Lymphocytic cholangitis in cats: a microbiological, histological and clinical approach

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Lymphocytic cholangitis in cats: a microbiological, histological and clinical approach

PhD thesis, Utrecht University, The Netherlands

The studies described in this thesis were performed at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, the Netherlands and the Laboratory of Microbiology, Agrotechnology and Food Sciences Group, Wageningen University, Wageningen, The Netherlands.

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Lymphocytic cholangitis in cats: a microbiological, histological and clinical approach

Een microbiologische, histologische en klinische benadering van lymfocytaire cholangitis bij de kat

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 10 oktober 2017 des middags te 12.45 uur

door

Cornelia Maria Adriana Otte

geboren op 19 maart 1970 te Halsteren

Promotoren:	Prof. dr. J.W. Hesselink
	Prof. dr. J. Rothuizen
Copromotoren:	Dr. L.C. Penning
	Dr. R.P. Favier

This thesis is dedicated to my brother,

who would have been proud

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CHAPTER 1 Aims and scope of the thesis

Lymphocytic cholangitis (LC) is one of the most common inflammatory hepatic diseases in cats.[1-3] Historically, less scientific research has been directed towards cats when compared to the amount of research in dogs. The dog may be man's best friend, but cats are currently the most popular pets in the Western world.[4] This thesis is dedicated to cats suffering from LC.

LC is a chronic disease in cats that affects the biliary tree and progresses slowly over months or years.[5] Histopathology is considered the gold standard for diagnosing and the hepatic lesions are characterized by aggregates of inflammatory cells in portal tracts, and in and around bile ducts.[3] Chronic inflammation in the bile ducts causes dilatations and strictures and may eventually lead to fibrosis and cirrhosis.[6, 7]

Early clinical signs include nausea and vomiting, changes in appetite, and gradual weight loss.[5] Jaundice is often present later in the course of disease. Blood analysis sometimes reveals elevated hepatic enzymes and bile acids, but hypergammaglobulinaemia is the most consistent finding.[5] Its aetiology is still unknown. Bacteria have been linked to the disease,[8-10] but an immune-mediated component has also been suggested.[5, 11]

In the general introduction of this thesis (**Chapter 2**), an overview is given of the healthy feline liver, its morphology, and its functions. The chapter continues with a description of feline diseases of the gall bladder and biliary tree, including aetiology, symptoms, diagnosis, treatments and prognosis. When necessary, comparisons to other species are included.

Past research has linked bacteria to the aetiology of LC.[8-10] **Chapter 3** describes the analysis of bile samples with molecular methods based on the amplification of the 16S ribosomal RNA gene. A 16S rDNA gene based PCR and denaturing gradient gel electrophoresis (DGGE), followed by direct sequencing, were used for this purpose.

LC is commonly treated with prednisolone or ursodeoxycholic acid (UDCA). The use of prednisolone has been warranted by the immune-mediated component suggested by the abundant presence of lymphocytes.[5, 11] UDCA has been proposed as supportive therapy based on its hepatoprotective properties, such as protection of cell membranes against the detergent properties of bile acids,[12] prevention of mitochondrial damage,[13] reduced cell destruction and inflammation, and choleresis.[14, 15] In **Chapter 4**, we compare the effect of these therapeutic agents on survival times of cats diagnosed with LC. Furthermore, prognostic factors were determined based on retrospective data.

Clinical evaluation of prednisolone and UDCA in cats with LC showed that prednisolone treatment resulted in a statistically significantly longer survival time than treatment with UDCA. However, it was undetermined if the improvement in clinical condition correlates with improvement in the underlying hepatic histological lesions.

The injured liver is capable of a remarkable and unparalleled regeneration, which is based on proliferation of both hepatocytes and cholangiocytes.[16] Only when regeneration capacity proves inadequate, hepatic progenitor cells (HPCs) will be activated.[16-18] Bipotent HPCs can give rise to both hepatocytes and cholangiocytes.[19] Upon activation, HPCs will proliferate, migrate from the canal of Hering to the site of injury, and differentiate into cholangiocytes or hepatocytes, depending both on the disease and on concurrent changes in their microenvironment (HPC niche).[17, 20, 21] The HPC niche, both a histological location and a functional unit, is of importance in maintaining and regulating HPCs behaviour, supporting self-renewal and balancing quiescence, proliferation and differentiation in response to injury.[21, 22]

Fibrosis occurs when the injury takes on a more chronic character, and the amount of collagen subsequently increases. The main source of collagen are Ito cells (also known as hepatic stellate cells (HSC), fat-storing cells, hepatic lipocytes or perisinusoidal cells) which are the main storage sites for vitamin A in non-diseased liver. Proliferating fibroblasts in the portal areas can also contribute to collagen deposits and fibrosis.

In **Chapter 5**, we describe how we evaluated liver histology during treatment with prednisolone or UDCA in cats with LC by analysing the proliferation of hepatocytes, lymphocytes, fibroblasts, bile ducts, activation of progenitor cells, fibrosis and the degree of inflammation.

Chapter 6 further elaborates on the regeneration of the injured liver in cats with LC by studying the activation of HPCs, and the HPC niche. Vimentin expression has been shown in hepatic progenitor cells of rats, mice, human beings, and dogs and has been shown to indicate proliferative activity of cells and an undifferentiated state of HPCs.[23-25] Remodelling of the extracellular matrix (ECM) and deposition of laminin have been shown to play an important role in HPC activation in hepatic injury in both rodents and human beings.[22, 26] It also promotes cholangiocyte differentiation of bipotent cells.[27] The Wnt/β-catenin signalling pathway has a central role in hepatic and bile duct development and regeneration, promoting gene activation, inhibiting apoptosis and increasing cellular proliferation.[28-30] It also guides

cells to a biliary phenotype.[24] Notch is involved in the proliferation, differentiation and apoptosis in all stages of organ development, including healthy and diseased livers.[31] Therefore, these commonly used immunohistochemical markers are investigated because a single specific HPC marker has not been identified yet, and many HPC markers have a shared expression with cholangiocytes.

Chapter 7 is a summarizing discussion.

Chapter 8 contains the first article published. This was aimed at practising veterinarians in the Netherlands.

Chapter 9 is a summary in Dutch.

Aims of the thesis

Pathogenesis (Chapter 3)

- Determine the presence of bacteria as an initiating factor for lymphocytic cholangitis

Treatment (Chapter 4, 5 and 6)

- Comparing life expectancies under different treatment regimens, *ie.* prednisolone or ursodeoxycholic acid
- Determining the effect of prednisolone and urodeoxycholic acid on liver histology
- Evaluating the role of hepatic progenitor cells in LC

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CHAPTER 2 General introduction: Feline diseases of the biliary tree and gallbladder

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The healthy liver

The first embryonic function of the liver is haematopoiesis.[1] Upon maturation the liver has critical functions in metabolism and detoxification. Examples are synthesis of proteins like albumin and blood clotting factors, maintenance of glucose homeostasis, lipid metabolism, conjugation and excretion of endogenous and exogenous toxins and drugs, and excretion of bile.[2, 3]

The feline liver is the largest internal organ and takes up approximately 3 to 4% of total body weight.[4, 5] Parenchymal liver diseases include lipidosis, amyloidosis and feline infectious peritonitis. In these diseases, the liver parenchyma is not the primary target, but is affected as part of the systemic disease. In cats, it is the biliary system rather than the liver parenchyma which is the primary target of disease.

Feline bile (pH 5.9 -7.8) consists of various (non-)sulphated bile acids, and cholesterol.[6, 7] Taurine is essentially the only nutrient used by cats to conjugate bile acids and because cats have limited taurine production capacity, it is considered an essential dietary component for cats.[7, 8] Therefore, taurine availability can also be considered a limiting factor in the production of conjugated bile acids.

Bile salts are excreted into specialised regions of canalicular membrane between hepatocytes, the bile canaliculi (Figure 1). The bile canaliculi drain into the canals of Hering, which merge to create interlobular bile ducts, and later fuse into intrahepatic bile ducts. These then join to form hepatic ducts which finally end in the common bile duct (CBD). Together with the cystic duct, this is considered to be the biliary tree. The biliary tree and gallbladder constitute the biliary system.
At Vater's papilla, the common bile duct enters the duodenum.[9-11] In cats, the major pancreatic duct usually joins the CBD before it enters the duodenum, and only 20% of cats are estimated to have an accessory pancreatic duct with its own separate opening into the duodenum.[12]

The hepatic ducts transport bile into the gallbladder, where bile is stored and concentrated during fasting. The duodenal mucosa produces cholecystokinin when food enters the intestines, the hormone which stimulates the gallbladder to contract. Bile enters the duodenum

via the CBD and mixes with food. Bile acids aid in digestion and absorption of lipids and fat-soluble vitamins. Bile is also used to eliminate many waste and toxic products, which are subsequently excreted in faeces. About 90% of bile acids are reabsorbed in the enterohepatic circulation.[6]

Felids have a low capacity for glucuronide conjugation of drugs and toxins, limiting their conversion to water soluble substances that can be excreted into urine or bile.[13] This makes cats more prone to suffer hepatotoxic effects when exposed to these drugs and toxins (see Box 1 for a list of common drugs that are hepatotoxic to felines).

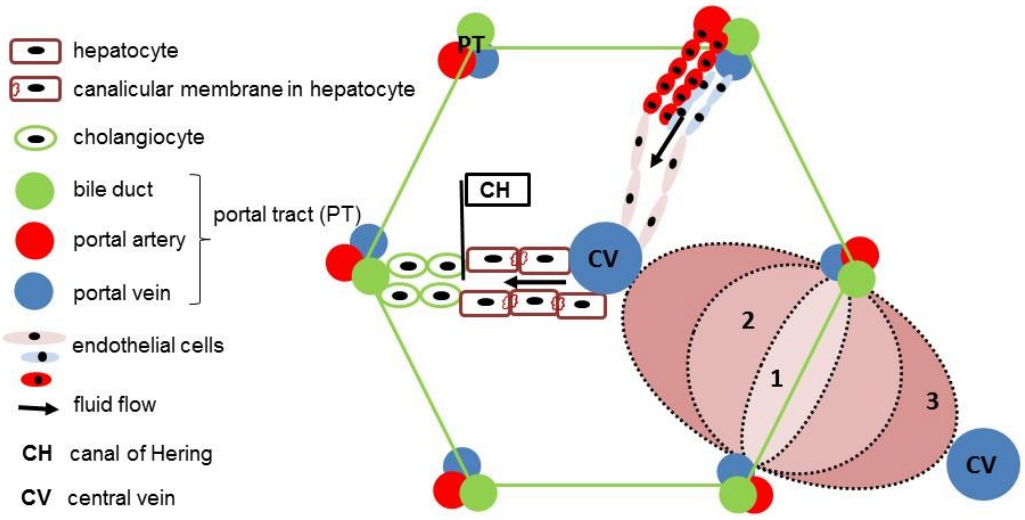

Figure 1. From a micro-anatomic point of view the liver is traditionally described as consisting of hexagonal hepatic lobules (Figure 1, left, hexagonal)[14]. In this view, blood flows from the portal vein (blue) and hepatic artery (red) to the central vein (CV). This clearly demonstrates a rather unique feature of the liver which is the dual blood supply: the liver receives venous blood from the gastrointestinal tract and arterial blood via the hepatic artery. The portal tracts (PT) are completed by a bile ductule (green).

A more functional representation of the liver is obtained by describing the smallest functional units of the liver: hepatic acini (Figure 1, right, oval)[14]. Zone 1 centres on the portal tract and receives the most oxygen-rich blood. Zone 3, on the other hand, receives less oxygen because it is further away from the portal tract. Therefore, zone 3 is more susceptible to hypoxia, while zone 1 is more likely to suffer direct toxic damages.

A detailed report by Boyden showed that over 12% of cats have some sort of accessory gallbladder (see ”diseases of the gallbladder”).[15] However, biliary disease and gall bladder anomalies are infrequently reported together which suggests that there is no association between the two.[16]

Cholestasis

An important clinical syndrome associated with biliary disease is cholestasis, defined as impaired bile flow.[17] Cholestasis is not a disease, but a pathophysiological phenomenon associated with dysfunction of the biliary system. Two forms are commonly distinguished:

1. intrahepatic cholestasis, which occurs predominantly at the level of hepatocytes, bile canaliculi, or in bile ductuli in zone 1 (see Figure 1)

   Intrahepatic cholestasis in cats is linked to the destruction of bile ductuli and hepatic fibrosis secondary to cholangitis.[16] Some cholestatic drugs, e.g. methimazole, cyclosporine, rifampicin and cloxacillin, have been associated with reduced bile flow at the canalicular level.[2, 15] In all species (including dogs and humans), endotoxins represent a major cause of intrahepatic cholestasis by impairment of the canalicular bile flow.[18]

2. extrahepatic cholestasis, due to obstruction of the CBD.

Extrahepatic bile duct obstruction

<u>Aetiology</u>

Bile sludge and choleliths, associated with cholangitis, cholecystitis, and impaired contractility of the gallbladder, have been implicated as the most common causes of extrahepatic bile duct obstruction (EHBDO).[18-21] Other causes include pancreatitis,[12, 19] biliary neoplasia,[19] foreign bodies,[21, 22] biliary mucoceles,[16] and gastrointestinal disease.[20]

<u>Symptoms</u>

Symptoms seem to be non-specific in relation to the cause of cholestasis, and include abdominal pain, vomiting, dehydration, nausea, anorexia, weight loss, lethargy, pyrexia, icterus, diarrhea, and polyuria-polydipsia.[12, 16, 19-22]

Although acholic faeces is always seen after experimental ligation of the CBD, it is not encountered in clinical practice.[12]

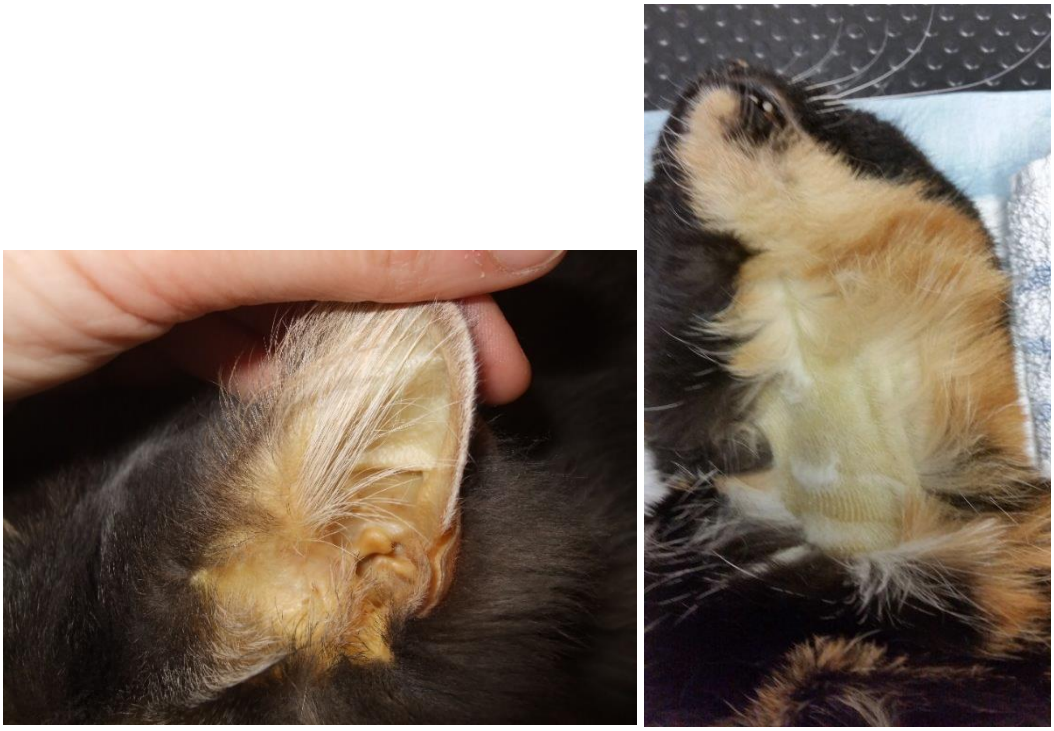

Figure 2. Icterus showing in the ear of a cat (left, courtesy of Dr. Robert Favier, Utrecht University) and on the skin of the ventral neck (right, courtesy of Marleen Assink, DVM).

Diagnosis

Increased activity of liver enzymes, especially the short-lived alkaline phosphatase (ALP), may indicate cholestasis (see Box 2 for an overview of commonly encountered laboratory findings).[6, 23] Elevations in total bilirubin and bile acids are also commonly seen.[6, 12, 20]

EHBDO can often be diagnosed with ultrasound. In normal cats, intrahepatic and extrahepatic ducts are not visible thus any duct visible on ultrasound is considered dilated.[19] The diameter of the CBD is a useful parameter in diagnosing EHBDO since it is greater than 5 mm in 97% of affected cats, with the upper limit for healthy cats established at 4 mm.[19, 21]

In contrast, gallbladder dilation is not a reliable sign of EHBDO in practice, although it is invariably seen after experimental bile duct ligation.[19]

Treatment

Treatment depends on the cause of the obstruction, and may involve the use of choleretics such as ursodeoxycholic acid (UDCA) (bile sludge), surgery (choleliths, foreign body, mucocele), praziquantel (liver flukes), prednisolone (cholangitis, gastrointestinal disease), antibiotics (suppurative cholangitis, gastrointestinal disease), low fat diet, anti-emetics and supportive fluids (pancreatitis).

Prognosis

Many of the above mentioned therapies can be very successful in relieving cholestasis and the prognosis depends heavily on the underlying cause.

In fact, there is often a combination of extra- and intrahepatic cholestasis. Obstruction of the CBD affects the entire biliary tree but is described as extrahepatic cholestasis. In all inflammatory biliary diseases, the entire bile system is involved but the predominant features occur in the smallest branches of the system so that these forms are described as intrahepatic cholestasis.

1. Inflammation of the biliary system

Inflammatory bile duct disease is amongst the most common hepatopathies in cats.[10, 24] Feline cholangitis may be accompanied by pancreatitis, inflammatory bowel disease, and cholecystitis.[25, 26]

1.1 Neutrophilic cholangitis (NC)

Aetiology

Enteric bacteria have been implicated in inflammatory liver disease, with translocation across the mucosal barrier the most likely aetiology.[26-28] NC may also be caused by ascending bacterial infection from the gut, or haematogenous dissemination of enteric bacteria.[27, 29, 30] In healthy cats, transient bacterobilia exists although the biliary tract is considered to be sterile.[31]

In cats, neutrophilic cholangitis is accompanied by cholecystitis.[27] In fact, cholecystitis and neutrophilic cholangitis are likely not distinct or different entities in cats.

Symptoms

The most noticeable symptoms are nausea, vomiting, anorexia, and fever. Icterus is present in a minority of cases of NC.

Diagnosis

Examination of a sample of bile is the most likely route to a diagnosis.[27, 32] Bile samples can be obtained by cholecystoscentesis with a 22 G needle under ultrasound guidance (see Box 3 for biopsy techniques, and Figure 2 for an example of a bile sample). Ultrasonography usually reveals no abnormalities of the gallbladder, although in acute, severe cases, oedema of the gallbladder wall may be seen. Cytology of bile will reveal neutrophils and sometimes bacteria. Bacterial culture will determine the bacterial species present, and their antimicrobial susceptibility. A biochemical profile is often within normal limits or may show an increased activity of alanine aminotransferase (ALT), while the most common finding on a complete blood count (CBC) is leucocytosis.

Figure 3. Bile sample from a patient with NC. The bile is yellow and turbid due to the presence of pus.

Treatment

Amoxicillin/clavulanate and ticarcillin/clavulanate for at least 4 weeks were used successfully in the treatment of NC and are the first choice treatment (see Box 4 for therapies used in feline biliary disease).[27, 33] Other antibiotics, such as chloramphenicol, fluoroquinolones, and metronidazole are well excreted in bile, have good anaerobic activity, and may be

considered.[6, 28] It is recommended to routinely determine the susceptibility for antibiotics and then select the best antibiotic. Long term treatment is essential to prevent recurrence.

Increased amounts of cyclooxygenase were detected in inflamed mucosa of feline gallbladders,[34] suggesting a role for NSAIDs in the treatment of cholecystitis.[35] Adjunctive therapies included UDCA and vitamin K.[27] However, there is no evidence for their beneficial effect and the authors limit the treatment of NC to the use of long term antibiotics as discussed above.[25]

Prognosis

The only study that reports on prognosis for cats with NC states that most cats regained their previous health after receiving the appropriate antibiotics.[27] However, if treatment is too late or unsuccessful the disease is often fatal.[25]/

1.2 Lymphocytic cholangitis (LC)

Aetiology

Bacterial and immunological aetiologies have been suggested for LC.[36-41] Hypothetically, an infectious agent could initiate the inflammation which could then be sustained as an immune-mediated disease while the infectious agent has already been eliminated. This would suggest that LC could be a chronic sequela to NC. However, such a connection has never been confirmed. LC and NC are presently considered to be two separate, unconnected diseases. Additionally, cholangitis has been diagnosed secondarily to bile duct obstruction by choleliths.[42] To date, however, no definite aetiology has been established.

Symptoms

Nausea, chronic vomiting, diarrhoea, anorexia, weight loss and icterus are seen in LC, as well as polyphagia and weight loss.[43, 44] The course of the disease is often protracted, spanning months to years. Icterus if present, usually indicates an advanced stage of the disease. Male cats appear to be affected more than females.[45]

Diagnosis

Hypergammaglobulinaemia is the most consistent abnormality on the biochemical profile.[33, 40] This, together with the chronicity of the disease often leads to a misdiagnosis of FIP.[46]

Ultrasonographically, dilated intra- and extrahepatic bile ducts are frequently seen (see Figure 4). Distinguishing between NC and LC based on these findings is impossible.[25] Dilation of the bile ducts excludes FIP. However, in many cases of LC there are no ultrasonographic changes in the bile ducts. Furthermore, ultrasound has a limited sensitivity for detecting infiltrative liver diseases.[19] Fine needle aspiration (FNA) is not diagnostic. Cytology may show lymphocytes and may lead to a misdiagnosis of malignant lymphoma.[47-50] The portal distribution of lesions in cats with LC cannot be identified with FNA cytology and there is a discrepancy between histology and cytology in 55% of cases.[51, 52] Histology is the gold standard for diagnosing LC (see Figure 5), using surgical or laparoscopic wedge biopsy procedures, or with larger needle biopsy techniques (discussed in Box 3) that obtain at least five portal triads, which is considered representative in humans.[53] In one stude a comparison between needle and wedge biopsies showed that only 2/13 cats and dogs received the same histological diagnosis,[53] this is likely due to the small needle diameter used for liver sampling. In the authors' experience the diagnosis can be accurately made with Tru-Cut liver biopsies of sufficient size. This is achieved using a 16G needle in a semi-automatic biopsy gun (see Box 3). Thinner needles lead to diagnostic inaccuracy, in which case surgical wedge biopsies should be preferred.

The typical histological lesions are lymphocytic inflammation of the biliary tree with fibrosis surrounding the bile ducts (an expression of the chronicity of the process). Lymphocytes are present inside the lumen, the wall, and around the bile ducts in the portal triads. In cats with LC the biliary system is prone to infection, leading to a mixed lymphocytic and neutrophilic inflammation, where both inflammatory cell types can be identified.

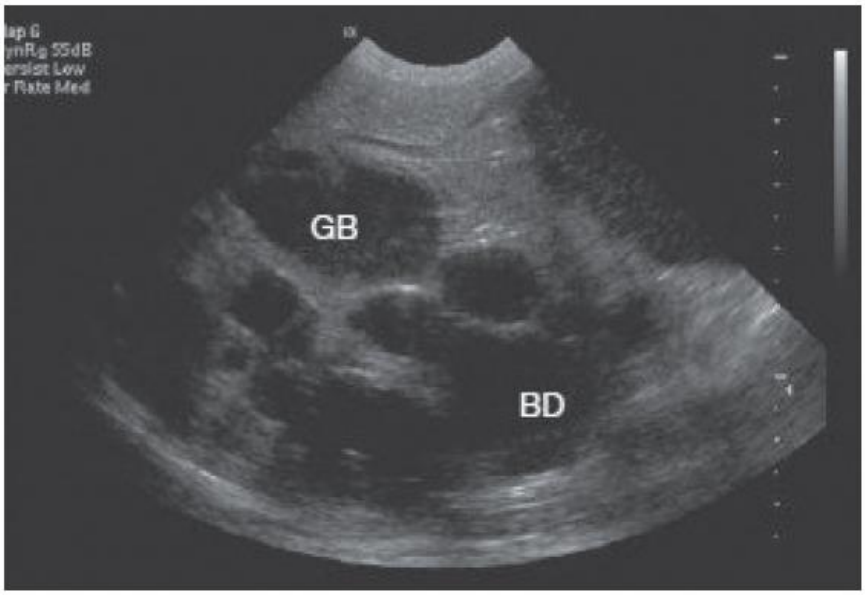

Figure 4. Distended bile ducts in a cat.[10] Reproduced with permission of Dr. George Voorhout in WSAVA Standards for Clinical and Histological Diagnosis of Canine and Feline Liver Diseases. Reprinted with permission.

Treatment

Corticosteroids, such as prednisolone (1-2 mg/kg/day), and UDCA (15 mg/kg/day) have been used as treatments for LC.[37, 40, 45] If there is a secondary bacterial infection, treatment should also include appropriate antibiotics for 4-5 weeks. The best sample for cytologic identification and culture of bacteria for sensitivity testing is bile, obtained by ultrasound-guided aspiration of the gall bladder.

Prognosis

Data from 23 cats showed that 70% survived for more than one year, with a mean survival time of 51.8 months.[24] Cats treated with prednisolone had better survival rates than cats treated with UDCA, with overall survival rates for 1, 2, and 3 years of 74%, 56%, and 35%, respectively, with a median overall survival time of 795 days.[45]

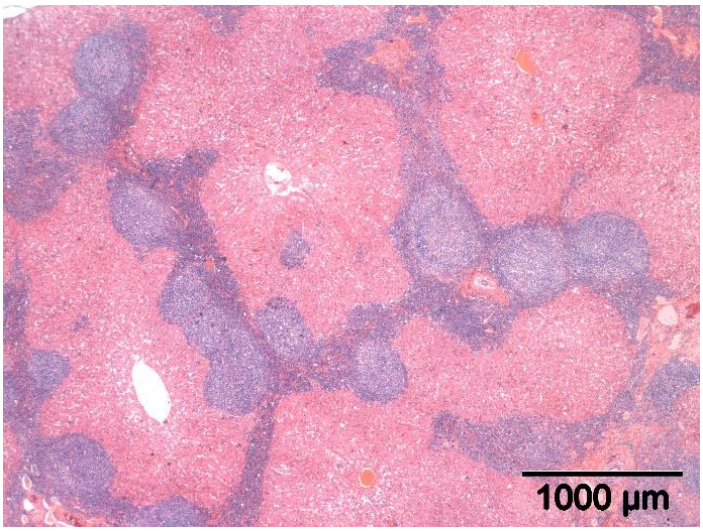

Figure 5. H&E staining of a liver biopsy of a cat with LC. Large infiltrates of small lymphocytes are present in portal areas, extending to portal-portal bridging.

1.3 Cholangitis due to liver flukes (LFC)

Aetiology

Liver flukes that cause feline cholangitis are members of the *Opisthorchiidae* or *Dicrocoeliidae*, and *Platynosomum spp.* are most common in (sub-)tropical regions.[10, 54, 55] Snails and lizards carry the liver fluke and cats become infected when they ingest these prey items.[10, 54] Adult flukes are present in the bile ducts and gallbladders of affected animals, which may lead to cholestasis (Figure 6).[6, 54]

Symptoms

Cats that suffer from liver fluke induced cholangitis have symptoms such as lethargy, weight loss, decreased appetite, painful abdomen, and vomiting.[55, 56] Icterus may be observed and is associated with cholestasis due to high numbers of parasites.[54]

Diagnosis

Microscopic examination of liver biopsies, bile, or faeces may sometimes show liver fluke eggs.[56, 57] Liver histology will show eosinophilic inflammation in the portal areas suggestive of LFC.[56] Chronic cholangitis caused by liver flukes leads to dilatation and fibrosis of bile ducts, which can be seen on ultrasound.[54] These images are very similar to those of chronic LC. A blood smear or CBC sometimes reveals eosinophilia, which is never seen in cats with LC.

Treatment

Liver flukes can be treated adequately with praziquantel, 20 mg/kg once daily for three days.[55]

Prognosis

With the correct therapy the prognosis is excellent.[58] Changes to the biliary tree, such as dilations, will remain and will increase the patient's sensitivity to ascending bacterial infections from the intestines.[33]

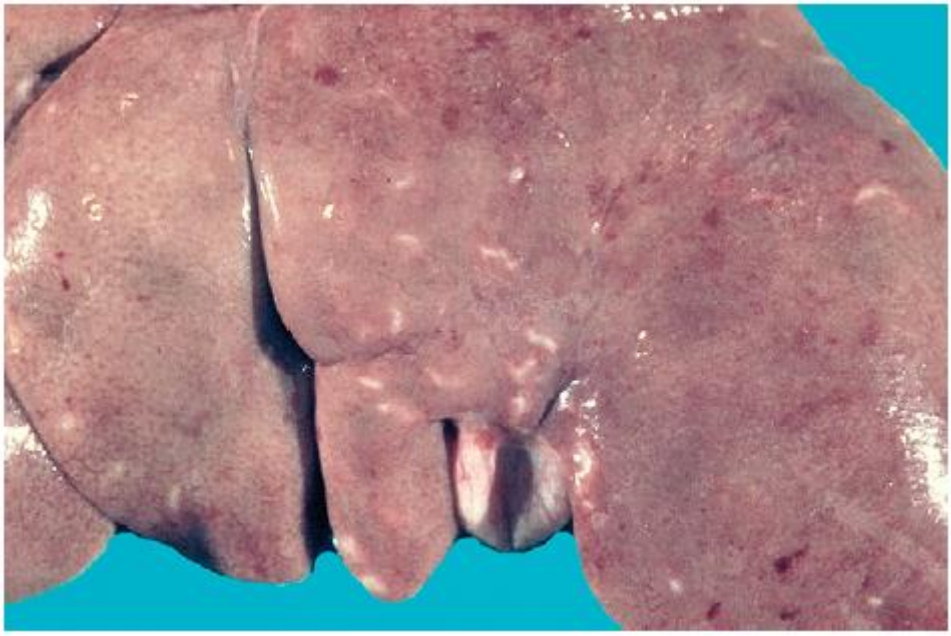

Figure 6. Cholangitis due to liver fluke infection.[55] (Reprinted with permission.)

1.4 Cholecystitis

Aetiology

Experimental feline cholecystitis was induced by platelet activating factor (PAF), *E. coli* lipopolysaccharide, and xenotransplantation of human gallstones into feline gallbladders.[34, 35] Furthermore, cytolytic agents in bile cause severe inflammation in cholestasis,[44] and bile salts cause tissue necrosis, aiding in bacterial colonization.[6] These bacteria may be either blood-borne or originate from the intestines.[6, 26] In cats, cholecystitis may be accompanied by neutrophilic cholangitis.[27] No bile peritonitis has ever been seen as a sequela to cholecystitis in cats.[59]

As discussed, there are no indications that cholecystitis and neutrophilic cholangitis are distinctly different entities in the cat.

Symptoms

Reported symptoms include inappetence, lethargy, vomiting, anorexia, diarrhoea, jaundice, cranial abdominal pain, weight loss, pyrexia, anaemia, and mild hyperbilirubinaemia.[27, 60]

Diagnosis

Ultrasound may demonstrate a thick, double-rimmed, oedematous gallbladder wall in acute forms of cholecystitis, and thinner, single-layered wall in chronic or mild inflammation.[61] In one study of six cats, cholecystoscentesis yielded bacteria (*E. coli, Streptococcus species, Clostridium species, Salmonella enterica*), as well as degenerate inflammatory cells in most cases.[44] Histology of the gallbladder wall shows neutrophils in the epithelium and/or wall.[10]

Treatment

Increased amounts of cyclooxygenase were detected in inflamed mucosa of feline gallbladders,[34] suggesting a role for NSAIDs in the treatment of cholecystitis.[35] Antibiotics, such as chloramphenicol, fluoroquinolones, amoxicillin/clavulanate, and metronidazole concentrate in bile and have good anaerobic activity.[6, 28]

Prognosis

Based on the only published study, the prognosis is good when the right antibiotic has been determined and administered.[27]

2. Diseases of the gallbladder

Anomalies in gallbladder shape are frequently seen on ultrasound and over 12% of cats have some sort of accessory gallbladder.[61, 62] Their existence is easily explained by the embryologic development of the feline gallbladder.[62] The partially subdivided gallbladder (*Vesica fellea divisa*) is most commonly seen, although a double biliary vesicle (*Vesica fellea duplex*, true duplex gallbladder) with two cystic ducts has been described in 10% of cases.[16, 62] Some feline gallbladders subdivide again, leading to a pseudo-multiplex form (*Vesica fellea multiplex*).[62] Most of the diagnosed aberrant gallbladders are incidental findings during

ultrasound, surgery, or at necropsy and cause no symptoms. Cholecystectomy can be performed if removal of the aberrant gallbladder is necessary.

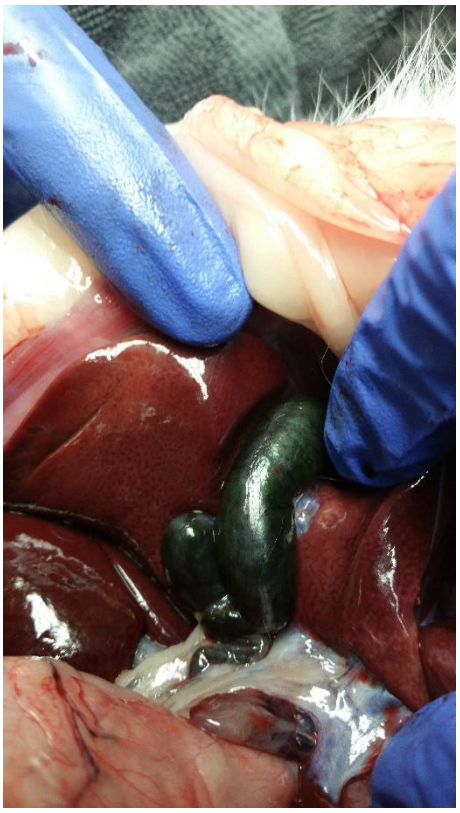

Figure 7. Accessory gallbladder, incidental finding at necropsy.

2.1 Cholelithiasis

Aetiology

Choleliths are encountered infrequently and account for less than 1% of canine and feline cases of liver disease combined.[6] They are much more common in humans and the majority of human gallstones are predominantly comprised of cholesterol, with pigment stones being much rarer.[63] Conjecturing from the knowledge of the pathogenesis of choleliths in man and prairie dog, contributing factors can be:

- bile supersaturation
- precipitation of a nucleus (nidi)
- mucin hypersecretion
- aberrant pH of bile

- biliary stasis or aberrant gallbladder motility
- altered bile composition
- cholecystitis
- cholangitis
- dietary factors
- biliary bacterial infections
- haemolysis.[42, 44, 64-66]

Gallstones, or choleliths, are sometimes encountered in cases of EHBDO.[19] They consist of calcium carbonate or palmitate, and amorphous plugs of bile salts and cholesterol.[19] Other studies have shown stones to be composed of bilirubin,[42] calcium carbonate, oxalate, phosphate, and magnesium,[12, 60] calcium carbonate and calcium bilirubinate,[44, 63] cholesterol, bilirubin derivatives, and calcium salts,[6] or,a combination of calcium carbonate, calcium bilirubinate and cholesterol.[64] Based on their composition, most feline stones are classified as black (based on bilirubin) or brown (bacterial degradation of biliary matter) stones, also known as pigment gallstones.[66]

Altered gallbladder motility, excess mucin production, and ascending bacterial infection might have contributed to stone formation in the case reported by Elwood and colleagues.[63] No definitive causal relationship has been determined between the presence of gallbladder sludge and the formation of stones.[20]

Symptoms

Symptomatic cholelithiasis is not encountered frequently in the cat,[6] and symptoms might be associated with the cause or consequence of cholelithiasis rather than the choleliths themselves.[63] Vomiting has been reported, as well as dehydration, anorexia, icterus, and lethargy.[42, 44, 64] Interestingly, most cats with choleliths are male.[64]

Diagnosis

Bile can be examined cytologically for cells, crystals, and bacteria.[63] Radiographs, ultrasound and cholecystography with contrast can be used to visualize gallstones.[6, 63, 64]

Treatment

Litholytic and choleretic agents, such as UDCA, are used in treating asymptomatic cholesterol gallstones in humans. UDCA has been used safely in cats at a dosage of 15mg/kg body weight once daily.[45] In contrast, pigment stones are removed surgically. Surgical removal of the gallbladder has been shown to prevent recurrence of stone formation.[63, 65] In cats, surgery to remove stones, the gallbladder or divert the biliary tract has been used successfully (see Box 5 for commonly used surgical techniques).[19, 42, 63, 64]

Prognosis

The prognosis is guarded since choleliths may be the cause, or the consequence of disease. The underlying or resulting disease will then determine the outcome. When cats survive surgery and recuperate well, the prognosis is favourable.

2.2 Gallbladder Mucocele

Aetiology

Mucous glands in the epithelium of the gallbladder protect against the deleterious effects of bile acids. Cats have fewer mucous glands than dogs, which may explain why mucoceles have been reported more frequently in dogs than in cats.[61] In total, three feline cases have been described.[16, 19, 67] In dogs, mucoceles have a distinct striated pattern but no such pattern was reported in the presentation of three cats.[10] Mucous gland hyperplasia was described in one feline case.[61]

In a case report by Bennett et al, concurrent hepatic lipidosis was reported.[67] Dyslipidaemias, possibly induced by excessive glucocorticoids, have been implicated in the formation of canine gallbladder mucoceles.[68] Certain breeds (Shetland sheepdog), gene mutations and endocrinopathies (hypothyroidism and hypercortisolism) have been associated with canine gallbladder mucoceles.[68, 69]

Symptoms

Symptoms may include vomiting, anorexia, lethargy, weight loss, and icterus.[16, 67] These could have been associated with concurrent diseases, such as EHBDO, hepatic lipidosis, and cholestasis, and not with the mucocele per se.

Diagnosis

Gallbladder mucoceles can been seen with abdominal ultrasound. Cholecystoscentesis usually does not yield any fluid because of the thick, inspissated bile.[67] Histology of the gallbladder wall would show cystic mucinous hyperplasia.[10, 67]

Treatment

Surgical removal of the gallbladder was described by Woods et al.[16] Other options include cholecystojejunostomy,[67] and choleretic drugs, such as UDCA.[16] For dogs, the prevailing opinion is that cholecystectomy is the most effective treatment.[70]

Prognosis

Two of the three cats reported in the literature did well.[16, 67] Possible complications include rupture of the gallbladder and recurrence. The prognosis is guarded in cases of feline EHBDO and hepatic lipidosis. High mortality rates have been reported with biliary diversion surgery in cats.[12]

2.3 Gallbladder Infarctions

Aetiology

Thrombosis of the cystic artery, a branch of the hepatic artery and the sole arterial blood supply to the gallbladder, may cause gallbladder infarcts.[10] Theoretically, torsion of the gallbladder may also cause arterial occlusion and infarction. The fundus of the gallbladder has been named explicitly as the site most prone to rupture as a sequela to ischemia.[59] Necrotic areas and ischemia were reported before, but the authors of that study linked them to anaerobic bacterial infection.[27] Gallbladder infarcts have been described in a series of dogs, but not in cats.[71]

The bile peritonitis that is expected to follow gallbladder infarction and necrosis is rarely encountered in feline practice and only reported once (0.3%) in 396 cases studied.[72] This might be due to excellent regenerative properties of the biliary epithelium, as has been shown in dogs.[67]

Symptoms

Symptoms reported (in dogs) include vomiting, icterus, anorexia, pyrexia, and diarrhoea.[71] Bile peritonitis in two cats was associated with anorexia and vomiting.[59]

Diagnosis

Ultrasound can help in identifying abnormalities in the gallbladder, as well as in showing poor abdominal detail suggestive of abdominal effusion.[59, 71] Surgery confirmed ruptured and distended gallbladders, but no torsions were encountered.[71] Histology of the gallbladder wall shows necrosis and thrombosis of the artery.[10]

Cytology of abdominal effusion in bile peritonitis has revealed predominantly neutrophils and macrophages with golden, green-brownish material in the cytoplasm of macrophages or in the background.[72] Bile leakage was shown to be diagnosed effectively by a more than two-fold increase in the bilirubin concentration of the effusion compared to serum.[59]

Treatment

Damaged gallbladders must be surgically removed and trauma causing bile peritonitis must be surgically repaired. Supportive therapies, such as fluid therapy and force feeding, might be required to assure the patient's recovery.

Prognosis

Conjecturing from the scant information available, the prognosis is guarded. Thrombosis might be caused by vascular problems which may have a far greater impact on the cat than gallbladder infarction. Biliary disease in cats is associated with nausea, vomiting and anorexia, which predisposes the patient to hepatic lipidosis. Bile salts are toxic to tissues leading to tissue necrosis and the growth of bacteria.[6] Furthermore, biliary diversion surgery has been shown to be associated with high mortality rates.[12, 73]

3. Neoplasia

Aetiology

In cats, neoplasms of the biliary system occur more frequently than neoplasms of hepatic cell origin.[74] Bile duct tumours were classified as (cyst)adenoma or adenocarcinoma, and gallbladder tumours were either adenomas or adenocarcinomas.[75] EHBDO was associated with neoplasia of biliary origin and tumour types found were carcinoma of the biliary tract and unclassified pancreatic/biliary tract carcinoma.[19] In another study, 41% of the tumours studied were cholangiocellular tumours,[76] although primary liver tumours account for only 1.0-5.5% of all tumours in cats.[75, 76]

Cholangitis was present concurrently in 3/25 cats suffering from cholangiocellular tumours, whilst another three cats were found to suffer from autosomal dominant polycystic kidney disease (ADPKD) concurrently.[76] It has been proposed that cholangitis may be implicated in the aetiology of cholangiocellular feline tumours.[76] Bile duct adenomas were also found to be associated with bile duct adenocarcinomas, suggesting that adenoma, metaplasia, and dysplasia may be viewed as precursor lesions in cats.[74] This same study found no choleliths in the cats with either gallbladder carcinoma or bile duct adenocarcinomas.[74]

Lymphoma and mast cell tumours were most frequently found as concurrent neoplasms, although lymphoma of the gallbladder is extremely rare.[47, 74] There is a single case report of urinary bladder lymphoma associated with concurrent involvement of the gall bladder.[77]

Feline neuroendocrine epithelial tumours have been reported, possibly the result of a malignant transformation of hepatic stem cells.[74, 75]

Symptoms

Male cats are overrepresented for biliary adenocarcinomas and cystadenocarcinomas, with up to 38% of cats being asymptomatic.[75] In symptomatic cases, clinical signs are similar to those seen in other hepatobiliary disorders, including icterus.[75] Paraneoplastic alopecia has been reported in two cats with hepatocellular carcinoma and bile duct carcinoma.[78, 79]

Diagnosis

Severe thickening of the gallbladder wall or common bile duct (CBD) was observed on ultrasound.[19, 77] Radiographs, cytology, and tissue biopsy may all be helpful in identifying these lesions.[75]

Treatment

A large part of the liver can be surgically resected without impairing hepatic function, so surgical removal of focal lesions seems to be the best choice. Systemic chemotherapy is relatively ineffective.[75, 80]

Prognosis

Up to 65% of primary hepatobiliary tumours in cats were found to be benign,[75] but malignant biliary tumours were found to have higher metastatic rates than hepatocellular carcinomas.[74]

4. Cystic diseases

Cysts in the liver always arise from the bile system, and are therefore also biliary diseases.

4.1 Solitary Biliary Cysts

Case reports have shown biliary cysts to be present in cougars, lions, leopards, as well as in domestic cats.[43, 44, 81, 82]

Aetiology

Extra-hepatic solitary cysts of the biliary system are rarely found in cats.[43] To our knowledge, no aetiology has been suggested. In humans, where the condition is also encountered, several aetiologies have been proposed:

- differences in epithelial cell proliferation which cause the area with faster proliferation rates to distend
- reflux of pancreatic enzymes into the cystic duct because of a defect in the pancreaticobiliary sphincter
- a localized weakness in the wall of the biliary duct.[83]

Experimentally, biliary cysts have been created in puppies, but not in adult dogs, by scraping the mucosa of the bile duct followed by CBD ligation.[83]

Biliary cystadenomas, an uncommon benign liver tumour of older cats, may be encountered as a focal or multifocal cystic lesion.[84] Their tissue of origin is still unknown and they are speculated to arise from "small retention cysts associated with embryologic bile ducts that do not connect to the main biliary tree".[84]

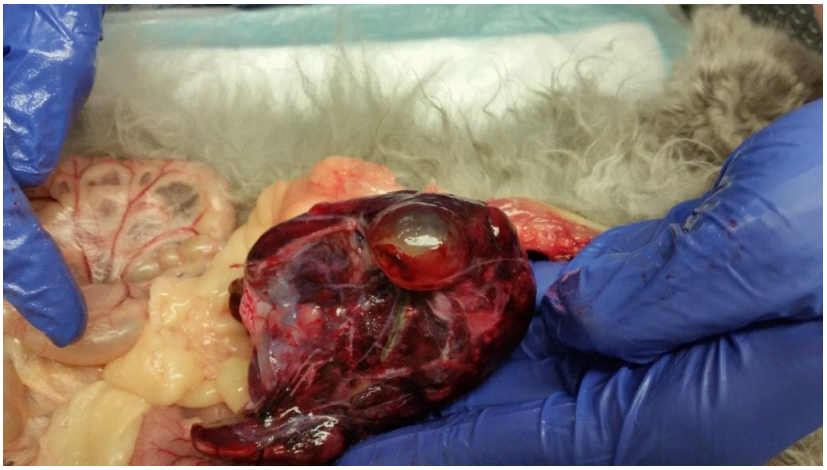

Figure 8. Liver cyst, incidental finding at necropsy. Courtesy Marleen Assink, DVM.

Symptoms

Reported symptoms include nervousness, polyphagia, weight loss, and urinating in the house due to increased pressure on the bladder by the cyst.[43] Chronic vomiting and anorexia have been reported in one case, although this cat also suffered from suppurative cholangitis which could cause these symptoms.[44]

Hepatobiliary cysts have been found as intrapericardial cysts following diaphragmatic hernias, causing progressive exercise intolerance and coughing.[82]

Biliary cystadenomas were associated with anorexia, lethargy, weight loss, occasional vomiting and weakness, but no abdominal pain or discomfort.[84]

Diagnosis

Large cysts have been found during clinical examinations.[43, 84] Diagnostic imaging might be helpful in locating biliary cysts, and some are found at necropsy.[81, 84] Clinical laboratory data

may be normal but elevations in liver enzymes, such as alanine aminotransferase, alkaline phosphatase and bilirubin have been reported.[43, 84] Clotting times were reported to be within normal reference values.[43] Fluid analysis revealed high protein content and limited mixed bacterial growth.[43] True cysts usually contain bile.[44, 84]

Treatment

Complete or partial surgical resection is the treatment of choice when the cyst needs to be removed because of mechanical compression of adjacent structures.[43, 84]

Prognosis

For biliary cystadenomas, the prognosis is good due to its benign character.[84] However, as the cause of solitary biliary cysts is usually unknown recurrence might occur and long-term outcome is uncertain.[43]

Figure 9. Biliary cyst.[10] (Reprinted with permission.)

4.2 Congenital Biliary Cystic Disease

Aetiology

Cystic liver disease in cats is most often multifocal and has been found in 68% of cats with polycystic kidney disease (PKD).[43, 85] Persian cats inherit PKD as an autosomal dominant trait. Besides hepatic cysts, liver fibrosis is also commonly seen in cats with PKD (Figure

8).[85] Increased epithelial proliferation is involved in the formation of renal cysts in Persian cats. This occurs intermittently and in the early stages of cyst formation.[86]

Symptoms

The disease in Persian cats is clinically similar to human autosomal dominant polycystic kidney disease (ADPKD).[10, 86] Clinical signs are mostly a result of the renal disease, and not the hepatic cysts.

Diagnosis

Both renal and hepatic cysts may be visualised ultrasonographically. The hepatic lesions of both cystic malformation and fibrosis are diagnosed by liver histology.

Treatment

There is no treatment for PKD. Breeding programmes and screening have contributed to reducing the number of affected cats over the years.

Prognosis

The prognosis is fair, since most cats only start showing clinical symptoms at an advanced age.

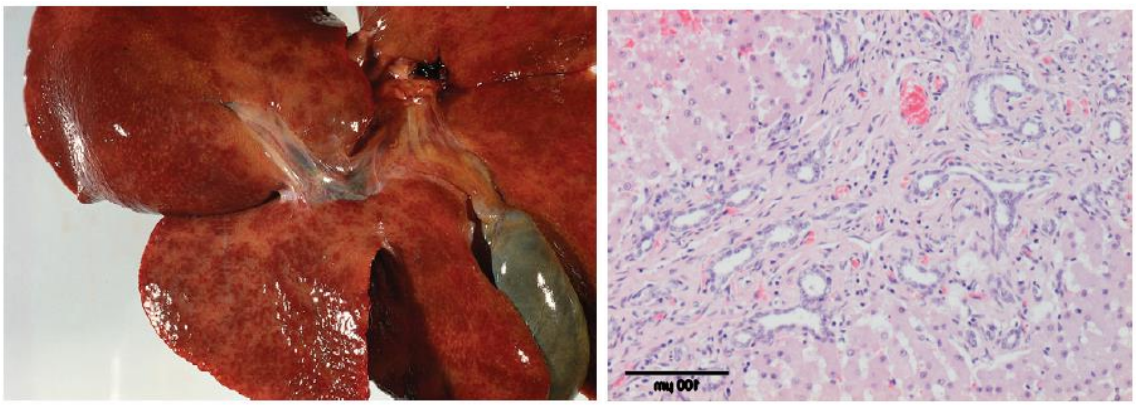

Figure 10. Juvenile biliary cystic disease with hepatic fibrosis (left: macroscopy; right: microscopy).[10] (Reprinted with permission.)

Key Points

- Biliary disease is more common in cats than diseases of the hepatic parenchyma.
- Due to limited glucuronide conjugation of drugs and toxins, cats are prone to hepatotoxic injury. Some frequently used veterinary drugs can be toxic to cats.
- Enteric bacteria have been implicated in inflammatory liver disease.
- Changes to the biliary tree, such as dilations, will remain and will increase the patient's sensitivity to ascending bacterial infections from the intestines.
- One in eight cats have some sort of accessory gallbladder. Usually, no clinical symptoms occur and these anomalies are found accidentally on ultrasound or at necropsy.
- Most cats with choleliths, lymphocytic cholangitis, and neoplasia are male.
- The gallbladder has only a single artery supplying blood, but infarcts have never been described in cats.
- Biliary cysts are encountered in many felines, not just domestic cats. In domestic cats, liver cysts are linked to polycystic kidney disease.
- Histology, cytology and culturing are valuable diagnostic tools in feline biliary disease.

Box 1: Hepatotoxic drugs in daily practice

methimazole[2]
carbimazole[2]
oral diazepam[2]
acetaminophen (paracetamol)[23]
cyclosporine A and cloxacillin[15]
rifampicin[15]
cloxacillin[15]
itraconazole[87]
ketoconazole[87]
tetracycline[87]
glipizide[87]
stanozolol[87]

Box 2: Biochemical profile: commonly encountered laboratory findings in biliary disease[23, 88]

Liver enzyme activity may be abnormally increased in liver disease, systemic diseases which influence the liver ("reactive hepatopathy"), and as a sequel to the administration of certain drugs.

Name	**Location**	**Increases with**	**$t_{1/2}$**	**Increases in**
ALT (alanine aminotransferase)[1]	Cytosol hepatocytes, cardiac muscle, kidney	Hepatocellular necrosis, inflammation, acetaminophen toxicosis	3.5 hours	The first 5 days after injury (within 24 hours in acetaminophen toxicosis)
AST (aspartate aminotransferase)[2]	Cytosol hepatocytes and mitochondria, skeletal heart muscle, kidney, brain, small intestine, spleen		1.5 hours	The first 3 days after injury
ALP (alkaline phosphatase)[3]	Membrane hepatocytes, bone, intestine, kidney, placenta	Neonates (colostrum), young animals (bone growth), hyperthyroidism, inflammation and neoplasia, late pregnancy, lipidosis	6 hours	Within 2 days of EHBDO

[1] Mostly located periportal
[2] Mostly located periacinar
[3] Does not increase in case of glucocorticoid use in cats

γGT (γ-glutamyltransferase)	Kidney, pancreas, intestine, gallbladder, and membrane cholangiocytes			Within 3 days of EHBDO
LDH (lactate dihydrogenase)[4]	Skeletal muscle, heart, kidney, intestine, cytosol hepatocytes, lung, pancreas	diffuse severe hepatic necrosis or inflammation; higher in kittens than adults		
arginase[4]	Cytosol hepatocytes and mitochondria			
SDH (sorbitol dihydrogenase)[4]	Cytosol hepatocytes			
γ-globulins				
Bile acids		loss of functional hepatic mass		
Cholesterol				
Bilirubin				

[4] Not used routinely

(Table continued)

Name	**Decreases in**	**Especially useful in**	**Biliary disease and diseases of the gallbladder (with supporting references)**
ALT (alanine aminotransferase)[5]	2 to 3 weeks after recovery (within 72 hours in acetaminophen toxicosis)		• Biliary cyst, cholecystitis and neutrophilic cholangitis[27, 44] • LC[33, 40, 44] • Choledochal cyst[43] • AD-PKD[85] • EHBDO[19, 22] • Gallbladder sludge[20] • Cholelith[12, 42, 63, 64] • Avulsion of CBD[12] • Cholestasis[12] • Hepatic lipidosis[16] • Non-specified hepatobiliary disease[28] • Cholangitis[25] • LFC[55, 56] • Gallbladder mucocele and lipidosis[67] • Cholecystitis, choleliths, gallbladder rupture and peritonitis[60] • Hepatocellular carcinoma[78]
AST (aspartate aminotransferase)[6]	2 to 3 weeks after recovery	hepatic necrosis, cholangitis, chronic bile duct obstruction	• Biliary cyst, cholecystitis and neutrophilic cholangitis[27, 44] • LC[44] • Cholangitis[25] • LFC[55, 56] • Cholelith[64] • Hepatocellular carcinoma[78]

[5] Mostly located periportal
[6] Mostly located periacinar

ALP (alkaline phosphatase)[7]	2 to 3 weeks after recovery	cholestasis	• Biliary cyst, cholecystitis and neutrophilic cholangitis[27, 44] • LC[33, 40, 44] • Choledochal cyst[43] • EHBDO[19] • Gallbladder sludge[20] • Cholelith[12, 42, 63, 64] • Cholestasis[12] • Non-specified hepatobiliary disease[28] • Normal in most cases of NC with cholecystitis[27] • Cholangitis[25] • LFC[55, 56] • Gallbladder mucocele and lipidosis[67]
γGT (γ-glutamyltransferase)		cholestasis, interpret together with ALP	• Biliary cyst, cholecystitis and neutrophilic cholangitis[44] • LC[44] • EHBDO[19] • Cholangitis[25] • Cholecystitis, choleliths, gallbladder rupture and peritonitis[60]
LDH (lactate dehydrogenase)[8]			
arginase[4]			
SDH (sorbitol dehydrogenase)[4]			
γ-globulins			• LC[40] • LFC (globulins unspecified)[55] • Bile duct carcinoma[79]

[7] Does not increase in case of glucocorticoid use in cats
[8] Not used routinely

Bile acids			• Normal in EHBDO (preprandial)[22] • Normal in cholecystitis[6] • Cholestasis[6] • Choleliths[6] • LFC[6] • EHBDO[6] • Normal in trauma of lower biliary tract[6]
Cholesterol			• EHBDO[19] • Cholelith[64]
Bilirubin			• LC[33, 40, 44] • Choledochal cyst[43] • EHBDO[19, 22] • Gallbladder sludge[20] • Gallbladder mucocele and lipidosis[67] • Cholelith[12, 42, 64] • Avulsion of CBD[12] • Cholestasis[12] • Non-specified hepatobiliary disease[28] • Neutrophilic cholangitis and cholecystitis[27] • Cholangitis[25] • LFC[55, 56] • Cholecystitis, choleliths, gallbladder rupture and peritonitis[60]

Box 3: Biopsies and cholecystoscentesis: techniques for obtaining liver tissue and bile samples

Histopathology is the gold standard for diagnosing hepatobiliary diseases.[10] Ultrasound may aid in establishing a diagnosis in cystic diseases, cholestasis, NC, LC, LFC, and gallbladder mucocele. Bile cytology and/or culture may help in NC and LC.[10]

Liver biopsy techniques[89]

Liver tissue can be sampled during surgery or with the help of a large bore needle. The different techniques yield different amounts of tissue: laparoscopic cup biopsy forceps around 45 mg, a 14G Tru-Cut–type biopsy needle around 15 to 20 mg, an 18G needle biopsy around 3 to 5 mg. At least two or three biopsies from separate liver lobes should be obtained. A 16G needle is preferred and five to six portal areas should be present in order to establish a good histopathological diagnosis.[53]

Precautions:

- Fast the patient for 12 hours so the stomach is empty
- Perform coagulation tests to ensure there is no bleeding disorder
- Do not take a liver biopsy when fibrinogen concentrations are lower than 50% of the lower reference range
- In prolonged and severe EHBDO, administer vitamin K1 before taking a biopsy (1–5 mg/kg subcutaneously daily for several days)
- Do not use an automatic spring loaded biopsy gun in cats[90]; this may lead to lethal shock reactions, especially in non-anesthetised cats!
- Manual Menghini technique can be used in cats
- take surgical liver biopsies early since hepatocellular changes may result from prolonged anaesthesia, vascular changes, and handling of the intestines

Neoplastic masses may have necrotic centres, which makes it best to sample from the periphery of the lesion. Diffuse liver disease, including neoplasia may be sampled with FNA.

FNA

Ultrasound may help in determining the exact location for FNA. A blind technique may be used with appropriate locations including caudal to the costal arch or on the right side at the 10th intercostal space at the level of the rib-cartilage junction. There is no need for local or general anaesthetics and 3-inch disposable 20G-22G needles are recommended.

Percutaneous ultrasound-guided cholecystocentesis (PUC)[30]

PUC is a minimally invasive technique for obtaining a sample of bile (0.9–3 mL). A 22-gauge 1.5-inch needle connected to a 12-mL syringe has been used successfully. Ultrasound helps in guiding the needle into the gallbladder.[89] To help prevent leakage of gall bladder contents into the abdomen, attempt to completely empty the gallbladder.Confirm this with ultrasound. Bile in the peritoneal cavity causes a chemical peritonitis and predisposes the cat to bacterial infection.[6] PUC could cause leakage if there is complete obstruction of the common bile duct and should be avoided in cases where EHBDO is likely.

Box 4: Reported use of medical therapies for feline biliary disease

Disease and references	**Therapy**
NC[44]	Metronidazole 30mg/kg SID Amoxicillin 10mg/kg BID for 2 months
NC[27, 33]	Amoxicillin/clavulanate or ticarcilline/clavulanate for at least 4 weeks
NC[27]	UDCA and vitamin K
NC and cholecystitis[27]	Enrofloxacin 2.5 mg/ kg q12h SC
NC and cholecystitis[27]	Amoxicillin/clavulanate 45 mg q12h SC initially, then 50 mg PO q12h for 4 weeks in total UDCA for 2 weeks
NC and cholecystitis[27]	Amoxicillin 100 mg q8h IV and ciprofloxacin 30 mg q24h PO for 6 weeks in total UDCA 30 mg q24h PO
NC and cholecystitis[27]	Doxycycline 50 mg q12h PO Cephalexin intravenously for 7 days then orally 112.5 mg q12h PO for 4 weeks in total
Cholecystitis[35]	NSAIDs
Cholecystitis[6, 28]	Chloramphenicol Fluoroquinolones Amoxicillin/clavulanate Metronidazole
Gallbladder rupture, NC and cholecystitis[27]	Cefoxitin 120 mg q8h IM Amoxicillin 100 mg q12h PO
Choledochal cyst[43]	Pre-surgery: intravenous amoxicillin/clavulanate 60 mg q8h and metronidazole 30 mg q12h Post-surgery: oral amoxicillin/clavulanate 50 mg q12h and metronidazole 33.3 mg q12h UDCA 37.5 mg q24h S-adenosylmethionine 50 mg q24h vitamin E 10 mg q24h vitamin K 0.05 mg q24h

EHBDO[22]	a 4-week course of orally administered potentiated amoxicillin 15 mg/kg metronidazole 15 mg/kg Samylin™ 1 g Sachet, once daily
LC[45]	Prednisolone 1-2 mg/kg/day for 4-6 weeks after which the dose was gradually tapered
LC[45], mucocele, choleliths, cholestasis[16, 67]	UDCA 15 mg/kg/day
LC[40]	prednisolone (up to 4 mg/kg) for an initial period of 3 weeks after which the dose was gradually decreased over 6-8 weeks
EHBDO[42]	Amoxycillin-clavulanate 20 mg/kg IV tid Metronidazole 10 mg/kg IV bid Buprenorphine 30 mg/kg IM tid vitamin K1 0.5 mg/kg SC bid Cephalexin 15 mg/kg bid UDCA 10 mg/kg PO sid (antibiotics for a total duration of 4 weeks)
Lipidosis[67]	Metaclopramide at 0.05 mg/kg/h IV Ranitidine 1.5 mg/kg twice daily IV
Hepatic encephalopathy[67]	Lactulose 1 mL, 3 times per day

Box 5: Surgical techniques employed in biliary surgery in cats[12]

When surgery is necessary, either to establish a diagnosis, to prevent leakage of bile or to repair bile flow from the gallbladder to the intestines, several techniques have been used successfully:

- cholecystoduodenostomy
- cholecystojejunostomy
- choledochojejunostomy
- choledochotomy, with or without tube stenting
- duodenotomy with CBD lavage or cholecystotomy
- tube cholecystostomy
- primary gallbladder repair
- cholecystectomy

Cholecystoenterostomy is the method of choice, due to the small diameter of the CBD. Vomiting and anorexia are commonly seen after biliary surgery. Therefore, it may be recommended to place a feeding tube while the cat is under anaesthesia.
Cholecystoenterostomy creates an open connection between the enteric lumen and the bile and pancreatic ducts. An inevitable complication is therefore infection of the bile ducts and pancreas, requiring repeated antibiotic treatment. This procedure often does not lead to significant improvement or prolongation of life expectation.

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CHAPTER 3 Detection of bacterial DNA in bile of cats with lymphocytic cholangitis

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1. Introduction

Lymphocytic cholangitis (LC) is a chronic inflammatory process in the biliary tree which causes dilatations of bile ducts, progressive fibrosis and may eventually end in cirrhosis. It is one of the most common inflammatory hepatic diseases in cats.[1, 2] Its etiology is still unknown, although bacteria such as *Bartonella* spp. and *Helicobacter* spp. have been linked to the disease.[3, 4]

Recently, molecular methods based on the amplification of the 16S ribosomal RNA gene have been used to determine bacterial presence in various substrates, such as contents of cecum and colon, coronary plaque, fecal samples, cerebrospinal fluid, and bone.[5-9] To our knowledge, these techniques have not been applied to bile samples of cats suffering from LC. Therefore, the aim of this study was to investigate the presence of bacteria in bile from LC patients by using a molecular screening method, and to determine their identity.

A 16S rDNA gene based PCR and denaturing gradient gel electrophoresis (DGGE), followed by direct sequencing, were used for this purpose.[10, 11] Additionally, we screened for Helicobacter spp. since its DNA was detected in patients' bile in an earlier study.[3]

2. Methods and materials

2.1. Collection of bile samples

Thirty-nine bile samples were collected by ultrasoundguided cholecystocentesis from 13 cats with LC between January 2000 and February 2009 at the University of Utrecht Clinic for Companion Animals in The Netherlands (UUCCA).

Samples were transferred to sterile cryovials and stored at -70 °C until further analysis. Diagnosis of LC was based on clinical presentation (n = 13), blood analyses (n = 13), liver biopsies (n = 10) and ultrasound examination (n = 13) (WSAVA Liver Standardization Group, 2006).

Control samples were collected from 14 cats at necropsy at the Pathobiology Department of Utrecht University between March and October 2009. All control animals presented with other pathology findings than LC. Samples were transferred to sterile tubes and stored at -20 °C until further analysis. Additionally, a bile sample from a cat with neutrophilic cholangitis where *E. coli* was isolated with culturing techniques was included as positive control.

All procedures were approved by the responsible ethical committees as required under Dutch legislation.

2.2. DNA isolation

Bile samples were thawed at room temperature and 200 μg was used for further analysis. DNA was isolated by using the FastDNA1 SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) and the Precellys1 24 Instrument (Bertin Technologies, Montigny-le-Bretonneux, France). A NanoDrop ND-1000 spectrophotometer (NanoDrop1 Technologies, Wilmington, DE, USA) was used to determine DNA concentration.

2.3. PCR amplification

Primers S-D-Bact-0968-GC and S-D-Bact-1401-a-A-17 were used to amplify the V6–V8 variable regions of the bacterial 16S rDNA (Table 1).[11] A GC rich sequence (‘‘GC clamp’’) was attached to the 50 end of the primer to improve detection of single-base changes with DGGE.[12]

Table 1. Characteristics of primer pairs.

Primer	**Species detected**	**F/R**	**Sequence (5’→ 3’)**
S-D-Bact-0968-GC	Eubacteria	F	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC[a]
S-D-Bact-1401-a-A-17		R	CGG TGT GTA CAA GAC CC
HelicoF	*Helicobacter* spp.	F	AAC GAT GAA GCT TCT AGC TTG CTA G
HelicoR		R	GTG CTT ATT CGT TAG ATA CCG TCA T
HP-ACT-1	*Helicobacter pylori*	F	CTT GCT AGA GTG CTG ATT A
HP-ACT-2		R	TCC CAC ACT CTA GAA TAG T

F/R, forward or reverse primer

[a] A GC clamp was attached to the 5’end of the primer to facilitate the distinction of single-base changes with DGGE

PCR was performed with 1 μl of sample (DNA concentrations varied between 1.2 and 44.1 ng/μl), 35.75 μl of H2O, 10 μl of Green GoTaq1 reaction buffer containing 7.5 mM MgCl2 (Promega Benelux, Leiden, The Netherlands), 1 μl of 10 mM dNTPs (Promega), 0.2 μM of each of the primers, forward primer S-D-Bact-0968-GC (Biolegio, Nijmegen, The Netherlands) and reverse primer S-D-Bact-1401-a-A-17 (Biolegio), and 0.25 μl of 5 U/μl GoTaq1 (Promega, Madison, WI, USA) using a PCR program specifically designed for DGGE (2 min initial denaturation at 94 °C, 35 cycles of 30 s denaturation at 94 °C, 40 s annealing at 56 °C, 1 min elongation at 72 °C, 5 min final elongation at 72 °C). All PCR reactions were performed in a 50 μl volume with a G-storm thermal cycler GS1 (Gene Technologies Ltd., Essex, UK). The integrity of the DNA was checked by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide. DNA concentration was determined with a NanoDrop ND1000 spectrophotometer (NanoDrop1 Technologies). Characteristics of primer pairs.

2.4. DGGE analysis of PCR amplicons

The microbial content of the bile samples was determined directly by DGGE.[10] We used the DCodeTM system (Bio-Rad, Veenendaal, The Netherlands) for separation of the amplicons. The analysis was performed in 8% (v/v) polyacrylamide gels containing 37.5:1 acrylamide–bisacrylamide. A 30–60% denaturing gradient was applied to separate the PCR amplicons. The electrophoresis ran for 16 h at 85 V in 0.5 TAE running buffer at a constant temperature of 60 °C. DNA bands were visualized by silver staining.[13] (Sanguinetti et al., 1994).

2.5. Amplification of Helicobacter DNA

Amplicons of *Helicobacter* spp. and *H. pylori* were generated as described previously (Table 1).[14, 15] A nested PCR was performed according to the protocol described by Boomkens *et al.*[3, 16, 17] *Helicobacter pylori* cultures (provided by Dr. H.Kusters, UMCU, The Netherlands) were used as positive controls.

2.6. Sequence analysis

The DGGE was repeated with the positive samples. The silver staining protocol was applied partly to enable sequencing of excised bands (Baseclear, Leiden, The Netherlands). The Basic Local Alignment Search Tool (BLAST) was used to align the sequences with known sequences in public databases (NCBI, via http://blast.ncbi.nlm.nih.gov/Blast.cgi). All samples

were tested blind. After obtaining the results, the blinding code was broken and samples were linked to identifying data.

2.7. Statistical analysis

Statistical analyses were performed with SPSS (version 16.0.1, SPSS Inc. Chicago). Chi-square tables and Fisher's exact tests were used to compare proportions between patients and controls. Differences in median ages between patients and controls were tested with the non-parametric Mann–Whitney U test. Results with $P < 0.05$ were considered to be statistically significant.

3. Results

The patients' median age was 11 years (range, 14 months to 14 years) and did not differ significantly ($P = 0.077$) from the median age of control animals (5 years; range, 6 months to 13 years). Among patients, ten were castrated males, two spayed females and one intact female. This did not differ significantly ($P = 0.462$) from the control group, which included seven castrated males, one intact male, two intact females, and three spayed females. Patient breeds included Domestic Shorthair ($n = 6$), Norwegian Forest Cat ($n = 2$), Siamese ($n = 2$) and one each of Cornish Rex, Persian and Sphynx, while the control breeds were Domestic Shorthair ($n = 9$), unknown ($n = 2$) and one each of Ragdoll, Siamese and Turkish Angora. The breed distributions did not differ significantly ($P = 0.387$) between patients and controls.

Nine cats supplied one sample each, two cats had two samples taken each and one cat had three samples taken. Additionally, one patient was followed for more than 4 years to determine the effect of repeated antibiotic treatments. As a result, 23 bile samples were collected from this cat over the years.

The sequence results of the DNA bands excised from the DGGE gels showed bacterial DNA to be present in seven bile samples (18%) from five LC patients (38%). None of the control samples showed any bacterial presence ($P < 0.001$). The positive control sample that contained *E. coli* was identified correctly. DGGE gels and subsequent sequencing revealed a 16S rDNA V6–V8 amplicon of only one single bacterial species in samples from four patients. In the fifth patient, however, 16S rDNA from two different bacterial species was detected. Three bile samples from one patient, collected during three successive visits to the UUCCA, harbored *E. coli* (Table 2, patient 1).

Table 2. Results of DGGE and Helicobacter spp. nested PCR for LC patients and controls.

LC patients	Results	Control animals	Results
1[a]	*E. coli*; *Helicobacter* spp.	Positive control with neutrophilic cholangitis and *E. coli* cultured from bile sample	*E. coli*
2	*Micrococcus* sp.	Positive control for *Helicobacter pylori*	*Helicobacter pylori*
3	*Streptococcus* so.	a	
4	*Jeotgallicoccus pinnipedialis*; *Citrococcus* sp.; *Helicobacter* spp.	b	*Helicobacter* spp.
5	*Brevibacterium casei*	c	*Helicobacter* spp.
6	*Helicobacter* spp.		*Helicobacter* spp.

[a] Three successive samples of this patient harbored *E. coli*

Interestingly, no 16S rDNA V6–V8 amplicons of *Helicobacter* spp. were identified with DGGE. Only by repeating the much more sensitive nested PCR described previously with all bile samples (n = 54; patient samples, n = 39; control samples, n = 15) were *Helicobacter* spp. DNA fragments shown to be present in six bile samples.[3, 14, 15] Three positive samples belonged to three LC patients (3/13 patients, 23%), and the other three positive samples belonged to three control animals (3/14 control animals, 21%) (Table 2). No significant differences were found between patients and control animals when these proportions were tested ($P = 0.535$).

4. Discussion

In the study reported here, analysis of the variable V6– V8 regions of the bacterial 16S rDNA revealed that bacteria from various species were present in bile of LC patients and not in bile of control animals.

E.coli from the positive control sample was correctly identified with the molecular technique used here. Furthermore, *E. coli* was detected in three successive bile samples of one cat with LC. These results were in accordance with earlier findings from culturing performed when this patient was first seen at the UUCCA.[18] The presence of *Streptococcus* sp. was also corroborated by traditional culturing techniques from when this cat was first seen (unpublished results from patients' medical file). Our results suggest that the molecular technique used here can successfully be used on feline bile samples.

E. coli and *Streptococcus* spp. are common bacteria in humans and animals. *Brevibacterium* spp. are traditionally found in dairy products and *B. casei* has also been found on human skin.[19, 20] *Jeotgalicoccus pinnipedialis* has only been isolated from the mouth of a southern elephant seal, although other *Jeotgallicoccus* spp. have been found in a traditional Korean fermented seafood condiment.[21, 22] Recently, it has been suggested that *Jeotgalicoccus* spp. might be abundant in our environment but unrecognized because of their resemblance to staphylococci.[23] *Micrococcus* spp. might be isolated from soil,water and human skin, while *Citrococcus* spp. have been isolated from soil and groundwater.[24-26] Thus, cats may have become infected by interacting with their owners, by consuming dairy products or seafood, or simply by roaming outdoors. Because cats are known to groom frequently, bacteria on their fur can easily be ingested.[27, 28] Thus, all bacteria identified in this study are likely to stem from the feline digestive tract. This suggests a retrograde invasion of the common bile duct, which might be facilitated by dilatations and reduced peristalsis due to chronic inflammation of the bile ducts.[29]

Based on the variety of organisms found and the presence of *Helicobacter* spp. DNA in both patients and controls, bacteriobilia in LC patients seems not to be the cause but rather a consequence of the disease. Therefore, treatment with antibiotics is unlikely to be a successful strategy, as was corroborated by the results of the longitudinal study of antibiotic treatment in the patient we studied for more than 4 years.[18]

Only one bacterial species could be detected in bile samples from four patients (67%), while two bile samples were invaded by two or three different bacterial species. To the authors'

knowledge, no studies have reported on using a molecular screening method for feline bile samples before. However, bacterial culture results from feline bile samples have been published.[30] Our results correspond with the results from this earlier study, where the majority of feline liver and bile samples also harbored a single bacterial species.[30] Wagner *et al.* found 36% of feline bile cultures positive for bacterial growth, whereas our study demonstrated the presence of bacteriobilia in 46% (6/13) of LC patients. Additionally, the bacterial species identified in our study differ from the bacteria found earlier. This may be due to the fact that Wagner *et al.* used traditional culturing techniques which may have prevented them to detect species that are not easily cultured, such as *Helicobacter* spp.[31] Additionally, bacterial overgrowth of one species can cause other bacterial species to remain undetected when culturing. Furthermore, the molecular technique used in this study can detect DNA of both living and dead bacteria, while culturing methods will only detect living bacteria. Therefore, based on the differences between traditional culturing techniques and bacterial DNA detection with PCR, a direct comparison of our results with the results from this earlier study is not possible.

5. Conclusion

In this study, we have successfully used molecular methods based on the amplification of the 16S ribosomal RNA gene on feline bile samples to show that bile of cats with LC is not sterile. This is probably due to the fact that the inflammatory process in the biliary tree causes dilatations. As a result, bacteria can easily migrate from the intestines via the common bile duct. The diversity of species identified and the presence of *Helicobacter* spp. DNA in both patients and controls suggest that bacteriobilia is secondary to the disease and is not the cause of LC.

Conflict of interest statement

The authors have declared that they have no conflicts of interests.

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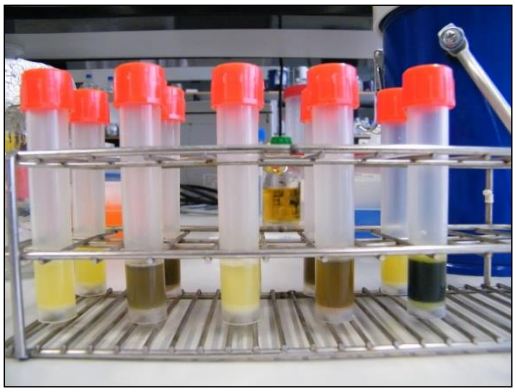
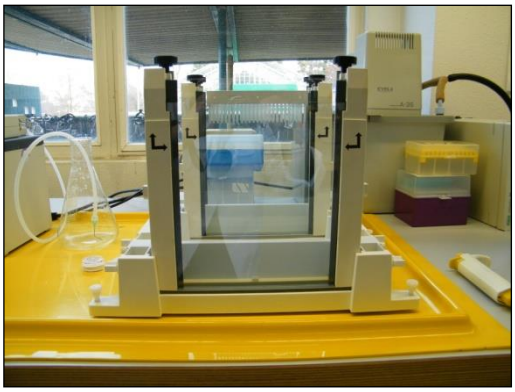
Appendix - Foto impression of equipment used in DGGE analysis

1. Bile samples for testing the DNA isolation kit

2. Test samples and contents of DNA isolation kit



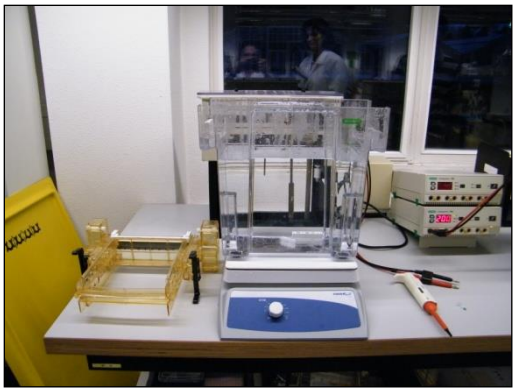
3. Bile samples being processed



4. Building a construction for casting DGGE gels

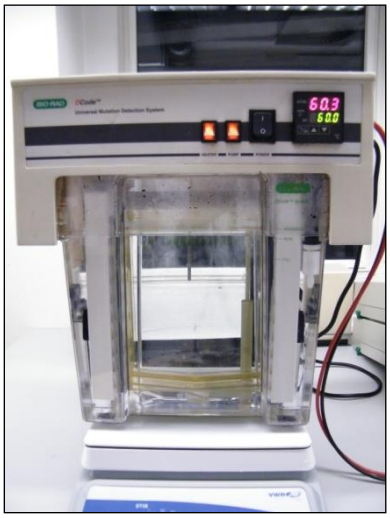
5. Pump for creating a denaturing gradient in gels

6. Casting the plug to prevent leakage

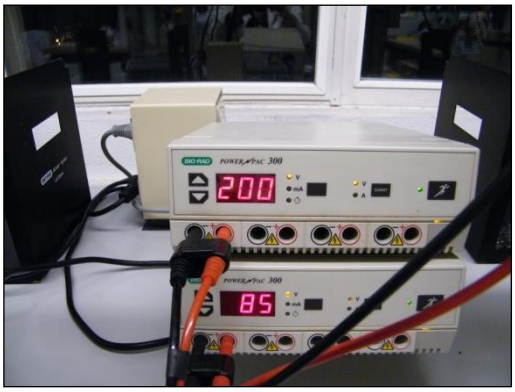


7. The electrophoresis machine

8. The samples have been pipetted in

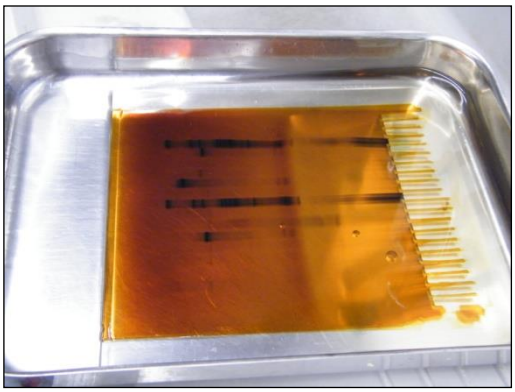


9. The electrophoresis machine running …



10. … at 85 Volt for 16 hours

11. The silver staining in progress



12. Developing the gel, bacterial DNA in black

CHAPTER 4 Retrospective comparison of prednisolone and ursodeoxycholic acid for the treatment of feline lymphocytic cholangitis

C.M.A. Otte, L.C. Penning, J. Rothuizen, R.P. Favier

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Introduction

The development of effective therapies for treating feline lymphocytic cholangitis (LC) is challenging, since its aetiology remains undefined. It is, however, one of the most common inflammatory hepatic diseases in cats, which are the most popular pets in the Western world.[1-4] Usually, LC is a chronic disease that affects the biliary tree and progresses slowly over months or years. The hepatic lesions are characterized by aggregates of inflammatory cells in portal tracts, and in and around bile ducts.[4]

Early signs include nausea and vomiting, changes in appetite, and gradual weight loss, and jaundice is often present later in the course of disease.[5, 6] Blood analysis sometimes reveals elevated hepatic enzymes and bile acids, but hypergammaglobulinaemia is the most consistent finding.[6] Chronic inflammation in the bile ducts causes dilatations and strictures and might eventually lead to fibrosis and cirrhosis.[7, 8]

Over the past decades, only a limited number of scientific articles have been published concerning feline LC and most of these have described individual cases and histopathological lesions.[3, 9-14] Helicobacter pylori DNA has been detected in the bile and liver tissue of some feline patients and LC has been successfully induced with *Bartonella* spp. in an experimental setting.[15-17] However, to date, no definitive cause for LC has been determined and so the treatment of LC remains challenging.

Traditionally, corticosteroids have been used, based on the inflammatory cell infiltrate seen with LC. To the authors' knowledge, there are no published studies that confirm the benefits of immunosuppressive therapy in feline LC. However, considerable interest has focused on therapies for human cholangitis. Interestingly, feline LC and primary sclerosing cholangitis (PSC) in humans have similar histopathological features, including inflammation of the portal tracts, bile duct proliferation and fibrosis.[6, 10, 18] Therefore, developments in the treatment of human PSC might also be of value to cats with LC.

Two placebo-controlled clinical trials reported promising results for ursodeoxycholic acid (UDCA) as a treatment for PSC and UDCA has been advocated as ancillary therapy in veterinary medicine based on its hepatoprotective properties.[19-21] These include protection of cell membranes, prevention of mitochondrial damage, reduced cell destruction and inflammation, and choleresis.[22-28] There were no adverse effects of UDCA treatment in healthy cats or in a Somali cat with pyruvate kinase deficiency, even when UDCA treatment was continued for 20 months.[29-31]

As far as we are aware, there are no published studies that compare the efficacy of prednisolone and UDCA in cats diagnosed with LC. The purpose of this retrospective study was to compare the effect of these therapeutic agents on survival times of cats diagnosed with LC and to determine prognostic factors.

Materials and methods

Animals

All cats diagnosed with LC between June 1996 and September 2008 were identified from patient registration systems at the University of Utrecht Clinic for Companion Animals (UUCCA). The diagnosis of LC was based on the presence of typical histopathological findings in liver biopsies according to WSAVA standards (WSAVA Liver Standardization Group, 2006). Liver biopsies were taken with a 16 G Tru-cut needle under ultrasound guidance.[32] No intestinal or pancreatic biopsies were collected. Cats were excluded from the study if follow-up data were insufficient to allow for survival analysis.

Data collection

Data were retrieved from medical records and the central patient registration system. Information obtained included breed, sex, age at the time of diagnosis, clinical signs, results of blood analyses, cytological and histopathological results, ultrasound findings, feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV) serology, details of comorbid conditions, and therapeutic protocol. If data could not be obtained from the medical records, referring veterinarians and owners were contacted by telephone in December 2009 and January 2010. Data collected included therapy compliance, medical problems encountered since the diagnosis of LC, and date and cause of death. Data concerning the total number of cats referred to the UUCCA were retrieved from the central patient registration system for 2002–2008.

Treatment regimen

Different treatment regimens were employed based on the individual clinical decisions of attending veterinarians. Cats presented to UUCCA from June 1996– October 2000 were treated with prednisolone only (1 mg/kg bodyweight/day in three cats and 2 mg/kg bodyweight/day in seven cats). Cats seen between March 2001 and September 2008 received UDCA only (15 mg/kg bodyweight/day). Two cats received a combination of prednisolone

and UDCA. They were treated in December 1997 and October 2006, respectively. UDCA therapy was typically continued for the rest of the cat's life since no adverse side effects were reported. Prednisolone therapy was administered for 4–6 weeks, after which time the dose was tapered. For cats with continuous clinical signs, this period was extended and the dosage was reduced.

Statistical analysis

Statistical analyses were performed with SPSS (version 16.0.1). Population data for the UUCCA were used for the evaluation of breed and sex proportions. The characteristics of the cats studied and their clinical signs were compared across the treatment groups with the Mann–Whitney *U* test. Proportions were compared using binomial tests and Fisher's exact tests. $P \leq 0.05$ was considered to indicate statistical significance.

Potential confounding factors, such as differences in breed distributions between the treatment periods, the increasing life expectancy of cats over time and the possibility that the aetiology of LC might have changed over time, prevent us from applying a Kaplan–Meier estimate to the data collected. Therefore, multivariable Cox regression models were used to evaluate the effect of confounding entry and follow-up variables.[33] Cox modelling is also very well suited for the correct modelling of survival times for cats that were treated in different periods of time, as in this study.[33] For each cat, we observe the time to death, loss to follow-up or censoring, regardless of when these events actually took place.[33, 34]

Significant variables were selected by a backward elimination method based on the 2 log likelihood statistic, with $P < 0.05$ for entry and $P > 0.1$ for removal of confounding variables from models. The assumption of proportional hazards in Cox regression was tested by plotting the log minus log of survival for both treatment groups. Multicollinearity was evaluated with the help of correlations, and tolerance and VIF statistics were evaluated from a linear regression model using the variables from the Cox model.[35, 36] Even though survival data do not meet the criteria for linear regression analysis, multicollinearity pertains to the relationships among the independent variables and is therefore independent of the exact modelling of the dependent variable.[37]

Results

Of the 26 cats identified with LC in this study, 20 were neutered males and six were spayed females. Ten cats were treated with prednisolone, 13 with UDCA, two cats received a

combination of prednisolone and UDCA. One cat died soon after admission to the hospital clinic due to cardiovascular problems and was not treated for LC. One cat in the UDCA group received additional treatment with intrabiliary antibiotics.[38]

Table 1. Characteristics of cats with lymphocytic cholangitis (LC) and the total referral population.

Variable	**LC 1996-2008 *n=26***	**LC 2002-2008 *n*=13**	**Referral population 2002-2008 *n*=7836**	***P* value**
Number of male cats (%)	20 (76.9%)	12 (92.3%)	4372 (55.8%)	0.006
Median age at diagnosis LC (range)	12.3 years (14-252 months)			
Median age at time of death (range)	13.3 years (20-272 months)			
Median interval diagnosis to death (range)	18 months (0-40 months)			
Breed, *n* (%)			3699	0.021
British Shorthair	3 (11.5%)		345 (9.3%)	
European Shorthair	17 (65.4%)	8 (61.5%)	2575 (69.6%)	
Norwegian Forest Cat	3 (11.5%)	3 (23.1%)	127 (3.4%)	
Persian	1 (3.8%)	1 (7.7%)	426 (11.5%)	
Siamese	1 (3.8%)	1 (7.7%)	209 (5.7%)	
Mixed Breed	1 (3.8%)		17 (0.5%)	
Blood analysis, median (range; reference interval)				
Gamma globulins (g/L)	21 (1-35; 4-8)			
Bile acids (μmol/L)	68 (4-541; < 13)			
ALT (alanine aminotransferase, U/L)	449 (44-768; 30-73)			

In the group treated with prednisolone, there was no difference in survival times between three cats treated with 1 mg/kg bodyweight/day and seven cats treated with 2 mg/kg bodyweight/day. Therefore, these subgroups were treated as one group in further analyses. There were no significant differences for the prednisolone (n = 10) and UDCA (n = 13) treatment groups when breed, sex, number of clinical signs, levels of gamma globulins, serum bile acids and liver enzymes, age at time of diagnosis, and age at time of death were compared.

Table 2 Prognostic variables from the multivariable Cox regression models for treatment groups prednisolone and ursodeoxycholic acid (UDCA).

Variable	**Category**	**Hazard ratio**	**95% CI**	***P* value**
Therapy	Prednisolone[a] UDCA	25.1	1.0-652.2	0.05
Breed	Domestic Shorthair[a] Purebred	84.0	3.1-2279.4	0.01
Sex	Male[a] Female	7.1	0.8-66.0	0.08
Clinical signs[b]	1-2[a] 3-5	24.4	0.8-764.1	0.07

[a]Reference group, [b]Clinical signs consist of the clinical signs most frequently reported i.e. weight loss, icterus, anorexia, vomiting, and listlessness.

The clinical signs most frequently mentioned were weight loss (18/23; 78.3%; 95% CI 61%, 95%), icterus (15/23; 65.2%; 95% CI 46%, 85%), anorexia (12/23; 52.2%; 95% CI 32%, 73%), vomiting (11/23; 47.8%; 95% CI 27%, 68%), and listlessness (11/23; 47.8%; 95% CI 27%, 68%). Cats commonly presented with more than one clinical sign (median 3; range 1–5). The median follow-up time was 97 months (range 15–162 months). At the time of follow-up, four cats were still alive, three were lost to follow-up, and 19 had died. Other important entry characteristics of the cats are shown in Table 1.

Between January 2002 and September 2008, 7,836 cats were referred to UUCCA. Of the referred cats, 55.8% were male. Male cats were at a significantly higher risk than females for the diagnosis of LC when the sex groups were compared to sex groups in the UUCCA feline population (P = 0.006). The distribution of feline LC patients by breed differed significantly

from the breed distribution in the referral population ($P = 0.021$) and Norwegian Forest cats were significantly overrepresented (Table 1).

All feline patients tested were negative on FeLV and FIV serology. Five cats had comorbid conditions, including atrioventricular valve insufficiency ($n = 1$), protein losing nephropathy ($n = 1$), struvite crystals in the urinary bladder ($n = 1$), and dental problems ($n = 2$), at the time of diagnosis with LC.

Survival analysis

Owners and referring veterinarians were contacted in December 2009 and January 2010. Therefore, the date of censorship was January 1, 2010. Only cats treated with either prednisolone ($n = 10$) or UDCA ($n = 13$) were included in this analysis.

Median overall survival time was 795 days. Survival rates for 1, 2, and 3 years were 74%, 56%, and 35%, respectively. Since extra explanatory data were collected for all feline patients, these were incorporated into the analyses by fitting multivariable Cox regression models with confounding factors.[33, 39] The multivariable model fitted included variables such as breed, sex, clinical signs, and therapy (Table 2). Purebreds (hazard ratio 84.0, $P = 0.01$) and cats treated with UDCA (hazard ratio 25.1, $P = 0.05$) were likely to have shorter survival times than Domestic shorthairs and cats treated with prednisolone.

The survival curves for prednisolone and UDCA are shown in Figure 1. The difference between the hazard functions for the two treatment groups (-2 Log Likelihood. 29.687) and the null model with no explanatory variables (-2 Log Likelihood. 41.885) has a chi-squared distribution on four degrees of freedom ($P = 0.015$).[40] Therefore, we conclude that the hazard functions for the two treatment groups differ significantly. No multicollinearity problems were encountered in the model.[35, 36, 41]

Additional analysis

Two cats were treated with a combination of prednisolone and UDCA. When included in survival analysis, the median overall survival time decreased to 755 days. One cat died after 407 days and the other after 697 days. Overall survival rates for 1, 2, and 3 years were 76%,

51%, and 32%, respectively when these cats were included. No multivariable Cox models were fitted because of the small number of cats treated with this combination therapy.

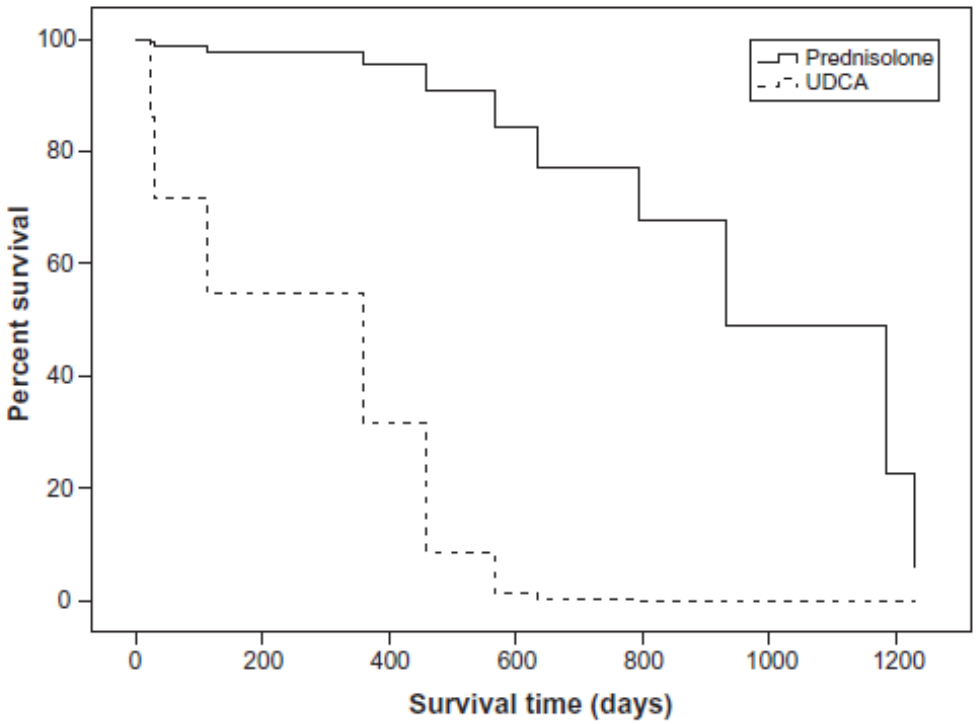

Figure 1. Survival curves for cats treated with prednisolone (solid line) or ursodeoxycholic acid (UDCA; dotted line).

Discussion

Since cats with LC were treated under standardized protocols within our institution, we were able to compare two distinctly separate treatment groups. Traditionally, corticosteroids have been used, but there is no published evidence confirming the efficacy of immunosuppressive therapy in feline LC. Since its aetiology is unknown, an infectious cause cannot be ruled out at this time. In human PSC, corticosteroids are not beneficial and can increase susceptibility to infections.[42] Therefore, alternative treatments with proven efficacy but without the risk of increased susceptibility to secondary infections were actively sought for cats with LC.

Promising results were reported for PSC treatment with UDCA.[19, 20] Based on similarities in anatomy and physiology between cats and humans, combined with similarities in histopathological features between feline LC and human PSC, UDCA was thought to have

potential as an effective treatment for feline LC. These considerations prompted the change in treatment protocol at the UUCCA from prednisolone only to UDCA only in 2001.

Even though LC is one of the most common inflammatory hepatic diseases in cats, only 26 cases were identifiedin the UUCCA database between June 1996 and September 2008. Therefore, a retrospective study was undertaken in an attempt to identify prognostic factors.[43] Our multivariable analysis confirmed that therapy is an important predictor of survival, even after adjusting for other prognostic variables such as breed, sex, and number of clinical signs. One explanation for the superior efficacy of prednisolone over UDCA could be that the appetite stimulating side effect of prednisolone protects the liver in cats that are sensitive to hepatic lipidosis associated with periods of anorexia. According to findings in human PSC, UDCA can improve blood parameters such as ALT. However, it is unknown whether UDCA exerts any effects on symptoms and quality of life in patients with PSC.[42]

The Persian breed predisposition reported in an earlier study was not replicated in our study, but most of our feline patients were Domestic shorthairs.[6] In our study population, Norwegian Forest cats were overrepresented with LC when compared to the referral population. Also, in the study population, purebred cats were likely to have a shorter survival time than Domestic shorthairs.

The majority of cats were male (76.9%, $P = 0.006$). Another published study found no gender predisposition among 21 cats with LC.[6] It is worth noting that the prevalence of PSC in humans is also higher in males.[18] Even though male cats were at a significantly higher risk than females for the diagnosis of LC, they also have longer survival times compared to females.

Cats with fewer than three clinical signs had longer survival times than cats with three or more clinical signs. Although the difference observed was not statistically significant ($P = 0.07$), it may possibly be of clinical relevance, since cats with fewer signs might be in an earlier stage of the disease, or the disease might be more severe in feline patients that present with more clinical signs.

The median age of the cats in this study was 12.3 years, while in previous studies, the majority of feline patients were reported to be 4 years old or younger.[6] Other researchers found LC was significantly more prevalent in cats >15 years.[44] Two recent studies report median ages of approximately 10 years.[9, 14]

To the authors' knowledge, no previous studies have reported median survival times for cats with LC, so a comparison of our data with published data is not possible. Based on our study, cats can live with LC for long periods of time, even if the disease was not cured.

A limitation of this study was limited statistical power because of the small number of cats included. As a rule of thumb, a ratio of events per variable (EPV) of 10 or more is deemed necessary to avoid modelling problems.[45] For our model, we would therefore need to observe 40 deaths. With an observed death rate of 73% (19/26) in this population, this means we would have needed to include at least 55 cats with LC (40/0.73). Other limitations of the study include the inherent disadvantages of retrospective data collection, which was necessary because of the relative scarcity of cats identified at UUCCA with LC. This problem was compounded by three cats being lost to follow-up. Furthermore, the limited number of cats treated with a combination of prednisolone and UDCA permits no definitive conclusions about the efficacy of the combination treatment. Treatment efficacy for prednisolone and UDCA might have been influenced by disease characteristics that changed during the study period, but further information on the likelihood of this is unavailable, mainly because of the unknown aetiology of the condition.

Larger blind randomized prospective studies of cats with LC are warranted to investigate the efficacy of different therapy regimens, including a protocol that combines prednisolone and UDCA. Given the small number of affected cats, this is likely to involve a multicentre research design.

Conclusions

Therapy regimen, sex, breed, and number of clinical signs are significantly associated with survival times for cats diagnosed with LC. Non-purebreds and cats treated with prednisolone had better survival times in the studied population. The majority of cats were males and a breed predisposition could be demonstrated for the Norwegian Forest cat.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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CHAPTER 5 A morphological and immunohistochemical study of the effects of prednisolone or ursodeoxycholic acid on liver histology in feline lymphocytic cholangitis

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Introduction

The increasing popularity of pet cats has heightened the need for specific knowledge about lymphocytic cholangitis (LC), a common inflammatory hepatic disease in cats.[1-3] Although bacterial DNA has been found in the bile of cats with LC, its aetiology remains enigmatic, and an immune-mediated component has also been suggested.[4-7]

Clinical signs include nausea and vomiting, changes in appetite, gradual weight loss and jaundice.[6] Blood analysis may reveal elevated activities of hepatic enzymes, such as alanine aminotransferase, reflecting damage to periportal hepatocytes, and increased levels of bile acids, while hypergammaglobulinemia is the most consistent finding.[6, 8, 9] Usually, LC is a chronic disease that can affect the entire biliary tree and progresses slowly during months or years. Aggregates of lymphocytes in portal tracts and in and around bile ducts are a hallmark of the disease.[3] The chronic inflammation in the bile ducts, characterised by dilations, strictures and proliferation of bile ducts may eventually lead to fibrosis and cirrhosis.[3] Histopathology is considered the gold standard for diagnosing LC.[3] The lesions are characterised by inflammatory cells in portal tracts, variable portal fibrosis and proliferation of bile ducts, which are considered the hallmarks of LC.[3, 5] These histopathological features exhibit similarities to those found in human primary sclerosing cholangitis (PSC).[5, 6, 10] To date, only a limited number of studies on the histological aspects of feline LC have been reported.[2, 5, 6, 11, 12]

LC is commonly treated with prednisolone or ursodeoxycholic acid (UDCA).[13, 14] The use of prednisolone has been warranted by the immune-mediated component suggested by the abundant presence of lymphocytes.[3, 5] UDCA has been proposed as supportive therapy based on its hepatoprotective properties.[14-20] As promising results were reported initially for UDCA as a treatment for human PSC, it was welcomed as a potential new treatment for cats with LC between 1998 and 2008.[21, 22] However, more recent data indicate a lower efficiency of UDCA in human PSC patients.[23-25]

Clinical evaluation of these therapeutic interventions in cats with LC showed that prednisolone treatment resulted in a statistically significantly longer survival time than treatment with UDCA.[9] Yet it is undetermined if the improvement in clinical condition correlates with improvement in the underlying hepatic histological lesions.

The liver is capable of a remarkable and unparalleled regeneration, which is based on proliferation of both hepatocytes and cholangiocytes.[26] Only when regeneration capacity

proves inadequate, progenitor cells (also known as 'oval cells' in rodents) will be activated.[26-29] Fibrosis occurs when the injury takes on a more chronic character, and the amount of collagen subsequently increases.[30] The main source of collagen is Ito cells, also known as hepatic stellate cells (HSC), fat-storing cells, hepatic lipocytes or perisinusoidal cells, which are the main storage sites for vitamin A in non-diseased liver.[31-34] Proliferating fibroblasts in the portal areas can also contribute to collagen deposits and fibrosis.

The purpose of this study was to evaluate liver histology during treatment with prednisolone or UDCA in cats with LC by analysing the proliferation of hepatocytes, lymphocytes, fibroblasts, bile ducts, activation of progenitor cells, fibrosis, and the degree of inflammation.

Materials and methods

All procedures were approved by the responsible ethical committees as required under Dutch legislation. All liver biopsies were taken for diagnostic purposes between 1998 and 2008. No tissue was taken for research or scientific purposes only.

Case selection

Twenty sequential archival biopsy materials (fixed in 10% neutral buffered formalin and embedded in paraffin) from nine cats diagnosed with LC were identified from the registration system used by the Department of Pathobiology, Faculty of Veterinary Medicine at Utrecht University (The Netherlands). The diagnosis of LC was based on the presence of typical histopathological findings combined with clinical signs, and elevated levels of bile acids and/or activities of liver enzymes.[3] The characteristics of the cats are shown in Table 1. The archives further showed that cats were treated with prednisolone (1–2 mg/kg/day) or UDCA (15 mg/kg/day).[35, 36] On average, biopsies were taken every two months (mode; range, 1–9 months) (Table 2). As a rule, a minimum of two biopsies was taken at any time from each patient by the attending clinician. Only cats with a diagnosis of LC that had serial biopsies taken were considered eligible for inclusion in this study.

Additionally, tissue samples from dogs and cats that did not suffer from LC were included to verify the correctness of the immunohistochemical staining processes.

Table 1 Characteristics of nine cats with lymphocytic cholangitis

	Prednisolone	**UDCA**
Breed		
European Shorthair	4	2
Mixed Breed*	1	0
Norwegean Forect Cat	0	1
Persian	0	1
Age at diagnosis, median (range), years	15 (14-21)	13 (9-14)
Age at death, median (range), years	18 (16-23)	13 (10-15)
Sex		
Male, castrated	2	4
Female, spayed	3	0

UDCA, ursodeoxycholic acid

* European Shorthair x Persian

Table 2 Description of biopsies and therapies in the different cats

Cat	**Number of biopsies taken**	**Time between biopsies (months)**	**Biopsy method**	**Therapy**
1	3	1; 6	Tru-cut	Prednisolone
2	2	2	Tru-cut	Prednisolone
3	2	7	Tru-cut	Prednisolone
4	2	1	Tru-cut	Prednisolone
5	2	2	Wedge	Prednisolone
6	2	9	Wedge	UDCA
7	2	4	Tru-cut	UDCA
8	2	2	Tru-cut	UDCA
9	3	1; 2	Tru-cut	UDCA

UDCA, ursodeoxycholic acid

Histopathology and immunohistochemistry

A routine haematoxylin and eosin (HE) staining was used to evaluate the degree of inflammation during therapy. Proliferation of cells was determined with Ki-67, a commonly used proliferation marker.[37] The activation of progenitor cells and proliferation of bile ducts was evaluated with immunohistochemical staining protocols for cytokeratin 19 (K19) as described previously.[38] Fibrogenesis was evaluated by applying a reticulin stain (Gordon and Sweet) to stain type III collagen (reticulin).[3, 39] Furthermore, we evaluated the activation of HSC, the main producers of collagen in the chronically injured liver, with a stain against α-smooth muscle actin (α-SMA).[26, 40-44]

Staining protocols

Archival formalin-fixed liver tissues were routinely processed to produce slides with freshly cut sections (3 µm) that were stained with HE. Additionally, freshly cut sections were mounted on silane-coated slides (Starfrost; Waldemar Knittel) and stained for reticulin according to Gordon and Sweet, Ki67, K19 and α-SMA. For these stainings, tissue sections were deparaffinised and rehydrated in a series of xylene, alcohol and aqua dest baths (5 mins each) before application of the following protocols.

Ki-67

Sections were incubated in pre-heated citrate buffer (10 mM, pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide (H_2O_2) in methanol. Sections were incubated with 10% normal horse serum to reduce background staining. Next, the sections were incubated with mouse anti Ki-67 (MIB1 clone, 1:50; Dako) overnight at 4°C in a humidified chamber in a refrigerator. After washing in phosphate-buffered saline (PBS) with Tween-20, sections were incubated in horse anti-mouse biotinylated immunoglobulin G (1:125; Vector Laboratories). Subsequently, avidin–biotin complex binding (ABC method; Vector Laboratories) and visualisation, with haematoxylin as counterstain, and 3-amino-9-ethylcarbazole (AEC; Dako) were performed according to the manufacturer's instructions. Jejunal samples from dog and cat were used as positive controls to check for the correctness of the staining protocol used.

K19

Tris-buffered saline (TBS; 0.01 M, 1.5 M sodium chloride, pH = 7.6) with Tween was used to wash sections before treating them with ready-to-use Proteinase K (Dako). Endogenous peroxidase activity was blocked with 0.1% H_2O_2 in TBS. Sections were then incubated with 10% normal goat serum to reduce background staining. Subsequently, sections were incubated with the primary antibody mouse anti human K19 (clone b170, 1:100; Leica Microsystems). After washing in TBS with Tween-20, sections were incubated with Envision (goat-anti-mouse; Dako). Visualisation with haematoxylin as a counterstain and AEC (Dako) were performed according to the manufacturer's instructions. Positive staining of bile ducts served as internal controls.

α-SMA

The ABC/peroxidase (PO) method for mouse anti-α-SMA was used. Endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol. PBS with Tween-20 was used to wash sections in between steps. Sections were then incubated with 10% normal horse serum to reduce background staining, and then with the primary antibody mouse-anti-SMA (MU128-UC, 1:1200; BioGenex Laboratories). After washing in TBS with Tween-20, sections were incubated with horse anti-mouse biotin (1:125; Vector Laboratories) for 30 mins. Then, the ABC method (Vector) and visualisation with AEC (Dako) were performed according to the manufacturer's instructions. HE was used as a counterstain. Arterial smooth muscle cells were used as internal controls.

Histological scoring and grading methods

HE and reticulin staining

A modified version of the grading system introduced by Gagne et al. for the number of lymphocytes, and fibrosis and bile duct proliferation was used to evaluate the biopsies (Tables 3 and 4).[2]

Table 3 Grading scheme for inflammation and fibrosis of haematoxylin and eosin- and reticulin-stained specimens

	Grade 0	**Grade 1**	**Grade 2**	**Grade 3**
Inflammatory infiltrate	0-10 lymphocytes	Mild inflammation, 11-50 lymphocytes	Moderate inflammation, 51-100 lymphocytes	Severe inflammation, > 100 lymphocytes
Fibrosis	No fibrosis	Mild fibrosis within the portal areas	Moderate fibrosis within portal areas and enlarging the portal areas	Severe fibrosis bridging between portal areas

Table 4 Scoring and grading scheme for immunohistochemically stained specimens

	Grade 0	**Grade 1**	**Grade 2**	**Grade 3**
Bile duct proliferation	1-2 bile ducts, normal	2-3 bile ducts, mild proliferation	4-5 bile ducts, moderate proliferation	>5 bile ducts, severe proliferation
Proliferation of hepatocytes	0	1-3	4-6	>7
Proliferation of lymphocytes	0	1-8	9-18	>19
Proliferation of fibroblasts	0	1-2	3-5	>6
Activation of progenitor cells	0	1-2	3-4	>5
Activation of hepatic stellate cells	Absent	Mild	Moderate	Strong

The percentage of the specimens infiltrated with inflammatory cells obscuring normal hepatic structure was measured. Two calibrated high-resolution microscopical images of randomly

selected areas in the biopsies were made using Cell^B software (version 3.0; Olympus Soft Imaging Solutions) with an Olympus ColorviewIIIu (Olympus) digital camera and an Olympus BX41 microscope at × 200 magnification. The average percentage of inflammation was calculated with Cell^B software (version 3.0; Olympus) (Table 5).

Immunoreactivity for Ki-67 was evaluated by counting the number of cells per high-power field (HPF; magnification × 400) and calculating the average of three HPFs (Table 4). K19 allowed for the enumeration of bile ducts and periportally located progenitor cells.

Immunoreactivity against α-SMA was evaluated by expressing the number of α-SMA positive cells graded as absent, mild, moderate or strong.

Table 5 Biopsy results for inflammation in nine different cats

Therapy	**Cat**	**Biopsy 1 (grade; % inflammation**	**Biopsy 2 (grade; % inflammation**	**Biopsy 3 (grade; % inflammation**
Prednisolone	1	3; 45	3; 41	3; 30
	2	3; 26	2; 13	
	3	3; 80	3; 57	
	4	3; 65	3; 11	
	5	3; 25	3; 34	
UDCA	6	1; 2	1; 2	
	7	2; 10	2; 3	
	8	1; 1	3; 5	
	9	2; 10	3; 15	

UDCA = ursodeoxycholic acid

Results

Inflammation

The percentage of the specimens infiltrated with inflammatory cells (Figures 1a,b) decreased in four and increased in one of the five cats treated with prednisolone. In contrast, the results for UDCA were less consistent (Table 5). As can be seen, the grade (*ie*, the number of lymphocytes per infiltrate) did not change, in the most part. Therefore, the largest contribution to changing the severity of inflammation was made by the total part of the liver biopsy being affected by inflammation (*ie*, percentage of biopsy). Overall, when comparing the two treatment groups, the group treated with UDCA was less severely affected by the disease than the group treated with prednisolone.

In two biopsies from cats treated with prednisolone, follicle formation of B-lymphocytes was seen. Seventeen biopsies were negative for follicle formation and one liver specimen could not be judged unambiguously.

Fibrogenesis and activation of HSC

The fibrosis score increased more frequently during treatment with UDCA (Table 6).

Table 6 Results for fibrosis in nine cats with lymphocytic cholangitis

Therapy	**Cat**	**Biopsy 1 (grade)**	**Biopsy 2 (grade)**	**Biopsy 3 (grade)**
Prednisolone	1	2	3	3
	2	2	2	
	3	3	3	
	4	3	2	
	5	1	2	
UDCA	6	1	2	
	7	1-2	2	
	8	1	1	
	9	1-2	2	3

UDCA = ursodeoxycholic acid

Sixteen of 20 biopsies were successfully stained against α-SMA. However, except for the positive results in blood vessel walls, no other cells stained positive in the needle biopsies (grade 0). The two wedge biopsies did contain α-SMA positive cells, that is, HSC (grade 1) (Figure 1d).

We also identified concentric fibrosis in three LC patients (Figure 1c).

Proliferation and regeneration

Eighteen out of 20 biopsies of feline patients were successfully stained for Ki-67 (Figure 1f). Increased proliferation of fibroblasts was seen in the UDCA group compared with the prednisolone group (Table 7).

Overall, 15 biopsies were successfully stained with K19 (Figure 1e). In both the prednisolone and UDCA treatment groups, the number of bile ducts and liver progenitor cells remained stable. Furthermore, there was no difference in absolute numbers of bile ducts or progenitor cells between the two treatment groups.

Table 7 Proliferation of different cell types in the different treatment groups based on Ki-67 staining

		Hepatocytes	**Fibroblasts**	**Lymphocytes**
Prednisolone (n=5 cats)	Increase	2	-	-
	Decrease	1	2	2
	Stable	2	3	3
UDCA (n=4 cats)	Increase	2	2	-
	Decrease	2	2	3
	Stable	-	-	1

UDCA = ursodeoxycholic acid

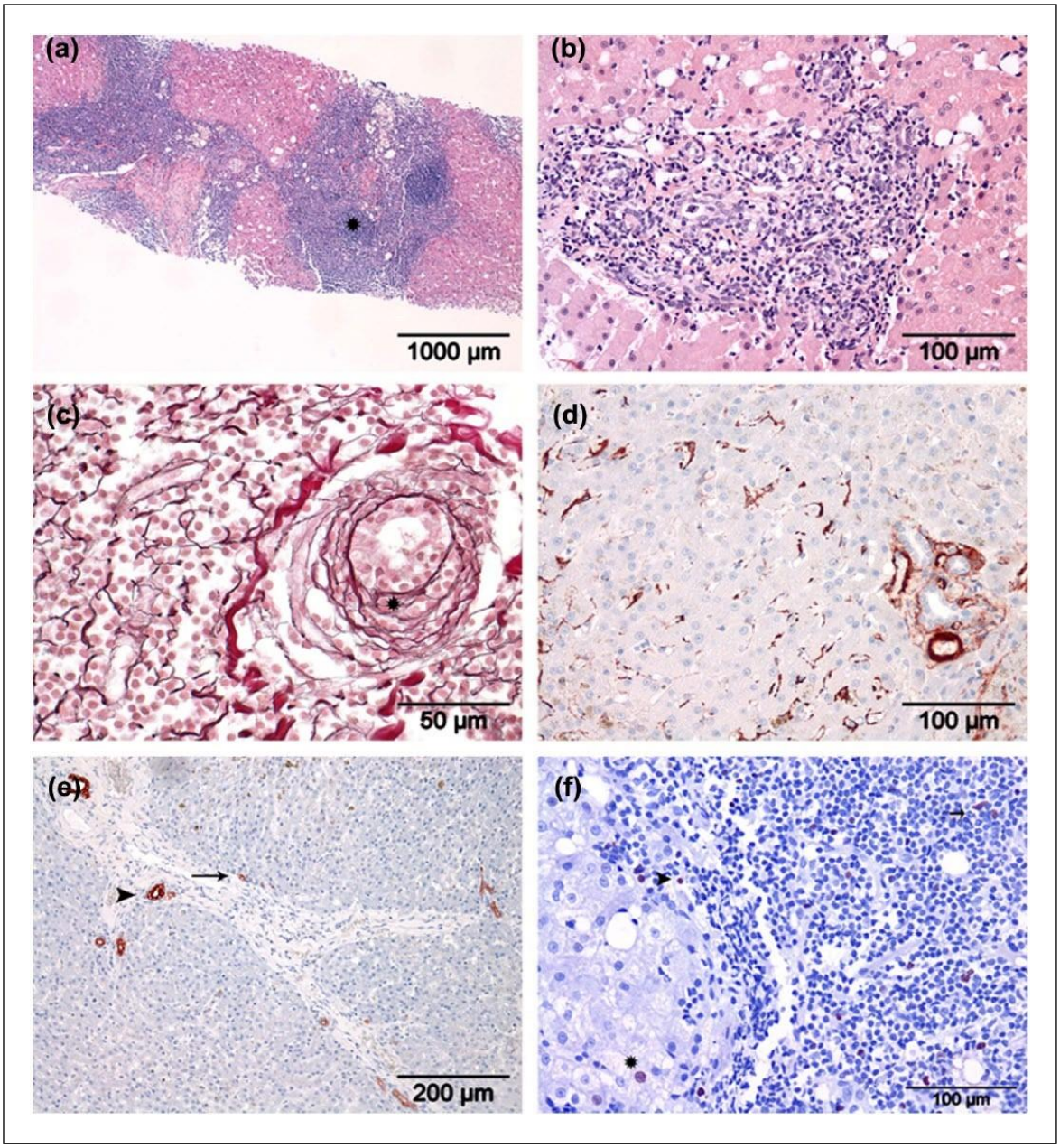

Figure 1 Histological examples of feline lymphocytic cholangitis. (a) Large infiltrates of small lymphocytes are present in portal areas (asterisk) extending to portal–portal bridging inflammation. Haematoxylin and eosin (HE) staining. (b) Portal areas are infiltrated by moderate numbers of small lymphocytes with moderate bile duct proliferation. HE staining. (c) Concentric bands of collagen surround the bile duct (asterisk). Collagen stain according to Gordon and Sweet. (d) Positive staining against α-smooth muscle actin is present around the bile ducts, in the arterial tunica media and in the wall of the portal veins. In addition, the perisinusoidal spaces throughout the parenchyma are moderately stained, which is most likely due to activated stellate cells. Immunohistochemistry (IHC). (e) Positive staining for K19 is present in the cytoplasm of bile duct (arrowhead) cells and other cells (most likely liver progenitor cells) located in the periportal parenchyma (arrow). IHC. (f) Positive Ki-67 staining is present in nuclei of proliferating lymphocytes (arrow), fibroblasts (arrowhead) in portal areas and in hepatocytes (asterisk).

Discussion

In liver disease, different cells (hepatocytes and cholangiocytes) can proliferate, and hepatocytes are the first to do so.[26] Only when regeneration capacity proves inadequate, progenitor cells (oval cells) residing in the canal of Hering will be activated.[26-29] All cats with LC showed proliferation of cholangiocytes and bile ducts, which may be considered a hallmark of LC.

The group treated with UDCA was less severely affected by LC than the group treated with prednisolone. As the treatment grouping was based on period in time and not on severity of the disease, this is not due to selection of cases and must be attributed to serendipity.

When LC was first reported, knowledge of the disease was scarce, and it is possible that only very severe cases were referred to the academic veterinary hospital after veterinarians tried to treat their patients and did not succeed. When, later on, more was learned about LC, referring veterinarians might have able to recognise the disease at an earlier stage. Furthermore, owners may have been more willing to be referred because of increased prosperity and their desire to obtain the best possible health care for their pets. As the cause of the disease is still unknown, we can also not rule out an external, yet unknown, factor that has diminished the severity of the disease over time. Owing to these differences in the two treatment groups, it may have been easier to score improvement or deterioration in the prednisolone group.

The suppression of proliferation of lymphocytes by prednisolone has been described previously.[45] One earlier study reported no effect of UDCA on the proliferation of lymphocytes in mice.[46] Follicle formation of B-lymphocytes was seen in two biopsies from cats treated with prednisolone. Newly formed lymphoid structures have often been associated with chronic inflammatory processes and have been previously observed in LC.[5, 47] Similarly, these structures have been detected in PSC.[48, 49] However, lymphoproliferative disorders have been linked to the prolonged use of immunosuppressive drugs in human research.[50, 51] More research is needed to establish the nature of the follicle formation, and specifically the role of prednisolone in this process.

Despite treatment with UDCA, the fibrosis score increased or remained stable in all cats. This may be explained by the fact that the number of fibroblasts increased in 50% of the cats treated with UDCA, while these numbers did not increase in any felines treated with prednisolone. Fibroblasts actively contribute to liver fibrosis.[52] Historically, HSC were considered to be the main source of fibroblasts, but epithelial-to-mesenchymal transition from

hepatocytes or biliary epithelial cells has been shown to be actively involved in accumulation of fibroblasts.[52, 53] Perhaps the main fibrotic component in cats with LC is not formed by HSC, but fibroblasts. Future research is needed to answer this question. Furthermore, the fixation method used does influence the quality of the liver tissues sampled as demonstrated before.[36] This might explain why no α-SMA-positive cells were identified in the needle biopsies of our feline patients. As a consequence, we were not able to confirm the positive correlation between the degree of fibrosis and α-SMA demonstrated previously.[54] Additionally, differences between diagnoses based on wedge biopsies or needle biopsies have been reported.[16, 55-57] Although no definitive agreement on required biopsy size has been reached, pathologists seem to agree that a diagnosis can be based on a specimen containing 4–10 portal triads.[55-57] Furthermore, chronic liver disease in rats has been shown to be equally well represented by needle biopsies as larger samples of tissue taken by laparotomy.[56] Wedge biopsies offer more material to be evaluated, but may misrepresent a disease when taken from a superficial part of the liver. Needle biopsies are smaller, but do also take tissue from the inner parts of the organ. In judging fibrosis, researchers found that the extent of fibrosis was overestimated in 40 cases, underestimated in 50 cases, and equally scored in 21 cases when compared with wedge biopsies when evaluating hepatic biopsies in both dogs and cats.[16] Based on these previous findings, we conclude that we may have encountered differences between the samples taken by needle biopsy and the samples taken by wedge biopsy. However, these differences may be small as LC is a chronic disease affecting the whole organ, and the fact that the wedge samples were equally distributed among both treatment groups. Comparisons of needle and wedge biopsies in larger numbers for LC specifically can be considered a much needed subject for future research.

Concentric fibrosis has been alternately absent and present in reports on feline LC and is considered to be a hallmark of the disease in humans.[5, 6, 58]

Ki-67 has mainly been used for the evaluation of neoplasms, but it has also been used successfully on inflammatory bile ducts and chronic liver injuries in humans.[59, 60] Historically, feline studies have employed the MIB-1 clone for Ki-67, but, recently, SP6 was judged to be equal or even superior to MIB-1 in human research.[61, 62] Unfortunately, our studies found that the SP6 clone did not work on feline hepatic and intestinal control samples. We used additional tissue samples from dogs' jejunum to verify our staining protocols, and these canine control samples were positive. Both feline and canine tissue samples were positive

when the MIB-1 clone was used. Therefore, this study shows that the MIB-1 clone can be used successfully on feline hepatic tissue to evaluate the proliferation of several cell types.

Although this is, to our knowledge, the first study of the effects of therapeutics for LC on the histopathological features of the liver, several limitations apply. First, the small number of cats and biopsies limits the study's power. Liver biopsies were taken to diagnose LC, but only a limited number of owners allowed multiple biopsies to be taken. In general, owners were primarily interested in the clinical improvement of their pet, and not in the histological development of the disease. The costs associated with taking liver biopsies might have attributed to this, as well as the perceived burden for the cat, including full anaesthesia. Second, low case numbers is a drawback observed in numerous feline studies, possibly associated with similar reasons as outlined above.[63-65] Extrapolation or generalisation of data must therefore be undertaken with caution. However, publications based on low numbers can still be beneficial for the clinician, owner and animal, for instance to prevent expensive overtreatment. Furthermore, it provides directions for future research with larger multicentred studies and facilitates meta-analyses.

Additionally, unequal numbers of sequential biopsies were available for various cats. Follow-up biopsies were also taken at different stages following the start of the therapy regimens. This complicates the process of comparison between the patients, biopsies, and efficacy of both treatment options. It would have been preferable to have identical groups of patients, with equal numbers of biopsies per patient, taken at set times from initiating the therapy in order to eliminate unwanted bias from the analyses. However, the retrospective nature of our research prevented this standardised set-up. Therefore, we must interpret the findings with caution as the duration of the therapy may have influenced the histopathological scores.

Unintentionally, all cats treated with UDCA had lower scores on percentage of inflammation at the start of the therapy than the cats treated with prednisolone. This, and the age difference between the two groups, might have biased outcomes. The retrospective character of the study further limits the possibilities for collecting additional data. Based on the outcomes of this study, future research could help in collecting sequential biopsies at set times in order to fully compare the effect of therapeutics on liver tissue in prospective randomised studies. Furthermore, more feline patients are needed to clarify the effects of therapeutics on liver histology.

Conclusions

Inflammation decreased more in the group treated with prednisolone, while the number of cholangiocytes, progenitor cells and fibroblasts did not differ between the treatment groups. No difference was found for the amount of fibrosis between the groups treated with either prednisolone or UDCA.

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Conflict of interest

The authors do not have any potential conflicts of interest to declare.

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CHAPTER 6 Immunohistochemical evaluation of the activation of hepatic progenitor cells and their niche in feline lymphocytic cholangitis

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Introduction

Lymphocytic cholangitis (LC) is a chronic disease in cats, which affects the biliary tree and progresses slowly. Early signs include nausea and vomiting, changes in appetite, and weight loss.[1, 2] Jaundice is often present later in the course of the disease.[1, 2] Hypergammaglobulinaemia is the most consistent finding and blood analysis sometimes reveals elevated hepatic enzymes and bile acids.[2] The WSAVA-liver standardization group described histological hallmarks of the disease, including hepatic lesions characterized by aggregates of inflammatory cells in portal tracts, and in and around bile ducts.[3] A ductular reaction (DR), also known as bile duct proliferation, is often seen.[3, 4] DR is defined as a proliferating and expanding progenitor cell compartment.[5]

Chronic inflammation in the bile ducts causes dilations and strictures and might eventually lead to fibrosis and cirrhosis.[6, 7] Bacterial and immune-mediated components have been suggested but the definitive aetiology of LC is still unknown.[8-12] This limits treatment options, which currently include corticosteroids and hepatosupportive drugs, such as ursodeoxycholic acid (UDCA).[13]

The injured liver is capable of a remarkable and unprecedented regeneration based on proliferation of all mature cell lines present in the liver.[14] Only when regeneration capacity proves inadequate, hepatic progenitor cells (HPCs) will be activated.[5, 14, 15]

Bipotent HPCs can give rise to both hepatocytes and cholangiocytes.[16] Upon activation, HPCs will proliferate, migrate from the canal of Hering to the site of injury, and differentiate into cholangiocytes or hepatocytes, depending both on the disease and on concurrent changes in their microenvironment (HPC niche).[17, 18] The HPC niche, both a histological location and a functional unit, is of importance in maintaining and regulating HPCs behaviour, supporting self-renewal and balancing quiescence, proliferation and differentiation in response to injury.[18, 19]

Putative HPCs have been demonstrated immunohistochemically before, both in healthy feline livers and in livers of cats suffering from cholangitis.[5, 20] In healthy feline livers, IJzer *et al.* demonstrated the presence of resident progenitor cells in "a small ductular structure just beyond the limits of the portal area".[5] This "suggests the presence of progenitor cells in the canal of Hering", as demonstrated with immunohistochemical (IHC) staining for cytokeratin-7 (CK7).[5] In diseased liver (from cats suffering from acute hepatitis, acute fulminant hepatitis, hepatic lipidosis, neutrophilic cholangitis, and lymphoma), a DR was demonstrated with

CK7.[5] Recommendations for future research included IHC staining with cytokeratin-19 (CK19).[5] CK-19 was later applied to livers of cats suffering from lymphocytic cholangitis and 15/20 samples stained positively, demonstrating a DR.[20]

It is likely that feline bile duct proliferation involves both cholangiocytes and HPCs.[17] To our knowledge, studies on feline HPCs and the HPC niche in feline LC are lacking. Therefore, the purpose of this study is to evaluate the activation of HPCs and the HPC niche in feline LC by comparing liver tissue of patients with liver tissue of unaffected cats. Commonly used immunohistochemical markers in other species, i.e. vimentin, laminin, β-catenin and Notch1 IntraCellular Domain (NICD), viz. the active form of Notch1, are investigated because a single specific HPC marker has not been identified yet, and many HPC markers have a shared expression with cholangiocytes.[21, 22]

The markers used for IHC were selected based on studies in other species. Vimentin expression has been shown in hepatic progenitor cells of rats, mice, human beings, and dogs.[22-25] It is a common mesenchymal marker and has been shown to indicate proliferative activity of cells and an undifferentiated state of HPCs.[24, 26]

Remodelling of the extracellular matrix (ECM) and deposition of laminin have been shown to play an important role in HPC activation in hepatic injury in both rodents and human beings.[19, 27] Furthermore, laminin was recently shown to promote cholangiocyte differentiation of bipotent cells in human liver disease.[28]

The Wnt/β-catenin signalling pathway has a central role in hepatic and bile duct development and regeneration, promoting gene activation, inhibiting apoptosis and increasing cellular proliferation.[29-31] Recently, it was shown to also be involved in homeostatic renewal of the liver.[32] It is believed to be involved in the proliferation, migration and differentiation of HPCs in mice, zebrafish, dogs and human beings.[17, 25, 29] Furthermore, active Wnt guides cells to a biliary phenotype.[25] When Wnt is activated, it shifts from a membranous staining pattern to a more cytoplasmic/nuclear staining pattern.

Notch is involved in the proliferation, differentiation and apoptosis in all stages of organ development, including healthy and diseased livers.[33] Notch1 was shown to be activated in human primary sclerosing cholangitis, feline mammary tumours, and in HPCs of dogs with lobular dissecting hepatitis (LDH).[25, 34, 35] The expression of the proteolytically processed, and therefore active, Notch intracellular domain (NICD) has been used to measure the activation of Notch1 in cats before.[35]

To date, no publications exist on the usage of these markers on healthy or diseased feline hepatic tissue. HPCs might provide new regenerative treatment options for LC in cats in the future.

Materials and methods

All liver biopsies from feline LC patients were taken for diagnostic purposes only between 1998 and 2010. Samples of unaffected control livers were obtained from two cats used in liver-unrelated research approved by the Animal Experiments Committee of Utrecht University, as required by Dutch legislation.

Case selection

Liver biopsies

Cats diagnosed with LC, based on typical histopathological findings, clinical signs, and elevated levels of bile acids and/or activities of liver enzymes, were identified from the registration system used by the Department of Pathobiology.[3, 20] Eight cats were identified. Four cats had second biopsies taken one to nine months after the first biopsy to evaluate the efficacy of therapies.[13] In total, 12 liver samples of cats diagnosed with feline LC and multiple samples (2 to 4 samples per cat per IHC staining) of control cats were available for this study (see table 1 for details). LC patient no. 3 was used as a negative control. Canine tissue samples were used as positive controls.[17, 18, 25]

All samples were fixed in 10% neutral buffered formalin and embedded in paraffin.

Table 1 – Characteristics of patients and biopsy materials.

Identification	**Sex**	**Age at diagnosis (in years)**	**Breed**	**Therapy**	**Biopsy type**	**Number of portal areas for evaluation**
LC1 A	M*	14	DSH	-	Needle	>5
LC1 B	M*	14	DSH	P	Needle	0
LC2 A	M*	13	DSH	-	Needle	>5
LC2 B	M*	13	DSH	U	Needle	=3
LC3 A	M*	9	NFC	-	Needle	=5
LC3 B	M*	9	NFC	U	Wedge	>5
LC4 A	M*	9	DSH	-	Needle	=4
LC4 B	M*	9	DSH	P	Wedge	>5
LC5	M*	13	DSH	-	Needle	>5
LC6	F*	14	DSH	-	Needle	>5
LC7	M*	12	DSH	-	Needle	=3
LC8	F*	14	DSH	-	Needle	=4
C1	M	1	DSH	-	Wedge	>5
C2	M	1	DSH	-	Wedge	>5

LC, LC patient; C, unaffected control; A, first biopsy; B, second biopsy; M, male; F, female; *, neutered; DSH, Domestic shorthair, NFC, Norwegian Forest Cat; U, ursodeoxycholic acid; P, prednisolone

Histology

A routine haematoxylin and eosin (HE) staining was used as reported before to diagnose the patients and determine the degree of inflammation.[20]

Immunohistochemistry (IHC)

Staining protocols

Tissue samples were routinely processed to produce slides with freshly cut sections (5 µm) and mounted on Polysine® slides. Deparaffinisation and rehydration was performed in a series of xylene, alcohol and milli-Q baths, 5 minutes each. Antigen retrieval was obtained with Tris/EDTA buffer (TE; for β-catenin and Notch1/NICD), 10 mM hot citrate buffer (for vimentin), and proteinase-K (PK; for laminin). TE and citrate antigen retrieval was performed by heat-induced epitope retrieval (98 °C water bath for 30 minutes, cooling down at room temperature (RT) for 30 minutes). Proteinase-K ready-to-use (Dako, Glostrup, Denmark), a proteolytic induced epitope retrieval method, was incubated for 10 minutes at RT. After rinsing in phosphate-buffered saline with 0.01 % Tween-20, pH = 7.4 (PBS/T; for β-catenin, vimentin and laminin) and Tris-buffered saline with 0.05% Tween-20, pH = 7.6 (TBS/T; for Notch1/NICD) for two minutes twice, endogenous peroxidase activity was blocked by incubating the slides with Dual Endogenous Enzyme Block (Dako) for 10 minutes at RT. A second rinsing step with PBS/T or TBS/T was performed (5 minutes, 3 times) and then background staining was reduced with 10% normal goat serum in PBS (for β-catenin, vimentin and laminin) and TBS (for Notch1/NICD) for 30 minutes at RT. Primary antibodies were diluted based on earlier findings and optimised for use on feline liver samples (see table 2 for details) in Antibody Diluent with background reducing components (Dako) and incubated in a humidified chamber at 4 °C overnight. Before incubating secondary antibodies of the EnVision+ System-HRP labelled polymer (Dako), slides were rinsed in PBS/T or TBS/T (5 minutes, 3 times). Then, slides were incubated with secondary goat-anti-mouse (for Notch1/NICD and vimentin) or goat-anti-rabbit (for β-catenin and laminin) antibodies for 45 minutes at RT and rinsed in PBS or TBS (5 minutes, 3 times). Slides were incubated with freshly made diaminobenzidine (DAB; Dako) as substrate for HRP for 5 minutes. After rinsing three times in milli-Q (5 minutes each), slides were counterstained with haematoxylin quickstain H-3404 (Vector Laboratories, Burlingame, CA, USA) for 10 seconds, whereupon the slides were placed under running tap water during 10 minutes. Dehydration was performed by 60%, 70%, 80%, 96% (twice) and 100% alcohol baths and two xylene baths. Finally, slides were covered with Vectamount (Vector Laboratories).

Secondary antibodies against β-catenin and laminin act as mutual internal controls since they both are polyclonal IgG rabbit antibodies and have a distinct staining pattern. In the same way, the antibodies against NICD and vimentin, both being mouse monoclonal IgG1

antibodies, presented as controls for a-specific secondary antibody binding. Non-specific binding and background staining was evaluated by comparing the staining patterns for β-catenin and laminin, and NICD and vimentin, respectively. For the negative control, the primary antibody was omitted.

Staining (yes, no), intensity of positive staining (weak, moderate or strong), and location of immunoreactivity were evaluated for all markers used.

Table 2 - Primary antibodies and antigen retrieval and washing buffer methods.

	Source	**Type**	**Clone**	**Company**	**Dilution**	**Antigen retrieval**	**Washing buffer**
Laminin	Rabbit	Polyclonal		Abcam	1:100	PK	PBS and PBS/T
β-catenin	Rabbit	Polyclonal		Abcam	1:2500	TE, pH 9.0	PBS and PBS/T
Vimentin	Mouse	Monoclonal	RV203	Abcam	1:300	Citrate, pH 6.0	PBS and PBS/T
Notch1/NICD	Mouse	Monoclonal	mN1a	Merck/ Millipore	1:200	TE, pH 9.0	TBS and TBS/T

TE, Tris/EDTA buffer; PK, proteinase-K; PBS, phosphate-buffered saline; PBS/T, phosphate-buffered saline with Tween-20; TBS, Tris-buffered saline; TBS/T, Tris-buffered saline with Tween-20

Results

Immunohistochemistry was performed on unaffected and diseased feline liver tissue due to LC to determine the expression and location of vimentin, laminin, β-catenin, and NICD. The results are summarized in table 3. Positive and negative controls stained appropriately for all of the IHC markers.

Vimentin

In unaffected liver tissue, vimentin staining was weak in the extracellular matrix and vascular smooth muscle (figure 1a). No staining was detected in the bile ducts in unaffected liver tissue. Occasionally, a stellate cell was seen in the hepatic parenchyma (table 3). In feline LC, portal structures including extracellular matrix and smooth muscle, stained strongly. Lymphocytes and bile ducts also stained strongly, and some individual cells in the DR stained positively, too (table 3 and figures 1b and 1c).

Table 3. Summary of immunohistochemistry in cats with LC and controls.

	Portal area			**Hepatic parenchyma**	
	Lymphocytes	Cholangiocytes	ECM	Hepatocytes	Stellate cells
Vimentin					
Unaffected	-, absent	-	+	-	+
LC	+++	+++	+++	-	+
Laminin					
Unaffected	-, absent	-	+	-	-
LC	-	++/+++	+	+(*)	-
β-catenin					
Unaffected	-, absent	++/+++, membrane	-	++/+++, membrane	-
LC	-	+++, cytoplasm/nucleus and ++/+++ membranes	-	++/+++, membrane and +, cytoplasm (**)	-
NICD					
Unaffected	-, absent	-	-	+, cytoplasm	-
LC	-	-/++	-	+, cytoplasm	-

LC, lymphocytic cholangitis; ECM, extracellular matrix; -, no staining; +, weak staining; ++, moderate staining; +++, strong staining; (*), non-specific background staining, (**), not observed in all slides

Laminin

The extracellular matrix stained weakly in unaffected liver tissue (table 3 and figure 1d). No staining was detected in the bile ducts in unaffected liver tissue. In diseased liver tissue, a moderate to strong expression of laminin was seen in the cytoplasm of cells in the DR (table 3 and figures 1e and 1f). A weak non-specific background staining in the cytoplasm of hepatocytes appeared in some slides of cats with feline LC.

β-catenin

In the unaffected liver samples, a moderate to strong staining of β-catenin in the membranes of hepatocytes and cholangiocytes was seen (table 3 and figure 1g), indicating a low activation status of the Wnt/β-catenin signalling cascade. In the LC samples, a moderate to strong staining for β-catenin was seen in a membranous pattern in hepatocytes. In some slides, cytoplasm of hepatocytes stained moderate to strong. Ductular structures in portal areas with cell infiltrates stained moderate to strong in the cytoplasm/nucleus of the cells (table 3 and figures 1h and 1j) while the membranes still exhibited moderate to strong staining, too.

NICD

In unaffected liver tissue (table 3 and figure 1k) NICD staining was minimal. In diseased liver, NICD showed strong staining results in some patients (table 3 and figures 1m and 1n). In other patients, the intensity varied from weak to moderate, with a propensity towards weak staining intensity. Expression was seen in the bile ducts as a staining around the nucleus of the cholangiocytes. A weak non-specific cytoplasmic staining in hepatocytes was observed in all slides (control and patients).

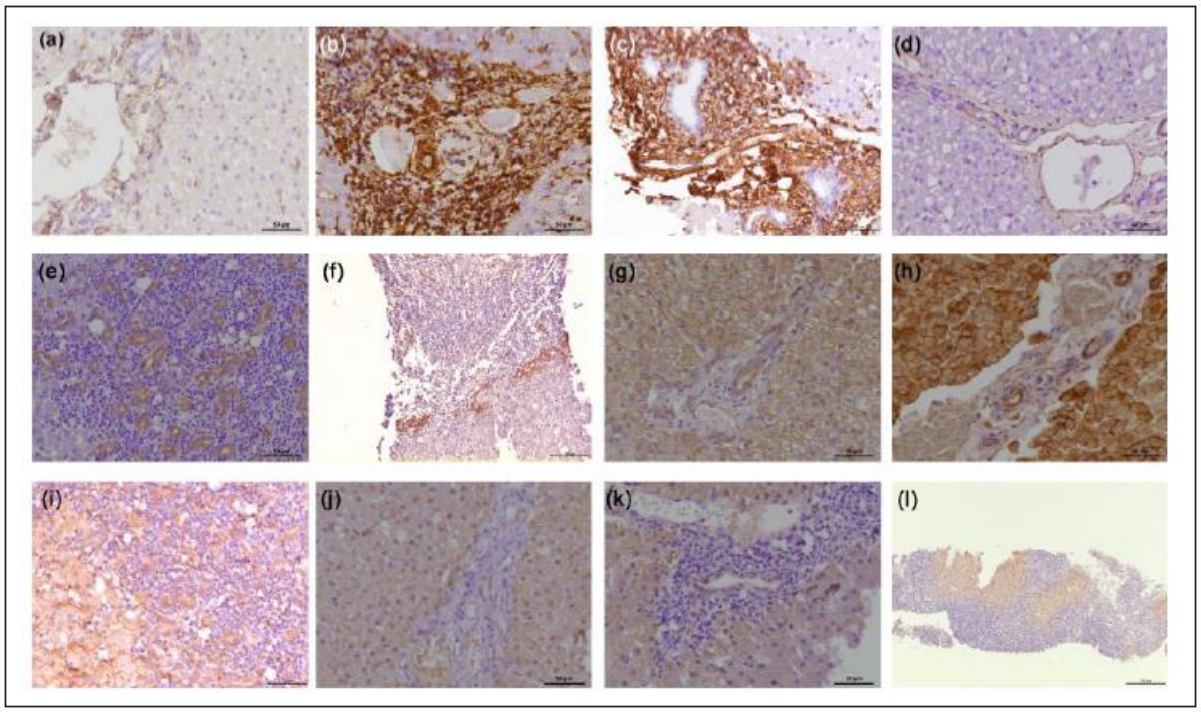

Figure 1. Immunolocalization of the expression of HPC markers in unaffected (a, d, g, k) and (b, c, e, f, h, j, m, n) liver tissue from LC patients. (a) weak vimentin expression in the extracellular matrix and vascular smooth muscle of unaffected liver (C2) and strong vimentin expression in portal structures in diseased liver (b, LC4 and c, LC5). (d) Weak laminin expression in the extracellular matrix of unaffected liver (C2) and moderate (e, LC6) to strong (f, LC2) expression of laminin in the DR in diseased liver. (g) Moderate to strong expression of beta-catenin in the membranes of hepatocytes and cholangiocytes in unaffected liver (C1) and moderate to strong expression of beta-catenin in membranes and cytoplasm/nucleus of hepatocytes and ductular structures in diseased liver (h, LC3 and j, LC6). (k) Weak Notch1/NICD expression in unaffected liver (C1) and weak to strong expression in the portal structures in diseased liver (m, LC5 and n, LC1).

Discussion

For this first and descriptive study of these immunohistochemical HPC markers on feline hepatic tissue, we used a small number of samples from LC patients and unaffected cats. All markers in our study showed markedly enhanced expression in livers of LC patients compared to unaffected liver samples. Additionally, the location of the expression differed between unaffected and LC liver tissue. This leads to our conclusion that the hepatic progenitor niche in cats with LC is remodelled and activated.

A limitation of our study is the small number of samples. This is a commonly encountered problem in many studies concerning cats, although it is also encountered in many liver research projects concerning human beings.[5, 20, 28, 33, 36] This made statistical analysis unsafe. Additionally, we have included one sample with no portal tracts in our analysis. Although this sample could not contribute to our study for the stainings that are aimed at portal tracts, we still obtained information from it about the hepatocytes and the staining with β-catenin. Furthermore, this specific sample was taken after treatment with prednisolone, which made it interesting to include for further evaluation. Therefore, this study describes the findings and future research is needed with larger sample sizes, including various feline liver diseases, such as hepatic lipidosis, and neutrophilic cholangitis in order to carry out statistical analyses.

Despite the small sample size, staining patterns were distinctly and consistently different in patients and unaffected cats. Although this is a much appreciated result, it made blinding of the authors for slide interpretation impossible.

Archival materials were used for patients. Fixation times and methods might have differed between these biopsies. Furthermore, unaffected samples were obtained from unrelated research which may have influenced the way liver biopsies were taken and stored in that study. None of the samples from patients or unaffected controls were optimized for immunohistochemical research and this impacts the staining results obtained. Additionally, cats had been treated by their local veterinarians before being referred to our referral clinic. Therefore, it was not possible to determine the chronicity of clinical disease. This may account for additional differences in staining results.

The control livers in this study expressed a low level of vimentin, suggesting the quiescent state of HPCs. In contrast, strong vimentin staining would indicate that cells are in an activated and proliferating state.[24, 26] Vimentin was expressed in lymphocytes, bile ducts and individual cells in the DR in the portal areas of the LC livers. Although vimentin is

traditionally regarded as a mesenchymal marker, it has been shown to be expressed in epithelial cells of human beings and rodents.[26, 37-40] More specifically, fibrosis of the hepatic tract has been attributed to cholangiocytes exhibiting mesenchymal characteristics, as demonstrated by the expression of vimentin.[39, 40] Since LC in cats may lead to fibrosis, our finding of vimentin positive cells in the DR may indicate that the same processes described in human beings and rodents take place in cats. This change in staining properties is attributed to epithelial-mesenchymal transition by some, although other researchers have concluded that vimentin is sometimes needed as a structural scaffold for cells to build their structures on.[26, 37-40]

The individual parenchymal cells in which vimentin was expressed, were most probably stellate cells since vimentin expression has been demonstrated in stellate cells before.[20, 41]

In this study, an expression of laminin in unaffected feline livers was seen in the ECM of portal areas, in and around vascular endothelium and bile ducts, and in sinusoidal endothelium adjacent to the portal area. This is similar to the results for healthy canine livers obtained in an immunofluorescence study.[18] In cats diagnosed with feline LC, an increase of laminin expression was seen in portal areas with many cell infiltrates, which is in accordance with earlier findings in cats and dogs.[18, 20] This may indicate activation of HPCs in cats, as it does in human beings and rodents.[19, 27] Furthermore, the role of activated stellate cells in promoting biliary differentiation of HPCs has been demonstrated recently for rodents.[42] Since LC is a biliary disorder, these findings correspond with our expectations.

The expression of β-catenin was evaluated because of its central role in the Wnt signalling pathway. In normal tissue, β-catenin is membrane-associated, but activation of the Wnt pathway leads to increased cytoplasmic/nuclear expression. The membranous expression of β-catenin in both hepatocytes and cholangiocytes in unaffected livers in the present study is in line with results from other mammals, indicating low activity of the Wnt signalling pathway in health.[25, 43] In the LC samples, an increase in cytoplasmic/nuclear staining was seen in ductular structures, together with co-expression in the membrane. In feline tumours of the uterus and mammary glands, a simultaneous staining of cytoplasm and membrane was observed previously.[31, 36] Thus, activated Wnt in cats may not shift completely from a membranous staining pattern to a cytoplasmic/nuclear staining pattern. Instead, the observed shift is more subtle as it includes a membranous staining pattern combined with a cytoplasmic/nuclear staining pattern. In our patients, the cytoplasm of cholangiocytes appeared to be darker than the cytoplasm of their hepatocytes. This is in line with the fact that

the Wnt/ β-catenin is involved in the development of bile ducts, the proliferation of HPCs and the promotion of a biliary phenotype. Furthermore, LC is not a liver disease, but a biliary disease.

NICD was expressed in several larger bile ducts in some LC samples, *ie.* wedge biopsies. As shown before, differences between needle and wedge biopsies exist and may be attributed to a change in epitopes as a result of formalin fixation ("overfixation").[44] Fixation routines and times influence immunohistochemical staining results by protein cross linking and masking of epitopes needed for IHC.[44]

Additionally, a weak cytoplasmic staining was seen in all hepatocytes. Boulter *et al.* showed Notch1 to be highly expressed in biliary regeneration in mouse models.[42] Variable Notch/NICD expression is also described in two earlier studies on human primary sclerosing cholangitis (PSC).[33, 34] Nijjar *et al.* found no expression of Notch1 in five PSC patients, while Ishimura *et al.* found Notch1 to be upregulated in the majority of PSC patients (75% of 16 patients). Our finding of variable upregulation of Notch1 in a series of similar size is consistent with findings in these two studies in humans with PSC.

Conclusion

Vimentin and laminin showed increased expression in portal areas of cats diagnosed with LC compared to unaffected feline livers. Additionally, β-catenin shifted from pure membranous to a more cytoplasmic/nuclear location. All markers showed enhanced expression in diseased livers, although NICD was not expressed in all patients.

These results indicate that the HPC-niche is remodelled and activated in feline lymphocytic cholangitis. Based on these and earlier found similarities between LC and PSC in men, feline LC might prove to be a suitable translational model for human biliary disease. Furthermore, HPCs might provide new regenerative treatment options for LC in cats in the future.

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Conflict of interest

The authors declare that they have no competing interests.

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CHAPTER 7 General discussion

Introduction

The feline liver is the largest internal organ and takes up approximately 3 to 4% of total body weight and consists of parenchyma, the biliary tree and vasculature like the portal vein and the hepatic artery.[1, 2] The liver has critical functions in metabolism and detoxification. Examples are synthesis of proteins like albumin and blood clotting factors, maintenance of glucose homeostasis, lipid metabolism, conjugation and excretion of endogenous and exogenous toxins and drugs, and excretion of bile.[3, 4]
Feline bile (pH 5.9 -7.8) consists of various (non-)sulphated bile acids, and cholesterol.[5, 6] Taurine is essentially the only nutrient used by cats to conjugate bile acids and because cats have limited taurine production capacity, it is considered an essential dietary component for cats.[6, 7] Therefore, taurine availability can also be considered a limiting factor in the production of conjugated bile acids. Felids have a low capacity for glucuronide conjugation of drugs and toxins, limiting their conversion to water soluble substances that can be excreted into urine or bile.[8] This makes cats more prone to suffer hepatotoxic effects when exposed to certain drugs and toxins.[9]

Parenchymal liver diseases in cats include lipidosis, amyloidosis and feline infectious peritonitis. In these diseases, the liver parenchyma is not the primary target, but is affected as part of the systemic disease. In cats, it is the biliary system rather than the liver parenchyma which is the primary target of disease.

Lymphocytic cholangitis (LC), one of the most common inflammatory hepatic diseases in cats, is a chronic disease that affects the biliary tree.[10, 11] It progresses slowly over months to years and its aetiology is still unknown. The hepatic lesions are characterized by aggregates of inflammatory cells in portal tracts, and in and around bile ducts.[11] Bacteria have been linked to the disease, but an immune-mediated component has also been suggested.[12-16]
Early clinical signs include nausea, vomiting and changes in appetite.[16, 17] Hepatic lipidosis is a complication that is best avoided but forcing an animal with sharp fangs and claws to eat poses threats for both owners and veterinarians.

Thus, determining the cause of this disease is desirable since this provides options to avoid the anorexic cat due to LC. Bacteria have been implicated in the past and that lead us to further investigate the role of bacteria. Additionally, treatment options are limited and more,

or better, treatment strategies are welcome.

Chapter 2 describes the current knowledge about feline diseases of the gallbladder and biliary tree. Inflammation, choleliths and neoplasms are described, together with rare conditions such as infarctions of the gallbladder. Interestingly, both LC and certain neoplasms occur more frequently in male cats.[18, 19] Feline LC and human PSC (Primary Sclerosing Cholangitis) have similar histopathological features, including inflammation of the portal tracts, bile duct proliferation, and fibrosis.[13, 16, 20] It is worth noting that prevalence of human PSC is also higher in males.[20-22]

Chapter 3 reports the screening of 39 bile samples of cats with LC. These samples were compared to 14 samples from feline controls. PCR analysis was performed, followed by DGGE analysis of the PCR amplicons. Additionally, *Helicobacter* spp. were used as positive control to screen for their presence in the bile samples.
All samples from controls were negative but in LC patients, bile was not sterile. Some controls and patients were positive for *Helicobacter* spp. Based on the variety of organisms found and the fact that both controls and patients were positive for *Helicobacter* spp., we concluded that bacteriobilia in LC patients seems not to be the cause but rather a consequence of the disease.
Chronic inflammation in the bile ducts causes dilatations and strictures and might eventually lead to fibrosis and cirrhosis.[23, 24] Stasis and altered propulsion in the biliary tract might provide an opportunity for ascending enteric bacteria.[10, 25-27] Based on these results, antibiotics are not indicated in the treatment of LC.

The advocated treatment for LC is corticosteroids, such as prednisolone. Since the aetiology of LC is unknown, an infectious cause cannot be ruled out. Corticosteroids can increase susceptibility to infections.[28] Therefore, alternative treatments with proven efficacy but without the risk of increased susceptibility to secondary infections were actively sought for cats with LC.
Recently, hepato-protective agents such as ursodeoxycholic acid (UDCA), have been added to the list of potentially beneficial treatment options.[29-31] In **Chapter 4**, we describe our analysis of retrospective data for both treatment options, *i.e.* prednisolone and UDCA. The results were based on analysing the data of 26 cats.
From an owner's perspective, the prognosis for LC is rather good, with a median overall

survival time of 795 days. Survival rates for 1, 2, and 3 years were 74%, 56%, and 35%, respectively. Purebreds and cats treated with UDCA were likely to have shorter survival times than Domestic shorthairs, and cats treated with prednisolone. Additionally, Norwegian Forest cats were overrepresented with LC when compared to the referral population.
Even though male cats were at a significantly higher risk than females for the diagnosis of LC, they also have longer survival times compared to females.

Cats with fewer clinical signs had longer survival times than cats with more clinical signs. This may be prognostically relevant, since cats with fewer signs might be in an earlier stage of the disease, or the disease might be more severe in feline patients that present with more clinical signs. From this study, we learned that cats with LC should preferably be treated with prednisolone.

Prednisolone's properties are well known. Its side effects are also numerous, and include polydipsia, polyuria, and polyphagia. Hepatic lipidosis is a complication that is life threatening in anorexic cats and should therefore be avoided. Thus, it might be that cats with LC that are treated with prednisolone eat well because of the polyphagia. This may lead to a better physical condition, no hepatic lipidosis, and a longer survival time. In **Chapter 5**, we explored this possible explanation by studying the effects of prednisolone and UDCA on hepatic tissue.
Twenty sequential archival biopsy materials from nine cats diagnosed with LC were used in this study. On average, biopsies were taken every two months and as a rule, a minimum of two biopsies was taken at any time from each patient by the attending clinician.
As mentioned before, cats have not received as much research attention as dogs. Therefore, we included tissue samples from dogs to verify the correctness of the immunohistochemical staining processes we used. As controls, tissue samples from cats that did not suffer from LC were included.

A routine haematoxylin and eosin (HE) staining was used to evaluate the degree of inflammation during therapy. Proliferation of cells was determined with Ki-67, a commonly used proliferation marker.[32] The activation of progenitor cells and proliferation of bile ducts was evaluated with immunohistochemical staining protocols for cytokeratin 19 (K19) as described previously.[33] Fibrogenesis was evaluated by applying a reticulin stain (Gordon and Sweet) to stain type III collagen.[11, 34] Furthermore, we evaluated the activation of HSC, the

main producers of collagen in the chronically injured liver, with a stain against α-smooth muscle actin (α-SMA).[35-40]

The severity of the inflammation decreased more when cats were treated with prednisolone. This was mostly due to the fact that the total part of the liver biopsy being affected by inflammation decreased. The fibrosis score increased more frequently during treatment with UDCA. This may be explained by the fact that the number of fibroblasts increased in 50% of the cats treated with UDCA, as fibroblasts actively contribute to liver fibrosis.[41] Concentric fibrosis was identified in three LC patients. This is a hallmark of the human disease Primary Sclerosing Cholangitis and has been seen in feline LC before, although it is not consistently present.
Based on these results, the survival results can now be explained in light of the effects of prednisolone and UDCA on liver tissue. We can confidently state that while longer survival times associated with prednisolone may be associated with polyphagia, a better body condition and the prevention of hepatic lipidosis, it is also due to decreased inflammation in the liver.

The injured liver is capable of a remarkable and unprecedented regeneration based on proliferation of all mature cell lines present in the liver.[38] Only when regeneration capacity proves inadequate, hepatic progenitor cells (HPCs) will be activated.[38, 42, 43]
Bipotent HPCs can give rise to both hepatocytes and cholangiocytes.[44] Upon activation, HPCs will proliferate, migrate from the canal of Hering to the site of injury, and differentiate into cholangiocytes or hepatocytes, depending both on the disease and on concurrent changes in their microenvironment (HPC niche).[42, 45, 46] The HPC niche, both a histological location and a functional unit, is of importance in maintaining and regulating HPCs behaviour, supporting self-renewal and balancing quiescence, proliferation and differentiation in response to injury.[46, 47] It is likely that the bile duct proliferation seen in LC involves both cholangiocytes and HPCs.[45]

Chapter 6 reports the results of our exploration of the activation of HPCs and the HPC niche in feline LC. Liver tissue of affected and unaffected cats was compared. The markers used for IHC were selected based on studies in other species, since most were not used on feline hepatic tissue before. A single specific HPC marker has not been identified yet, and many HPC markers have a shared expression with cholangiocytes.[45, 48, 49]

Vimentin is a common mesenchymal marker and has been shown to indicate proliferative activity of cells and an undifferentiated state of HPCs.[50, 51] Remodelling of the extracellular matrix (ECM) and deposition of laminin have been shown to play an important role in HPC activation in hepatic injury in both rodents and human beings.[47, 52] Furthermore, laminin was recently shown to promote cholangiocyte differentiation of bipotent cells in human liver disease.[53]

The Wnt/β-catenin signalling pathway has a central role in hepatic and bile duct development and regeneration, promoting gene activation, inhibiting apoptosis and increasing cellular proliferation.[54-56] Recently, it was shown to also be involved in homeostatic renewal of the liver.[57] Furthermore, active Wnt guides cells to a biliary phenotype.[58] When Wnt is activated, it shifts from a membranous staining pattern to a more cytoplasmic/nuclear staining pattern.

Notch is involved in the proliferation, differentiation and apoptosis in all stages of organ development, including healthy and diseased livers.[59] Notch1 was shown to be activated in human Primary Sclerosing Cholangitis, feline mammary tumours, and in HPCs of dogs with lobular dissecting hepatitis (LDH).[58, 60, 61] The expression of the proteolytically processed, and therefore active, Notch intracellular domain (NICD) has been used to measure the activation of Notch1 in cats before.[61]

All markers showed markedly enhanced expression in livers of LC patients compared to unaffected cats. Additionally, the location of the expression differed between unaffected and LC liver tissue. This leads to our conclusion that the hepatic progenitor niche in cats with LC is remodelled and activated. Therefore, HPCs might provide new regenerative treatment options for LC in cats in the future.

Conclusions of the thesis

- in LC patients, bile is not sterile
- *Helicobacter* spp. can be found in bile of cats suffering from LC and in unaffected cats
- bacteriobilia in LC patients seems not to be the cause but rather a consequence of the disease
- Norwegian Forest cats were overrepresented when compared to the referral population
- male cats were at a significantly higher risk than females for the diagnosis of LC, but they have longer survival times than females
- cats with fewer clinical signs had longer survival times than cats with more clinical signs
- purebreds and cats treated with UDCA were likely to have shorter survival times than Domestic shorthairs, and cats treated with prednisolone
- the severity of the inflammation decreased more when cats were treated with prednisolone
- liver fibrosis increased more frequently during treatment with UDCA
- concentric fibrosis, a hallmark of the human disease Primary Sclerosing Cholangitis, was identified in three LC patients
- longer survival times in the group treated with prednisolone may be associated with polyphagia, a better body condition and the prevention of hepatic lipidosis, but it is also due to decreased inflammation in the liver
- the hepatic progenitor niche in cats with LC is remodelled and activated

Directions for future research

Primary Sclerosing Cholangitis in humans has received considerable attention. The disease can occur at any age, but has a peak incidence at around 40 years of age.[21, 22] Liver transplantation is currently the only known treatment for halting the disease, although recurrence occurs in 15-25% of patients.[21, 22, 62]

The pathogenesis is still unknown, although genetics and environment are both suspected to play a role.[22] Currently, four hypotheses have been postulated:

1. the toxic bile hypothesis
2. leaky gut hypothesis
3. aberrant gut lymphocyte homing hypothesis
4. modification of pathways involved in liver fibrogenesis.[62]

Based on these hypotheses, research is aimed at modifying the bile acid pool, modulation of the intestinal microbiome, expression of signalling molecules on liver endothelium, and antifibrogenic therapies.[62] In one study, NSAID use was associated with a decreased risk for PSC patients for needing a liver transplantation.[21] Interestingly, NSAID use has been linked to more preferable outcomes in chronic liver disease before.[63]

Animal models and organoids are desperately needed to investigate the pathogenesis and new potential treatment options.[22] However, an ideal model for PSC has not yet been found.[64, 65]

Our studies show that cats with LC are a naturally occurring animal model for PSC. Although we are used to animal models fitting in one hand, *i.e.* rats and mice, this larger animal model might prove to be needed in order to further the research into human PSC.

Furthermore, feline liver organoids have been described recently.[66] This will facilitate the study of the influence of bile acids and intestinal bacteria on feline hepatic tissue. It would also be desirable to create feline LC organoids to study therapies.

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CHAPTER 8 Cholangitis bij katten: symptomen, oorzaak, diagnose, therapie en prognose

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Samenvatting

Ontsteking van de galwegen komt bij katten vaak voor. In dit artikel wordt een overzicht gepresenteerd van de huidige wetenschappelijke kennis over de verschillende vormen van cholangitis die worden onderscheiden, te weten neutrofiele cholangitis, lymfocytaire cholangitis en cholangitis door leverbot. Voor zowel cholangitis door leverbot als neutrofiele cholangitis bestaan passende therapieën die leiden tot een goede prognose voor de patiënt. Voor lymfocytaire cholangitis is echter tot op heden geen oorzaak vastgesteld waardoor een tegen de oorzaak gerichte therapie ontbreekt. In de literatuur worden verschillende mogelijke oorzaken beschreven maar het is noodzakelijk de onderzoeksinspanningen op te voeren om de etiologie vast te stellen en een therapie te bepalen.

Summary

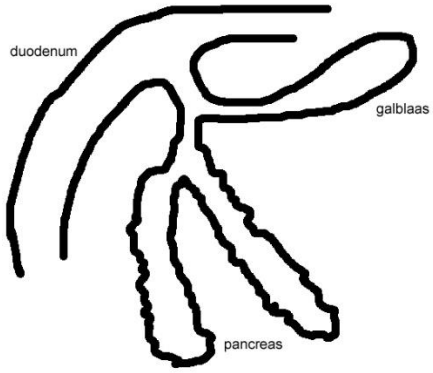
Inflammation of the bile ducts is a disease that frequently occurs in cats. In this review article, the current scientific knowledge about all types of cholangitis (i.e. cholangitis caused by liver flukes, neutrophilic cholangitis and lymphocytic cholangitis) is presented. For both cholangitis caused by liver flukes and neutrophilic cholangitis, therapies exist which ensure a good prognosis for the patient. However, an etiology for lymphocytic cholangitis is still missing and so is an evidence based therapy. Scientific literature suggests a number of possible etiologies, but it is necessary to increase the research efforts in order to establish the etiology of this disease and develop an appropriate therapy.

Introductie

De kat neemt een steeds prominentere plaats in binnen de diergeneeskunde. Op dit moment zijn katten de meest gehouden huisdieren in Nederland.[1] Bovendien worden zij steeds ouder als gevolg van een veranderende houding ten opzichte van huisdieren, toegenomen welvaart en verbeterde veterinaire zorg. Katten met cholangitis worden vaak bij de dierenarts aangeboden met aspecifieke verschijnselen als algehele malaise, koorts, misselijkheid, braken en afgenomen of wisselende eetlust.[2-4]

Bij de kat komen, in tegenstelling tot bij de hond, ziektes van de galwegen vaker voor dan ziektes van het leverparenchym zoals hepatitis. Het is onbekend waarom cholangitis bij de kat geregeld wordt vastgesteld. Een van de hypotheses is dat katten door hun specifieke

anatomische configuratie van de afvoergangen van de galblaas en de pancreas een verhoogde gevoeligheid voor cholangitis hebben.[5] Beide afvoergangen komen immers samen alvorens gezamenlijk uit te monden in het duodenum, zoals weergegeven in figuur 1. Dit is mogelijk ook de oorzaak van het feit dat cholangitis samen wordt gezien met pancreatitis.[6]



Figuur 1. Anatomische relatie van de galgang en de ductus pancreaticus bij de kat.

Bij de mens komt een dergelijke anatomische configuratie ook voor. Zij is hier echter, in tegenstelling tot bij de kat, afwijkend en wordt pancreatobiliary maljunction (PBM) genoemd.[7] Er zijn aanwijzingen dat bij PBM pancreassap terug kan vloeien in de galwegen waardoor PBM-patiënten last kunnen krijgen van steeds terugkerende episoden van cholangitis.[7, 8]

In dit artikel wordt een overzicht gepresenteerd van de huidige kennis op het gebied van cholangitis bij de kat. Na een korte uitleg van cholangitis worden drie vormen beschreven conform de huidige indeling volgens de liver standardization group of the World Small Animal Veterinary Association (WSAVA).[9] Dit zijn neutrofiele cholangitis, lymfocytaire cholangitis en cholangitis door leverbot. Deze vormen verschillen in etiologie, behandeling en prognose. Voor de prognose is het daarnaast mede van belang of de aandoening heeft geleid tot littekenweefsel of obstructie van de extrahepatische galwegen.[10, 11] Bij katten is het chirurgisch opheffen van een dergelijke obstructie via cholecystoduodenostomie, cholecystojejunostomie of met stents namelijk niet zonder risico.[12, 13]

Neutrofiele cholangitis

Symptomen

De klinische verschijnselen van neutrofiele cholangitis (NC) passen bij een (sub)acute ontsteking (algehele malaise en koorts), welke in sommige gevallen leidt tot een extrahepatische cholestase (icterus en acholische feces).[14] De meest opvallende symptomen van galwegziekten zijn echter misselijkheid, braken en anorexie.

Oorzaak

NC wordt meest waarschijnlijk veroorzaakt door een ascenderende bacteriële infectie vanuit de darmen. Een hematogene infectie is echter ook mogelijk.[15] Er zijn slechts twee studies die uitspraken doen over bacteriële veroorzakers van NC uitgevoerd bij katten. Wagner *et al.* rapporteren in zeven leverbiopten van 49 katten en vijf galmonsters van 14 katten een positieve bacteriekweek, waarbij in 83% van de gevallen slechts één organisme werd gekweekt.[16] In 19% van de gevallen betrof dit E. coli. Door Brain *et al.* werd uit de gal van zes katten in vier gevallen *E. coli* gekweekt.[17] De ontsteking gaat gepaard met pusvorming (foto 1).

Foto 1. Een galmonster van een patiënt met neutrofiele cholangitis. De gal in de spuit is geelgekleurd door de aanwezigheid van pus.

Diagnose

Bloedonderzoek toont verhoogde waarden van leverenzymen en vaak ook hyperbilirubinemie.[18]

De diagnose wordt gesteld met behulp van echografie van het abdomen en galonderzoek. Vooral in het subacute-chronische stadium toont echografie soms een galblaas met een verdikte wand en een verwijde ductus choledochus. In acute gevallen kan galblaasoedeem als een zwarte lijn in de galblaaswand worden gezien. In sommige gevallen is tijdens echografie echter niets afwijkends te zien.[9]

Een galmonster kan onder anesthesie worden genomen met een 22G naald. Savary-Bataille *et al.* betogen dat echogeleide percutane cholecystocentese een veilige, eenvoudige, minimaal invasieve en effectieve manier is om galmonsters te verkrijgen van gezonde katten.[19] Een kleinschalige rapportage over cholecystocentese bij katten met NC rapporteert geen directe negatieve gevolgen voor de patiënt.[17] Ook in de universiteitskliniek (UKG) worden galmonsters op deze wijze als standaardonderdeel van de onderzoeksprocedure probleemloos genomen. Op grond van ervaringen bij andere zoogdieren wordt geadviseerd de galblaas grotendeels te legen om lekkage van gal en gallige peritonitis te voorkomen.[19] De gepuncteerde gal moet zowel cytologisch als microbiologisch worden onderzocht.[9] Bij cytologie zijn neutrofielen, en soms ook bacteriën, zichtbaar. Tevens moet een bacteriekweek (aeroob en anaeroob) worden ingezet om de verantwoordelijke bacterie en zijn gevoeligheid voor antibiotica te bepalen.[9] Het nemen van een leverbiopt is bij NC niet nodig, want niet alle katten waarbij het bacterieel onderzoek positief is vertonen het karakteristieke histologische beeld met portaal oedeem en invasie van neutrofiele granulocyten in het epitheel en lumen van de galwegen.[9] Op basis van de aanwezige neutrofiele granulocyten kan dit beeld worden onderscheiden van extrahepatische cholestase. Echter, de histologische kenmerken zijn niet altijd zo specifiek. Histologisch kan er ook een niet-specifieke reactieve hepatitis zijn, zonder bovengenoemde veranderingen. De gouden standaard voor de diagnostiek is dan ook cytologisch en bacterieel onderzoek van de gal, inclusief een antibiogram.[9]

Therapie

Op de UKG starten wij in afwachting van de uitslagen van kweek en antibiogram met een behandeling met amoxicilline/clavulaanzuur (zie Tabel 1 voor een samenvatting van de verschillende therapieën bij cholangitis).[16, 17] Echter, resistentie tegen dit antibioticum is vastgesteld bij *E. coli*.[16] Het is verstandig om de behandeling minimaal drie à vier weken te laten duren.[17, 20] Om er zeker van te zijn dat de infectie geheel verdwenen is, is het aan te raden om na het beëindigen van de behandeling een tweede galmonster af te nemen en dit opnieuw cytologisch en microbiologisch te evalueren.

Prognose

De enige gepubliceerde studie betreft zes katten en toont aan dat in vrijwel alle gevallen (83%) de kat met het juiste antibioticum volledig herstelt.[17] Op grond daarvan kan de prognose zeer gunstig worden genoemd voor gevallen waarbij geen complicaties optreden.

Lymfocytaire cholangitis

Lymfocytaire cholangitis (LC) is ook bekend als lymfocytaire cholangiohepatitis, lymfocytaire portale hepatitis en niet-pusvormende cholangitis.[9] De ontstekingsreactie wordt gekenmerkt door lymfocytaire infiltratie in het lumen en de wand van portale galgangen en portale gebieden, tot in het parenchym.[9] Er treedt een diffuus verspreide intra- en extrahepatische cholestase op. De groepjes ontstekingscellen zijn in de actieve fase van de ziekte vooral T-cellen, omringd door B-cellen en plasmacellen.[21] In de chronische fase van de ziekte treedt er duidelijk fibrosering op.[21] Op grond van bovenstaande heeft de liver standardization group of the World Small Animal Veterinary Association (WSAVA) geoordeeld dat de beste naam voor deze ziekte is lymfocytaire cholangitis.[9]

Ondanks dat de kat een zeer populair huisdier is, is er in wetenschappelijk onderzoek weinig aandacht voor deze specifiek feliene aandoening. Sinds 1982 zijn er slechts een beperkt aantal artikelen gepubliceerd over LC. Deze artikelen zijn vooral gericht op mogelijke oorzaken.[22-24] In andere artikelen worden vooral individuele gevallen en histopathologische laesies beschreven.[15, 21, 25-27] Een duidelijke etiologie ontbreekt echter, evenals een goede, retrospectieve evaluatie van mogelijke therapieën. Ook prognostische indicatoren bestaan nog niet. Hierdoor is behandeling en preventie op grond van evidence based veterinary medicine tot op heden niet mogelijk.

Symptomen

De meeste eigenaren zullen op enig moment in het leven van hun kat verschijnselen als misselijkheid, wisselende eetlust, braken en een geleidelijk gewichtsverlies melden. Dit zijn symptomen die bij vele ziekten kunnen passen, waaronder LC.[2] De eerste studie naar LC in 1984 rapporteerde dat LC vooral werd gezien bij katten jonger dan 4 jaar, maar Weiss *et al.* concludeerden later dat LC vooral voorkomt bij katten die ouder zijn dan 10 jaar.[3, 6]

LC is een chronische ziekte die alle galwegen aantast en langzaam progressief voortschrijdt gedurende maanden of jaren. De ziekte openbaart zich niet onmiddellijk en wanneer uiteindelijk

symptomen optreden heeft de patiënt vaak al het chronische stadium bereikt. De aanhoudende ontstekingsreactie in de galwegen veroorzaakt verwijdingen, stricturen en fibrosering van de galwegen. In het meest chronische stadium is de ontsteking en fibrosering ook in het leverparenchym doorgedrongen. Geelzucht is vaak aanwezig in een later stadium van de ziekte. Andere symptomen, zoals misselijkheid, braken, verminderde eetlust en geleidelijk gewichtsverlies treden al eerder op. De lever is meestal vergroot en is bij lichamelijk onderzoek achter de ribboog voelbaar. Soms is een eiwitrijke vloeistof aanwezig in de buikholte (ascites) als gevolg van portale hypertensie.

LC geeft dus een duidelijk ander ziektebeeld dan NC. De klinische verschijnselen en de histopathologische veranderingen vertonen volgens Boomkens et al. veel gelijkenis met primary sclerosing cholangitis (PSC) bij de mens.[22] Li *et al.* associëren een histologisch patroon dat zij lymfocytaire cholangitis noemen met meerdere klinische aandoeningen, waaronder PSC.[28] Lucke en Davies betogen dat progressieve LC in de kat oppervlakkige gelijkenissen vertoont met PSC.[3] Center concludeert daarentegen dat het destructiepatroon van galwegen bij de kat opvallende gelijkenissen vertoont met bepaalde galwegziektes bij de mens, waaronder PSC.[18]

Oorzaak

In de literatuur worden enkele oorzaken gesuggereerd voor LC. Zo kan LC mogelijk worden veroorzaakt door een bacteriële ontstekingsreactie.[22-24] Sommige auteurs speculeren dat LC een immunologische basis heeft.[3, 21] Echter, een ondubbelzinnige oorzaak voor LC in katten is niet aangetoond en hierdoor ontbreekt een etiologisch gebaseerde behandeling.

In de UKG wordt uit de gal van patiënten met LC met de huidige laboratoriumprotocollen meestal geen bacteriën gekweekt. Een hypothese is dat een infectieuze oorzaak de aandoening initieert, waarna deze in stand wordt gehouden als immuungemedieerde aandoening, zoals wordt gesuggereerd door het type ontstekingscellen dat wordt gevonden.

Een andere mogelijke oorzaak van de schijnbare steriliteit van de gal van patiënten is de aanwezigheid van een persisterend infectieus agens dat de ziekte aanwakkert, maar dat *in vitro* niet of alleen onder zeer specifieke omstandigheden te kweken is. Zo zijn *Helicobacter spp.* en *Bartonella spp.* in verband gebracht met LC.[22-24] Greiter-Wilke *et al.* vonden *Helicobacter spp.* in 6% van de onderzochte katten.[23] Boomkens *et al.* tonen aan dat 26% van de katten met LC DNA-fragmenten van *Helicobacter spp.* in de gal heeft tegenover 16% katten zonder LC.[22] Over de aanwezigheid van *Helicobacter spp.* bij chronische lever- en/of galwegproblemen bij

verschillende diersoorten zijn vele artikelen gepubliceerd. Enkele voorbeelden betreffen onderzoek bij de mens, hond en knaagdieren.[29-32] Kordick *et al.* hebben experimenteel LC weten op te wekken in negen van 13 katten (69%) door hen in te spuiten met kattenbloed dat besmet was met Bartonella spp.[24] Voor zowel *Helicobacter spp.* als *Bartonella spp.* geldt dat zij zeer moeilijk te kweken zijn in het laboratorium.[33, 34]

Diagnose

Bloedonderzoek toont vaak verhoogde activiteit van leverenzymen en galzuren. Hypergammaglobulinemie treedt in bijna alle (9/14) gevallen op.[3, 20] Op basis van de hypergammaglobulinemie en de eventueel aanwezige ascites in de buikholte is het mogelijk om onterecht de waarschijnlijkheidsdiagnose FIP te stellen.[35]

Echografisch zijn in de meeste gevallen verwijde intra- en extrahepatische galwegen zichtbaar. Dit beeld wordt echter ook bij de veel zeldzamere gevallen van galwegobstructie en chronisch verwaarloosde NC gezien. Om LC definitief te kunnen vaststellen is dus ook nog een leverbiopt, waarin de architectuur van de lever zichtbaar is, noodzakelijk.[9] In de UKG is de ervaring dat het histologisch beeld van LC heel duidelijk kan zijn terwijl de galwegen echografisch niet afwijkend waren. Omgekeerd komen katten voor met onregelmatige dilatatie van de galwegen waarbij een obstructie kan worden uitgesloten maar waar de histologische kenmerken van LC niet overtuigend aanwezig waren. Een dunnenaaldaspiratiebiopt (DNAB) van de lever toont bij LC alleen lymfocyten aan en kan eventueel soms aanleiding geven tot een onjuiste diagnose van maligne lymfoom.[36-38] Een studie door Wang toonde aan dat cytologie slechts één van acht gevallen van LC correct kon diagnosticeren.[39] Roth betoogt dat 55% van de gevallen waarin histologie niet overeenstemt met cytologie worden veroorzaakt door ontstekingsverschijnselen die cytologisch niet en histopathologisch wel zichtbaar waren.[11] De WSAVA concludeert dat histologie noodzakelijk is om LC vast te stellen.[9]

Voordat histologische leverbiopten kunnen worden genomen moet worden vastgesteld dat de stollingsparameters (APTT PT, fibrinogeen en thrombocyten) van de patiënt binnen de referentiewaarden vallen. Bovendien is het van belang dat de patiënt nuchter is. Leverbiopten worden genomen onder echogeleide en algehele anesthesie om complicaties te voorkomen.[40, 41]

Het stellen van de juiste diagnose is van groot belang omdat de prognose voor patiënten met maligne lymfoom en FIP aanzienlijk slechter is dan voor patiënten met LC. In een onderzoek onder katten waarbij FIP met zekerheid was vastgesteld werd een mediane overlevingsduur van

9 dagen (interval: 3-200 dagen) gevonden.[42] Slechts één van de 37 katten overleefde langer dan drie maanden.[42] Een evaluatie van chemotherapie bij katten met maligne lymfoom toonde een mediane overlevingsduur van 116 dagen (interval: 7-1491+ dagen) aan.[43] Andere auteurs stelden een mediane overlevingstijd van 26 dagen vast voor maligne lymfoom in de lever bij acht katten.[44] Weiss *et al.* hebben 23 katten met LC gevolgd en kwamen tot de conclusie dat 30% minder dan één jaar, 44% tussen één en vijf jaar, en 26% langer dan vijf jaar overleefde.[4] De katten die langer dan één jaar overleefden behaalden een gemiddelde overlevingstijd van 51.8 maanden.[4]

Therapie

Tot 2003 werd op de UKG, op basis van literatuur en persoonlijke ervaringen, voornamelijk immuunsuppressie met prednison ingezet. De dosis en therapieduur werden bepaald op basis van de verschijnselen en de respons. Deze behandeling komt voort uit het feit dat LC vooralsnog als een immuungemedieerde ziekte wordt beschouwd.[3, 21] Er is geen gepubliceerd onderzoek dat een klinisch effect aantoont van systemische steroïden bij feliene LC. Aangezien er aanwijzingen zijn gevonden voor een infectieuze oorzaak is het onderdrukken van het immuunsysteem niet zonder risico's.[22-24] Bovendien zijn er de bekende bijwerkingen en contra-indicaties van corticosteroïden.[45]

In de Chinese geneeskunde wordt al sinds de vijfde eeuw ursodeoxycholzuur (UDCA) toegepast bij lever- en galwegziekten.[46] Sinds 1980 wordt het ook in de Westerse humane geneeskunde gebruikt bij diverse lever- en galwegziekten.[10, 47-49] Op grond van overeenkomsten in anatomie en fysiologie tussen kat en mens worden patiënten met LC in de UKG sinds 2000 ook behandeld met UDCA (15 mg/kg/dag).

Lichaamseigen galzuren zijn hydrofoob en bezitten een zeepachtige werking. Hierdoor veroorzaken zij beschadigingen van de celmembraan van hepatocyten en galwegepitheel, met apoptose als gevolg, zoals aangetoond in in vitro studies aan cellen van rat, muis en mens.[50-52] Ook is beschreven hoe endogene galzuren schade toebrengen aan mitochondriën en zo tot celdood leiden bij *in vitro* studies aan mitochondriën van ratten.[50] Het is bovendien aangetoond bij de mens dat endogene galzuren zorgen voor een verhoogde expressie van MHC I op hepatocyten en galwegepitheel, waardoor celdestructie wordt aangewakkerd en een ontstekingsreactie in stand wordt gehouden.[53, 54] Hydrofiele galzuren zoals UDCA stabiliseren juist de mitochondriale membraan en voorkomen daarmee celdood. De verwachting is dat door toxische, lichaamseigen galzuren te vervangen door UDCA het proces van apoptose wordt

afgeremd. UDCA stimuleert ook galsecretie (cholerese), waardoor cholestase en de daaruit voortvloeiende schade aan hepatocyten en galgangepitheel worden beperkt.[47] Katten met LC worden voor de rest van het leven behandeld met UDCA om schade aan hepatocyten blijvend te minimaliseren.

Prognose

Een prognose kan niet worden gegeven omdat er momenteel geen goede indicatoren zijn op basis waarvan de resterende levensduur voor de patiënt kan worden voorspeld.

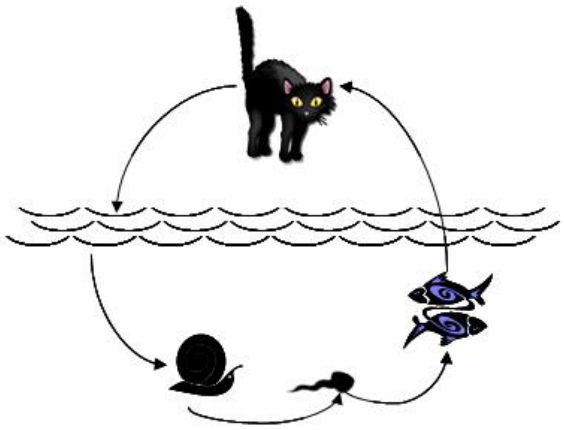
Cholangitis door leverbot

Symptomen

Katten met cholangitis door leverbot vertonen symptomen zoals lethargie, gewichtsverlies, verminderde eetlust, een pijnlijke buik, braken en icterus.[55, 56]

Oorzaak

Cholangitis door leverbot wordt bij de kat veroorzaakt door leverbotten uit de familie Opisthorchiidae of Dicrocoeliidae.[9, 57] Infecties met Dicrocoeliidae (*Platynosomum spp.*) komen voor in tropische en subtropische streken en worden in Nederland niet gezien.[57] Infecties met Opistorchiidae worden zelden waargenomen in Nederland. Een retrospectieve evaluatie van patiëntendossiers van de UKG uit de periode 1996-2009 toonde twee gedocumenteerde gevallen van cholangitis door leverbot aan.



Figuur 2. De levenscyclus van Opistorchiidae.

Volwassen leverbotten bevinden zich in de galwegen van de kat. Eieren worden met de gal uitgescheiden in de feces. Wanneer feces terechtkomt in zoetwaterplassen kunnen slakjes de eieren opnemen. In de slak ontwikkelen de miracidiën uit de eieren zich tot sporocysten en redia. Uiteindelijk komen cercariae vrij in het water. Hier infecteren zij het spierweefsel van zoetwatervissen.[58] De kat raakt vervolgens besmet door het eten van rauwe zoetwatervis (zie figuur 2). Besmetting is dus eenvoudig te voorkomen door geen rauwe zoetwatervis te voeren aan katten.

Diagnose

Leverboteieren zijn soms zichtbaar bij het histologisch beoordelen van leverbiopten.[55] Ook kunnen zij worden aangetoond door middel van microscopisch onderzoek van de gal.[55] Met behulp van concentratietechnieken kunnen zij tevens worden aangetoond in de feces.[59] De gevoeligheid van deze detectiemethoden is echter laag, zodat deze oorzaak moeilijk met zekerheid kan worden uitgesloten. Wanneer op basis van de anamnese het eten van rauwe zoetwatervis met absolute zekerheid kan worden uitgesloten mag cholangitis door leverbot wél uit de lijst met differentiaaldiagnoses worden verwijderd.

De infectie met leverbot kan een eosinofiele ontstekingsreactie in en rond de galgangen veroorzaken. Een histologisch beeld met een eosinofiele ontsteking in de portale gebieden is suggestief voor cholangitis door leverbot.[55] Chronische cholangitis door leverbot leidt tot dilatatie en fibrosering van de galwegen, hetgeen echografisch aantoonbaar is.

Therapie

Opistorchiasis is goed te behandelen met praziquantal, 20 mg/kg eenmaal daags gedurende drie dagen.[56] Onderzoek wijst uit dat het wegnemen van de oorzaak een belangrijke factor is in het beperken van leverfibrose, zelfs wanneer fibrosering al is opgetreden.[60] Bovendien kan voortgang naar levercirrhose worden voorkomen.[60]

Prognose

De prognose is goed wanneer de therapie correct wordt ingezet en uitgevoerd.[61] Veranderingen aan de galwegen, zoals dilatatie, zullen aanwezig blijven en zorgen voor een verhoogde gevoeligheid voor ascenderende bacteriële infecties vanuit de darmen.[20]

Conclusie en toekomstig onderzoek

Voor de drie vormen van feliene cholangitis is de huidige kennis op het gebied van symptomen, oorzaak, diagnose, therapie en prognose beschreven. Neutrofiele cholangitis en cholangitis door leverbot kennen een duidelijke etiologie waardoor een doeltreffende therapie voorhanden is. Voor lymfocytaire cholangitis is echter nog geen definitieve oorzaak vastgesteld. Daarom is meer onderzoek nodig naar deze vorm.

Het DNA dat door Boomkens *et al.* (2004) is aangetoond in de gal van katten is identiek aan het DNA van de Helicobacter soort die wordt gezien als oorzaak voor hepatocellulair carcinoom bij de mens.[22] De kat is mogelijk dus niet alleen zelf slachtoffer, maar misschien ook zoönotisch reservoir. Door de etiologie van LC op te helderen kan ook een gerichte therapie worden bepaald.

Bovendien zijn er overeenkomsten tussen galwegproblemen bij kat en mens.[3, 18, 22, 28] Het is belangrijk om LC en PSC met elkaar te vergelijken om vast te stellen in hoeverre deze ziektebeelden overeenkomen. Op basis hiervan kan dan uit de overvloedig beschikbare literatuur over onderzoeken bij de mens aanvullende kennis worden opgedaan over het feliene ziektebeeld.

Cholangitis door leverbot	Praziquantel (20 mg/kg eenmaal daags, drie dagen)
Neutrofiele cholangitis	Antibiotica op geleide van ABG, minimaal 3 à 4 weken
Lymfocytaire cholangitis	Ursodeoxycholzuur (15 mg/kg/dag, levenslang)

Tabel 1. Overzicht therapieën voor cholangitis.

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CHAPTER 9 Nederlandstalige samenvatting

De lever is een belangrijk orgaan in het kattenlichaam. Qua grootte neemt het ongeveer 3-4% van het totale lichaamsgewicht in beslag. Het is van belang voor diverse kritieke processen in het lichaam, zoals de productie van eiwitten en bloedstollingsfactoren, het bewaken van de bloedglucosespiegel, de vethuishouding, het onschadelijk maken van medicijnen/toxines en de productie en afvoer van gal.
De lever bestaat uit twee verschillende typen weefsel:

– het functionele leverweefsel (parenchym)
– en het systeem van galwegen.

Vooral de galwegen zijn bij de kat aangedaan door ziektes.

Ontstekingen van de galwegen kunnen we onderscheiden in drie verschillende vormen:

- neutrofiele cholangitis: een ontstekingsvorm waarbij bacteriën een belangrijke rol spelen
- cholangitis door leverbot: een ontstekingsvorm waarbij wormen een belangrijke rol spelen
- **lymfocytaire cholangitis**: een chronische ontstekingsvorm waarvan de oorzaak onbekend is maar waarin een rol is weggelegd voor bacteriën en het afweersysteem van de kat.

Omdat de oorzaken divers zijn verschilt ook de behandeling van de drie vormen. Daarom is het van belang om met behulp van beeldvormende technieken, zoals echo, en het nemen van kleine stukjes weefsel (biopt) te komen tot een diagnose. In het Nederlandstalige artikel uit het Tijdschrift voor Diergeneeskunde, **hoofdstuk 8**, worden de verschillende vormen, met hun therapie en prognose, beschreven.
In **hoofdstuk 2** wordt op basis van de beschikbare literatuur een overzicht gegeven van de verschillende aandoeningen die de galwegen en de galblaas kunnen treffen bij de kat.

Cholestase treedt op wanneer de afvoer van gal wordt belemmerd. Dit is geen ziekte, maar een fenomeen dat kan optreden in het verloop van verschillende aandoeningen aan de galwegen of lever. Een dikke, slijmachtige gal (*sludge*) en galstenen kunnen zorgen voor een belemmerde galafvoer. Ook ontstekingen en een verminderde samentrekkingskracht van de galblaas kunnen er toe leiden dat de gal niet correct wordt afgevoerd. De symptomen zijn vaag, maar meestal zijn de katten misselijk en stoppen zij met eten. Geelzucht kan optreden.

De galblaas van katten heeft vaak (bij 12% van de katten) een afwijkende vorm. Dit betekent

niet zoveel en vaak wordt zo'n afwijkende galblaas bij toeval gediagnosticeerd. Galstenen kunnen worden aangetroffen in de galblaas, maar zorgen zelden voor klinische klachten. Wanneer stenen vastlopen in de afvoerwegen kan natuurlijk cholestase ontstaan en dan zien we vaak wel klachten. Gal kan worden onderzocht door met een naald een galmonster uit de galblaas te nemen. Verschillende onderzoeksmogelijkheden (kweek, cytologie) zijn hiervoor beschikbaar.
Bij de hond zien we soms een slijmerige inhoud in de galblaas, maar dat is bij de kat uiterst zeldzaam. Een infarct, waarbij de galblaasslagader wordt afgesloten door een bloedstolsel, is beschreven bij de hond maar zijn nog niet aangetroffen bij katten.

Tumoren tasten bij de kat vaker het systeem van de galwegen aan dan het leverparenchym. Vooral katers zijn aangedaan. Een groot deel van de lever kan worden verwijderd zonder dat de leverfunctie er onder lijdt. Chirurgie is dan ook vaak de beste keuze. Gelukkig zijn tumoren zeldzaam (1 tot 5,5% van alle tumoren in katten).

Cystes (onnatuurlijke lichaamsholtes, bekleed door epitheelcellen) komen voor in de galwegen. Niet alleen bij onze huiskatten, maar ook bij grote katachtigen zoals leeuwen en luipaarden. Meestal veroorzaken deze cystes geen klachten, maar soms zijn zij het gevolg van PKD (*polycystic kidney disease*). Deze ziekte, die vooral bij Perzische katten bekend is, treft voornamelijk de nieren, maar kan ook leiden tot cystes in de lever. Door goede fokprogramma's waarbij tevoren gekeken werd of de ouderdieren het PKD-gen bezaten en niet te fokken met deze dieren is deze ziekte tegenwoordig ver terug gedrongen.

In de tabellen van hoofdstuk 2 worden medicijnen besproken die bij katten schadelijke effecten op de lever (kunnen) hebben. Ook de verschillende laboratoriumwaarden die uit bloedonderzoek kunnen blijken worden opgesomd en besproken. Technieken voor het nemen van biopten of het uitvoeren van chirurgie worden samengevat, als ook de verschillende therapieën die succesvol zijn en worden gebruikt in de praktijk.

In het vervolg van het proefschrift wordt nader ingegaan op het onderzoek dat is verricht naar lymfocytaire cholangitis (LC).

Hoofdstuk 3 is gewijd aan het onderzoek dat in samenwerking met Wageningen Universiteit is uitgevoerd. Omdat studies in het verleden bacteriën aanwezen als mogelijke veroorzaker

van LC zijn galmonsters van LC-patiënten en katten die niet leden aan de ziekte onderzocht. Hiervoor is het 16S ribosomaal RNA gen gebruikt. Dit materiaal is aanwezig in bijna alle bacteriën en verschilt sterk van soort tot soort. Daarom kan het goed worden gebruikt om bacteriën te identificeren en te classificeren.

De galmonsters van patiënten en gezonde katten werden gebruikt om het DNA van dit gen te isoleren. Daarna werd met een polymerase kettingreactie (*PCR*) het gevonden DNA vermenigvuldigd. Onderzoekers hebben gedacht dat Helicobacter bacteriën mogelijk een rol spelen bij het ontstaan van LC. Daarom is specifiek ook gezocht naar de aanwezigheid van Helicobacter in de galmonsters van patiënten en niet-patiënten.

Gel electroforese is een methode waarmee DNA-moleculen op grootte kunnen worden gescheiden. DNA-moleculen zijn namelijk negatief geladen en zullen zich van de negatieve kant van een gel naar de positieve kant van een gel bewegen. Wanneer DNA van het 16S ribosomaal RNA gen wordt gebruikt heeft dit echter altijd dezelfde grootte. Daarom maken wij gebruik van DGGE.

DGGE (*denaturing gradient gel electrophoresis*) is een techniek waarbij gebruik wordt gemaakt van het feit dat de opgevouwen vorm van DNA onder invloed van chemische stoffen kan worden verbroken (denaturatie). Bovendien wordt hitte ingezet om het DNA te smelten waardoor zij hun opgevouwen vorm verliezen. Normaliter heeft opgevouwen DNA een mooie helix-vorm (dubbele spiraal). Deze is redelijk aerodynamisch. Door het ontvouwen wordt de vorm veel minder aerodynamisch. De gel kan door de aerodynamische vorm gemakkelijk worden doorkruist, maar de minder aerodynamische vorm komt ergens in de gel tot stilstand. De plaats waar het DNA tot stilstand komt is afhankelijk van de temperatuur waarbij het smelt. Dit verschilt van DNA-molecuul tot DNA-molecuul en dus van bacterie tot bacterie.

De verschillende bandjes in de gel vertegenwoordigen DNA-moleculen van verschillende samenstelling. Door deze bandjes uit te snijden en verder te analyseren kan de volgorde van het DNA worden bepaald. Daarna kan in een grote databank met bekende DNA-volgordes worden opgezocht welke bacteriën aanwezig waren in de galmonsters. In de appendix achter hoofdstuk 3 wordt het hier beschreven proces in beeld gebracht.
In de galmonsters van katten die niet leden aan LC werd geen enkel stuk bacterieel DNA aangetroffen. Maar in de galmonsters van katten met LC werden wel bacteriële DNA-stukken

aangetroffen. De gevonden stukken bacterieel DNA behoorden tot normale bacteriën waarmee de kat in aanraking kan zijn gekomen door het eten van bepaald voer (vis, zuivel), het buiten rondlopen (grond en water) en door het contact met de baasjes (huid). Omdat katten zich vaak wassen kunnen zij gemakkelijk via de vacht bacteriën op likken. Deze kunnen dan via het maagdarmkanaal in de kat terecht komen. Wanneer de galwegen zijn aangedaan door een chronische ontsteking zoals LC zal de normale voortstuwende beweging (peristaltiek) worden belemmerd en kunnen bacteriën tegen de richting in omhoog kruipen om de gal te koloniseren.

Helicobacter, de bacterie die bij de mens kan leiden tot maagzweren en die verdacht werd van een rol bij LC, werd niet aangetroffen in de galmonsters. Daarom hebben wij een nog gevoeliger techniek toegepast om specifieker te zoeken naar deze bacterie. Na toepassing hiervan werd zowel bij patiënten als bij katten die niet leden aan LC DNA van Helicobacter bacteriën aangetroffen. Het is dus niet heel waarschijnlijk dat deze bacterie een specifieke oorzaak van LC is.

Helicobacter is een lastige bacterie om te kweken in een kweekschaaltje in het laboratorium. Zo kan zij moeilijk groeien bij teveel zuurstof. Moleculaire technieken, zoals hier toegepast, zijn dus voor Helicobacter een uitkomst. De techniek is ook zeer goed toepasbaar wanneer er wordt gezocht naar onbekende bacteriën. Immers, het is dan ook onbekend welke kweekvoorwaarden gehanteerd moeten worden en dat maakt het haast onmogelijk om voor alle mogelijke aanwezige bacteriën de juiste voorwaarden te scheppen. De hier toegepaste moleculaire techniek gaat volledig hieraan voorbij en kan op grond van het 16S ribosomaal RNA gen dat in bijna alle bacteriën aanwezig is bepalen met welke bacteriën men van doen heeft.

De veelheid aan gevonden organismen geeft aan dat een behandeling met antibiotica als primaire strategie waarschijnlijk niet zoveel zin heeft. Wel kunnen antibiotica worden ingezet wanneer de secundaire infecties leiden tot klinische problemen bij de patiënt. In het algemeen wordt bij LC vaak gebruik gemaakt van corticosteroïden zoals prednison omdat het immuunsysteem van de patiënt waarschijnlijk een rol speelt.

Omdat de oorzaak van LC nog niet is vastgesteld is het lastig om effectieve behandelingen te bepalen. Omdat er bijna uitsluitend lymfocyten worden gezien als ontstekingscellen in het

leverweefsel van katten met LC wordt vaak aan een auto-immuun of immuun-gemedieerd ontstekingsproces gedacht. Daarom worden vaak corticosteroïden (zoals prednison) als therapie gebruikt. Bij mensen met cholangitis (primair scleroserende cholangitis, PSC) wordt ook vaak gebruik gemaakt van UDCA (ursodeoxycholzuur, ursochol). Omdat er overeenkomsten bestaan tussen het ziektebeeld bij de mens en bij de kat is nader onderzoek verricht naar UDCA. Het beschikt over beschermende eigenschappen waarbij vooral ontsteking wordt geremd, cellen worden beschermd en de afvoer van gal wordt gestimuleerd. Op de Faculteit Diergeneeskunde te Utrecht zijn een aantal katten behandeld met prednison en ook UDCA is toegepast. In **hoofdstuk 4** worden beide behandelwijzen met elkaar vergeleken, waarbij vooral wordt gekeken hoe lang patiënten overleven wanneer zij met een van beide therapieën worden behandeld.

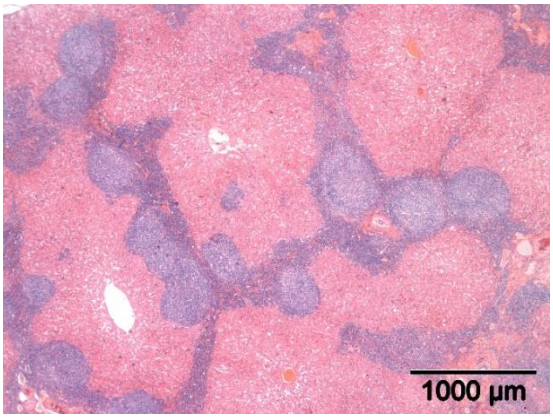
Voor deze studie waren de gegevens van 26 katten beschikbaar (20 katers en zes poezen). Tien katten werden behandeld met prednison en 13 met UDCA. Twee katten kregen een combinatietherapie van prednison en UDCA, één kat kreeg ook nog antibiotica in de gal ingespoten en één kat stierf kort na opname in de kliniek aan hartproblemen.
Opvallend was het grote aantal mannelijke patiënten. Dit zien we niet alleen bij katten, maar ook bij de mens zijn het voornamelijk mannen die door LC/PSC worden getroffen. De meeste katten zijn wat ouder (gemiddeld 12 jaar) wanneer de ziekte wordt geconstateerd en leven na diagnose gemiddeld nog 18 maanden. De Noorse Boskat was oververtegenwoordigd ten opzichte van andere rassen. Over het algemeen hadden raskatten een kortere overlevingstijd dan gewone huiskatten (Europese Korthaar). De gemiddelde levensverwachting na diagnose is 795 dagen en na 1, 2 en 3 jaar is nog respectievelijk 74%, 56% en 35% van de patiënten in leven. Katers overleven wat langer dan poezen en katten behandeld met prednison overleven langer dan katten behandeld met UDCA.

Een mogelijke verklaring is dat de vergrote eetlust die optreedt bij het gebruik van prednison de patiënten goed doet. Immers, een kat die stopt met eten loopt kans op leververvetting en dat is een ernstige aandoening die het leverweefsel aantast en het functioneren van de lever beperkt. Blijven eten heeft dus een gunstig effect op de lever en deze bijwerking van prednison is voor katten met verminderde eetlust door LC een zeer welkome bijwerking. Om te onderzoeken of dit de reden is dat prednison beter werkt dan UDCA is gekeken naar het leverweefsel van patiënten. In **hoofdstuk 5** wordt dit onderzoek beschreven.

De lever is een uniek orgaan in meerdere opzichten. Zo is de lever in staat om bij beschadiging zichzelf te herstellen: een eigenschap waarover de meeste andere organen niet beschikken. Dit herstel vindt plaats met de al aanwezige lever- en galwegcellen. Pas wanneer dit mechanisme tekortschiet worden voorlopercellen (stamcellen) geactiveerd.
Wanneer leverweefsel chronisch wordt beschadigd kan fibrosering optreden. Hierbij wordt steeds meer steunweefsel (collageen) in de lever aangelegd waardoor het sponzige weefsel verandert in een taaiere massa die niet langer alle functies goed kan uitvoeren (levercirrhose).

De gouden standaard om LC vast te stellen is het beoordelen van een stukje leverweefsel (biopt). Tijdens de behandeling van patiënten met prednison of UDCA zijn meerdere biopten genomen. Door deze biopten met elkaar te vergelijken kan worden vastgesteld welk effect de therapie heeft op leverweefsel. Hierbij kijken we specifiek naar herstel door te zien of levercellen, galwegcellen en voorlopercellen worden geactiveerd. Ook kijken we of de ontsteking vermindert, verergert of hetzelfde blijft door de mate van ontsteking en het aantal lymfocyten te beoordelen. Daarnaast wordt het aantal cellen dat tot fibrosering kan leiden beoordeeld.

Wanneer een biopt wordt ingezonden voor beoordeling door de patholoog wordt altijd een standaard kleuring toegepast op het weefsel. Deze geeft een paars-roze beeld en stelt de patholoog in staat de verschillende structuren en cellen onder de microscoop te onderscheiden en te beoordelen.

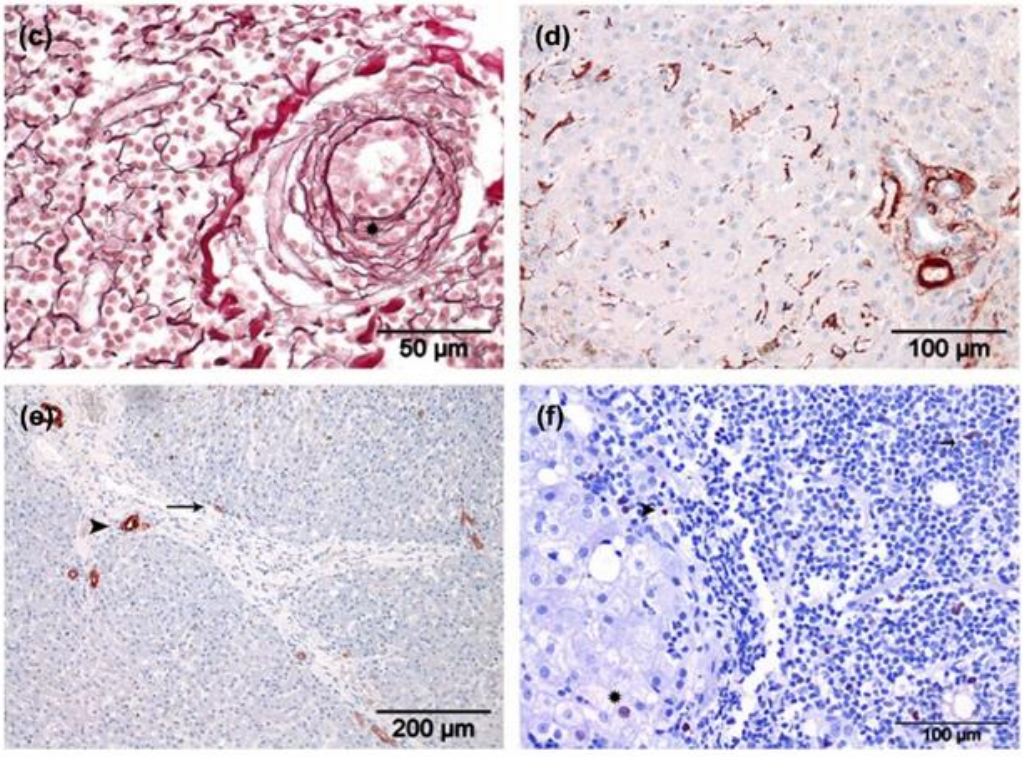


Hematoxyline en eosine (H&E) kleuring van een leverbiopt van een kat met LC. Lymfocyten (paars) zijn in grote aantallen aanwezig in de portale gebieden en overbruggen de afstand van portaal gebied naar portaal gebied.

Helaas geeft deze kleuring niet voldoende mogelijkheid tot het beoordelen van de cellen waarin wij geïnteresseerd zijn. Daarom hebben wij immunohistochemische kleuringen ontwikkeld en toegepast.
Immunohistochemie (*IHC*) verwijst naar een techniek waarmee met behulp van antilichamen antigenen in weefsels kunnen worden aangetoond. Omdat specifieke cellen beschikken over specifieke antigenen kan met een bijbehorend specifiek antilichaam een kleurtje worden aangebracht op deze cellen. Hierdoor zijn zij onder de microscoop identificeerbaar.
Wij hebben gebruik gemaakt van de volgende immunohistochemische kleuringen:

- Ki-67: hiermee kunnen delende cellen zichtbaar worden gemaakt
- K19: om de activering van voorlopercellen en de toename vangalwegen zichtbaar te maken
- α-SMA: om collageenproducerende cellen zichtbaar te maken.

Om collageen in bindweefsel aan te kleuren hebben we een reticuline kleuring gebruikt. In onderstaande figuur worden enkele resultaten getoond.



In (c) is een galweg aangegeven met de asterisk. Daaromheen zien we ringen van collageen (reticuline kleuring). In (d) zien we de aankleuring van α-SMA rond de galwegen, in bloedvaten en in collageenproducerende cellen. Met de pijlpunt wordt in (e) gewezen naa aankleuring van K19 rondom een galgang. Voorlopercellen (aangegeven met de hele pijl) zien we in het leverparenchym. In (f) zien we een toename van lymfocyten (pijl), fibroblasten (pijlpunt) en levercellen (asterisk) die met Ki67 aankleuren.

De resultaten van deze studie laten zien dat prednison de ontsteking sterker remt dan UDCA. Bij behandeling met UDCA neemt fibrosering bovendien sterker toe dan tijdens behandeling met prednison. Het positieve effect van prednison op de levensverwachting van katten, zoals beschreven in hoofdstuk 4, is dus niet alleen toe te wijzen aan de vergrote eetlust. Prednison heeft ook een positief effect op het leverweefsel zelf, met name op de remming van de ontsteking en het tegengaan van fibrosering.

Hoofdstuk 6 beschrijft aanvullend onderzoek naar de activering van voorlopercellen (*hepatic progenitor cells, HPC*). In hoofdstuk 5 zijn deze al beoordeeld met behulp van de K19 kleuring, maar kon geen verschil worden aangetoond tijdens de twee verschillende behandelingen (met prednison of UDCA). HPC zijn bipotent en kunnen zowel levercel als galwegcel worden. Als de schade aan de lever zo groot is dat deze niet met de aanweizge cellen kan worden gerepareerd worden de HPC geactiveerd. Normaal gesproken houden zij zich op in het kanaal van Hering, maar bij activering migreren zij naar de plaats waar zij nodig zijn. De omgeving, HPC niche genoemd, speelt hierbij een belangrijke rol.

Bij LC zien we een toename van het aantal galwegen in de leverbiopten van patiënten. Het is waarschijnlijk dat zowel galwegcellen als HPC hierbij betrokken zijn. Helaas zijn er geen specifieke kleuringen beschikbaar die HPC zichtbaar maken onder de microscoop. Daarom is een combinatie van kleuringen toegepast zodat met meer zekerheid kan worden bepaald of het om HPC gaat. Omdat dit nog niet eerder voor katten is gedaan hebben wij gebruik gemaakt van studies in andere diersoorten. Op basis van die resultaten hebben we gekozen voor toepassing van de volgende kleuringen:

- vimentine: deze kleuring is succesvol gebruikt voor HPC in ratten, muizen, mensen en honden
- laminine: deze kleuring toont bij knaagdieren en mensen een hermodellering van de extracellulaire matrix en activering van HPC aan
- Wnt/β-catenin: bij muizen, zebravisjes, honden en mensen is deze kleuring toegepast om toename, migratie en differentiatie tot galwegcellen van HPC aan te tonen
- Notch1/NICD: deze kleuring is o.a. succesvol toegepast bij mensen met PSC en Notch1/NICD is betrokken bij de ontwikkeling van de lever, zowel wanneer deze gezond is als wanneer deze wordt aangedaan door ziekte.

Naast leverweefsel van patiënten is ook leverweefsel van gezonde katten gebruikt. Zo kan een vergelijking worden gemaakt tussen ziek en gezond.
Bij katten met LC waren alle kleuringen sterker aanwezig dan bij de gezonde katten. Bovendien verschilde de locatie waar de kleuringen geactiveerd werden tussen patiënten en gezonde katten. Op grond hiervan hebben wij geconcludeerd dat bij een chronische ziekte als LC de HPC niche wordt geactiveerd en dat het herstel van leverweefsel dus mogelijk mede door HPC wordt bewerkstelligd. Het activeren van HPC is dus mogelijk een interessante optie voor toekomstige therapieën voor katten met LC.

Naast deze resultaten, die helpen in het verder karakteriseren en begrijpen van LC bij katten, heeft het onderzoek ook immunohistochemische protocollen opgeleverd. Voor honden waren deze protocollen wel aanwezig, maar voor katten moest een en ander nog worden aangepast en verder ontwikkeld. In **hoofdstuk 5 en 6** worden de protocollen dan ook uitgebreid beschreven zodat zij ook door andere onderzoekers kunnen worden toegepast.

In **hoofdstuk 7** worden alle resultaten samengevat en bediscussieerd. Er worden bovendien enkele aanbevelingen gedaan voor toekomstig onderzoek.

Concluderend kan worden gezegd dat dit onderzoek heeft bijgedragen aan de kennis van LC bij katten. Door de ontwikkeling van immunohistochemische kleuringen kan in de toekomst eenvoudiger en gerichter onderzoek worden gedaan naar de lever van katten in het algemeen. Voor practici is het duidelijk geworden welke therapie het beste kan worden ingezet en welke prognoses zij aan de eigenaren van patiënten kunnen geven. Hoewel LC een belangrijke leverziekte bij katten is, worden weinig patiënten hiermee naar de universiteitskliniek voor gezelschapsdieren doorgestuurd. Grotere aantallen zijn nodig om dit onderzoek een vervolg te geven in prospectieve klinische trials.

Acknowledgements

For what it's worth: It's never too late to be whoever you want to be.
I hope you live a life you're proud of, and if you find that you're not,
I hope you have the strength to start over- F. Scott Fitzgerald

I would like to show my gratitude to all individuals who helped me during the adventure that becoming a veterinarian and getting a PhD has been. Thank you!

When I started as a new student at the Faculty of Veterinary Medicine after spending 11 years in various offices, there was a bit of shock involved. Especially when the biochemist put some chicken livers in the blender, turned on the machine and said: "this is biochemistry". Surrounded by other new students, who thought I was old enough to be their mother, I did wonder what I got myself into. But then I walked into the pathobiology department and attended a lecture about dementia in dogs. And I was totally blown away by veterinary medicine. That feeling of wanting to know more, wanting to learn more, and becoming an excellent veterinarian has been there ever since. So thank you, Dr. Jaime Rofina: I will always be grateful that you did that for me, even though you never knew it. Please go on inspiring others.

Enthusiasm is one of the most powerful engines of success.
When you do a thing, do it with all your might.
Put your whole soul into it.
Stamp it with your own personality.
Be active, be energetic and faithful, and you will accomplish your object.
Nothing great was ever achieved without enthusiasm - Ralph Waldo Emerson

I am heartily thankful to all cats, their owners and veterinarians for providing the information and samples needed for this research.

A big thank you to Robert Favier and Jan Rothuizen for their support and the fact that they entrusted their patient data and samples to me. Jan, whenever I thought that my research project would not be very interesting to other people, you managed to convince me that it

would. This thesis is proof that you were right all along. I am very proud that I was one of your students.

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I owe a big thank you to all the nice people at the Laboratory of Microbiology, Wageningen, and especially Hauke Smidt, Hans Heilig, and Odette Pérez Gutiérrez. They endured my presence and all my questions on where to find things. Odette, you found the time to spend many days answering my questions even though you were completing your own thesis. ¡Muchos gracias! The fact that you are crazy about your own two cats must have helped in getting the nice results we did, and we had a good time as well.

Cats are the central theme of this thesis, and the next two that crossed my path are owned by Sandra Vreman. So, when Sandra heard about the research project and the fact that we would be adding to knowledge about felines, she happily jumped on board. We worked together closely in the lab and spent many hours looking through the microscope. Sandra, thank you for helping me with all the histological challenges.

Also, thanks to Ted van den Ingh, whose knowledge about histopathology and cats helped a lot. Thank you, Andrea Gröne, for participating in this project and supervising the histopathological diagnoses. Guy Grinwis, thank you for collecting bile samples of control cats. Ellen Martens, thanks for helping me with the PCR, capillary electrophoresis and sequencing.

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A big thank you to Siobhan Hubers, Monique van Wolferen, and Loes Oosterhof for helping with immunohistochemical stainings and the intricate workings of the microscope. Although we touched some buttons that were definitely a no-go area, we did manage to get the pictures and avoid the wrath of other users of this expensive machine.

Designer Ronald Jeans created the feline design used throughout my report and presentations. Thanks Ronny, for spending your precious time on this wonderful design.

If we knew what we were doing
it would not be called research, would it? – Albert Einstein

Thanks to all my fellow ET-students and Cathelijn for all the fun we had. Soccer games near the pond (which proved to be a less than excellent combination…), picnics under trees (was the ice cream really stored in the -70 freezer?) and our many doggy walking sessions (love you, Kai!) made sure that my laughter lines grew much more than my quadragenarian wrinkles. Our pizza sessions also helped, as did the blondmokkel-discussions and getting haircuts.

To be normal is the ideal aim of the unsuccessful – Carl Jung

Thanks to all members of the Rotary Club Heusden, who invited me to speak about my research. The year theme was "high flyers: people who make deviant choices" and in preparing this lecture, I learned that I was never a middle of the road kind of girl. And that that is perfectly okay. Thank you for being proud of me and appreciating my choices for what they are.

Time spent with cats is never wasted – attributed to Sigmund Freud,
although he was more of a dog person and never actually said this

Thank you Marleen Assink and Nel Voerman for being there, always and on this big day of getting my PhD. Dear Marleen, we haven't known each other that long, but ever since we found out that we were born in the same village we have been finding more and more similarities. You too started of in a business environment and switched to veterinary medicine later in life. And you too adore cats. Thank you, for everything, and for collecting liver photos and tissues. The fact that they are marked "lever-voor Corma" says it all. For me, you will always be "DE kattendokter".

Dear Nel, when I asked you to be my paranimf today, I did not expect your enthusiastic reaction. Although you assured me several times that you would not be of any help in answering questions, your support cannot be valued enough. We both earned some scars and bruises throughout life. They cannot be undone and made us who we are today. I know that we both have some angels rooting for us, today and always.

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Curriculum Vitae

Corma Otte was born on March 19, 1970 in Halsteren, the Netherlands. She graduated from her secondary school, het Mollerlyceum in Bergen op Zoom, in 1988. She successfully completed the integrated bachelor's and master's programme in Business Administration at Tilburg University in 1994. In 1999, she studied at Lowry Mays College and Graduate School of Business, Texas A&M University, in College Station (TX), USA.

She worked for well-known international companies, such as SPSS, ING Bank, Center Parcs, and Essent.

In 2005, she fulfilled her lifelong dream of becoming a student of Veterinary Medicine, at the Department of Companion Animals, Utrecht University. She graduated in June 2012 and has been working as a small animal veterinarian ever since. Her main interests include feline medicine, internal medicine, and dermatology.

She has been studying lymphocytic cholangitis since 2009, when she started her research master (excellent tracé, 2009-2010). The results are described in this thesis.

Plans for the future include travelling, acquiring more knowledge about feline and internal medicine, and writing a novel.

Our prime purpose in this life is to help others.
And if you can't help them at least don't hurt them – Dalai Lama

List of Publications and Conference Proceedings

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Feline diseases of the biliary tree and gallbladder, Corma M.A. Otte, Louis C. Penning, and Jan Rothuizen. 2017, Journal of Feline Medicine and Surgery, 2017, 19(5), pp. 514-528

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