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Betina Fabis Lautert

# MASTOCITOMAS CANINOS DIAGNOSTICADOS NA REGIÃO CENTRAL DO RIO GRANDE DO SUL: GRADUAÇÃO HISTOLÓGICA, PADRÃO DE EXPRESSÃO DA PROTEÍNA KIT E INVESTIGAÇÃO DE MUTAÇÕES

Santa Maria, RS
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Dissertação apresentada ao Curso de Pós-Graduação em Medicina Veterinária na Universidade Federal de Santa Maria (UFSM, RS), como requisito para a obtenção do título de **Mestre em Medicina Veterinária.**

Orientadora: Prof.ª Dr.ª Mariana Martins Flores

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## RESUMO

### MASTOCITOMAS CANINOS DIAGNOSTICADOS NA REGIÃO CENTRAL DO RIO GRANDE DO SUL: GRADUAÇÃO HISTOLÓGICA, PADRÃO DE EXPRESSÃO DA PROTEÍNA KIT E INVESTIGAÇÃO DE MUTAÇÕES

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O mastocitoma é um neoplasma maligno originário de mastócitos que pode se originar de diferentes tecidos. Seu comportamento biológico, principalmente quando se origina na pele, é altamente variável, o que tem estimulado diversos estudos acerca de fatores prognósticos. Além dos sistemas de graduação histológica propostos por Kiupel (2011), o índice Ki67, o padrão de imunomarcação para KIT e o status para mutações nos éxons 8 e 11 do gene *c-kit* são algumas das ferramentas prognósticas mais recentemente estabelecidas. A prevalência de mutações nos éxons 8 e 11 do *c-kit*, quando somadas, foram observadas em até 45% dos casos em mastocitomas cutâneos caninos. Este estudo teve como objetivo principal caracterizar os aspectos anatomopatológicos e moleculares de mastocitomas cutâneos em biópsias e mastocitomas em necropsias de cães provenientes da rotina diagnóstica do Laboratório de Patologia Veterinária (LPV) da Universidade Federal de Santa Maria (UFSM). Com estas informações, dois artigos científicos foram elaborados. O primeiro artigo caracterizou 16 cães com mastocitomas metastáticos submetidos à necropsia no intervalo 2012-2022. Os mastocitomas foram avaliados histologicamente e submetidos à coloração de Azul de Toluidina (AT), imuno-histoquímica (IHQ) para KIT e Reação em Cadeia da Polimerase (PCR) para os éxons 8 e 11. As localizações primárias observadas foram pele e/ou tecido subcutâneo (13), intestino (2), e tecido periocular (1). Cinco cães haviam sido submetidos à ressecção do neoplasma antes da disseminação sistêmica e seis foram submetidos à quimioterapia. As metástases foram mais comuns nos linfonodos locais (16), fígado (16) e baço (15). Histologicamente, os mastocitomas variaram de bem diferenciados, com grânulos abundantes, a pouco diferenciados, com escassos grânulos. O padrão de KIT de todos os casos foi aberrante, sendo 9 classificados como padrão II e 7 em padrão III. O sítio primário e metastático foram idênticos. Os éxons 8 e 11 foram amplificados em sete neoplasmas de quatro cães, sem nenhuma mutação detectada. Espera-se que esse estudo venha a auxiliar oncologistas veterinários no diagnóstico, prognóstico e terapia de mastocitomas com comportamento biológico mais agressivo. O segundo artigo teve como objetivo principal investigar a presença de mutações nos éxons 8 e 11 do *c-kit* em 45 mastocitomas cutâneos caninos (MCCs) diagnosticados no sul do Brasil. Todos os MCCs foram avaliados histologicamente com os métodos de Patnaik e Kiupel, submetidos a IHQ para KIT e a PCR em busca de mutações nos éxons 8 e 11. Vinte e cinco tumores foram classificados em baixo grau, e vinte em alto grau. Quanto aos padrões de expressão do KIT, padrão I (12), II (29) e III (2), além de dois tumores que não apresentaram células imunomarcadas. Treze casos foram positivos para mutações do éxon 11, sendo a maioria de amostras congeladas. A amplificação da região de DNA para o éxon 8 ocorreu em apenas treze casos e todos foram negativos para mutação. Este estudo foi importante na caracterização dos aspectos imuno-histoquímicos e moleculares dos mastocitomas cutâneos diagnosticados na região sul do Brasil.



**Palavras-chave:** Mutação. Imuno-histoquímica. PCR. Prognóstico.

## ABSTRACT

### CANINE MAST CELL TUMORS DIAGNOSED IN THE CENTRAL REGION OF RIO GRANDE DO SUL: HISTOLOGICAL GRADING, KIT PROTEIN EXPRESSION PATTERN AND INVESTIGATION OF MUTATIONS

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Mast cell tumor (MCT) is a malignant neoplasm originating from mast cells that can arise in different tissues. Its biological behavior is highly variable, especially when it originates in the skin, which has stimulated several studies about prognostic factors. In addition to the histological grading system proposed by Kiupel (2011), the Ki67 index, the immunostaining pattern for KIT and *c-kit* mutation status for exons 8 and 11 are some of the most recently mentioned prognostic tools. The prevalence of mutations in *c-kit* exons 8 and 11, when added together, were observed in up to 45% of cases in canine cutaneous mast cell tumors. The main objective of this study was to characterize the anatomopathological and molecular aspects of mast cell tumors in biopsies and mast cell tumors in necropsies of dogs from the diagnostic routine of the Laboratory of Veterinary Pathology (LPV) of the Federal University of Santa Maria (UFSM). With this information, two scientific articles were prepared. The first article characterized 16 dogs with metastatic MCTs submitted to necropsy between 2012-2022. Mast cell tumors were evaluated histologically and submitted to Toluidine Blue (TB) staining, immunohistochemistry (IHC) for KIT and Polymerase Chain Reaction (PCR) for exons 8 and 11. The primary locations observed were skin and/or subcutaneous tissue (13), intestine (2), and periocular tissue (1). Five dogs resected the neoplasm before systemic dissemination and six had undergone chemotherapy. Metastases were most common in local lymph nodes (16), liver (16) and spleen (15). Histologically, mast cell tumors ranged from well differentiated, with abundant granules, to poorly differentiated, with scarce granules. The KIT pattern of all cases was aberrant, in 9 cases were pattern II and 7 cases were pattern III. The primary and metastatic site pattern was identical. Exons 8 and 11 were amplified in seven neoplasms from four dogs, with no mutation detected. It is hoped that this study will help veterinary oncologists in the diagnosis, prognosis and therapy of MCTs with a more aggressive biological behavior. The second article aimed to investigate the presence of mutations in exons 8 and 11 of *c-kit* in 45 canines cutaneous MCTs diagnosed in southern Brazil. All MCTs were evaluated histologically with the methods of Patnaik and Kiupel, submitted to IHC for KIT and PCR in search of mutations in exons 8 and 11. Twenty-five tumors were classified as low grade, and 20 as high grade. As for the KIT expression patterns, pattern I (12), II (29) and III (2), in addition to two tumors that did not show immunolabelled cells. Thirteen cases were positive for exon 11 mutations, the majority being from frozen samples. Amplification of the DNA region for exon 8 occurred in only thirteen cases and all were mutation negative. This study was important in characterizing the immunohistochemical and molecular aspects of cutaneous mast cell tumors diagnosed in southern Brazil.



Keywords: Mutation. Immunohistochemistry. PCR. Prognostic

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## 1 INTRODUÇÃO

O mastocitoma cutâneo é o câncer de pele mais frequente em cães na rotina de biópsias do LPV-UFSM (SOUZA et al., 2006). Devido a seu comportamento biológico altamente variável (KIUPEL et al., 2011), métodos complementares à histopatologia têm sido utilizados na determinação do prognóstico (BROCKS et al., 2020). A descoberta de que mastocitomas cutâneos caninos (MCCs) com mutações internas em sequência no éxon 11 do gene *c-kit* são mais agressivos, e que ao mesmo tempo, respondem melhor ao tratamento com inibidores da tirosinaquinase (LONDON, 1999) motivou vários estudos quanto a este tema nos últimos anos e revolucionou a forma como os MCCs são tratados pelo clínico e cirurgião veterinários (DOWNING et al., 2002; GIANTIN et al., 2012; HAHN, 2008; MARCONATO et al., 2014; TAKEUCHI et al., 2013; THAMM et al., 2020; VOZDOVA et al., 2019; VOZDOVA et al., 2020). Além disso, o estabelecimento da imuno-histoquímica para KIT como um marcador prognóstico independente para MCCs também foi um marco, levando essa técnica a ser oferecida na rotina diagnóstica em muitos laboratórios ao redor do mundo como ferramenta auxiliar na investigação prognóstica. Apesar disso, a busca por novos marcadores prognósticos para MCCs segue ativa, e a cada ano, novos estudos surgem indicando possíveis ferramentas adicionais na investigação do seu comportamento biológico.

Com base nisso, os objetivos principais desta dissertação foram: (1) através de um estudo retrospectivo e prospectivo, graduar de acordo com o tecido de origem os mastocitomas caninos, definir o grau de granulação citoplasmática, bem como determinar o padrão de imunomarcação para KIT e estabelecer a prevalência de mutações nos éxon 8 e 11 do gene *c-kit* em cães submetidos à necropsia; (2) através de um estudo retrospectivo e prospectivo, caracterizar o padrão de imunomarcação para KIT e investigar a presença de mutações nos éxons 8 e 11 do gene *c-kit* em espécimes de biópsia.

A metodologia, resultados, discussão e conclusões que fazem parte desta dissertação serão apresentados na forma de dois artigos científicos (disponíveis no Capítulo 3) a serem submetidos para publicação nas revistas The Veterinary Journal e Ciência Rural, respectivamente.

# 2 REVISÃO BIBLIOGRÁFICA

A pele é o maior órgão do corpo e serve como limite anatômico entre o corpo e o meio ambiente. É constituída pela epiderme, derme, anexos cutâneos, tecido subcutâneo, nervos, vasos sanguíneos e linfáticos (CONCEIÇÃO; LOURES, 2016; MAULDIN; KENNEDY, 2016). Os mastócitos são uma população heterogênea de células redondas com origem na medula óssea. Residem em tecidos vascularizados (SNYDER, 2017), todavia, são particularmente mais abundantes na derme, tratos digestório e respiratório (DALECK; ROCHA; FERREIRA, 2016; JUNQUEIRA; CARNEIRO, 2012). Sua morfologia é característica: um pequeno núcleo central e citoplasma preenchido por finos grânulos, sendo facilmente observados em condições não patológicas nas colorações de rotina, como a Hematoxilina e Eosina (EROSCHENKO, 2008; SNYDER, 2017).

A localização estratégica dos mastócitos combinada com os mediadores inflamatórios, citocinas e fatores quimiotáticos contidos em seus grânulos, permite uma rápida interação com células dendríticas e endoteliais (ACKERMAN, 2017; WELLE et al., 2008). Por isso, estão diretamente envolvidos nas respostas inflamatórias aguda, crônica, reparação tecidual, reações de hipersensibilidade e processos proliferativos (SNYDER, 2017).

Mastocitoma é um neoplasma maligno com origem em mastócitos (DALECK; ROCHA; FERREIRA, 2016; LONDON; THAMM, 2013). Em cães, a literatura aponta o mastocitoma como a neoplasia cutânea maligna mais frequentemente observada (FIGHERA et al., 2008; LONDON; THAMM, 2013; SOUZA et. al, 2006). Os membros torácicos, pélvicos, região cervical, tórax, dorso, escroto e boca são os locais mais acometidos (WELLE et al., 2008). A média de idade dos cães é de 8,5 anos e não há predisposição sexual. Na literatura brasileira há uma maior propensão em cães sem raça definida (DALECK; ROCHA; FERREIRA, 2016). Quanto trata-se de cães com raça, Boxer, Boston Terrier, Bulldog, Labrador Retriever, Golden Retriever, Beagle, Teckel e Shar-Pei estão entre os mais frequentemente descritos (DALECK; ROCHA; FERREIRA, 2016; LONDON; THAMM, 2013).

## 2.1 DIAGNÓSTICO

### 2.1.1 Técnicas histoquímicas

O exame citológico é utilizado como uma ferramenta diagnóstica de triagem pois é um método rápido, de baixo custo e invasividade (SOUZA et al., 2006). A avaliação das

características morfológicas das células neoplásicas e classificação em alto (figuras mitóticas, células binucleadas ou multinucleadas, pleomorfismo nuclear ou > 50 % anisocariose) e baixo grau, quando as amostras não se enquadram em algum destes critérios, se mostrou um preditor útil para o planejamento de tratamento e prognóstico. Apesar disto, diferenças no tipo de corante utilizado, que podem ou não facilitar a visualização dos grânulos citoplasmáticos, e a impossibilidade de estabelecer a origem em dérmica ou em tecido subcutâneo (KIUPEL; CAMUS, 2019), atributos extremamente importantes para o prognóstico, demonstram a necessidade da utilização de outros métodos de diagnóstico mais acurados (CAMUS et al., 2016).

Desta forma, a utilização de sistemas visando uma padronização na graduação histológica do mastocitoma cutâneo canino (MCC) por meio da coloração de Hematoxilina e Eosina é a ferramenta mais empregada atualmente na rotina diagnóstica no Brasil. O primeiro sistema de graduação foi proposto em 1973 por Bostock, seguido pelo de Patnaik e colaboradores em 1984. Ambos apresentam três categorias de classificação, critérios de morfologia semelhantes, mas em ordem inversa de classificação (BOSTOCK, 1973; PATNAIK, 1984). A subjetividade de alguns parâmetros na graduação associada a diferenças nos critérios avaliativos entre patologistas ainda era algo frequente (NORTHRUP et al., 2005). Em 2011, Kiupel e colaboradores propuseram um novo sistema com menos critérios histológicos a serem avaliados e apenas dois graus de classificação, o que diminuiu consideravelmente este problema (KIUPEL et al., 2011).

Apesar disto, em casos de mastocitomas pouco diferenciados, há dificuldade na visualização dos grânulos citoplasmáticos em colorações de rotina (hematoxilina e eosina). A técnica histoquímica de azul de toluidina facilita sua visualização devido a suas características metacromáticas, isto é, a capacidade de alterar a coloração sem alterar a estrutura química da célula (CULLING, 1985), que pode ser graduada em leve, moderada ou acentuada (RECH et al., 2004).

Em virtude da alta variabilidade no comportamento biológico do mastocitoma (GOLDSCHMIDT; HENDRICK, 2002; GROSS et al., 2005, KIUPEL, 2017), ferramentas auxiliares são utilizadas com objetivo de melhor definir o comportamento deste neoplasma. O principal critério utilizado na histopatologia e que visa estimar a proliferação tumoral é a contagem mitótica (BOSTOCK, 1973; KIUPEL et al., 2011; PATNAIK, 1984), entretanto, apenas este critério pode levar à super ou subestimação da fração de crescimento total (SLEDGE; WEBSTER; KIUPEL, 2016). Uma segunda técnica histoquímica bastante empregada e que auxilia na interpretação da proliferação celular é o AgNOR (regiões

organizadoras nucleolares argirofílicas), que são subestruturas nucleolares envolvidas na transcrição do RNA ribossomal (DERENZINI, 2000). O que se evidencia são células neoplásicas progredindo em seu ciclo celular (DERENZINI, 2000) por meio de pontos pretos ou marrom-escuros intranucleares (RECH et al., 2004) devido à afinidade das proteínas pela Prata (WEBSTER et al., 2007). A contagem é feita de maneira manual em 100 mastócitos neoplásicos e dividida por esta mesma quantidade. Um estudo classificou medias iguais ou menores que 1,5, como grau I, grau II quando 1,85 e grau III quando 3,25 (RECH et al., 2004). Quanto maior o número de NORs observadas no núcleo das células neoplásicas, maior é a atividade proliferativa do tumor, o que está associado a um pior prognóstico (BOSTOCK et al., 1989, DERENZINI, 2000).

### 2.1.2 Imuno-histoquímica

A técnica imuno-histoquímica para detecção de Ki67, uma proteína nuclear não histona, altamente sensível à protease disposta por uma cadeia polipeptídica (GERDES et al., 1991), é utilizada com a mesma finalidade que a de AgNOR. A contagem dos núcleos positivos também é realizada de maneira manual e a imunomarcação intranuclear é interpretada como positiva (WEBSTER et al., 2007). Entretanto, o tempo de fixação da amostra em formol pode apresentar resultados não confiáveis (SANTOS et al., 2019) devido às alterações na integridade do epítopo, o que pode levar a resultados imuno-histoquímicos falso-negativos ou fracamente positivos (RAMOS-VARA; MILLER, 2014).

Não há um valor bem determinado para classificar os resultados em graus, apesar disto, sabe-se que cães com Ki67 elevado tem pior prognóstico (LELYVELD et al., 2015; MAGLENNON et al., 2008). Para um valor de proliferação tumoral mais fidedigno, recomenda-se a combinação dos resultados dos marcadores de proliferação celular, multiplicando-se o escore AgNOR pelo índice Ki67, resultando no escore Ag67 (BROCKS et al., 2020).

A propensão à proliferação celular descontrolada é a principal característica do câncer e tem sido amplamente utilizada para prognosticar doenças neoplásicas humanas e veterinárias (WEBSTER et al., 2007). O receptor transmembrana tipo III, KIT, é responsável pela diferenciação, migração, proliferação e sobrevivência dos mastócitos (DAHLIN, 2021; LENNARTSSON; RÖNNSTRAND, 2012) e em uma variedade de células, incluindo melanócitos e precursores eritroides (GALLI; ZSEBO; GEISSLER, 1994; KIUPEL et al., 2004). Três padrões de imunomarcação podem ser observados em mastocitomas: padrão I –

marcação associada à membrana, padrão II – marcação citoplasmática focal ou pontilhada, e padrão III – marcação citoplasmática difusa (KIUPEL et al., 2004). Apesar disto, um fator que pode ser limitante é a oscilação na intensidade da imunomarcação. Há algumas hipóteses sugerindo diferença fenotípica entre mastócitos de diferentes localizações (THOMPSON et al., 2011), grau histológico do neoplasma (REGUERA, 2000) e tempo prolongado de fixação no formol (RAMOS-VARA et al., 2014). Os padrões de expressão do KIT por mastócitos neoplásicos (KIUPEL et al., 2004), bem como a contagem de Ki67 (SCASE et al., 2006; VASCELLARI et al., 2012; WEBSTER et al., 2007) mostraram indicadores prognósticos correlacionados, como tempo de sobrevivência e recorrência neoplásica.

### 2.1.3 Reação em cadeia de polimerase

Estruturalmente, o KIT consiste em vários domínios, que são codificados por 21 éxons do proto-oncogene *c-kit*. A porção extracelular (codificada pelos éxons 1–9) consiste em 5 domínios semelhantes a imunoglobulinas, o domínio transmembrana é codificado pelo éxon 10, justamembrana pelo éxon 11 e porção intracelular pelos éxons 12–20 (LENNARTSSON; RÖNNSTRAND, 2012; STANKOV et al., 2014). Em circunstâncias normais, a ativação do *c-kit* é fortemente regulada e sua ativação inadequada está associada ao desenvolvimento de uma série de doenças malignas em medicina humana (STANKOV et al., 2014). Na medicina veterinária, estudos estabelecem correlações indiretas entre a presença de mutações e o grau histológico (GIANTIN et al., 2012; MARCONATO et al., 2014; TAKEUCHI et al., 2013; TAMLIN et al., 2017; THOMPSON et al., 2016; VOZDOVA et al., 2019; ZEMKE et al., 2002), outros marcadores prognósticos (GIANTIN et al., 2012; WEBSTER et al., 2007) ou diretamente com o desfecho clínico do paciente (DOWNING et al., 2002; GIANTIN et al., 2012; HAHN, 2008; MARCONATO et al., 2014; TAKEUCHI et al., 2013; THAMM et al., 2020; VOZDOVA et al., 2019; VOZDOVA et al., 2020).

A mutação mais observada até o presente momento são duplicações internas em sequência (*Internal Tandem Duplications* - *ITDs*) no éxon 11 (GIANTIN et al., 2012; HAHN et al., 2008; LETARD et al., 2008; VOZDOVA et al., 2019; VOZDOVA et al., 2020), enquanto as mutações no domínio da quinase são raras (LETARD et al., 2008; VOZDOVA et al., 2020; WEBSTER; KIUPEL; YUZBASIYAN-GURKAN, 2006). Apenas um estudo identificou as mutações do éxon 5 como as mais comuns, e ITDs dos éxons 8 e 9 foram relatados com prevalência de 2% a 7% (GIANTIN et al., 2012; HAHN, 2008; LETARD et al., 2008; MARCONATO et al., 2014; TAKEUCHI et al., 2013; VOZDOVA et al., 2020). Além disso,

as mutações de outros éxons, incluindo 2, 6, 7, 10, 15 (TAKEUCHI et al., 2013), 12 (DOWNING et al., 2002), 14 (NAKANO et al., 2017) e 17 (HAHN et al., 2008; LETARD et al., 2008) foram relatadas com baixa frequência.

A prevalência de mutações no éxon 8 e 11 do *c-kit*, quando somadas, foram observadas em até 45% dos casos em MCCs (BROCKS et al., 2020; DOWNING et al., 2002; LETARD et al., 2008; LONDON, 1999; TAKEUCHI et al., 2013, TAMLIN et al., 2017, VOZDOVA et al., 2019 ZEMKE, et al., 2002). As ITDs na região do éxon 11 foram associadas à fosforilação do KIT apesar da falta de ligação ao receptor, o que sugere que as mutações no *c-kit* levam à proliferação celular independentemente do fator de crescimento das linhagens celulares (DOWNING et al., 2002; LONDON, 1999; MA et al., 1999). Os parâmetros prognósticos em MCCs com mutações no éxon 11 foram comumente associados a uma alta atividade proliferativa e mau prognóstico (DOWNING et al., 2002; ZEMKE, et al., 2002). Apesar disto, sabe-se que mesmo com características de malignidade mais acentuadas, estes neoplasmas respondem melhor ao tratamento com inibidores da tirosinaquinase (LONDON, 1999). O que gera uma esperança quanto ao tratamento quimioterápico nestes casos.

Artigos recentes têm pesquisado e demonstrado a importância da ITD na região do éxon 8. Em mastocitomas cutâneos caninos, os índices proliferativos demonstraram-se mais baixos quando comparados a casos positivos no éxon 11, sugerindo que a presença dessas mutações esteja associada a um melhor prognóstico. Estes neoplasmas teriam comportamento biológico, então, semelhante ao observado em MCCs sem mutações nos éxons 8 ou 11 do *c-kit* (BROCKS et al., 2020). Este mesmo dado foi demonstrado em mastocitomas subcutâneos (CHEN et al., 2022).

## 2.2 TRATAMENTO

O tratamento para o mastocitoma pode ser realizado utilizando uma técnica isolada, ou mesmo a associação de abordagens terapêuticas (excisão cirúrgica, quimioterapia antineoplásica, eletroquimioterapia, fármacos inibidores dos receptores tirosinaquinase) e radioterapia. A escolha da abordagem terapêutica depende, em grande parte, dos fatores prognósticos e o estadiamento clínico (DALECK; ROCHA; FERREIRA, 2016). Por isto recomenda-se uma combinação de testes prognósticos, incluindo o status mutacional do *c-kit* (KIUPEL; CAMUS, 2019; SLEDGE; WEBSTER; KIUPEL, 2016).

Na medicina humana, é bem estabelecido o tratamento quimioterápico com inibidores da tirosinaquinase (HAN et al., 2011) e drogas citotóxicas convencionais que induzem autofagia

(YANG et al., 2011), entretanto, este mesmo processo protege as células tumorais contra a radiação (PAGLIN et al., 2001). Estudos recentes demonstram que a combinação de inibidores da tirosinaquinase com quimioterápicos convencionais abriu uma nova via de tratamento para pacientes que não respondem aos medicamentos existentes (TAMLIN et al., 2020). As taxas de terapias combinadas com inibidores da tirosinaquinase chegam a 90% de eficácia (OLSEN et al., 2018). O tratamento exclusivo com estes fármacos também tem sido indicado para MCCs em que não há possibilidade de excisão cirúrgica ou metástases estejam presentes (BURTON et al., 2015; HORTA et al., 2018a).

# 3 ARTIGOS

## 3.1 METASTATIC MAST CELL TUMORS IN DOGS SUBMITTED TO NECROPSY: PATHOLOGY, KIT EXPRESSION PATTERN AND MUTATION INVESTIGATION IN 16 CASES (2012–2022)

Artigo a ser submetido à revista The Veterinary Journal.

**Original Article**

# Metastatic mast cell tumors in dogs submitted to necropsy: pathology, KIT expression pattern and mutation investigation in 16 cases (2012–2022)

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**Abstract**

Mast cell tumors (MCT) are among the most frequent malignant neoplasms in dogs. Due to their unpredictable biological behavior, several prognostic tools, such as immunohistochemistry (IHC) and mutation status, have been developed over the years. Necropsy studies on dogs with metastatic MCTs are scarce, and rarely characterize the KIT expression pattern and mutation status of these tumors. This study aimed to characterize the clinical, anatomopathological aspects, KIT expression pattern and *c-kit* mutation status in 16 dogs with metastatic MCT submitted to necropsy in a Brazilian Veterinary Pathology service (2012-2022). MCTs were histologically evaluated and submitted to Toluine blue special stain, IHC using an anti-KIT antibody and Polymerase Chain Reaction for exons 8 and 11. Sixteen dogs with primary MCTs in the skin and/or subcutaneous tissue (13) intestine (2), and periocular tissues (1) were included. Five dogs had been submited to neoplasm ressection before systemic dissemination and six had been submitted to chemotherapy. Metastasis were most common in the local lymph nodes (16), liver (16) and spleen (15). Histologically, MCTs ranged from well-differentiated neoplasms with abundant granules to poorly differentiated and scarcely granulated ones. All MCTs had an aberrant KIT expression pattern characterized by cytoplasmic labelling, which was similar between primary and metastatic sites. Exons 8 and 11were amplificated in seven neoplasms from four dogs, with no mutations detected. This study characterized KIT expression pattern and investigated exon 11 and 8 mutations in fatal metastatic cases of MCT. Our results may help further characterizing the use of these prognostic factors in metastatic MCTs of dogs, assisting veterinary oncologists with the diagnosis, prognostication and therapy of these aggressive neoplasms.

*Keywords: dog; mast cell tumor; metastasis foci, immunohistochemistry*

## Introduction

Mast cell tumors (MCT) are malignant neoplasms originating from mast cells (Kiupel 2017). In dogs, MCTs are most frequent in the skin, where they correspond to 16 to 21% of all cutaneous neoplasms (London et al., 2013, Kiupel, 2017). Other primary locations such as gastrointestinal tract and other visceral organs are infrequent (Patnaik et al., 1982; Kiupel, 2017). MCTs have a variable biologic behaviour, and some of these neoplasms may behave aggressively, recurring and metastasizing to local lymph nodes and distant organs (Kiupel, 2017).

Due to the highly variable biological behaviour of MCTs in dogs, complementary methods to histopathology have been used to determine the prognosis and help choosing the more adequate systemic therapy when necessary (Kiupel et al., 2011; Brocks et al., 2020). These methods are mainly aimed at cutaneous MCTs (cMCTs), although some studies have applied these tools to tumors from other locations as well (Kobayashi et al. 2012, Larsen et al. 2022). They include investigating the Ki-67 index, the KIT expression pattern and the mutation status of the *c-kit* gene (Kiupel et al. 2004, Webster et al. 2007, Brocks et al. 2020). These ancillary techniques have been largely explored over the last decades, which has improved the diagnosis and treatment of MCT patients.

Necropsy studies of dogs dying due to MCT-related disease are scarce (Kiupel, 2017). Most of these references are antique and have not applied more modern prognostic techniques such as KIT immunohistochemistry (IHC) and Polymerase chain reaction (PCR) for mutation investigation. In addition, most of them do not characterize the pathologic aspects of metastatic lesions in different organs (Hottendorf et al.; 1968, O'Keefe et al., 1987). Studying these aspects of MCTs in necropsies may help improving the antemortem diagnosis, mainly

through imaging techniques and biopsies (London et al., 2013). Additionally, investigating the immunohistochemical KIT pattern and mutation status in metastatic lesions may be useful for oncologists and pathologists handling aggressive cases of MCT with metastatic lesions. Based on this scenario, the aim of this study was to characterize the clinical, anatomopathological aspects, KIT immunostaining pattern and investigate the presence of *c-kit* mutations in 16 dogs with metastatic MCT submitted to necropsy.

## Materials and methods

A retrospective study of the necropsy database from a Brazilian pathology service was conducted (2012-2022), and dogs with a cause of death or euthanasia attributed to metastatic MCT were studied. Only cases with available paraffin-embedded tissues were included. Data concerning age, breed, clinical history and necropsy findings were retrieved from the necropsy reports.

New histological slides from all cases were obtained from the paraffin blocks, and cases were histologically evaluated. Cutaneous MCTs were graduated according to a three-tier (Patnaik et al. 1984) and a two-tier (Kiupel et al. 2011) grading systems. Tumors from other anatomical locations, including subcutaneous MCTs, were not graded. Cytoplasmic granulation on HE was evaluated in all tumors and classified as scarce, moderate or abundant at a higher magnification (400 X). All tissues affected by primary or metastatic MCTs were submitted to Toluidine Blue (TB) special stain. For TB staining, 3µm-thick histologic sections were deparafinized, hydrated, and stained in a TB solution (0.1%) for 3 minutes. The slides were then washed in tap water and dehidrated. The amount of stained granules was classified as scarce, moderate and abundant at a higher magnification (1000 X).

One section of the primary MCT and at least one section of a metastatic site (preferentially the regional lymph node) were submitted to IHC using a rabbit polyclonal anti-KIT antibody (CD117, Dako, A4502)[I]. Antigen retrieval was performed by microwaving (10 min at full power) in TRIS–EDTA pH 9. Sections were incubated with the primary antibody diluted in phosphate-buffered saline with Tween 20 (PBST) (1:150) for 1h at 37 °C. A polymer-HRP system (Easy Link One, EasyPath)[II] was used, followed by substrate development with 3,3′diaminobenzidine (DAB; EasyPath)I[I]. One cutaneous mast cell tumor from a dog was used as positive control. For negative controls, the same test sections were used, and the primary antibody was replaced with PBST. The MCTs were classified according to KIUPEL et al. (2004) into three patterns of KIT immunostaining according to location of antigen expression: pattern I, characterized by membrane staining; pattern II, characterized by focal or stippled cytoplasmic staining; or pattern III, characterized by diffuse cytoplasmic immunostaining. Additionally, the percentage of immunolabeled cells was estimated.

All primary neoplasms and two metastatic sites were submitted to polymerase chain reaction (PCR) for exons 8 and 11. Extraction was performed using a commercially available kit (Qiagen QIAamp DNA FFPE Tissue Kit®, Quiagen, 56404)[III]. Four 5µm sections of each paraffin-embedded tissues were cut and placed in 2ml tubes. Paraffin was removed by treatment with xylene and ethanol. From frozen samples at least 0.5 $cm^3$ of tissue from each case were subjected to the extraction protocol. The following extraction phases were performed according to the manufacturer's protocol. After extraction, samples were stored in a -20°C refrigerator. A negative extraction control (NEC) was included in the extraction procedure. The PCR protocol for exon 11 and intron 11 was performed using the following primers: PE1/PE2 (PE1: CCCATGTATGAAGTACAGTGGAAG; PE2: GTTCCCTAATCATTGTTACACG) (JONES et al., 2004). Primers used to exon 8 and intron

8: PE1/PE2 (PE1: GTCCTCTTCAAACTCAAGAAGG; PE2: CCAAAATAATCCTCTCACCTCTGC) (VOZDOVA et al., 2019). PCR reaction was performed using the Taq DNA Polymerase, Recombinant (5U/µL) (Thermo Fisher ScientificTM®). Both reaction were optimized for annealing temperature (52 to 62°C, with a 2°C interval) and $MgCl_2$ concentration (1.5, 2, and 2.5mM). The PCR reaction was performed by using PE1/PE2 for initial denaturation at 94ºC for 3 minutes, followed by 35 cycles of 94ºC for 45 seconds, 58ºC for 30 seconds, 72ºC for 30 seconds and then a 72ºC for 10 minutes step. A non-diluted sample and a 1:10 sample were tested. The PCR product was analyzed by using agarose gel electrophoresis (agarose gel in Tris-acetate-EDTA buffer, 4%). The PCR product was analyzed by using agarose gel electrophoresis (agarose gel in Tris-acetate-EDTA buffer, 4%). Positive cases for exon 11 ITDs were cut from the electrophoresis gel and purified with a commercial kit (PureLink™ Quick Gel Extraction and PCR Purification Combo Kit) following the manufacturer's instructions and sent to ACTGene Análises Ltda. [IV] for Sanger sequencing. ACTGene performs sequence analysis using the Applied Biosystems® (ABI) 3130 Genetic Analyzer (ABI standard protocols).

**Results**

From 1497 dogs being necropsied in this period, 16 died or were euthanised due to metastatic mast cell tumor. The primary MCTs were solitary (n=15) or multifocal (n=1). The primary locations of the different MCTs included were skin (n=6), subcutaneous tissue (n=6), skin and subcutaneous tissue simultaneously (n=1), intestine (n=2) and periocular tissues (n=1). The affected dogs developed metastasis to the lymph nodes (n=16), liver (n=16), spleen (n=15), kidney (n=1), heart (n=1) and mediastinum (n=1) (Table 1). The major primary cutaneous tumors, when present during necropsy, were graduated as grade III and high grade (n=3) or grade II and high grade (n=2). One dog (n° 13) had numerous cMCTs and

scMCTs disseminated throughout the body; and in this case cMCTs had variable grading, ranging from grade II/low grade to grade III/high grade (Table 2). The MCT in the periocular tissues invaded the ocular nerve, frontal bone and brain. Seven dogs were submitted to surgical resection of the primary tumor before systemic dissemination, two (n° 5 and n° 12) with complete resection and, thus did not have this tumor available for reassessment making naccessible. Six dogs were submitted to chemotherapy (Table 3).

Macroscopically, the primary cMCTs and scMCTs ranged from small nodules (0,5 cm in diameter) to large masses (27 x 20 x 5 cm) with occasional ulcers (n=4) (Figure 1A). These tumours invaded the subjacent adipose tissue and muscles. One dog had more than twenty nodules disseminated in different body regions (Figure 1B). One of the intestinal masses affected duodenum and was relatively small (1,5 cm diameter) and diffusely hemorrhagic, which did not rise a suspicion of mast cell tumor during necropsy (Figure 1C). It had not been visualized during an abdominal ultrasound, being only detected during necropsy. The other intestinal mass affected the ileum and was a large (12 x 6.5 x 3.5 cm) whitish mass with hemorrhagic areas and was adhered to multiple enlarged mesenteric lymph nodes affected by metastasis (Figures 1D, 1E). The intestinal mucosa was ulcerated at the site of both intestinal MCTs (Figure 1F). The periocular MCT was characterized by a poorly demarked mass affecting the retrobulbar area, invading soft tissues and bone and reaching the olfactory bulb. The ocular globe was not invaded. Metastasis to the lymph nodes were mainly characterized by organomegaly (n=15) (Figures 2A and 2B) but also occasionally caused grossly visible nodules (n=6). Splenic metastases were observed in fourteen dogs, in which splenomegaly (n=8) (Figure 2C) and/or splenic nodules (n=4) (Figure 2D) were observed. Hepatic metastasis caused hepatomegaly in only 6 cases, sometimes with additional white nodules

(n=8) (Figure 2E, 2F). Renal, cardiac, and mediastinal metastases were characterized by multiple white nodules.

Histologically, the primary and metastatic MCTs ranged from well-differentiated (Figure 3A) and granulated neoplasms that were easily recognized (8 dogs) to poorly differentiated and scarcely granulated round cell tumors (Figure 3B) that depended on TB to be confirmed as MCTs (8 dogs). Metastatic foci varied from obvious areas that were easily recognized on HE sections (12 dogs) to more discrete areas that demanded TB stain to be recognized (4 dogs). The degree of differentiation was similar between the primary and metastatic lesions, and the number of stained granules on TB stain varied from scarce (n=7) (Figure 3C) to moderate (n=5) (Figure 3D) or abundant (n=4). Several dogs had numerous neoplastic cells filling the liver sinusoids (13), pulmonary capillaries (9), glomerular capillaries (5) and splenic sinusoids (5) in TB stained sections. In dog n° 11, circulating mast cells had been visualized in the blood smear, facilitating the suspicion of systemic mastocytosis. In other tissues, circulating neoplastic mast cells were seen in multiple small blood vessels in the pancreas, intestine, urinary bladder, brain and heart.

All primary and metastatic lesions submitted to IHC showed some degree of KIT antigen expression. However, the amount of immunolabeled neoplastic cells varied greatly. Antigen expression always predominated in the cytoplasm, and cases were classified in pattern II (9) (focal or stippled labelling) (Figure 3E) or pattern III (7) (diffuse labelling) (Figure 3F). The percentage of immunolabeled neoplastic cells in the primary tumors varied from approximately 90% (n=3) to 50% (n=9) and 30% (n=4). The percentage of immunolabeled cells and the pattern of immunolabelling was always similar between the primary and

metastatic lesion in each dog. Nine of the 16 MCTs was successfully amplification of exons 8 and 11 but no case were positive for ITDs.

**Discussion**

MCTs should always be considered potentially malignant, since they occasionally behave unpredictably, with local reccurence and metastases to lymph nodes and distant tissues (Kiupel, 2017). Although several investigations concerning grading and use of ancillary techniques on prognostication of biopsied MCTs have been published over the last few decades (Patnaik et al., 1984; Reguera et al., 2000; Kiupel et al., 2004; Webster et al., 2007; Kiupel et al., 2011; Brocks et al., 2020), studies focused on necropsies of dogs dying of MCT-related disease are uncommon (Hottendorf et al.; 1968, O'Keefe et al., 1987). In this investigation, dogs dying or being euthanized due to metastatic MCT corresponded only to 0.52% of all necropsies in this species. MCTs with an aggressive behaviour correspond to a subset of the hole group, which may partially explain this low prevalence. Additionally, several patients with a previously confirmed high-grade MCT are probably not submitted to necropsy by the owners, since their cause of death is already expected to be MCT-related. This possibly contributes to decrease the prevalence of MCT-related disease among necropsies. It is known that disseminated forms of the disease usually result from a primary cutaneous tumor (O'Keefe et al., 1987; Takahashi et al, 2000), and this agrees with our results, where six cutaneous and six subcutaneous MCTs were considered the primary lesion. The equal number of cutaneous and subcutaneous tumors was an interesting finding. Although older literature does not differentiate cutaneous and subcutaneous MCTs (Hottendorf et al.; 1968, O'Keefe et al., 1987), it is currently suggested that subcutaneous MCTs tend to be less aggressive when compared to their cutaneous counterparts (Newman et al., 2007; Thompson et al., 2011), which would make us expect a lower prevalence of subcutaneous primary

lesions in this study. Unfortunately, based on the relatively small number of cases included in this investigation, we cannot explain this similar prevalence between cutaneous and subcutaneous MCTs.

Extracutaneous MCTs are uncommon in dogs and can develop in any conjunctive tissue containing resident mast cells, including gastrointestinal tract, oral cavity, conjunctiva, salivary gland, nasopharynx, larynx, spinal cord, urethra, liver, spleen and lung (Kiupel, 2017). Three dogs in our study developed MCTs in infrequent locations. Dogs n°11 and 16º had intesinal tumors affecting the duodenum and ileum, respectively, and dog n°4 had periocular tissue involvement with consequent protrusion of the ocular globe and invasion of the optic nerve and frontal lobe. Prognostic studies on canine extracutaneous MCTs are scarce, making it difficult to predict their biological behaviour. However, the few studies available show that MCTs arising from mucosal surfaces (oral cavity, mucocutaneous junctions and gastrointestinal tract) are generally prone to metastasize, showing a more aggressive behaviour when compared to their cutaneous counterparts (Ozaki et al., 2002; Hillman et al., 2010; Larsen et al., 2022). KIT location and *c*-kit status are uncommonly evaluated in these studies since most of them were published before the establishment of these prognostic factors (Takahashi et al., 2000; Ozaki et al., 2002). One of the rare studies investigating these two prognostic factors in extracutaneous MCTs status showed that more aggressive nasal MCTs tended to have an atypical KIT location, which agrees with the IHC patterns observed in the extra cutaneous cases from this study, while mutations at exons 8 seemed to be uncommon (Larsen et al., 2022). Dog nº16 was the only one with atypical MCT location and successful PCR amplification, and its intestinal tumor was negative for mutations in both exons 8 and 11.

The histologic arrangement of metastatic neoplastic cells was variable in this study. While in some organs, the metastatic lesions were quite large and obvious in HE sections, in others, they were more difficult to identify, being constituted of small clusters of poorly granulated neoplastic cells. The metachromatic characteristics of mast cell granules evidenced by TB proved to be extremely important and decisive in the searching for neoplastic cells, particularly in poorly differentiated cases. It is not uncommon for cutaneous or extracutaneous MCTs to be constituted of poorly granulated mast cells, which may impair the histologic diagnosis (Ozaki et al., 2002; Kiupel, 2017; Larsen et al., 2022). Additionally, the cytoplasmic granules in some MCTs, particularly in gastrointestinal sites, often show weak metachromasia, leading to a predominantly negative TB (Ozaki et al. 2002). This was observed in both intestinal neoplasms from this study. In the cases with poor granularity and/or weak metachromasia, slide evaluation at a higher magnification (1000 X), as performed in this study, may aid in the diagnosis by facilitating the visualization of granules within neoplastic cells, either in HE or in TB sections. Additionally, some IHC antibodies, such as the combination of mast cell tryptase and KIT, may help confirming a MCT diagnosis (Ozaki et al., 2002; Kiupel, 2017).

In several cases from this study, circulating neoplastic mast cells were seen in multiple small blood vessels of the spleen, liver, lung, glomerulus, pancreas, intestine, urinary bladder, brain and heart. The embolization of neoplastic cells is less commonly observed in round cell tumors when compared to carcinomas and is generally not associated with blood vessel occlusion and infarctions, mainly because round cell tumors do not form cell clusters within the blood stream, facilitating cell entrapment, as observed with neoplastic epithelial cells (Pazzi et al., 2022). Neoplastic mast cells within the blood stream are an important prognostic factor in dogs with metastatic MCTs, being generally associated with a poor prognosis

(Kiupel, 2017). In dog n° 11, these cells were visualized in the blood smear, aiding in the clinical suspicion of systemic mastocytosis.

The KIT location was considered atypical in all tumors from this study. This pattern of KIT expression, at least in cMCTs, was previously linked to a more aggressive behaviour, to the presence of exon 11 mutations in the *c-kit* gene and to a better response to therapy with tyrosine kinase inhibitors (Webster et al., 2007; Kiupel, 2017). Unfourtunately, DNA amplification was unsuccessful in most tumors testes in this study. This was probably atributed to DNA damage induced by formalin-fixation (Dietrich et al., 2013). All nine tumors where it was possible to amplificate exon 11 and/or exon 8 were negative for ITDs. This was unexpected reusult, since most of the tumors with patterns 2 and 3 of KIT immunolabeling are postitive for exon 11 ITDs (Webster et al., 2007).

While patients baring cMCTs with *c-kit* mutations at exon 11 are generally treated with tyrosine kinase inhibitors, dogs baring cMCTs without mutations have better response rates to a combination therapy of vinblastine/prednisone (Webster et al., 2007). Unfortunately, prognostic tools for MCT evaluation and selection of systemic treatment protocols are still uncommonly applied in veterinary medicine in some countries. The dogs from this study were not submitted to mutation screening before the establishment of a systemic therapy, and we were not able to establish which criteria led clinicians to choose certain treatment protocols. Regardless of the treatment option in these dogs, they ended up dying of MCT-related disease.

Although KIT immunolabeling is traditionally used as a prognostic tool for cMCTs, it has been shown to be relevant in the prognosis of scMCTs as well (Kiupel, 2017). Recent

studies have been suggesting it may also have a prognostic importance in some mucosal MCTs (Kiupel, 2017; Larsen et al., 2022). As mentioned above, most cMCTs with atypical KIT location are positive for mutations at exon 11 of the *c-kit* gene (Webster et al., 2007), however, this association does not seem to exist in nasal MCTs. A recent study did not find any association between atypical KIT pattern and *c-kit* mutations in these tumors (Larsen et al., 2022).

Reguera (2000) described that the number of immunolabelled cells was lower in low-grade cMCTs, in which immunolabeling was also more intense. In our study, high grade MCTs had a weak to almost absent immunolabeling, which hampered the IHC evaluation. However, the low number of cases did not allow any statistical analysis of these findings. Thompson (2011) suggests that phenotypic differences of mast cells with different primary locations may result in protein expression variabilities in profiles or responses to local signals. This theory could possibly explain the low percentage of immunolabelled cells in several cases included in our study. Another possibility is the influence of formalin-fixation times on epitope integrity, which may lead to false-negative or weakly positive immunohistochemical results for different proteins (Ramos-Vara et al., 2014). Regardless of the cause, the weak to nearly absent KIT expression observed in several MCTs from this investigation is a reason for concern, since it may misdirect pathologists to other diagnoses, particularly in poorly-differentiated MCTs. In addition to these variations in IHC intensity, it is important to remember that KIT protein is not completely specific for MCTs (Morini et al. 2004). For these two reasons, it should not be used for diagnostic purposes.

## Conclusions

Subcutaneous MCTs were relatively common in this study. This was unexpected, since they are generally less prevalent, and most of them have a less aggressive behavior. Primary and metastatic MCTs have generally the same pattern of KIT expression in this investigation. In addition, there was a significant oscillation in the number of immunolabelled cells in the MCTs from this study, which may be a limiting factor for KIT evaluation in routine samples. In the few samples with a succesful DNA amplification, no ITDs were detected. Most tested tumors in this study did not allow DNA amplification for mutation detection. PCR for exons 8 and 11 in formalin-fixed samples may represent a challenge, mainly due to formaline-induced DNA damage.

**Conflict of interest statement**

The authors report no conflicts of interest. The authors alone are responsible for the contents and writing of the paper.

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**Sources and manufactures**

[I] Dako, Carpinteria, California, United States.

[II] Easy Path, São Paulo, São Paulo, Brazil.

[III] Qiagen, Valencia, California, United States.

[IV] ACTGene Análises Moleculares, Alvorada, Rio Grande do Sul, Brazil.

## References


Brocks, B.A., Bertram, C.A., Bartel, A., Kirpensteijn, J., Collins-Webb, A., Catlin C., Thaiwong, T., Kiupel, M., 2020. Internal Tandem Duplication of Exon 8 of c-kit Is Associated With Longer Total Survival in Canine Cutaneous Mast Cell Tumors. Veterinary Pathology, 58, 315-324.

Dietrich, D., Uhl, B., Sailer, V., Holmes, E. E., Jung, M., Meller, S., & Kristiansen, G., 2013. Improved PCR performance using template DNA from formalin-fixed and paraffin-embedded tissues by overcoming PCR inhibition. PloS one, 8, e77771.

Jones, C.L.R., Grahn, R.A., Chien, M.B., Lyons, L.A., London, C.A.,2004. Detection of c-kit mutations in canine mastcell tumors using fluorescent polyacrylamide gel electrophoresis. Journal of Veterinary Diagnostic Investigation, 16, 95-100.

Kiupel, M., 2017. Mast cell tumors. In: Meuten, D.J., Tumors in Domestic Animal. 5 ed., John Wiley & Sons, Ames, Iowa, EUA, 176-202.

Kiupel, M., Webster, J.D., Bailey, K.L., Best, S., DeLay, J., Detrisac, C.J., Fitzgerald, S.D., Gamble, D., Ginn, P.E., Goldschmidt, M.H. et al., 2011. Proposal of a 2-tier histologic grading system for canine cutaneous mast cell tumors to more accurately predict biological behaviour. Veterinary Pathology, 48, 147-155.

Kiupel, M., Webster, J.D., Kaneene, J.B., Miller, R., Yuzbasiyan-Gurkan, V., 2004. The use of KIT and tryptase expression patterns as prognostic tools for canine cutaneous mast cell tumors. Veterinary Pathology, 41, 371-377.

Hillman, L.A., Garrett, L.D., de Lorimier, L.P., Charney, S.C., Borst, L.B., Fan, T.M., 2010. Biological behavior of oral and perioral mast cell tumors in dogs: 44 cases (1996–2006). Journal of American Veterinary Medical Association, 237, 936-942.

Hottendorf, G.H., Nielsen, S.W., 1968. Pathologic report of 29 necropsies on dogs with mastocytoma. Pathologia veterinaria, 5, 102-121.

Kobayashi M., et al. 2012. Canine intestinal mast cell tumor with c-kit exon 8 mutation responsive to imatinib therapy. The Veterinary Journal, 193(1):264-267.

Larsen, E., Watson, A.M., Muñoz Gutiérrez, J.F., 2022. Intranasal mast cell tumors: Clinical, immunohistochemical, and molecular features in 20 dogs. Veterinary Pathology, july, 1-7.

London, C.A., Thamm, D.H., 2013. Mast Cell Tumors. In: Withrow, S.J. MacWen, E.G., Small Animal Clinical Oncology, 3rd ed., Saunder, Philadelphia, EUA, p. 335-355.

Morini, M., Bettini, G., Preziosi, R., Mandrioli, L., 2004. C-kit Gene Product (CD117) Immunoreactivity in Canine and Feline Paraffin Sections. Journal of Histochemistry & Cytochemistry, 52, 705-708.

Newman, S.J., Mrkonjich, L., Walker, K.K., Rohrbach, B.W., 2007. Canine subcutaneous mast cell tumor: diagnosis and prognosis. Journal of Comparative Pathology, 136, 231-239.

O'Keefe, D.A., Couto, C.G., Burke-Schwartz, C., Jacobs, R.M., 1987. Systemic Mastocytosis in 16 Dogs. Journal of Veterinary Internal Medicine, 1, 75-80.

Ozaki, K., Yamagami, T., Noruma K, Narama I., 2002. Mast cell tumors of the gastrointestinal tract in 39 dogs. Veterinary Pathology, 39, 557-564.

Patnaik, A.K., Ehler, W.J., MacWen, E.G., 1984. Canine Cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. Veterinary Pathology, 21, 469-474.

Patnaik, A.K., MacEwen, E.G., Black A.P., Luckow, S., 1982. Extracutaneous mast-cell tumor in the dog. Veterinary Pathology, 19, 608-615.

Pazzi, P., Celliers, A., du Plessis, E.C., Kristensen, A.T., Goddard, A. 2022. The prevalence of intra-tumoral and distant thrombi, as well as tumor-cell emboli in canine neoplasia. Veterinary Comparative Oncology, 20, 154-163.

Ramos-Vara, J.A., Miller, M.A., 2014. When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry – the red, brown, and blue technique. Veterinary Pathology, 51, 42-87.

Reguera, M. J., Rabanal, R. M., Puigdemont, A., Ferrer, L., 2000. Canine mast cell tumors express stem cell factor receptor. American Journal of Dermatopathology, 22, 49-54.

Thompson, J.J., Yager, J.A., Best, S.J., Pearl, D.L., Coomber, B.L., Torres, R.N., Kiupel, M., Foster, R.A., 2011. Canine Subcutaneous Mast Cell Tumors: Cellular Proliferation and KIT Expression as Prognostic Indices. Veterinary Pathology, 48, 169-181.

Takahashi, T., Kadosawa, T., Nagase, M., Matsunaga, S., Mochizuki, M., Nishimura, R., Sasaki, N., 2000. Visceral mast cell tumors in dogs: 10 cases (1982-1997). Journal of American Veterinary Medical Association, 216, 222-226.

Vozdova, M. et al. Prevalence and prognostic value of c-kit and TP53 mutations in canine mast cell tumours. Veterinary Journal, v. 247, p. 71-74, 2019.

Webster, J.D., Yuzbasiyan-Gurkan, V., Miller, R.A., Kaneene, J.B., Kiupel, M., 2007. Cellular proliferation in canine cutaneous mast cell tumors: associations with c-KIT and its role in prognostication. Veterinary Pathology, 44, 298-308.

**Table 1. Metastatic mast cell tumors leading to euthanasia or death: 16 cases. Organs affected by the primary and metastatic lesions, clinical signs and cause of death**

| *Nº* | *Age* | *Breed* | *Clinical evolution* | *Primary lesion* | *Metastasis* | *Clinical signs* | *Cause of death* | *Surgery* | *Systemic therapy* |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 16 y | Labrador Retriever | NI | SC tissue | Lymph node, liver, spleen | Anaemia | Perforated gastric ulcer | No | No |
| 2 | 10 y | Cocker Spaniel | NI | SC tissue | Lymph node, liver, spleen, lungs, kidneys, SC tissue | Sub-mandibular volume increase | Euthanasia | Yes | No |
| 3 | 8 y | Miniature Pinscher | 10 days | SC tissue | Lymph node, liver, spleen, lungs, skeletal muscle, mediastinum | Dyspnoea, inappe-tence, apathy | Euthanasia | Yes | No |
| 4 | 10 y | Mixed breed | 1 month | Periocular tissue | Lymph node, liver, spleen, bone marrow, brain | Convulsion, dyspnoea | Euthanasia | No | No |
| 5 | 5 y | Boxer | 4 days | Dermis | Lymph node, liver, | Uraemia | Euthanasia | No | No |
| 6 | 10 y | Mixed Breed | 2 months | Dermis | Lymph node, liver, spleen, urinary bladder | Ascites | Euthanasia | Yes | Yes |
| 7 | 13 y | Dachshund | 12 months | Dermis | Lymph node, liver, kidneys, bone marrow | Vomiting and anorexia | Euthanasia | Yes | Yes |
| 8 | NI | American Staffordshire Terrier | NI | Dermis | Lymph node, liver, spleen, lungs, heart | NI | NI | NI | NI |
| 9 | 14 y | Mixed breed | 2 months | SC tissue | Lymph node, liver, spleen, lungs | Apathy | Euthanasia | Yes | No |
| 10 | 12 y | Mixed breed | NI | Dermis | Lymph node, liver, spleen, lungs, kidney, bone marrow, intestine | NI | Euthanasia | No | No |
| 11 | 10 y | Australian Cattle Dog | 3 days | Small intestine | Lymph node, liver, spleen, kidneys, heart, urinary bladder, pancreas | Acute vomiting, and diarrhoea | Gastric ulcer | No | No |
| 12 | 11 y | Chinese Shar-Pei | 6 months | Dermis | Lymph node, liver, spleen, lungs | NI | Hepatic failure | No | Yes |
| 13 | 4 y | American Staffordshire Terrier | 36 months | Dermis and SC tissue | Lymph node, liver, spleen, lungs, kidneys, bone marrow, heart, testicle, adrenal glands | Apathy, anorexia, and anaemia | Euthanasia | No | Yes |
| 14 | 11 y | Mixed breed | 2 months | SC tissue | Lymph node, liver, spleen, lungs, kidneys, skeletal muscle, brain | Syncope, salivation, tremors, vomiting, and melena | Euthanasia | Yes | Yes |
| 15 | 13 y | Mixed breed | 30 months | SC tissue | Lymph node, liver, spleen, lungs, bone marrow, skeletal muscle | Anorexia, apathy, and vomiting | Euthanasia | Yes | Yes |
| 16 | 12 y | Mixed breed | 3 days | Colon | Lymph node, liver, spleen | Vomiting | Gastric ulcer | No | No |

y: years, NI: not informed, SC: subcutaneous tissue.

**Table 2. Cutaneous mast cell tumours leading to euthanasia or death due to metastatic spread: information regarding surgery, chemotherapy and death regarding surgery, chemotherapy and death**

| *Nº* | *Clinical evolution since 1st diagnosis* | *Grade (Two-tier grading system)* | *Grade (Three-tier grading system)* | *KIT IHC pattern of primary tumor* | *Surgical removal of primary tumor* | *Systemic therapy surgery* | *Metastasis following surgery and systemic therapy* | *Local recurrence following surgery and systemic therapy* | *Cause of death* |
|---|---|---|---|---|---|---|---|---|---|
| 5 | NI | NI | NI | NI | No | No | Lymph node, liver | No | Euthanasia |
| 6 | 2 months | High | Three | III | Yes | Yes | Lymph node, liver, spleen, urinary bladder | Yes | Euthanasia |
| 7 | 12 months | High | Three | III | Yes | Yes | Lymph node, liver, spleen, kidneys, bone marrow | No | Euthanasia |
| 8 | NI | High | Two | II | NI | NI | Lymph node, liver, spleen, lungs, heart | No | NI[1] |
| 10 | NI | High | Two | II | No | No | Lymph node, liver, spleen, lungs, kidney, bone marrow, intestine | No | Euthanasia |
| 12 | 6 months | NI | NI | NI | No | Yes | Lymph node, liver, spleen, lungs | No | Systemic mastocytosis |
| 13 | 36 months | Low and high | Two and three | II | No | Yes | Lymph node, liver, spleen, lungs, kidneys, bone marrow, heart, testicle, adrenal glands | No | Euthanasia |

NI: not informed

**Table 3. Dogs with cutaneous or subcutaneous mast cell tumours submitted to systemic treatment (chemotherapy)**

| *Nº* | *Surgery* | *Systemic treatment* | *Used drug(s)* | *Number of sections* | *Death* |
|---|---|---|---|---|---|
| 6 | Yes | Yes | Prednisolone<br>Vinblastine | 4 | Euthanasia |
| 7 | Yes | Yes | Prednisolone<br>Vinblastine | 7 | Euthanasia |
| 12 | No | Yes | Prednisolone<br>Vinblastine<br>Cyclophosphamide<br>Toceranib | 6 | Spontaneous |
| 13 | No | Yes | Prednisolone<br>Lomustine<br>Famotidine<br>Vinblastine | 6 | Euthanasia |
| 14 | Yes | Yes | Prednisolone<br>Vinblastine | 2 | Euthanasia |
| 15 | No | Yes | Prednisolone<br>Vinblastine | 6 | Euthanasia |

**Figure Legends**

**Figure 1A.** Primary dermic mast cell tumour (dog n°8). A large ulcerated mass is observed in the axillary area.

**Figure 1B.** Multiple primary dermic and subcutaneous mast cell tumours (dog n°13). Several alopecic nodules are distributed in different body regions.

**Figure 1C.** Primary duodenal mast cell tumour (dog n°14). A hemorrhagic nodule is observed in the intestinal wall. Surrounding mesentery is hemorrhagic and edematous.

**Figure 1D.** Primary ileum mast cell tumour (dog n°16). A multilobulated mass arrising from the ileum wall and invading adjacent mesenteric lymph nodes is observed.

**Figure 1E.** Primary ileum mast cell tumour (dog n°16). On cut surface, the intestinal mass is transmural, whithish with hemorrhagic areas. Some circular nodules observed in this picture are invaded lymph nodes.

**Figure 1F.** Primary ileum mast cell tumour (dog n°16). The intestinal mucosa in the area affected by the tumor is multifocally ulcerated.

**Figure 2A.** Submandibular lymph node with mast cell tumor metastasis (dog n°7). The lymph node is diffusely enlarged.

**Figure 2B.** Submandibular lymph node with mast cell tumour metastasis (dog n°7). On cut surface, it is diffusely white with loss of the nodal architeture.

**Figure 2C.** Liver and spleen metastasis in a dog with mast cell tumour (dog n°12). The liver and spleen are diffusely and severely enlarged.

**Figure 2D.** Splenic metastasis in a dog with mast cell tumour (dog n°8). The spleen is diffusely enlarged, with multifocal to coalescent white nodules, occasionally containing an hemorrhagic center.

**Figure 2E.** Liver metastasis in a dog with mast cell tumour (dog n°12). The liver has an exacerbation of the lobular pattern, and a pinkish metastatic nodule is observed in the parenchyma.

**Figure 2F.** Liver metastasis in a dog with mast cell tumour (dog n°15). Multiple pinpoint whit areas are observed in the liver capsular surface. They were confirmed as metastasis.

**Figure 3A.** Metastatic mast cell tumour (dog n°11). A cluster of metastatic cells showing moderate amount of methachromatic cytoplasmic granules is observed. Some cells are within sinusoids. Toluine Blue (TB).

**Figure 3B.** Poorly differentiated mast cell tumour (dog n°13). The neoplastic cells are arranged in solid sheaths supported by a scarce collagenous stroma. These cells have a scarce to moderate amount of eosinophilic cytoplasm with rare basophilic granules. HE.

**Figure 3C.** Mast cell tumour embolization (dog n°3). Several atypical mast cells containing metachromatic granules are observed filling the liver sinusouds. TB.

**Figure 3D.** Metastatic mast cell tumour (dog n°11). A cluster of metastatic cells showing moderate amount of methachromatic cytoplasmic granules is observed. Some cells are within sinusoids. Toluine Blue (TB).

**Figure 3E.** Mast cell tumour with aberrant KIT expression (dog n°3). Some neoplastic cells have a weak and focal cytoplasmic labelling, showing an atypical KIT protein location compatible with pattern II. IHC.

**Figure 3F.** Mast cell tumour with aberrant KIT expression (dog n°5). Most neoplastic cells have a diffuse cytoplasmic labelling, showing an atypical KIT protein location compatible with pattern III. Immunohistochemistry (IHC).

**Figure 1**

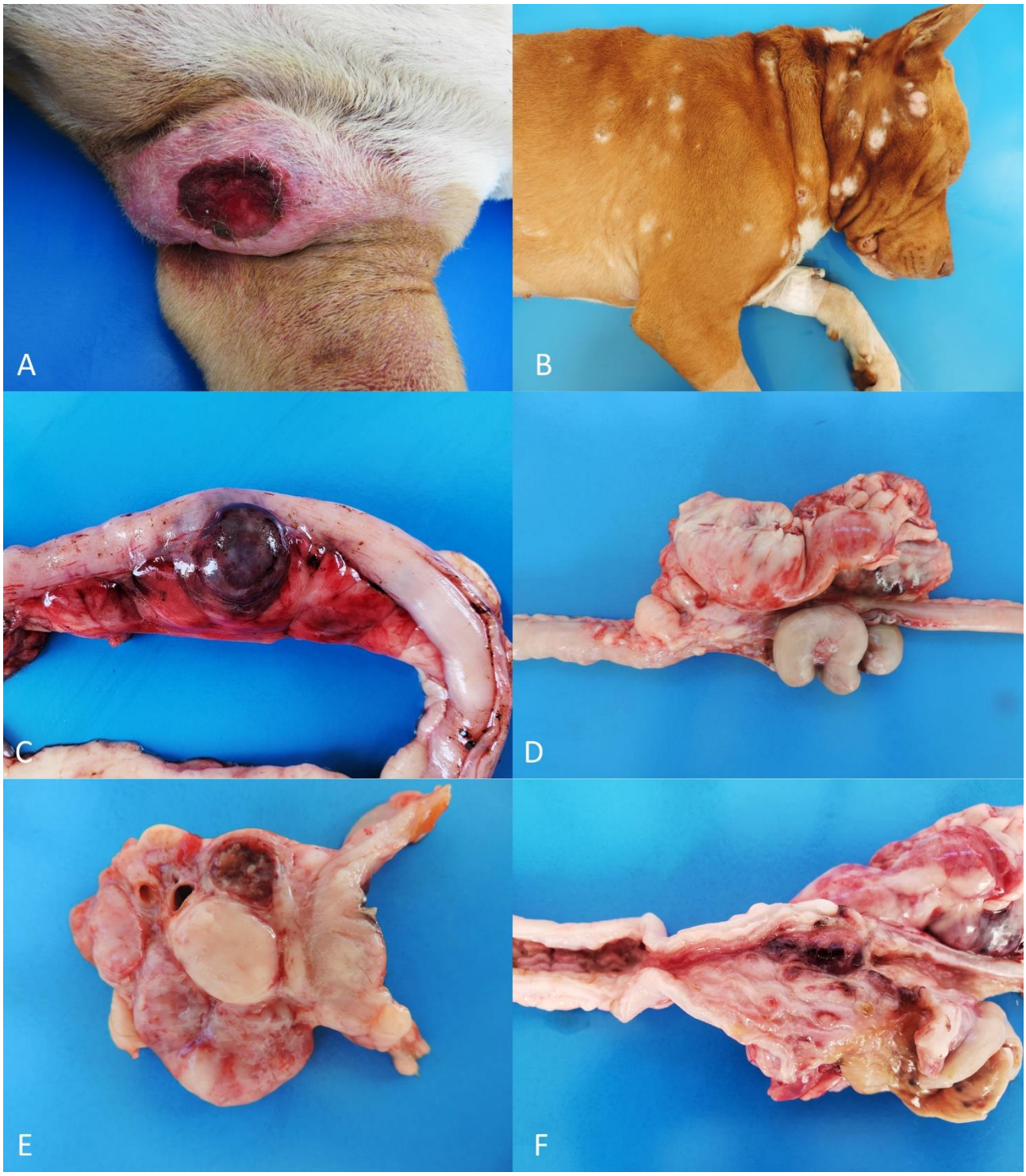


**Figure 2**

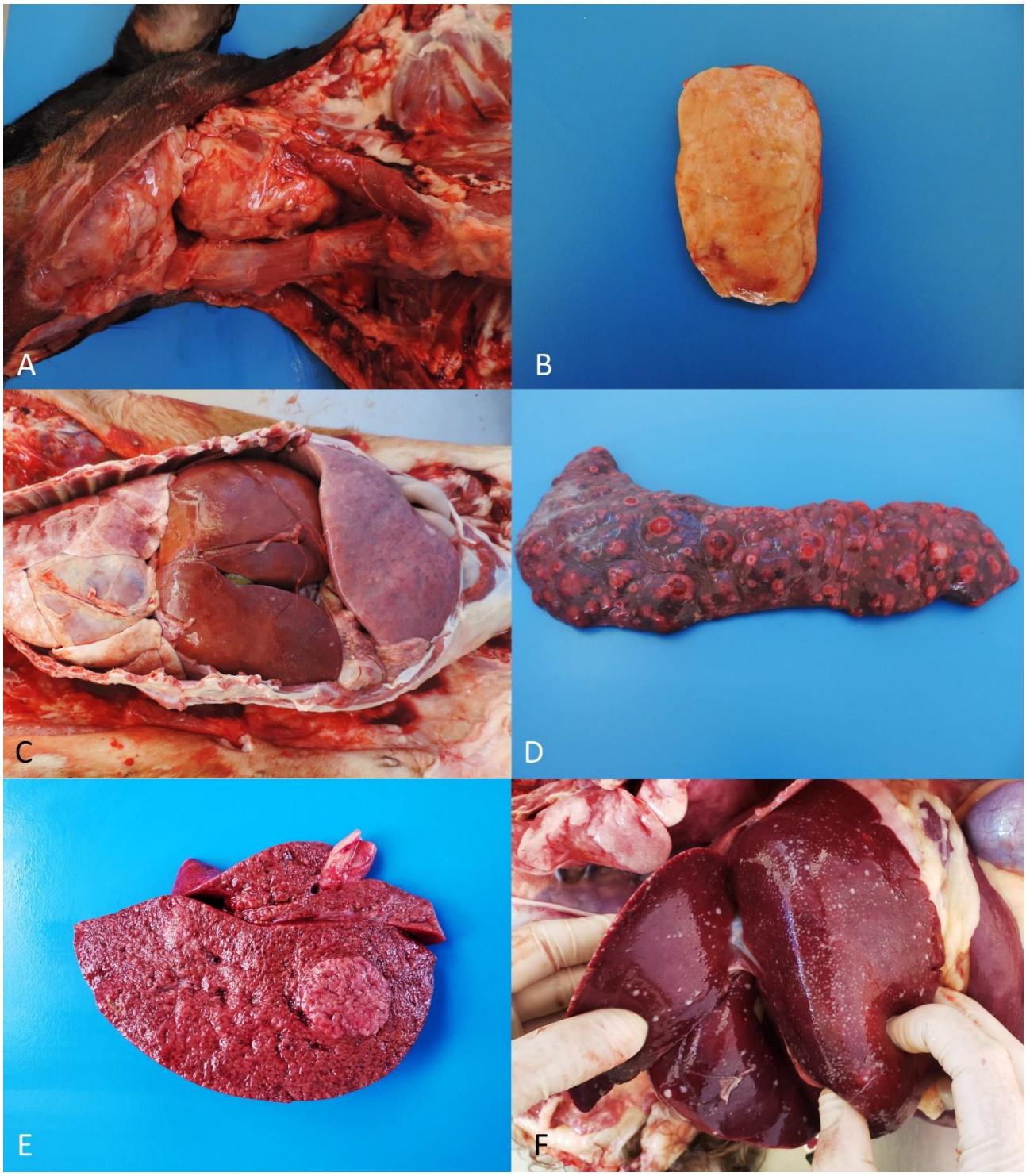
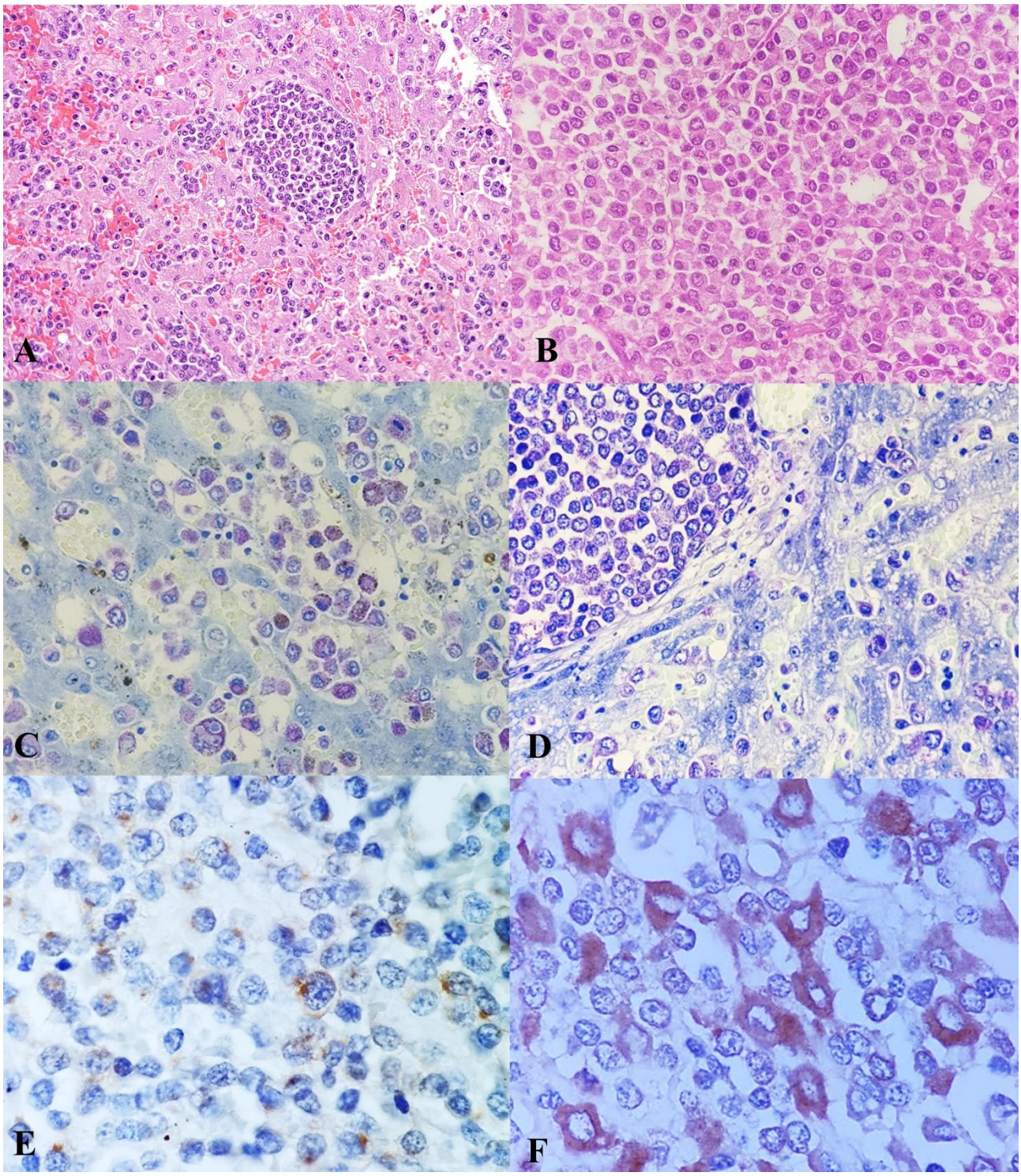


**Figure 3**



## 3.2 INTERNAL TANDEM DUPLICATIONS OF *C-KIT* EXONS 8 AND 11 IN CANINE CUTANEOUS MAST CELL TUMORS FROM BRAZIL

Artigo a ser submetido à revista Ciência Rural.

# Internal Tandem Duplications of *c-kit* exons 8 and 11 in canine cutaneous mast cell tumors from Brazil

# Duplicações internas em sequência nos éxons 8 e 11 do gene *c-kit* de mastocitomas cutâneos caninos diagnosticados no Brasil

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## ABSTRACT

Cutaneous mast cell tumor (MCT) is a very frequent malignant skin neoplasm in dogs and has a highly variable biological behavior. Because histopathology alone cannot predict it prognostic, the search for ancillary methods is essential. Recent studies show that internal tandem duplications (ITDs) in *c-kit* exons 8 and 11 are correlated with their biological behavior. The main objective of this note was to investigate the presence of *c-kit* exons 8 and 11 mutations in 45 canine cutaneous mast cell tumors diagnosed in Southern Brazil. All MCTs were histologically evaluated with both Patnaik and Kiupel grading systems, submitted to immunohistochemistry (IHC) using an anti-KIT antibody and PCR for exons 8 and 11. The histological grade varied among the five possible method combinations as well as all KIT location patterns in the IHC. Additionally, two cases did not have any immunolabeled cells. Thirteen cases were positive for exon 11 ITDs, most from frozen samples. Amplification of DNA region for exon 8 occurred in only thirteen cases, none of which were ITD positive. This study was important to identify limitations in this technique and may help on the further standardization of PCR for exon 8 in formalin-fixed samples.

**Keywords:** KIT, immunohistochemistry, mutation, PCR

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**RESUMO**

O mastocitoma cutâneo (MC) é um neoplasma cutâneo maligno muito frequente em cães e possui comportamento biológico altamente variável. Como a histopatologia sozinha não pode prever seu prognóstico, a busca por métodos auxiliares é essencial. Estudos recentes mostram que as *internal tandem duplications* (ITDs) nos exons 8 e 11 do *c-kit* estão correlacionados com seu comportamento biológico. O objetivo principal desta nota foi investigar a presença de mutações dos éxons 8 e 11 do *c-kit* em 45 MCs caninos diagnosticados no sul do Brasil. Todos os MCs foram avaliados histologicamente, graduados pelos métodos de Patnaik e Kiupel, submetidos a imuno-histoquímica (IHQ) usando um anticorpo anti-KIT e PCR para exons 8 e 11. O grau histológico variou entre as cinco combinações de métodos possíveis, assim como todos os padrões de localização de KIT na IHQ. Além disso, dois casos não apresentaram células imunomarcadas. Treze casos foram positivos para ITDs do exon 11, a maioria de amostras congeladas. A amplificação da região de DNA para o exon 8 ocorreu em apenas treze casos, nenhum dos quais com ITD positivo. Este estudo foi importante para identificar as limitações desta técnica e pode auxiliar na futura padronização da PCR para o exon 8 em amostras fixadas em formol.

**Palavras-chave:** KIT, imuno-histoquímica, mutação, PCR

Cutaneous mast cell tumor (MCTs) is a very frequent malignant skin neoplasm in dogs (KIUPEL et al., 2011, SLEDGE et al., 2016). It has a highly variable biological behavior, which is not always predictable by histopathology alone (KIUPEL et al., 2011). In this sense, several additional prognostic markers are used to access biologic behavior, and sometimes help choosing the best therapeutic option (BROCKS et al., 2020; HÄMÄLÄINEN et al., 2021; KNIGHT et al., 2022). The KIT expression pattern (WEBSTER et al., 2006) and the mutation status of *c-kit* exon 11 (LONDON et al., 1999) are considered important prognostic factors for MCTs. The predominance of cytoplasmic KIT labelling (WEBSTER, et al., 2006) and the presence of internal tandem duplications (ITDs) in exon 11 of *c-kit* (LONDON et al., 1999) are generally correlated with a more aggressive

behavior. More recently, a new set of mutations affecting exon 8 of *c-kit* has been proposed as a new prognostic marker (BROCKS et al., 2020). This first study suggests that ITDs of *c-kit* exon 8 are associated with a longer survival in dogs with MCT. Studies exploring ITDs in exon 8 are still scarce, since the prognostic importance of this set of mutations has been only recently proposed (BROCKS et al., 2020). The main objective of this note was to investigate the presence of *c-kit* exons 8 and 11 mutations in 45 canine cutaneous mast cell tumors diagnosed in Southern Brazil.

A retrospective study of the biopsy database from a Brazilian pathology service was conducted (2018-2022), and dogs diagnosed with cutaneous mast cell tumors were studied. Only cases with availabile paraffin-embedded tissues and that had a succesful amplification DNA region of exon 11 in a previous PCR protocol were included. Sixteen cases included had frozen samples available for PCR. The data obtained from the biopsy reports were: sex, breed, age, tumor location and previous treatment. New histological slides from all cases were obtained from the paraffin blocks, and cases were evaluated histologically. All cutaneous MCTs were graduated according to a three-tier (PATNAIK et al., 1984) and a two-tier (KIUPEL et al., 2011) grading systems.

One histological section from each MCT was submitted to IHC with protocol adapted from WEBSTER (2007) using a rabbit polyclonal anti-KIT antibody (CD117, Dako, A4502)[I] incubated with the primary antibody diluted in phosphate-buffered saline with Tween 20 (1:150) for 1h at 37 °C. A polymer-HRP system was used, followed by substrate development with 3,3′diaminobenzidine (DAB; EasyPath)[II]. Positive control was a cutaneous mast cell tumor from a dog. Negative control was the same test section, but the primary antibody was replaced with phosphate-buffered saline with Tween 20. KIT expression patterns were classified into three according to criteria previously established by KIUPEL (2004): pattern I (membrane staining); pattern II (focal or stippled labelling) and pattern III (diffuse labelling) .

All cases were submitted to polymerase chain reaction (PCR) for exons 8 and 11. Thirty one samples were from paraffin-embedded tissues and foutteen from frozen samples. Extraction was performed using a commercially available kit (Qiagen QIAamp DNA FFPE Tissue Kit®, Quiagen,

56404)[III]. Fifty two samples were from paraffin-embedded tissues and sixteen from frozen samples. Four 5μm sections of each paraffin-embedded tissues were cut and placed in 2ml tubes. Paraffin was removed by treatment with xylene and absolute ethanol. From frozen samples at least 0.5 $cm^3$ of tissue from each case were subjected to the extraction protocol. The following extraction phases were performed according to the manufacturer's protocol. After extraction, samples were stored in a -20°C refrigerator. A negative extraction control (NEC) was included in the extraction procedure. The PCR protocol for exon 11 and intron 11 was performed using the following primers: PE1/PE2 (PE1: CCCATGTATGAAGTACAGTGGAAG; PE2: GTTCCCTAATCATTGTTACACG) (JONES et al., 2004). Primers used to exon 8 and intron 8: PE1/PE2 (PE1: GTCCTCTTCAAACTCAAGAAGG; PE2: CCAAAATAATCCTCTCACCTCTGC) (VOZDOVA et al., 2019). PCR reaction was performed using the Taq DNA Polymerase, Recombinant (5U/μL) (Thermo Fisher ScientificTM). Both reaction were optimized for annealing temperature (52 to 62°C, with a 2°C interval) and $MgCl_2$ concentration (1.5, 2, and 2.5mM). The PCR reaction for exon 11 was performed by using PE1/PE2 for initial denaturation at 94ºC for 3 minutes, followed by 35 cycles of 94ºC for 45 seconds, 58ºC for 30 seconds, 72ºC for 30 seconds and then a 72ºC for 10 minutes step. The PCR reaction for exon 8 was performed by using PE1/PE2 for initial denaturation at 94ºC for 5 minutes, followed by 35 cycles of 94ºC for 30 seconds, 64ºC for 40 seconds, 72ºC for 30 seconds and then a 72ºC for 7 minutes step. A non-diluted sample and a 1:10 sample were tested for both exons. The PCR product was analyzed by using agarose gel electrophoresis (agarose gel in Tris-acetate-EDTA buffer, 4%). Positive cases for exon 11 ITDs were cut from the electrophoresis gel and purified with a commercial kit (PureLink™ Quick Gel Extraction and PCR Purification Combo Kit) following the manufacturer's instructions and sent to ACTGene Análises Ltda. [IV] for Sanger sequencing. ACTGene performs sequence analysis using the Applied Biosystems® (ABI) 3130 Genetic Analyzer (ABI standard protocols).

A total of 45 MCTs were included. The 45 samples came from 36 dogs, 24 females and 12 males. Mixed breed dogs predominated (n= 14), followed by Boxer (n= 6), Dachshund (n= 4), Pit

Bull (n= 3) and other breeds (n=9). The average age was 9 years. The most frequent anatomical locations were thorax (10/14) and limbs (8/14) in females, and the scrotum (4/12) in males. Other locations were vulva (n= 5), abdomen (n=4), head (n= 3), and back, neck and tail (n=1 each). Histologal grades, KIT pattern expression and PCR results about the MCTs are concisely described in Table 1.

Positivity for exon 11 duplication was considered when two bands were observed, occurring in 10 paraffin-embedded tissue samples (10/31 [32,26 %]) and 3 frozen samples (3/14 [21,43%]). The average results obtained from both samples (26,5%) are similar to those observed in some studies (DOWNING et al., 2002; WEBSTER et al., 2008; ZEMKE, et al., 2002). The PCR for exon 8 was succeeded in 13 samples, all of them frozen tissue. However, no case was positive for mutations.

Several studies have identified the ITDs of exon 11 as the most prevalent (HAHN et al., 2008; LETARD et al., 2008; GIANTIN et al., 2012; VOZDOVA et al., 2019; VOZDOVA et al., 2020). These ITDs were associated with KIT phosphorylation despite the lack of receptor binding, with consequent high proliferative activity and poor prognosis (DOWNING et al., 2002; ZEMKE, et al., 2002). In the other hand, these MCTs respond better to treatment with tyrosine kinase inhibitors (LONDON et al., 1999). Interestingly, in only one of the cases with mutation, the dog had a clinical history of treatment with ITKs. After six chemotherapy sessions, the patient stopped responding. This raises a question: why this patient stopped to respond to chemotherapy at some point?

Exon 8 ITDs are reported with a prevalence ranging from 2% to 7% (HAHN, 2008; GIANTIN et al., 2012; LETARD et al., TAKEUCHI et al., 2013; MARCONATO et al., 2014; VOZDOVA et al., 2020), and affected MCTs exhibiting lower proliferative indices, similar to those observed in MCTs without mutations in *c-kit* exons 8 or 11 (BROCKS et al., 2020). The characterization of the MCTs reported in this note ranged from well-differentiated, low-grade cases, to high-grade neoplasms. This population incites an expectation of the presence of mutations in exon 8. The low number of cases with a successful exon 8 amplification possibly contributed to the non-observance of ITDs in this region.

The major limitation of this study was the failure of the DNA amplification of exon 8. Amplification failure was essentially observed in paraffin-embedded tissues, which are widely used as source for biomarker studies (DIETRICH et al., 2013). Factors such as fixation protocol, type of fixative solution, use of unbuffered formalin, age of the paraffin block, and endogenous or exogenous inhibitors can affect tissue integrity and make PCR unsuccessful (AN & FLEMING, 1991; GREER et al., 1991). The fixation time of the samples in this study is unknown, but all were fixed in buffered formalin. Correction possibilities in some cases may be changing the concentration of polymerase and dNTP, and PCR extension time (GREER et al., 1991). These configurations were performed and unfortunately did not generate best results. Another possibility is that the amplicon size of exon 8 is larger than that of exon 11 (VOZDOVA et al., 2019), which may be a limiting factor (HAMATANI et al., 2006).

The techniques traditionally used for the routine diagnosis of MCTs were easily applied with results clearly interpreted. On the other hand, exon 8 DNA amplification was substantially impaired in cases from paraffin-embedded tissues, just the opposite of frozen tissue samples, what was not observed for exon 11. This study was important to identify the main limitation of the technique; and it may be useful for new studies and laboratories in Brazil to standardize this auxiliary prognostic technique.

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**DECLARATION OF CONFLICTS OF INTEREST**:

The authors declare no conflict of interest.

## SOURCES AND MANUFACTURES

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## REFERENCES

AN, S. F., & FLEMING, K. A. Removal of inhibitor(s) of the polymerase chain reaction from formalin fixed, paraffin wax embedded tissues. **Journal of clinical pathology**, v.44, p.924-927. 1991. Available at <http://dx.doi.org/10.1136/jcp.44.11.924> Accessed: Nov. 19, 2022.

BROCKS, B. A. et al. Internal Tandem Duplication of Exon 8 of c-kit Is Associated with Longer Total Survival in Canine Cutaneous Mast Cell Tumors. **Veterinary Pathology**, v.58, p.315-324. 2020. Available at <https://doi.org/10.1177/0300985820973463> Accessed: Nov. 19, 2022.

DE CÁSSIA-PIRES, R. et al. Multiplex PCR as a tool for the diagnosis of *Leishmania* spp. kDNA and the gapdh housekeeping gene of mammal hosts. **PloS One**, v.12, p.e0173922. 2017.Available at <https://doi.org/10.1371/journal.pone.0173922> Accessed: Nov. 19, 2022.

DIETRICH, D. et al. Improve PCR performance using template DNA from formalin-fixed andparaffin-embedded tissues by overcoming PCR inhibition. **PloS one**, v.8, p.e77771. 2013Available at <https://doi.org/10.1371/journal.pone.0077771> Accessed: Nov. 19, 2022.

DOWNING, S. et al. Prevalence and importance of internal tandem duplications in exons 11 and 12 of c-kit in mast cell tumors of dogs. **American Journal of Veterinary Research**, v.63, p.1718-1723. 2002. Available at <https://doi.org/10.1177/0300985820973463> Accessed: Nov. 19, 2022.

GIANTIN, M. et al. c-KIT messenger RNA and protein expression and mutations in canine cutaneous mast cell tumors: correlations with post-surgical prognosis. **Journal of Veterinary Diagnostic Investigation**, v.24, p.116-126. 2012. Available at <https://doi.org/10.1177/1040638711425945> Accessed: Nov. 19, 2022.

GREER, C. E. et al. PCR amplification from paraffin-embedded tissues: recommendations on fixatives for long-term storage and prospective studies**. PCR methods and applications**, v.1, p.46–50. 1991. Available at <https://doi.org/10.1101/gr.1.1.46> Accessed: Nov. 19, 2022.

HÄMÄLÄINEN, S. et al. Carboxypeptidase A3 expression in canine mast cell tumors and tissue-resident mast cells. **Veterinary pathology**, v.59, p.236-243. 2022. Available at <https://doi.org/10.1177/03009858211062636> Accessed: Nov. 19, 2022.

HAMATANI K, et al. Improved RT-PCR Amplification for Molecular Analyses with Long-term Preserved Formalin-fixed, Paraffin-embedded Tissue Specimens. **Journal of Histochemistry & Cytochemistry**. v. 54, p.773-780, 2006. Available at <https://doi.org/10.1369/jhc.5A6859.2006> Accessed: Nov. 19, 2022.

HAHN, K. et al. Masitinib is safe and effective for the treatment of canine mast cell tumors. **Journal of Veterinary Internal Medicine**, v.22, p.1301–1309. 2008. Available at <https://doi.org/10.1111/j.1939-1676.2008.0190.x> Accessed: Nov. 19, 2022.

JONES, C. L. R. et al. Detection of c-kit mutations in canine mastcell tumors using fluorescent polyacrylamide gel electrophoresis. **Journal of Veterinary Diagnostic Investigation**, v.16, p.95-100. 2004. Available at <https://doi.org/10.1177/104063870401600201> Accessed: Nov. 19, 2022.

KIUPEL, M. et al. Proposal of a 2-tier histologic grading system for canine cutaneous mast cell tumors to more accurately predict biological behaviour. **Veterinary Pathology**, v.48, p.147-155. 2011. Available at <https://doi.org/10.1177/0300985810386469> Accessed: Nov. 19, 2022.

KIUPEL, M. et al. The use of KIT and tryptase expression patterns as prognostic tools for canine cutaneous mast cell tumors. **Veterinary Pathology**, v.41, p.371-377. 2004. Available at <https://doi.org/10.1354/vp.41-4-371> Accessed: Nov. 19, 2022.

KNIGHT, B. J. et al. Beclin-1 is a novel predictive biomarker for canine cutaneous and subcutaneous mast cell tumors. **Veterinary Pathology**, v.59, p.46-56. 2022. Available at <https://doi.org/10.1177/03009858211042578> Accessed: Nov. 19, 2022.

LETARD, S. et al. Gain-of-function mutations in the extracellular domain of KIT are common in canine mast cell tumors. **Molecular Cancer Research**, v.6, p.1137-1145. 2008. Available at <https://doi.org/10.1158/1541-7786.MCR-08-0067> Accessed: Nov. 19, 2022.

LONDON, C. A. et al. Spontaneous canine mast cell tumors express tandem duplications in the proto-oncogene c-kit. **Experimental Hematology**, v.27, p.689-697. 1999. Available at <https://doi.org/10.1016/S0301-472X(98)00075-7> Accessed: Nov. 19, 2022.

MARCONATO, L. et al. Concordance of c-kit mutational status in matched primary and metastatic cutaneous canine mast cell tumors at baseline. **Journal of Veterinary Internal Medicine**, v.28, p.547-553. 2014. Available at <https://doi.org/10.1111/jvim.12266> Accessed: Nov. 19, 2022.

PATNAIK A.K. et al. Canine Cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. **Veterinary Pathology**, v.21, p.469-474. 1984. Available at <https://doi.org/10.1177/030098588402100503> Accessed: Nov. 19, 2022.

SHI C. et al. Evaluation of Housekeeping Genes for Quantitative Real-Time PCR Analysis of Bradysia odoriphaga (Diptera: Sciaridae). **International Journal of Molecular Sciences**, v.17, p.7. 2016. Available at <https://doi.org/10.1371/journal.pone.0240972> Accessed: Nov. 19, 2022.

SLEDGE, D. G. et al. Canine cutaneous mast cell tumors: a combined clinical and pathologic approach to diagnosis, prognosis, and treatment selection. **Veterinary Journal**, v.215, p.43-54. 2016. Available at <https://doi.org/10.1016/j.tvjl.2016.06.003> Accessed: Nov. 19, 2022.

STEPHENS, A.S. et al. Internal control genes for quantitative RT-PCR expression analysis in mouse osteoblasts, osteoclasts and macrophages. **BMC Research Notes**, v.4, p.410. 2011.Availeble at <https://doi.org/10.1186/1756-0500-4-410> Accessed: Nov. 19, 2022.

TAKEUCHI, Y. et al. Validation of the prognostic value of histopathological grading or c-kit mutation in canine cutaneous mast cell tumours: a retrospective cohort study. **Veterinary Journal**, v.196, p.492-498. 2013. Available at <https://doi.org/10.1016/j.tvjl.2012.11.018> Accessed: Nov. 19, 2022.

VOZDOVA, M. et al. Mutation and methylation status of KIT and TP53 in canine cutaneous and subcutaneous mast cell tumours. **Veterinary Comparative Oncology**, v.18, p.438-444. 2020. Available at <https://doi.org/10.1111/vco.12543> Accessed: Nov. 19, 2022.

VOZDOVA, M. et al. Prevalence and prognostic value of c-kit and TP53 mutations in canine mast cell tumours. **Veterinary Journal**, v.247, p.71-74. 2019. Available at <https://doi.org/10.1016/j.tvjl.2019.03.005> Accessed: Nov. 19, 2022.

WEBSTER, J. D. et al. Cellular proliferation in canine cutaneous mast cell tumors: associations with c-Kit and its role in prognostication. **Veterinary Pathology**, v.44, p.298-308. 2007. Available at <https://doi.org/10.1354/vp.44-3-298> Accessed: Nov. 19, 2022.

WEBSTER, J.D. et al. Evaluation of prognostic markers for canine mast cell tumors treated with vinblastine and prednisone. **BMC Veterinary Research**, v.4, p.32. 2008. Available at <https://doi.org/10.1186/1746-6148-4-32> Accessed: Nov. 19, 2022.

WEBSTER, J. D. et al. Evaluation of the kinase domain of c-KIT in canine cutaneous mast cell tumors. **BMC Cancer**, v.6, p.1-8. 2006. Available at <https://doi.org/10.1186/1471-2407-6-85> Accessed: Nov. 19, 2022.

ZEMKE, D. et al. Mutations in the juxtamembrane domain of c-KIT are associated with higher grade mast cell tumors in dogs. **Veterinary Pathology**, v.39, p.529–535. 2002. Available at <https://doi.org/10.1354/vp.39-5-529> Accessed: Nov. 19, 2022.

**Table 1. Mast cell tumors with successful amplification of exon 11: histologic grade, kit pattern and tumor location**

| | Positive for ITD exon 11 | Negative for ITD exon 11 | Total |
|---|---|---|---|
| **Low grade** | 5 | 20 | 25 |
| **High grade** | 8 | 12 | 20 |
| | | | |
| **Grade I** | 0 | 1 | 1 |
| **Grade II** | 9 | 27 | 36 |
| **Grade III** | 4 | 4 | 8 |
| | | | |
| **Kit pattern I** | 1 | 11 | 12 |
| **Kit pattern II** | 8 | 21 | 29 |
| **Kit pattern III** | 2 | 0 | 2 |
| **Kit negative** | 2 | 0 | 2 |
| | | | |
| **Thorax** | 2 | 9 | 11 |
| **Limbs** | 3 | 7 | 10 |
| **Vulva** | 1 | 4 | 5 |
| **Not informed** | 0 | 5 | 5 |
| **Scrotum** | 3 | 1 | 4 |
| **Abdomen** | 0 | 4 | 4 |
| **Head** | 2 | 1 | 3 |
| **Back** | 1 | 0 | 1 |
| **Neck** | 0 | 1 | 1 |
| **Tail** | 1 | 0 | 1 |

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## 4 DISCUSSÃO

Por meio deste estudo foi possível caracterizar os aspectos anatomopatológicos e moleculares de mastocitomas em biópsias e necropsia de cães provenientes de aproximadamente 8 anos da rotina diagnóstica do Laboratório de Patologia Veterinária (LPV) da Universidade Federal de Santa Maria (UFSM). Além disso, foram realizadas novas técnicas auxiliares ainda pouco estudadas. Todos estes dados resultaram em dois artigos que constituem esta dissertação

O primeiro artigo caracterizou 16 cães com mastocitoma metastático submetidos à necropsia no intervalo 2012-2022. Apesar de ser o neoplasma cutâneo mais frequente na rotina diagnóstica do LPV (SOUZA et al., 2006) esta população representa aproximadamente 0,52% dos cães que são necropsiados no LPV. Possivelmente isto se deve a mastocitomas com comportamento agressivo correspondem a um subconjunto do grupo. Outra hipótese é que os tutores destes cães bem como os clínicos já saibam o diagnóstico e escolham não submeter o paciente ao exame necroscópico.

O padrão de expressão de KIT idêntico no neoplasma primário e metástases pode auxiliar clínicos que não tenham acesso ao sítio primário para entender melhor o comportamento biológico do neoplasma primário, caso este já esteja ausente. Além disso, houve uma oscilação significativa no número de células neoplásicas imunomarcadas neste estudo, o que pode ser um fator limitante para a avaliação do KIT em amostras de rotina oriundas de material de necropsia. Nas poucas amostras com amplificação de DNA bem-sucedida, nenhuma ITD foi detectada.

O segundo artigo, em formato de nota, teve como objetivo principal investigar a presença de mutações no exon 8 do *c-kit* em 45 mastocitomas cutâneos caninos diagnosticados no sul do Brasil. O critério de inclusão de apenas os MCCs que amplificaram o DNA para a região do exon 11 do *c-kit* objetivou selecionar amostras com material genético preservado. Apesar disso, houve uma grande dificuldade na padronização da técnica para a região do exon 8. A pequena quantidade de amostras amplificadas foi oriunda apenas de material congelado, sendo que nenhuma delas foi positiva para a ITD. Este estudo foi importante para identificar a principal limitação da técnica

## 5 CONCLUSÃO

Este estudo abordou os aspectos histoquímicos, imuno-histoquímicos e moleculares do mastocitoma em cães. Observou-se uma prevalência semelhante de mastocitomas cutâneos e subcutâneos no estudo de necropsias, o que sugere que os mastocitomas subcutâneos não sejam tão incomuns como a literatura estabelece. O fato desses tumores estarem associados a metastatização e morte/eutanásia também chama atenção, já geralmente se espera que eles tenham um comportamento biológico menos agressivo. Na imuno-histoquímica podem ocorrer casos que as células neoplásicas não demonstrem qualquer padrão de imunomarcação, o que pode ser uma limitação no uso desta técnica na rotina oncológica. Mutações no exon 11 foram relativamente comuns neste estudo, o que vai ao encontro do que já é descrito. Entretanto, uma limitação observada na investigação molecular de mutações foi a dificuldade de amplificação de do exon 8. Acredita-se que ela seja atribuída à fixação dos tecidos em formol. Isso pode representar um desafio para laboratórios brasileiros que pretendem oferecer a técnica na rotina diagnóstica.

## REFERÊNCIAS BIBLIOGRÁFICAS

ACKERMAN, M. R. Inflammation and Healing. In: Zachary, J.F. **Pathologic Basis of Veterinary Disease**. 6th. ed. St Louis: Elsevier, 2017, cap. 3, p. 73-131.

BOSTOCK, D. E. The prognosis following surgical removal of mastocytomas in dogs. **Journal of Small Animal Practice**, v. 14, n. 1, p. 27-41, 1973.

BOSTOCK, D. E.; CROCKER, J.; HARRIS, K.; SMITH, P. Nucleolar organiser regions as indicators of post-surgical prognosis in canine spontaneous mast cell tumours. **British Journal of Cancer**, v. 59, n. 6, p. 915-918, 1989.

BROCKS, B. A. W.; BERTRAM, C. A.; BARTEL, A.; KIRPENSTEIJN, J.; COLLINS-WEBB, A.; CATLIN, C.; THAIWONG, T.; KIUPEL, M. Internal Tandem Duplication of Exon 8 of c-kit Is Associated with Longer Total Survival in Canine Cutaneous Mast Cell Tumors. **Veterinary Pathology**, v.58, p.315-324. 2020.

BURTON, J. H.; VENABLE, R. O.; VAIL, D. M.; WILLIAMS, L. E.; CLIFFORD, C. A.; AXIAK-BECHTEL, S. M.; AVERY, A. C.; THAMM, D. H. Pulse-Administered Toceranib Phosphate Plus Lomustine for Treatment of Unresectable Mast Cell Tumors in Dogs. **Journal of Veterinary Internal Medicine**, v. 29, n. 4, p. 1098-1104, 2015.

CAMUS, M. S.; PRIEST, H. L.; KOEHLER, J. W.; DRISKELL, E. A.; RAKICH, P. M.; ILHA, M. R.; KRIMER, P. M. Cytologic Criteria for Mast Cell Tumor Grading in Dogs With Evaluation of Clinical Outcome. **Veterinary Pathology**, v. 53, n. 6, p. 1117-1123, 2016.

CHEN, P.; MARCONATO, L.; SABATTINI, S.; KIUPEL, M. Mutations in Exons 8 and 11 of c-kit Gene in Canine Subcutaneous Mast Cell Tumors and Their Association with Cell Proliferation. **Veterinary Science**, v. 9, n. 493, 2022.

CONCEIÇÃO, L. G.; LOURES, F. H. Sistema tegumentar. In: SANTOS, R. de L.; ALESSI, A. C. (Org.). **Patologia veterinária**. 2. ed. Rio de Janeiro: Roca, 2016. cap. 7, p. 407-413.

CULLING, C. F. A. **Cellular Pathology Technique**. Butterworths: London, 4th ed, cap. 6, p. 118-119, 1985.

DAHLIN, J. S.; MAURER, M.; METCALFE, D. D.; PEJLER, G.; SAGI-EISENBERG, R.; NILSSON, G. The ingenious mast cell: Contemporary insights into mast cell behavior and function. **Allergy**, May 6. 2021.

DALECK, C. R.; ROCHA, N. S.; FERREIRA, M. G. P. A. **Oncologia em cães e gatos**. 2. ed. Rio de Janeiro: Roca, 2016. cap. 50, p. 955-971.

DERENZINI, M. The AgNORs. **Micron**, v. 31, n. 2, p. 117-120, 2000.

DOWNING, S.; CHIEN, M. B.; KASS; P. H.; MOORE, P. E.; LONDON, C. A. Prevalence and importance of internal tandem duplications in exons 11 and 12 of c-kit in mast cell tumors of dogs. **American Journal of Veterinary Research**, v.63, p.1718-1723. 2002.

EROSCHENKO, V. P. In: **Di Fiore's atlas of histology with functional correlations**, 11 ed. Lippincott Williams & Wilkins, 2008. 552 p.

FIGHERA, R. A. **Causas de morte e razões para eutanásia de cães**. 2008. 171 p. Tese (Doutorado em Patologia Animal) – Universidade Federal de Santa Maria, Santa Maria, RS, 2008.

GALLI, S. J; ZSEBO, K. M; GEISSLER, E. N. The kit ligand, stem cell factor. **Advances in Immunology**, v. 551, p. 1-96, 1994.

GERDES, J.; LI, L.; SCHLUETER, C.; DUCHROW, M.; WOHLENBERG, C.; GERLACH, C.; STAHMER, I.; KLOTH, S.; BRANDT, E.; FLAD, H. D. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. **The American journal of pathology**, v. 138, n. 4, p. 867-73, 1991.

GIANTIN, M.; VASCELLARI, M.; MORELLO, E. M.; CAPELLO, K.; VERCELLI, A.; GRANATO, A.; LOPPARELLI, R. M.; NASSUATO, C.; CARMINATO, A.; MARTANO, M.; MUTINELLI, F.; DACASTO, M. c-KIT messenger RNA and protein expression and mutations in canine cutaneous mast cell tumors: correlations with post-surgical prognosis. **Journal of Veterinary Diagnostic Investigation**, v.24, p.116-126. 2012

GOLDSCHMIDT, M. H; HENDRICK, M. J. Tumors of the skin and soft tissues. In: Meuten, D.J. **Tumors in domestic animals**: 4.th. Ames: Iowa State, 2002. cap.2, p. 44-117.

HAN, W.; PAN, H.; CHEN, Y.; SUN, J.; WANG, Y.; LI, J.; GE, W.; FENG, L.; LIN, X.; WANG, X.; WANG, X.; JIN, H. EGFR Tyrosine Kinase Inhibitors Activate Autophagy as a Cytoprotective Response in Human Lung Cancer Cells. **PLoS One**, v. 6, n. 6, e18691, 2011.

HAHN, K. A.; OGILVIE, G.; RUSK, T.; DEVAUCHELLE, P.; LEBLANC, A.; LEGENDRE, A.; POWERS, B.; LEVENTHAL, P. S.; KINET, J. P.; PALMERINI, F.; DUBREUIL, P.; MOUSSY, A.; HERMINE, O. Masitinib is safe and effective for the treatment of canine mast cell tumors. **Journal of Veterinary Internal Medicine**, v.22, p.1301–1309. 2008.

HORTA, R. D. S.; GIULIANO, A.; LAVALLE, G. E.; COSTA, M. P.; DE ARAÚJO, R. B.; CONSTANTINO-CASAS, F.; DOBSON, J. M. Clinical, histological, immunohistochemical and genetic factors associated with measurable response of high-risk canine mast cell tumours to tyrosine kinase inhibitors. **Oncology Letters**, v. 15, n. 1, p. 129-136, 2018.

JUNQUEIRA, L. C. U; CARNEIRO, J. **Histologia básica: texto e atlas**. L. C. Junqueira e José Carneiro. 13. ed. Rio de Janeiro: Guanabara Koogan, 2012, 554 p.

KIUPEL, M., 2017. Mast cell tumors. In: Meuten, D.J. **Tumors in Domestic Animal**. 5 ed., John Wiley & Sons, Ames, Iowa, EUA, 176-202.

KIUPEL, M.; WEBSTER, J. D.; BAILEY, K. L.; BEST, S.; DELAY, J.; DETRISAC, C. J.; FITZGERALD, S. D.; GAMBLE, D.; GINN, P. E.; GOLDSCHMIDT, M. H.; HENDRICK, M. J.; HOWERTH, E. W.; JANOVITZ, E. B.; LANGOHR, I.; LENZ, S. D.; LIPSCOMB, T. P.; MILLER, M. A.; MISDORP, W.; MOROFF, S.; MULLANEY, T. P.; … MILLER, R.

Proposal of a 2-tier histologic grading system for canine cutaneous mast cell tumors to more accurately predict biological behaviour. **Veterinary Pathology**, v.48, p.147-155. 2011.

KIUPEL, M.; WEBSTER, J. D.; KANEENE, J. B.; MILLER, R.; YUZBASIYAN-GURKAN, V. The use of KIT and tryptase expression patterns as prognostic tools for canine cutaneous mast cell tumors. **Veterinary Pathology**, v.41, p.371-377. 2004

KIUPEL, M; CAMUS, M. Diagnosis and prognosis of canine cutaneous mast cell tumors. **Veterinary Clinics of North America : Small Animal Practice**, v. 49, n. 5, p. 819–836, 2019.

LELYVELD, S.; WARLAND, J.; MILLER, R.; MAW, H.; FOALE, R.; GOODFELLOW, M.; DOBSON, J. Comparison between Ki-67 index and mitotic index for predicting outcome in canine mast cell tumours. **Journal of Small Animal Practice**, v. 56, p. 312-319, 2015.

LENNARTSSON, J; RÖNNSTRAND, L. Stem cell factor receptor/c-Kit: from basic science to clinical implications. **Physiological Reviews**, v. 92, n. 4, p. 1619-1649, 2012.

LETARD, S.; YANG, Y.; HANSSENS, K.; PALMÉRINI, F.; LEVENTHAL, P. S.; GUÉRY, S.; MOUSSY, A.; KINET, J. P.; HERMINE, O.; DUBREUIL, P. Gain-of-function mutations in the extracellular domain of KIT are common in canine mast cell tumors. **Molecular Cancer Research**, v.6, p.1137-1145. 2008

LONDON, C. A.; GALLI, S. J.; YUUKI, T.; HU, Z. Q.; HELFAND, S. C.; GEISSLER, E. N. Spontaneous canine mast cell tumors express tandem duplications in the proto-oncogene c-kit. **Experimental Hematology**, v.27, p.689-697. 1999.

LONDON, C. A.; THAMM, D.H. 2013. Mast Cell Tumors. In: Withrow, S.J. MacWen, E.G., **Small Animal Clinical Oncology**, 3rd ed., Saunder, Philadelphia, EUA, p. 335-355.

MA, Y.; LONGLEY, B. J.; WANG, X.; BLOUNT, J. L.; LANGLEY, K.; CAUGHEY, G. H. Clustering of activating mutations in c-KIT's juxtamembrane coding region of canine mast cell neoplasms. **Journal of Investigative Dermatology**, v. 112, p. 165-170, 1999.

MAGLENNON, G. A; MURPHY, S.; ADAMS, V.; MILLER, J.; SMITH, K.; BLUNDEN, A.; SCASE, T. J. Associations of Ki67 index with prognosis for intermediate-grade canine cutaneous mast cell tumors, **Veterinary Comparative Oncology**, v. 6, p. 268-274, 2008.

MARCONATO, L.; ZORZAN, E.; GIANTIN, M.; DI PALMA, S.; CANCEDDA, S.; & DACASTO, M. Concordance of c-kit mutational status in matched primary and metastatic cutaneous canine mast cell tumors at baseline. **Journal of Veterinary Internal Medicine**, v.28, p.547-553. 2014.

MAULDIN, E. A.; KENNEDY, J. P. Integumentary system. In: MAXIE, M. G. **Jubb, Kennedy, and Palmer's pathology of domestic animals**, 6th. ed. Saint Louis: Elsevier, 2016. v. 1, cap. 6, p. 726-728.

NAKANO, Y.; KOBAYASHI, M.; BONKOBARA, M.; TAKANOSU, M. Identification of a secondary mutation in the KIT kinase domain correlated with imatinib-resistance in a canine mast cell tumor. **Veterinary Immunology and Immunopathology**, v. 188, p. 84-88, 2017.

NORTHRUP, N. C.; HOWERTH, E. W.; HARMON, B. G.; BROWN, C. A.; CARMICHEAL, K. P.; GARCIA, A. P.; LATIMER, K. S.; MUNDAY, J. S.; RAKICH, P. M.; RICHEY, L. J.; STEDMAN, N. L.; GIEGER, T. L. Variation among pathologist in histologic grading of canine cutaneous mast cell tumors with uniform use of a single grading reference. **Journal of Veterinary Diagnostic Investigation**, v. 17, n. 6, p. 561-564, 2005.

OLSEN, J. A.; THOMSON, M.; O'CONNELL, K.; WYATT, K. Combination vinblastine, prednisolone and toceranib phosphate for treatment of grade II and III mast cell tumours in dogs. **Veterinary Medicine and Science**, v. 24, n. 4, p. 237–251, 2018.

PAGLIN, S.; HOLLISTER, T.; DELOHERY, T.; HACKETT, N.; MCMAHILL, M.; SPHICAS, E.; DOMINGO, D.; YAHALOM, J. A. novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. **Cancer Research**, v. 61, n. 2, p. 439–444, 2001.

PATNAIK, A. K.; EHLER, W. J.; MACWEN, E. G. Canine Cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. **Veterinary Pathology**, 21, 469-474, 1984.

RAMOS-VARA, J.A., MILLER, M.A. When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry – the red, brown, and blue technique. **Veterinary Pathology**, 51, 42-87, 2014.

RECH, R. R.; GRAÇA, D. L.; KOMMERS, G. D.; SALLIS, E. S. V.; RAFFI, M. B.; GARMATZ, S. L. Mastocitoma cutâneo canino: estudo de 45 casos. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 56, n. 4, p. 441-448, 2004.

REGUERA, M. J., RABANAL, R. M., PUIGDEMONT, A., FERRER, L., Canine mast cell tumors express stem cell factor receptor. **American Journal of Dermatopathology**, v. 22, p. 49-54, 2000.

SANTOS, A.; NASCIMENTO, H. H. L.; FLORES, M. M.; KOMMERS, G. D. Use of different fixation times and application of two immunohistochemical methods for detection of KIT and Ki67 proteins in canine cutaneous mast cell tumors. **Pesquisa Veterinária Brasileira**, v. 39, n. 1, p. 52-60, 2019.

SCASE, T. J.; EDWARDS, D.; MILLER, J.; HENLEY, W.; SMITH, K.; BLUNDEN, A.; MURPHY, S. Canine mast cell tumors: correlation of apoptosis and proliferation markers with prognosis. **Journal of Veterinary Internal Medicine**, v. 20, p.151-158, 2006.

SLEDGE, D. G.; WEBSTER, J.; KIUPEL, M. Canine cutaneous mast cell tumors: a combined clinical and pathologic approach to diagnosis, prognosis, and treatment selection. **Veterinary Journal**, v.215, p.43-54. 2016.

SNYDER, P. W. Diseases of Immunity. In: Zachary, J.F. **Pathologic Basis of Veterinary Disease**. 6ª th. St Louis: Elsevier, 2017, cap. 5, 242-285, 2017.

SOUZA, T. M.; FIGHERA, R. A.; IRIGOYEN, L. F.; BARROS, C. S. L. Estudo retrospectivo de 761 tumores cutâneos em cães. **Ciência Rural**, v. 36, n. 2, p. 555-560, 2006.

STANKOV, K.; POPOVIC, S.; MIKOV, M. C-KIT signalling in cancer treatment. **Current Pharmaceutical Design**, v. 20, n. 17, p. 2849-2880, 2014.

TAKEUCHI, Y.; FUJINO, Y.; WATANABE, M.; TAKAHASHI, M.; NAKAGAWA, T.; TAKEUCHI, A.; BONKOBARA, M.; KOBAYASHI, T.; OHNO, K.; UCHIDA, K.; ASANO, K.; NISHIMURA, R.; NAKAYAMA, H.; SUGANO, S.; OHASHI, Y.; TSUJIMOTO, H. Validation of the prognostic value of histopathological grading or c-kit mutation in canine cutaneous mast cell tumours: a retrospective cohort study. **Veterinary Journal**, v.196, p.492-498. 2013.

TAMLIN, V. S; BOTTEMA, C. D. K; PEASTON, A. E. Comparative aspects of mast cell neoplasia in animals and the role of KIT in prognosis and treatment. **Veterinary Medicine and Sciences**, v. 6, n. 1, p. 3-18, 2020.

TAMLIN, V. S.; KESSELL, A. E.; MCCOY, R. J.; DOBSON, E. C.; SMITH, T. S.; HEBART, M.; BROWN, L., MITROVIC, D.; PEASTON, A. E. Prevalence of exon 11 internal tandem duplications in the C-KIT proto-oncogene in Australian canine mast cell tumours. **Australian Veterinary Journal**, v. 95, n. 10, p. 386-391, 2017.

THAMM, D. H.; WEISHAAR, K. M.; CHARLES, J. B.; EHRHART, E. J. Phosphorylated KIT as a predictor of outcome in canine mast cell tumours treated with toceranib phosphate or vinblastine. **Veterinary Comparative Oncology**, v. 18, n. 2, p. 169-175, 2020.

THOMPSON, J. et al. Receptor tyrosine kinase expression profiles in canine cutaneous and subcutaneous mast cell tumors. **Veterinary Pathology**, v. 53, n. 3, p. 545-558, 2016.

THOMPSON, J.J., YAGER, J.A., BEST, S.J., PEARL, D.L., COOMBER, B.L., TORRES, R.N., KIUPEL, M., FOSTER, R.A. Canine Subcutaneous Mast Cell Tumors: Cellular Proliferation and KIT Expression as Prognostic Indices. **Veterinary Pathology**, v. 48, p. 169-181, 2011.

VASCELLARI, M.; GIANTIN, M.; CAPELLO, K.; CARMINATO, A.; MORELLO, E. M.; VERCELLI, A.; GRANATO, A.; BURACCO, P.; DACASTO, M.; MUTINELLI, F. Expression of Ki67, BCL-2, and COX-2 in canine cutaneous mast cell tumors: association with grading and prognosis. **Veterinary Pathology**, v. 50, p.110-121, 2012.

VOZDOVA, M.; KUBICKOVA, S.; FICTUM, P.; FRÖHLICH, J.; JELINEK, F.; RUBES, J. Prevalence and prognostic value of c-kit and TP53 mutations in canine mast cell tumours. **Veterinary Journal**, v. 247, p. 71-74, 2019.

VOZDOVA, M.; KUBICKOVA, S.; FICTUM, P.; CERNOHORSKA, H.; FRÖHLICH, J.; RUBES, J. Mutation and methylation status of KIT and TP53 in canine cutaneous and subcutaneous mast cell tumours. **Veterinary Comparative Oncology**, v.18, p.438-444. 2020.

WEBSTER, J.D.; YUZBASIYAN-GURKAN, V.; MILLER, R.A.; KANEENE, J.B.; KIUPEL, M. Cellular proliferation in canine cutaneous mast cell tumors: associations with c-KIT and its role in prognostication. **Veterinary Pathology**, 44, 298-308, 2007.

WELLE, M. M.; BLEY, C. R.; HOWARD, J.; RÜFENACHT, S. Canine mast cell tumours: a review of pathogenesis, clinical features, pathology and treatment. **Journal of Veterinary Oncology**, v. 19, n. 6, p. 321-339, 2008.

YANG, Z. J.; CHEE, C. E.; HUANG, S.; SINICROPE, F. The role of autophagy in cancer: therapeutic implications. **Molecular Cancer Therapeutics**, v. 10, n. 9, p. 1533-1541, 2011.

ZEMKE, D. YAMINI, B.; YUZBASIYAN-GURKAN, V. Mutations in the juxtamembrane domain of c-KIT are associated with higher grade mast cell tumors in dogs. **Veterinary Pathology**, v.39, p.529–535. 2002