β-catenin, Notch1/NICD, vimentin and laminin as immunohistochemical markers for hepatic progenitor cell activation in feline lymphocytic cholangitis

Three months research - Faculty of Veterinary Medicine, Utrecht University

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Summary

Feline lymphocytic cholangitis (LC) is a common hepatobiliary disease in cats. Its aetiology is unclear and as a result, treatment is challenging and symptomatic. The liver is able to regenerate in case of hepatobiliary diseases, due to the proliferation of mature hepatocytes or cholangiocytes. When this regeneration capacity is inadequate, bipotent hepatic progenitor cells (HPCs) can be activated. These cells are a reserve population for maturated cells in the liver and can differentiate into hepatocytes or cholangiocytes. HPCs have just recently been described histologically in feline livers and knowledge about mechanisms affecting proliferation and/or differentiation of HPCs in feline LC is lacking. Therefore, the expression of β-catenin, Notch1/NICD, vimentin and laminin, well known for their involvement in HPC activation, in twelve samples of cats diagnosed with feline LC was evaluated and compared to healthy livers. The diseased livers showed enhanced expression of laminin and vimentin in portal areas with numerous cell infiltrates. However, the activation of the Wnt/β-catenin and Notch pathways could not be proven unequivocally. More research is needed for further understanding of the pathophysiological effects of feline LC and for developing new rational therapeutic solutions.

Introduction

Cholangitis and cholangiohepatitis are the second most common types of liver diseases in cats, following hepatic lipidosis (1). Of these inflammatory hepatobiliary diseases, feline lymphocytic cholangitis (LC) is one of the most common (2). Characteristic of feline LC are the aggregates of lymphocytes in portal tracts and in and around bile ducts (3), but solitary plasma cells and eosinophils may also be present (4). LC is a chronic disease, that can affect the entire biliary tree and can lead to dilatations, strictures and bile duct proliferation. The disease often progresses slowly over months or years and fibrosis and cirrhosis can eventually arise (3). Its aetiology is unknown, but bacterial and immune-mediating components are suggested (1,5-8). As a result, treatment of LC is challenging and symptomatic. Immune-mediative corticosteroids, hepatoprotective ursodeoxycholic acid (UDCA) and antibiotics are common recommend therapies, as well as supportive therapies like tube feeding, intravenous fluid and antioxidants such as S-adenosylmethionine (SAMe) and vitamin K (9,10).

Cats suffering from LC usually expose rather non-specific signs such as nausea and vomiting, changes in appetite, gradual weight loss and icterus (3). Some cats also expose high-protein ascites, which makes differentiation from feline infectious peritonitis (FIP) important, because the diagnosis of FIP often results in the decision to euthanize (10). Hepatic histopathology is considered as the gold standard for diagnosis of feline LC (4) and besides FIP, also lymphoma has to be ruled out because of its predilection for portal areas (1,10). Typically, there are few symptoms during the initial stages, but at the time of diagnosis, the disease has often progressed into a chronic form (1). The number of clinical signs seems to influence survival times, just as therapy regimen, sex and breed (2). The prognosis for cure is poor due to the chronical progress despite of treatment. However, the mortality rate of cats suffering from LC is low, because the disease does generally not process to end-stage cirrhosis. Cats suffering from LC that do die, often have a concurrent pancreatic or intestinal disease, which may be responsible for the poor outcome (10).

In case of hepatobiliary diseases, the liver is able to regenerate due to the proliferation of mature hepatocytes and cholangiocytes. When this regeneration capacity is inadequate, for example in chronic hepatopathies, bipotent hepatic progenitor cells (HPCs) can be activated (3,11). These cells are a reserve population for adult, so fully maturated, cells in the liver. They are located in the terminal branch of the biliary tree, which is known as the canal of Hering (figure 1), and are demonstrated in rodents, humans, dogs and cats (12). In rodents, HPCs are known as 'oval cells' and are characterized by their small size (7-10 μm, while hepatocytes are around 50 μm), epithelial type, high nucleus to cytoplasm ratio, ovoid nucleus and scat cytoplasm (13). Upon activation, HPCs will proliferate, migrate to the site of injury and differentiate into cholangiocytes or hepatocytes,

depending on the disease and the concurrent changes in the microenvironment (14). Among others, hepatic stellate cells, Kupffer cells (liver specific macrophages) and the extracellular matrix (ECM) are thereby of importance (12). These and other cells and stroma located in the immediate environment of the HPCs, the microenvironment, are referred to as HPC niche (15). This niche, both a histological location and a functional unit, is of importance in maintaining and regulating HPC behaviour, supporting self-renewal and balancing quiescence, proliferation and differentiation in response to injury. Growth factors (e.g. hepatocyte growth factor and fibroblast growth factor) and cytokines (e.g. TNF-like weak inducer of apoptosis and transforming growth factor β) produced by non-parenchymal cells play a role thereby (14,16).

In all species, the HPC response takes place at the site of disease activity (12). The process of HPC activation is often called a ductular reaction, because of the forming of structures with a ductular phenotype. The ductular reaction may be composed of immature small progenitor cells and intermediate hepatobiliary cells (13). These intermediate cells are HPCs differentiating into hepatocytes or cholangiocytes and are larger than the small progenitor cells, but still smaller than mature hepatocytes. Furthermore, they have a lower nucleus to cytoplasm ratio compared to progenitor cells (14). In veterinary literature, the ductular reaction is also often referred as 'bile duct proliferation'. This does not suggest activation of stem cells and indicates that the presence of HPCs is not generally recognized in veterinary medicine (12). In human medicine it is suggested that a regenerative response of bile duct proliferation comprises of both HPC activation and proliferation of cholangiocytes (12,17).
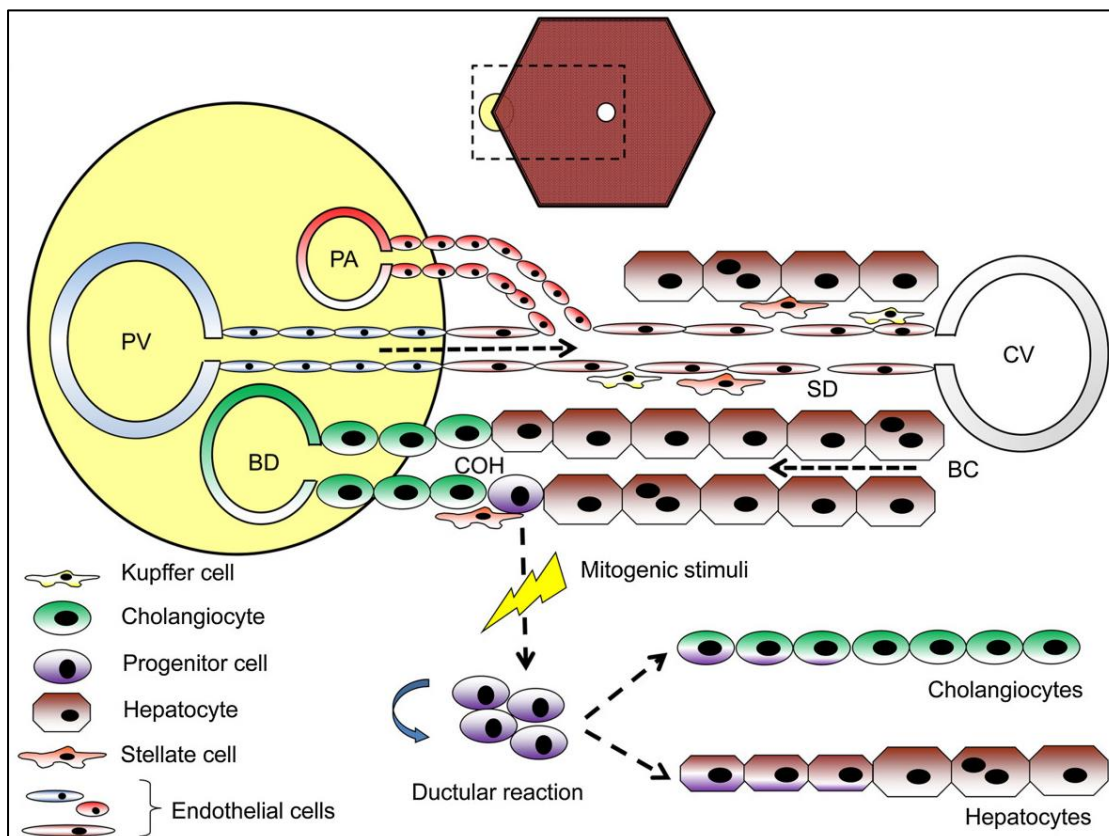
Figure 1 - Schematic representation of the localization and activation of HPCs in the canal of Hering. PV: portal vein; BD: bile duct; PA: portal artery; COH: canal of Hering; SD: space of Disse; BC: bile canaliculus; CV: central vein (12)

HPCs have just recently been described histologically in feline livers. The first evidence was presented in 2009 by IJzer et al., who immunohistochemically evaluated the expression of, among others, cytokeratin-7 (K7) in feline livers (18). K7 is a frequently used, but rather non-specific HPC

marker. Feline HPCs, which morphologically resemble those in humans and dogs and which were situated in canals of Hering, were reported in livers of healthy cats. In acute and chronic feline liver diseases furthermore, a ductular reaction was seen (18). In feline LC specifically, bile duct proliferation was demonstrated in the past (19). Gagne et al. observed bile duct proliferation in 26 out of 27 cats with feline LC in a study published in 1999 (19). Kruitwagen et al. suggested that, again similar to humans and dogs, bile duct proliferation seen in cats involves both cholangiocytes and HPCs (12). The real proof of HPCs in livers of cats suffering from feline LC is, however, scarce. Otte et al. (3) showed HPCs in histological sections of livers from cats diagnosed with feline LC, but they only mentioned the absence of difference in absolute numbers of HPCs between the prednisolone and UDCA treatment groups. Information of signalling pathways affecting proliferation and/or differentiation of HPCs in feline LC is, to our knowledge, lacking.

Yet, detailed information about the HPC niche and the regulation of the HPC activation in feline LC is of crucial importance for understanding the pathophysiological effects of this disease and could be of importance in new rational therapeutic solutions. Moreover, cats could be a good model for biliary diseases in humans; diseases that are not frequently seen in dogs (12). Therefore, the purpose of this research is to evaluate the activation of the HPC niche in feline LC, by means of immunohistochemistry. Because a specific HPC marker has not been identified yet (13) and many HPC markers have a shared expression with cholangiocytes (12), multiple markers are used in HPC immunohistochemistry. In this study, the expression of β-catenin, Notch1/NICD, vimentin and laminin, all factors involved in HPC activation, were evaluated and compared with liver samples of healthy cats. Vimentin and laminin expression showed to be enhanced in portal areas of cats diagnosed with feline LC. However, activation of the Wnt and Notch pathways in diseased livers could not be proven.

Materials and methods
Liver samples
Liver samples were obtained from eight cats (seven European shorthairs and one Norwegian Forest; both castrated males and spayed females) diagnosed with feline LC, according to the WSAVA criteria by a European board-certified veterinary pathologist (S. Vreeman) (4). From four of these cats, a second biopsy sample was available, taken one to nine months after the first one. Therefore, in total twelve liver samples of cats diagnosed with feline LC were available. The WSAVA-guided diagnosis of feline 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 (3,4). The patients were identified from the registration system used by the Department of Pathobiology, Utrecht University and the samples were previously used in a study of Otte et al (3).
All these tissue samples were obtained from liver biopsies which were taken for diagnostic purposes between 1998 and 2010. There was no tissue taken for research or scientific purposes only (3).

Next to the samples of cats diagnosed with feline LC, liver samples of two healthy cats were evaluated. They were included to check the correctness of the used staining protocols and were therefore considered as positive controls; except for the Notch1/NICD marker, for which no expression was expected in the healthy liver. Next to it, the healthy liver samples were used to make a comparison with the diseased livers.
The liver samples of healthy cats were obtained from cats of a control group in a study of Corbee et al. (20). Al the experiments of this study were approved by the Animal Welfare Committee on Experimental Animal Use, as required by Dutch legislation (DEC 2011.III.01.008) (20). It is the university 3R-policy to hand over surplus material to other researchers.

Both the healthy liver samples as the liver samples of cats diagnosed with feline LC were fixed in 10% neutral buffered formalin and embedded in paraffin.

Immunohistochemistry

Liver samples were cut as 5 μm slices 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) or proteinase-K (PK; for laminin). TE and citrate antigen retrieval was performed by heat-induced epitope retrieval (HIER), by means of a 98 °C water bath during 30 minutes, after which it was cooled down at room temperature (RT) for another 30 minutes. Proteinase-K ready-to-use (Dako, Glostrup, Denmark), a proteolytic induced epitope retrieval (PIER) 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) or Tris-buffered saline with 0.05% Tween-20, pH = 7.6 (TBS/T; for Notch1/NICD) for 2 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 for 5 minutes 3 times and after that, background staining was reduced with 10% normal goat serum in PBS or TBS for 30 minutes at RT. Primary antibodies were diluted (β-catenin 1:2500, Notch1/NICD 1:200, vimentin 1:300 and laminin 1:100) in Antibody Diluent with background reducing components (Dako) and incubated in a humidified chamber at 4 °C overnight. For negative controls, the primary antibody was omitted and only the Antibody Diluent used was subsequently incubated. Before incubating secondary antibodies of the EnVision+ System-HRP labelled polymer (Dako), slides were rinsed in PBS/T or TBS/T for 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 after that, they were rinsed in PBS or TBS for 5 minutes 3 times. Diaminobenzidine (DAB; Dako) was used as substrate for the secondary HRP antibodies, so slides were incubated with freshly made DAB substrate for 5 minutes. After 3 times rinsing in milli-Q for 5 minutes, slides were counterstained with haematoxylin quickstain H-3404 (Vector Laboratories, Burlingame, CA, USA) for 10 seconds, whereupon the slides were placed under running tapwater during 10 minutes. Dehydration was performed by 60%, 70%, 80%, 96%I, 96%II and 100% alcohol baths and two xylene baths. At the end, slides were covered with Vectamount (Vector Laboratories).

Details of the primary antibodies as well as antigen retrieval and washing buffer methods are described in table 1. A summary of the immunohistochemical protocols is added in appendix 1.

Immunoreactivity was evaluated by describing the localization and intensity (minimal, mild, moderate or strong) of expression and by comparing the healthy and diseased livers.

**Table 1 - Details of the primary antibodies and antigen retrieval and washing buffer methods**

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

Results

β-catenin

In the healthy tissue samples, a membranous expression of β-catenin in hepatocytes and cholangiocytes was seen (figure 2), indicating a low activation status of the Wnt/β-catenin signalling cascade. Next to it, there was a slight background staining of the cytoplasm of these cells and in the matrix of the portal area. Expression was absent in the negative control slide (data not shown).
In the samples of cats diagnosed with feline LC, β-catenin was also expressed in a membranous staining pattern in hepatocytes. In some slides, cytoplasm of hepatocytes stained moderate to strong. Ductular structures in portal areas with cell infiltrates stained quite intense in most of the diseased livers (figures 3 and 4). At this location, expression was present in both membranes and cytoplasm, but membranes seem to stain stronger than cytoplasm in these structures. No nuclear staining was present. A semi-quantitative scoring system for the four antigens is presented in appendix 2.
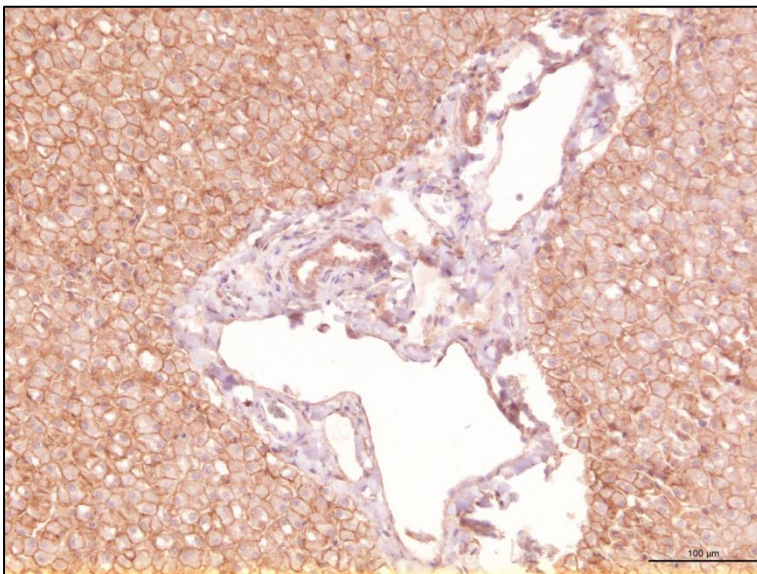
Figure 2 - Expression of β-catenin in a healthy liver. Positive staining is most obvious in membranes of hepatocytes and cholangiocytes.
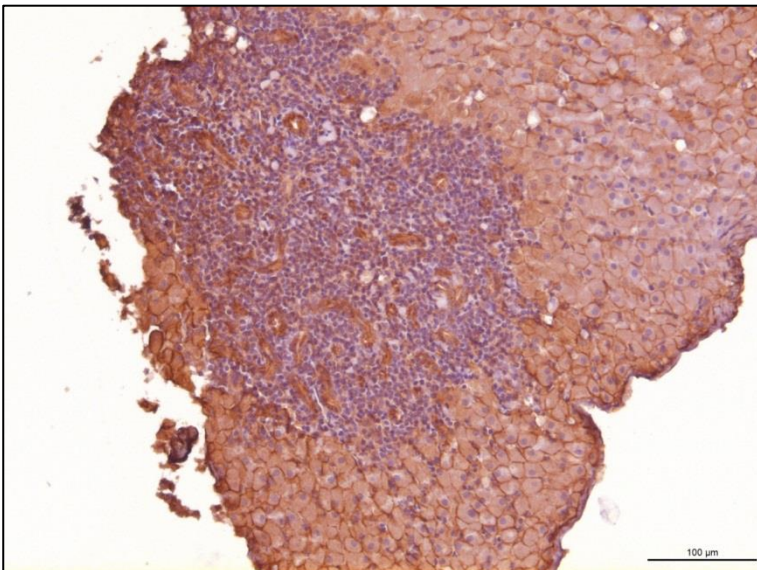

Figure 3 - Expression of β-catenin in a liver of a cat diagnosed with feline LC. Strong positivity in membranes of hepatocytes and cells of ductular structures in the area with cell infiltrates. An increase of positivity is also seen in the cytoplasm of cells in the ductular structures.
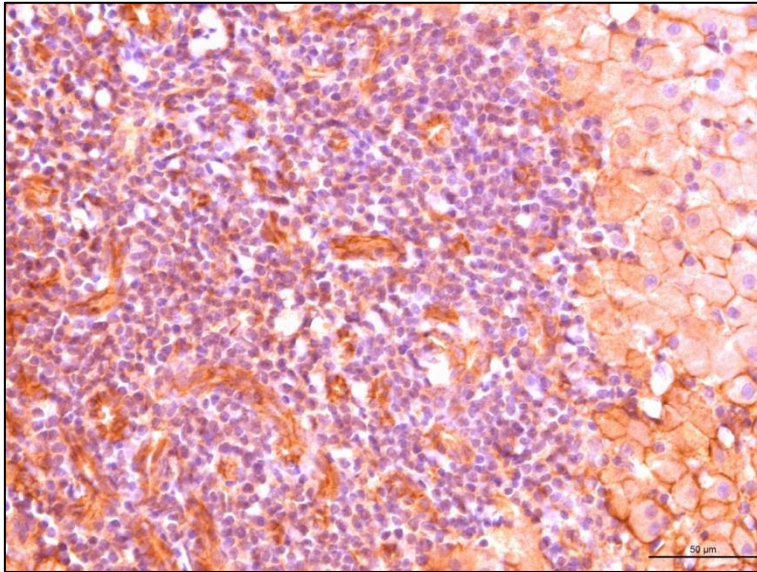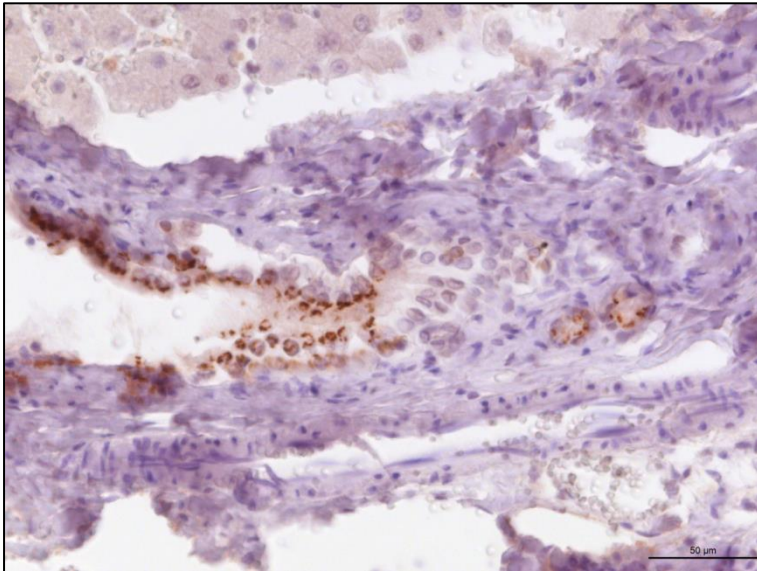
Figure 4 - Expression of β-catenin in a liver of a cat diagnosed with feline LC. Strong positivity of both membranes and cytoplasm of ductular structures in a portal area with numerous cell infiltrates. Hepatocytes expressed a membranous staining pattern. Enlargement of figure 3.

Notch1/NICD

The Notch1/NICD staining resulted in minimal positivity (figure 5). Only one out of the twelve samples of cats diagnosed with feline LC showed Notch1/NICD expression. There, positivity was present in some of the large bile ducts, where a clear peri-nuclear staining was seen (figure 6). The other slides of cats diagnosed with feline LC did not show clear staining. However, a light, unclear cytoplasm staining of all hepatocytes was seen (figure 7), which was in contrast to the negative control slide, that remained completely negative (data not shown).
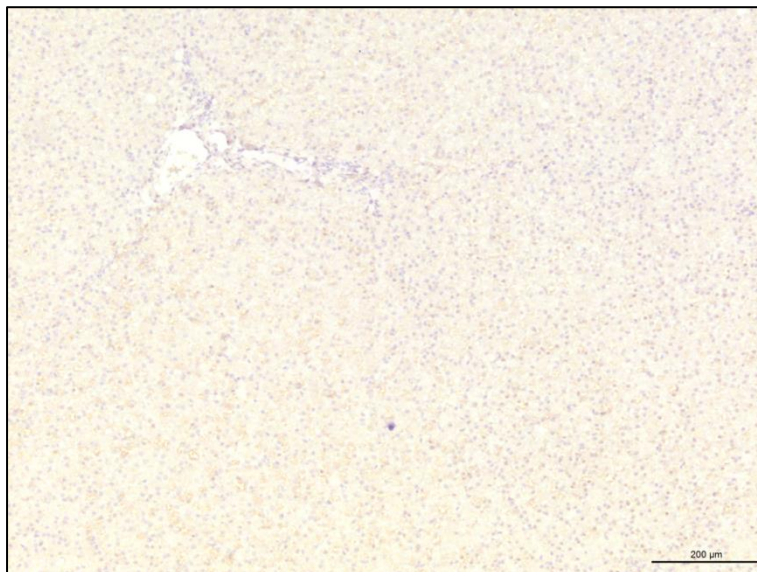
Figure 5 - No expression of Notch1/NICD in a healthy liver.

Figure 6 - Expression of Notch1/NICD in a liver of a cat diagnosed with feline LC. Positivity is seen above the nucleus in the cytoplasm of bile ducts.
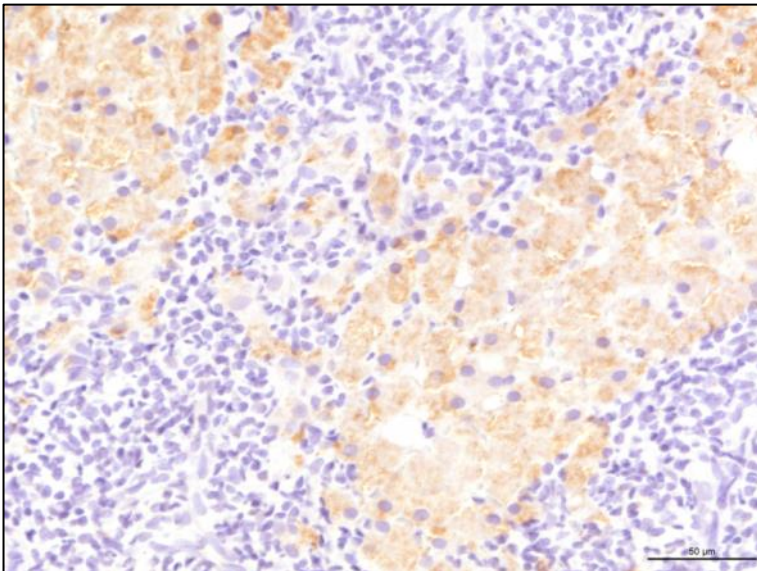

Figure 7 - Slight Notch1/NICD cytoplasm staining of hepatocytes in a liver of a cat diagnosed with feline LC.

Vimentin

Expression of vimentin, a mesenchymal marker, was seen in the ECM of portal areas and in vascular endothelium. Next to it, mesenchyme of sinusoids and individual cells, most likely stellate cells, stained positive. In slides of healthy livers, the staining intensity was minimal (figure 8), but a clear increase of intensity was seen in all cats diagnosed with feline LC (figures 9 and 10), except in one biopsy. In the diseased livers, portal areas with numerous cell infiltrates showed a strong expression of vimentin. Besides the ECM of these portal areas, also inflammation cells stained positive. One slide was excluded because the slide stained so diffusely and strong positive, that it was not possible to distinguish individual cells. No positivity was present in the negative control slide (data not shown).
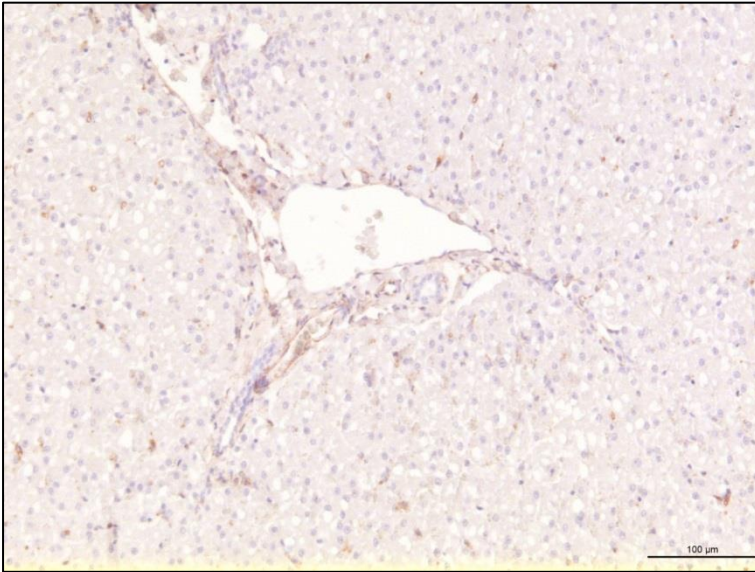
Figure 8 - Expression of vimentin in a healthy liver. Positive staining is present in the ECM of the portal area, vascular endothelium, mesenchyme of sinusoids and individual cells.
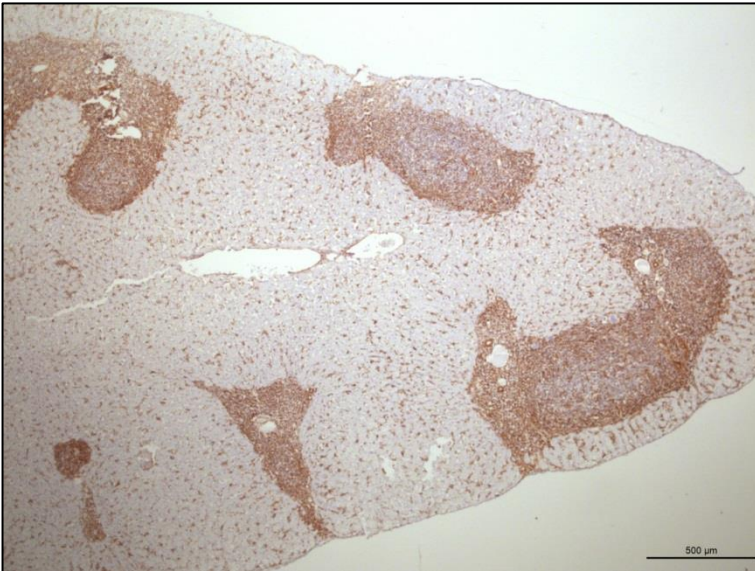
Figure 9 - Expression of vimentin in a liver of a cat diagnosed with feline LC. Strong positivity of the ECM of portal areas, vascular endothelium, mesenchyme of sinusoids and individual cells.
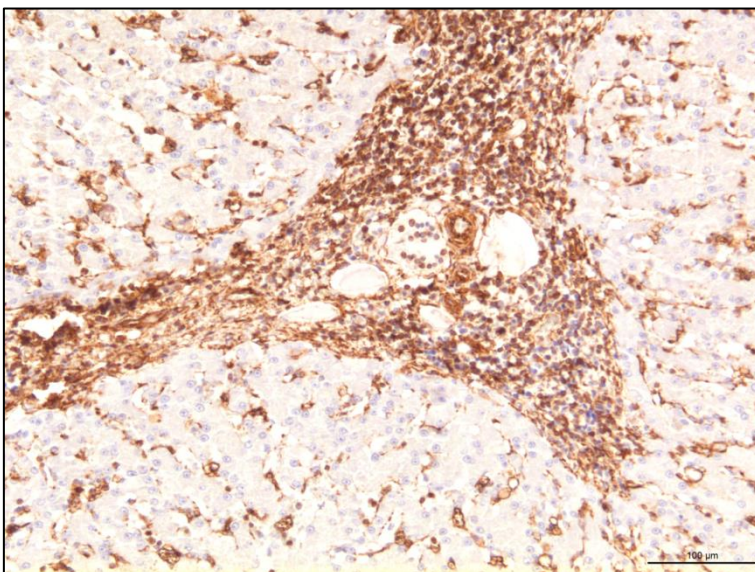
Figure 10 - Expression of vimentin in the liver of a cat diagnosed with feline LC. Strong positivity of the ECM of a portal area, vascular endothelium, mesenchyme of sinusoids and individual cells. Also positivity of inflammatory cells. Enlargement of figure 9.

Laminin

Staining of laminin, a glycoprotein that is a major component of the basement membrane, was present in the matrix of portal areas and, more specifically, in or around vascular smooth muscle cells and bile ducts in all slides (figure 11). There was also a mild expression of laminin in sinusoidal endothelium seen; mainly adjacent to portal areas. In diseased livers, an increase of expression was seen in portal areas with cell infiltrates. Both cytoplasm of reactive ductules and/or the basement membrane showed strong positivity in these areas (figures 12 and 13). However, in around four of the diseased livers, also areas with cell infiltrates were present where no or just minimal positivity was seen. A slight background staining in the cytoplasm of hepatocytes appeared in some slides of cats with feline LC. The negative control remained negative (data not shown).
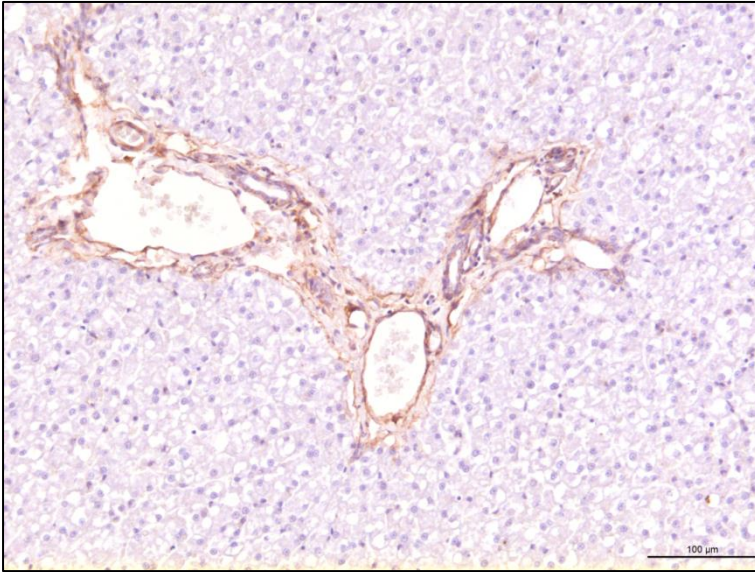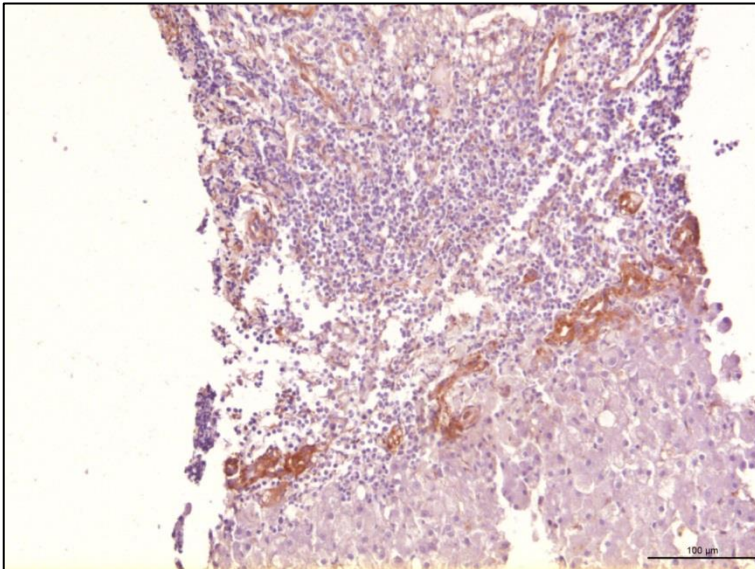

Figure 11 - Expression of laminin in a healthy liver. Positive staining is present in vascular smooth muscle cells, bile ducts and sinusoidal endothelium in and around the portal area.

Figure 12 - Expression of laminin in a liver of a cat diagnosed with feline LC. Moderate to strong positivity of ductular structures in a portal area with numerous cell infiltrates.
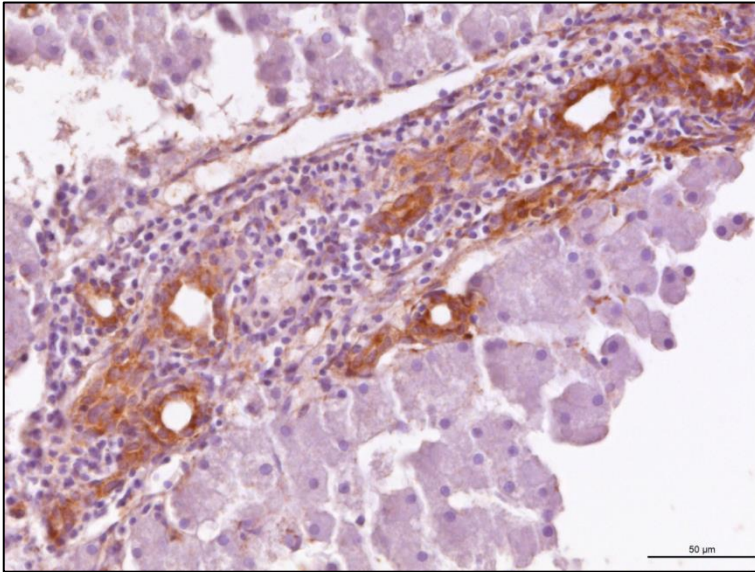
Figure 13 - Expression of laminin in a liver of a cat diagnosed with feline LC. Strong positivity of ductular structures and basement membranes in a portal area with cell infiltrates.

Discussion

The expression of β-catenin, Notch1/NICD, vimentin and laminin in livers of cats diagnosed with feline LC was evaluated in the present study. All four markers showed to be expressed in the diseased livers, although Notch1/NICD was only clearly expressed in just one of these livers. Because the negative controls, in which the primary antibody was omitted, all remained negative, non-specific binding of the secondary antibody to certain antigens in the sample could be excluded. Since both β-catenin and laminin markers are polyclonal IgG rabbit antibodies and have a distinct staining pattern, these two primary antibodies acted as each other's control. In the same way, Notch1/NICD and vimentin antibodies, which are both mouse monoclonal IgG1 antibodies, did. This made non-specific staining improbable.

The expression of β-catenin was evaluated because of the central role of this molecule in the Wnt signalling pathway, in which it promotes activation of several genes, apoptosis inhibition, increased cellular proliferation and migration. Next to a role in tissue growth, survival and differentiation, the pathway plays a role in the maintenance of progenitor cells (21). Specifically for HPCs, the Wnt/β-catenin pathway is believed to be involved in the (induction of) proliferation, migration and differentiation of HPCs in, among others, dogs and human (11,12). β-catenin is membrane-associated, but activation leads to decreased membrane expression and an increased cytoplasmic or nuclear expression (11,22). The membranous expression of β-catenin in hepatocytes and cholangiocytes in healthy livers in the present study indicates a low activation status of the Wnt signalling pathway (11).

The results of β-catenin expression in the feline LC samples, however, were more difficult to interpret. An increase of cytoplasmic staining was seen in ductular structures in areas with numerous cell infiltrates. However, membranous expression was still present in these cells. Although not clear in the pictures, this membranous expression was more obvious in all diseased livers, when compared to the cytoplasmic expression. Next to it, also cytoplasm of hepatocytes stained more intense, so it is difficult to conclude whether the Wnt/β-catenin pathway is activated in livers of cats diagnosed with feline LC.

Normally, increased cytoplasmic expression of β-catenin is inversely related to decreased membranous expression, which is also seen in feline tumours (21,22). In dogs with lobular dissecting hepatitis (LDH), this was present in non-differentiated cells of the ductular reaction, whereafter the activation of the Wnt/β-catenin pathway in activated HPC niches was concluded (11). Because previous (immuno)histochemical findings of HPCs in cats are in accordance with those in humans, dogs and rodents (18), the same expression pattern was expected in feline LC. It is not clear why this

was not found in the present study. Probably, the used antibody concentration was too high and as a result, there was too much background staining, after which distinction between cytoplasmic and membranous staining was difficult. Another options are that there was indeed no activation of the Wnt/β-catenin pathway or that activated HPCs were not present in the present liver samples. It is possible that, in our diseased samples, HPCs already became intermediate cells, in which usually no activation of the Wnt/β-catenin pathway is present (11). This could probably due to the chronic establishment at the time of diagnosis or the establishment of treatment. The suggested role of the Wnt/β-catenin pathway in differentiation of HPCs toward a biliary phenotype (11) cannot explain why activation is absent, because especially in a biliary disease as feline LC, HPC differentiation to the biliary phenotype could be expected.

In accordance to β-catenin, Notch is involved in the proliferation, migration and differentiation of HPCs (11). Of various Notch-receptor proteins, Notch1 has shown to be activated in HPCs of dogs with LDH (11) and in feline mammary tumours (23). Upon binding of ligand proteins expressed on the surface of neighbouring cells to the Notch1 extracellular domain, a proteolytic cleavage leads to the release of Notch1 intracellular domain (Notch1/NICD). The intracellular domain then enters the nucleus to alter gene transcription ('established NICD metabolic intranuclear pathway'; figure 14) or accumulates in the cytoplasm in proximity of the nuclear membrane to further stimulate the Notch1 pathway ('aberrant Notch1 pathway'; figure 15) (23). In this way, immunohistochemical expression of the Notch1/NICD molecule can show the activation of the Notch pathway.
In the present study, hardly any Notch1/NICD expression was seen. The absence of expression in healthy livers could be explained by the high affinity of the antibody for only the activated, intracellular form of Notch1, the Notch1/NICD. Although, in an immunofluorescence study in which another clone of the Notch1/NICD antibody was used, a canalicular staining pattern in hepatocytes and bile duct cells was found in normal liver tissue in dogs (11).

It is not clear why there was no positivity in eleven out of twelve livers of cats diagnosed with feline LC. In dogs suffering from LDH, activated Wnt and Notch pathways were found at the same histological location, so a simultaneous act of the Wnt and Notch pathways was suggested (11). The absence of Notch1/NICD expression is therefore in accordance to the probable absence of β-catenin expression. As shown in dogs with LDH (11), a strong cytoplasmic and/or nuclear staining of non-differentiated cells would be expected in the diseased livers in the present study. As is the case with β-catenin, the absence of this expression could be explained by the absence of undifferentiated HPCs in these slides. However, it remained unclear why there was expression in some large bile ducts in one sample of a cat diagnosed with feline LC. Probably, this is aberrant staining, for which the absence of cytoplasmic or nuclear β-catenin expression at the same location in that slide makes it more likely.

Vimentin is a mesenchymal marker and was therefore used to evaluate whether the HPCs were able to obtain mesenchymal characteristics. In earlier studies, vimentin expression has been shown in undifferentiated oval cells of rats (24) and in human HPCs (25). In dogs with LDH, furthermore, vimentin has been shown to be expressed in ductular structures on a subset of undifferentiated HPCs. These positive HPCs also showed to exhibit Wnt and Notch signalling and therefore, a causative relation in canine HPCs was suggested (11).
It is indicated that a mesenchymal capability is necessary for stem cells to proliferate, loosen cell-cell connection, migrate and differentiate. It is suggested that during HPC differentiation, cells change from a mesenchymal, de-polarized, proliferative and migrating state to an epithelial, polarized, differentiated, adhesive and inactivation state (26); a process that is known as mesenchymal epithelial transition (MET). Therefore, vimentin could also be used as indicator for cell proliferate activity and an undifferentiated status of HPCs (26).
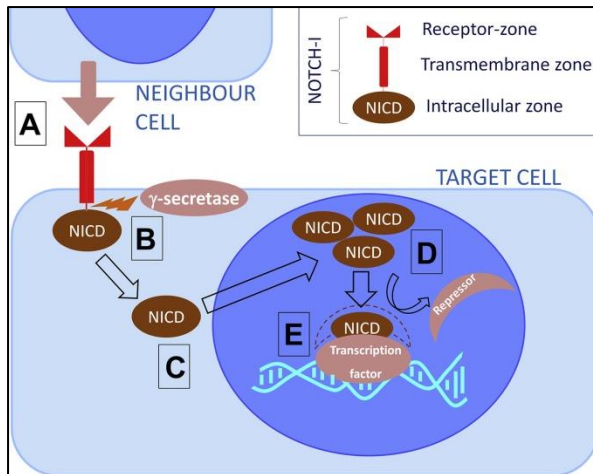
Figure 14 - Established NOTCH1 pathway. (A) NOTCH1 transmembrane protein is activated on the target cell by the interaction with a membrane-bound ligand on a neighbouring cell. (B) This process leads to cleavage of NICD by the action of gamma-secretase and (C) release of free NICD into the cytoplasm. (D) This active form enters the nucleus and accumulates inside it. NICD binds to a repressor and removes it, promoting transcription by (E) activation of a transcription factor. These molecular events lead to cell proliferation and survival (23).
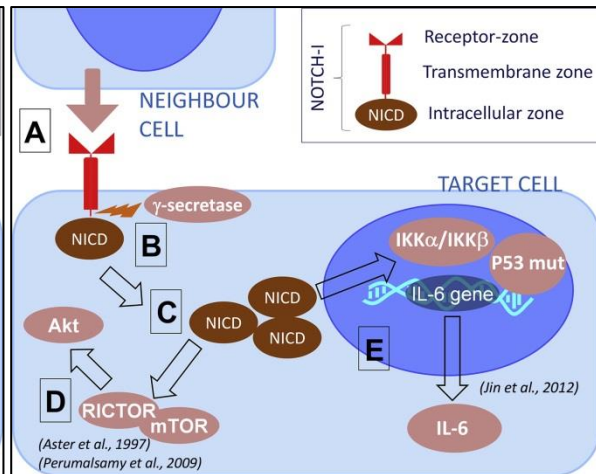
Figure 15 - Alternative aberrant NOTCH1 pathway. (A) NOTCH1 transmembrane protein on the target cell is activated by the interaction with a membrane-bound ligand on a neighbouring cell. (B) This process leads to cleavage of NCID by the action of gamma-secretase and (C) release of free NICD into the cytoplasm, which accumulates in the proximity of nuclear membrane. (D) NCID activates Akt in a RICTOR‑mTOR-dependent manner, leading to cell proliferation and survival. (E) NICD stimulates the increase of interleukin (IL)-6 production through activation of IKK alpha/IKK beta and mutated p53 (23).

The healthy feline livers in this study expressed a low level of vimentin, suggesting that these cells are in quiescent state. In contrast, strong vimentin staining would suggest that cells are in a activation and proliferation state (26). In this study, vimentin was mainly expressed in mesenchyme of portal areas in the diseased livers. No positive undifferentiated HPCs were found, which makes it difficult to interpretate these findings, because most literature of vimentin expression is about expression in cells rather than the ECM. The individual parenchymal cells in which vimentin was expressed, were most probably stellate cells. Vimentin expression has been demonstrated in stellate cells earlier (27).

In one liver biopsy of a cat with feline LC, there was just minimal positivity. In an earlier biopsy of the same cat, a strong positivity was seen. Besides a failed staining in only this slide, it could be suggest that the cells of the ductular reaction are in a more differentiated state in the last biopsy; probably due to treatment. The presence of much lower numbers of lymphocytes in the portal areas support this option. However, an activation and undifferentiated state of the cells in the ductular reactions in all the other diseased liver biopsies is in contrast to the absence of Wnt/β-catenin and Notch pathway activation. Next to it, it remains unclear why inflammatory cells stained positive for vimentin in almost all diseased livers.

Laminin was evaluated because it has been considered as a major component of the HPC niche (28). It has been shown that laminin plays an important role in HPC activation in mouse models and human fibrotic liver disease (12,28). For example, mice which had an failure to deposit laminin after chronic fibrotic liver injury due to degradation resistant collagen I, showed an impaired ability of the liver to initiate and maintain the HPC response (28). Another study concluded that deposition and remodelling of laminin around the ductular reaction is required for HPC proliferation and migration (16). In addition, *in vitro* studies showed that laminin is of importance in maintaining the undifferentiated phenotype of HPCs and only when HPCs escape from the laminin matrix and enter the parenchyma, they can differentiate into a hepatocyte phenotype (12,16).

In this study, an expression of laminin in healthy feline livers was seen in the matrix of portal areas, in and around vascular smooth muscle cells and bile ducts, and in sinusoidal endothelium adjacent

to the portal area. This is in similarity with what was seen in healthy dog livers in an immunofluorescence study (14). In cats diagnosed with feline LC, an increase of laminin expression was seen in portal areas with numerous cell infiltrates, which is in accordance with the increased immunofluorescence for laminin particularly surrounding the DR in dogs with LDH (14).

There is not much knowledge about the interaction between HPCs and the ECM, laminin in particular, in cats. However, recent unpublished data showed a clear co-localization of activated HPCs with laminin in cats diagnosed with feline chronic neutrophilic cholangitis [Unpublished observations section: Valtolina et al.] (12). Data acquired in the present study also suggest a role of laminin in the ductular reaction. The cytoplasmic laminin in ductular structures might even suggest that HPCs actively produce laminin. Next to it, it is shown that laminin is involved in more than just migration of HPCs, taken into account that in case of biliary diseases the main site of injury is nearby the HPC niche. It is not clear why some areas with cell infiltrates showed ductular structures, but no laminin positivity.

Whether the ductular reactions, in and around which expression was seen for laminin and vimentin, are composed of HPCs remained unclear. As earlier mentioned, Kruitwagen et al. suggested the involvement of both cholangiocytes and HPCs in bile duct proliferation (12). The absence of the activation of Wnt and Notch pathways could indicate that the involved HPCs probably already became intermediate cells.

For all four markers, there were no specific characterized, undifferentiated HPCs recognized. Next to the possible differentiation toward a more biliary phenotype, this could be due to the absence of undifferentiated HPCs in the biopsy tissues. Although HPCs were demonstrated by cytokeratin 19 (K19) immunohistochemistry in the same tissue samples a few years ago, there were only one or two HPCs per tissue sample demonstrated in four out of the twelve samples of cats diagnosed with feline LC used in the present study (3).; Unfortunately this observation can, by definition, only be done in hindsight. Out of these four samples, three were small needle biopsy tissues and less tissue remained after earlier use of these samples in another studies. It is possible that there were no HPCs remained in these samples. Only one of the biopsy tissues in which HPCs were demonstrated, was a larger wedge biopsy. It could be that the one or two HPCs that would be present in this sample, were missed.

For future, a gene expression study or a double immunohistochemistry study with a HPC marker like keratin 7 (K7) could be valuable for interpreting results of a study to the same markers with more certainty. Next to it, a study with more samples included would be recommend. The small number of samples used in the present study limits the power of this study. However, biopsy tissues of cats diagnosed with feline LC are scarce because most veterinarians do not send tissue samples of their patients suspected from feline LC to the department of Pathobiology of the University of Utrecht. Furthermore, samples studied before by Otte et al. (3) could not be used again because there was not enough tissue remaining.

It is important to further study the HPC niche and regulation of HPC activation in feline LC. Based on the chronical establishment of feline LC despite of treatment, it could be concluded that HPCs fail to regenerate the diseased liver. More information is needed to understand this pathophysiology. When it is known at which phase of activation (proliferation, migration or differentiation) HPCs fail to regenerate, new therapeutic strategies could be developed, because currently, only symptomatic options are available (11,12). In the new therapeutic options, the own HPCs of the patient in vivo can be target for specific drugs or small molecules, with the aim to activate the HPC niche and trigger proliferation and/or differentiation, or (either autologous or allogeneic) proliferated and differentiated HPCs can be used for cell transplantation into the patient's liver (12). Before this can be used in a clinical setting, detailed knowledge of the activation mechanisms of HPCs is essential, because overstimulation can lead to cancerous development (11,13).

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Unpublished observations

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Appendix 1

Immunohistochemistry protocol

1. Deparaffinising and rehydrating
   - xylene   2 x 5 min.
   - alcohol 96%   5 min.
   - alcohol 80%   5 min.
   - alcohol 70%   5 min.
   - alcohol 60%   5 min.
   - MQ   5 min.
2. Antigen retrieval   variable
                                                   (see table 1)
3. Rinsing (in PBS/T or TBS/T)   2 x 2 min.
4. Inhibiting endogenous peroxidase activity   10 min. RT
5. Rinsing (in PBS/T or TBS/T)   3 x 5 min.
6. Incubating with 10% normal goat serum (in PBS or TBS)   30 min. RT
7. Incubating with first antibody   3 x 5 min.
8. Incubating with second antibody   45 min. RT
9. Rinsing (in PBS or TBS)   3 x 5 min.
10. Incubating with freshly made DAB substrate   5 min.
11. Rinsing (in MQ)   3 x 5 min.
12. Counterstaining with haematoxylin   10 sec.
13. Rinsing (in running tapwater)   10 min.
14. Dehydration
    - alcohol 60%   5 min.
    - alcohol 70%   5 min.
    - alcohol 80%   5 min.
    - alcohol 96%I   5 min.
    - alcohol 96%II   5 min.
    - alcohol 100%   5 min.
    - xylene   2 x 5 min.
15. Covering with vectamount

Appendix 2

Table 2 - Scoring β-catenin

Cat	Treatment	Biopsy	Hepatocytes	Ductular structures
FLC 1	Prednisolone	1	Membrane: +	Indistinct: +/-
		2	Membrane: +++ Cytoplasm: ++	Indistinct: ++
FLC 2	UDCA/ab-ib	1	Membrane: +++ Cytoplasm: ++/+++	Membrane: +++ Cytoplasm: ++/+++
		2	Membrane: ++ Cytoplasm: +	Indistinct: ++
FLC 3	UDCA	1	Membrane: +++ Cytoplasm: +/++	Membrane: +/-
		2	Membrane: ++	Membrane: +++ Cytoplasm: ++/+++
FLC 4	Prednisolone	1	Membrane: +/++	Indistinct: +/++
		2	Membrane: +	Membrane: +/-
FLC 5	UDCA	1	Membrane: + to ++	Membrane: ++ Cytoplasm: +/++
FLC 6	Prednisolone	1	Membrane: ++ Cytoplasm: +	Membrane: ++/+++ Cytoplasm: +
FLC 7	UDCA	1	Membrane: +++ Cytoplasm: ++	/
FLC 8	?	1	Membrane: +++ Cytoplasm: ++/+++	Indistinct: +/++
Healthy 1	/	1	Membrane: +	Membrane: +/-
Healthy 2	/	1	Membrane: +	Membrane: +/-
Negative control (FLC 3)	UDCA	2	-	-

Table 3 - Scoring Notch1/NICD

Cat	Treatment	Biopsy	Hepatocytes	Ductular structures
FLC 1	Prednisolone	1	Cytoplasm: +/-	-
		2	Cytoplasm: +/-	-
FLC 2	UDCA/ab-ib	1	Cytoplasm: +/-	-
		2	Cytoplasm: +/-	-
FLC 3	UDCA	1	Cytoplasm: +/-	-
		2	Cytoplasm: +/-	Cytoplasm above nucleus: +++
FLC 4	Prednisolone	1	Cytoplasm: +/-	-
		2	Cytoplasm: +/-	-
FLC 5	UDCA	1	Cytoplasm: +/-	-
FLC 6	Prednisolone	1	Cytoplasm: +/-	-
FLC 7	UDCA	1	Cytoplasm: +/-	-
FLC 8	?	1	Cytoplasm: +/-	-
Healthy 1	/	1	Cytoplasm: +/-	-

Cat	Treatment	Biopsy		
Healthy 2	/	1	Cytoplasm: +/-	-
Negative control (FLC 3)	UDCA	2	-	-

Table 4 - Scoring vimentin

Cat	Treatment	Biopsy	ECM portal areas	Mesenchyme sinusoid	Stellate cells	Vascular endothelium
FLC 1	Prednisolone	1	+ to +++	+	+	+/-
		2	/	/	/	/
FLC 2	UDCA/ab-ib	1	+ to +++	++	++	+/-
		2	++/+++	+/++	+/++	+/-
FLC 3	UDCA	1	+++	++	++	+/-
		2	+/-	+/-	+/-	+/-
FLC 4	Prednisolone	1	+++	+++	+++	+/-
		2	+++	++/+++	++/+++	++/+++
FLC 5	UDCA	1	+++	+	+	+/-
FLC 6	Prednisolone	1	+ to +++	++	++	+/-
FLC 7	UDCA	1	+++	++	++	+/-
FLC 8	?	1	++ to +++	++	++	+/-
Healthy 1	/	1	+/-	+/-	+/-	+/-
Healthy 2	/	1	+/-	+/-	+/-	+/-
Negative control (FLC 3)	UDCA	2	-	-	-	-

Table 5 – Scoring laminin

Cat	Treatment	Biopsy	Cytoplasm ductular structures	Basement membrane	Vascular endothelium	Cytoplasm hepatocytes
FLC 1	Prednisolone	1	- to +/++	- to +/++	+/-	-
		2	+ to ++	+/- to +	+/-	+
FLC 2	UDCA/ab-ib	1	+/- to +++	++	+/-	+
		2	- to +	+	+/-	+/-
FLC 3	UDCA	1	+/-	+	+	-
		2	+	+++	++	+
FLC 4	Prednisolone	1	+ to ++	+	+/-	+ to ++
		2	- (most) to +	+/- to +	+/-	-
FLC 5	UDCA	1	++	+	+/-	-
FLC 6	Prednisolone	1	- to ++	+/-	++	-
FLC 7	UDCA	1	+	+++	+/-	-
FLC 8	?	1	+	+/++	+/-	+/-
Healthy 1	/	1	+/-	+/-	+	-
Healthy 2	/	1	+/-	+/-	+	-
Negative control (FLC 3)	UDCA	2	-	-	-	-

Legend to appendix 2:
-: negative
+/-: minimal
+: mild
++: moderate
+++: strong
/: excluded

UDCA = ursodeoxycholic acid