The prevalence and increased malignancy of Keratin 19 positive hepatocellular tumors in the dog and cat

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Content

Summary

Hepatocellular carcinoma is a malignant tumor of the liver. It is known that a proportion of the hepatocellular carcinomas show positivity for K19. In several studies across multiple species these K19 positive tumors showed a more malignant nature compared to K19 negative tumors. During this study we used 96 dog liver samples and 20 cat liver samples to examine the prevalence of K19 positivity in hepatocellular tumors and determined the malignancy of K19 positive tumors in the dog and the cat.

General Introduction

Liver tumors

Hepatocellular carcinoma

Hepatocellular carcinomas (HCC) are seen in both dogs and cats [34]. They may occur as a large solitary structure [20, 34] that resembles normal liver tissue, but also may be seen widespread throughout the liver (Fig. 1). [34]

Histologically, they form irregular trabeculae separated by sometimes markedly dilated sinusoids and cystic blood and/or serum filled spaces, or form acinar or large solid structures. In many cases lipidoses or glycogen accumulation in the neoplastic hepatocytes can be seen. [34]. The hepatocytes in HCC can be well-differentiated, but also can be very poorly differentiated. This leads to a heterogeneous histological picture. Staging systems can help to predict the prognosis of patients with HCC and guide the therapeutic approach. After treatment it can assist in the evaluation of the results of treatment. It also can help the exchange of information among researchers [32]. The tumors can be classified by:

- Infiltration at the periphery of the tumor
- Diffuse or cellular pleiomorfism
- Variation in cell and nuclear size
- One or more nucleoli varying in size
- Frequent presence of mitotic figures

There are different kinds of classification systems of HCC. They are extensively described in the markers chapter.

In humans, there is an underlying cause for HCC. Often there will be liver cirrhosis in combination with a hepatocellular carcinoma. Cirrhosis can be the result of a viral infection with hepatitis B and C virus. This is a virus that replicates in hepatocytes. Sometimes it is seen in combination with chronic hepatitis. [1, 33] In dogs there is no association between the incidence of liver tumors and a viral infection. Cirrhotic livers in canine seem insensitive for the development of HCC.

The clinical signs for HCC in dogs look like general liver disease symptoms such as lethargy, anorexia, weight loss, jaundice and abdominal distention from either hepatomegaly or effusion.(24) Therefore hepatocellular carcinoma is often recognized in a late stadium [12].

Fig.1: Dog, Hepatocellular carcinoma [34]

Cholangiocellular carcinoma

Cholangiocellular carcinomas are seen in both dogs and cats [34]. There is no breed or sex predisposition [23]. It is a malignant neoplasm of biliary epithelium. Usually they arise from the intrahepatic ducts, but the extrahepatic bile ducts can be affected as well. The tumor can be a large single mass [20, 34], but is often seen as irregularly formed multiple nodules within the liver (Fig. 2) [34]. The masses are firm, raised, often with a central depression, pale gray to tan and unencapsulated. The cells seen in the tumor resemble biliary epithelium. Well defined carcinomas are organized into a tubular or acinar pattern. Poorly differentiated carcinomas are composed of patches, islands or cords and areas of squamous differentiation can occur [20]. In well differentiated areas, mucin can be seen in the lumen of these structures. The differentiation of neoplastic cells varies. Usually mitotic figures and pleiomorfism is seen. [34] The epithelium components are separated by fibrous connective tissue. This fibrous connective tissue provides the tumor the firm texture. At the margin of the cholangiocellular carcinoma there are multiple sites where the tumor invades in the surrounding liver parenchyma. Multiple necroses in adjacent parenchyma can also be seen. [20]

The cholangiocellular carcinoma is very malignant. Metastases to other parts of the body are common, particularly to the adjacent lymph nodes of the cranial abdomen, lungs or into the abdominal cavity. Metastasis into the peritoneal cavity is also been seen. [20]
In cats chronic cholangitis has been associated with the development of intrahepatic or extrahepatic cholangiocellular carcinomas [34].

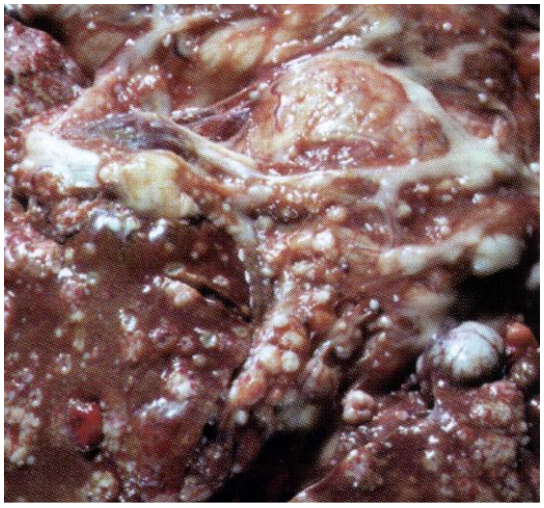

Fig. 2: Dog, cholangiocellular carcinoma. Multiple nodulus are seen on the surface of the liver. [20]

Nodular hyperplasia

Hepatocellular nodular hyperplasia is normally only seen in the dog and occurs less often in the cat [20, 34]. The hyperplasia develops from the age of 6, after which the incidence increases [20]. Almost all dogs over the age of 10 show multiple hyperplastic nodules. There is no predilection for sex or breed. [34]

Multiple hyperplastic nodules are present. The nodules can be seen on the livercapsular surface (Fig. 3) and are typically raised and hemispherical, yellow to tan (Fig. 4). When you look at the histology of the nodules they contain all the elements of normal liver tissue, but the lobular pattern is distorted. Compared with normal liver, the nodules contain an increased proportion of hepatocytes and decreased numbers of portal tracts and central veins [20]. These last two can be seen within or at the periphery of the nodule [34]. Hepatocytes are variable in size and frequently contain cytoplasmatic lipid- or glycogen-containing vacuoles. [20, 34]. The surrounding parenchyma can show a slight compression [34].

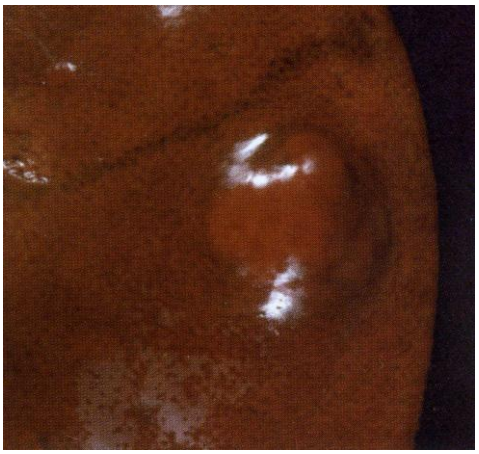

Fig. 3: Dog, Nodular hyperplasia. The nodule protrudes above the surface of the liver. [20]

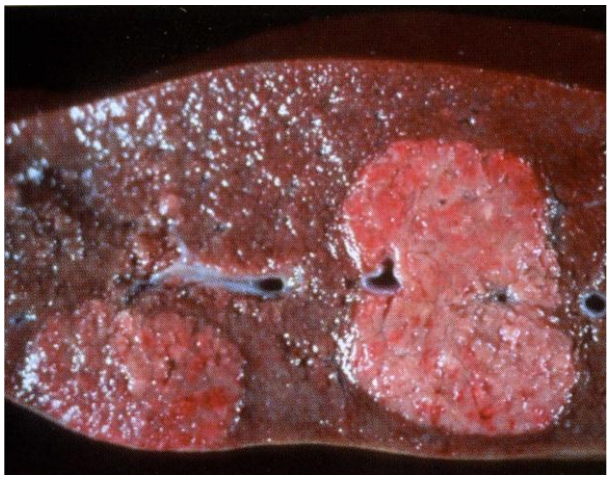

Fig. 4: Dog, Nodular hyperplasia. Cut surface of the liver.Two nodules are visible on the surface of this liver. [20]

Hepatocellular adenoma

Hepatocellular adenomas are seen in dogs and cats [34]. Hepatocellular adenomas are benign neoplasms of hepatocytes [34, 31]. In general they are restricted to one or two liver lobes (Fig. 5)[34].The neoplasms are usually single, unencapsulated, well demarcated [34], variably sized red or brown masses that compress adjacent parenchyma (Fig. 6).
Histologically, the hepatocellular adenomas consist of well differentiated hepatocytes. The hepatocytes have a uniform appearance, nucleoli may be prominent. Macrovesicular or mixed type lipidosis or marked glycogen accumulation of the neoplastic hepatocytes can be observed. [34] The hepatic cords are separated by sometimes markedly dilated sinusoids and cystic and/or serum filled spaces [34]. Portal tracts and central veins are not seen very often in the neoplasm and sometimes not present at all. [20]
It is difficult to distinguish hepatocellular adenomas from hepatocellular nodular hyperplasia. Histologically, adenomas are characterized by only one or few portal tracts, whereas hyperplastic nodules retain normal lobular architecture elements, although the portal tracts are more separated than normal. [20]

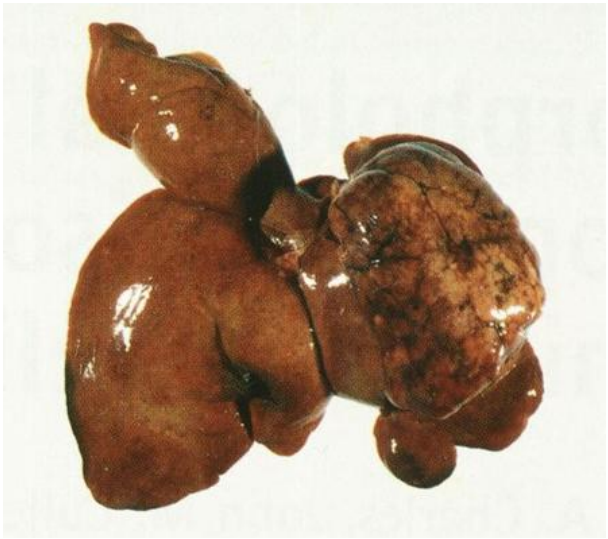

Fig. 5: Cat, Hepatocellular adenoma [34]

Fig.6: Dog, Hepatocellular adenoma on cut surface of the liver. [20]

Carcinoids

Hepatic carcinoids are not often seen in dogs and cats. They arise from neuroendocrine cells, which embryologically are of neural crest origin. Most carcinoids arise from neuroendocrine cells that migrate from the neural crest to the forgut, midgut and hindgut. They are normally found throughout the gastrointestinal tract from mouth to anus. The pancreas contains a lot of these cells, the biliary tree contains a few of these cells, whereas the liver normally contains none. [25] So, primary hepatic carcinoids in the liver arise from cells that lie in biliary epithelium [20, 34], but they may also possibly arise from hepatic progenitor cells (HPC) [34]. They can form in the intrahepatic and extrahepatic biliary system. Often they form a single mass [20, 25], but by intrahepatic metastasis multiple nodules can occur (Fig. 7). [20] Cells tend to be small, elongated or spindle-shaped [20, 34] and form cords [34], ribbons [20, 34] or rosettes [20, 34].

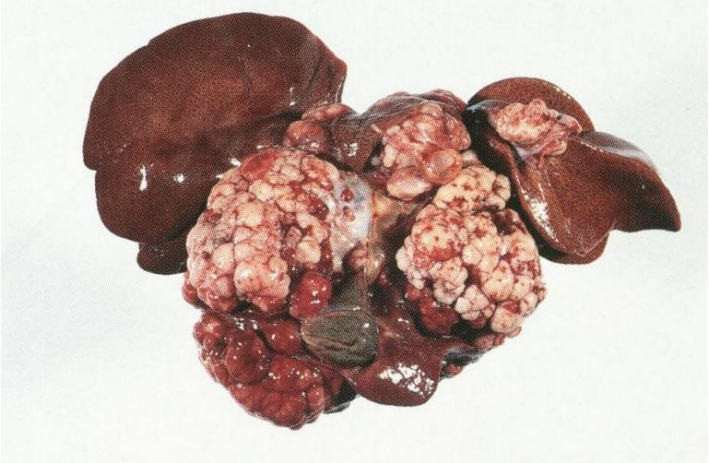

Fig. 7: Cat, Hepatic carcinoid [34]

Stage

A staging system describes in which state the cancer is and if the cancer is spread to other parts of the body. Staging is based on the knowledge of the way cancer develops. Neoplastic cells can invade into vascular structures or the lymphatic system. In this way the tumor can spread from the primary side to other organs in the body. This is called metastasis.

There are different kinds of classification systems of HCC. Three of these systems have been validated: the Cancer of the Liver Italian Program (CLIP) score, the Japan Integrated Staging (JIS) system and the Barcelona-Clinic Liver Cancer (BCLC) staging system.
The CLIP system looks to portal invasion, if there is more or less than 50% of liver area involved, to alfa-fetoprotein (AFP)(a protein than is produced by cancer cells in the liver, prostate or ovaries). For the liver function this system uses the child-pugh score (total bilirubin, serum albumin, ascites, hepathic encephalopathy and protrombin time) and alkaline phosphatase. The BCLS system includes parameters related to the liver functional status (Child-pugh, bilirubin and portal hypertension). It uses portal invasion, presence of metastasis, morphology and Okuda (more or less than 50% of liver area involved, albumin, bilirubin and ascites) to determine the stage of the tumor. It has been suggested that this classification is best suited for treatment guidance, and particularly to select early stage patients who could benefit from curative therapies. Another system, JIS, uses TNM to determine the tumor stage and the child-pugh method to determine the liver function. [32]
The Tumor Node Metastasis system (TNM) is one of the most commonly used systems. The T in this system represents the extent of the tumor, the N indicates the extent of spread to the lymph nodes and the M indicates the presence of metastasis. Each letter gets a number. [7]. These numbers provide more details about each of these factors. The T can be staged 0, 1, 2, 3a, 3b and 4: the higher the staging how more the extent of the tumor. The N and M can be steged 0 or 1. Zero means that the cancer did not spread to regional (N) or distant (M) lymph nodes or other organs (M). One means that the cancer has spread to regional (N) or distant (M) lymph nodes or other organs (M), The T, N, and M groups are then combined to give an overall stage, using Roman numerals I to IV [44]:

- Stage I: T1, N0, M0: There is a single tumor that hasn't spread to others parts of the body.
- Stage II: T2, N0, M0: There is a single tumor that grows into blood vessels or there is more than one tumor where no tumor is larger than 5 cm. across. There is no metastasis to other parts of the body.
- Stage IIIA: T3a, N0, M0: There is more than one tumor and at least one tumor has a diameter that is larger than 5 cm. The cancer has not spread to nearby lymph nodes or distant sites.
- Stage IIIB: T3b, N0, M0: At least one tumor is growing into a branch of the major veins of the liver (portal vein or hepatic vein). The cancer has not spread to nearby lymph nodes or distant sites.
- Stage IVA: Any T, N1, M0: The cancer has invaded nearby lymph nodes but has not spread to distant sites. Tumors in the liver can be any size or number and they may have grown into blood vessels or nearby organs.
- Stage IVB: Any T, Any N, M1: The cancer has spread to other parts of the body. (Tumors can be any size or number, and nearby lymph nodes may or may not be involved.)

The staging system used for this study is a summary of the TNM system. It is only based on the spread and invasion of the tumor. The tumors are staged in three categories instead of the original four, because stage II and III are combined to one stage*:*

- **Stage 0**: In this stage there is no indication for metastasis found. There is only one tumor process found in the liver. The tumor is well encapsulated or circumsribed.
- **Stage 1** : to be marked as a stage one tumor, the tumor has to meet at least one of the three following conditions:
    - Multiple tumor processes are present in the liver
    - Spread beyond the primary site to adjacent tissue/ bloodvessels (Fig. 8).

    - Microsatellites can be observed
- **Stage 2**: In this stage there was metastasis found in the lymph nodes or other organs of the body, this is also called distant metastasis

All the tumors used for this study are microscopically examined. This way the tumors are all placed in the staging category were they belong. Information received during the anamneses helped to determine if there was a metastasis found in the patient.

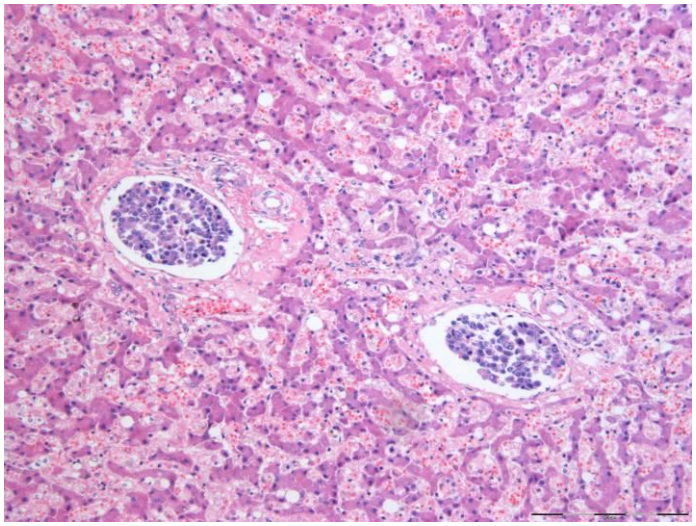

Fig. 8: Hepatocellular tumor with invasion of tumors cells in the portal veins

Grade

The histological grading system of tumors is based on the principle that those that look worse, based on the cell and nuclear pleiomorfism, number of mitosis and lack of differentiation, will grow faster.
During this study we used a grading system based on the classification of Edmondson and Steiner (ES differentiation grade). Edmondson and Steiner designed a grading system for hepatocellular carcinomas on a scale of 1 to 4, the higher the tumor is classified on the scale, the more nuclear irregularity and hyperchromatism is seen. The nuclear/cytoplasmic ratio, associated with decreasing cytological differentiation will be increased for each successively higher grade [7].
The system used in this study is a modified four category grading system based on:

- Anisocytosis (cell morphology)
- Anisokaryosis (nuclear morphology) and nucleolar morphology (Fig. 9A)
- presence of multinucleated tumor cells
- mitotic activity (Fig. 9B)

All the slides were scored for the cell, nuclear and nucleolar morphology, mitotic figures and presence or absence of multinucleated cells. The nucleolus is a basic organelle of the nucleus and is composed of RNA and protein. It function is the synthesis of rRNA, essential in protein synthesis [20]. For the cell and nuclear morphology the slides were scored on a scale with a range from zero to three. If the slides were scored at zero the cells and nuclei were uniform of size and form, if the slides were scored at three there was a lot of pleiomorfism in the cells and nuclei seen. All the tumors were also scored for the amount of mitotic figures. This was scored on a scale with a range from zero to three. If the slides were scored zero there were no mitotic figures present and if the slides were scored at three there were many mitotic figures present in the slides. The presence of multinucleated cells was not scored on a scale from zero to three, but if they were absent (0) or present (1). The tumor was placed in a group based on the sum of grades they get for the amount of anisocytosis, anisokaryosis, multinucleated cells and mitotic activity.

- Group 0: 0
- Group 1: 1-2
- Group 2: 3-4
- Group 3: 5-6

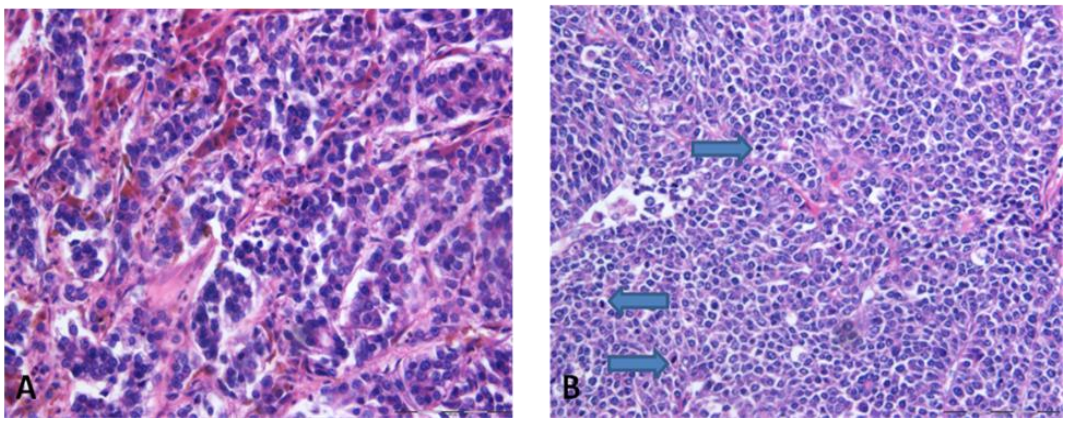

Fig. 9: In photo A there is cell and nuclei pleiomorfism seen in a hepatocellular tumor. In B there are different mitotic figures seen in a hepatocellular tumor, the arrows give some examples of mitotic figures.

Markers

Clinicopathological and prognostic markers

In man HCC is an aggressive cancer and has a bad prognosis if it is not detected in an early stage [1]. It is increasing in incidence worldwide [14]. For an effective treatment the tumor has to be detected when they are smaller than 3 cm [1]. The first step to diagnose a HCC is a screening. If there is a mass in the liver, it will be identified by radiological techniques.

In man the only potentially curative modality for HCC is surgical resection or a liver transplantation. The survival of patients can be quite different even if they had the same tumor stage and received the same treatment. This suggests that tumor stage based on clinicopathologic findings is not sufficient to differentiate HCC subtypes. [43]
Liver transplantation has been shown the best outcome for patients with early tumor stage but its application is limited by the shortage of donor livers. After hepatic resection prognosis remains unsatisfactory due to a high incidence of tumor recurrence. Selective internal radiation therapy is emerging as promising loco-regional treatment for patients with advanced HCC having good performance status and liver reserve but not amenable to surgery 10].

Prognostic markers for HCCs can help to determine the prognosis for a patient. Furthermore, they can help to diagnose the disease earlier. Nowadays staging and grading systems are used to determine the prognosis of a cancer patient. The histological assessment of a tumor will take some time and every pathologist examines the histological slide on his own way. With a prognostic marker, the interpretation will be a lot easier and faster.

Different kind of prognostic markers are investigated for HCC. One example is serum alpha fetoprotein (AFP). This is a good marker for the detection and monitoring of HCC. However, this is a marker with poor sensitivity and specificity. Even in patients with advanced HCC, the AFP levels may remain normal in 15%-30% of the patients. Other biomarkers, such as lens culinaris agglutinin-reactive AFP and des-gamma carboxyprothrombin, glypican-3, human hepatocyte growth factor, and insulin-like growth factor, have been proposed as markers for HCC detection. [38, 42]

Keratin 19 (K19) can be a prognostic marker for HCC. Tumors with K19 expression seems to be more aggressive than HCCs without K19 expression. These K19 expressing HCCs had a higher rate of recurrence after liver transplantation [31, 39]. The five year recurrence rate after resection is as high as 40-70% [15]. Other studies also linked increased K19 expression in HCC with a worse prognosis and faster recurrence after surgical treatment [39]. In another study they observed a significantly shorter survival in patients with HCC expressing K19 without any treatment [41]. These studies in humans validate K19 as a clinical important and prognostically relevant marker for hepatocellular carcinoma.

Keratin 19 (K19)

The cellular structure of cells is maintained by a major filament cytoskeletal protein network. The three major filaments are actin microfilaments, tubulin microtubules and tissue specific intermediate filament (IF) proteins. The majority of the IF protein group consist of keratins. [21] In an early stage of development the liver exist of hepatoblasts which express keratin (K)8, K18 and K19. Intrahepatic biliary epithelial cells develop from hepatoblasts surrounding portal veins [16]. Cholangiocytes express K7 and K19 [14]. Bipotential hepatic progenitor cells (HPCs) also express K7 and K19 [14]. During differentiation from hepatic progenitor cells to hepatocytes the K19 expression is lost and at the same time they acquiring further parenchymal cell markers. [16]. Normally, healthy hepatocytes express K8 and K18 [14, 27]. Keratins are also expressed in malignant epithelial cells [14].

K19 is normally seen in ductal epithelium [16], bile ducts [16], less-differentiated cells of the ductal tree in the pancreas [4] and renal collecting tubules [16], and in the mucosa of the gastrointestinal tract [16].
Keratins have different functions in the cell:

- Provide mechanical strength
- Participate in response to stress
- Cell signaling
- Participate in apoptosis by playing a role in movement of organelles and substrates within cells

K19 expression in the liver is a marker of biliary differentiation and progenitor cell phenotype and is also a promising prognostic marker for HCC. [16]

Hepatocyte paraffin 1 (HepPar-1)

Hepatocyte paraffin 1 is a monoclonal antibody that recognized a component of the membrane of hepatocellular mitochondria. This epitope appears not present in mitochondria of other normal tissue. HepPar-1 can be used for immunohistochemistry on formalin-fixed/paraffin-embedded tissue. [19] The staining shows a distinctly granular, occasionally ring like, cytoplasmatic staining of hepatocytes. [40] HepPar-1 expression is diffusely seen throughout the hepatocyte cytoplasm [19, 40] and is often only focally present is poorly differentiated tumors [19]. Non-parenchymal liver cells and bile ducts will not be stained by HepPar-1. [40] This marker can be used to distinguish different tumors, like hepatocellular carcinomas, cholangiocellular carcinomas, hepatic carcinoids and other kinds of tumors in the liver [19].

Differentiation markers

There are different types of neoplastic disorders which can develop in the liver. Sometimes it is very difficult on histological basis to distinguish these different neoplastic disorders. To categorize tumors in the group were they belong differentiation markers can be used. Chromogranin-A and Neuron-specific Enolase can help to distinguish hepatocellular carcinomas from hepatic carcinoids.

Chromogranin-A (CgA)

Chromogranin A is a member of the granin family, which constitutes of three members: Chromogranin A, Chromogranin B and Chromogranin C [2]. It is a water soluble glycoprotein [5] that is present in the large dense core vesicles (LDCV) of neuroendocrine tissue [5, 26]. CgA can be used as a biomarker in immunohistochemistry of neuroendocrine tumors. [2, 5, 26].

Neuron-specific Enolase (NSE)

Neuron-specific Enolase is a glycolytic enzyme that is produced by neuronal and neuroendocrine cells. NSE is a neuronal isomer of the glycolytic enzyme enolase, This enzyme is seen in all organs, except in striated muscles, in three dimeric isoenzymes: αα, αт and тт. The тт form is the form present in neuronal tissue, where it responds to neuron-specific protein. [11]
NSE is not secreted unlike chromogranin A. The presence of NSE in blood is thought to be related to a high cell death of neuroendocrine cells [2]. NSE can be used as a biomarker in immunohistochemistry for neuroendocrine tumors [2, 11]

Keratin 19 and Hepatocellular carcinomas

Cells of origin

Stem cells are functionally defined as self-renewing and multipotent cells. If they are more differentiated, they will be called progenitor cells. Hepatic progenitor cells (HPCs) are bipotential stem cells residing in human and animal livers that are able to differentiate towards the hepatocytic or cholangiocytic lineages. [12, 31] In healthy liver tissue HPCs are quiescent. HPCs are found in the most peripheral branches of the biliary tree, the ductules and the canal of Hering. (Fig. 10) [31, 33] They are activated in the majority of liver diseases when hepatocytes or cholangiocytes are damaged or inhibited in their replication. [39] Since they are present during liver diseases, they might also be prone to carcinogenesis.

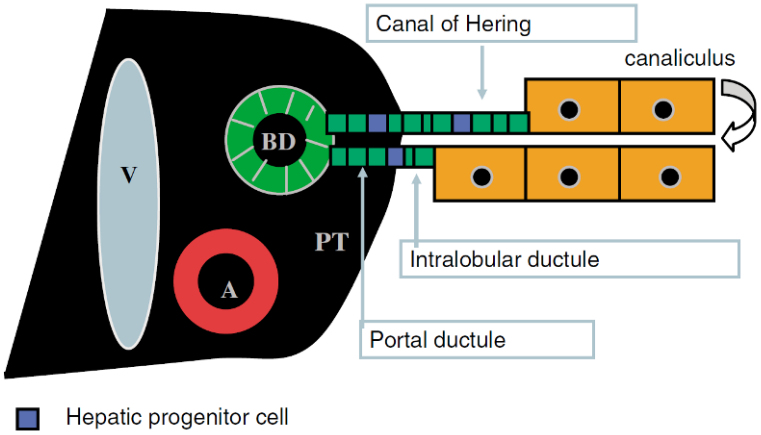

Fig. 10: Localization of progenitor cells in the liver. The left part illustrates the portal tract containing a portal vein (V), a hepatic artery (A) and a bile duct (BD). HPCs are found in the most peripheral branches of the biliary tree, the ductules and the canal of Hering. [33]

It is still unclear which cell can be considered as the origin of HCC. There are three kinds of cells in the liver that live long enough to be a possible cell of origin of a cancer: hepatocytes, hepatic progenitor cells and cholangiocytes [33]. There are different observations to propose that hepatocellular carcinomas arise from hepatic progenitor cells. If hepatocellular carcinomas developed from hepatocytes and the keratin pattern was invariable during neoplastic transformation, HCC would only express K8 and K18. Some HCC express K7 and K19 which are considered HPC as well as cholangiocyte markers. [14, 21, 31, 39]. K7 positive and K19 positive neoplastic cells often show a morphological similarity to non-neoplastic HPCs and intermediate cells [14]. Also, there are animal models of carcinogenesis were it is been shown that HPCs are the cells of origin of HCC [6, 13, 31].

The presence of cells expressing K7 and/or K19 in HCC might be the result of dedifferentiation of mature hepatocytes to a progenitor cell/biliary phenotype (dedifferentiation theory) or of a maturation arrest of progenitor cells during the carcinogenic process (maturation arrest theory). (Fig. 11) HPCs are a potential cell of origin for a percentage of hepatocellular carcinomas. [14, 21 , 31, 33]. Although there is no definitive proof of the maturation arrest or dedifferentiation theory, it is clear that HCCs with progenitor cell features are highly malignant and warrant extensive research.

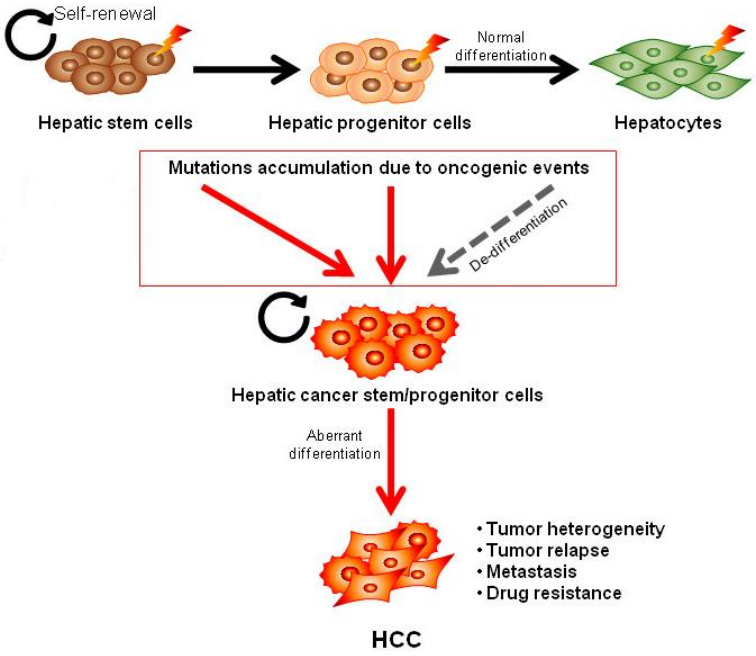

Fig. 11: Maturation arrest theory: the maturation of progenitor cells will stop so they can give rise to a tumor.
Dedifferentiation theory: mature hepatocytes dedifferentiated to hepatic progenitor cells that give rise to a tumor. [18]

Aim of the study

This study is a sequel to the study of Renee van Sprundel performed in 2008. She concluded in her study that the prevalence of Keratin 19 in hepatocellular carcinomas was 12 percent in the dog. She also found that K19 positive hepatocellular tumors were more malignant than K19 negative tumors. The K19 positive tumors were poorly differentiated and showed the presence of vascular invasion and metastasis. [39]

The aim of this study is to get to know more about the prevalence of Keratin 19 positive hepatocellular tumors in dogs and cats in a European cohort study with a large validation group of tumors. Histology (staging and grading) of the tumors will be assessed to get more understanding of the malignancy of K19 positive tumors.
The hypotheses of this study are:

- The prevalence of keratin 19 positivity in all hepatocellular tumors is between the 10 and 20 percent
- Keratin 19 positive tumors have a more malignant nature compared to keratin 19 negative tumors.

Materials and methods

Samples

In this study liver paraffin material of 96 dog patients and 20 cat patients with primary liver tumors was used. They were collected from Valuepath, Laboratory of pathology, Hoensbroek, The Netherlands (dog n=22, cat n=6), department pathobiology, Faculty of Veterinary Medicine, Utrecht University (dog n=20, cat n=9), Zurich University, (dog n=35) and Berlin University (dog n=12, cat n=5). In addition frozen material (dog n=7) was available from the tissue bank present at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University.

Healthy paraffin liver material was available from the department of clinical sciences of companion animals, Faculty of Veterinary medicine, Utrecht University.
All liver samples were routinely embedded in paraffin. The paraffin sections (5 µm) were mounted on poly-L lysine coated slides and silane coated slides.

Immunohistochemistry for K19, NSE, CgA and HepPar-1 was performed on all tumor samples. All samples were also stained with haemotoxylin and Eosin (H&E) for histological determination.

Immunohistochemistry

Immunohistochemistry is used for detecting a protein in tissue sections. A specific antibody is coupled to an enzyme that converts a colorless substrate into a colored reaction product. The antibody binds stably to its antigen preventing the antibody from being washed away. [17]

There are different methods for immunohistochemistry. The oldest technique is the direct method (Fig. 12). In this method an enzyme-labeled primary antibody reacts with the antigen in the tissue.[3]

The method used in this study is the two-step indirect method (Fig. 13). In this method a primary antibody first binds to the antigen. An enzyme-labeled secondary antibody directed against the first antibody, now the antigen, is then added. After this step a substrate-chromogen solution will be added. This procedure is more sensitive than the direct method, because the signal is amplified. This is because of several secondary antibodies are likely to react with a number of different epitopes of the primary antibody, thus more enzyme molecules are attached per each target site. [3]

Another method is the three-step indirect method (Fig 14). In this method there is a second enzyme-conjugated antibody added to the two-step indirect method. This third antibody uses to amplify the signal more. This is helpful when the antigen that is stained has a limited number of epitopes. [3]

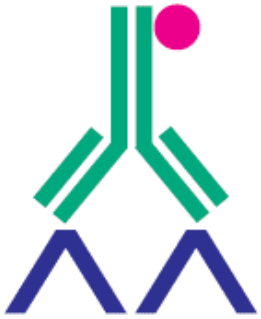

Fig. 12: direct method [3]

Fig. 13: Two step indirect method [3]

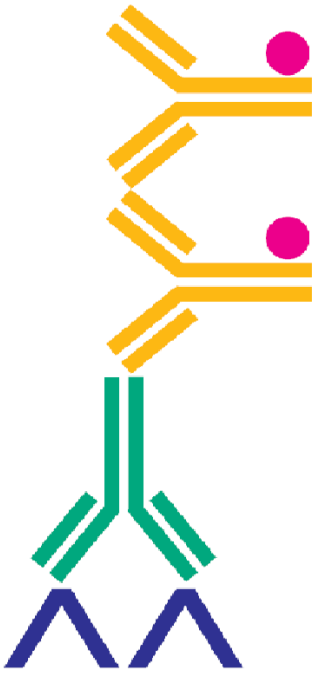

Fig. 14: Three step indirect method [3]

For IHC tissue has to be fixed on a slide. This fixation can have a negative impact on the IHC detection. Fixation can alter the protein biochemistry such that epitopes of interest are masked and can no longer bind to the primary antibody. Antigen retrieval is the technique used to reverse this and epitope-antibody binding is restored.
Antigen Retrieval can be achieved in two ways: heat-induced-epitope-retrieval (HIER) and protease-epitope-induced-retrieval (PIER). In PIER the mechanism of action is thought to be the cleavage of peptides that may be masking the epitope. In HIER the slides are put in heat

in an aqueous medium for a period of time. Some of the bridges formed by the formalin fixation are reversible (Schiff bases), while others are not (methylene bridges). The reversible cross bridges will be restored. In this way the integrity of the protein will be restored and so the epitope- antibody binding.
In most of the time heat-induced epitope retrieval has been more successful than protease-epitope-induced-retrieval. [3, 36]

Immunohistochemistry methods

Most of the steps in the protocols for K19, NSE, CgA and HepPar-1 are the same. A standard protocol is depicted in table 1.The steps that differ between the different markers will be explained for each marker.

Step	Act	Time
1	Deparaffinize and rehydrate sections in a series of: Xylene: 2x5 ; Alc. 96% 2x5; Alc. 80% ; Alc. 70%; Alc. 60%; Alc. 30%; MQ 5'	Each step for 5 min.
2	Antigen retrieval	
3	Rinse in **PBS/T** or **TBS/T** buffer solution	2x2 min
4	Inhibit endogenous peroxidase activity by incubating the slides in Dako Ready to use peroxidase block	10 min, RT
5	Rinse in **PBS/T** or **TBS/T** buffer solution	3x5 min.
6	Incubate in 10 percent normal goat serum	30 min.
7	Incubate with antibody (in ab-diluent, DAKO)	
8	Rinse in **PBS/T** or **TBS/T** buffer solution	3x5 min.
9	Incubate in Envision Goat anti Mouse/rabbit HRP	45 min.
10	Rinse in **PBS** or **TBS** buffer solution	3x5 min.
11	Incubate the sections in freshly made DAB substrate (result: brown)	5 min.
12	Rinse the sections in mQ	3x5 min.
13	Counterstain the sections haematoxylin QS-Dako	10 sec.
14	Rinse sections in running tapwater	10 min.
15	Dehydrate section and cover in vectamount: 30% Alc; 70% Alc; 96% Alc 2x5; 100% Alc; Xylene 2x5	Each step for 5 min.

Table 1: general protocol for immunohistochemical staining

Keratin 19

For the staining of K19 Proteinase K (DAKO) is used to perform antigen retrieval. This will be used for 15 minutes. Then it is tapped of and washed for 2x2 minutes. For washing these slides TBS/T or TBS is used. The antibody used is Keratin 19, manufacturer: Novocastra Laboratories Ltd, type: mouse monoclonal, clone: B170. (Table 2)

Step	Act	Time
2	Proteinase K	15 min.
3,5,8, 10	Use TBS/T or TBS to rinse	
7	Incubate with antibody (in ab-diluent, DAKO), Dilution: 1:100	1 h, RT

Table 2: steps in the K19 protocol that differs from other markers

Chromogranin-A

For the staining of CgA there is no antigen retrieval step necessary. After deparaffinisation the slides will be washed for 2x2 minutes. PBS/T or PBS can be used for this. The antibody used is chromogranin-A, manufactured: MP Products, type: rabbit monoclonal, clone: 20086 SP-1. (Table 3)

Step	Act	Time
2	-	-
3,5,8, 10	Use PBS/T or PBS to rinse	
7	Incubate with antibody (in ab-diluent, DAKO) Dilution: 1:800	4^{o}C O/N

Table 3: steps in the CgA protocol that differs from other markers

Neuron-Specific Enolase

Antigen retrieval by NSE staining is achieved by a hot citrate bath for 40 minutes at 98 degrees. After 40 minutes the slides will come out of the water bath, but still in the hot buffer to cool down for 30 minutes. The buffer used to rinse the slides is PBS/T or PBS. The antibody Neuron-Specific Enolase is manufactured by Dakocytomation, type mouse monoclonal, clone: BBS/NC/VI-H14. (Table 4)

Step	Act	Time
2	Antigen retrieval in 10 mM hot citrate buffer pH 6.0 Cool down at room temperature (still in hot buffer outside water bath)	40 min. 98^{o}C 30 min.
3,5,8, 10	Use PBS/T or PBS to rinse	
7	Incubate with antibody (in ab-diluent, DAKO) Dilution 1:400	4^{o}C O/N

Table 4: steps in the NSE protocol that differs from other markers

HepPar-1

Antigen retrieval by HepPar-1 staining is achieved by a hot Tris-EDTA (TE) bath for 40 minutes at 98 degrees. After 40 minutes the slides will cool down in the TE buffer outside the water bath for 30 minutes. The buffer used to rinse the slides is PBS or PBS/T. The antibody HepPar-1 is manufactured by Dakocytomation, type: mouse monoclonal, clone: OCH1E5. (Table 5)

Step	Act	Time
2	Antigen retrieval in 10 mM hot TE buffer pH 9.0 Cool down at room temperature (still in hot buffer outside water bath)	40 min. 98^{o}C 30 min.
3,5,8, 10	Use PBS/T or PBS to rinse	
7	Incubate with antibody (in ab-diluent, DAKO) Dilution: 1:50	4^{o}C O/N

Table 5: steps in the HepPar-1 protocol that differs from other markers

Results

In this study liver material of 96 dogs and 20 cats was used. Of the dogs, 39 were male, 44 were female and from 13 dogs the gender was not provided. Of the cats, 9 were male, 10 were female and of one cat the gender was not provided. The age range of the dogs was between 6 to 18 years. For the cats it was between 2 to 20 years. The dogs were of different kinds of breeds: Mixed, Labrador, Munsterlander, Setter, Appenseller Senner, (mini) Schnauzer, Viszla, Airedale terrier, Englisch Cocker Spaniel, Eurasier, (Shorthair) Teckel, Yorkshire Terrier, German Shepherd, West Highland White, Hovawart, Rottweiler, Fox Terrier,New Foundland, Golden Retriever, Basset hound, Silky terrier, Berger des Pyrenee, Jack Russel Terrier, Flat coated retriever, Alaskan Malumute, Maltheser and Pit bull. For the cats the following breeds were included: mixed, Persian, Siamese and European Shorthair.

The liver tumors were microscopically examined and classified as followed:

- Nodular hyperplasia (dog:13 (14%), cat:1(5%))
- Hepatocellular tumors (dog: 70 (73%), cat: 8 (40%))
- Cholangiocellular carcinoma (dog: 7(7%), cat: 4 (20%))
- Hepatic carcinoid (dog: 6 (6%), cat:7 (35%))

All the hepatocellular tumors were classified in different groups based on the K19 positivity. After the classification there were four groups:

- Nodular hyperplasia (group A) (dog n=13, cat n=1)
- Hepatocellular tumor negative for K19 (group B) (dog n=44, cat n=5)
- Hepatocellular tumor with 5-10 % positivity for K19 (group C) (dog n=12, cat n=3)
- Hepatocellular tumor with more than 30% positivity for K19 (group D) (dog n=14)

There were different kinds of paraffin blocks collected that were not included in this study. From the dog samples this was 17 blocks. 15 blocks did not contain primary liver tumors. These blocks contained metastasized tumors to the liver or no liver tissue at all. Two blocks were autolytic or necrotic and were difficult to assess.
11 paraffin blocks of cat tissue were not included in this study. For 10 of these blocks the pathologist had some difficulties to interpret the slides, because the staining for NSE was positive in almost all samples. This staining has to be repeated. It was also difficult to determine if these slides were primary liver tumors or not. One of the blocks only contained normal lung tissue.

In the following sections the results on all groups of primary liver tumors (group A to D for hepatocellular carcinomas as well as cholanchiocellular carcinomas as hepatic carcinoids) are described in full.

Primary Liver tumors

Group A: Nodular hyperplasia

Histologically, the hepatocytes are well-differentiated. The cells and cell nuclei are all of the same size, so there is no pleiomorfism seen in the tumors. There are no mitotic figures seen in the nuclei of the neoplastic hepatocytes. No multinucleated hepatocytes were present.

Nodular hyperplasia	K19 expression	Grading	Staging	HepPar -1 expression	NSE expression	CgA expression
Healthy liver	0%	0	0	100%	0%	0%
Dog (n=13)	0%	0 (n=12) 1 (n=1)	0	100%	0%	0%
Cat (n=1)	0%	0	0	100%	0%	0%

Table 6: Nodular hyperplasia and the compared with histological, clinicopathological and neuroendocrine markers

The K19, Neuron-Specific Enolase and Chromogranin A expression is negative in all nodular hyperplasia samples. All but one of the nodular hyperplasias were classified in group zero of the grading and staging system. This is because one nodular hyperplasia found in the dog had some mitotic figures in the neoplastic hepatocytes. This neoplasm was therefore classified as a grade one tumor. In all nodular hyperplasias was the HepPar-1 expression 100%. Nodular hyperplasias have the same characteristics as healthy liver. (Table 6)

Group B: Hepatocellular tumors K19 negative

Histologically, this tumors formed well-differentiated hepatocytes. In the dog the cells and nuclei were uniform from size, so almost no pleiomorfism was seen in these hepatocellular tumors. In the cat there is more pleiomorfism seen. Sometimes there is a mitotic figure present, but in most of the hepatocellular tumors mitotic figures were absent. Multinucleated cells are very rare seen in the hepatocellular tumors without K19 expression. (Example provided in Fig. 15A)

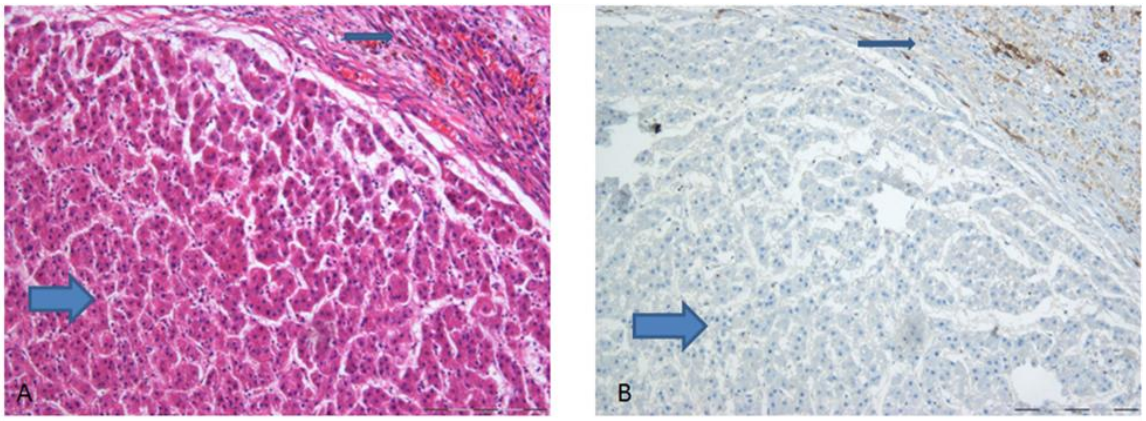

Fig. 15: ***Hepatocellular adenoma****. The histological structure of a hepatocellular tumor, in this case a hepatocellular adenoma, without K19 expression is seen in A. There is no K19 expression in this kind of tumor seen in B. On both photos there is a transition from neoplastic to non-neoplastic tissue in the upper right corner*

Hepatocelllar tumor group B	K19 expression	Grading	Staging	HepPar-1 expression	NSE expression	CgA expression
healthy	0%	0	0	100%	0%	0%
Dog (n=44)	0%	0 (n= 7) 1(n= 27) 2(n= 10)	0 (n=42) 1 (n= 1) 2 (n= 1)	100% (n= 42) 60 (n= 1) <5% (n=1)	0% (n= 39) 5% (n=2) 10-20% (n=2) 50% (n=1)	0%
Cat (n=5)	0%	1 (n= 2) 2 (n= 2) 3 (n=1)	0	100%	0%	0%

Table 7: Hepatocellular tumors with 0% positivity for K19 in the dog and the cat compared with histological, clinicopathological and neuroendocrine markers

Expression of K19 in 0% of the tumor cells was seen in 44 of the 70 hepatocellular tumors in the dog and 5 of 8 hepatocellular tumors in the cat. This is 63% in the dog and 62% in the cat. (Example provided in Fig. 15B)
In the dog none of these tumors are categorized in the most malignant group of the grading system (group 3). There are 7 tumors categorized in group zero, 27 in group 1 and 10 in group two. All the tumors in dogs and cats, except two tumors, are categorized in group zero from the staging system. This means that there is only one single mass and there is no metastasis found in other tissue or in the liver itself. In the cat there is more pleiomorfism seen than in the dog. In the cat one tumor is graded is the highest group of the grading system, but is staged in the lowest group of the staging system. The grading and staging system the cat are not corresponding to each other, because the grading system is classified in grade 1, 2 and 3. The staging system is staged in group 0. This means that there is no metastasis seen in the liver or in other parts of the body. Than you would expect that the staging of these tumors will be in the lower groups of the grading system. (Table 7)

All the tumors in this group are strongly positive for HepPar-1. All tumors are positive for 100%, except two tumors in the dog. In one of these tumors the HepPar-1 positivity is 60%, but in the other tumor it is below 5%. (Table 7)

In the dog, five tumors are positive for Neuron-Specific Enolase. These tumors are not categorized as a hepatic carcinoid. There is no positivity found in al the hepatocellular tumors in both dog and cat for Chromogranin-A. (Table 7)

Group C: Hepatocellular tumor 5-10% K19 positive

Hepatocelllar tumor group C	K19 expression	grading	Staging	HepPar-1 expression	NSE expression	CgA expression
healthy	0%	0	0	100%	0%	0%
Dog (n=12)	5-10%	0(n=2) 1(n= 8) 2(n= 2)	0	50% (n=1) 90-100% (n=11)	0% (n=10) <5% (n=2)	0% (n=11) 5% (n=1)
Cat (n=3)	5-10%	2 (n= 2) 3 (n=1)	0	90-100%	0%	0%

Table 8: Hepatocellular tumors with 5-10% positivity for K19 in the dog and the cat compared with histological, clinicopathological and neuroendocrine markers

Expression of K19 in 5-10% in the tumor cells is observed in 12 of the 70 hepatocellular tumors in the dog (17%). In the cat 3 out of 8 hepatocellular tumors have 5-10% positivity found for K19 (38%). (Table 8)

All the tumors show a strong positivity for HepPar-1. One tumor in the dog has a positivity of 50%. The other 14 tumors are 90-100% positive for HepPar-1. (Table 8)

The tumors differ in histology. Tumors are categorized in group zero to two in the grading system in dogs. In cats they are categorized in group two and three of the grading system. In

cats the pleiomorphy varies more than in dogs. All the tumors are categorized in group zero of the staging system. (Table 8)

Two of the hepatocellular tumors in the dog is less than 5% positive for Neuron-Specific Enolase. One of the hepatocellular tumors in the dog is 5 % positive for Chromogranin-A. All the hepatocellular tumors from group C in the cat are negative for Neuron-Specific Enolase and Chromogranin-A. (Table 8)

Group D: Hepatocellular tumor >30% K19 positive

Histologically, these tumors are poorly differentiated. The cells and nuclei in the neoplastic hepatocytes differ in shape and size. The uniformity disappeared in the tumors. There was much cell pleiomorfism. Some multinucleated cells can be seen in this kind of tumors. The mitotic activity was very high. (Example provided in Fig. 16A and 16B)

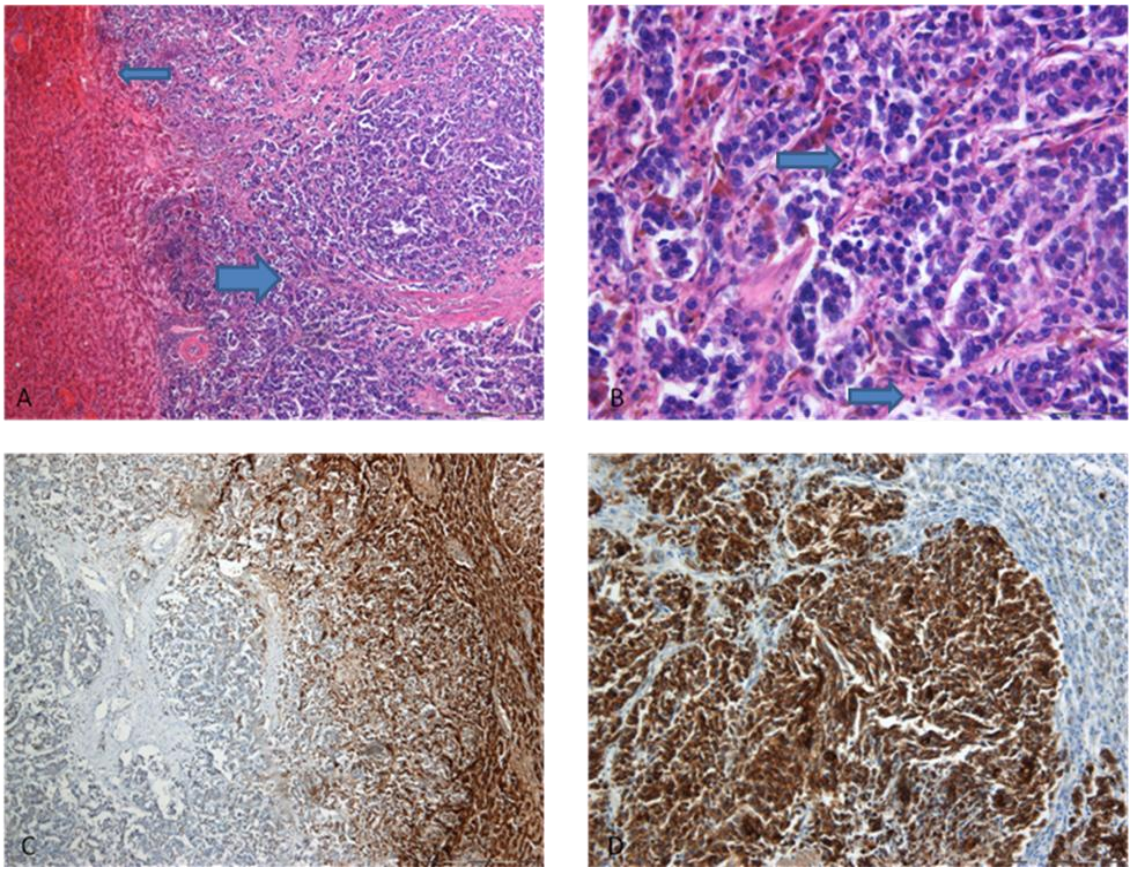

Fig. 16: ***Hepatocellular carcinoma****. Histological structure of a hepatocellular carcinoma in a dog is shown in A. On the right side of this picture neoplastic tissue is seen. On the left side there is a transition to non-neoplastic tissue. Cell and nuclei pleiomorfism and mitotic figures can be seen in B. Negativity for HepPar-1 staining in a hepatocellular carcinoma in the dog is seen in C. 100% positivity for K19 in a hepatocellular carcinoma in the dog is seen in D*

In the table there is no data shown of the cat. This is because we found no hepatocellular tumors in the cat with a K19 expression above 10%.

Hepatocelllar tumor group D	K19 expression	grading	Staging	HepPar-1 expression	NSE expression	CgA expression
healthy	0%	0	0	100%	0%	0%
Dog (n=14)	30-100%	1 (n=3) 2 (n=4) 3 (n= 7)	1 and 2	0% (n=11) 50-80% (n=3)	0%	0% (n= 12) 30% (n= 2)

Table 9: Hepatocellular tumors with >30% positivity for K19 in the dog compared with histological, clinicopathological and neuroendocrine markers

Expression of K19 in 30-100% of the neoplastic cells was found in 14 of the 70 hepatocellular tumors (20%) (Example provided in Fig. 16D). Seven of these tumors were categorized in the most malignant group of the grading system (group three). Four were grouped in group two of the grading system and the other three tumors were categorized in group one of the grading system. All of these tumors are staged in group one or two. This means that these tumors had spread in the liver or to other organs in the body. This shows the malignant character of K19 positive tumors. (Table 9)

HepPar-1 was negative in 11 of the 14 tumors (Example provided in Fig. 16C). One tumor was 50% positive, one was 70% positive and the last one was 80% positive for HepPar-1. (Table 9)

In these tumors there is a strong correlation between the grading and staging system and K19 expression. There is a negative correlation between the HepPar-1 expression and K19 expression. (Table 9)

In two of the 14 hepatocellular tumors with more than 30% K19 expression is 30% positivity seen for Chromogranin-A. All the tumors of this group were negative for Neuron-Specific Enolase. (Table 9)

Cholangiocellular carcinoma

Histologically, there are a lot of mitotic figures seen in this kind of tumor, so the mitotic activity is very high. The cells and nuclei are not uniform in size. There is a lot of cell pleiomorfism seen in cholangiocellular carcinomas. There are no multinucleated cells. (Example provided in Fig. 17A)

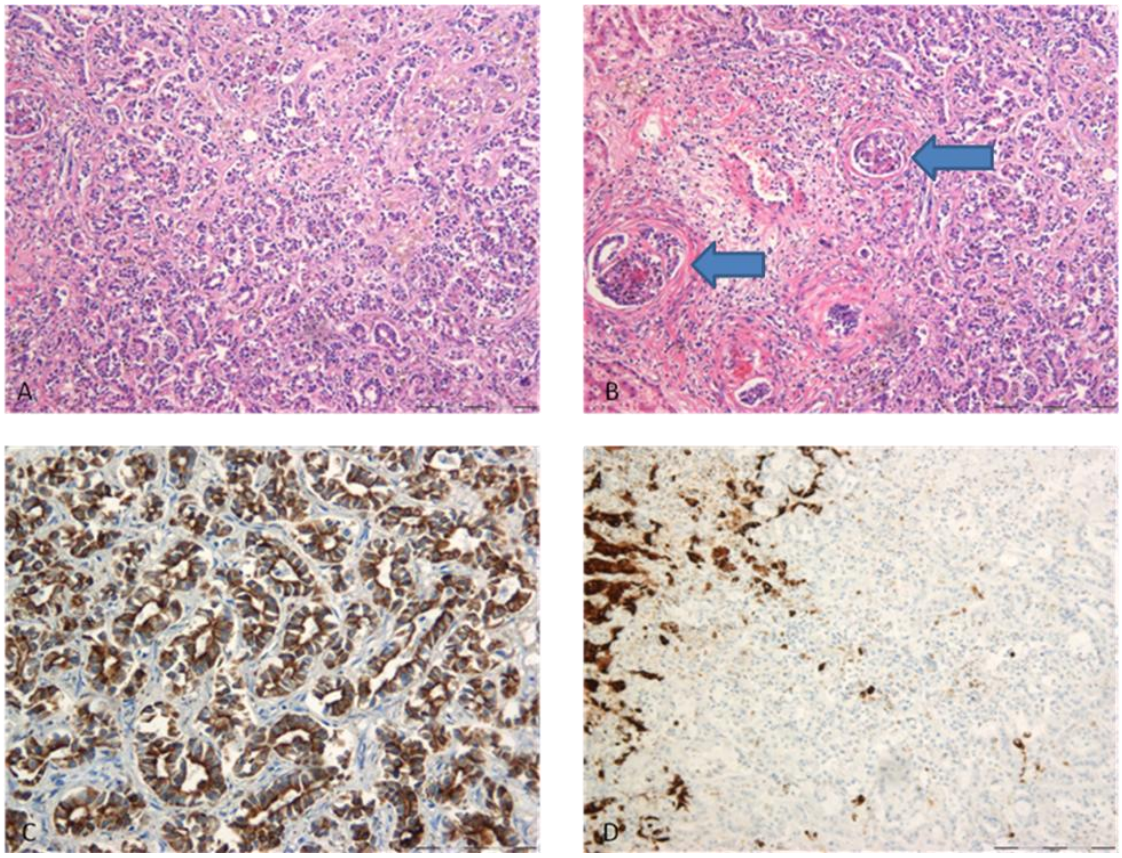

*Fig. 17: **Cholangiocellular carcinomas**. Histological structure of a cholangiocellular carcinoma in the dog is shown in A. The malignant character of cholangiocellular tumors showed with metastatic properties in the dog shown in B. 100% K19 positive cells in a cholangiocellular carcinoma in the dog shown in C. HepPar-1 negative cells in a cholangiocellular carcinoma in the cat shown in D.*

The cholangiocellular carcinomas are not distributed in groups, because of their limited number in this study. The cholangiocellular carcinomas will be shown separately in the tables.

Cholangiocellular carcinoma	K19 expression	Grading	Staging	HepPar-1 expression	NSE expression	CgA expression
1	90%	3	2	0%	0%	0%
2	100%	3	2	0%	0%	0%
3	100%	3	2	0%	0%	0%
4	100%	2	2	0%	0%	0%
5	100%	2	1	0%	0%	0%
6	60%	2	1	0%	0%	0%
7	100%	2	1	0%	100%	0%

Table 10: Cholangiocellular carcinomas in the dog compared with histological, clinicopathological and neuroendocrine markers

Cholangiocellular carcinoma	K19 expression	Grading	Staging	HepPar-1 expression	NSE expression	CgA expression
1	100%	3	1	0%	0%	0%
2	100%	3	1	0%	<1%	0%
3	100%	2	1	0%	20%	0%
4	100%	2	1	0%	100%	0%

Table 11: Cholangiocellular carcinomas in the cat compared with histological, clinicopathological and neuroendocrine markers

All cholangiocellular neoplasms show positivity for K19. In the cat all the cholangiocellular tumors are 100% positive for K19. In the dog two of the seven tumors are not 100% positive for K19. One tumor shows 90% positivity and the other tumor shows 60% positivity. The rest of the cholangiocellular carcinoma's are 100% positive for K19. (Example provided in Fig. 17C) (Table 10 and Table 11)

Grading: Three cholangiocellular carcinomas of the dog and two cholangiocellular carcinomas of the cat are categorized in group three of the grading system. The other four tumors in the dog and the other two in the cat are categorized in group two.
Staging: All cholangiocellular tumors in the cat were grouped in group one of the staging system. In the dog there are four cholangiocellular tumors categorized in group two and three tumors are categorized in group one of the staging system. This shows the high malignant characteristics of cholangiocellular tumors. (Example provided in Fig. 17B) (Table 10 and Table 11)

In one cholangiocellular tumor of the dog 100% positivity of the neoplastic cells for Neuron-Specific Enolase is found. In the cat there are three tumors with Neuron-Specific Enolase positivity. One is for 100% positive, the other is for 20% positive and the last one is less than 1% positive. (Table 10 and Table 11)

No expression of HepPar-1 (Example provided in Fig. 17D) and Chromogranin-A is seen in the neoplastic cells of the cholangiocellular tumors. (Table 10 and Table 11)

Carcinoid

In generally, cells in carcinoids tend to be small, elongated or spindle-shaped [20, 34]. There are three different patterns which the tumor cells can form:

- Cords [34],
- ribbons [20, 34]
- rosettes [20, 34]

In this study these three patterns were found in carcinoids. (Example provided in Fig. 18A, 18B and 18D)

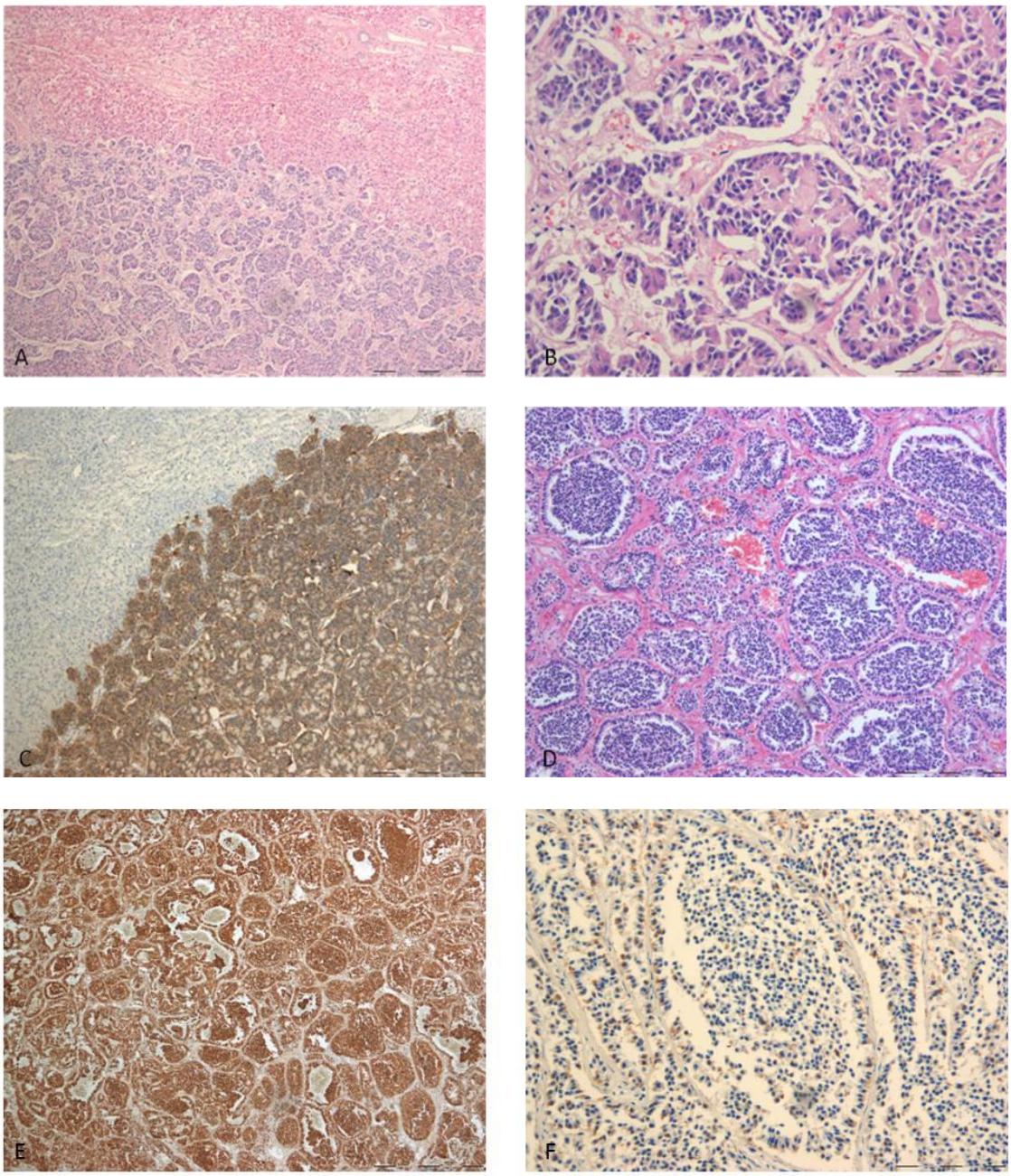

Fig. 18: ***Carcinoid****. Histological structure of a carcinoid in the dog is shown in A and B.100% positivity for Neuron-Specific Enolase staining in the dog is shown in C. The histological structure of a carcinoid in the cat is shown in D. 100% positivity for Neuron-Specific Enolase staining in the cat is shown in E. Chromogranin A positivity in a carcinoid in the cat is shown in F.*

The hepatic carcinoids are not distributed in groups, because of their limited number in this study. The hepatic carcinoids will be shown separately in the tables. In the tables the mitotic index will be shown. This is integrated in the grading system, but in carcinoids the cells are uniform. This means that these tumors could be categorized in grade zero although carcinoids are high malignant.

carcinoids	K19 expression	Mitotic index	Grading	Staging	HepPar-1 expression	NSE expression	CgA expression
1	5%	3	1	1	80%	>90%	30%
2	5%	2	0	1	100%	90%	30%
3	0%	1	1	1	0%	100%	0%
4	5%	1	1	1	0%	100%	40%
5	100%	3	3	1	0%	30%	5%
6	100%	3	3	1	80%	40%	0%

Table 12: Hepatic carcinoids in the dog compared with histological, clinicopathological and neuroendocrine markers

carcinoids	K19 expression	Mitotic index	Grading	Staging	HepPar-1 expression	NSE expression	CgA expression
1	0%	1	1	2	0%	100%	70%
2	40%	2	2	1	0%	80%	40%
3	0%	3	1	2	0%	100%	0%
4	0%	3	1	2	0%	>90%	0%
5	0%	3	1	1	0%	>90%	>90%
6	50%	2	1	2	0%	>90%	<5%
7	>90%	3	1	2	0%	>90%	0%

Table 13: Hepatic carcinoids in the cat compared with histological, clinicopathological and neuroendocrine markers

All of the carcinoids are positive for Neuron-Specific Enolase (Example provided in Fig. 18C and 18E). 11 of the 13 carcinoids in both the cat and the dog are more than 80% positive for NSE. The other two carcinoids are 30% respectively 40% positive for Neuron-Specific Enolase. In some of the hepatic carcinoids is also positivity seen for Chromogranin-A (Example provided in Fig. 18F). In the dog and the cat there is one carcinoid 100% positive for Neuron-Specific Enolase, but no positivity for Chromogranin A. In the dog one carcinoid is 40% positive for Neuron-Specific Enolase, but it shows no positivity for Chromogranin-A. There are two other carcinoids in the cat that are more than 90% positive for Neuron-Specific Enolase that show no positivity for Chromogranin-A. (Table 12 and Table 13)

In the cat there are no hepatic carcinoids positive for the HepPar-1 marker. Remarkably, in the dog there are three hepatic carcinoids positive for the HepPar-1 marker. Two show an 80% positivity and the other one shows 90% positivity for HepPar-1. (Table 12 and table 13)

In the dog, 5% positivity for K19 is seen in three of the four hepatic carcinoids .These K19 positive tumors are grouped in group 1 of the grading and staging system. In the cat three of the seven carcinoids have K19 positivity for more than 40%. In one carcinoid there is more than 90% positivity seen in the neoplastic cells. These tumors are grouped in group 1 of the grading system en group 2 of the staging system. The carcinoids that show no expression for K19 are also categorized in group 2 of the staging system, so there is no correlation between K19 expression and the grading of hepatic carcinoids. (Table 12 and Table 13)

The grading system for both dogs and cats is categorized from zero to three. One carcinoid in the dog is categorized as a grade zero and two as a grade three. In the cat one carcinoid is categorized as a grade two. The other nine neoplasms are grouped in group 1 of the grading system. The mitotic index both in the dog and cat varies from one to three. In the cat there are four carcinoids that scored a scale three in mitotic figures. These tumors are graded in group one of the grading system. This shows the discrepancy between the pleiomorfism and the mitotic index in carcinoids. (Table 12 and Table 13)

The hepatic carcinoids in the dogs are all categorized in group 1 of the staging system. In the cat there is one carcinoid of stage 1 and the other eight are stage 2 carcinoids. This means that in the cat eight of the nine hepatic carcinoids have metastasized to other parts of the body. The other carcinoids in both dogs and cats are grouped in group 1 of the staging system. This still shows the malignancy of hepatic carcinoids. (Table 12 and Table 13)

Conclusion

In this study we examined two hypotheses. These hypotheses were:

- The prevalence of keratin 19 positivity in all hepatocellular tumors is between the 10 and 20 percent
- Keratin 19 positive tumors have a more malignant nature compared to keratin 19 negative tumors.

From this study we can conclude that the prevalence of keratin 19 positivity in more than 30% of the neoplastic cells in hepatocellular tumors is 20 percent. This number falls in range of the first hypotheses. So from this data we can assume that the first hypothesis holds true.

The biggest part of the K19 positive hepatocellular tumors is graded in the most malignant group (group three) and all K19 positive tumors are staged in group one and two.
The conclusion from this data is that cells do not have the uniformity anymore of healthy liver tissue and the tumor is poorly differentiated. Because all these tumors fall in stage one or two all these tumors show infiltrative growth into surrounding liver tissue, vascular structures or other organs in the body. Data about metastasized tumors can be derived from the anamnesis that accompanied liver samples. This will give a patient a poor prognosis. From this data we can conclude that K19 positive hepatocellular tumors in dogs have a more malignant nature compared to K19 negative tumors.

In the cat there were no hepatocellular tumors with more than 30% positive cells for K19, but 38% of the tumors showed 5-10% positivity for K19. These tumors were categorized in the higher groups of the grading system (groups two and three), but were staged in group zero. This means that the tumors in the cat show more cell and nucleus pleiomorfism compared to the dog and that there are more mitotic figures compared to the dog. There is no metastasis found in these tumors in the cat, so the grading system seems not to correspond with the staging system. These K19 positive tumors are not metastasized to other parts of the body, so there needs to be more data collected before there can something be said about the malignancy of K19 positive tumors in cats.

Discussion

Hepatocellular tumors

One study reported K19 expression in 5-100% of the neoplastic cells in 16% of the hepatocellular carcinomas in man [14]. Jain et al. reported an incidence of 10- 27% of K19 positive hepatocellular carcinomas in man [16]. Sprundel et al. showed a K19 expression in 12% of the hepatocellular tumors in the dog [39]. This study demonstrates the expression of K19 in more than 30% of the tumor cells in 20% of the hepatocellular tumors in the dog. In the cat there were no tumors found with K19 expression in more than 10% of the tumor cells.

Hepatocellular tumors with K19 expression would have a poorer prognosis than hepatocellular tumors without K19 expression. Durnez et al. studied the recurrence after liver transplantation in human patients with hepatocellular carcinomas. She reported that the incidence of recurrence was significant higher and occurred earlier in the K19 positive patients. [14] Another study observed a shorter survival rate in patients with HCC expressing K19 without any treatment. The higher recurrence rate of K19 positive hepatocellular carcinomas after transplantation suggest a worse prognosis for HCC expressing K19 compared to K19 negative HCC. [41]

In our study, the biggest part of the 30-100% positive hepatocellular tumors is graded in the most malignant group (group three). Some of the hepatocellular tumors positive for K19 fall in group one or two of the grading system. The hepatocellular tumors are all staged in group one and two. Eleven of the 14 tumors do not show any positivity for HepPar-1. In these tumors there are no normal hepatocytes present in the tumors. The conclusion from this data is that cells do not have the uniformity anymore of healthy liver tissue and that the tumor is poorly differentiated. All these tumors show infiltrative growth into surrounding liver tissue, vascular structures or other organs in the body. This will give a poor prognosis.
K19 expression in 30-100% of the neoplastic cells in the hepatocellular tumor has a positive correlation with the histological staging and grading system. It can be concluded that there is a positive correlation between these histological markers and the prognosis of a patient. Therefore, K19 can be used as a malignancy marker for hepatocellular tumors in dogs. K19 stainings on a hepatocellular tumor can be used as a prognostic marker and clinicopathological marker in the histological diagnosis of hepatocellular carcinomas.

In the cat there are no hepatocellular carcinomas found with more than 10% K19 positivity. There are a few possibility's why we didn't found 30-100% positivity for K19 in primary liver tumors of the cat. First of all, it is possible that there are no 30- 100% positive K19 hepatocellular carcinomas in the cat. Second, there are K19 positive HCC in the cat, but we didn't collect a K19 positive HCC in the samples we used for the cat. In the dog we found a expression of K19 in more than 30% of the tumor cells in 20% of the hepatocellular tumors. From the dog we collected 96 liver samples with primary liver tumors. From the cat we have only 20 liver samples with primary liver tumors. Maybe we used too less cat samples to detect a more than 30% positivity for K19 in a hepatocellular tumor. The third options is that there is a K19 positive HCC in our samples, but the staining we performed on the blocks of the cat didn't work that well.

Carcinoids

Hepatic carcinoids are very rare in humans and in animals. Carcinoid tumors in the liver are most of the time metastases from the gastrointestinal tract. In English literature there are 65 cases of true hepatic carcinoids reported in humans. [25] These tumors were found mostly in middle- aged patients and more frequently in females. [30] From the gastrointestinal tumors 1 to 2% is a carcinoid. These carcinoids metastasized in approximately 5-10% to the liver. [25] Hepatobiliary carcinoids are mostly located in the gall bladder and extrahepatic bile ducts. There are a few reports of carcinoids arising from the intrahepatic tissue. The source of these neoplasms is most likely to be the intrahepatic bile ducts. [29]
In humans, carcinoid tumors are frequently found in the gastrointestinal system (74%) and the bronchopulmonary system (25%). Within the gastrointestinal tract, the most common sites include the small bowel (29%), appendix (19%), and rectum (13%). [22] These intestinal tumors may be associated with a pattern of clinical symptoms referred to as the carcinoid syndrome. This pattern of symptoms includes diarrhea, episodic flushing, bronchospasm, cyanosis, telangiectasia and skin lesions. [35]
In dogs and cats neuroendocrine carcinomas has been reported in the liver [22,30], intestines [22, 30], nasal cavity [30], bile ducts and gallbladder [30], stomach [30], esophagus [30], skin and lungs [22, 30]. Intestinal carcinoids seem to affect dogs that are at least 9 years old. Intestinal carcinoids usually occur in the large intestine (rectum, colon, and cecocolic junction); they are rarely found in the stomach and duodenum.[35]
The first reports of neuroendocrine carcinoma in the liver of dogs and cats were published in 1981 [29] and 1992 [28], respectively. No other reports have been published in these animals except for one case report in a dog. [9, 30]

In humans and animals, carcinoid tumors arise from dispersed cells of the neuoroendocrine system in the gastrointestinal tract, biliary system, pancreas, and lung. [22]
Neuroendocrine cells are embryologically derived from the gut and are widely distributed in various tissues such as the tracheobronchial tree, liver, pancreas, and genitourinary system. [35] These neuroendocrine cells belong to one of two functional groups: 1) the amine precursor uptake and decarboxylation cells (APUD) that produce serotonin and adrenocorticotrophic hormone, or 2) those cells capable of synthesizing low molecular weight polypeptide or protein hormones such as chromogranin, cholecystokinin and secretin. [22]

In this study, six hepatic carcinoids in the dog and seven hepatic carcinoids in the cats are found. Al carcinoids showed some positivity for Neuron-Specific Enolase and most of them also for Chromagranin A. These markers are used as neuroendocrine markers.

Cholangiocellular carcinomas

Cholangiocellular carcinomas occur less often as hepatocellular carcinomas in human. It accounts 15% of all liver tumors. [8] Intrahepatic cholangiocellular carcinomas arises from intrahepatic bile ducts and is the second most common primary liver cancer behind hepatocellular carcinoma. Extrahepatic cholangiocellular carcinomas arise from the biliary tree outside the liver parenchyma. [37]
The cholangiocellular carcinoma is very malignant. Metastasis to other parts of the body is common, particularly to the adjacent lymph nodes of the cranial abdomen, lungs or into the abdominal cavity. Metastasis into the peritoneal cavity is also been seen. [20]
Extensive and complex resections are often required in human, because of the advanced stage at presentation. After resection the outcome of this treatment is uncertain. [8]
It is known that cholangiocytes are positive for K19. [14]

In this study, the cholangiocellular tumors showed positivity for K19. Al the tumors in the cat were 100% positive for K19. In the dog five of the seven carcinomas were 100% positive for K10. The other two were 60% respectively 90% positive for K19.

The high malignant character of this tumor can be seen in the results of the grading and staging system. Neoplastic cells of cholangiocellular carcinomas show a high cell pleiomorfism and no cell uniformity. There are a lot of mitotic figures seen in this kind of tumor, so the mitotic activity is very high. All the cholangiocellular tumors are grouped in group one or two of the staging system. This means that they are all metastasized in surrounding liver tissue, vascular structures or other organs in the body.

References

1. Arias, I.M., Alter, H.J., Boyer, J.L., Cohen, D.E., Fausto, N., Shafritz, D.A. AND Wolkoff, A.W. (2009) The liver biology and pathobiology. West Sussex: Wiley-Blackwell
2. Bajetta**,** E., Ferrari, L., Martinetti, A., Celio, L., Procopio, G., Artale, S., Zilembo, N., Di Bartolomio, M., Seregni, E. AND Bombardieri, E. (1999) Chromogranin A, Neuron-Specific Enolase, Carcinoembryonic antigen, and Hydroxyindole acetic evaluation in patients with neuroendocrine tumors. *Cancer* **89**(5): 858-865
3. Boenisch, T.M.S. (2001) Immunical staining methods. California: Carpinteria, 3rd edition
4. Bouwens, L. (1999) Cytokeratins and cell differentiation in the pancreas. *The journal of pathology* 184**(3):** 234-239
5. Borges, R., Diaz-Vera, J., Dominguez, N., Arnau, M.R. AND Machado, J.D. (2010) Chromogranins as regulators of exocytosis. *Journal of neurochemistry* **114**: 335-343
6. Braun, L, Mikumo, R. AND Fausto, N. (1989) Production of hepatocellular carcinoma by oval cells: cell cycle expression of c-myc and p53 at different stages of oval cell transformation. *Cancer Res.* 49: 1554-1561
7. Burt D, Portmann C, Ferrel D. (2007) MacSween's Pathology of the liver. Churchill Livingstone: Elsevier
8. Chen, M (1999). Peripheral cholangiocarcinoma (cholangiocellular carcinoma): clinical features,diagnosis and treatment. *Journal of gastroenterology and hepatology* **14**: 1144-1149
9. Churcher, R.K. (1999) Hepatic carcinoid, hypercortisolism, and hypokalemia in a dog. *Aust. Vet. J.* **77**(10)**:**641–645
10. Cicinnati, V.R., Sotiropoulos, G.C. AND Beckebaum, S. (2010) Established and emerging therapies for hepatocellular carcinoma. *Minerva Med.* 101**(6):** 405- 418
11. Collazos, J., Genolla, J. AND Ruibal, A. (1991) Neuron-Specific Enolase concentrations in serum benign liver diseases. *Clinical chemistry* **37**(4):579-581
12. Cogliati, B., Aloia, T. P. A., Bosch, R.V., Alves, V. A. F., Hernandez-Blazquez, F. J. AND Dagli, L. Z.(2010) Identification of hepatic stem/progenitor cells in canine hepatocellular and cholangiocellular carcinoma. *Veterinary and comparative oncology* **8** (2): 112-121
13. Dumble, M.L., Croager, E.J., Yeoh, G.C. AND Quail, E.A. (2002)Generation and characterization of p53 null transformed hepatic progenitor cells: oval cells give rise to hepatocellular carcinoma. *Carcinogenesis* 23: 435-445
14. Durnez, A., Verslype, C., Nevens, F., Fevery, J., Aerts, R., Pirenne, J., Lesaffre, E., Libbrecht, L., Desmet, V., AND Roskams, T (2006) The clinicopathological and prognostic relevance of cytokeratin 7 and 19 expression in hepatocellular carcinoma. A possible progenitor cell origin. *Histopathology* **49**: 138-151
15. Imamura, H., Matsuyama, Y., Tanaka, E., Ohkubo, T., Hasegawa, K., Miyagawa, S., Sugawara, Y., Minagawa, M., Takayama, T., Kawasaki, S. AND Makuuchi, M. (2003) Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. *J. Hepatol* 38: 200-207
16. Jain, R., Fischer, S., Serra, S. AND Chetty, R (2010) The Use of Cytokeratin 19 (CK19) Immunohistochemistry in Lesions of the Pancreas, Gastrointestinal Tract, and Liver. *Appl. Immunohistochem. Mol. Morphol.* **18** (1): 9-15
17. Janeway, C.A.,Travers, P., Walport, M. AND Shlomchik, M.J. (2005) Immunobiology:The immune system in health and disease. New-York: Garland Science Publishing
18. Kumar, M., Zhao, X. AND Wang, X.Y. (2011) Molecular carcinogenesis of hepatocellular carcinoma and intrahepatic cholangiocarcinoma: one step closer to personalized medicine? *Cell & Bioscience* **1**(5): 1-13
19. Lamps, L.W. AND Folpe, A.L. (2003) The Diagnostiv Value of Hepatocyte Paraffin antibody 1 in differentiating hepatocellular neoplasms from nonhepatic tumors: A review. *Advances in anatomic pathology* **10**(1): 39-43
20. Mc Gavin, M.D. AND Zachary, J.F. (2007) Pathologic basis of veterinary disease. Missouri: Elsevier

21. Mishra, L., Banker, T., Murray, J., Byers, S., Thenappan, A., He, A.R., Shetty, K, Johnson, L AND Reddy, E.P. (2009) Liver Stem Cells and Hepatocellular Carcinoma. *Hepatology* **49** (1): 318-329
22. Morrel,C.N., Volk, M.V.,and Mankowski, J.L. (2002)A Carcinoid Tumor in the Gallbladder of a Dog. *Vet. Pathol*. **39**:756–758
23. Moulton, J.E., Tumors in domestic animals (1978) California: Berekely and Los Angeles Press, 2nd edition
24. Nelson, R.W. and Couto, C.G. (2003)Small animal internal medicine. Mosby, Missouri
25. Nikfarjam, M.,Muralidharan, V. AND Christophi, C. (2004)Primary hepatic carcinoid tumours. *HPB* **6**(1):13-17
26. Nobels, F.R.E., Kwekkeboom, D.J., Coopmans, W., Schoenmakers, C.H.H., Lindemans, J., De Herder, W., Krenning, E.P., Bouillon, R., AND Lamberts, S.W.J. (1997) Chromogranin A as serum marker for neuroendocrine neoplamsia: comparison with Neuron-Specifin Enolase and the α-subunit of glycoprotein hormones. *Journal of clinical endocrinology and metabolism* **82**(8): 2622-2628
27. Omary, M.B., Ku, N. AND Toivola, D. M. (2002) Keratins:Guardians of the Liver. *Hepatology* **35** (2): 251-257
28. Patnaik, A.K.(1992) A morphologic and immunocytochemical study of hepatic neoplasms in cats. *Vet. Pathol.* **29**(5) 405– 415
29. Patnaik,A. K., Lieberman,P. H., Hurvitz, A.I. AND Jhonson, G.F. (1981) Canine Hepatic Carcinoids. *Vet. Pathol*. **18***:* 445-453
30. Paknaik,A.K., Lieberman, P.H.,. Erlandson, R.A. and Antonescu, C. (2005)Hepatobiliary Neuroendocrine Carcinoma in Cats: A Clinicopathologic, Immunohistochemical, and Ultrastructural Study of 17 Cases. *Vet. Pathol.* **42**: 331–337
31. Papoulas, M. AND Theocharis, S. (2009) Primary liver tumors: origin and target therapy. *Expert Opin. Ther. Targets* **13**(8):957-965
32. Pons, F., Varela, M. AND Llovet, J.M. (2005) Staging systems in hepatocellular carcinoma. *HPB* **7**: 35–41
33. Roskams, T. (2006) Review: Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. *Oncogene* **25**: 3818-3822
34. Rothuizen, J., Bunch, S.E., Charles, J.A., Cullen, J.M., Desmet, V.J., Szarmari. V., Twedt, D.C., van den Ingh, T.S.G.A.M., van Winkle, I AND Washabau, R.J. (2006) WSAVA standards for clinical and histological diagnosis of canine and feline liver disease. Saunders Elsevier
35. Sako, T., Uchida, E., Okamoto, M., Yamamoto, E.,. Kagawa, Y., Yoshino, T., Hirayama, K. and Taniyama, H. (2003) Immunohistochemical Evaluation of a Malignant Intestinal Carcinoid in a Dog. *Vet. Pathol.* **40**: 212–215
36. Shi, S., Cote, R.J. and Taylor, C.R. (2001) Antigen Retrieval Techniques: Current Perspectives. *The Journal of Histochemistry & Cytochemistry* **49**(8): 931–937
37. *Shimoda,M., Farmer,D.G., Colquhoun,S.D., Rosove, M.,Ghobrial,R.M., Yersiz,H., Chen, P.AND Busuttil, R.W.(2001)*Liver Transplantation for Cholangiocellular Carcinoma:Analysis of a Single-Center Experience and Review of the Literature. *Liver Transplantation* **7**(12): 1023-1033
38. Spangenberg, H.C.,Thimme, R. AND Blum, H.E. (2006) Serum Markers of Hepatocellular Carcinoma. *Semin Liver Dis* **26**(4): 385-390
39. Van Sprundel, R.G.H.M., Van den Ingh, T.S.G.A.M., Desmet, V.J., Katoonizadeh, A., Penning, L.C., Rothuizen, J., Roskams, T AND Spee, B (2010) Keratin 19 marks poor differentiation and a more aggressive behaviour in canine and human hepatocellular tumours. *Comparitive Hepatology* **9** (4): 1-11
40. Wennerberg, A.E., Nalesnik, M.A. AND Coleman, W.B. (1993) Hepatocyte paraffin 1: A monoclonal antibody that reacts with hepatocytes and van be used for differential diagnosis of hepatic tumors. *American Journal of Pathology* **143**(4): 1050-1054
41. Wu,P.C., Fang, J.W., Lau,V.K.,Lai, C.L., Lo,C.K. AND Lau, J.Y.(1996)Classification of hepatocellular carcinoma according to hepatocellular and biliary differentiation markers. Clinical and biological implications. *Am. J. Pathol*. **149**(4): 1167-1175
42. Yao, D., Dong, Z. AND Yao, M. (2007) Specific molecular markers in hepatocellular carcinoma. *Hepatobiliary Pancreat Dis Int* **6:** 241-247
43. Zhuang, P., Zhang, J., Zhu, X., Zhang, W., Wu, W., Tan, Y., Hou, J., Tang, Z., Qin, L., AND Sun, H. (2008) Two Pathologic Types of Hepatocellular Carcinoma With Lymph

Node Metastasis With Distinct Prognosis on the Basis of CK19 Expression in Tumor. *Cancer* **112**(12): 2740-2748

Website:

44. http://www.cancer.org/cancer/livercancer/detailedguide/liver-cancer-staging

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Appendix

Two step IHC protocol: Keratin 19

- Deparaffinize and rehydrate sections in a series of Xylene: 2x5' ; Alc. 96%; Alc. 80%; Alc. 70%; Alc. 60%; Alc. 30% (each step 5'); MQ 5'
- Proteinase-K (Dako) — 15 min. RT
- Rinse in **TBS/T** buffer solution — 2 x 2 min.
- Inhibit endogenous peroxidase activity by incubating the slides in Dako Ready to use peroxidase block — 10 min. RT
- Rinse in **TBS/T** buffer solution — 3 x 5 min.
- Incubate in 10 percent normal goat serum — 30 min. RT
- Incubate with (in ab-diluent, DAKO) — O/N at 4°C
- K19 – #29 – 1:100 (mouse)
- Rinse the sections in **TBS/T** buffer solution — 3 x 5 min.
- Incubate in
- Envision Goat anti mouse HRP — 45 min. RT
- Rinse the sections in **TBS (NO TWEEN)** — 3 x 5 min.
- Incubate the sections in freshly made DAB substrate (result: brown) — 5 min.
- Rinse the sections in mQ — 3 x 5 min.
- Counterstain the sections haematoxylin QS-Dako — 10 sec.
- Rinse sections in running tapwater — 10 min.
- Dehydrate section and cover in vectamount:
- 30% Alc.;70% Alc.; 96% Alc.; 96% Alc.; 100% Alc.; 2x5' xylene (each step 5 min.)

Two step IHC protocol: Neuron-specific Enolase (NSE)

- Deparaffinize and rehydrate sections in a series of:
- Xylene: 2x5' ; Alc. 96% 2x5; Alc. 80% ; Alc. 70%; Alc. 60%; Alc. 30% (each step 5'); MQ 5'

- Antigen retrieval in 10 mM hot citrate buffer pH 6.0 (3.36 gr in 1600 ml mQ, adjust pH to 6) — 40 min. 98°C
- Cool down at room temperature (still in hot buffer outside water bath) — 30 min.

- Rinse in **PBS/T** buffer solution — 2 x 2 min.

- Inhibit endogenous peroxidase activity by incubating the slides in Dako Ready to use peroxidase block — 10 min. RT

- Rinse in **PBS/T** buffer solution — 3 x 5 min.

- Incubate in 10 percent normal goat serum — 30 min. RT

- Incubate with (in ab-diluent, DAKO) NSE – 36 – 1:400 (mouse) — 4°C O/N

- Rinse the sections in **PBS/T** buffer solution — 3 x 5 min.
- Incubate in envision Goat anti mouse HRP — 45 min

- Rinse the sections in **PBS (NO TWEEN)** — 3 x 5 min.

- Incubate the sections in freshly made DAB substrate (result: brown) — 5 min.

- Rinse the sections in mQ — 3 x 5 min.

- Counterstain the sections haematoxylin QS-Dako — 10 sec.

- Rinse sections in running tapwater — 10 min.

- Dehydrate section and cover in vectamount:
- 30% Alc.;70% Alc.; 96% Alc.; 96% Alc.; 100% Alc.; 2x5' xylene(each step 5 min.)

10 mM Citrate buffer (pH 6.0)
Add 2.1 gram citric acid to 1000 ml mQ
Adjust pH with NaOH

Two step IHC protocol: Chromogranin-A

- Deparaffinize and rehydrate sections in a series of:
- Xylene: 2x5' ; Alc. 96%; Alc. 80% ; Alc. 70%; Alc. 60%; Alc. 30% (each step 5'); MQ 5'

• Rinse in **PBS/T** buffer solution	2 x 2 min.
• Inhibit endogenous peroxidase activity by incubating the slides in Dako Ready to use peroxidase block	10 min. RT
• Rinse in **PBS/T** buffer solution	3 x 5 min.
• Incubate in 10 percent normal goat serum	30 min. RT
• Incubate with (in ab-diluent, DAKO) Glypican-3 – 33 – 1:800 (rabbit)	4^{o}C O/N
• Rinse the sections in **PBS/T** buffer solution	3 x 5 min.
• Incubate in Envision Goat anti mouse HRP	45 min. RT
• Rinse the sections in **PBS (NO TWEEN)**	3 x 5 min.
• Incubate the sections in freshly made DAB substrate (result: brown)	5 min.
• Rinse the sections in mQ	3 x 5 min.
• Counterstain the sections haematoxylin QS-Dako	10 sec.
• Rinse sections in running tapwater	10 min.

- Dehydrate section and cover in vectamount:
  30% Alc.; 70% Alc.; 96% Alc.; 96% Alc.;100% Alc.; 2x5 xylene (each step 5 min.)

10 mM Citrate buffer (pH 6.0)
Add 2.1 gram citric acid to 1000 ml mQ
Adjust pH with NaOH

Two step IHC protocol HepPar-1

- Deparaffinize and rehydrate sections in a series of:
- Xylene: 2x5' ; Alc. 96% 2x5; Alc. 80% ; Alc. 70%; Alc. 60%; Alc. 30% (each step 5'); MQ 5'

• Antigen retrieval in 10 mM hot TE buffer pH 9.0	40 min. 98°C
• Cool down at room temperature (still in hot buffer outside water bath)	30 min.
• Rinse in **PBS/T** buffer solution	2 x 2 min.
• Inhibit endogenous peroxidase activity by incubating the slides in Dako Ready to use dual endogenous enzyme block	10 min. RT
• Rinse in **PBS/T** buffer solution	3 x 5 min.
• Incubate in 10 percent normal goat serum	30 min. RT
• Incubate with mouse anti-HepPar-1 (#9) 1:50 (in ab-diluent)	O/N at 4°C
• Rinse the sections in **PBS/T** buffer solution	3 x 5 min.
• Incubate in Envision Goat anti rabbit HRP	45 min. RT
• Rinse the sections in **PBS**	3 x 5 min.
• Incubate the sections in freshly made DAB substrate (result: brown)	5 min.
• Rinse the sections in mQ	3 x 5 min.
• Counterstain the sections haematoxylin QS-Dako	10 sec.
• Rinse sections in running tapwater	10 min.

- Dehydrate section and cover in vectamount:
  30% Alc.; 70% Alc.; 96% Alc.; 96% Alc.; 100% Alc.; 2x5 xylene (each step 5 min.)

Buffers
Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0):
Tris Base ---------- 1.21 g
EDTA ---------- 0.37 g
Distilled water ---------- 1000 ml (100 ml to make 10x, 50 ml to make 20x) Mix to dissolve. pH is usually at 9.0 and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4 C for longer storage.
Tris-EDTA buffer: Place slides in the preheated buffer in the water bath, and heat bath to 98ºC. Incubate for 20 min.