Characterisation of the Feline Progenitor Cell Niche

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Date: 22 February 2013
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Introduction

Liver disease in Europe

Liver disease is a serious problem in Europe, according to the European Association for the Study of the Liver nearly 29 million people in the European Union are suffering from liver disease, where this statistic is not accurate since alcohol related disease and liver cancer are not included in the data. Therefore in reality the number of patients is much higher. Cirrhosis and liver cancer are the two most prominent end-stage liver diseases a result of a group of four frequently seen causes: alcohol consumption, hepatitis B&C and/or metabolic disorders due to overweight/obesity. At this moment liver transplantation is the only solution to for end-stage liver disease but the amount of donor livers available is far to few to supply the demand.

The Hepatic Progenitor cell

When there is liver disease there is always a degree of damage to the liver tissue, sometimes a little sometimes quite extensive. To restore the damage the liver relies on its regenerative capabilities. The regenerative capabilities are based on the activation of one or more mechanisms. In the case of parenchymal liver disease one of these mechanisms is the proliferation of adult hepatocytes, which replenishes the lost hepatocytes. In cases where the hepatocyte regeneration is impaired, this can happen during extensive liver damage or a chronic disease, the hepatic progenitor cell (HPC) niche is activated[1]. After activation of the HPC niche the progenitor cells proliferate. HPCs are able to differentiate bidirectionally, depending on the liver disease, into hepatocytes or cholangiocytes.

In humans, rodents and canine similar hepatic stem cells have been found, named oval cells in rodents and hepatic progenitor cells in humans and canines[2,3,4].

De hepatic progenitor cells (HPCs) are found in the smallest branches of the bile ducts, known as the Channels of Hering[3]. The activation of HPCs in the liver has been termed ductular reaction referring to its duct like appearance[5]. The ductular reaction is seen in both parenchymal liver disease as well as in biliary liver disease[6]. The HPCs along with the neighbouring cells and stroma make up de hepatic progenitor cell niche[4].

The HPC niche

"A stem cell niche is the restricted compartment in a tissue that maintains and regulates stem cell behaviour, supporting self-renewal and maintaining the balance between quiescence, proliferation and differentiation required in response to injury.'[7]

Cells in the liver, other than hepatocytes, which are components in the HPC niche (non-parenchymal cells, NPCs) are hepatic stellate cells (HSCs)/myofibroblasts and macrophages. In the healthy liver HSCs are responsible for the storage of vitamin A in lipid vacuoles and in the diseased liver they become activated and increase their production of collagen. Macrophages, first and for most crucial in the immune response, are also involved in tissue remodelling and fibrosis resolution in the case of extensive damage.

All these NPCs produce cytokines and growth factors that influence the HPC and hepatocyte proliferation[7,8,9].

During HPC activation the ductular reaction has been described to be always surrounded by a laminin matrix. This laminin matrix is suggested to allow the maintenance of the HPC's

progenitor/biliary phenotype and inhibits the differentiation into hepatocytes. Once the HPCs have left the laminin enveloped niche they differentiate into a hepatocyte phenotype[7]. In 2009 during a cross-species investigation into the activation of the HPC niche B. Schotanus showed the reaction in the liver was 'highly similar in man and dog'.

The HPC niche in cats

After research of the HPC in the human and the dog the first evidence of a similar HPC in the cat was published by J. Ijzer et al. Ijzer describes a hepatic progenitor cell in the cat that morphologically resembles the HPC in human and dog. Ijzer selected samples after staining with CK19 (marker for cholangiocytes and HPCs) to study the morphology of the HPCs. Ijzer's study included healthy liver, fulminant hepatitis, hepatic lipidosis, acute hepatitis, neutrophilic cholangitis, chronic hepatitis and t-cell lymphoma[10]. What still remains to be characterized is the HPC niche in cats, and the relation of HPC activation to disease severity (inflammation, potentially fibrosis) in cats. Since the use of the HPC niche is promising to play a role in new therapeutic solutions to liver disease, it is important that the HPC and its activation mechanisms are identified and characterized[1].

Lipidosis and cholangitis are diseases found in humans but not in dogs, therefore the cat would be a good model for these diseases.

Liver disease occurs frequently in animals. The causes of liver disease in dogs can be similar to the causes of liver disease in humans, being micro-organisms, toxins and drugs and immune-mediated reactions. Histologically and molecularly the canine and human livers and the pathologies of the liver diseases are similar and therefore the canine 'situation' could serve as an appropriate model for the human clinical situation[6]. Where dogs often present with parenchymal disease, cats often present with diseases of the biliary tree.

The two most common liver diseases in cats are hepatic lipidosis (parenchymal) and cholangitis [11,12,13].

The most common form of lipidosis in cats is also known as 'secondary lipidosis' where a cat suffers from anorexia due to a primary disease. Primary lipidosis can develop when the cat's dietary intake is insufficient because of forced underfeeding during an incorrect weight loss diet, periods of accidental food deprivation or selective anorexia due to change in food, changes in lifestyle or other stress factors[12].

Cholangitis occurs in three different forms, a neutrophilic cholangitis (caused by an ascending infection from the intestines), lymphocytic cholangitis and chronic cholangitis due to liver fluke infection[11].

These two groups of liver disease are rarely seen in the dog, therefore the cat could be a suitable model for (non)-alcohol induced steatohepatitis and primary sclerosing cholangitis in humans. Since diseases of the bile ducts occur considerably more often in cats than in dogs, cats could be used for the characterisation of the differentiation of the hepatic progenitor cell to cholangiocyt. There is no known treatment for lymphocytic hepatitis[14] and the HPC niche could be a route to a new therapeutic strategy. At this moment strategies considered are transplantation of proliferated and differentiated HPCs into the patient's liver or the stimulation of the activation mechanisms of the HPC niche to trigger proliferation and regeneration.

Characterisation of the feline HPC niche

To take the next steps in characterising the feline HPC niche immunohistochemical staining was used to examine the pattern of activation in the HPC niche in the different diseases (lipidosis and cholangitis). All study and control samples were examined immunohistochemically for the expression of CK19 (bile duct and progenitor cell marker)[4] , MAC387 (myeolomonocytic antigen)[15,16], αSMA (Smooth Muscle Actin antibody for Myofibroblasts[4,7], and laminin[7] . Immunoflourescent double staining was performed using the before mentioned markers and an additional marker panCK. PanCK is a wide spectrum cytokeratin marker which is a validated HPC marker[8].

The aim was to explore which markers are expressed in healthy liver tissue and which markers are expressed in the diseased liver. Staining was performed on liver tissue from healthy cats and from cats who suffered from liver disease or in which a liver disease was found post mortally.

Materials and Methods

Formalin fixed paraffin embedded normal liver samples were obtained from seven cats with histologically normal livers which were euthanized for reasons unrelated to liver disease.
Formalin fixed paraffin embedded liver samples with lipidosis (n=7) or with cholangitis (n=11) were obtained from cats submitted for post-mortem examination at the University of Utrecht. Cats were ages 5 months- 14 years.
Not all cats displayed clinical signs of liver disease and the cause of death could not always be linked to liver disease. In all samples the liver disease was histologically diagnosed/confirmed during post-mortem examination.

The samples were divided into groups based on the histologically diagnosed liver disease. The lipidosis group was composed of seven samples containing varying degrees of lipidosis. In this group one lipidosis sample had been diagnosed with peracute hepatitis with extensive secondary lipidosis. For the cholangitis group the ten samples were divided into the following groups: neutrophilic cholangitis (n=7), subdivided into neutrophilic cholangitis (n=4), chronic neutrophilic cholangitis (n=2) and neutrophilic cholangiohepatitis (n=1); lymphocytic cholangitis (n=2) neutrophilic lymphocytic cholangitis (n=1) and biliary cirrhosis (n=1). The control group consisted of seven healthy liver samples.
From each sample's paraffin block multiple sections (4µm) were cut and mounted on charged slides.

Immunohistochemistry

All study and control samples were examined immunohistochemically for the expression of CK19 (bile duct and progenitor cell marker), MAC387 (myeolomonocytic antigen), αSMA (Smooth Muscle Actin antibody for Myofibroblasts), and Laminin. These were examined using single staining.

All paraffin-embedded samples were de-waxed and rehydrated and IHC was performed using optimalised protocols (see addendum), specific for each antibody (for details see Table 1).

Table 1: Antibodies used in IHC staining

	Source	Type	Clone	Antigen Retrieval	Incubation	Company	Product Code	Dilution
CK19	Mouse	Monoclonal	b170	Prot. K	O/N 4°C	Novocastra	NCL-CK19	1:100
MAC387	Mouse	Monoclonal	MAC387	Prot. K	O/N 4°C	Abcam	ab22506	1:1000
αSMA	Mouse	Monoclonal	1A4	None	60min RT	BioGenex	MU128-UC	1:200
LAM	Rabbit	Polyclonal		Prot. K	O/N 4°C	Abcam	ab11575	1:100

Immunoflourescence

IF double staining was performed on a selection of samples, lipidosis (n=3), chronic neutrophilic cholangitis (n=1) and neutrophilic cholangiohepatitis (n=1). The antibody combinations used were: MAC387 – panCK; αSMA – panCK and LAM – CK19.

Sections were dewaxed and rehydrated; antigen retrieval was either obtained using the proteolytic-induced epitope retrieval method (proteinase K for 10 min) or heat-induced epitope retrieval method (citrate bath for 30 min at 98°C). The samples were then washed in PBS buffer solution. Non-specific sites were blocked using Normal Goat Serum (5% or 10% for 1 hour at room temperature). The samples were incubated overnight with a primary antibody mix (dilutions see table 2) at 4°C. The sections were then washed in PBS and incubated with the secondary antibody mix for 1 hour at room temperature with goat-anti-mouse AlexaF-488 (Invitrogen) 1:100 in antibody diluent (DAKO) and goat-anti-rabbit AlexaF-568 (Invitrogen) 1:100 in antibody diluent (DAKO). Sections were then washed in PBS and counterstained using DAPI (1:2000) in filtered PBS for 10min at room temperature. The sections were washed in PBS and mounted using Fluorsave.

Table 2: Antibodies used in Immunoflourescent double staining

	Source	Type	Clone	Antigen Retrieval	Incubation	Company	Product Code	Dilution
MAC387	Mouse	Monoclonal	MAC387	Prot. K	O/N 4°C	Abcam	ab22506	1:500
αSMA	Mouse	Monoclonal	1A4	Citrate 98°	O/N 4°C	BioGenex	MU128-UC	1:200
LAM	Rabbit	Polyclonal		Prot. K	O/N 4°C	Abcam	ab11575	1:100
pan_CK	Rabbit	Polyclonal		P.K/citr	O/N 4°C	Dako	Z0622	1:400
CK19	Mouse	Monoclonal	b170	Prot. K	O/N 4°C	Novocastra	NCL-CK19	1:100

Markers Used and why

CK19 is a generally accepted marker of HPCs; it has concurrent expression on bile duct cells and bile canaculi. They can be distinguished by morphological appearance and histological location[4]. During the immunoflourescence staining panCK was also used as a marker for HPCs[8].

MAC387 is a marker for both resident and infiltrating macrophages. Macrophages are inflammatory cells but they are also involved in tissue remodelling and fibrosis resolution after extensive damage next to their inflammatory properties[7]. In rodents, humans and dogs with normal liver tissue MAC387 positivity is only found as diffusely spread macrophages, with no clear spatial proximity to the bile ducts. In injured liver tissue macrophages are found clustered in close proximity to the ductular reaction and an increase in cells in the parenchyma.[7]

αSMA is a marker for smooth muscle and activated stellate cells. Hepatic stellate cells are believed to play an important role in the activation of HPCs by the expression of GFs and cytokines. The HSCs express αSMA (a smooth muscle actin) upon activation[4] In rodents, humans and dogs with normal liver tissue αSMA positivity is only found in very low intensity throughout the hepatic lobule, next to bile ducts and in vessel walls. In injured tissue the αSMA positivity increases and the amount of αSMA positive cells increases periportally (myofibroblasts) and in the parenchyma (HSCs).[7]

Macrophages and stellate cells serve as a key source of cytokines and growth factors crucial to the behaviour of the HPCs[2,7,9]. Where stellate cells produce collagen and macrophages could be involved in the differentiation of HPCs into hepatocytes.

Laminin marker binds to laminin in the tissue. In past research it has been seen that a laminin matrix always forms around a HPC response, this laminin matrix is theorised to be responsible for the maintenance of the bipotential nature of the HPCs. As long as the HPCs are in the laminin sheath they do not differentiate. Stellate cells/myofibroblasts are main producers of collagen and could be the origin of the laminin deposition[7]. Another theory is that the laminin is produced by the activated HPCs[17]. In rodents, humans and dogs with normal liver tissue laminin is prominent around portal vessels with weak staining in the hepatic sinusoids. In injured liver tissue laminin positivity increases due to increased laminin deposition around the ductular reaction.[7]

Results

Immunohistochemistry

In table 3 the semiquantative scoring is shown where (-) stands for no up regulation of positivity, (+/-) stands for a slight increase in posivity, (+) stands for an increase in marker positivity, (++) stands for a extensive increase in positivity. For inflammation (-) stands for no presence of inflammatory signs in the tissue, (+) stands for the presence of an inflammatory response, (++) stands for a severe increase in inflammatory cells in the tissue.

Table 3. Semiquantative scoring of immunohistochemical stainings

Sample	Diagnosis	CK19	MAC387	αSMA	LAM	Inflammation
1	L	+	+	+	+	+
4	L	+/-	+	+	+	+
9	L	+	+	+	+	+
3	L	+	+	+	+	++
6	L	-	-	-	-	-
7	L	-	-	-	-	-
8	L	-	-	-	-	-
15	NC	+	+	+	+	+
18	NC	-	+	+	-	+/-
25	NC	-	+	+	-	+/-
26	NC	-	+	+	-	+/-
14	CNC	+	+ (only PA)	+	+	++
27	CNC	+/-	+	+	+	++
19	NCH	++	++	+	+	++
16	LC	+	-	+/- (not PA)	+	+ (PORT)
20	LC	+	-	+	+	+ (PORT)
17	LNC	+	+/-	+	+	+
21	BC	++	++	++	++	++

Legend: Legend: L = Lipidosis, NC = Neutrophilic cholangitis, CNC = Chronic neutrophilic cholangitis, NCH = Neutrophilic cholangiohepatitis, LC = Lymphocytic cholangitis, LNC = lymphocytic neutrophilic cholangitis, PA = located in parenchyma, PORT= located portally

CK19

In the healthy samples the CK19 positivity was only in the cells of the bile bucts and small single lying lightly staining cells in the periportal areas, the HPCs. See fig 1 of the addendum. This was not the case in all the control samples, some samples displayed more CK19 positivity than others.

In the lipidosis group it was apparent that there were samples which had increased CK19 positivity and samples without an increase in CK19 positivity. Three of the seven samples displayed a definite increase in CK19 positivity in comparison to healthy samples including the sample diagnosed with peracute hepatitis with extensive lipidosis. One sample displayed

an intermediate increase in positivity, and three of the samples did not display an increase in CK19 positivity.
Photos of the lipidosis staining can be found in the attached addendum, fig 2 is the sample lipidosis with no CK19 increase, fig 3 is the sample lipidosis with increased CK19 and fig 4 is the sample peracute hepatitis with extensive lipidosis.
In the group neutrophilic cholangitis one of the four samples displayed a clearly increased CK19 positivity in and around the portal areas. Three of the four samples displayed a minimal increase in CK19 positive cells.
In the chronic neutrophilic cholangitis group one sample had a substantial increase in CK19 positive cells around the portal area and the other sample had a slight increase in CK19 positivity.
The case diagnosed with neutrophilic cholangiohepatitis had a high increase in CK19 positivity in the portal area indicating a severe ductular reaction.
Two cases diagnosed with lymphocytic cholangitis both displayed an increase in CK19 positivity.
One sample contained both lymphocytic and neutrophilic cholangitis, in this sample there was a substantial increase in CK19 positivity around the portal area.
The biliary cirrhosis case displayed a severe ductular reaction.
In the addendum are photos of the IHC staining of the sample neutrophilic cholangitis with increased CK19 staining, the sample with chronic neutrophilic cholangitis, the sample lymphocytic cholangitis and the sample containing lymphocytic neutrophilic cholangitis.

Relationship between Histopathology and CK19
Based on the CK19 staining a relationship can be seen between the histopathologic disease activity and the up regulation of CK19. In the lipidosis group the presence of lipidosis was diagnosed but some of the samples contained signs of inflammation present in the liver tissue, for example the presence of lymphocytes, macrophages or neutrophiles. It seems that in the group lipidosis it can be said that those samples with an inflammatory response displayed an increase in CK19 positivity and those in samples with no additional liver damage besides lipidosis there was no increase in CK19 positivity.
In the group neutrophilic cholangitis the sample with increased CK19 positivity was also the sample with the most inflammation compared to the other samples, histologically there was a diffuse ductular reaction with inflammatory infiltrate with neutrophiles in the bile ducts. The samples without CK19 up regulation contained a few neutrophiles in the bile duct lumen and some inflammatory cells in the parenchyma or periportal areas.
In the group chronic cholangitis the sample with substantial increase in CK19 positivity was also the sample with the most inflammation, fibrosis and cholestasis.
The sample with neutrophilic cholangiohepatitis contained a high amount of CK19 positivity also contained many inflammatory cells in the parenchyma, the periportal areas and inside the bile ducts.
The lymphocytic cholangitis samples both displayed an increase in CK19 positivity, their histopathology stated that there were infiltrations of lymphocytes and plasma cells but these were restricted to the periportal areas, indicating that the inflammation was restricted to the portal area.
The sample with combined lymphocytic and neutrophilic cholangitis displayed a definite increase in CK19 positivity also contained a diffuse inflammatory response.

The biliary cirrhosis sample contained a high amount of CK19 positivity, the histopathology stated this sample was a severe chronic case with extensive fibrosis formation.

MAC

In the healthy samples MAC387 positive cells were found spread throughout the tissue as single cells with no clear spatial relationship with the portal areas.

In the lipidosis group with increased CK19 staining there was also an increase in the number of MAC387 positive cells. In these samples the MAC387 positive cells formed clusters varying in number from 2-3 or 4-6 cells per cluster. The clusters were mostly found in close proximity to the portal areas, these being the areas where there was increased CK19 positivity.

In the lipidosis group with no increase in CK19 positivity the MAC387 staining resembles that of healthy liver tissue. The cells are diffusely spread throughout the tissue and do not appear as clusters.

In the group neutrophilic cholangitis all samples displayed an increase in the number of MAC387 positive cells throughout the tissue. In all the samples there was also the formation of clusters of MAC387 positive cells, all of samples the clusters were located in spatial proximity to the portal areas. In one of these samples K19 positivity was increased.

In both of the chronic neutrophilic cholangitis samples there was an increase in the number of MAC387 positive cells. Only the sample with the definite increase in CK19 positivity also displayed a slight cluster formation of two-three cells, these few clusters were located in the parenchyma.

In the neutrophilic cholangiohepatitis case there was a definite increase in number of MAC387 positive cells and these cells were clustered around the areas of increased CK19 positivity, the ductular reaction.

In the lymphocytic cholangitis samples neither of the samples displayed an increase in number of MAC387 positive cells.

In the lymphocytic and neutrophilic cholangitis sample there was a slight increase in the number of MAC387 positive cells throughout the tissue and there was minimal cluster formation of only two to four cells found near the portal areas but also in the parenchyma.

The biliary cirrhosis case displayed a severe increase in the amount of MAC387 positive cells and these were al clustered around the ductular reaction forming an almost continuous layer.

αSMA

In the healthy samples αSMA positivity was found in the portal triads around the bile ducts and in vessel walls. The parenchyma was negative for αSMA.

In the lipidosis group with increased CK19 positive staining there was increased positivity of αSMA in the parenchyma. The αSMA positivity around the portal triads and the ductular reaction was also increased.

In the lipidosis group without increased CK19 positivity the αSMA positivity was not increased.

In the neutrophilic cholangitis group the sample with increased CK19 positivity also displayed an increase in αSMA activity. The increase in αSMA activity was concentrated around the portal areas but there was also a definite increase of activity in the parenchyma.

The other three samples all displayed an increase in positivity in the parenchyma and in and around the portal areas. In these samples the positivity around the portal areas was more prominent than the positive staining in the parenchyma.
In both of the chronic neutrophilic cholangitis samples there was an increase in αSMA positivity in the parenchyma and an increase around the portal areas.
In the neutrophilic cholangiohepatitis sample there was a definite increase in αSMA activity in the parenchyma and around the portal areas where the CK19 positivity was.
In the lymphocytic cholangitis group one of the samples displayed a slight increase in αSMA positivity only around the portal area. The other sample displayed an increase in activity around the portal areas, and an increase in activity in the parenchyma.
In the lymphocytic and neutrophilic cholangitis sample there was an increase in positivity in the parenchyma as well as around the portal areas.
In the biliary cirrhosis sample the αSMA activity was highly increased, the activated areas surrounded the ductular reaction.

Laminin
In healthy tissue laminin positivity was found to be located in and around the portal areas. The laminin formed a layering around these portal areas, and was negative in the parenchyma. The thickness of the layering differed throughout the healthy samples and it could be suggested that this difference coincided with the difference in the degree of CK19 positivity.
In the lipidosis group with increased CK19 staining there was always increased laminin positivity. The positivity in this group was focused around the portal area and decreased in intensity precipitating to the central areas. The central areas in all samples were negative for laminin staining. The sample with an intermediate increase in CK19 positivity lacked an increase in laminin positivity; the laminin staining closely resembled that in healthy tissue.
In the lipidosis group where there was no increased CK19 positivity there was also no increase in laminin staining.
In the neutrophilic cholangitis samples the sample which displayed an increased CK19 staining also displayed an increase in laminin positivity around the portal areas. The three samples which did not contain an increase in CK19 staining also did not display an increase in laminin staining.
In both of the chronic neutrophilic cholangitis there was increased laminin positivity in the portal areas.
The case with neutrophilic cholangiohepatitis displayed an increase in laminin positivity around the areas where there was also CK19 positivity and MAC387 positive cell clustering.
In the lymphocytic cholangitis group one of the two cases displayed a definite increase in laminin positivity the other displayed an increase in laminin positivity, both samples displayed up regulated CK19 positivity.
In the lymphocytic and neutrophilic cholangitis sample there was an increase in laminin positivity in the portal area.
The biliary cirrhosis sample contained a high amount of laminin all concentrated around the ductular reaction forming a continuous sheath around these areas.

The laminin staining brought a peculiar positivity to light in four samples from different groups. Besides the increase in positivity in laminin layering around the ductular reactions in all these samples there was also laminin positive staining of the cytoplasma of adult

hepatocytes lying next to these ductular reactions. The staining in the cytoplasma of these hepatocytes was less intense than the laminin layered around the ductular reaction and in all samples the staining became less to nonexistent towards the parenchyma. The area of this hepatocyte positivity was three to four hepatocytes thick and outside this area the hepatocytes were negative. See figure 6D of addendum. The samples in which this occurred were one lipidosis, both chronic neutrophilic cholangitis and one lymphocytic cholangitis sample.
In all these samples all investigated markers were up regulated, except for the MAC387 staining in the lymphocytic cholangitis sample. At this moment one can only speculate about an explanation for hepatocyte positivity. The laminin positivity cannot be considered a-specific because it is located so specifically in the samples and in some hepatocytes (those located adjacent to the ductular reaction) but not in others. Where one hepatocyte's cytoplasm stains positive but a neighbouring hepatocyte is completely negative for laminin. One explanation could be that a change has occurred in these specific hepatocytes to make them stain positively for laminin, these changes could include induced degeneration, they could be differentiating HPCs, with remnant laminin expression, which is also seen for other markers in the dog (thesis Schotanus), or other responses to the liver disease.

Double IF staining

Immunoflourescent staining was done to compare the locations of positivity between the activated progenitor cells and the three different niche markers.

For the lipidosis group three samples were selected, one sample with increased CK19 positivity, one sample without increased CK19 positivity and the sample diagnosed with peracute hepatitis with extensive lipidosis. The lipidosis sample with increased CK19 positivity displayed a spatial relationship between the location panCK positivity and MAC387 positivity and between panCK and αSMA positivity. The staining of CK19 and laminin also displayed a spatial relationship between the two.
The IF double staining of the sample without increased CK19 confirmed the findings from IHC staining, that there was no increase in positivity for any of the markers. In these samples the CK19 or panCK positivity was restricted to the bile ducts in the portal areas and some single lying cells in the periportal areas. The MAC387 cells were diffusely spread throughout the tissue as single lying cells, the aSMA positivity was only located around the vessels and bile ducts in the portal area and the laminin was only thinly deposited around the portal triads.
The IF double staining of the peracute hepatitis with extensive lipidosis also confirmed the findings from the IHC staining. There was a spatial relationship between panCK positive cells and MAC387 positive cells. See fig 10 of the addendum. The αSMA staining displayed a spatial relationship between the panCK positive cells but also the positivity in the parenchyma was clearly visible. See figure 11 of the addendum. The laminin – CK19 staining demonstrated an increase in thickness of the layer of laminin around the CK19 positive cells and the spatial relationship between the two.

For the cholangitis group a sample from the chronic neutrophilic cholangitis group (with increased CK19 staining) and the sample with neutrophilic cholangiohepatitis were double stained.

The double staining for panCK and MAC387 confirmed that there was no MAC387 positive clustering in close proximity to the areas of panCK positivity in the chronic neutrophilic cholangitis sample. The clustering was only found in the parenchyma.
The double staining for panCK and αSMA confirmed the increase in αSMA activity around the area of panCK positivity and also the increase in αSMA positivity in the parenchyma where there was no panCK positivity. The double staining for CK19 and laminin displayed a spatial relationship between cells positive for CK19 and laminin positivity. The laminin positivity was layered around the CK19 positive cells.

The panCK – MAC387 staining for the neutrophilic cholangiohepatitis displayed the increase in MAC387 cells and the clustering of these cells in high amounts around the areas of panCK positivity. The panCK – αSMA staining confirmed the increase in αSMA positivity around the area of panCK positivity and also the presence of αSMA positivity in the parenchyma where there was no panCK positivity. The double staining for CK19 and laminin displayed the spatial relationship between the laminin positivity and the CK19 positive cells. The laminin positivity is seen to layer around the CK19 positive cells.

Discussion

The liver is usually capable of regenerating through adult hepatocyte replication after injury but in some liver diseases the damage can be so severe that hepatocyte replication falls short. In these cases the hepatic progenitor cells and their niche are activated to take over the regeneration.
This research has shown that the feline HPC niche consists of the same cellular and extracellular components that are found in activated HPC niches in canines and humans.
The activated feline HPC niche consists of HPCs, macrophages (resident Kupffer cells and invading macrophages), activated hepatic stellate cells/myofibroblasts and laminin. Though it can be suggested that not all components are activated simultaneously.
This research has confirmed that not all liver disease leads to HPC activation. In the lipidosis group there was lipidosis in all the samples but the amount of inflammation of the liver after histological examination differed, ranging from none to extensive. Four out of seven samples displayed an increase in CK19 positivity indicating HPC activation. In these four samples the HPC activation was accompanied by an up regulation of the activity of the other three HPC niche components suggesting that in these cases full activation was accomplished. Those samples which displayed an increase in CK19 positivity the pathology report mentioned inflammation in the tissue occurring alongside the lipidosis. This was also displayed by an increased presence of MAC387 positive cells.
This could support the idea that the amount of CK19 positivity and thus the amount of HPC activation correlates with degree of histological disease found in these lipidosis samples. Samples with less inflammation displayed less HPC activation, suggesting that the lipidosis was not causing a reaction in the liver.
In the cholangitis group the absence of CK19 positivity only occurred within the neutrophilic cholangitis group. This could indicate that the severity of the cholangitis in these samples was not substantial enough to induce HPC activation, this was seen in three out of four samples. In the pathology reports of these three samples the amount of liver inflammation was minimal compared to the other samples.

All the other samples in the cholangitis groups displayed an increase in CK19 positivity indicating HPC activation.
This also supports the conclusion that the amount of CK19 positivity (HPC activation) correlates with the degree of liver disease.

After this research similar conclusions could be made about the role of the macrophage and hepatic stellate cell/myofibroblasts.
In the lipidosis group all HPC activation was accompanied by an up regulation of αSMA and MAC387 positivity indicating activation of hepatic stellate cells/myofibroblasts, both in the parenchyma and around the ductular reaction, and an increase in number of macrophages with cluster formation, respectively.
In the neutrophilic cholangitis group in three of the four samples CK19 positivity wasn't increased indicating the absence of HPC activation. In these three samples the MAC387 was increased with cluster formation and these three samples the αSMA was also increased indicating that the macrophages and hepatic stellate cells *were* activated. This means that the parenchyma in these samples was involved in the liver disease. This potentially suggests that HSC/myofibroblast activation and macrophage recruitment occurs before HPC activation. This could mean that the components of the HPC niche were being activated and recruited and that these components, the macrophages and hepatic stellate cells, were required to trigger HPC activation, or that the liver's capacity is still enough to support full liver function and HPC activation is not yet necessary. Hepatic macrophages and myofibroblasts are responsible for a variety of signals which are crucial in the control of liver development and HPC behaviour[7]. HPCs themselves are a source of chemokines responsible for the recruitment of infiltrating macrophages[9]. It would be interesting to use a specific kupffer cell marker to distinguish between resident and infiltrating macrophages. The MAC387 marker does not specify between resident and infiltrating macrophages so no conclusion can be formed on the type of macrophages present in the neutrophilic cholangitis group without increased CK19 positivity but it could be speculated that these are resident macrophages. Since there is no HPC activation HPCs are also not responsible for the macrophage infiltration.
Contrarily in the lymphocytic cholangitis groups both samples displayed HPC activation with an increased αSMA suggesting HSC/myofibroblast activation but there was no increase in MAC387 positivity. Suggesting that HSC/myofibroblast activation has occurred but the recruitment of macrophages has not. The reason is that in lymphocytic cholangitis the inflammatory cells are lymphocytes and not monocytes therefore there is no increase in amount of macrophages. Also lymphocytic cholangitis is a portal disease with little to no involvement of the parenchyma. Additionally in recent study it has been suggested that macrophages play a role in the differentiation of HPCs into hepatocytes, in a biliary disease there is no need for new hepatocytes, the HPCs need to regenerate into cholangiocytes[21].
All the other cholangitis groups displayed HPC activation with increased macrophage presence and HSC/myofibroblast activity. This could also be an explanation for the abscence of macrophage clustering around the ductular reaction in the chronic neutrophilic cholangitis case.
There was also a spatial relationship between the location of HPC activity and macrophage and HSC/myofibroblast activity. Both cell types were located near or around the HPCs. This supports the idea that macrophages and HSC/myofibroblasts are important to the HPC niche and its behaviour. MAC and aSMA were however also found at sides unrelated to the

ductular reaction. This indicates the role the macrophages and HSCs play during disease independent of HPCs. Their activation at sites currently distant from HPCs could well prime the liver tissue for HPCs to migrate into the tissue. It is in line with the hypothesised bidirectional signalling of inflammatory cells-macrophages/HSCs-early fibrosis and HPC activation .
The patterns of macrophage and HSC/myofibroblast activation are similar to the activation found in diseased liver in humans and canines.

This research confirmed that laminin is an important component in the feline HPC niche. Similarly to canine and human disease liver a laminin sheath surrounds every activation of HPCs. Only in samples where there was an increase in CK19 positivity (HPC activation) there was also an increase in laminin deposition, this applied to all the disease groups. The theory is that the laminin is produced by the HPCs and is needed to retain the bipotential qualities of the newly formed HPCs[17]. This way HPCs are allowed to proliferate but the laminin sheath keeps them in an undifferentiated state[17]. Whether this function holds true in the cat liver should be functionally investigated, e.g. using liver organoid cultures.

Based on this research it could be suggested that the feline HPC niche resembles that of the canine and the human in healthy and in diseased livers. The fact that this research included samples with varying degrees of the investigated diseases it was possible to see correlations between the histological disease severity and the reaction of the HPC niche. Cats could serve as models for HPC niche research of biliary disease or for (non) alcoholic steatohepatitis in humans.

Further research is needed based on cats clinically suffering from liver disease to make a definitive link between the findings of the HPC niche and the clinical presentation of the liver disease.

As far as therapeutic opportunities one must bear in mind that (once possible) transplantation of HPCs into the diseased feline liver or the therapeutic activation of the niche to induce HPC proliferation only solves part of the problem. This would replace the diseased liver cells, being hepatocytes or cholangiocytes but the source of liver inflammation would remain. The focus must therefore also lie in a complementary therapy which can cure the inflammation in the liver for example: the use of anti-inflammatory mesenchymal stem cells in addition to the stem cells.

Addendum

Photos

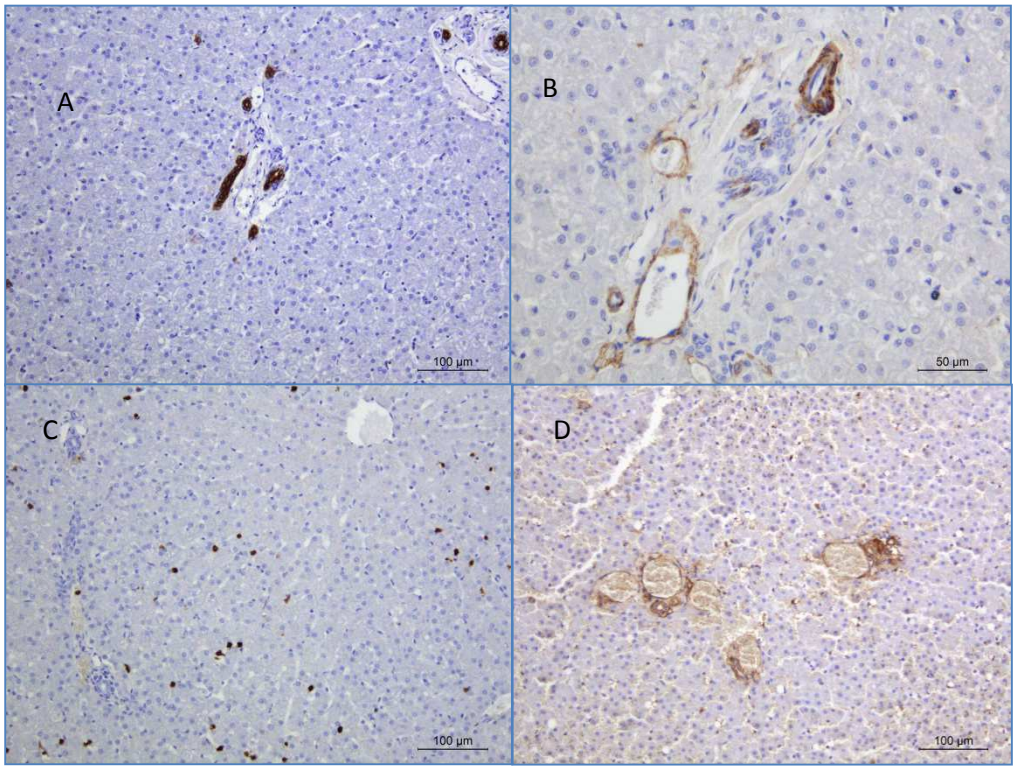


Figure 1 – Immunohistochemistry of healthy liver
The healthy liver shows positive CK19 staining of bile ducts in the portal triads and an isolate small cell in the periportal area (A). The healthy liver shows positive αSMA staining in the portal triad surrounding the vessels and the bile ducts (B). The healthy liver shows single macrophages diffusely spread throughout the liver tissue (C). The healthy liver shows laminin positivity surrounding the portal areas (D).

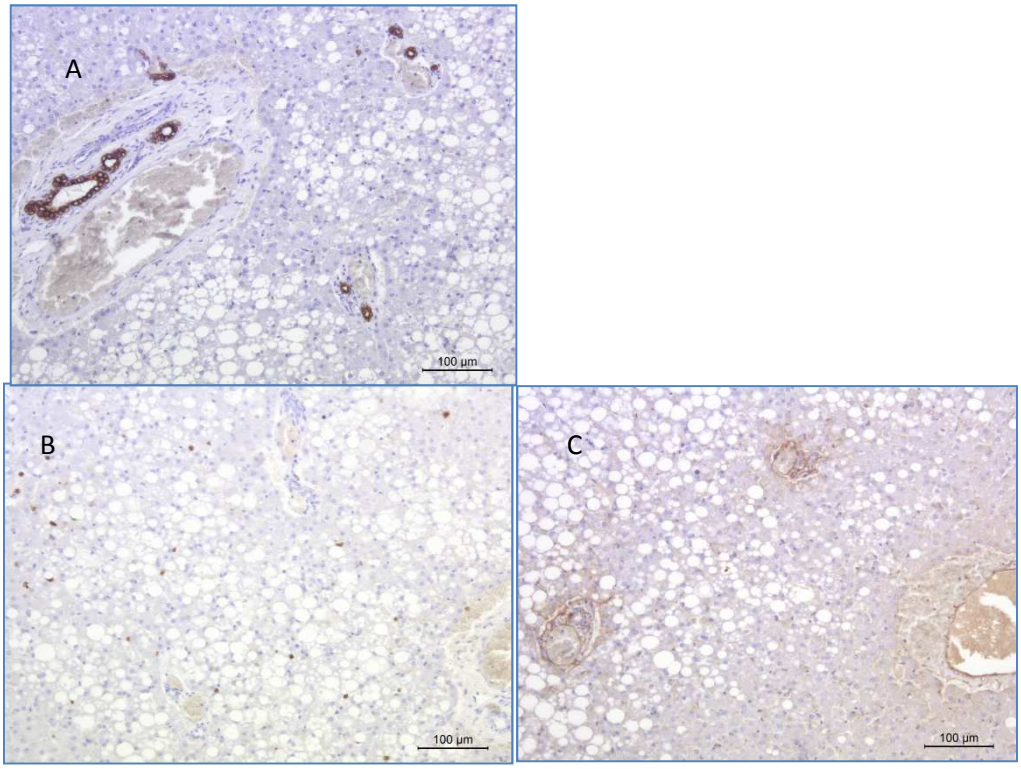


Figure 2 – Lipidosis with little CK19 positivity
The extensive amount of lipidosis can be seen in all the pictures. The CK19 positivity is similar to that in healthy tissue (A). The MAC387 staining shows single cells diffusely spread throughout the tissue (B). The laminin positivity is only found surrounding the portal areas. (C)

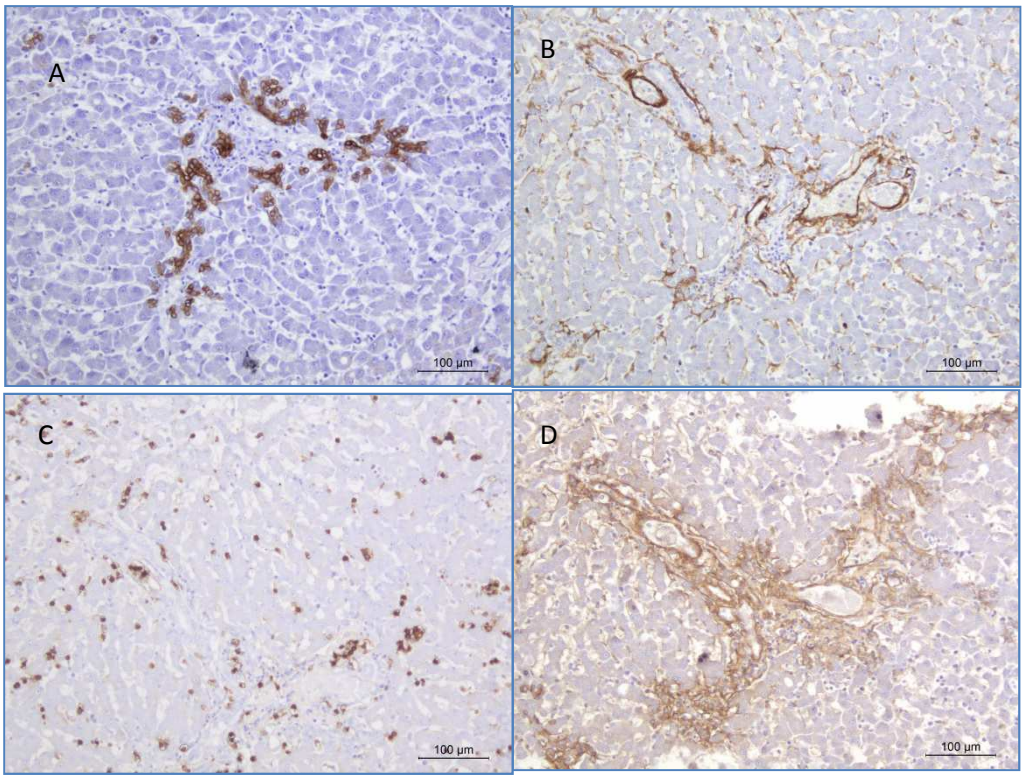


Figure 3 Immunohistochemistry of lipidosis with increased CK19
The increase in CK19 positivity indicated HPC activation (A). The αSMA positivity has increased around the portal areas and in the parenchyma; the staining around the portal areas is more intense than in the parenchyma (B). The number of MAC387 macrophages has increased and there is cluster formation in proximity to the portal area (C). The laminin sheath has increased in thickness around the portal area and the ductular reaction.

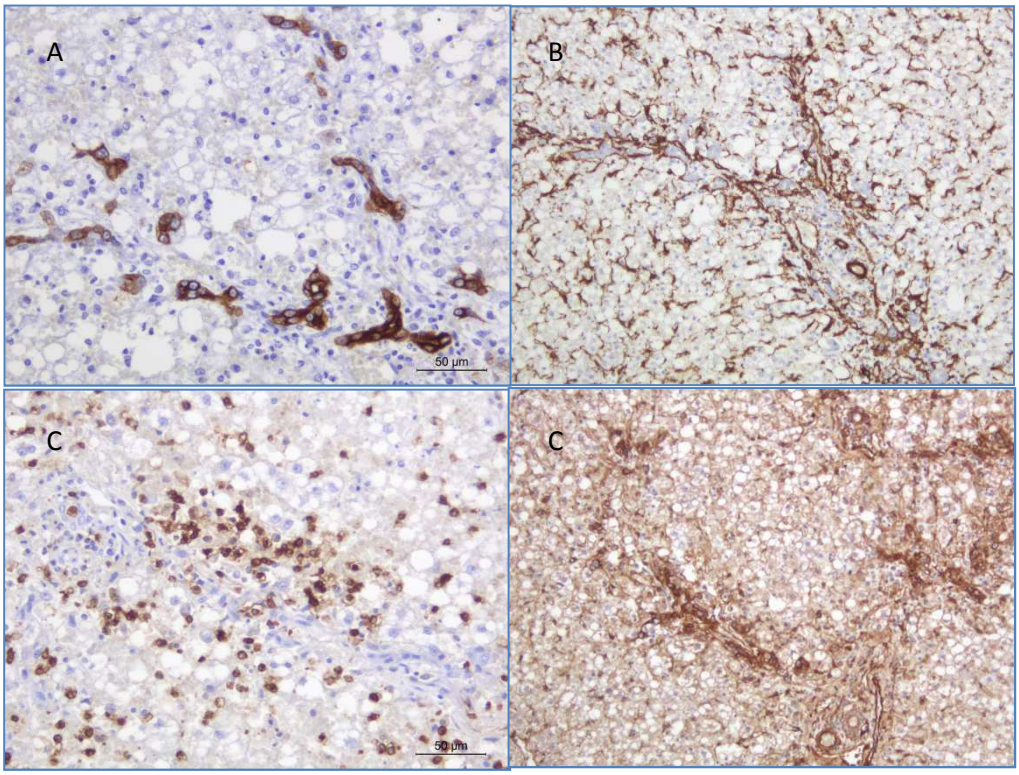


Figure 4 – Immunohistochemistry of peracute hepatitis with extensive lipidosis
An increase in CK19 positivity in a ductular fashion (A). Dark staining αSMA around the ductular reaction and in the parenchyma (B). Cluster formation of macrophages around the ductular reaction (C). Increase in laminin deposition around the ductular reaction and increased positivity in the parenchyma (D).

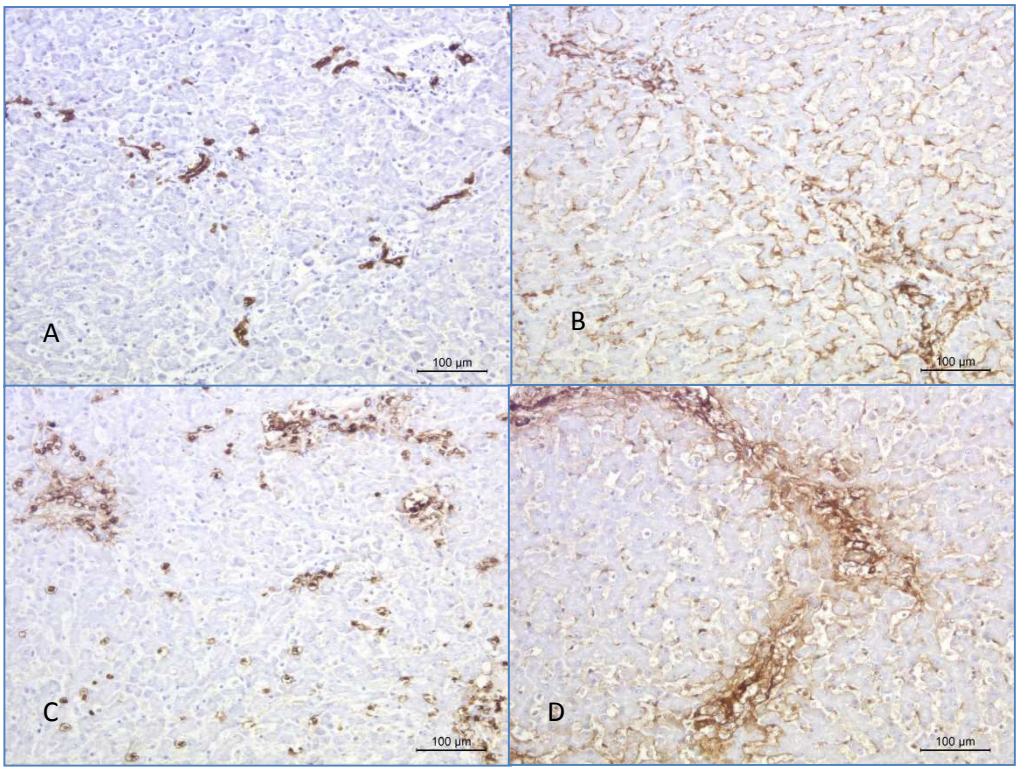


Figure 5: Immunohistochemistry of neutrophilic cholangitis
An increase in CK19 positivity in the liver tissue indicates a ductular reaction (A). The increase in αSMA activity in the parenchyma can be seen the increase of αSMA activity around the ductular reaction (B). Cluster formation by the macrophages in spatial proximity to the HPC (C) and the increase in laminin matrix (D).

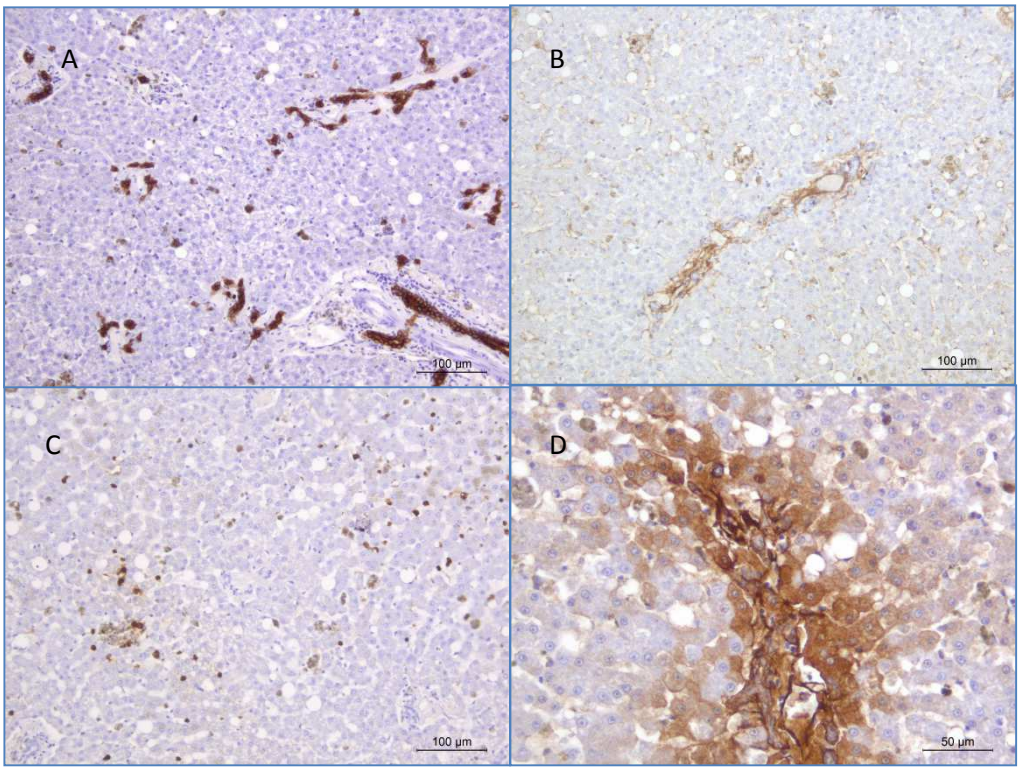


Figure 6 Immunohistochemistry of chronic neutrophilic cholangitis
Substantial increase in CK19 positivity (A). Increase in αSMA activity around the portal area and in the parenchyma (B). Cluster formation of two-three macrophages concentrated in proximity to the ductular reaction (C). Increase of laminin positivity around the portal area, also the cytoplasma of the hepatocytes adjacent to the portal area show positivity for laminin (D)

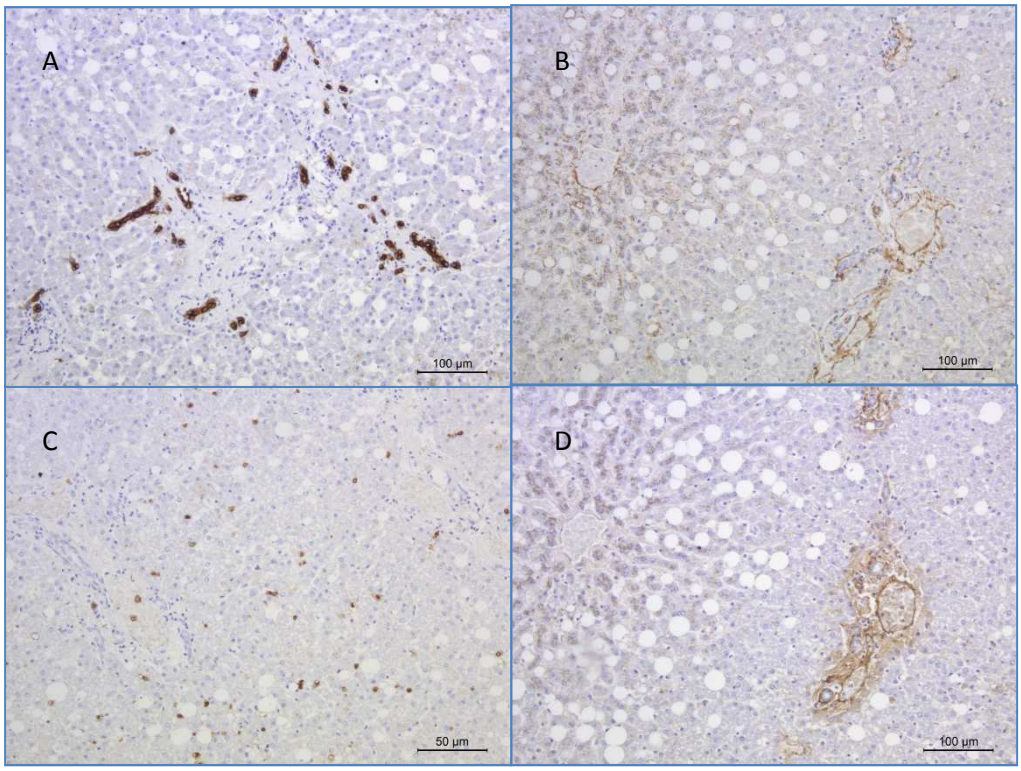


Figure 7: Immunohistochemistry of lymphocytic cholangitis
Increase in CK19 positivity (A), joined by an increase in αSMA activity and laminin activity (B&D). The macrophages are not increased in number (C).

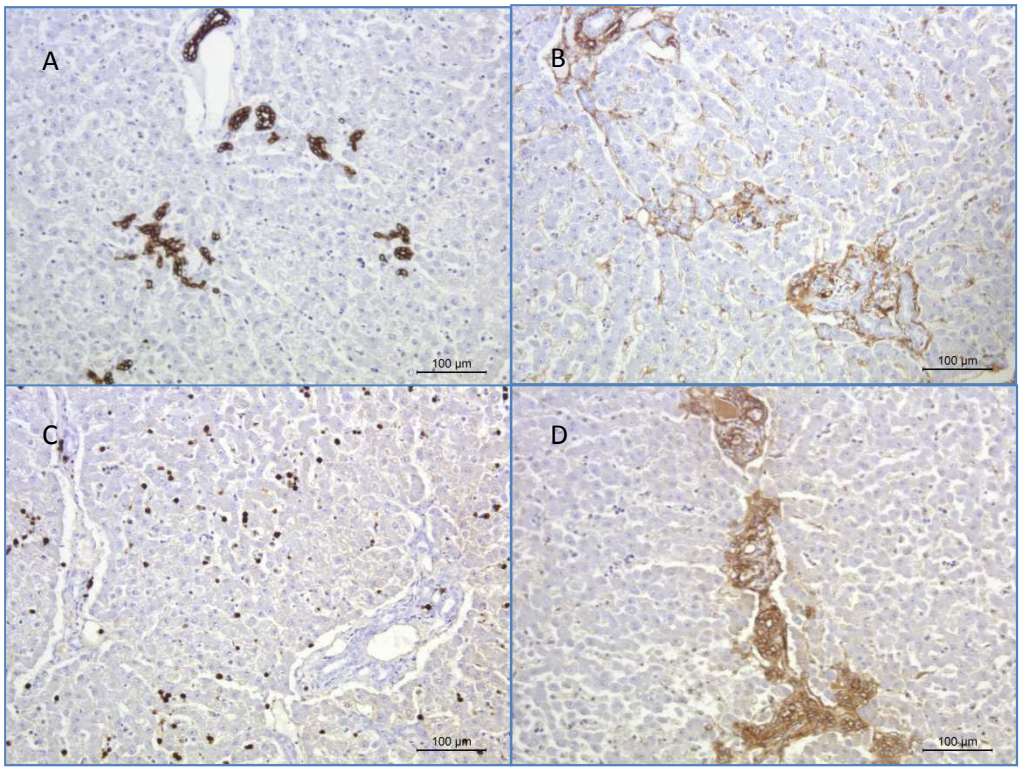


Figure 8: Immunohistochemistry of Lymphocytic and neutrophilic cholangitis.
Substantial increase in CK19 positivity in the periportal areas, a ductular reaction (A). αSMA activity is increased in the parenchyma and around the portal areas (B). The number of macrophages has increased slightly and there are formations of clusters consisting of two to four cells near the portal areas but also in the parenchyma (C). The laminin sheath is increased in thickness and surrounds the portal area (D)

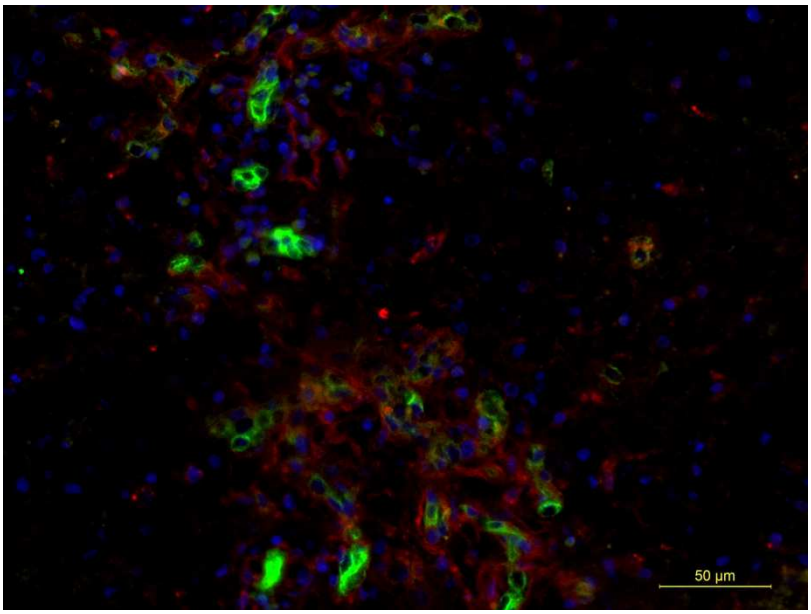


Figure 9 - IF CK19-Laminin double staining
The laminin (red) can be seen as sheets around the HPCs (green). There is a definite spatial relationship between the two. As seen in peracute hepatitis with extensive lipidosis sample.

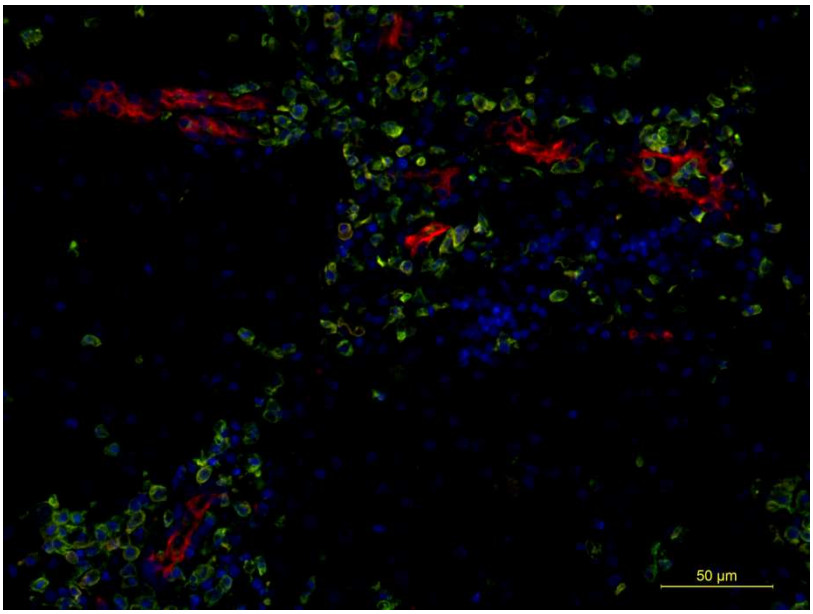


Figure 10 - IF pan-CK – MAC387 double staining

Increase in macrophages (green) and clustering of these cells in close proximity to the HPCs (red). As seen in neutrophilic cholangiohepatitis.

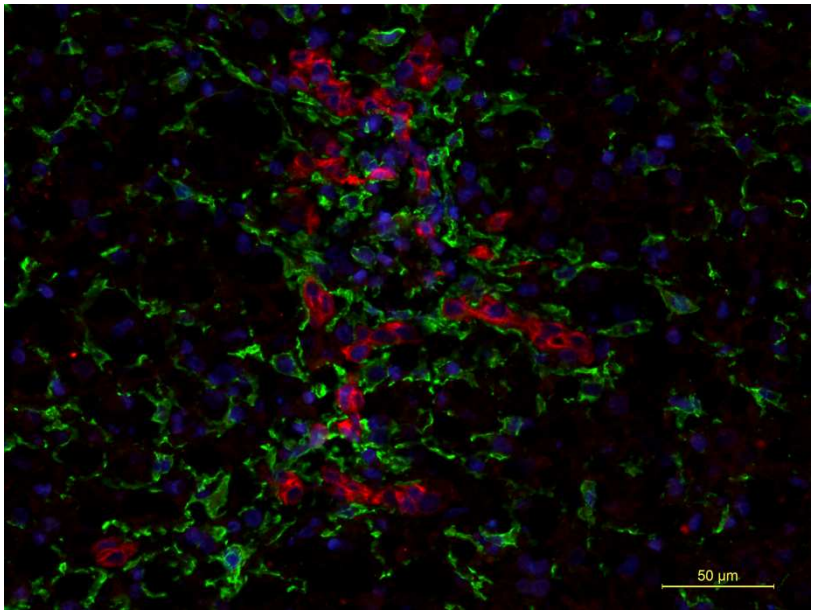


Figure 11– IF pan_CK – aSMA double staining

The increase in aSMA (green) positivity in parenchyma, also positivity around HPCs (red) is continuous with few gaps. As seen in peracute hepatitis with extensive lipidosis.

Side step IHC staining

Basic Protocol

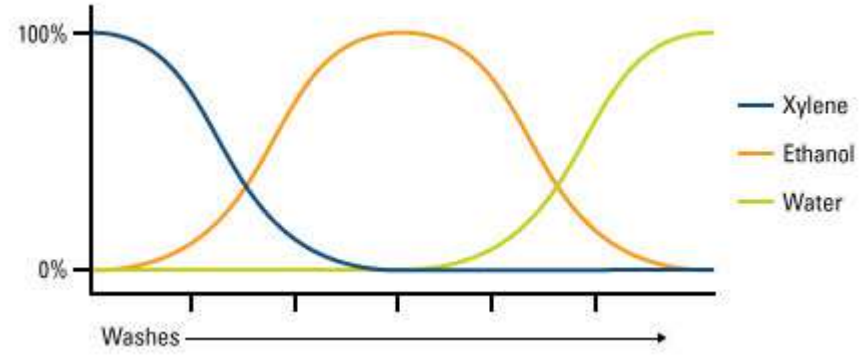
1. Deparaffinise and rehydrate sections in a series:
    a. Xylene: 2x5'; Alc. 96%; Alc. 80%; Alc. 70%; Alc. 60% (each step 5'); MQ 5'
2. Antigen retrieval
    a. Proteinase-K — 15 min
3. Rinse in TBS/T buffer solution — 2x2min
4. Inhibit endogenous peroxidase activity
    a. Dako Dual Endogenous Enzyme block — 10 min RT
5. Rinse in TBS/T buffer solution — 3x5 min
6. Incubate in 10% Normal Goat Serum — 30 min RT
7. Incubate with (in ab-diluent, DAKO)
    a. CK19 - #29 – 1:100(mouse) — O/N at 4C
8. Rinse the sections in TBS/T buffer solution — 3x5 min
9. Incubate in
    a. Envision Goat anti mouse HRP — 45 min RT
10. Rinse the sections in TBS — 3x5 min
11. Incubate the sections in freshly made DAB substrate (result: brown) 5 min
12. Rinse the sections in mQ — 3x5 min
13. Counter stain the sections in haematoxylin QS-Dako — 10sec.
14. Rinse the sections in funning tap water — 10 min
15. Dehydrate section and cover in vectamount:
    a. 60% alc.; 70% alc.; 80% alc.; 96% alc.; 96% alc.; 2x3'xylene

Fixation

Fixation is obtained by placing the tissue sample in 4% formaldehyde solution and then the tissue sample is embedded in paraffin.

After cutting sections of the paraffin block containing the tissue sample these are mounted onto charged microscope slides.

The paraffin covers the antigens on the tissue making it impossible for the antibodies to bond; therefore the paraffin is removed using a Xylene wash, consisting of two successive five minute baths. The Xylene is then removed from the tissue in an ethanol bath of 96%. The tissue is then slowly rehydrated in graded washes of ethanol in water, 80% then 70% then 60% and then in distilled water[18].



Antigen retrieval:
The formaldehyde used for fixating the tissue forms methylene bridges between proteins. These bridges can block the epitopes on the tissue which makes these bridges can block the epitopes making detection by the primary antibodies impossible.
In this study two different methods were used two remove the methylene bridges, heat-induced epitope retrieval (HIER) and proteolytic- induced epitope retrieval (PIER).
In het HIER method heat and a buffer is used to remove the methylene bridges. In this study citrate buffer (pH6) was used at a temperature of 98C[19].
In the PIER method the enzymatic properties of pronase, pepsin, ficin, trypsin or proteinase K can be used to digest the protein bridges covering the epitopes[18]. In this study Proteinase K was used.

Blocking Endogenous enzymes
IHC uses enzymes to generate chromogenic signals; examples of these enzymes are horseradish peroxidase (Envision HRP) and alkaline phosphatase. These enzymes are both found as endogenous activities in cells and tissues. Problems occur when the endogenous enzymes in the samples are similar in specificity to the enzymes being used in the IHC protocol. This can result in false-positive signals which could interfere with the desired IHC reactions.
To inhibit the activity of these enzymes in the cells and tissue of the sample blockers can be used, so that when the IHC enzymes are used the endogenous enzymes will not interfere with the reaction.
In a majority of the protocols used in this study DAKO Dual endogeneous Enzyme Block was used; this enzyme block contains horseradish peroxidase and alkaline phosphatase labels.
Another enzyme blocking reagent used was hydrogen peroxide which blocks horseradish peroxidase labels.[20]

Blocking non-specific sites
Antibodies normally show a preference for specific epitopes. It may occur that antibodies partially bind to reactive sites that are similar to the binding site of the target epitope, when this occurs there is a high quantity of background staining and detection of the target antigen is not possible. To block these non-specific reactive sites 10% normal goat serum was used.[18]

Antibody Incubation Time:
The incubation time for the primary antibody can vary up to 24 hours, it depends on the antibody titre, higher the antibody titre the shorter the incubation time.
If an antibody needs to react in a short time the concentration of the antibody and the affinity for the antibody must be high and environment must be optimal. When incubation time is lengthened the concentrations of the primary antibody can be much lower and higher dilutions ensure better economy. Long incubation times also ensure sufficient bonding between antibody and target antigen.[18]

Incubation temperature
The higher the incubation temperature the sooner the antibody reactions reach their equilibrium. This means that at a higher temperature the concentration of antibody can be

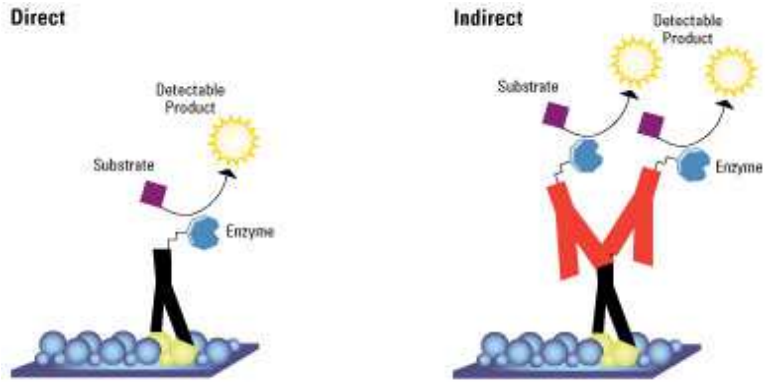
lower and the incubation time can be shorter. The higher the incubation temperature is the more critical the incubation time becomes where an incubation time that is too long could ruin the staining. Usually 4°C is used when incubating overnight or longer.[20]

Antibody Titre

Antibody titres used for polyclonal antibodies are usually 1:100 to 1:2000 and 1:10 to 1:1,000 for monoclonal. An optimalisation in antibody titre allows for a high specificity in staining and the least amount of background staining.[18]

Secondary Antibody & DAB

The indirect method of detection was used in this study. This method utilizes a secondary antibody which has specificity against the unlabeled primary antibody. Multiple secondary antibodies can bind to the primary antibody thus amplifying the primary signal. After multiple secondary antibodies bind to each primary antibody, the enzyme label on the secondary antibody is then reacted with substrate to give the chromogenic response.



The enzymes in this study were horseradish peroxidase and the substrate used was DAB. During immunoflourescent staining differs because the secondary antibody is conjugated with flourophore. Therefore an enzyme label and substrate reaction is not needed. [18]

Counter stain

To make sure the antigen of interest can be viewed and judged in proper context a counter stain is preformed to dye the surrounding tissue. Haematoxylin is the counter stain used most and was also used in this study. In the immunoflourescent staining DAPI was used as a nuclear counter stain.[20]

Washing Buffers

During the protocol it is important to wash the sample between steps, this ensures that any unbound antibody is removed but also that any antibodies that may have weakly bound to non-specific sites are removed. The most common washing buffers are phosphate buffered saline (PBS) or tris buffered saline (TBS). Sometimes a small amount of detergent, like Tween 20, is added to the washing buffer to allow uniform spreading of the antibody treatment across the tissue.[18]

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