are weaned and may account for the increase. It is presently unclear if similar changes occur in humans at weaning, however, the fecal EL-1 levels in juvenile ferrets are three to four times higher than typically found in humans. Thus, this change may be species-specific. CF ferrets have been shown to have glucose excursions >200 mg/dL during the 1- to 2-month period when weaning occurs, and it is possible that a decline in endocrine function during this time window is linked to increased pressure on the pancreas to produce pancreatic enzyme.

In summary, our findings demonstrate that the lack of CFTR function leads to gastrointestinal disease in juvenile and adult ferrets in a similar fashion to humans. Interestingly, CF ferrets demonstrated phenotypic variability in the extent of disease in the liver, pancreas, and gallbladder similar to that observed in CF patients. The CF ferret model may be useful in determining the genetic and environmental influences responsible for disease variability in CF patients, and offer an important modality for testing therapies targeting pancreatic, gallbladder, intestinal, and liver disease.

**Acknowledgment**

We thank Robyn L. Marsh for performing the clustering analyses for ecological associations in the fecal microbiome deep sequence data.

**Supplemental Data**

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2014.01.035.

**References**