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Mutational evaluation of TP53 gene in the canine hepatocellular carcinoma: a comparative approach

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CERTIFICADO

Certificamos que a proposta intitulada "Avaliação mutacional do gene p53 no carcinoma hepatocelular em cães: uma abordagem comparativa ", protocolada sob o CEUA nº 7153030217 (ID 003372), sob a responsabilidade de **Bruno Cogliati** *e equipe; David Salas Gómez* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 15/03/2017.

We certify that the proposal "Mutational evaluation of p53 gene in the canine hepatocellular carcinoma: a comparative approach", utilizing 80 Dogs (males and females), protocol number CEUA 7153030217 (ID 003372), under the responsibility of **Bruno Cogliati** *and team; David Salas Gómez* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 03/15/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 03/2017 a 03/2019 Área: Patologia Experimental E Comparada

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Espécie:	Cães	sexo:	Machos e Fêmeas	idade:	0 a 20 anos	N:	30
Linhagem:	Diversas			Peso:	1 a 100 kg		
Origem:	Amostras biológicas estocadas						
Espécie:	Cães	sexo:	Machos e Fêmeas	idade:	0 a 20 anos	N:	50
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São Paulo, 31 de janeiro de 2019

Profa. Dra. Anneliese de Souza Traldi
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ABSTRACT

SALAS-GÓMEZ, D. **Mutational evaluation of TP53 gene in the canine hepatocellular carcinoma:** a comparative approach. [Avaliação mutacional do gene TP53 no carcinoma hepatocelular em cães: uma abordagem comparativa]. 2019. 78f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

Hepatocellular carcinomas (HCC) represent an important part of hepatic diseases in dogs, correspond to more than 50% of primary neoplasms of the liver in this species. HCC affects, mainly, male dogs over 10-years-old, presenting poor prognosis in patients not suitable for surgery. Latest years, studies about comparative oncology have acquired great importance, since dogs and humans share diverse cellular and molecular features. In humans, HCC is associated with chronic liver injuries, caused by viral infections (hepatitis C and B), fatty liver disease (alcoholic and non-alcoholic), chronic intoxication (drugs, mycotoxins, etc.), among others. The consumption of aflatoxin B1 (AFB1) is associated with a specific mutation in codon 249 of the TP53 gene, causing HCC in part of the patients exposed to this mycotoxin. In case that dogs do not present chronic viral hepatitis or fatty liver diseases, environmental and feeding factors, such as the possible presence of aflatoxin B1 in pet food, may be directly related to the etiology of HCC in this species. Firstly, this study performed a comparative literature review of epidemiological, clinicopathological and anatomo-pathological aspects of HCC in dogs and humans. Subsequently, the presence of mutations in the TP53 gene were evaluated in canine HCC samples (n = 24), in order to determine a possible association of AFB1 in hepatocarcinogenesis. Clinical-epidemiological data observed in canine HCC are in agree with the literature reviewed. However, mutations in the TP53 gene or p53 immunoexpression were not observed. Thus, due to the absence of mutations in the TP53 gene in the analyzed samples, the participation of AFB1 as an etiological factor in HCC remains open.

Keywords: Canine, hepatocellular carcinoma, Mutation, TP53 gene, aflatoxin B1

RESUMO

SALAS-GÓMEZ, D. **Avaliação mutacional do gene TP53 no carcinoma hepatocelular em cães:** uma abordagem comparativa [Mutational evaluation of TP53 gene in the canine hepatocellular carcinoma: a comparative approach]. 2019. 78f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

Os carcinomas hepatocelulares (CHC) representam uma parte importante das doenças hepáticas em cães, correspondendo a mais de 50% das neoplasias primárias do fígado nesta espécie. O CHC afeta, principalmente, cães machos com mais de 10 anos de idade, apresentando prognóstico ruim em pacientes não elegíveis para cirurgia. Nos últimos anos, os estudos em oncologia comparada têm adquirido grande importância, uma vez que cães e humanos compartilham diversas características celulares e moleculares. Em humanos, o CHC é associado às lesões hepáticas crônicas, causadas por infecções virais (hepatites C e B), doença hepática gordurosa (alcoólica e não alcoólica), intoxicações crônicas (medicamentos, micotoxinas, etc.), dentre outras. O consumo da aflatoxina B1 (AFB1) está associado a uma mutação especifica no códon 249 do gene TP53, ocasionando o CHC em uma parcela dos pacientes expostos à esta micotoxina. Como os cães não apresentam hepatites virais crônicas ou doenças gordurosas hepáticas, fatores ambientais e alimentares, como a possível presença de aflatoxina B1 nas rações, podem estar diretamente relacionados à etiologia do CHC nesta espécie. Assim, inicialmente este estudo realizou uma revisão de literatura comparada sobre os aspectos epidemiológicos, clinicopatológicos e anatomopatológicos do CHC em cães e humanos. Posteriormente, avaliou-se a presença de mutações no gene TP53 em amostras de CHC canino (n=24), com o objetivo de determinar uma possível associação da AFB1 na hepatocarcinogênese. Os dados clínico-epidemiológicos observados nos CHCs caninos apresentaram-se dentro do relatado na literatura. No entanto, não foram observadas mutações no gene TP53 ou imunoexpressão de p53. Assim, devido à ausência de mutações no gene TP53 nas amostras analisadas, a participação da AFB1 como fator etiológico no CHC permanece em aberto.

Palavras-chave: Cães, carcinoma hepatocelular, mutação, gene TP53, aflatoxina B1

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1 INTRODUCTION

Primary hepatic neoplasms represent between 0.6 to 1.3% of canine tumors (PATNAIK; HURVITZ; LIEBERMAN, 1980) and constitute up to 21% of liver diseases, being hepatocellular carcinoma (HCC) the most common hepatic neoplasm in dogs (HIROSE et al., 2014; CONSTANT et al., 2016). Canine HCC is more recurrent in males, usually in animals older than 10 years (BURITICÁ; BARBOSA; ECHEVERRY, 2009). There is no well-established breed predisposition, however, one study indicates that Shih Tzu breed has an important predisposition to this tumor (HIROSE et al., 2014). The etiology of canine CHC has not yet been established, but potential causes such as aflatoxins, nitrosamines, hepatic parasites and radioactive components have been reported in experimental studies and spontaneous findings (CULLEN, 2017). Generally, the prognosis of HCC is restricted due to its diagnosis at advanced stages or presence of non-operable lesions (KAWARAI et al., 2006). Meanwhile, when neoplasia is detected early by imaging and occurs separately in a hepatic lobe, partial hepatectomy is effective and the prognosis is good (MICHISHITA et al., 2014).

Macroscopically, CHC can be massive, which usually involves just one hepatic lobe; nodular, when it presents dispersed nodules affecting several lobes or diffuse, being characterized by indistinct masses scattered throughout the liver parenchyma. Metastases are rare, but may occur in lymph nodes near the tumor, lungs, or may spread into the peritoneal cavity. Vascular invasion and intrahepatic metastasis is more usual (CULLEN; STALKER, 2016). Histologically, HCC presents variable patterns such as solid, pseudoglandular and trabecular, the last one being the most common pattern (CULLEN; BROWN, 2013). HCC in dogs is usually not associated with other liver lesions and is subclassified in HCC with 0 to 5% cytokeratin 19 (CK19) positive cells, HCC with more than 5% CK19 + cells or scirrhous HCC with tubular structures positive for CK19. This classification considers the origin of neoplastic hepatocytes from the differentiation of hepatic progenitor cells, and these lastest two subtypes present a more aggressive biological behavior (VAN SPRUNDEL et al., 2013; TU et al., 2014).

In recent years, HCC has become a widely studied disease in dogs and humans due to its similar clinical and histological characteristics (MICHISHITA et al., 2014). Human HCC is also the most common primary hepatic neoplasia and the fifth most frequent cancer in the world population (LAFARO; DEMIRJIAN; PAWLIK, 2015),

characterized by high mortality and poor prognosis, with around 600,000 deaths per year (TU et al., 2014)). The vast majority of cases are related to chronic liver diseases (KUMAR et al., 2016), such as viral hepatitis C and B, representing 75% to 85% of the causes, in addition to other etiologies, such as alcoholic or non-alcoholic steatohepatitis (ASH/NASH), aflatoxins, and other drugs (TU et al., 2014; SINGAL; EL-SERAG, 2015). In humans, the most notable macroscopic feature is the presence of chronic hepatitis, which is not common in dogs. Although, histological characteristics of HCC are very similar between humans and dogs, only differ in the fibrolamellar variable that is absent in dogs (FERREL; KAKAR, 2007). Similarly, prognosis is poor if it is not early identified, with only 40-50% of cases being diagnosed early (SU et al., 2014). Serum alpha-feto protein (AFP) dosage is used for early diagnosis, in along with other markers such as prothrombin, glypican 3, serum golgi protein 73 and osteopontin (KNUDSEN; GOPAL; SINGAL, 2014). AFP has also been used in dogs, with promising results (KITAO et al., 2006a). Imaging techniques such as ultrasonography, tomography and magnetic resonance imaging are routinely employed in the early diagnosis of HCC in humans (TU et al., 2014).

Although the incidence of HCC in South America is low, Brazil has one of the highest incidences in the region (LAFARO; DEMIRJIAN; PAWLIK, 2015). In some regions of Africa and Asia, a critical cause of HCC is associated with the consumption of food containing mycotoxins, mainly aflatoxin B1 (AFB1), which are known as a potent hepatic carcinogen (KEW, 2013; TU et al., 2014). Aflatoxins originate principally from the *Aspergillus Flavus fungi*, which produce the toxins B1 and B2 and *Aspergillus parasiticus*, which generate G1 and G2 toxins (DING et al., 2015). Aflatoxins were first identified in peanut meal, which caused deaths due to acute toxicosis in turkeys, chickens and ducks being liver critical target organ (WOGAN; KENSLER; GROOPMAN, 2015). AFB1 has also been identified in oilseeds, nuts, meat and milk. These fungi growth in conditions of 85% relative humidity and temperatures between 25°C and 32°C, affecting foods stored under poor conditions (KEW, 2013; MAGNUSSEN; PARSI, 2013).

In a study conducted in Vietnam, the authors demonstrated that AFB1 can be present in up to 85.7% of staple foods evaluated in local markets in that country, such as rice, wheat, corn, sorghum, meat and milk, resulting in a daily exposure of 39.4 ng/kg (HUONG et al., 2016). On the other hand, there are regions in China where the national maximum permissible aflatoxin limit in rice is greater than 10 µg/kg (SUN; SU;

SHAN, 2017). Similar studies have been carried out on commercial food for breeding animals in Brazil, where 12% of the analyzed foods presented AFB1 contamination, of which 5% had values higher the maximum permitted limit of 50 µg/kg (MAIA; PEREIRA BASTOS DE SIQUEIRA, 2002). Another study, also conducted in Brazil, showed that up to 50% of commercial pet mainly corn, showed high aflatoxin contamination (WOUTERS et al., 2013a). In addition, a study carried out in Peru showed that 100% of the commercial dog food, sold in bulk were exposed to environmental conditions, showed aflatoxin contamination in amounts of 0.2 to 8 ppb. It is worth mentioning that this type of food sale practice is frequent in some countries of South America (PERALES VIZCARRA; CAMACHO PERALES; MORALES CAUTI, 2014).

The most frequently affected molecular pathways in human HCC are represented by the genes TP53, retinoblastoma, kinase-dependent cyclin A2 inhibitor (KNUDSEN; GOPAL; SINGAL, 2014) and via Wnt-β-catenin, by loss of AXIN1 and AXIN2 functions. These pathways are responsible for the functions of regulation, differentiation, proliferation and cell death, as well as the activation of proto-oncogenes and tumor suppressor genes (SU et al., 2014). Aflatoxins are liposoluble and readily absorbed by the gastrointestinal system (STENSKE et al., 2006). Aflatoxin is metabolized in the liver by cytochrome P450 (CYP1A2, CYP2A6, CYP3A4), producing an unstable reactive, called AFB1 8,9 epoxide (WU; SANTELLA, 2012; YANG et al., 2016). This makes covalent bonds to the DNA, generating two main adducts AFB1 guanine N7 and AF lysine. These products are detected in the urine and serum, being useful as biomarkers as a result of interaction of toxins with the genetic material (WOGAN; KENSLER; GROOPMAN, 2015; JU et al., 2016).

In humans the HCC caused by aflatoxin contamination, is identified because it has a punctual mutation (G to T transversion) at codon 249 (exon 7) of the TP53 gene (KEW, 2013), which is present in more than 50 % of cases of HCCs associated with AFB1 (SU et al., 2014). This mutation is known as R249S and results in a substitution of essential amino acids such as arginine for serine, generating inhibition of apoptosis (YANG et al., 2014). In addition, TP53 mutations affect histones and nucleosomes; proteins with important functions in DNA structure, replication and gene expression (NISHIDA; KUDO, 2016). TP53 normally acts in the G1-phase, synthesis phase and mitosis of the cell cycle, acting with pro-apoptotic and anti-apoptotic proteins (STOCKMANN et al., 2011). It is also known that these mutations favor the increase of reactive oxygen species (ROS) and the reduction of antioxidant and detoxifying

enzymes, such as superoxide dismutase and glutathione transferase (QIN et al., 2016). These interactions were demonstrated in experimental studies of rodents with AFB1-induced HCC, finding a suggestive feature of cellular damage by ROS, such as lipid peroxidation, besides hypermethylation, increased expression of anti-apoptotic genes (Bcl-2, MAPK8, NFKb1), and diminution of pro-apoptotic genes (Casp1, Il4, MPO) (REBBANI et al., 2015; SHI et al., 2016). In a study carried out in Brazil, the R249S mutation was demonstrated in 28% of the analyzed HCC, indicating that there is high aflatoxin exposure in the country (NOGUEIRA et al., 2009).

Likewise, this exposure to high concentrations of AFB1 in inappropriately maintained food and/or raw materials could be related to the development of HCC in dogs. Canine TP53 gene shows high similarity with the human gene, also containing 11 exons, but 381 amino acids; while the human gene has 13 additional amino acids in Exon 4. With this small difference, the homology in its structure is 81% (YORK et al., 2012). The conserved evolutionary domains II are 94% homologous, which is the region in which most of the mutations of the TP53 gene occur in humans. These domains differ in only one amino acid in dogs, with tryptophan in place of cysteine at codon 124 (KRAEGEL; PAZZI; MADEWELL, 1995). Several mutations in the TP53 gene have been described in other canine neoplasms, such as osteosarcomas, which present point mutations in exons 4-9. Most of these mutations are identical to the human of the same tumor type, such as those occurring in codons 176, 214 and 273 (KIRPENSTEIJN et al., 2008). On the other hand, canine brain tumors only showed a mutation identical to the equivalent tumors in humans, a mutation located in the codon 233 of the canine TP53 gene in an oligodendroglioma corresponding to the codon 245 in the same tumor in humans, both in exon 7 (YORK et al., 2012).

Animals, especially dogs, share the same food base with humans and may be exposed to the same risks as aflatoxin consumption; or even larger, if methods of storage of grains and food intended for animal consumption are considered. Considering the several macroscopic, histological and molecular similarities of HCC in dogs and humans, besides the absence of a well-defined etiology in dogs, the association of AFB1 contamination and hepatocarcinogenesis could be a valid hypothesis in dogs. Therefore, this study aimed to assess whether or not canine HCC presents mutations in TP53 gene, especially homologous to 249 codon in humans, or that it would be an indirect indicative source of contamination by AFB1 as the main etiological agent of this neoplasia.

2 LITERATURE REVIEW

2.1 COMPARATIVE ASPECTS OF CANINE AND HUMAN HEPATOCELLULAR CARCINOMA

2.1.1 EPIDEMIOLOGY

In dogs, primary hepatic neoplasms represent 21% of all hepatic diseases (HIROSE et al., 2014), being the hepatocellular carcinoma (HCC) responsible for up to 50% of all primary hepatic neoplasms. In an overall approach, HCC constitutes around 0.6 to 1.3% of total canine neoplasms (PATNAIK; HURVITZ; LIEBERMAN, 1980; TRIGO et al., 1982). In humans, hepatic neoplasms represent 5.7% of cancer being considered the sixth more frequent tumor (PARKIN et al., 2005). All human hepatic neoplasms, 70-85% are HCC (AHMED et al., 2008). However, differently from humans, chronic hepatic disease and cirrhosis is just present in less than 8% of canine HCC (PATNAIK; HURVITZ; LIEBERMAN, 1980; TRIGO et al., 1982; LAMOUREUX et al., 2012).

HCC affects mainly male dogs with more than 10 years (PATNAIK et al., 1981a; LODI et al., 2007; HIROSE et al., 2014). However, cases in young animals were reported without apparent cause (TESHIMA et al., 2013a)In humans, this cancer is more frequently to middle-aged men (ALTEKRUSE; MCGLYNN; REICHMAN, 2009). Nevertheless, HCC is also reported in children and human younger than 35 (range 2-35 years) and an average age of 17.4 years, in addition, it is hard to diagnose because a relative preservation of hepatic functions (ARAMAKI et al., 2005). Dogs do not display breed predilection, however one retrospective study reported a higher prevalence of HCC in Shih Tzu and Yorkshire Terriers (HIROSE et al., 2014). In a study in the United States, in relation to human races, Asian race presents HCC twice meanwhile Black race presents it four times than White race (SERAG; RUDOLPH, 2007).

Necropsy reports showed that between 22.22 to 61% of canine HCC cases outcome in metastases, being the lungs, small intestine, mandible and submandibular lymph nodes the most common local for dissemination (PATNAIK et al., 1981a; TRIGO et al., 1982). Comparatively, in a study of human HCC including 513 patients, 42% presented metastasis, being more frequently affected in thoracic organs (21%),

abdominal organs (16%) and bone (10%) (SI et al., 2003). Human HCC, just between 3 to 5% of patients survive the disease course becoming the third cause of death in cancer diseases (DONATO; BOFFETTA; PUOTI, 1998; PARKIN et al., 2005). On the other hand, most cases of dogs treated surgically have a satisfactory resolution with average survival times longer than 1400 days against less than 300 days in non-operated dogs (LIPTAK et al., 2004a)

2.1.2 ETIOLOGY

Several etiologies that lead to chronic liver disease and cirrhosis have been implicated in human hepatocarcinogenesis, such as viral infections (B and C hepatitis), alcoholic and non-alcoholic steatohepatitis (ASH and NASH, respectively), aflatoxin B1, carcinogenic drugs, iron/copper accumulation, among others (SERAG; RUDOLPH, 2007). In decreasing order of importance, hepatitis virus B (HVP) affecting more than 300 million of people worldwide. HVB increases 5 to 15 times the predisposition to develop HCC in contrast the rest of population. HVP-related HCC is especially high in countries where the virus is endemic, for example, in China and Africa (MCMAHON et al., 1990; BLOCK et al., 2003; FERENCI et al., 2010a). Otherwise, hepatitis virus C (HVC) is found, being the most important cause of HCC in Japan, Latin America, the United States and Europe (YOSHIZAWA H., 2002; FERENCI et al., 2010a). Viral hepatitis lead to chronic inflammation, fibrosis and cirrhosis promoting HCC development (CRAMP, 1999). Chronic intoxication with aflatoxin B1, which lead to 249 codon TP53 gene mutation, is an important cause o HCC in subtropical countries in Asia, Africa and South America (SERAG; RUDOLPH, 2007; NOGUEIRA et al., 2009). Other risk factors related are Diabetes mellitus and obesity which progress to NALFD (non-alcoholic liver fatty disease) and subsequently to NASH, being a chronic liver disease also related to cirrhosis and HCC (BUGIANESI et al., 2002; MARRERO et al., 2002; REGIMBEAU et al., 2004). Finally, alcohol intake with amounts higher than 50 g per day only or along with viral hepatitis is considered an important risk factor to human hepatocarcinogenesis (DONATO; TAGGER; GELATTI, 2002).

As far we know there are no identified etiologies to spontaneously canine HCC (PATNAIK; HURVITZ; LIEBERMAN, 1980; TRIGO et al., 1982; LAMOUREUX et al., 2012). Experimentally, hepatic tumors were induced in dogs using several chemical

compounds as such 1- and 2-Naphthylhydroxylamina (DEWA et al., 2009), 2-Acetylamino fluorene, 4- and 2-Acetylaminobiphenyl (JABARA, 1963; DEICHMANN; RADOMSKI, 1969), diethylnitrosamine (HIRAO et al., 1974) and 3,3'dichlorobenzidine (STULA et al., 1978). Moreover radioactive compounds such as 144CeCl also was capable to induce liver neoplasms in beagles subjected to its inhalation (HAHN et al., 1997). In addition, hepatic neoplasm were induced in dogs during a long exposure to gamma radiation (IAKOVLEVA, 1984). In counterpart, gamma radiation is not a risk factor to develop HCC in humans, by the other way, alpha radiation has low participation in that carcinogenesis (TOKARSKAYA et al., 2006).

Adverse long-term effect leading to HCC was identified by prolonged Danazol therapy in a 2-years female beagle to treat non-regenerative immune-mediated anemia. In this case, two hepatic masses appeared in different moments during immunosuppressive treatment (KOBAYASHI et al., 2016). This adverse effect also has been observed in human in few cases where danazol is used to treatment of endometriosis, lupus erythematosus, idiopathic thrombocytopenic purpura and other autoimmune conditions. Danazol is well known for its adverse effects in liver (PEARSON; ZIMMERMAN, 1980; CONFAVREUX et al., 2003).

Another condition related is the Von Gierke disease or glycogen storage disease type 1a leading to accumulation of glycogen, mainly in liver, caused by deficiency of glucose 6-phosphatase. This condition is recognized to progress to hepatocellular adenoma (HCA) and HCC (CHEN, 2001). In dogs, surveillance of 5 animals undergoing glycogen storage disease type 1a revealed that HCC is a long-term effect suggesting that canine and human share some carcinogenesis mechanisms although these keep unknown (BIANCHI, 1993; BROOKS et al., 2018). In a retrospective study, (CORTRIGHT et al., 2014) observed high prevalence of HCC (34%) in Scottish terrier breed undergoing progressive glycogen-associated vacuolar hepatopathy. This condition is frequently attributed to high endogen production of steroidogenic molecules or exogenous glucocorticoid therapies (SEPESY et al., 2006). Moreover, this study suggested hepatocellular dysplastic foci in dogs such pre-neoplastic lesions (CORTRIGHT et al., 2014), which could be comparable with dysplastic foci identified in humans associated with glycogen storage and other chronic liver diseases (LIBBRECHT et al., 2000).

2.1.3 CLINICAL AND LABORATORIAL FINDINGS

2.1.3.1 Clinical sings and paraneoplastic syndromes

The main clinical signs find in dogs are hepatomegaly, followed by anorexia, lethargy and weakness. Contrary to what is believed jaundice is not so common and it represent 18% of the cases (PATNAIK; HURVITZ; LIEBERMAN, 1980). Some studies suggest that more than 30% of cases present palpable abdominal mass; however, ascites, diarrhea and emesis are less common findings (LIPTAK et al., 2004a; LODI et al., 2007). In humans, early small hepatocellular carcinomas are mostly asymptomatic cases and jaundice is a rare finding (SHINAGAWA et al., 1984). In contrast, advanced tumors associated to chronic liver disease shows common symptoms referent to cirrhosis and hepatic failure such, hepatomegaly, splenomegaly, abdominal pain, ascites and jaundice along with paraneoplastic syndrome, weakness, loss weight, and others (FERENCI et al., 2010a).

Besides HCC induces unspecific clinical sings also, it is capable to induce several paraneoplastic syndromes. For example, hypoglycemia was reported in a male beagle with HCC, resulting in diabetes *mellitus* subsequent to surgical excision. This syndrome can be related with high glucose consumption by the HCC cells or by production of insulin-like molecules (SAKAI et al., 2006). In humans, these two scenarios are described such hypoglycemia type A and B, respectively (YEUNG, 1997). Hypoglycemia induced by HCC was demonstrated by measurement of serum insulin-like growth factor type-II levels, which was higher in comparison to healthy dogs (ZINI et al., 2007). In humans, hypoglycemia represents up to 12% of HCC-related paraneoplastic syndromes and it is associated to large HCC or end-stage liver disease. In type B patients there are high levels of serum insulin-like growth factor type-II in comparison to no hypoglycemic patients; furthermore, it is well recognized to play a role in the abnormal glucose consumption (DAUGHADAY et al., 1988; WU et al., 1988; LUO et al., 2002; HUH et al., 2005).

The hyperalbuminemia is a rare paraneoplastic syndrome in domestic animals, as well as in humans (NIZAM; AHMED, 1995). A 12-years mixed breed male dog presented hyperalbuminemia concomitant with HCC, which was resolved after surgical resection. The authors suggested that this syndrome was a consequence of a high albumin production originated from neoplastic hepatocytes by some mRNA unbalance;

however, the exact mechanism is unknown (COOPER; WELLMAN; CARSILLO, 2009). Additionally, a case of hypertrophic osteopathy was described in a 9-years mixed breed female dog presenting HCC, which was solved after tumor resection (RANDALL et al., 2015). In counterpart, human hypertrophic osteopathy is related with hepatic dysfunction originated from several causes, including HCC. In cases of human hypertrophic osteopathy associated to HCC, surgical resection resolves the syndrome as it occurred in the dog case (MORGAN, 1972; PITT et al., 1994).

2.1.4 LABORATORY FINDINGS

Liver enzymes as such alkaline phosphatase (AP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are usually increased in dogs HCC (TRIGO et al., 1982). Serum ALT is higher in more than 70% of dogs with HCC (LODI et al., 2007) and presents a prognostic value in human patients with HCC-related HCV (TARAO et al., 2000). A variety of AP isoenzymes is used to discriminate among different liver conditions in humans, including HCC (ĐOKIĆ-LIŠANIN et al., 2013). In comparison, a canine thermostable AP isoenzyme appears only in HCC assessed along with a series of hepatic neoplasm which seems to be fairly specific of HCC and absent in other conditions turned an useful diagnosis tool (FUKUI et al., 2006).

Recently, the use of serum Endothelin-1 demonstrated to be a feasible biomarker to identify HCC in dogs (FUKUMOTO et al., 2014). In human, biomarker has been used to show the neovascularization process in HCC (KAR; YOUSEM; CARR, 1995).

Other important serum biomarker in HCC is alpha-feto protein (AFP), which is widely applied as a screening tool in human patients with chronic liver diseases, promoting an early diagnosis and, eventually, predicting their prognosis and surveillance (FARINATI et al., 2006). For example, patients with high serum AFP levels after HCC resection treatment alert about possible recurrence tumor and fatal outcome (TORO et al., 2014). In dogs, the serum level of AFP is usually high in HCC, elseway non-neoplastic hepatic diseases and cholangiocarcinoma shows mild increasing and normal values, respectively. In this context, the serum AFP could be useful to discriminate these conditions (LOWSETH et al., 1991; YAMADA et al., 1999). AFP is also steadly related to occurrence of primary canine hepatic neoplasm, moreover this marker is independent of others hepatic enzymes assessed (LOWSETH et al., 1991;

HAHN; RICHARDSON, 1995). In addition, one study noted reestablishment of serum AFP to normal values two months after HCC surgical resection(KITAO et al., 2006a). Finally, canine HCC cell lines can also produce high levels of AFP in the culture medium (KAWARAI et al., 2006). Despite of serum AFP measurements be widely used in HCC approaches, especially in human patients, there are authors that question its sensitivity and specificity, and suggest that AFP high levels could be inherent to underlying liver disease in some cases and not necessarily related to HCC (SHERMAN et al., 2010)

In human HCC, overexpression of α1-acid glycoprotein lead to suppression of diverse inflammatory mechanisms, thus tumor microenvironment is maintained and patient is more susceptible to tumor progression and opportunistic infections (TAMURA et al., 1981). In a study, 5 dogs with HCC presented the same protein at high levels suggesting similar disease mechanisms among both species (YUKI et al., 2011). In other human studies, α1-acid glycoprotein displayed more sensitivity and specificity than AFP to identified patient with HCC, as some tumors do not lead to AFP elevation. Then, the combination of α1-acid glycoprotein and AFP measurements could increase its diagnostic accuracy (BACHTIAR et al., 2009, 2010) .

2.1.5 IMAGE TECHNIQUES

2.1.5.1 Ultrasonography

As the ultrasonography (US) is a widely used diagnostic technique in veterinary medicine, some attempts had been performed to characterize hepatic neoplastic lesions in dogs. However, this technique has low accuracy to locate diverse nodules in the same organ, as well as, to discriminate between malignant and benign masses. The main features of malignant hepatic lesions are solitaire/unique nodule, hypoechoic halo by tumor compressing of normal hepatocytes, and hyperechoic center by degenerative/necrotic process (WHITELEY et al., 1989; CUCCOVILLO; LAMB, 2002). In 2004, researches assessed 35 canine hepatic nodules using commercial contrast medium and harmonic software to digital analysis to improve the conventional ultrasound technique. The overall outcome was hypoechogenicity aspect in malignant nodules and isoechogenicity in benign lesions (O´BRIEN et al., 2004).

2.1.5.2 Magnetic resonance imaging

Magnetic resonance imaging (MRI) with paramagnetic gadolinium such contrast medium offers a whole vision regard of tumor vascularity and necrosis areas. In a study with dogs, 25/27 nodular hepatic lesions were differentiated between benign and malignant nodules by MRI, showing 100% of sensitivity and 86% of specificity. The main findings of HCC assessed by MRI are tissue heterogeneity and hypervascularity in malignant nodules, which are not found in benign nodules and normal liver (CLIFFORD et al., 2004). The MRI with Gadoxetate disodium contrast obtained fluctuant results being confident to identify lesions but not to draw a distinction line between benign or malignant findings (CONSTANT et al., 2016).

2.1.5.3 Computerized tomography

Canine HCC shows an improved contrast by computerized tomography (CT), with central and margins more defined in arterial phase compared with nodular hyperplasia, which presents a diffuse contrast in later phases (FUKUSHIMA et al., 2012). The triple-phase helical CT along with Iohexol such contrast was used to show a 3D image of vascularity in hepatic masses in dogs. This technique showed principally heterogeneous enhancement of tumor pattern in arterial and venous phases of HCC, homogeneous pattern in venous and delay phases of nodular hyperplasia, and homogeneous pattern in arterial and venous phases of metastatic masses (KUTARA et al., 2014). Others contrast image techniques also can be used to establish the neoplasms limits with more accuracy to improve surgical resection. This can be performed by intravenous administration of fluorescent contrasts (*i.e.* indocyainine green), which must be eliminated by hepatocytes through bile in normal conditions; however, the HCC neoplastic cells tend to retain the contrast being feasible its visualization by high sensitivity near-infrared fluorescence equipment. Yet in veterinary medicine, there are some limitations regard the presence of no-fluorescent HCC, decreasing sensitivity of this test (IIDA et al., 2013). Independently from the image technique selected for screening the hepatic neoplastic lesions, the histopathologic analysis is fundamental to guarantee accuracy in the definitive diagnosis (WARREN-SMITH et al., 2012).

2.1.6 PATHOLOGICAL FINDINGS

2.1.6.1 Gross pathology

Veterinary pathologists have been applied human HCC gross classification, which can present massive, nodular or diffuse distribution. (EGGEL, 1901; OKUDA; PETERS, 1976; PATNAIK et al., 1981a). In humans, several proposals to classify macroscopically the HCCs were performed. Thus, some authors considered tumor capsule, presence of cirrhosis and thrombus in portal vein to classify in infiltrative, expansive, mixed and diffuse type (OKUDA; PETERS; SIMSON, 1984; NAKASHIMA; KOJIRO, 1986). Thereafter, some researches added also shape tumor and histological features, currently, medical pathologists try to use these classification system with some adjustments and correlate the gross tumor aspect with prognosis and predictive response to therapeutic approaches, by multivariable analysis of several tumor findings (KANAI et al., 1987; SHIRABE et al., 2011; HE et al., 2015).

In agreement with the current classification in veterinary medicine, most of HCC shows a massive distribution (54-60%), defined as a large neoplastic formation commonly in just one hepatic lobe, which most of times is the left lateral lobe (66%) (PATNAIK; HURVITZ; LIEBERMAN, 1980; LIPTAK et al., 2004a). Other less common distribution are nodular (29% of cases), characterized by discrete nodules distributed randomly in several lobes and diffuse (10% of cases), compromising almost completely the liver (PATNAIK et al., 1981b). In humans, the hepatic left lobes are also the most frequently lobe affected (RINGE; PICHLMAYR; WITTEKIND, 1991). In addition, there are reports of ectopic HCC in both species. In dogs, one case of ectopic HCC was described in gastrosplenic ligament and great omentum (BURTON et al., 2005). In humans, several reports include ectopic HCC in pancreas (KUBOTA et al., 2007), bile duct (TSUSHIMI et al., 2005), diaphragm (TAKAYASU; ITABASHI; MORIYAMA, 1994) and jejunum (SHIGEMORI et al., 2006).

The surface appearance of HCC is often smooth or nodular. On cut surface, a multinodular aspect is seen, furthermore with a wide color variation; brown in most of tissue but also gray to white, yellow and green, finally, grey color explained by fibrosis or cirrhosis concomitance. Necrotic areas, as well as reddish foci are described usually (MULLIGAN, 1949; PATNAIK et al., 1981b; TRIGO et al., 1982).

2.1.6.2 Cytological features

Cytological diagnosis focused mainly to differentiate benign proliferations and well- differentiated malignant lesions. The last one presents almost lack of cellular atypia and morphologic alterations, leading to problems to classify and discordance between pathologist´s diagnosis. To improve the cytological diagnosis, in a study was assessed 33 cytological features of canine HCC and demonstrated that hepatocellular arrange, naked nuclei along with mild cellular atypia were the only significate criteria (MASSERDOTTI; DRIGO, 2012; MASSERDOTTI et al., 2014). Others authors suggest that high nucleus:cytoplasm proportion, cells bigger than normal, nucleoli number increased, few vacuolated cytoplasm and lymphocytes infiltration are important cytological criteria to diagnosis HCC (STOCKHAUS et al., 2004). In humans, the foremost criteria proposed to discriminate between HCC and non-neoplastic process are trabecular arrangement, atypical naked hepatocyte nuclei and high nuclear/cytoplasm proportion (COHEN et al., 1991). However, accuracy depends critically on a good quality samples and enough cellular atypia to be detected (SOLÉ et al., 1993).

Intracitoplasmatic inclusions of hyaline bodies are rarely reported in canine HCC, which is associated with altered protein production or transport. The same features are more common in humans, being present between 50 to 66% of HCC (MASSERDOTTI et al., 2014).

2.1.6.3 Histological subtypes and HCC grading

In veterinary were described 11 canine HCC histological subtypes, where the most frequent is the trabecular one (PATNAIK et al., 1981a; TRIGO et al., 1982). The trabecular pattern is arranged in thick trabeculae with a variable amount of well-differentiated neoplastic hepatocytes (more than 3-4), few vacuolated cells and rare intracytoplasmic bile pigment (PATNAIK et al., 1981a). Impregnation silver histological techniques are useful to define the trabecular limits in this pattern, by staining of reticulin fibers located in perisinusoidal spaces; however, those fibers seems to be scarcer in less-differentiated tumor grade (SHIGA; SHIROTA; NOMURA, 1996). The peliod pattern is characterized by extensive areas of vascular dilatations, occupied by erythrocytes, sometimes without presence of endothelium (ROONEY, 1959). The

pseudogladular subtype is composed of well distinguishable neoplastic hepatocytes creating pseudo acini that are capable to store proteinaceus material (PATNAIK et al., 1981a). This pattern can be confused with combined hepatocellular and cholangiocellular carcinoma, it is differentiated by Alcian Blue staining, which is positive to intraluminal Mucin in genuine tubular cholangiocellular structures (SHIGA; SHIROTA; ENOMOTO, 2001a). The pleomorphic subtype is defined through groups of pleomorphic cells with absent of trabeculae arrange but separated by stroma of connective tissue. The anaplastic subtype displays trabeculae arrange but reflecting a more aggressive behavior of anaplastic cells with infiltrative growth and high mitotic activity (PATNAIK et al., 1981a)The scirrhous subtype shows extensive areas of fibrous tissue that delimited groups of neoplastic hepatocytes, often this desmoplastic reaction is associated with extensive areas of necrosis (VAN SPRUNDEL et al., 2013). Other subtypes include cobblestone, peritheliomatous, clear-cell, solid and combined hepatocellular and cholangiocellular carcinomas. Results obtained in this study suggested that dogs with pleomorphic and anaplastic HCC subtypes are more susceptible to metastases (PATNAIK et al., 1981a). Regarding the histological grade, there is one study that adopt an Edmonson and Steiner's classification to separate canine HCC in well-differentiated (grade I-II), moderately differentiated (grade II-III) and poorly differentiated (grade III-IV) (SHIGA; SHIROTA; NOMURA, 1996). However, authors did not show a relation between prognosis and histological grade.

In humans, contrary to veterinary medicine, there is a clear distinction between growth patterns and HCC subtypes. For example, trabecular, pseudo acinar, solid and macrotrabecular are considered as well-defined growth patterns in human HCC (NZEAKO; GOODMAN; ISHAK, 1995). HCC subtype should have enough specific histological findings to diagnosis in combination with an exclusive molecular profile and a strong clinical correlation involving prognosis (WOOD et al., 2013). In this context, human HCC can be defined in 12 subtypes, as such carcinosarcoma, carcinosarcoma with osteoclast-like giant cells, cirrhotic mimetic, clear cell, combined hepatocellular carcinoma-cholangiocarcinoma, combined hepatocellular and neuroendocrine carcinoma, fibrolamellar carcinoma, granulocyte colony-stimulatin factor producing hepatocellular carcinoma, lymphocyte-rich and lymphoepithelioma-like, sarcomatoid, scirrhous, and steatohepatitic. Another 6 HCC variants are waiting for additional criteria to be considered as a tumor subtype, including combined hepatocellular carcinoma-cholangiocarcinoma with stem cell features, chromophobe subtype, hepatocellular

carcinoma with syncytial giant cells, lipid rich variant, myxoid and transitional (TORBENSON, 2017).

2.1.6.4 Immunohistochemical markers

Most of canine and human HCC are positive to monoclonal marker Heppar1, that also stain the cytoplasm of normal and hyperplastic hepatocytes and is useful to differentiate the hepatocellular origin (VAN SPRUNDEL et al., 2013). However, Heppar1 presents rare positivity to canine intestinal tumors; thus, it is not fully reliable in poorly differentiated liver tumors (RAMOS-VARA; MILLER, 2002). Furthermore, cytokeratin (CK) 7 marker is very sensible to identify bile duct cells (RAMOS-VARA; MILLER; JOHNSON, 2001). In routine, there is difficulty to differentiate among pseudoglandular HCC and canine cholangiocarcinoma, claudin-7 marker, which is protein of membrane tight junction, demonstrated be very specific to normal and neoplastic cholangiocytes cells and negative to normal and malignant hepatocytes, even to poorly differentiated (JAKAB et al., 2010). Conversely, in human researches was observed that caludin-7 stain weakly in hepatocytes cytoplasm which become a not feasible marker to differentiate these two tumors (HOLCZBAUER et al., 2014). For human liver neoplasms, many attempts had been made to differentiate HCC and Cholangiocarcinoma by immunohistochemistry such as AFP and carcinoembryonic antigen like hepatocyte and cholangiocyte marker respectively, however these markers resulted less specific. Otherwise, CK19 was seem such a very sensitive and specific marker to normal and neoplastic cholangiocytes (TSUJI et al., 1999).

Canine HCC presents progressive loss of CK 5/8 immunoexpression in accordance with a decreasing in cell differentiation. By the other way, AFP immunoexpression shows an opposed behavior, increasing its expression along with less cellular differentiation (MARTÍN DE LAS MULAS et al., 1995). AFP in canine neoplastic hepatocytes has an inverse correlation with its serum levels (KITAO et al., 2006b). In contrast, AFP have been related with equivalent immunostaining and serum levels in humans. Additionally, AFP stain has an inverse correlation with albumin stain, as neoplastic hepatocytes can loss the features to produce albumin and then adopt embryonic features with AFP production (KOJIRO et al., 1981).

HCC can present foci of hepatocytes-like cells with vimentin/cytokeratin co-expression. These foci are related to bipotential stem/progenitor cells proliferation in

both species, seen such oval, ductular or small hepatocytes (SARRAF et al., 1994; SHIGA; SHIROTA, 2000). In humans, HCC vimentin immunoexpression is related with poor prognosis and high metastasis risk, being usually associated with epithelial to mesenquimal transitional process (HU et al., 2004; ZHAI et al., 2014). In dogs, this feature was described in HCC, however the authors did not find any relation to prognosis (SARRAF et al., 1994; SHIGA; SHIROTA, 2000).

Recently, a new classification for canine HCC was proposed, in which a criterion of malignancy also explored in human HCCs is adopted (VAN SPRUNDEL et al., 2013) Criterion is based in the positivity of neoplastic hepatocytes to the CK19 Immunohistochemical antibody. This marker under normal conditions has reactivity to normal bile duct cells, hepatic progenitor cells with bipotential features and it is negative in normal hepatocytes (LIBBRECHT; ROSKAMS, 2002). These authors suggest that HCC positive in more than 10% of cells showed a more aggressive behavior than CK19 negative HCC, this occurs because neoplastic hepatocytes acquire bipotential capacities (DURNEZ et al., 2006; VAN SPRUNDEL et al., 2010)

2.1.7 MOLECULAR HEPATOCARCINOGENESIS

Some human HCC carcinogenic mechanisms were also identified in dogs by *in vitro* and *ex vivo* tests. In both species, HCC displays mutations in the c-MET, a proto-oncogene closely involved with growth hepatocyte factors. These mutations are located in different gene positions, but both are involved with the tyrosine kinase domain, which could suggests similar carcinogenic mechanisms (PARK et al., 1999; BOOMKENS et al., 2004). Additionally, canine HCC shows a similar expression of P-glycoprotein, which is linked to multidrug resistance protein (MRD)-1 gene and it is involved in chemotherapy resistance (TASHBAEVA et al., 2007). Finally, AFP, albumin and ceruloplasmin production were also identified in cell lines of canine HCC (BOOMKENS et al., 2004).

The maintenance and progression of HCC is conditioned to its microenvironment, in which converge several growth factors and its receptor, such as platelet-derived growth factor-β (PDGF-β), transforming growth factor-α (TGF-α), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF). Growth factors have an important role in the hepatocarcinogenesis and they can be related to tumor progression, metastasis and

therapeutic response (FURUSE, 2008). It is the case of the insulin-like growth factor (IGF)-II, which is involved also in vascular proliferation of HCC through stimulation VEGF in a hypoxic microenvironment (KIM et al., 1998). Instead of several growth factors are interconnected in HCC progress, VEFG has been enrolled as an independent prognosis value in humans (POON et al., 2004) and it is used as one of the first therapeutic targets in non-resectable HCCs (THOMAS et al., 2007). In addition, angiopoietin 2 have a direct influence in the microvessel density and tumor size in human HCC (MOON et al., 2003). Similarly, one case of canine HCC showed higher expression of angiopoietin 2 in comparison to normal canine tissue and others neoplasms, becoming a potential biomarker predictor (KATO et al., 2006). Also, the gene expression of PDGFR-β was overexpressed in canine HCC in comparison to normal liver and nodular hyperplasia. Although high levels of this growth factor was identified, the exactly mechanisms and how they contribute to hepatocarcinogenesis remains unclear (IIDA et al., 2014).

It is well known that progenitor cells are able to differentiate in hepatocytes or cholangiocytes, which have capacity to self-renew, accumulating mutations therefore being possibly implicated in HCC carcinogenesis (MA et al., 2010; FUJIMOTO et al., 2013; MICHISHITA et al., 2014)**.** Cancer stem/progenitor cells or tumor-initiating cells have been reported in canine HCC cell lines by CD90, CD133, CD34 and CD13 markers. *In vivo* study also detected the CK19 and CD44-positive progenitor cells in canine tissue of HCC (COGLIATI et al., 2010). In addition, the co-expression of HepPar1 and CK7 in canine HCC cell lines suggested its capacity of stem/progenitor cells as well its role in the carcinogenic process (BOOMKENS et al., 2004).

MicroRNAs (miRNAs) are a class of small noncoding RNAs of ∼22nt in length which are involved in the regulation of gene expression at the posttranscriptional level (KROL; LOEDIGE; FILIPOWICZ, 2010). Several miRNAs were described in human HCC and have an important role in the carcinogenic process (TOMIMARU et al., 2012). In canine HCC, miR200c was overexpressed in comparison to other hepatobiliary diseases (DIRKSEN et al., 2016). Additionally, a decreasing of MiR-1 levels was linked to c-MET overexpression in canine HCC cell lines. These cells acquired extra proliferation and metastasis features (LAI et al., 2018). In human HCC cell lines, the c-MET activation also showed a negative correlation with miR-1 expression, with a reduction of proliferation and apoptosis activation in these cells (DATTA et al., 2008).

2.1.8 THERAPEUTIC APPROACHES

In dogs, total surgical resection is an effective treatment with a survival time longer than 756 days. On the other hand, no complete resection increases the recurrence and death indices in about 100%, related with tumor progression (MATSUYAMA et al., 2017). In addition, some authors reported 4.8% of deaths related to surgical procedure in massive HCC (LIPTAK et al., 2004a). Usually deaths are not linked to the hepatic disease in HCC-bearing dogs; however, animals that are not submitted to surgical approach has a 15 times increased risk of death (LIPTAK et al., 2004a). Normally the surgical resection in human HCC is not enough successful, mainly because most times it is associated with end-stage liver disease. Therefore, tumor recurrence after partial or total hepatectomy is frequent, besides the impaired liver function. In the majority of cases, the best way is the liver transplantation, which has additional problems related to organ availability and increased risk of death due other factors such bleeding and transplant rejection. In humans, the majority of HCC are not resectable, of the group of patients who undergo surgery, 35% survive more than five years in the case of partial hepatectomy and only 12% of liver transplant cases have similar survival rates. (RINGE; PICHLMAYR; WITTEKIND, 1991).

Despite lobectomy being considered the golden standard for canine HCC therapeutic, the surgery approach can be more complex if critical anatomical structures are involved, such as the cava caudal vein(LIPTAK et al., 2004a). En bloc technique is the term used to liver neoplasms resection which includes another organ or structure. In humans, it is a method used with inferior caudal vein resection but it is recommended just in few case due the high risk of death (HEMMING et al., 2004). In veterinary, there is one case described in a female Shih Tzu, which was submitted to a successful resection en bloc of a massive HCC involving the cava caudal vein (SEKI et al., 2011).

Non-resectable canine HCC has poor prognosis and chemotherapy protocol with gemcitabine showed low efficacy against these tumors (ELPINER et al., 2011). The same difficulty was found in human HCC, where the improvement in patient condition is poor to moderated even using chemotherapy combinations. Currently, some of these combinations in human patients includes gemcitabine, oxaliplatin and sorafenib. The last one is known as tyrosine kinase inhibitor widely used to non-resectable human HCC (PATRIKIDOU et al., 2014; SRIMUNINNIMIT; SRIURANPONG; SUWANVECHO, 2014).

Previously, dogs was used such experimental model to asses hepatic tissue response induced by direct application of ethanol in healthy animals (KAWANO, 1989). Then pharmacologic therapies were applied in HCC-induced dogs to produces direct necrosis in neoplastic nodules (IWASAKI et al., 1995). Even direct heat application in hepatic nodules was tried in dogs by needle-type heater (HARIHARA, 1988). In one study, the percentage of necrosis obtained, in small human HCC, with two different techniques was evaluated: direct ethanol injection vs radiotherapy frequency ablation. Results showed a total tumor necrosis in 80% of cases using ethanol injection and 90% with the radiotherapy frequency ablation technique (LIVRAGHI et al., 1999).

In veterinary, the three dimensional radiotherapy was applied in six dogs with non-resectable HCC, showing minimal improvement in tumor size, However, a high tolerability was observed at radiation doses applied in all patients, this opens the possibility to new studies to reach more effective doses improving survival time in dogs with non-resectable HCC. (MORI et al., 2015). In humans, conventional radiotherapy is considered harmful to non-neoplastic liver tissue and the survival time is slightly increased (LAWRENCE et al., 1992; ROBERTSON et al., 1993).

Direct microwave ablation was used recently in one dog with hepatic neoplasm and showed positive results with reduction of nodule before resection (YANG et al., 2017). In human, the microwave ablation improved the survival expectations in patients with solitary tumors smaller than 4 cm and cirrhosis classified as Child-Pugh A score (LIANG et al., 2005).

The chemoembolization is used to keep located and concentrated the anticancer drug by occlusion of specifically vessels. In hepatic nodules, hepatic artery should be occluded without affect non-neoplastic liver since normal liver receives most of blood supply by portal vein (VALJI; MARONEY, 1999; DYET et al., 2002). Guide chemoembolization was performed in a HCC-bearing dog; however the results were not satisfactory (WEISSE et al., 2002). Furthermore, human HCC subjected to transarterial chemoembolization just represent a slight improvement in 2 years of overall survival in comparison with patients without active treatment (CAMMA et al., 2002).

3 OBJETIVES

3.1 GENERAL OBJETIVES

To assess the presence of mutations in the TP53 gene in canine hepatocellular carcinoma, indirectly determining whether prior exposure to aflatoxin B1 would be involved in hepatocarcinogenesis in dogs

3.2 SPECIFIC OBJETIVES

3.2.1 To determine the presence of specific mutation in the canine TP53 gene, corresponding to codon 249 in human hepatocellular carcinomas.

3.2.2 To evaluate the p53 immunuoexpression in canine hepatocellular carcinomas.

4 MUTATIONAL ASSESSMENT OF TP53 GENE IN CANINE HEPATOCELLULAR CARCINOMA

4.1 INTRODUCTION

Liver canine neoplasms represent between 0.6 to 1.3% of canine tumors (PATNAIK; HURVITZ; LIEBERMAN, 1980; PATNAIK et al., 1981a; TRIGO et al., 1982), being hepatocellular carcinoma (HCC) the most common hepatic neoplasm (HIROSE et al., 2014; CONSTANT et al., 2016). Despite there is no clear evidence of a gender-related incidence and breed predisposition, higher risk to HCC has been related in male dogs older than 10 years and Shih-Tzu (Patnaik et al., 1981; Hirose et al., 2014). Prognosis is usually poor when the tumor is lately diagnosed (KAWARAI et al., 2006). In early diagnosis, the prognosis depends on surgical viability and/or the complete resection of the tumor (MICHISHITA et al., 2014). The etiology of HCC remains unclear; nevertheless, potential agents such as aflatoxins, nitrosamines, hepatic parasites and radioactive compounds were suggested formerly (NEWBERNE; BUTLER, 1969; HIRAO et al., 1974; IAKOVLEVA, 1984; HAHN et al., 1997).

In human, HCC is also the most frequent liver neoplasm, being the fifth more frequent cancer worldwide (OZAKYOL, 2017)HCC is related with high mortality and poor prognosis with 600.000 deaths per year (ALTEKRUSE; MCGLYNN; REICHMAN, 2009). Recognized etiologies leading to human HCC are viral C (YOSHIZAWA H., 2002; FERENCI et al., 2010b) and B (BURNS; THOMPSON, 2014), alcohol abuse (MASSARWEH; EL-SERAG, 2017),(CHOLANKERIL et al., 2017) non-alcoholic steatohepatitis (NASH) , aflatoxins chronic intoxication (NOGUEIRA et al., 2009), and others.

Several HCC similarities have been related in both species, such as gross pathology (EGGEL, 1901; PATNAIK et al., 1981a; OKUDA; PETERS; SIMSON, 1984), histopathology (PATNAIK et al., 1981a; NZEAKO; GOODMAN; ISHAK, 1995; TORBENSON, 2017) and molecular mechanism (DATTA et al., 2008; LAI et al., 2018). However, canine HCC usually lacks a background chronic liver disease (PATNAIK et al., 1981a; TRIGO et al., 1982) and some risk factors, as chronic viral hepatitis and alcohol intake. In this context, the exposure to environmental toxins common to both species, in special the mycotoxins, could be involved in the hepatocarcinogenesis.

Aflatoxin B1 (AFB1) is a mycotoxin produced mainly by the fungus *Aspergillus flavus and Aspergillus parasiticus* (WILLIAMS et al., 2004). First identified in peanut flour that produced acute hepatic toxicity with high mortality in chickens, ducks and turkeys (BLOUNT, 1961; LANCASTER; JENKINS; PHILP, 1961; SARGEANT et al., 1961). AFB1 has been identified in oilseeds, dried fruits, meat and milk. Moreover, *Aspergillus flavus* growths in conditions of relative humidity of 85% and temperatures of 25-32°C concerning food storage in deficient conditions (WILLIAMS et al., 2004; STROSNIDER et al., 2006). AFB1 is found in many countries, especially in Asian and African continents (OZAKYOL, 2017). In other subtropical countries such Brazil, AFB1 is also an important health problem and it is involved in 28% of human HCC cases (NOGUEIRA et al., 2009). In addition, 12-50% of commercial animal food in Brazil is contaminated with AFB1, with quantities ranging to 50 µg/Kg (MAIA; PEREIRA BASTOS DE SIQUEIRA, 2002; WOUTERS et al., 2013b). One study described that 100% of pet food sold in bulk, a practice widely used in Peru and other South American countries, could be contaminated with AFB1 (PERALES VIZCARRA; CAMACHO PERALES; MORALES CAUTI, 2014). Due to the facts previously exposed we hypothesize that AFB1 contamination is a shared risk for HCC in both species and it could be a potential etiology candidate to canine HCC.

The carcinogenic mechanisms of AFB1 is mediated by its absorption through gastrointestinal system by the aflatoxin fat-soluble nature (AGAG, 2004). After, AFB1 is metabolized by P450 (CYP1A2, CYP2A6, CYP3A4) cytochrome action in liver leading to production of unstable molecules called 8,9 AFB1 epoxides (FORRESTER et al., 1990). These molecules realize covalent links affecting DNA and producing adduct AFB1-N7-guanine, which generate TP53 punctual mutation (ESSIGMANN et al., 1977; KENSLER et al., 1986). The HCC developed due to aflatoxin chronic intoxication is produced by a punctual mutation (R249S), which lead to a transversion G>T in the codon 249 (exon 7) of TP53 gene. This mutation causes an amino acid substitution of Arginine to Serine and, consequently, loss of pro-apoptotic P53 functions (BRESSAC et al., 1991; HSU et al., 1991). Finally, the R249S punctual mutation is considered an indirect marker of human HCC-induced carcinogenesis(GOUAS; SHI; HAINAUT, 2009).

The most of human cancer presents TP53 mutations among codons 120 to 290 (LEVINE et al., 1994), localized among exon 4 and 8 (WALKER et al., 1999). In comparison to dogs, TP53 gene has several similarities with the human gene, with

81% of homology. Both species contain 11 exons in the TP53 gene; however, the human gene count 13 additional amino acids in the exon 4 (CHU et al., 1998; VELDHOEN; MILNER, 1998). In addition, both species conserved evolutionary domains II with 98% of homology. Interestingly that regions show the most of mutations found in human cancers (KRAEGEL; PAZZI; MADEWELL, 1995). Regarding the point mutation, human 249 codon (AGG) is equivalent to (CGG) in canine TP53 gene, which lead to production of the same protein Arginine (SOUSSI; DEHOUCHE; BÉROUD, 2000; MAYR; REIFINGER, 2002)

TP53 gene has been assessed in several neoplastic diseases in dogs, as circumanal gland tumors (MAYR et al., 1997), cecum anaplastic carcinoma (MAYR; REIFINGER, 2002), hemangiosarcoma (MAYR et al., 2002), and mammary tumors (MUTO et al., 2000). In addition, a detected germline p53 mutation could be a predisposal factor in Bull Mastiffs to lymphomas (VELDHOEN et al., 1998). As well, several canine tumors have been submitted to p53 immunohistochemistry, as in colorectal tumors (WOLF et al., 1997), lymphomas B and T-cells (SUEIRO; ALESSI; VASSALLO, 2004), multicentric lymphosarcomas (DHALIWAL et al., 2013), mast cell tumors (GINN et al., 2000), and melanomas (ROELS; TILMANT; DUCATELLE, 2001; KOENIG et al., 2002). P53 overexpression have no prognostic value in these studies; however, the TP53 gene mutation has been associated as a tumor initiator (GINN et al., 2000).

The present study aims to evaluate the canine TP53 gene mutations in hepatocellular carcinomas and its possible association with AFB1 intoxication. In addition, the p53 and cellular proliferation will be assessed by immunohistochemistry.

4.2 MATERIALS AND METHODS

4.2.1 Sample collection and inclusion criteria

Twenty-four liver tumor samples from dogs that underwent surgical treatment at PROVET Veterinary Hospital, São Paulo, Brazil, were collected in a prospective study from May 2017 to November 2018, after the owner's dog permission. This study was approved by the institution's ethics committee (CEUA nº 7153030217) and it was performed at the Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo (FMVZ-USP), São Paulo, Brazil.

The liver tumors were analyzed immediately after the surgical removal, which were photographed and measured by an automatic pachymetry in three dimensions. Then, the volume was calculated in cm³ and it was normalized by the “dog’s size”. After, tumors were systematically sectioned and fragments were immersed in 10% neutral buffered formalin for 24 h. Other fragments were preserved in RNAlater® (Qiagen) for 24 h and were storage at -80ºC freezer for posterior DNA extraction and molecular analysis.

Criteria for inclusion were (1) a diagnosis of resectable liver nodules by computerized tomography (CT), (2) absence of other neoplasm evident at the time of diagnosis, (3) histopathology diagnosis of pure or mixed hepatocellular carcinoma (HCC), and (4) follow-up was available for at least 6 months post-surgery. For each animal, the following clinicopathological features were recorded: age, breed, number and hepatic location of tumor lesions, tumor size, and overall survival (OS) time.

4.2.2 Histologic methods and criteria

Each liver tumor was fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5 µm-thick sections for Hematoxylin-Eosin (HE) and reticulin staining. Liver carcinomas were classified according with the most relevant references in canine HCC regard histological analysis (PATNAIK et al., 1981; MEUTEN; CAPEN, 2002; JUBB; STENT, 2016) . The recorded histological types included trabecular, pseudoglandular, peliod, solid, cobblestone, peritheliomatous, clear-cell, anaplastic, pleomorphic, and combined hepatocellular-cholangiocellular (HCC-CC) carcinomas. In these instances, the largest growth pattern of the tumor determined the subtype (more than 80%). The mitotic index was determined by the count of neoplastic hepatocyte mitosis in 10 high-power fields (400x, field diameter 0.625 mm).

4.2.3 Immunohistochemistry

Manual immunohistochemistry was used to detect p53, cytokeratin 19 (CK19) and the proliferation marker Ki67 in hepatic liver tumors. Further details on the IHC protocol are recorded in Table 1. FFPE tissues were sectioned at 5 µm, deparaffinized in xylene, hydrated in graded ethanol, and washed in *tris-buffered saline*, 0.1% *Tween* 20 (TBST). Heat-induced antigen retrieval (HIAR) was carried out by boiling sections

in a pressure cooker in citric acid buffer (pH 6.0) or EDTA buffer (pH 9.0). Endogenous peroxidase activity was blocked by incubating the tissue sections in 3% hydrogen peroxide for 30 min at room temperature. To reduce non-specific staining, sections were incubated with protein block for 10 min at room temperature. Sections were then incubated with the primary antibodies overnight (16 hours) at 4ºC (Table 1). The staining was performed using a modified streptavidin-peroxidase conjugate method based on the poly-HRP anti-rabbit IgG detection system (Reveral polyvalent HRP-DAB detection System SPD-125 - Spring BioScience), following the manufacturer's guidelines. The peroxidase activity was developed with DAB chromogen (3,3 diaminobenzidine) for 5 min. Finally, tissue sections were counterstained with Mayer's hematoxylin (Merck, New Jersey, USA), dehydrated and mounted with Entellan® mounting medium (Merck Millipore, Darmstadt, Germany). Positive controls were described in Table 1 and negative controls were obtained using a mouse/rabbit isotype control antibody.

The Ki-67 proliferation index was determined by dividing the number of tumoral cells showing positive nuclear immunostaining per 1000 tumor cells analyzed over at least three high-amplified microscopic fields (400×). For CK19 immunoexpression, liver tumors were considered positive when more than 10% of the neoplastic cell cytoplasm expressed CK19, as previously reported by van Sprundel et al. (2013).

Table 1: Summary of antibodies and conditions of use.

Antigen	**Clone (Manufacturer)**	**Dilution**	**Antigen retrieval**	**Positive control**
p53	Pab240 (BD Biosciences)	1:100	EDTA buffer (pH 9.0); 20 min in high pressure cooker.	Canine gastric carcinoma
CK19	B170 (Novocastra)	1:500	EDTA buffer (pH 9.0): 20 min in high pressure cooker.	Bile duct cells (internal control)
Ki67	MIB-1 (Dako/Agilent)	1:1000	Citrate buffer (pH 6.0): 20 min in high pressure cooker.	Canine lymph node

4.2.4 DNA extraction and PCR protocol

Approximately 50 mg of frozen tumor tissue was used for DNA extraction by salting out method (MILLER; DYKES; POLESKY, 1988). DNA pellets were dissolved in ultra-pure water. DNA concentrations and purity were determined by the ratio of O.D. at 260/280 nm in a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reactions were carried out with Super Master Mix (Life Technologies, Carlsbad, CA, USA) containing 200 nM of primers (Table 2) and 5 µl of DNA in a final volume of 25 µl. The following amplification conditions were used: initial step of 94ºC for 3 min; 34 cycles at 94ºC for 1 min, 62ºC (exons 3-4), 61.6ºC (exons 7-8) or 63.3ºC (exons 5-6) for 1 min, 72ºC for 1 min and then a final extension step at 72ºC for 10 min in a thermocycler (Eppendorf Mastercycler 5332 thermocycler Ag 22331 Hamburg, Germany). PCR products were electrophoresed in 1% agarose gel, stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and visualized under UV illumination (ChemiDoc™ MP Imaging System, Bio-Rad Laboratories, Inc., Hercules, California, EUA). The negative control used was double-distilled water.

Table 2: The primers set used for PCR, according to York et al. (2012).

Exons	**Primers sequences**	**Molecular weight**
Exons 3-4	CCCTCTGAGCCAGGAGAC *forward* GAAAGCCCAAGGTCAAGG *reverse*	748bp
Exons 5-6	CGTCTGCCTTTGGTTCAG *forward* TGCCTCTGCACTCCTCAC *reverse*	700 bp
Exons 7-8	AGGTGGGTCATCCCATTC *forward* GAGGCAGGCTCCCTACAG *reverse*	736bp

4.2.5 Sequencing protocol

PCR products were purified with ExoProStar™ S 500 (Sigma-Aldrich, St. Louis, Missouri, USA) and the precipitation process was carried out with the ethanol-EDTA

protocol. For sequencing, 1 µl of BigDye 3.1 (*BigDye Terminator v3.1 Cycle Sequencing Kit*, Applied Biosystems, USA), 5 µl of 5X buffer, 0.5 µL of each primer (Table 2) in separate reactions, 40 ng of target DNA, and RNase- and DNase-free ultra-pure water were used for a final reaction volume of 20 µL in a capillary automated sequencer (*ABIPrism 3730 DNA Analyser*, Applied Biosystems, USA). The following was used: initial step of 96ºC for 1 min; 40 cycles at 96ºC for 15 sec, 62ºC (exons 3-4), 61.6ºC (exons 7-8) or 63.3ºC (exons 5-6) for 15 sec, and 60ºC for 4 min. The quality of the chromatograms generated for each primer (forward and reverse) of each sample was evaluated using the online application Phred (http://asparagin.cenargen.embrapa.br/phph/1) and then manually edited using the Chromas Lite v. 2.1.1. software (http://technelysium.com.au/?page_id=13). The final sequence of each sample was obtained using the application Cap-contig of the BioEdit v.5.0.9 software (www.mbio.ncsu.edu/bioedit/bioedit.html) and subjected to a homology search for other sequences deposited in GenBank using the Blast 2.2.29 software (http://www.ncbi.nlm.nih.gov/blast/).

4.2.6 Statistical analysis

Kruskall-Wallis test was applied for comparisons between more than two groups; the significant results under this technique were complemented with the Hoc Dunn test for multiple comparisons with Bonferroni correlation for P value. The comparison between only two groups was made applying the Mann Whitney test considering *p* and *r* value. For the correlations in relation to the frequency of patients with tumor in the right lobes and frequency of deaths, the Fisher exact test was applied. When survival was analyzed in relation to time, Kaplan Meier curves were applied with calculation of the *p* value by log rank. Finally, the numerical variables were analyzed with Spearman's correlation test (P value < 0.05). Statistical analyzes were carried out in the R language (v3.4.3, open source) and supported by the Apache OpenOffice software (v.4.1.3, open source) for data processing. R packages used were hhhplot2, Hmisc, coin, FSA, ggsignif, survival, survminer and ggfortify.

4.3 RESULTS

4.3.1 HCC Patients

Regarding the breeds, Golden Retriever and mixed breeds were more affected with 5/24 (21%) each (Figure 1A). There is no sex predilection, being 11/24 (46%) males and 13/24 (54%) females. The affected animals aged 11.16 ± 2.0 years (range from 6 to 16 years) (Figure 1B). All data patient is summarized in Appendix A.

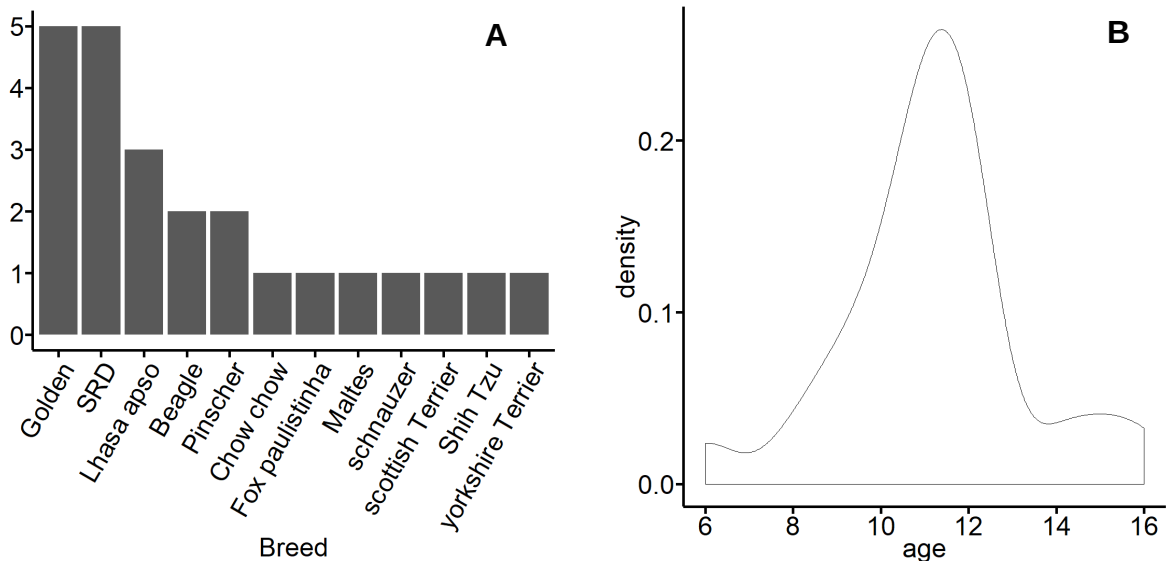


Figure 1: (A) Distribution of breeds in relation to the number of cases. **(B)** Population density in relation to age.

The majority of animals (20/24, 83.4%) presented solitary tumors, 3/24 (12.5%) had 2 nodules and just 1/24 (4.1%) dog had six neoplastic nodules, totalizing 32 hepatic nodules. These lesions were distributed in the hepatics left lobe (13/32, 41%), right lobe (9/32, 28%), caudate lobe (7/32, 22%) and quadrate lobe (3/32, 9%) (Figure 2).

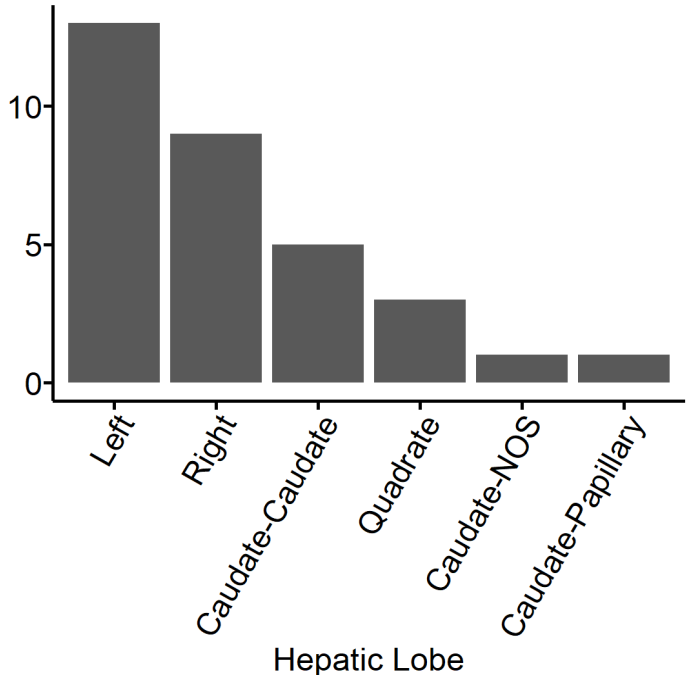


Figure 2: Different liver lobes affected in association with number of cases.

4.3.2 Gross and microscopical pathology

Considering the largest tumor size in each case, toy breeds were consistently affected by small tumors (212.6 ± 219.3 cm^3). On the other hand, contradictory to the expectations the large breeds were affected by small nodules and the small breed by large nodules (Figure 3). In addition, large tumors were more frequently involved with less degree of differentiation and vascular invasion (Figure 4). Macroscopically, massive pattern was the most common being found in (17/24, 70%) cases, followed by the nodular pattern and diffuse pattern, in 6/24 (25%) and 1/24 (4%) of cases, respectively.

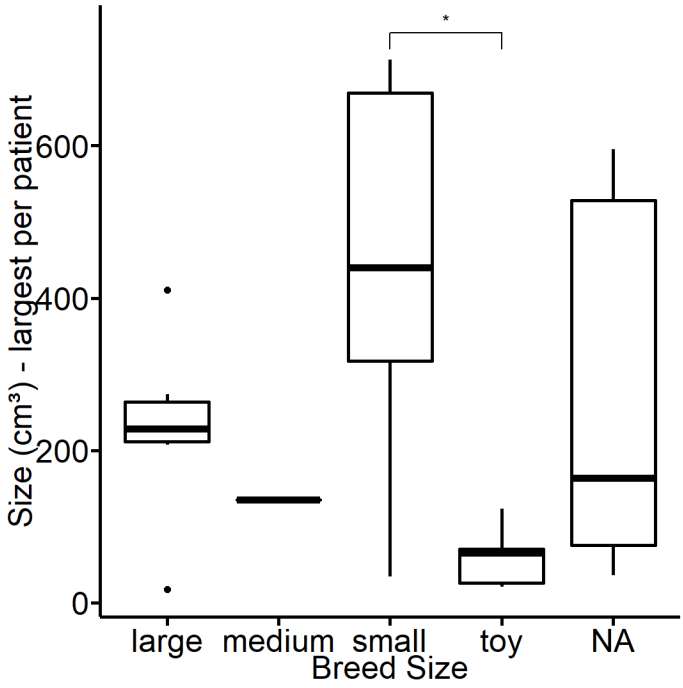


Figure 3: Distribution of the largest tumor volume per case in relation to the groups of breed sizes.

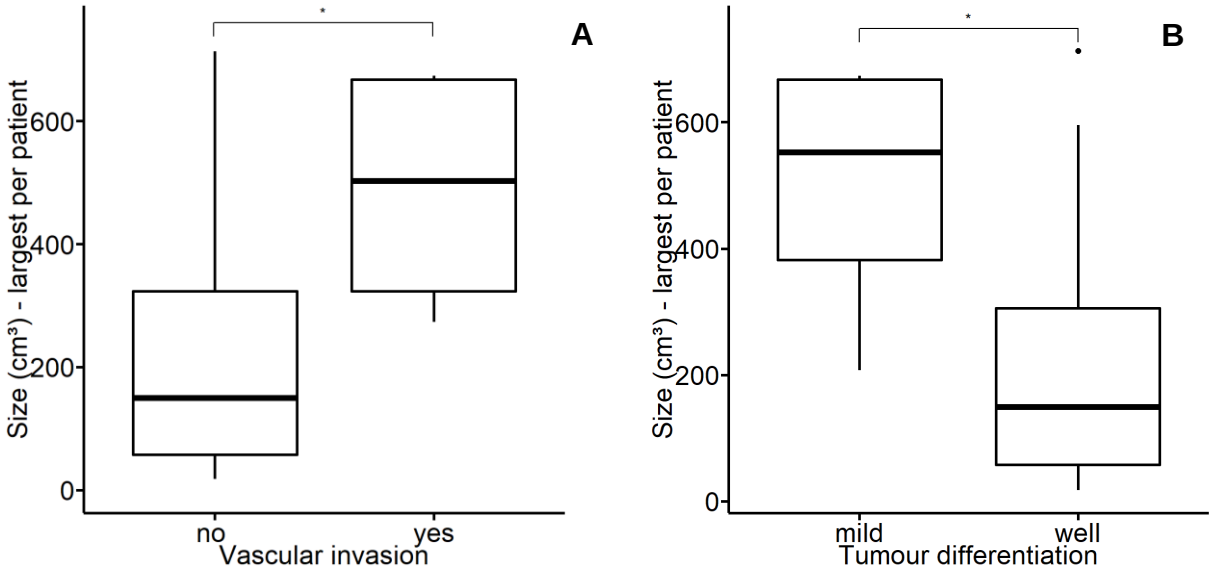
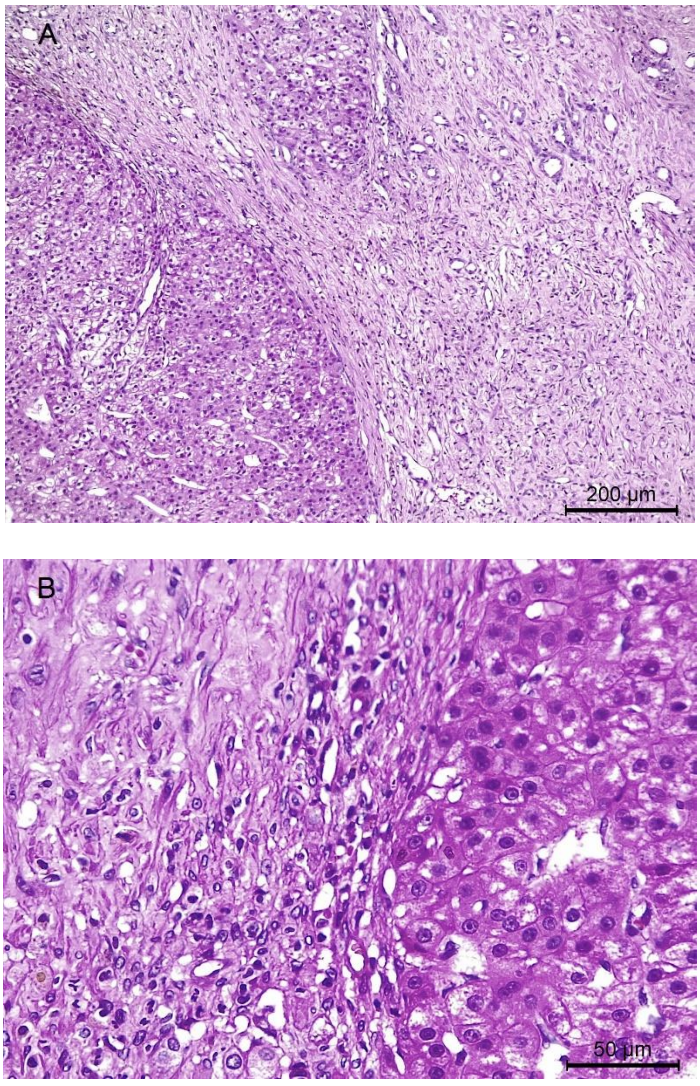


Figure 4: Association of tumor size with degree of cellular differentiation (A) and vascular invasion (B).

Microscopically, the trabecular growth pattern was the most frequent detected, being present in (10/24, 42%) cases of hepatocellular carcinomas. The other HCC cases were classified as SSD subtypes in (4/24, 17%) (Figures 5A-C), clear cells (3/24,

13%), pelioid (3/24, 13%), solid (2/24, 8%), pseudoglandular (1/24, 4%) and mixed tumor (1/24, 4%). Regard cellular differentiation (4/24, 17%) of cases were moderately differentiated and (20/24, 83.3%) were well differentiated. The mitotic index was relatively low in all HCC cases, (3±2.88 mitosis, ranged from 1 to 14 mitosis). It was observed that the highest mitotic index corresponded to moderately differentiated tumors (Figure 6A), SSD group and solid patterns (Figure 6B), and tumors with vascular invasion (Figure 7).



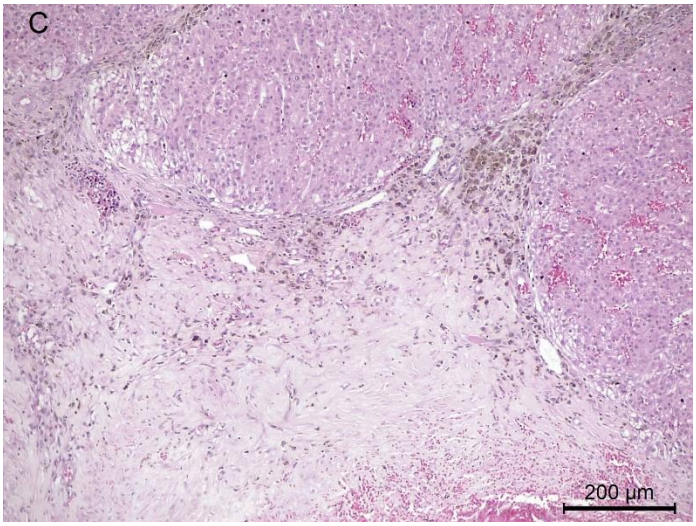


Figure 5: (A) Scirrhous pattern, extensive areas of fibrous tissue separating neoplastic hepatocytes in nests and sheets. 100×, HE. **(B)** Sarcomatoid pattern, HCC concomitant with fibrous tissue with high-cell density. 400×, HE. **(C)** Desmoplastic areas with low cell density and presence of brown pigments 100×, HE.

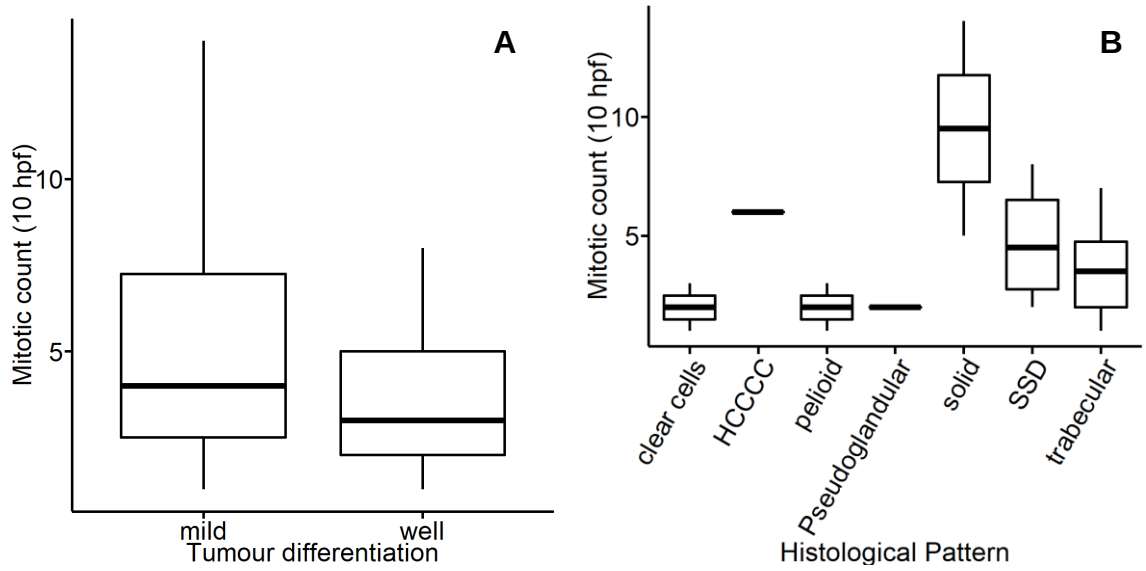


Figure 6: (A) Mitotic index according to the degree of cellular differentiation. **(B)** distribution of mitotic indices according to pattern or histological subtype.

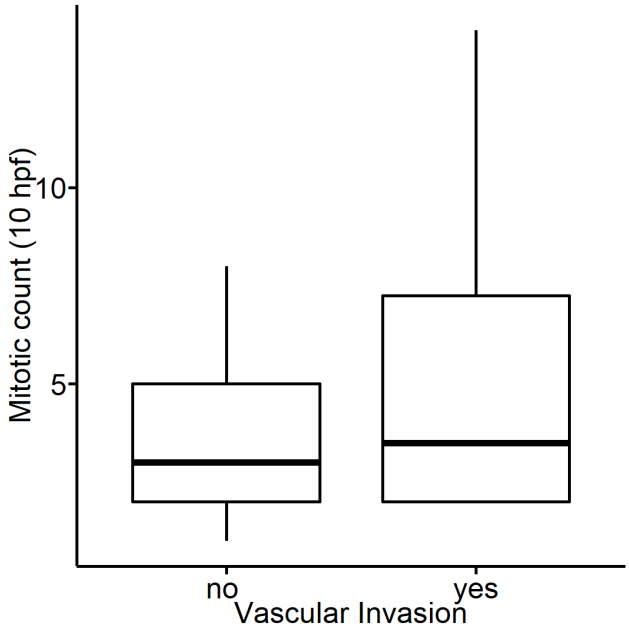


Figure 7: High mitotic indices related to a greater frequency of vascular invasion.

The proliferation index, measured by counting the Ki67-positive nuclear neoplastic hepatocytes, showed low values for all HCC (2.57 ± 0.04 %, ranging from 0 to 18.6%). Similar to the mitotic index, the cell proliferation index was higher in SSD subtypes (18.6%) (Figure 8A). However, no statistical correlation was found between the two variables (Figure 8B). Finally, the intensity of the Ki-67 staining varied being intense staining in some cases and weak in others (Figure 9A-B).

Moreover, the CK19 immunoexpression was negative in the neoplastic hepatocytes from all tumors. On the other hand, CK19 staining was limited to small foci of normal cells, probably liver progenitor cells (Figure 10A). One exception was demonstrated in the case of combined hepatocellular-cholangiocellular carcinoma, where the CK19 expression was present in the cholangiocellular compartment (Figure 10B).

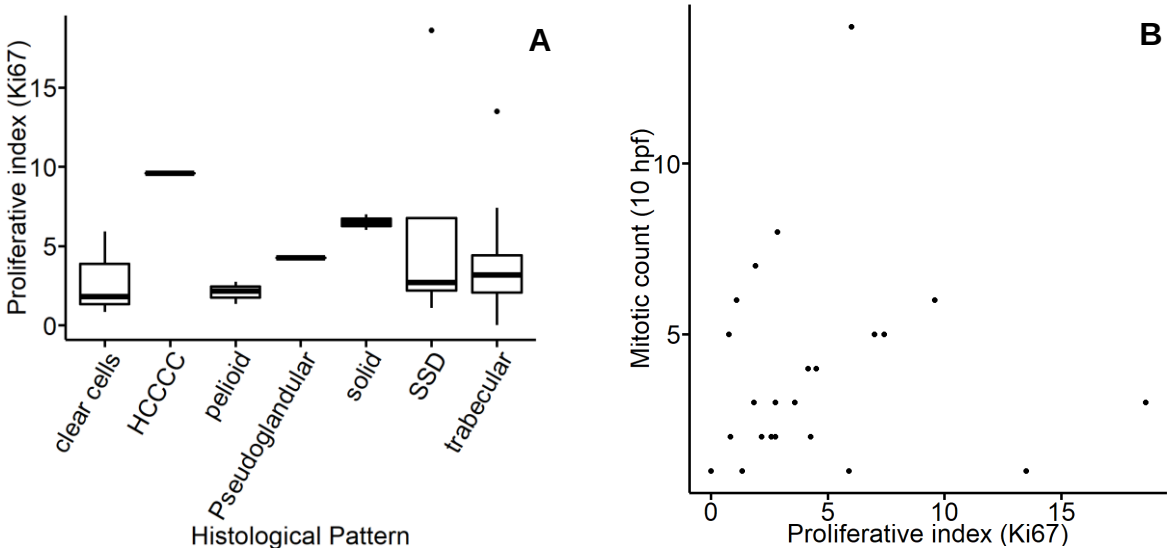


Figure 8: (A) Ki67 index in each histological different subtype and pattern. **(B)** Lack of distribution linear between ki67 and mitosis indices.

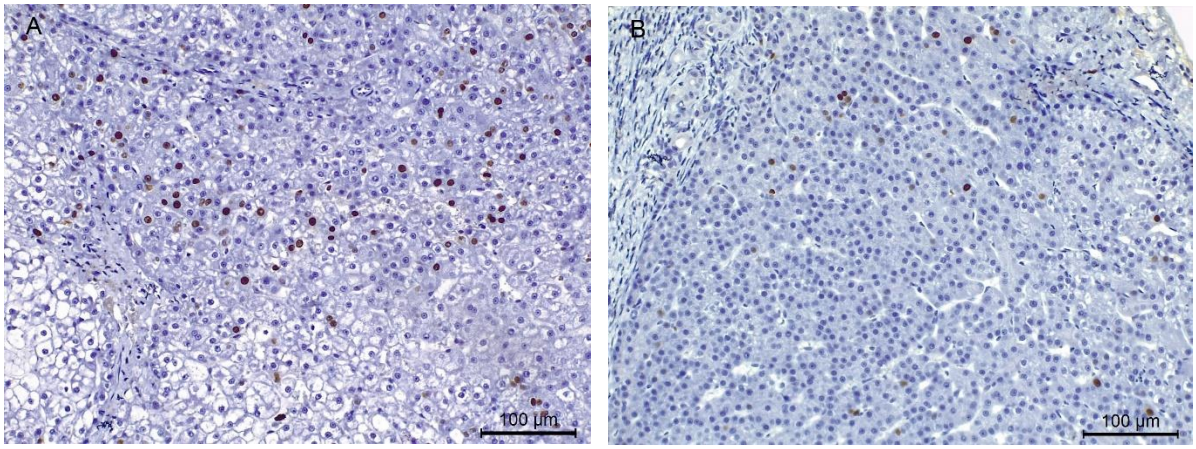


Figure 9: (A) High and strong nuclear staining for Ki-67 in canine HCC. 200×. **(B)** Low nuclear staining index for Ki-67 in canine HCC. 200×. Immunohistochemistry for Ki-67.

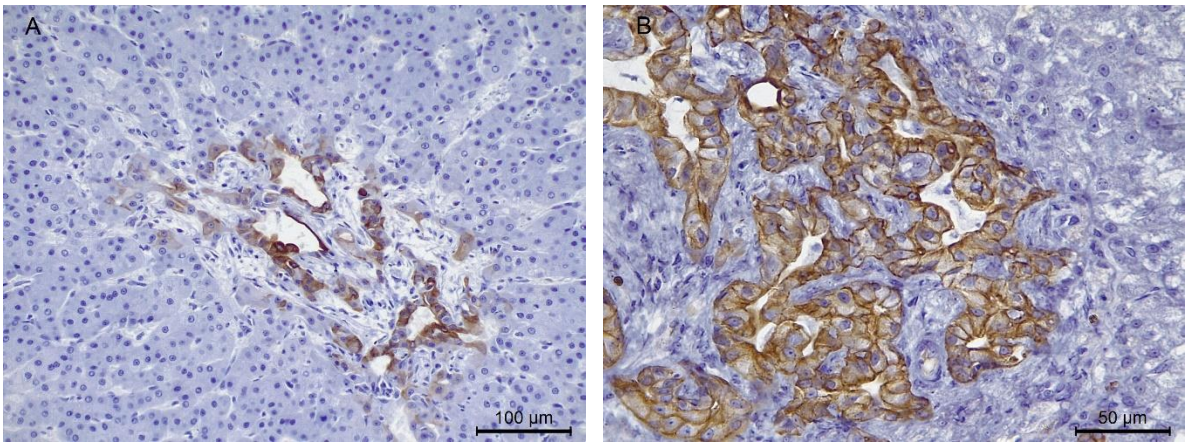


Figure 10: (A) Small group of normal cells positive for CK19 into the canine HCC. 200×. **(B)** Intense staining for CK19 in the cholangiocellular component in a mixed HCC tumor. 400×. Immunohistochemistry for CK19.

4.3.3 p53 assessment

All cases of HCC were negative for p53 immunohistochemistry and no TP53 gene mutation was found. The positive control of canine gastric carcinoma showed intense nuclear staining for p53, showing a positive immunoreaction with the canine tissue (Figure 11). The majority of chromatograms had a good quality for genetic analysis; however, some cases showed some technical issues (Figure 12A-B).

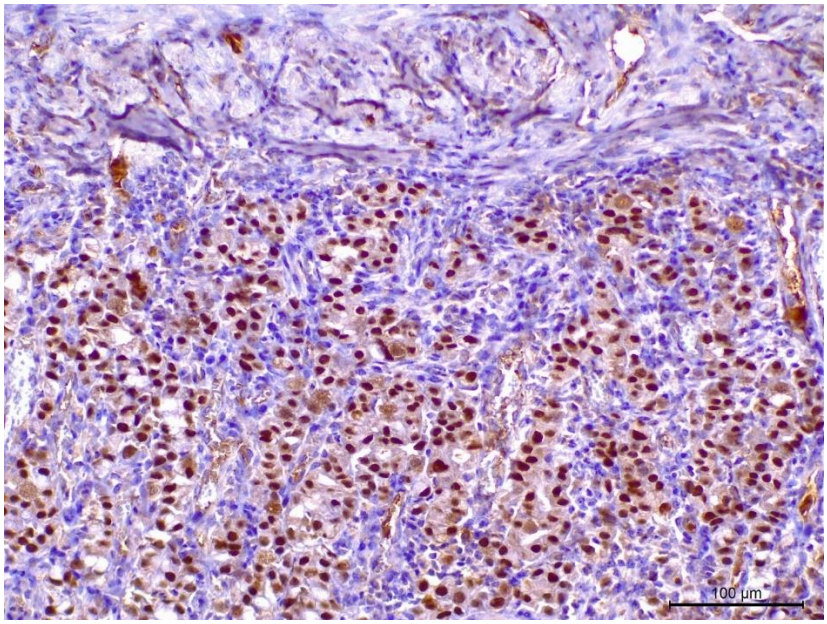
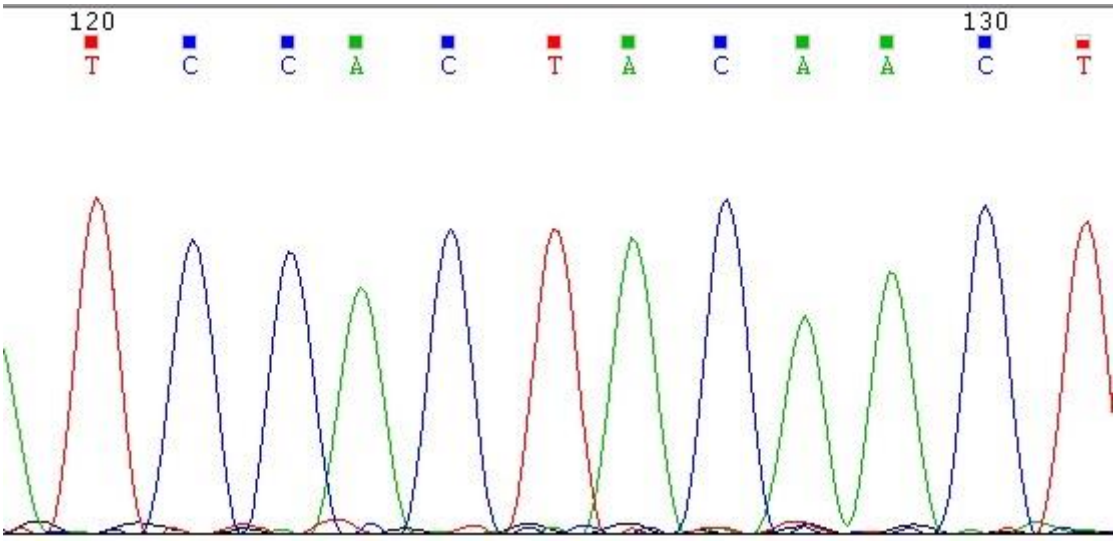


Figure 11: Positive nuclear label for p53 in a canine gastric carcinoma used as a positive control of the antibody. 200×. Immunohistochemistry for p53.

A



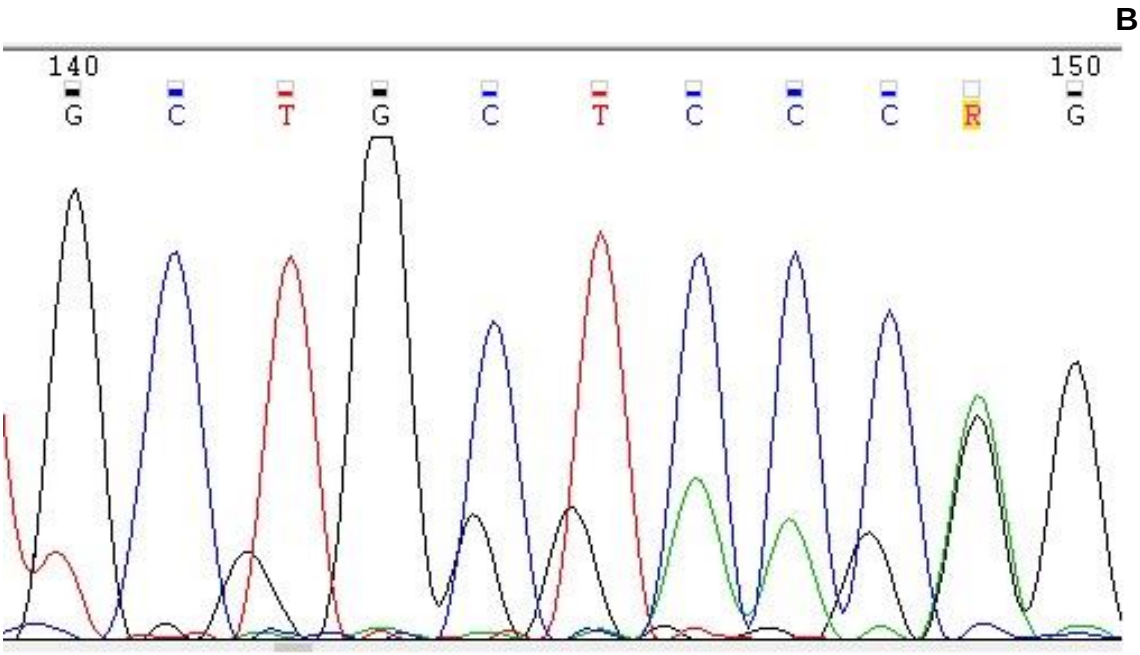


Figure 12: (A) High quality chromatograms showing unique and well-defined peaks **(B)** high and low peaks of different nucleotides in the same positions considered as noise in the sequence.

4.3.4 Follow-up

The overall survival (OS) time of HCC-bearing dogs was 281.04 ± 179.61 days. Three dogs died in the first 24 hours after surgery by post-surgical complications, which had tumors in the right hepatic lobes. However, there is no statistical difference related to the tumor location (p=0.0573). Others findings without statistical significance were decreased survival probability and OS in patients with vascular invasion and moderately differentiated tumors (p=0.45, p=0.089, respectively).

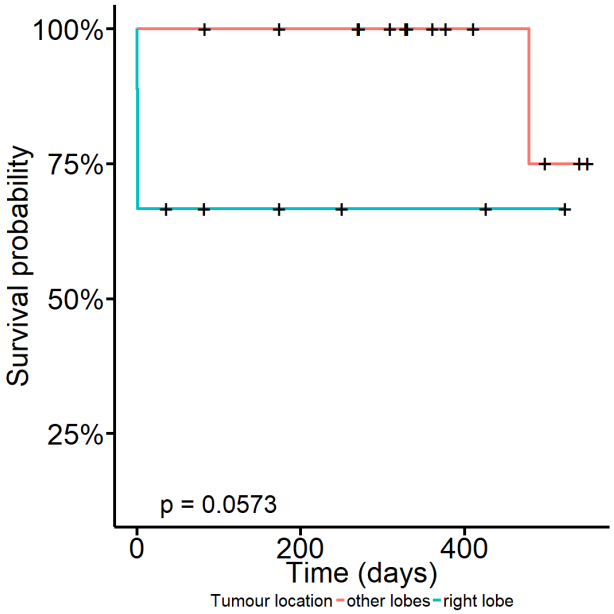


Figure 13: Decrease in survival probability in patients with HCC in right hepatic lobes.

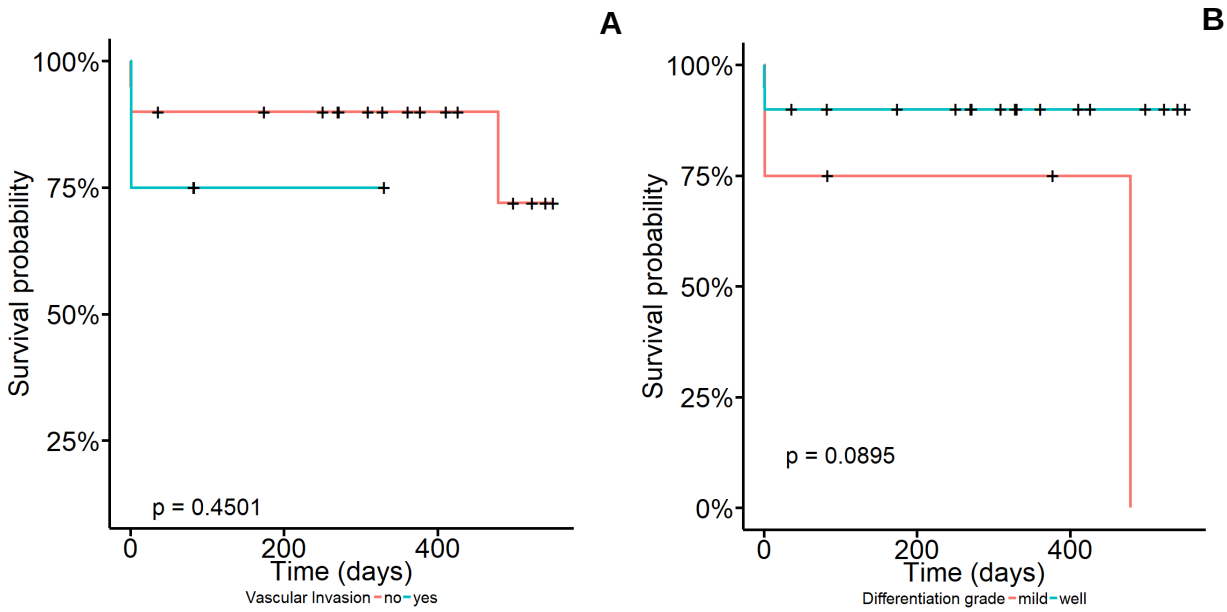


Figure 14: (A) less probability of survival and life time in patients with vascular invasion compared with patients without vascular invasion. **(B)** probability of decreased survival in patients with moderately differentiated tumors compared with patients with well-differentiated tumors.

5 DISCUSSION

In relation to the breeds, we observed that Golden Retriever and mixed breeds were more affected by HCC than others; however, without significative difference. The data related to breed predisposition in the literature is still contradictory. Hirose et al. (2014) analyzed a large casuistic in the city of Tokyo and showed that Shih Tzu dogs were the most affected by HCC. This finding would be expected to be a local breed; differently, we found the Shih Tzu breed only in 2/24 (8%) cases. In opposition, other researches have not described racial predisposition (PATNAIK et al., 1981; TRIGO et al., 1982). Regarding sex predisposition, we did not find a significant difference in the proportion of cases. This result was in accordance to some researches (TRIGO et al., 1982; LIPTAK et al., 2004a), but in disagreement with other ones (PATNAIK et al., 1981; HIROSE et al., 2014), which claim that male dogs are more frequently affect by HCC. Finally, we obtained an average age consistent with several authors which report an average close to age of 11 years (PATNAIK et al., 1981; TRIGO et al., 1982; LIPTAK; DERNELL; WITHROW, 2004; HIROSE et al., 2014). In our casuistic, we obtained a minimum value of 6 years, which would be considered as an out of normal patient to present HCC, meanwhile there is a case report of an even younger dog presenting HCC with 2 years (TESHIMA et al., 2013).

In a retrospective study, hepatic left and right lobes were affected by HCC in 68.3% and 12% of dogs, respectively, and the mortality rate was 40% (LIPTAK et al., 2004a). In contrast, our study presented a proportion of 41% and 28% of HCC in hepatic left and right lobes, with a mortality rate of 33%. In both studies, these deaths can be associated with complications during or just after the surgical procedures (LIPTAK et al., 2004b). Few studies have correlated the tumor size with other criteria of malignancy and prognosis in dogs (CARVALHO et al., 2016). Here, we decided to use the tumor volume, a measurement presuming the elliptical format of the tumors, to offer a more reliable variable (CARLSSON; GULLBERG; HAFSTRÖM, 1983). In humans, the tumor volume is considered a predictive factor for some neoplastic diseases (PAWLIK et al., 2005). We obtain data related to correlation of tumor size with differentiation degree and vascular invasion. Larger tumors were less differentiated and more susceptible to present vascular invasion.

In general, our canine HCC samples demonstrated low Ki-67 proliferation index (2.57 ± 0.04 %). In human HCC, only proliferation index more than 10% are considered

positive cases and presented prognostic value (KING et al., 1998; GUZMAN et al., 2005). This suggests that canine HCCs may be less aggressive than human HCCs. In this series of cases, a mixed hepatocellular-cholangiocellular carcinoma tumor was included. According some authors, this tumor presents a more aggressive behavior than conventional HCCs (VAN SPRUNDEL et al., 2010). In fact, this tumor had a higher mitotic index and Ki-67 proliferation index in comparison to other tumors. The immunoexpression of CK19 in canine HCC was evaluated according to Van Sprundel et al. (2013) and all cases were negative for this prognostic marker (less than 5-10% of positive cells). In addition, these cells were morphological similar to progenitor cells, showing cholangiocytes and hepatocytes in intermediate stages of differentiation in a repair process. Finally, the positivity of the cholangiocellular compartment in the mixed tumor was greater than 90%, as reported before (VAN SPRUNDEL et al., 2010, 2013).

For immunohistochemical analysis of the p53 protein, the clone Pab240 (monoclonal antibody) was chosen, which detects only the p53 mutant-type . This means that it offers a low sensitivity and a very high specificity (ZACCHETTI; VAN GARDEREN; RUTTEMAN, 2007). In contrast, the majority of studies applied the clone CM-1 (polyclonal antibody) to evaluate p53 in canine tumors, which detects p53 wild-type and mutant-type. CM-1 antibody has been positive in 44% of canine colorectal tumors (WOLF et al., 1997), in 17% of hemangiosarcomas (YONEMARU et al., 2007), in 60% of lymphomas (SUEIRO; ALESSI; VASSALLO, 2004) and 44.6% in mast cell tumors (GINN et al., 2000). Meanwhile, a study showed higher scores of CM-1 immunoexpression in benign tumors (15.38%) in comparison to malignant tumors (5.26%) (BERTAGNOLLI et al., 2009). Additionally, there is not a direct relationship with the labeling of the clone CM-1 and the presence of TP53 gene mutations (YONEMARU et al., 2007).

In this context, the clone Pab240 has been identified in 61% of canine gastric carcinomas (CARRASCO et al., 2011) and in 30% of mammary carcinomas, showing a positive correlation with TP53 gene mutations (LEE et al., 2004). That result would be comparable with ours since the absence of p53 staining in canine HCC tissues were also reflected in the absence of TP53 gene mutations. A further disadvantage reported is the affinity of the antibody with a specific region of the protein. For example, Pab240 detects intermediate regions of p53, and other markers such as bp53-12 is linked to the N-terminal region and pab122 that binds to the C-terminal region. Then, some authors suggests the use of a mixture of several antibodies to detect p53 and increase

the their sensitivity (HAGA et al., 2001). Also, due to the challenge that exists in veterinary medicine with the use of antibodies for immunohistochemistry specific for mice and humans, critical investigation evaluated the cross reaction of these antibodies with canine proteins. Four human-specific antibodies were used against canine p53, as such DO-7, Ab-7, CM-1 and pab240. The authors concluded that only CM-1 and pab240 had a cross-reaction with the canine p53 (KELLER et al., 2007).

We performed the sequencing technique based on the results and methodology reported by York et al. (2012). We analyzed the exons 4 to 8 exons, where the the majority of TP53 gene mutation was related in humans and dogs (MAYR et al., 2002; YONEMARU et al., 2007; KOSHINO et al., 2016). Our results showed no TP53 gene mutations in all canine HCC cases; however, we detect some artefacts (noises) in the chromatograms generated by the Sanger technique. Muto et al. (2000) also reported the same noise in chromatograms sequencing product of TP53 gene in canine mammary carcinomas. In this case, the authors interpreted this noise as polymorphisms due to the presence of both wild and mutant-types. However, Muto et al. (2000) reported a very high frequency of mutations in the TP53 gene, contrary to other investigations (VELDHOEN et al., 1999; LEE et al., 2004). In our case, these polymorphisms were not present in the two chains, thus we interpret as noise. This may be due to the fact that if p53 is mutated in low frequency in the canine HCC, it is expected that the mutant type loses by competition against the wild type in techniques such as PCR and Sanger sequencing technique (CIBULSKIS et al., 2013). Hypothesizing that the mutation is in a low percentage of cells, other techniques could be contemplated as the Single Stranded Conformational Polymorphism (HAYASHI, 1991; VELDHOEN et al., 1999; YONEMARU et al., 2007; KOSHINO et al., 2016).

Our research was carried out in Brazil where moderate exposure to aflatoxin B1 has been reported (DE CARVALHO et al., 2013). In our study local São Paulo , human HCC has been related to the R249S mutation produced by chronic aflatoxin intoxication, in 28% of analyzed cases (NOGUEIRA et al., 2009). Our results show that dogs do not suffer such mutation, it is possible that there is low exposure in this group of patients or that the species is resistant to the toxin. For example, studies in woodchucks which despite to have high exposure to AFB1 along with the aggravation of chronic viral hepatitis, do not present mutations in the TP53 gene however this species develop HCC (DUFLOT et al., 1994; RIVKINA et al., 1994, 1996). This is not a definitive negative result since even in humans not all cases associated with AFB1

contain R249S mutation. Additionally to AFB1, other etiologies related to human HCC in non-cirrhotic livers should be more studied in dogs (EVERT; DOMBROWSKI, 2008; TREVISANI et al., 2010). For Example, two metabolic syndromes associated to human HCC, vacuolar liver disease which also affects Scottish terrier dogs and suggest predisposition of this breed to HCC (SEPESY et al., 2006; CORTRIGHT et al., 2014) and alpha-antitrypsin deficiency known to be Involved in human HCC growth and also associated with hepatic diseases of the dog (SEVELIUS; ANDERSSON; JÖNSSON, 1994).

6 GENERAL CONCLUSIONS

In conclusion, we did not find any TP53 gene mutations in our casuistic of canine HCC, as well as no p53 immunoexpression was found in the neoplastic hepatocytes. Then, due to the absence of mutations in the TP53 gene, the participation of aflatoxin B1 as an etiological factor in HCC remains open in dogs.

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Appendix A: Summary of clinical, anatomopathological and molecular aspects of HCC-bearing dogs.

Case	Breed	Age	Sex	Number nodules	Volume of nodules (cm^3)	Gross pattern	Liver lobes affected	Micro pattern	Ki-67 (%)	CK19	p53 (%)	Mitosis count	Vascular invasion	Cellular differentiation	TP53 gene mutation	Outcome after surgery	Overall Survival (days)
1	Scottish Terrier	11	male	1	65.25	nodular	right	trabecular	2.75%	<10%	negative	2	no	well	no	alive	36
2	Yorkshire Terrier	12	female	1	124.35	massive	left	squirrous	1.08%	<10% (6 foci)	negative	6	no	well	no	alive	550
3	Schnauzer	9	male	1	257.74	massive	quadrate	sarcomatoid	18.6%	<10%	negative	3	no	well	no	alive	540
4	Golden Retriever	11	male	1	445.5	nodular	right	trabecular	-	negative	negative	1	no	well	no	alive	523
5	Golden Retriever	15	female	2	(396) (92.6)	massive	caudate (caudate), caudate (papillary)	cear cells	1.83%	negative	negative	3	no	moderate	no	death (sudden death)	479
6	Beagle	6	male	1	1361.9	massive	left	sarcomatoid	2.83%	negative	negative	8	no	well	no	alive	498
7	Golden Retriever	8	male	1	783.36	massive	right	trabecular	1.9%	negative	negative	7	no	well	no	alive	426
8	Pinscher	12	male	1	236	massive	left	squirrous	2.58%	<5%	negative	2	no	well	no	alive	411
9	Beagle	12	male	1	840	massive	quadrate	clear cells	5.9%	<5%	negative	1	no	moderate	no	alive	377
10	Mixed	9	female	1	1,008.412	massive	left	peliod	2.75%	<5%	negative	3	no	well	no	alive	361
11	Golden Retriever	12	female	6	(197.75) (522.15) (165.12) (44.4) (33.07) (23.80)	diffuse	quadrate (1), left (4), caudate(caudate) (1)	clear cells	0.83%	negative	negative	2	yes	well	no	alive	330
12	Chow-chow	10	female	1	33.81	nodular	left	trabecular	0.75%	negative	negative	5	no	well	no	alive	328
13	Mixed	11	male	1	1137.12	massive	left	trabecular	2.58%	negative	negative	2	no	well	no	alive	309
14	Shih Tzu	14	male	1	133.92	massive	right	trabecular	13.5%	negative	negative	1	no	well	no	death (immediately after surgery)	0
15	Lhasa apso	16	female	1	1286.5	massive	right	Solid	6%	>5%	negative	14	yes	moderate	no	death (24h after surgery)	1
16	Pinscher	10	male	1	40.43	nodular	left	trabecular	7.41%	negative	negative	5	no	well	no	alive	271
17	Mixed	11	female	1	313.22	massive	caudate	pelioid	1.33%	negative	negative	1	no	well	no	alive	271
18	Lhasa Apso	12	female	1	562.02	massive	left	pelioid	2.16%	negative	negative	2	no	well	no	alive	270
19	Maltes	11	female	1	48.70	nodular	right	Combined HCC-CC	9.58%	100% ductular CC areas	negative	6	no	well	no	alive	250
20	Golden Retriever	11	female	2	(427.46) (199.64)	massive	right, caudate (caudate process)	(pelioid), (clear cells)	4.16% (1) 1.41% (2)	negative (1), <5% (2)	negative negative	4 (1), 4 (2)	no	well	no	alive	174
21	Mixed	12	female	1	68.54	massive	caudate (caudate process)	trabecular	4.5%	negative	negative	4	no	well	no	alive	174
22	Fox paulistinha	10	female	2	(1271.47) (80.73)	massive	left, caudate (caudate process)	solid	7%	<5%	negative	5	(no) (yes)	moderate	no	alive	83
23	Lhasa apso	12	female	1	649	massive	right	pseudoglandular	4.25%	negative	negative	2	yes	well	no	alive	82
24	Mixed	11	male	1	144.21	nodular	right	trabecular	3.58%	negative	negative	3	no	well	no	death (24h after surgery)	1