FACULDADE DE CIÊNCIAS
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BIOMÉDICAS ABEL SALAZAR
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Canine cutaneous tumors: epidemiological and molecular insights

Ana Luísa Mendes Pereira Martins

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/ _____ / _____

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RESUMO

Uma das principais causas de mortalidade em cães é o cancro. Os tumores cutâneos estão entre os tumores caninos mais frequentemente encaminhados para avaliação histopatológica e, entre eles, os mastocitomas são uma das neoplasias mais diagnosticadas, correspondendo de 7% a 21% de todas as ocorrências. Os objetivos deste estudo foram relatar a prevalência de tumores cutâneos caninos no Laboratório de Patologia Veterinária (LPV) do ICBAS-UP juntamente com caracterizar e categorizar o local anatómico, raça, idade e sexo de histotipos tumorais distintos; realizar uma análise epidemiológica do risco de desenvolvimento de mastocitomas em cães em relação a outros tumores cutâneos e, por último, analisar as correlações entre dois imunomarcadores, nomeadamente as proteínas Ki-67 e KIT, com a graduação histológica dos mastocitomas e outros parâmetros clínico-patológicos.

Cerca de 1.185 casos de tumores cutâneos caninos foram recuperados do banco de dados do LPV e os mais frequentemente encontrados foram mastocitomas (22,70%). O maior risco de desenvolver mastocitomas foi encontrado em Labrador Retrievers (OR = 2,063), Boxers (OR = 2,004), Bulldog Franceses (OR = 3,071) e Pugs (OR = 9,561). Além disso, os Boxers mostraram uma maior predisposição para tumores de baixo grau (grau I, II de Patnaik e baixo grau de Kiupel) (OR = 5,902, OR = 1.989 e OR = 2,616, respetivamente). Labrador Retrievers e Pugs apresentaram um alto risco para lesões de grau II de Patnaik (OR = 2,128 OR = 12,873, respetivamente) e baixo grau de Kiupel (OR = 2,306 e OR = 17,084, respetivamente). French Bulldogs (OR = 7,878) tiveram um alto risco para lesões de grau III e os Pit Bulls uma predisposição para o grau III de Patnaik (OR = 4,434) e tumores de alto grau de Kiupel (OR = 4,962).

A área perigenital e o tronco foram identificados como regiões de alto risco para o desenvolvimento de tumores grau III (OR = 6,615 e OR = 1,868, respetivamente). Os membros apresentaram o maior risco para lesões grau II (OR = 1,648). A região das nádegas, cabeça e pescoço apresentaram risco diminuído para tumores grau II (OR = 0,071 e OR = 0,396, respetivamente). Os grupos de idades de 4-6 anos e 7-10 anos apresentaram maior risco quando comparados ao grupo mais velho do estudo. Esses grupos apresentaram maior risco para a ocorrência de lesões grau II de Patnaik (OR = 2.680 e OR = 1.629, respetivamente). Para os tumores de baixo grau de Kiupel, foi observado um risco maior para o grupo entre 4 e 6 anos (OR = 2.647).

O índice de Ki67 mostrou uma relação dependente e negativa com a idade, bem como uma relação positiva e moderada com a graduação histológica de Kiupel. Os resultados para a correlação entre o padrão KIT e os parâmetros clínico-patológicos

destacaram uma correlação positiva moderada a forte entre a imunoexpressão KIT e os graus histológicos de Patnaik e Kiupel.

As informações epidemiológicas obtidas neste estudo podem auxiliar os médicos veterinários regionais, auxiliando no diagnóstico preliminar ou suspeita de tumores cutâneos caninos e fornecer informações prognósticas mais adequadas e contextualizadas em relação aos mastocitomas caninos.

Palavras-chave: Oncologia; cão; tumores cutâneos; mastocitoma; raças; localização anatómica; idade; Ki67; KIT.

ABSTRACT

One of the leading causes of mortality in dogs is cancer. Skin tumors are amongst the most frequent canine tumors sent for histopathological evaluation and amongst these, mast cell tumors (MCTs) are one of the most frequently diagnosed neoplasms accounting for 7% to 21% of all occurrences. The aims of this study were to report the prevalence of canine cutaneous tumors in the LPV from ICBAS-UP along with categorize and characterize the anatomical site, breed, age and sex of distinct tumor histotypes; to conduct an epidemiological analysis of the risk of MCT development in dogs in relation to other skin tumors and lastly to analyze the correlations between two immunomarkers, namely Ki-67 and KIT proteins, with MCTs histological grading and other clinicopathological parameters.

About 1,185 cases of canine cutaneous tumors were retrieved from LPV databases and the most often encountered were MCTs (22.70%). The highest risk for developing MCTs was found in Labrador Retrievers (OR= 2.063), Boxers (OR= 2.004), French Bulldogs (OR=3.071) and Pugs (OR=9.561). Plus, Boxers had a higher predisposition to lower grade tumors (Patnaik's grade I, II and Kiupel's low-grade) (OR= 5.902, OR=1,989 and OR=2.616, respectively). Labrador Retrievers and Pugs presented a high risk for Patnaik's grade II lesions (OR=2.128 OR=12.873, respectively) and Kiupel's low-grade (OR=2.306 and OR=17.084, respectively). French Bulldogs (OR= 7.878) had a high risk for grade III lesions and Pit Bulls have a noted predisposition to Patnaik's grade III (OR= 4.434) and Kiupel's high-grade tumors (OR=4.962).

The perigenital area and trunk were identified as high-risk regions for grade III tumors development (OR=6.615 and OR=1.868, respectively). The limbs had the highest risk for grade II lesions (OR=1.648). Buttock area, head and neck showed a decrease risk for grade II tumors (OR=0.071 and OR=0.396, respectively). The aged groups of 4-6 years and 7-10 years had the higher risk when compared to the older group in the study. These groups depicted higher risk for Patnaik's grade II lesions occurrence (OR=2,680 and OR=1,629, respectively). For Kiupel's low-grade tumors a higher risk for the group between 4 and 6 years old (OR=2,647) was noted.

Ki67 index showed a dependent and negative relationship with age as well as a moderate positive relationship with Kiupel's histological grading. Results for the correlation between KIT pattern and the clinicopathological parameters have highlighted a moderate to strong positive correlation between KIT immunoexpression and both Patnaik's and Kiupel's histological grades.

The epidemiological information achieved in this research can assist regional veterinarians, favoring a preliminary diagnosis or suspicion of canine cutaneous tumors and provide more adequate and contextualize prognostic information regarding canine mast cell tumors.

Keywords: Oncology; dog; cutaneous tumors; mast cell tumors; breeds; anatomical location; age; Ki67; KIT.

TABLE OF CONTENTS

ILLUSTRATION LIST

ABREVIATIONS

FCUP – Faculdade de Ciências, University of Porto

ICBAS – Institute of Biomedical Sciences Abel Salazar

MCT – Mast cell tumor

STS - Soft tissue sarcomas

SCC – Squamous cell carcinoma

NOS – not otherwise specified

NOC – not otherwise classified

CI - Confidence Interval

OR - Odds ratio

LPV-ICBAS - Laboratory of Veterinary Pathology from Institute of Biomedical Sciences Abel Salazar

HE/H&E- Hematoxylin and eosin stain

Ki-67 - The Ki-67 protein (also known as MKI67) is a cellular marker for proliferation.

IP – Proliferative index

KIT - Tyrosine-protein kinase receptor

SCF – Stem cell factor

IHC – Immunohistochemistry

HPF – High-power field

RTK - Receptor tyrosine kinases

UN - Unknown

MI - Mitotic index

AgNORs - Argyrophilic Nucleolar Organizer Region

NORs – Nucleolar Organizer Region

PCNA - Proliferating cell nuclear antigen

NSC – Not subjected to this classification system

(N:C) – Nucleus to cytoplasm ratio

DAB - 3,3′-Diaminobenzidine is a derivative of benzene

TBS - Tris-HCl Buffered Saline

NR – No record

SD – Standard deviation

MAPK – Mitogen-activated protein kinase

SFK - Src family of protein tyrosine kinases

CHAPTER 1

General introduction

CHAPTER 1 - General Introduction

Normal histology and cytology of the skin

The skin is the largest organ of the body. It is a multifaceted, integrated, and dynamic organ that has functions beyond its role as a barrier against the environment.[1,2] Consists of epidermis, dermis, subcutis and adnexa (hair follicles and sebaceous and sweat glands) – **Figure 1-A**.[2–6]

Overall, the basic architecture of the skin is similar in all mammals, but the histologic structure differs significantly by anatomic site and amongst different species. In general, haired skin is thicker over the dorsal aspect of the body and on the lateral aspect of the limbs and its thinner on the ventral aspect of the body and the medial aspect of the thighs. Besides, haired skin has a thinner epidermis, while non-haired skin as nose and paw pads have a ticker epidermis. Hence, exists differences in the thickness of the epidermis and dermis in various regions of the body between species and within the same species.[1,3,4,6,7]

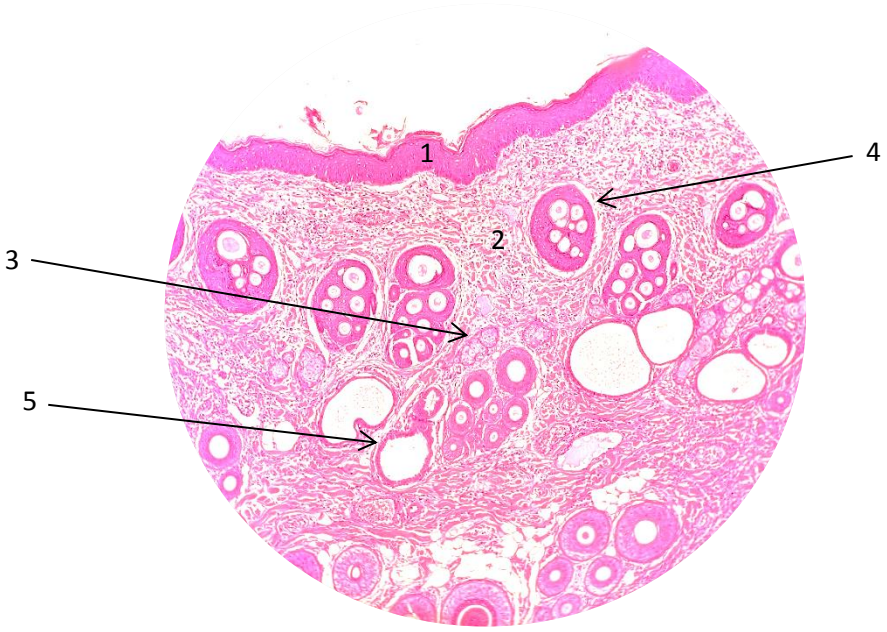


Figure 1-A: Example of histology of the skin and some of the constituents. (1) Epidermis; (2) Dermis; (3) Sebaceous gland; (4) Hair Follicles and (5) Apocrine sweat gland.

Epidermis

The epidermis is the outermost layer of the skin. It's a keratinized stratified squamous epithelium and it's originated from ectoderm.[1–4,7] In regions with a heavy shielding coat of hair, the epidermis is thinner and in non-haired skin, the epidermis is

thicker.[8–13] In the epidermis, the cells go through an orderly pattern of proliferation, differentiation, and keratinization.[4,7] Mast cells are not present in normal epidermis.[6]

The epidermis of haired skin consists of four basic layers, from the deepest to the outermost layers: *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*.[1,2,4,7] On the other hand, the epidermis of hairless skin has an additional layer, the *stratum lucidum*, located between the *stratum granulosum* and *stratum corneum*.[1,4,6,7]

The *stratum basale* is the deep germinative layer of the epidermis and it's made of a single layer of cuboidal to low columnar cells resting on the basement membrane zone or the basal lamina. These cells are attached to the underlying basement membrane by hemidesmosomes and connected to each other and overlying keratinocytes by desmosomes (anchoring structures that mediate adhesion between cells).[1] These cells functioned heterogeneously, some can act as stem cells, having the ability to divide and produce new cells, whilst others mainly help to anchor the epidermis.[2,4,6,7]

A specific feature of the *stratum spinosum* is the prominent intercellular bridges that form the desmosomal attachments between cells. The spinous appearance a result of the shrinkage artifact that happens during tissue processing.[1,6] The cells are polyhedral to slightly flattened and in haired skin are arranged in 1 or 2 layers in dogs with regard to non-haired skin, this layer is thicker and may be up to 20 cells in footpads and nasal planum.[4,7]

The *stratum granulosum* in haired skin appears only with 1-2 cells tick[1], however in non-haired skin, this layer is more distinguished, averaging 4-8 layers in thickness. It is composed of numerous layers of flattened parallel cells to the epidermal-dermal junction with shrunken nuclei. This layer has irregularly shaped nonmembrane-bounded eletrondense basophilic keratohyalin granules and also lamellar granules.[2,4,6,7] The *stratum granulosum* doesn't exist in all stratified squamous epithelia for example, the mucous membrane of the mouth.

The *stratum lucidum* can only be found in certain areas with a very thick skin and in hairless regions, like plantar and palmar surfaces or *planum nasale*. This stratum is a thin, translucent homogeneous border formed of numerous fully keratinized, layers of compacted dense cells, lacking a nuclei and cytoplasmic organelles.[2,4,7]

The outermost layer of the epidermis is the *stratum corneum* and consists of numerous layers of entirely keratinized dead cells, that are constantly being shed.[1,6] This layer doesn't contain nuclei or cytoplasmatic organelles and appears clear. This stratum differs in thickness in different regions of the body and between species. Where considerable abrasive action occurs, like for example on the palmar and plantar surfaces, the stratum is thicker. The cells are flattened, polyhedral, anucleated, highly organized

and form vertical interlocking columns. This subtle layer acts as an active and strong hydrophobic barrier regulating water movement in and out of the skin as well as avoids both the infiltration of substances from the environment and the loss of body fluids.[2,4,6,7]

Germinal cells in the stratum basal originate keratinocytes. They make up about 85% of the epidermal cells.[4,7] The keratinocytes undergo a process of differentiation and proliferation that helps to repair after trauma.[2,4,7]

Non keratinocytes are distributed through the epidermis.[4,7] Three different types of non-keratinocytes exist: melanocytes, tactile epithelioid cells (Merkel cells) and intraepidermal macrophages (Langerhans cells). Regarding melanocytes, they are derived of neural crest cells and can be found in lower layers of the *stratum spinosum* and in the basal layer.[1,6] They also can appear in the external epithelium root sheath and hair matrix of hair follicles, in sweat glands ducts and in sebaceous glands.[2] The melanocyte contains intracytoplasmic pigment granules, named melanosomes. The melanosome produces melanin pigment, providing skin and hair colour whose intensity is determined by numerous factors, such as the size, number, distribution and also the degree of melanization of melanosomes (typically it exists 1 melanocyte per 10-20 keratinocytes).[2,4,7] To help protect the cell nucleus from UV light-induced injury, melanocytic granules are transferred to and distributed in keratinocytes and form a cluster of granules between the nucleus and the external surface of the skin. Tactile epithelioid cells also known as Merkel cells, are localized in the basal region of the epidermis in both hairy or hairless skin, mainly in areas of the body with tactile sensitivity like digits and lips and also in the external section of the hair follicles.[1,2,4,6,7] Merkel cells are connected to keratinocytes by desmosomes and express keratin proteins. A specific feature of these cells is a region of vacuolated cytoplasm close to the dermis, that has spherical dense granules that contain specific chemical mediators. As well as functioning as mechanoreceptors, Merkel cells can also stimulate and maintain hair follicle stem cells, influence keratinocyte proliferation, blood flow and sweat production.[4,7] Langerhans cells, otherwise known as intraepidermal macrophages, are dendritic cells derived of bone marrow cells associated to monocyte-macrophage cells at a functional and immunological level.[1,2,4,6,7] In routine H&E sections they appear as clear cells and can be scattered from the *stratum basale* to the *stratum spinosum* reliant on species and also specific region of the skin. These cells can also be present in dermal lymph vessels, in lymph nodes and in the dermis. A unique characteristic of those is the presence in the cytoplasm of rods or rocket shaped granules, referred as intraepidermal macrophage (birbeck) granules. These granules, depending on the species can contain langerin, a Ca^{++} dependent type II lectin. These cells are held to be the primary receptors for cutaneous immune responses being capable of presenting antigen to lymphocytes.[4,7]

Dermis

The dermis is responsible for the maintenance and repair of the skin and also for the elasticity of the skin and tensile strength.[2] The thickness of the skin is determined mostly by the thickness of the dermis which is composed mainly of dense irregular connective tissue.[2,4,7,10,12,13] This layer consists of a glycosaminoglycan ground substance with collagen and elastic fibers, blood and lymphatic vessels, nerves and low number of lymphoid cells.[3]

The superficial dermis meets the contour of the epidermis and usually supports the upper portion of the hair follicle and sebaceous glands, being constituted of fine collagen fibers. The deep dermis supports the lower portion of the hair follicle and apocrine glands and it consists of thicker densely arranged collagen fibers than those present in the superficial dermis.[6] Elastic fibers are less numerous, thicker, and parallel to the skin surface in the deep dermis.[10,12]

Mast cells, macrophages, plasma cells, lymphocytes and rarely eosinophils and neutrophils can be found in normal dermis. Fibroblasts are dispersed in low numbers through the dermis. They synthesize majority of the fibrillar and ground substance proteins of the dermis along with various growth factors and cytokines. Melanocytes in the dermis are typically positioned near superficial dermal vessels.[4,7]

Subcutis

Is the deepest layer of the skin and it is mainly constituted by loose connective tissue.[2] The subcutis connects the dermis to the underlying muscle or bone, there are some exceptions where the subcutis may be absent like the lip, cheek, eyelid, external ear, and anus. Its composition consists of adipose tissue and collagenous and elastic fibers.[3,6] The skin flexibility and free movement over the underlying structures is due to the loose organization of the collagen and elastic fibers. In terms of the adipose tissue it protects against temperature variation and in paw pads, helps with shock absorvation.[2,4,6,7]

Skin Appendages - Adnexa

The skin appendages are the hair follicle and the sebaceous and sweat glands.

The entire body in domestic animals is cover with hair, apart from some regions like the foot pads, hoofs and mucocutaneous junctions. There are several types of hair follicles such as primary or secondary, and simple or compound. A primary hair follicle has a large diameter, is root is deep in the dermis, and is frequently associated with

sebaceous and sweat glands and *arrector pili* muscle.[2,4,6,7] Single or simple hair follicles have a unique hair shaft that arises from the follicle opening through an external orifice of the epidermis.[2,4,6,7] A diversity of hair types can be found in different breeds of dogs such as tactile hairs, including sinus (whiskers or vibrissae) which commonly arise on the nose, over the eyes, lips and tylotrich hairs that have the function of mechanoreceptors.[2,4,6,7,10,12]

When talking about sebaceous glands we may say that they are simple, branched, or compound alveolar glands and that they release their secretory product by the holocrine mode with the ducts opening into the hair follicles, with the exception of some mucocutaneous junctions where the glands open on the surface of the skin.[1,10,12] The secretory product of the gland is the sebum, an oily secretion containing a mixture of lipids and disintegrated cells.[2,4,7] It works as an antibacterial agent as well as in hairy mammals, as a waterproofing agent.[4,7]

Based on their mode of release of the secretory product, sweat (sudoriferous) glands are classified into two types: apocrine and merocrine (eccrine).[1] The structure of apocrine sweat glands varies significantly between species. They produce a viscous secretion containing a scent that is linked to communications among species, probably as a sex attractant or a territorial marker.[2,4,7] The apocrine glands in the domestic animals are located through most of the skin. The merocrine (eccrine) sweat glands are found mainly in distinct skin areas, for instance, in the foot pads. They are simple tubular glands, relatively straight that open straight into the skin surface instead of into hair follicles. [4,6,7]

Neoplasia's

Normal tissue is usually composed of mainly mature cells which show homogeneity nuclear and cellular size and shape.[3] Contrary to normal cells, the development of neoplastic cells is: (1) autonomous, the growth is independent of growth factors and/or regulatory mechanisms functioning in the normal tissues; (2) excessive, this can be visible in the size of the outgrowths and the duration of the proliferation; (3) and finally is disorganized, the structures formed by the tumor cells vary from normal tissues and aren't suitable in the overall organization structure of the healthy tissue.[14] Therefore, a neoplasm is a type of abnormal and excessive growth, that usually forms a mass.[15,16]

In veterinary medicine, in terms of cancer incidence in dogs, skin tumors are some of the most common neoplasms observed. Since they are easily detected by the

owner, they are commonly brought to the attention of the veterinarian. Thus, they are, possibly, the most common histopathology specimen sent to diagnostic laboratories.

Cytomorphologic features

Neoplasm can be divided into categories.[17–19] These categories are based in the origin and on their general cytomorphologic characteristics that include their association to one another (**Table 1-A**). The first two terms, epithelial and mesenchymal, are derived from their histogenesis histology.[20]

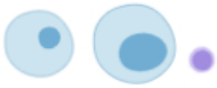
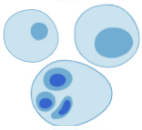
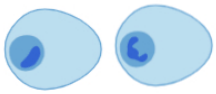
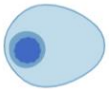
Table 1-A: General cytomorphologic characteristics of the three categories of neoplasm. Based of Cowell and Tyler's Diagnostic cytology and hematology of the dog and cat (2014) and Canine and feline cytology (2015).

	EPITHELIAL	MESENCHYMAL	ROUND CELL
CELLULARITY	High	Low	High
CELLS	Clusters	Individual	Individual
SHAPE	Cuboidal	Spindle	Round
ORIGIN	Glandular, parenchymal tissue and lining surfaces	Connective tissue elements	Hematopoietic cells
ARRANGEMENT	Polyhedral shapes and monolayer sheets	Loosely arranged	
SPECIAL FEATURES	Adhere to each other; Cells exfoliate into tight clumps or sheets Cells are large and round to polygonal Distinct intact cytoplasm borders Nuclei round to oval	Exfoliate individually although aggregations can be seen bound by an extracellular matrix Great percentage of extracellular matrix Cytoplasmic borders unclear Nuclei are round to elliptical	Cells exfoliate individually Distinct cytoplasmic borders Nuclei are round to indented Cells are usually smaller than epithelial cells
EXAMPLES	Apocrine adenoma; Trichoepithelioma	Hemangiosarcoma, Perivascular Wall tumor	Lymphoma, Mast cell tumors, Plasmacytoma

Biological behavior

The division into benign and malignant is based on the cytomorphologic characteristics exhibited by the neoplastic cells and on the infiltrative and metastatic power. Benign cells display uniformity in nuclear and cell size, nuclear-to-cytoplasmic ratio, and other nuclear features. Malignant cells often display three or more criteria of cellular immaturity or atypia, which should be identified before a diagnosis of malignancy is made. For that, histopathologic examination is recommended.[3] Malignant cells have a tendency to be morphologically different from the progenitor cell population. Malignant cells present one or more of the following features described in the **Table 1-B** below.

Table 1-B: General Criterial of Malignancy.

	Criteria	description
	Anisocytosis and Macrocytosis	Variation in cell size, with some cells ≥ 2 times larger than normal
	Pleomorphism (except in lymph nodes)	Variable size and shape in cell of the same type
	Macrokaryosis (Karyomegaly)	Increased nuclear size; cell with nuclei larger than 20μm in diameter suggest malignancy
	Increased nucleus-to-cytoplasm ratio (N:C)	Normal non lymphoid cells usually have a N:C of 1:3 to 1:8, depending on the tissue
	Anisokaryosis	Variation in nuclear size; especially important if the nuclei of multinucleate cells vary in size
	Multinucleation	Multiple nuclei in a cell; especially important if the nuclei vary in size
	Increased mitotic figures	Mitosis is rare in normal tissue
	Abnormal mitosis	Abnormal chromosomal fragments may appear with uneven length of chromatin strands and as isolated or lag chromatin. Improper alignment of chromosomes
	Coarse chromatin pattern	The chromatin pattern is coarser than normal; may appear ropy or cordlike
	Nuclear molding	Deformation of nuclei by other nuclei within the same cell or adjacent cells
	Macronucleoli	Nucleoli are increased in size; nucleoli ≥ 5μm strongly suggest malignancy. For reference, RBCs (red blood cell) are 5-6 μm in the cat and 7-8μm in the dog
	Angular nucleoli	Nucleoli are fusiform or have other angular shapes instead of their normal round to slightly oval shape
	Anisonucleoliosis	Variation in nucleolar shape or size
	Phagocytic activity	
	Heterotopia	Presence of a given cell type where it is not found anatomically

Illustrations made in Adobe Illustrator. Based of Cowell and Tyler's *Diagnostic cytology and hematology of the dog and cat and canine and feline cytology (2014).*

Skin tumors

A broad range of neoplasia's can be found in the skin, hypoderm and adnexa.[21] Skin tumors are amongst the most frequent canine tumors that enter in laboratories for histopathologic diagnose.[9,22] The skin is the most common site of tumor occurrence in dogs (≈30% of the total).[10,12,23,24] In males and female dogs, these neoplastic diseases are the most and second-most frequently reported tumors, respectively.[25–30]

A skin tumor diagnosis typically comprises the assessment of cells using cytology and histopathology through a biopsy retrieved from the patient. It is also required a search for metastasis which aims the grading and staging of the neoplastic disease. The treatment of preference for skin tumors, in specific malignant tumors, in the great majority of cases involves surgical excision but this decision depends on the type of cancer, stage, grade, and location.[21,31] In malignant tumors, radiation or chemotherapy can be used on his own or as adjunctive therapy.

In dermatology, the term tumor is presently used to express an increase in the volume of the skin that may have a hyperplastic, dysplastic, metaplastic or neoplastic origin.[10,12] The term hyperplasia implies the increase in the volume of a given tissue due to the increase in the quantity of some of the cells that form the specific tissue.[32–36] Different from neoplasia, in these cases, at least in theory, exists a purpose for this growth.[33,34,36] Dysplasia denotes a situation in which there is a loss of normal tissue architecture, that is, dysplastic tissue in comparison to normal tissue there is a inadequate distribution of its elements.[32,34–36] For several authors, dysplasia can be considered a pre-neoplastic form..[32,35,36] The use of the term neoplasia designate an abnormal and uncontrolled tissue growth.[5,34] Conform to this terminology, proliferative skin disorders have been classified by numerous authors as neoplastic tumors and non-neoplastic tumors.[10,12] Other experts have used distinct expressions, such as lesions "like-tumors" and lesions "similar to tumors".[37–39]

The skin components are derived from two of the three germ layers, the ectoderm and the mesoderma.[40] The epidermis and, therefore, the adnexa develop from the ectoderm; the dermis and hypodermis are derived from the mesoderm.[40] Hence, tumors originating from cells of the epidermis, hair follicle and attached glands have epithelial origin, while neoplasms derived from cells of the dermis and hypodermis are mesenchymal.[10,12] Melanocytic tumors do not fit into this categorization, since they derive from melanocytes that originate from the neural crest.[10,12,36,37,41]

Benign neoplasms carry the suffix "oma", for example, fibroma (benign neoplasm derived from fibroblasts) or adenoma (benign neoplasm of glandular origin).[5,34–36] Some exceptions happen, such as lymphoma, mastocytoma and melanoma, all potentially

malignant tumors in the dog.[10,12,32,37–39,42] The sarcoma suffix implies that, in adding up to being malignant, the tumor originates from the mesenchymal tissue; the word carcinoma defines a malignant tumor with epithelial origin.[5,34–36] So, neoplastic skin disorders can be divided into their tissue origin such as epithelial, mesenchymal and melanocytic.[42]

The most widespread skin tumor in dogs and cats differs to some extent among researches. In general, canine cutaneous tumors may be roughly categorized as approximately 55% mesenchymal, 40% epithelial, and 5% melanocytic in origin, and feline skin tumors as 50% epithelial, 48% mesenchymal, and 2% melanocytic.[10,12] There is another group whose neoplastic cells derive from round cells, like mast cells, lymphocytes, plasma cell… such tumors can be mast cell tumors, lymphomas, plasmacytomas, histiocytoma…

Canine cutaneous tumors epidemiologic data is limited and the results differ amongst the different geographic locations often reflecting distinct conditions such as breed preferences, prevalent environmental influences, living conditions and practices that can vary significantly and somewhat influence the outcomes and variables within this studies.[31,45] When considering all these points, it becomes opportune to determine the prevalence of skin tumors that affect dogs and thus assist veterinarians in the diagnosis of these routine conditions.

Mast Cell Tumor

Mast cell tumors (MCTs) are the most common cutaneous tumor in dogs, accounting for 7 to 21% of all skin tumors. [24,26,43,46–48,49,50] The biological behaviour of canine MCTs is highly variable, ranging from a single surgically resectable benign nodule to a fatal metastatic cancer[45,46,51–54] and most pathologists efforts are focused on accurately identifying the most aggressive forms.[55]

Mast cell numbers in the skin differ significantly depending on body location. In dogs, 4 -12 mast cells per 400× field is considered within the normal accepted range. For instance, in dogs the nasal planum has the lowest density and the highest is in the pinnae and interdigital skin. Mast cells are concentrated around blood vessels, especially postcapillary venules.

Mast cell tumors are neoplastic proliferations[56,57] that commonly appear in the dermis as solitary lesions, although it is not unusual to find them in the subcutis or as multiple tumors.[58,59] Their appearance might vary from hairless, raised, erythematous masses to nodular rashes or diffuse swellings. All canine cutaneous mast cell tumors should be considered potentially malignant.

Although all breeds are affected, several racial predispositions have been reported[32,60,61]: boxer, Boston terriers, bull terriers, bullmastiff, Staffordshire terries, Fox Terriers, English Bulldogs, Dachshunds, Labrador and Golden Retrievers, Beagles, Pugs, Chinese Shar-Peis, Rhodesian Ridgebacks and Weimaraner's. The average age of affected dogs is 8 years, but MCTs are occasionally found in dogs as young as 4 months. There is no gender predisposition reported.

Cytologically, MCTs can be characterized by a dominance of individualized, monomorphic round cells, that have a central to slightly eccentric round nucleus and typically large numbers of magenta granules.[32] In hematoxylin-eosin (H&E) stained sections, neoplastic mast cells have moderate quantities of pale pink cytoplasm that often contains abundant light gray/blue granules. Neoplastic mast cells can be packed into dense sheets or occur individualized, usually creating rows that intrude between collagen bundles.

In poorly differentiated MCTs, neoplastic mast cells ca be extremely anaplastic, leading to different staining features, including a lack of granules in both the cytoplasm and in the background. Instead, neoplastic mast cells might degranulate, either related to trauma or the tumor microenvironment, which results in the lacking of heavily stained granules within the cytoplasm and plenty of detectable granules in the background.[60] Analogous MCTs can represent a diagnostic challenge in surgical biopsy samples, including ancillary diagnostic techniques, in particular special stains and immunohistochemistry (IHC).

A major part of MCTs are effectively treated by surgery and/or radiotherapy, but a percentage of these tumors spread to a regional lymph node, spleen and/or liver, and with local therapy alone death usually follows quickly. Adjunctive chemotherapy[56] is often used when dogs have metastatic disease, or are believed to have a high risk of developing metastasis.

Mast cell granules contain histamine, heparin, and various proteases. Histamine is a vasoconstrictor that triggers permeability of small venules, thus allowing leakage of plasma, causing tissue edema helping to dispose of foreign antigens quickly. Histamine also stimulates smooth muscle contraction in small airways. Heparin acts as an anticoagulant and is believed to stimulate angiogenesis.[4,7] These cells also produce interleukin 5, a cytokine that induces eosinophil migration.[32,62] Hence, in most canine MCTs, the neoplastic cells are mixed with huge numbers of eosinophils. Besides eosinophils, MCTs frequently have abundant fibroblasts. Other trait frequently linked to MCTs is the presence of collagen. The appearance of collagen is credited to tryptase-containing and chymase-containing mast cells that induces collagen synthesis.[32,62]

Secondary inflammation is normal and frequently associated with severe ulceration and necrosis.

Microscopic evaluation

Histological grade

Histologic grading has been the method most commonly used to predict the biological behavior of canine cutaneous MCTs and is the most important single prognostic factor.[57] Currently, two grading systems are used. The Patnaik grading system[45] is well established and assigns the mast cell tumors (MCTs) to one of 3 grades according to descriptive histological criteria (**Table 1-C**). The more recent binary Kiupel histologic grading[63] system utilizes more numerical and fewer descriptive criteria.

The system proposed by Patnaik *et al*.[45] (1984) divides MCTs in three histological grades: well-differentiated tumors (Grade I) – **Figure 1-B**, intermediately differentiated tumors (Grade II), and poorly differentiated tumors (Grade III) – **Figure 1-C**.[45,60,64] The grading system by Patnaik *et al*. has been referenced most widely by pathologists when providing a histologic grade however, the system has been criticized for a high degree of inconsistency among pathologists[63,65,66] in accurately applying the criteria; for ascribing excessive importance to tumor depth, for including grade II[56,63,65] (moderately differentiated lesions) that creates a vast and heterogeneous group of lesions with different biological behaviours[67] and because it does not predict metastasis. Finally, this 3-tier grading system determines the grade of a canine cutaneous MCT in order to predict survival times rather than predicting biological behavior.[68]

Table 1-C: Patnaik grading system for canine mast cell tumors (MCTs) - (1984)

GRADE	MICROSCOPIC DESCRIPTION
I – LOW GRADE; WELL-DIFFERENTIATED	Round nuclei with condense chromatin; Mitotic figures are absent; Large, well staining cytoplasmatic granules; Tumors are confined to the dermis
II – INTERMEDIATE GRADE; INTERMEDIATELY DIFERENTIATED	**Moderately pleomorphic cells;** **Mitotic figures are infrequent;** **Tumors in the dermal and subcutaneous tissues;** **Tumors extended to the skeletal muscle or surrounding tissues;** **Edema is preset in some tissues;**

Table 1-C: (Continued)

III – HIGH GRADE; POORLY DIFFERENTIATED	**Highly pleomorphic cells; Binucleated cells are common; Frequent mitosis; Low number of cytoplasmatic granules; Tumors has replaced subcutaneous tissues; Edema, hemorrhage and necrosis are common in and around the tumor**

A new histological grading system was proposed by Kiupel *et al*.[63] (2011) in a way to overcome these limitations. This is a two-tiered system, thereby avoiding the problems related to Patnaik's intermediate grade, and offers important prognostic information and was established not only to increase interobserver consistency but also, above all, to identify more precisely those MCTs that have a high risk of aggressive biological behavior, or for instance, of metastatic disease.[63] All grading criteria are based on nuclear morphology and mitotic activity, except tumor depth. The 2-tier system divides canine cutaneous MCTs into low-grade (**Figure 1-B**) and high-grade (**Figure 1C**) MCTs (**Table 1-D**). High-grade MCTs were significantly associated with shorter time for a new tumor development and for metastasis. Dogs with low-grade MCTs had a median survival time of more than 2 years, whereas dogs with high-grade MCTs had a median survival time of less than 4 months .[63,69]

Table 1-D: Kiupel grading system for canine mast cell tumors (MCTS) - (2011)

GRADE	MICROSCOPIC DESCRIPTION
HIGH GRADE	Any 1 of the following criteria: • at least 7 mitotic figures in 10 high-power fields (HPFs); • at least 3 multinucleated (3 or more nuclei) cells in 10 HPFs; • at least 3 bizarre nuclei in 10 HPFs; • karyomegaly (nuclear diameters of at least 10% of neoplastic cells vary by at least 2 times).
LOW GRADE	None of the above criteria

In order to assess to assess the different parameters, fields with the highest mitotic activity or with the highest degree of anisokaryosis should be selected. High powerful fields should be measured with an ocular with a field number of 22 and a 40-times objective.[32,55,62]

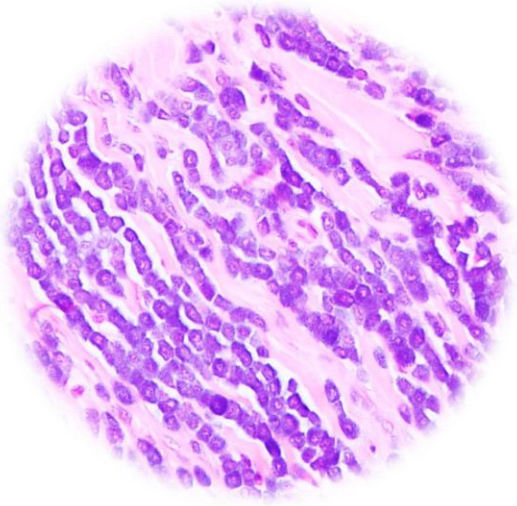


Figure 1-B: Dog, skin: Mast cell tumor: Low grade/Grade I (HE; 200X)

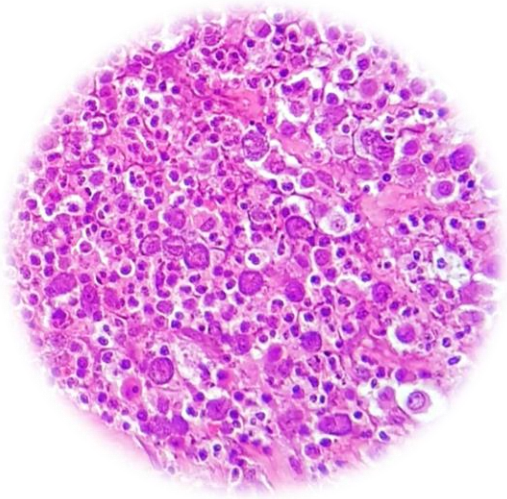


Figure 1-C: Dog, skin: Mast cell tumor High grade/Grade III (HE; 200X)

Margins

The prognosis of a mast cell may be influenced by whether or not exists an adequate portion of tissue free of neoplastic cells at the excision margin. Determining cleanliness of surgical margins is an important part in the evaluation of excisional biopsies of canine cutaneous MCTs. Margins should be inked, and surgical sutures are most frequently used to help the pathologist tissue orientation. Canine MCTs often are surrounded, by edema, reactive stromal cells and inflammatory cells, including non-neoplastic mast cells, that can form a halo of several-centimeter thickness.[60]

Differentiating neoplastic from non-neoplastic mast cells when examining surgical margins usually is based on the arbitrary decision that clusters of 3 or more mast cells are considered neoplastic, while individual, well-granulated mast cells are considered inflammatory mast cells.[32,55,62] The method used to cut MCTs for microscopic examination has a major impact on the ability of pathologists to determine cleanliness of margins as well as distance of neoplastic cells to those margins.

Routinely, most MCTs are radially sectioned (halves and quarters). This method only examines neoplastic mast cells along the radii of the 4 quarters. A single mast cell measures around 10 mm. In order to detect all clusters of 3 or more mast cells spreading to the margins, more than 500 radial sections would be required. Examining such huge numbers would be technically and economically impracticable. Hence, combined radial and tangential sectioning is the ideal method to assess MCT margins and has a more than 20% higher sensitivity in spotting dirty margins. The radial sections are based on palpation of the mass and offer high-quality samples for the diagnosis and future

prognostic testing, along with some information about the distance of the tumor to the margins. Therefore, the tangential sections give the most accurate information about margin cleanliness

Considering the technical problems in determining clean margins, innovative methods, based on molecular pathology, have been more recently applied to determine the need for supplementary surgery or radiation therapy.[55,70–73] For example, high-grade MCTs as well as MCTs with a mutation in exon 11 on *c-kit* have a high likelihood (usually up to 40%) of local recurrence, despite clean surgical margins.[55,73] On the other hand, low-grade MCTs with a low proliferation activity, as determined by the Ki67 index are highly unlikely (less than 10%) to recur, regardless of the cleanliness of margins (usually more than 90% of these do not recur).[71,72] Currently, the combination of radial and tangential margin examination with these molecular methods represents the most accurate method to determine the likelihood of local recurrence.

Specific complementary tests

These additional tests are extremely important since recent studies demonstrate relevant correlations between certain MCTs characteristics and the biological behavior of these neoplasms. The results herein obtained can help predict the clinical behavior in the patient in question and influence decisions regarding the most appropriate treatment plan.

Proliferation markers

Uncontrolled cellular proliferation is considered a hallmark of cancer. Numerous studies have assessed cellular proliferation in canine MCTs and have shown that cellular proliferation remains a solid prognostic parameter. To assess proliferation markers, it is vital to understand some key concepts of tumor growth.

Tumor growth is the consequence of a disturbed balance between cell proliferation and cell death. Any disturbance that outcomes in an increased ratio of proliferation with cell death, can result in tumor growth. The number of cells within the cell cycle (growth fraction) and the rate (speed) of cells advancing through the cycle (proliferation rate) determines the degree of cellular proliferation.

The most used proliferation markers in veterinary medicine include counting of nucleolar organiser regions (NORs) detected on the basis of their argyrophilic properties (AgNORs), the immunohistochemical detection of the proliferating cell nuclear antigen (PCNA), Ki67, and the mitotic index (MI). These proliferation markers characterize different aspects of proliferation.[55] Usually, areas with the highest proliferation activity

must be used to precisely determine the MI, the Ki67 index, or the number of AgNORs in any given MCT.[32,55,62]

Mitotic index

As grade is so variable and subjective, several other prognostic factors have been assessed in an effort to better predict the behaviour of canine MCT.[74] Mitotic index (MI) is an indirect measure of cell proliferation.[75,76] It is characterized as the percentage of cells suffering mitosis in a certain population of cells (usually count in 10 HPF). Mitosis is the division of somatic cells into two daughter cells and the duration of the cell cycle and mitosis differ according with cell types. An indication that higher number of cells are dividing is provided by an elevated mitotic index. In cancer cells, the mitotic index is usually elevated when compared to normal growth of tissues or cellular repair processes of the injury site.

Ki67 index

Ki-67 is a more sensitive proliferation marker than PCNA, since PCNA is present only in cells during the S phase of the cell cycle, while Ki-67 is a nuclear protein that is expressed in all phases of the cell cycle but is not expressed in noncycling cells like those that are only resting (i.e., G0) cells.[77,78] It is, hence, a marker of the growth fraction. The relative number of Ki67- positive cells in a certain tissue is used to determine the proliferation index or the relative number of cells actively involved in the cell cycle (growth fraction).[77–81]

A standardized method for determining the Ki67 index has been based on the use of an ocular grid and, in this process, an average of more than 23 Ki67–positive cells per grid area is associated with shorter survival times, MCT-related mortality and an increased in disease progression and risk of systemic disease.[82] On the other hand, a lower Ki67 index indicates that these are highly unlikely to recur, even if their surgical excision has been incomplete, indeed only a small percentage of them may present local recurrence (about 11%). When using a chromogen to label Ki67-positive cells, examination of slides at low magnification allows much easier identification of the areas with the highest index and, therefore, a higher consistency among pathologists.

Molecular markers

KIT

KIT (CD117), type III tyrosine kinase protein[83,84] (**Figure 1-D**) acts as a receptor for stem cell factor (SCF), otherwise known as mast cell growth factor, kit ligand, or steel factor.[85–91] The activation of this receptor tyrosine kinase (RTK) leads to cell survival, proliferation and motility through multiple intracellular signaling pathways such as the RAS/mitogen-activated protein kinase (MAPK) pathway, the PI3-kinase and the Src family of kinases (SFK) pathway.[92–94] So it takes a major role in the regulation of cell survival and proliferation, gametogenesis, hematopoiesis, melanogenesis, stem cell maintenance and mast cell development, migration and function. The growth and differentiation of mast cells and the growth factor activation in MCTs has this cytokine as an essential factor.[83,95–97] The activation of KIT causes an upregulation of DNA replication and consequent mast cell survival, differentiation, maturation, proliferation, chemotaxis, degranulation, suppression of apoptosis and adhesion to fibronectin.[46,86,96,98–101]

The receptor tyrosine kinase encoded by the KIT gene is a transmembrane protein with an extracellular domain comprised of five immunoglobulin-like domains, followed by a single spanning transmembrane region. The intracellular part of c-Kit starts with the juxtamembrane region, a region of great importance for regulation of c-Kit kinase activity. The kinase domain is comprised of two subdomains, tyrosine kinase domain 1 and 2, which is interrupted by a kinase insert sequence. Finally, the COOH-terminal tail ends the protein.

The tyrosine kinase receptor KIT plays an essential role in the survival, proliferation, differentiation, and migration of mast cells. With immunohistochemistry (IHC) it's possible to detect an aberrant expression of KIT protein that has been shown a negative prognostic indicator for canine cutaneous MCTs.[102] Also, a correlation between aberrant KIT localization and activating mutations was found.[103] This discovery is probably due to activated KIT molecules being detached from the cell membrane and internalized more quickly than inactivated KIT.[104] However aberrant KIT localization can as well occur without a detectable c-kit mutation, suggesting an alternate means of constitutive activation among which gene duplication or autocrine/paracrine production of KIT's ligand, stem cell factor.

In terms of the KIT protein immunoexpression three different KIT expression patterns are recognized (Pattern I, II and III).[92,103,105,106] Pattern I, namely membrane-associated, consists in perimembranous labeling of 90% of neoplastic cells, commonly found in well differentiated MCTs and it is not related to an aggressive biological behavior.[102] A KIT expression with pattern II (paranuclear or Golgi-like) is characterized by focal perinuclear or stippled cytoplasmic labelling and loss of perimembranous labeling in at least 10% of neoplastic cells. Finally, diffuse cytoplasmic labeling in at least 10% of neoplastic cells is consistent with pattern III. The membrane-associated pattern is observed in normal mast cells and the presence of cytoplasmic KIT immunoexpression correlates with reduced post-surgical survival an increased incidence of local recurrence, higher histological grade and increased cell proliferation [92,102,106–109]

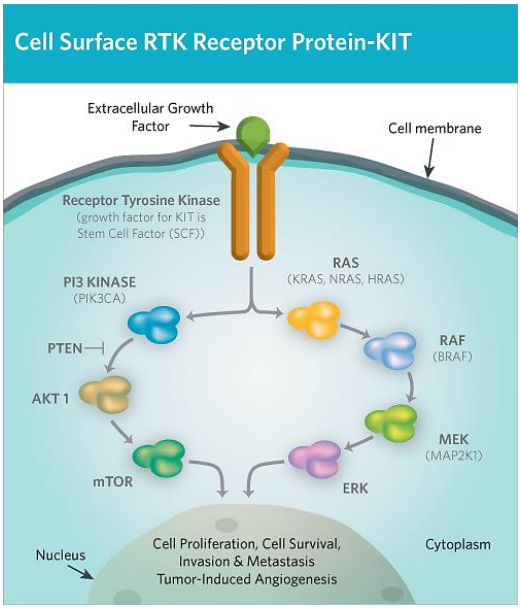


Figure 1-D: Schematic representation of KIT expression. The KIT gene (also known as CD117) encodes a transmembrane receptor that binds to the ligand known as, stem factor (SCF). Binding of the SCF ligand outside the cell leads to activation of the KIT's tyrosine kinase receptor inside the cell. When tyrosine kinases are activated, they become phosphorylated, meaning that they acquire a phosphate that is added to specific locations in the portion of the receptor that is inside the cell. These phosphorylation sites serve as anchor points for the assembly of signal proteins, which then cause the activation of several signaling pathways. When activated, these signal pathways promote cell proliferation and survival.
http://targetedcancercare.massgeneral.org/My-Trial-Guide/Genes/KIT.aspx

C-kit

This molecular marker is important since activating *c-kit* mutations have been described in canine mast cell tumors, therefore implicating *c-kit* in their pathogenesis. It consists of studying the sequence of this gene in mast cell tumors using gel

electrophoresis and DNA sequencing techniques.[52,83,102,103,105–107,110–112] The study of this molecular marker can help the development and application of c-kit inhibitors to treat canine mast cell tumors representing an important example of how translational research can create a new targeted therapy for use in veterinary oncology.[92]

C-kit gene

Proto-oncogenes regulate cell growth and differentiation. Oncogenes are genes that usually cause cancer. The proto-oncogene *c-kit* was first identified as the oncogenic constituent of the highly transforming, replication-deficient Hardy-Zuckerman 4-feline sarcoma virus.[113] This proto-oncogene has been implicated in the pathogenesis of numerous neoplastic diseases such as gastrointestinal stromal tumors and mast cell tumors in dogs. It is extremely conserved among mammals. In dogs this gene is in the chromosome 13 and has 21 exons.

The KIT gene encrypts a transmembrane type III tyrosine kinase which is the receptor for the stem cell factor (SCF).[114] The receptor KIT can be expressed by several cell types, including hematopoietic progenitor cells and mast cells and has been linked to cell survival, proliferation, and differentiation.[115–120] Like several other receptor tyrosine kinases, ligand binding instigates receptor dimerization, that activates the tyrosine kinase, which leads to autophosphorylation and the phosphorylation of exogenous substrates. These phosphorylations then lead to downstream signal transduction.[121] In various neoplasms, mutations in the KIT gene have been identified, and can result in the production of an altered protein that cannot be regulated normally. The mutated KIT protein found in tumors does not need to bind the SCF ligand and stays in an endless activated state (uncontrolled proliferation).[122] This relentless stimulation of growth and survival signaling pathways can result in the development of cancer.[123]

Mutations are connected with higher grade, more aggressive behavior, and a poorer outcome.[82,103,124] Activated RTKs have the potential to increase tumor aggressiveness via numerous mechanisms, for example, increased cell proliferation, invasion, apoptosis avoidance, migration and angiogenic growth factor production.[125] The most commonly *c-kit* mutations are located on exon 11 impair the regulatory function of the juxtamembrane domain, causing constitutive *c-kit* activation, independent of SCF binding.[103,126–129] However, canine mast cell tumors also have other mutations, that affect exons 2, 5, 6, 7, 8, 9, 10 and 15.[129,130] Some known KIT mutations linked with canine mast cell tumors includes internal tandem duplications (ITD), small insertions and deletions in the intracellular juxtamembrane region (exons 11 and 12).[83,89,126,130,131]

Aim/Purpose

The aims of this epidemiological study were:

(1) To report the prevalence of canine cutaneous tumors in the Laboratory of Veterinary Pathology from Institute of Biomedical Sciences Abel Salazar, University of Porto, Portugal as well as characterize and categorize the anatomical distributions, breed, age, and sex of different cutaneous tumor histotypes.

(2) To conduct an epidemiological analysis of the risk of MCT development in dogs in relation to other skin tumors.

(3) To study the role of Ki67 index and KIT immunoexpression in canine MCTs by analysing the correlations between one proliferation marker (Ki67), one molecular marker (KIT immunoexpression patterns), histological grading, and other clinicopathological parameters.

The epidemiological information achieved can act as a reference for regional veterinarians, in order to favour a preliminary diagnosis of canine cutaneous tumors and provide more adequate and contextualize prognostic information regarding canine mast cell tumors.

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CHAPTER 2

Canine cutaneous tumors

CHAPTER 2 - Canine cutaneous tumors

Retrospective study of canine cutaneous tumors in LAB-PAT ICBAS 2014-2020, University of Porto

Introduction

A broad range of neoplasia's can be found in the skin, hypoderm subcutis and adnexa.[12] Skin tumors are amongst the most frequent canine tumors that enter in laboratories for histopathologic diagnose. Since they are easily visualized by the owner, they are brought to the attention of the veterinarian.[57] In males and female dogs, these neoplastic diseases are the most and second-most frequently reported tumors, respectively.[11,18,19,22,32,58] A skin tumor diagnosis typically comprises cellular evaluation through cytology and histopathology. That will further allow search for metastasis aiming grading and staging of the malignant neoplastic lesion. In the great majority of cases, the preference treatment for cutaneous tumors requires surgical excision. However, this decision depends on the type of neoplasm as well as its stage, grade, and location.[12,37] In malignant tumors, radiation or chemotherapy can be used either as isolated or as adjunctive treatment.

Numerous retrospective studies have been performed to probe canine cutaneous tumors epidemiology.[12,15,28,37,46,59] Notwithstanding, data collection, study population, inclusion criteria, sample size, geographical location and outcomes often fluctuate between studies. Tumor incidence in retrospectives studies could be calculated based on population data from the national canine cancer registries and veterinary authorities or in some cases, information from diagnostic laboratories can be used and in the end the relative frequency of cutaneous tumors is reported.[12,15,21,28,37,46,59] Regardless the differences in data collection and study population, all these investigations share the mutual aim of clarifying and contributing to cutaneous tumors occurrence knowledge, in conjunction with the relationships stablished with anatomical location, breed, age and sex in a certain region in the world.

Canine cutaneous tumors epidemiological data is limited worldwide and according with the different geographic locations, distinct conditions such as breed preferences, environmental influences, living conditions and practices can significantly vary and influence the outcomes and conclusions of these studies.[37,47] The aim of the present study was to report the prevalence of canine cutaneous tumors in this laboratory and region as well as characterize and categorize the anatomical distributions, breed,

age and sex of different cutaneous tumor types. The epidemiological information achieved with this study can serve as a beneficial reference for regional veterinarians to utilize and determine a preliminary diagnosis of canine cutaneous tumors.

Materials and Methods

Study population

From January 2014 to June 2020, tissue biopsies from 2,291 dogs were submitted to the Laboratory of Veterinary Pathology from Institute of Biomedical Sciences Abel Salazar, University of Porto for histopathological examination. Surgical biopsies of canine cutaneous tumors diagnosed during this time interval were selected from the laboratory database and details such as breed, age, and sex were recorded. Exclusion criteria include cases with more than one missing clinical information (*e.g.*, sex, age, breed or anatomical locations) and cases with a diagnosis of epithelial cyst, ceruminous gland tumor, mammary gland tumor, anal sac gland tumor, and meibomian gland tumor. Core biopsies were considered whenever a definitive diagnosis was possible to reach. A tumor with multicentric development was regarded as a single tumor event. Concurrent manifestation of different tumor types and tumor recurrence in one patient were considered multiple separate events.[28,37] In the present retrospective analysis, 1.185 cases of canine cutaneous tumors and tumor-like lesions were included based on these criteria. The relative frequency (% of all cutaneous tumor types), prevalence rate (% of the total canine population in the database during the study period), and breed-specific prevalence of cutaneous tumors were calculated. Anatomical sites were labeled as cranial, facial, ear, neck, shoulder, pectoral, costal, dorsal, pelvic, buttock, tail, forelimb, forepaw, hindlimb, hindpaw, perigenital area, multicentric, and skin not otherwise specified (NOS). Anatomical location was recorded as multicentric when a particular tumor type developed in multiple cutaneous regions also when a tumor such as cutaneous mast cell tumor, in one patient had more than one MCT diagnosed with the same grading this was considered one MCT with multicentric growth. The term “skin (NOS)” was used when the precise site of tumor development was not known.

Tumor diagnosis and classification

The tumor data originated from the LPV from ICBAS-UP. This registry comprises diagnostic records of canine neoplasms classified according to the World Health Organization (WHO) International Histological Classification of Tumors of Domestic Animals and its updates by four Veterinary Pathologists (one also a board-certified

pathologist: Fátima Gärtner). Additionally, cutaneous mast cell tumors were graded according to the Kiupel's 2-tier grading system and Patnaik classification system.[36,47] The dataset used for this study included only surgical biopsy cases.

Results

Canine population in the database, 2014-2020

A total of 2,291 dogs, with a median age of 9 (range= 0-20), were obtained from LPV archive. Female to male ratio was 1:0.70 (female 58.83% and male 41.17%). The most common breeds in the database were Mixed-breed dogs (39.63%), Labrador Retriever (13.74%), Boxer (5.94%), German Shepherd (3.92%), Yorkshire Terrier (3.27%), Poodle (3.06%), Golden Retriever (2.26%), Cocker Spaniel (2.05%), Pinscher (2.01%), French Bulldog (1.78%), Beagle (1.35%), Pit Bull (1.22%), Rottweiler (1.1%), Siberian Husky (0.96%) and Estrela Mountain dog (0.86%).

Study population

During the 7-year study period, 1,185 cases from 937 dogs were diagnosed as cutaneous tumors. Two or more tumor types were diagnosed in 162 dogs. The median age of the affected dogs at the time of diagnosis was 10 (range = 0-18, median = 10). Most cutaneous tumors were present in female dogs (n=609; 51.39%) and in 576 males (48.60%) (female to male ratio of 1:0.90). Among the 78 dog breeds presented with cutaneous tumors, the most affected were mixed-breed dogs (n=404; 34.09%), Labrador Retriever (n=214; 18.05%), Boxer (n=116; 9.78%), Cocker Spaniel (n=41; 3.5%), Golden Retriever (n=39; 3.29%), German Shepherd (n=30; 2.53%), Yorkshire Terrier (n=20; 1.68%), Pit Bull (n=20; 1.68%), Poodle (n=18; 1.51%) and French Bulldog (n=18; 1.51%), accounting for 77.63% of the total cases.

Anatomical distribution of skin tumors

The anatomical sites where cutaneous tumors frequently developed, and the 10 most diagnosed tumor types are depicted in **Figure 2-A and Table 2-A**. Skin tumors were mostly found on the hindlimb (n=143; 12.07%), forelimb region (n=102; 8.61%), buttock area (n=84; 7.09%), abdominal (n=77; 6.50%) and costal (n=62; 5.23%), with more than 60 cases being recorded in each body region. One hundred and seventy-three (14.60%) cutaneous tumor cases showed multicentric development.

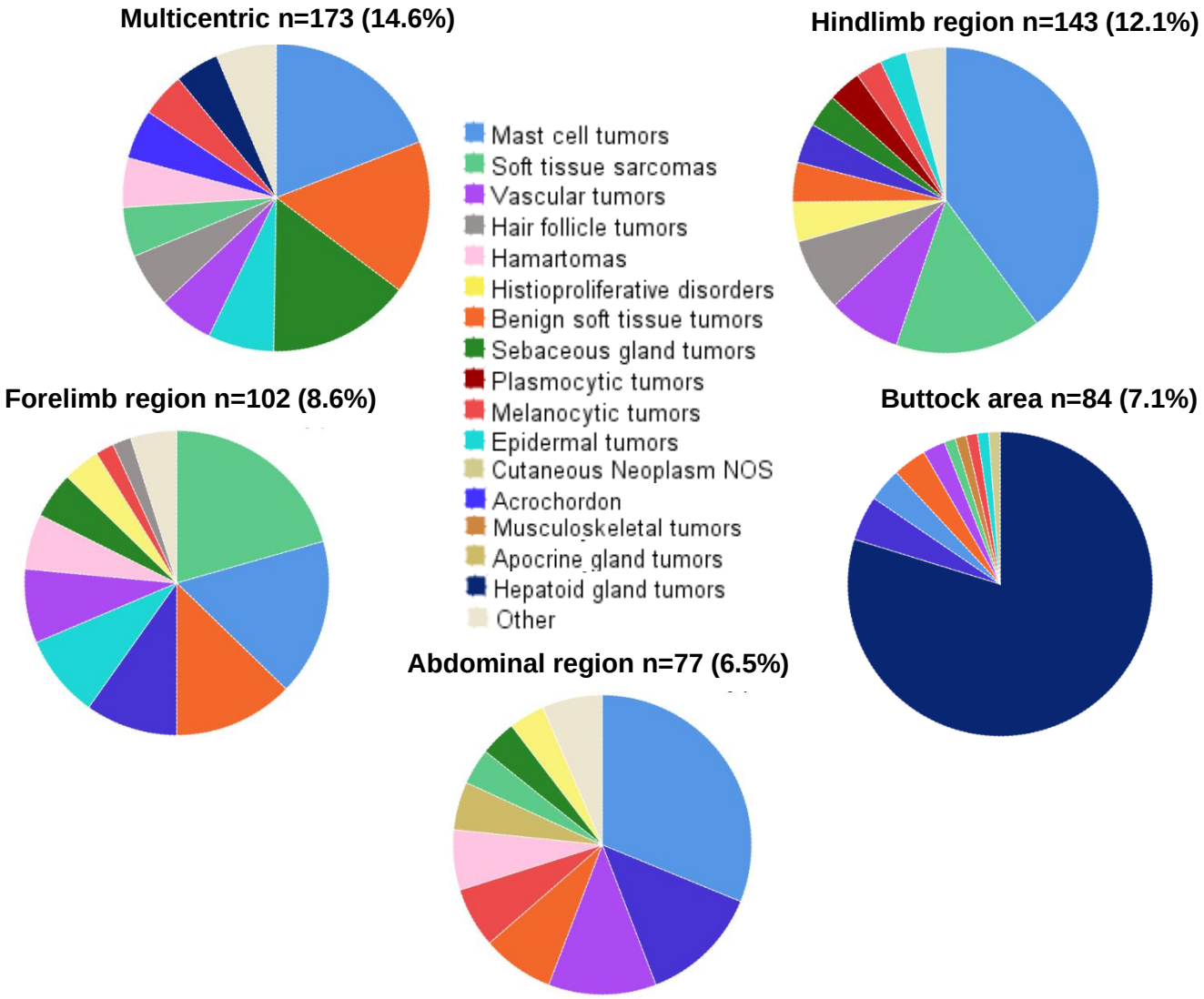

Figure 2-*A*: Five most common anatomical locations of canine cutaneous tumors (n=numbers of tumors) and the relative frequency (%) of the 10 most frequently encountered tumor histotypes in each site

Table 2-A: Frequencies of the most common tumor types in the five most common anatomical distributions.

Tumor histotypes vs location	Mast cell tumor (%)	Soft tissue sarcomas (%)	Vascular tumors (%)	Hair follicle tumors (%)	Hamartomas (%)	Histioproliferative disorders (%)	Benign soft tissue tumors (%)	Epidermal tumors (%)	Sebaceous gland tumors (%)	Plasmocytic tumors (%)	Melanocytic tumors (%)	Musculoskeletal tumors (%)	Cutaneous neoplasm NOS	Acrochordon (%)	Hepatoid gland tumors (%)	Apocrine gland tumors (%)	Others (%)
Multicentric	19.08	5.20	5.78	5.78	5.20	-	16.18	6.94	15.03	-	4.62	-	-	5.20	4.62	-	6.36
Hindlimb	39.86	15.38	7.69	7.69	6.29	4.19	4.19	2.79	3.49	3.49	2.79	-	-	4.19	-	-	4.19
Forelimb	16.67	20.59	7.84	1.96	5.88	3.92	12.75	8.82	4.90	-	1.96	-	-	9.80	-	-	4.90
Buttock	3.57	1.19	2.38	-	-	-	3.57	1.19	-	-	1.19	1.19	1.19	4.76	79.76	-	-
Abdominal	31.17	3.90	11.69	-	6.49	3.90	7.79	-	3.90	-	6.49	-	-	12.98	-	5.19	6.49

Tumor types

The relative frequency and prevalence rate of cutaneous tumors (calculated based on the total canine population of the database, 2014-2020) are shown in **Table 2-B**. Of the 1.185 cases, 745 (62.86%) cases were diagnosed as benign, while 440 (37.13%) were malignant. Broadly, mast cell tumors (n=269; 22.70%) were mostly frequent diagnosed, followed by benign soft tissue tumors (n=115; 9.70%), sebaceous gland tumors (n=96; 8.10%), vascular tumors (n=94; 7.93%) and soft tissue sarcomas (n=90, 7.59%). These comprised 56.02% of the total cutaneous tumor cases. The breed, age and sex distribution of these patients are summarized in **Table 2-E.**

In the present study, **mast cell tumors** (n=269, 22.70%) were the most common type of tumor. These were categorized into **cutaneous** (n=244, 90.71% of all MCT cases), **subcutaneous** types (n=21; 7.81%) and **mast cell tumors NOC** (n=4; 1.49%). MCTs had a higher number of cases concentrated in dogs with ages between 7- and 11-years-old, thus representing almost 60% (59.76%) of all cases with registered age. MCTs were often found on the hindlimb region (n=57; 21.19%), abdominal region (n=24, 8.92%), costal region (n=23; 8.55%), forelimb region (n=17; 6.32%) and perigenital area (n=14; 5.20%) and 33 cases (12.27%) had multicentric development. Labrador retrievers (n=72; 26.77% of all affected breeds), mixed-breed dogs (n=69; 25.65%) and boxers (n=41; 15,24%) were most affected. Labrador retriever and boxers were overrepresented, with a breed-specific prevalence of 22.86% and 30.15%, respectively in comparison with mixed-breed dogs that had a breed-specific prevalence of 7.60%. In specific, cutaneous MCT had an anatomical distribution similar to all mast cell tumors, with the most common anatomical sites being the hindlimb region (n=50; 20.49%), abdominal region (n=21, 8.61%), costal region (n=21; 8.61%), forelimb region (n=16; 6.56%) and perigenital area (n=14; 5.74%) and 30 cases (12.30%) had multicentric development. The most common breeds affected were identical to those of the generic group, Labrador Retrievers (n=67; 27.47% of all breeds; 21.27% of breed-specific prevalence), mixed-breed (n=64; 26.23% of all breeds; 7.05% of breed-specific prevalence) and Boxer (n=37; 15.16% of all breeds; 27.21% of breed-specific prevalence). In this study, two classification systems were used to classified cutaneous mast cell tumors, namely: Patnaik47 and/or Kiupel36. According with Kiupel classification system, 111 cutaneous MCT cases were diagnosed as low-grade and 45 cases were high-grade (88 cases were not subjected to this classification evaluation). With Patnaik system, 29 cases were diagnosed as grade I, 146 cases as grade II and 50 cases as grade III (19 cases were not subjected to this classification evaluation).

Table 2-B: Histopathological diagnosis, relative frequency, and prevalence rate (total population=2.291 dogs) of the 1.185 canine skin tumors recorded in the database of the Laboratory of Veterinary Pathology of ICBAS-UP (Institute of Biomedical Sciences Abel Salazar, University of Porto).

DIAGNOSIS	NUMBER OF CASES	RELATIVE FREQUENCY (% OF ALL SKIN TUMORS)	PREVALENCE RATE (%)
EPITHELIAL TUMORS	339	28.61	14.79
EPIDERMAL TUMORS	60	5.06	2.62
BASAL CELL CARCINOMA	6	0.51	0.26
PAPILLOMA	22	1.86	0.96
SQUAMOUS CELL CARCINOMA	32	2.70	1.40
HAIR FOLLICLE TUMORS	69	5.82	3.01
INFUNDIBULAR KERATINIZING ACANTHOMA	10	0.84	0.44
TRICHOLEMMOMA	2	0.17	0.09
TRICHOBLASTOMA	20	1.69	0.87
TRICHOEPITHELIOMA	27	2.28	1.18
PILOMATRICOMA	6	0.51	0.26
SUBUNGUAL KERATOACANTHOMA	3	0.25	0.13
KERATOMA PAW PAD	1	0.08	0.04
SEBACEOUS GLAND TUMORS	96	8.10	4.19
SEBACEOUS ADENOMA	62	5.23	2.71
SEBACEOUS EPITHELIOMA	34	2.87	1.48
APOCRINE GLAND TUMORS	18	1.52	0.79
APOCRINE ADENOMA	8	0.68	0.35
APOCRINE CARCINOMA	6	0.51	0.26
CUTANEOUS CLEAR CELL ADNEXAL CARCINOMA	1	0.08	0.04
APOCRINE ADENOCARCINOMA	1	0.08	0.04
APOCRINE CISTOADENOMA	1	0.08	0.04
APOCRINE NEOPLASM NOS	1	0.08	0.04
HEPATOID GLAND TUMORS	88	7.43	3.84
HEPATOID ADENOMA	57	4.81	2.49
HEPATOID EPITHELIOMA	14	1.18	0.61
HEPATOID CARCINOMA	10	0.84	0.44
HEPATOID NEOPLASM	7	0.59	0.31
EPITHELIAL TUMORS NOS	8	0.68	0.35
ADENOCARCINOMA	1	0.08	0.04
ADENOMA NOS	1	0.08	0.04
CARCINOMA NOS	4	0.34	0.17
ANAPASIC CARCINOMA	1	0.08	0.04
CARCINOSARCOMA	1	0.08	0.04
MELANOCYTIC TUMORS	43	3.63	1.88
MELANOCYTOMA	23	1.94	1.00
MELANOMA	20	1.69	0.87
MESENCHYMAL TUMORS	311	26.24	13.57
BENIGN SOFT TISSUE TUMORS	115	9.70	5.02
LIPOMA	86	7.26	3.75
INFILTRATIVE LIPOMA	6	6.98	0.26
SPINDLE CELL LIPOMA	1	1.16	0.04
FIBROMA	25	2.11	1.09
FIBROLIPOMA	1	0.08	0.04
MYXOMA	3	0.25	0.13

Table 2-B: (continued)

DIAGNOSIS	NUMBER OF CASES	RELATIVE FREQUENCY (% OF ALL SKIN TUMORS)	PREVALENCE RATE (%)
SOFT TISSUE SARCOMAS	90	7.59	3.93
FIBROSARCOMA	2	0.17	0.09
PERIVASCULAR WALL TUMOR	20	1.69	0.87
PERIPHERAL NERVE SHEATH TUMOR	39	3.29	1.70
LIPOSARCOMA	2	0.17	0.09
SARCOMA NOS	21	1.77	0.92
SINOVIAL SARCOMA	3	0.25	0.13
MYXOSARCOMA	3	0.25	0.13
VASCULAR TUMORS	94	7.93	4.10
HEMANGIOMA	68	5.74	2.97
LYMPHANGIOMA	3	0.25	0.13
HEMANGIOSARCOMA	23	1.94	1.00
MUSCULOSKELETAL TUMORS	8	0.68	0.35
LEIOMYOMA	1	0.08	0.04
RHABDOMYOSARCOMA	1	0.08	0.04
OSTEOSARCOMA	4	0.34	0.17
CONDROMA	1	0.08	0.04
LEIOMYOSARCOMA	1	0.08	0.04
MIXED MESENCHYMAL NEOPLASM			
ANGIOLIPOLEIOMYOMA	1	0.08	0.04
MESENCHYMAL NEOPLASM NOS	3	0.25	0.13
HEMOLYMPHATIC TUMORS	357	30.13	15.58
MAST CELL TUMORS	269	22.70	11.74
MAST CELL TUMORS NOC	4	0.34	0.17
CUTANEOUS MAST CELL TUMORS	244	20.59	10.65
SUBCUTANEOUS	21	1.77	0.92
PLASMOCYTIC TUMORS	18	1.52	0.79
PLASMOCYTOMA	17	1.43	0.74
INDOLENT PLASMOCYTOMA	1	5.88	0.04
ANAPLASTIC PLASMOCYTOMA	1	5.88	0.04
MULTIPLE MYELOMA	1	0.08	0.04
LYMPHOMAS	6	0.51	0.26
EPITHELIOTROPIC LYMPHOMA	1	16.67	0.044
NONEPITHELIOTROPIC LYMPHOMA	1	16.67	0.044
LYMPHOMA NOS	1	16.67	0.044
T- CELL LYMPHOMA	2	33.33	0.087
LYMPHOBLASTIC LYMPHOMA	1	16.67	0.044
HISTIOPROLIFERATIVE DISORDERS	61	5.15	2.66
HISTIOCYTOMA	58	4.89	2.53
HISTIOCYTIC SARCOMA	3	0.25	0.13
ROUND CELL NEOPLASM NOS	3	0.25	0.13
HAMARTOMAS	56	4.73	2.44
HAMARTOMA	56	4.73	2.44
TUMOR LIKE LESIONS	71	5.99	3.10
ACROCHORDON	71	5.99	3.10
CUTANEOUS NEOPLASM NOS	8	0.68	0.35
UNDIFFERENTIATED MALIGNANCY	8	0.68	0.35
TOTAL	1.185	100.00	51.72

NOS - not otherwise specified

NOC - not otherwise classified

In boxers (n=37), regarding Patnaik classification the most common grading was grade I and grade II (27.02% and 59.46%, respectively), with Kiupel's classification system low grade cases had the highest frequency (56.76%). In mixed breed dogs the most common grade classified was grade II (59.38%) in the Patnaik's classification system. Regarding Kiupel's classification low grade cases were most common for this breed (43.75%). With mixed-breed dogs was observed a higher percentage of females affected (n=44; 68.8%) compared to males (n=20; 31.3%). Labrador Retrievers (n=67) had a similar distribution as mixed-breed dogs, with a higher number of cases classified as grade II and grade III (61.19% and 17.91%, respectively). Similarly, in Kiupel's system most cases were classified as low-grade (49.3%). All the details of the frequency distribution for the classification system for the three most common breeds with MCTs is compiled in **Tables 2-C** and **2-D**.

Benign soft tissue tumors (n=115; 9.70% of all skin tumors) were the second most common tumor type group, consisting in lipomas (7.26% of all tumor diagnoses), fibromas (2.11%), myxomas (0.25%) and fibrolipoma (0.08%). **Lipomas** were the most common entity with 86 cases which represent 74.78% of the total group, followed by fibromas (n=25; 21.74%), myxomas (n=3; 2.61%) and finally fibrolipoma with only 1 case (0.89%). The three most common breeds in this group were mixed-breed dog (n=55; 47.83%), Labrador Retrievers (n=27; 23.48%) and Golden Retrievers (n=3; 2.61%). The most common sites for tumor development were forelimb (n=13; 11.30%), pectoral region (n=11; 10.43%) and costal region (n=10; 8.70%). However, 28 cases displayed multicentric development, corresponding to 24.35% of all the cases. Female dogs had a higher frequency (n=81; 70.43%) compared to male dogs (n=34; 29.56%) and in terms of age, the highest number of cases concentrated in 10-year-old dogs, although the age interval of most of the cases was found between 7- and 12-years-old. Lipomas were the most observed neoplasm in this group and within lipomas, specific variants were observed such as infiltrative lipomas (n=6; 6.98%) and one case of spindle cell lipoma (1.16%). Lipomas presented breed distribution identical to the main group, affecting mixed-breed dogs (47.67%), Labrador Retrievers (24.42%) and Golden Retrievers (3.49%), and these had a prevalence of 4.52%, 6.67% and 5.77%, respectively. In descending order, the anatomical distribution was multicentric development (n=27; 31.40%), pectoral region (n=10; 11.63%), costal region (n=n=9; 10.47%) and forelimb region (n=9; 10.47%). Females (72.09%) and dogs with 10-years-old were more frequently affected and the age interval with highest frequency was 8- to 12- years old. Fibromas most often occurred in the abdominal region (n=5; 20.00%) and forelimb (n=3; 12.00%) and breeds commonly affected were mixed-breed dogs, Labrador Retrievers, Poodles and Rottweilers with the last two having a higher breed-specific prevalence

(2.86%; 8.00% respectively) than the first two breeds (1.21%; 1.59%). Females (64.00%) and 7-year-old dogs had the highest frequency (25.00%) followed by 10- year-old dogs (16.66%).

Table 2-C: Frequency distribution of the Patnaik's classification system in the three most common breeds with mast cell tumor.

Breeds	Patnaik's classification system						NSC*		Total
	Grade I		Grade II		Grade II				
	n	%	n	%	n	%	n	%	n
Boxer	10	27.02	22	59.46	3	8.11	2	5.41	37
Mixed breed	4	6.25	38	59.38	17	26.56	5	7.81	64
Labrador Retriever	8	11.94	41	61.19	12	17.91	6	8.96	67

*NSC, not subjected to this classification system

Table 2-D: Frequency distribution of the Kiupel's classification system in the three most common breeds with mast cell tumor.

Breeds	Kiupel's classification system				NSC*		total
	Low grade		High grade				
	n	%	n	%	n	%	n
Boxer	21	56.76	4	10.81	12	32.43	37
Mixed breed	28	43.75	14	21.88	22	34.38	64
Labrador Retriever	33	49.25	9	13.43	25	37.31	67

*NSC, not subjected to this classification system

Benign sebaceous tumors were often reported as **sebaceous adenomas** (n=62; 64.58% within the group) and **sebaceous epitheliomas** (n=34; 35.42%). This group was most often found in mixed-breed dogs (n=24, 25,00%), Labrador Retrievers (n=23; 23.96%) and Cocker Spaniels (n=16;16.67%). Cocker Spaniels recorded a high breed-specific prevalence (34.04%), while mixed-breed dogs and Labrador Retrievers had a value quite lower (2.64% and 7.30%, respectively). Tumors occurred commonly on the neck (n=9; 9.38%) and ear (n=8; 8.33%) regions and a higher proportion showed multicentric development (n=26; 27.08%). For sebaceous adenoma, which comprised 5,23% of all tumor types, multicentric development (n=21; 33.87%) had a higher proportion in comparison to the remaining locations, but in singular anatomical sites, neck (n=5; 8.06%) and cranial region (n=4; 6.45%) were the most common. Sebaceous epithelioma (2.87% of all tumor types) had his development more commonly in the ears (n=8; 23.53%), followed by multicentric development (n=5; 14.71%) and facial region

Table 2-E: Breed, age, and female to male ratio of the most common skin tumor types.

Tumor histotypes (n=number of cases)	3 most affected breeds				Age	Female to male ratio
	Breed	**n**	**% of all affected breeds**	**Prevalence within breed, %**		
Mast cell tumor (n=269)	Labrador Retriever	72	26.77	22.86	Range = 1 – 16	1: 0.81
	Mixed	69	25.65	7.60	Median = 9	
	Boxer	41	15.24	30.15	Mode = 10	
Cutaneous mast cell tumor (n=244)	Labrador Retriever	67	27.46	21.27	Range = 1 – 16	1: 0.83
	Mixed	64	26.23	7.05	Median = 9	
	Boxer	37	15.16	27.21	Mode = 10	
Benign soft tissue tumor (n=115)	Mixed	55	47.83	6.06	Range = 0 - 17	1: 0.42
	Labrador Retriever	27	23.48	8.57	Median = 10	
	Golden Retriever	3	2.61	5.77	Mode = 10	
Lipomas (n=86)	Mixed	41	47.67	4.52	Range = 1 - 17	1: 0.39
	Labrador Retriever	21	24.42	6.67	Median = 10	
	Golden Retriever	3	3.49	5.77	Mode = 10	
Sebaceous gland tumor (n=96)	Mixed	24	25.00	2.64	Range = 4 - 18	1: 0.88
	Labrador Retriever	23	23.96	7.30	Median = 12	
	Cocker Spaniel	16	16.67	34.04	Mode = 13	
Sebaceous adenoma (n=62)	Mixed	18	29.03	1.98	Range = 4 - 18	1: 0.72
	Labrador Retriever	11	17.74	3.49	Median = 12	
	Cocker Spaniel	8	12.90	17.02	Mode = 13	
Vascular tumor (n=94)	Mixed	33	35.11	3.63	Range = 3 – 14	1: 0.92
	Labrador Retriever	15	15.96	4.76	Median = 8.5	
	Boxer	13	13.83	9.56	Mode = 7/8	
Hemangioma (n=68)	Mixed	20	29.41	2.20	Range = 3 – 14	1: 0.94
	Labrador Retriever	13	19.12	4.13	Median = 9	
	Boxer	12	17.65	8.82	Mode = 7/10	
Soft tissue sarcomas (n=90)	Mixed	41	45.56	4.52	Range = 2 - 18	1: 0.96
	Labrador Retriever	11	12.22	3.49	Median = 10	
	Boxer	9	10.00	6.62	Mode = 10	
Peripheral nerve sheath tumor (n=39)	Mixed	19	48.72	2.09	Range = 2 - 18	1: 0.86
	Boxer	4	10.26	2.94	Median = 10	
	German Shepherd	4	10.26	4.44	Mode = 12	
Hepatoid gland tumor (n=88)	Mixed	46	52.27	5.07	Range = 2 - 18	1: 6.33
	Labrador Retriever	6	6.82	1.90	Median = 11	
	Estrela Mountain Dog	4	4.55	20.00	Mode = 9/10/11	
Hepatoid adenoma (n= 57)	Mixed	30	52.63	3.30	Range = 2 - 18	1: 4.18
	Labrador Retriever	5	8.77	1.59	Median = 10	
	Cocker Spaniel	3	5.26	6.38	Mode = 9	
Tumor like lesions (n=71) ***Acrochordon***	Boxer	15	21.13	11.03	Range = 1 - 17	1: 0.82
	Mixed	14	19.72	1.54	Median = 9	
	Labrador Retriever	10	14.08	3.17	Mode = 11	
Hair follicle tumor (n=69)	Mixed	28	41.18	3.08	Range = 2 - 18	1: 1.13
	German Shepherd	7	10.29	7.78	Median = 8	
	Basset Hound	5	7.35	38.46	Mode = 6	
Trichoepithelioma (n=27)	Mixed	16	57.14	1.76	Range = 4 - 14	1: 0.87
	Basset Hound	2	7.14	15.38	Median = 7	
	Boxer	2	7.14	1.47	Mode = 6	
Histioproliferative disorders (n=61)	Mixed	20	32.79	2.20	Range = 0 - 14	1: 0.85
	Boxer	10	16.39	7.35	Median = 2	
	French Bulldog	7	11.48	17.07	Mode = 1	
Histiocytoma (n=58)	Mixed	20	34.48	2.20	Range = 0 - 14	1: 1.07
	Boxer	9	15.52	6.62	Median = 2	
	French Bulldog	7	12.07	17.07	Mode = 1	
Hamartomas (n=56) ***Hamartoma***	Mixed	18	32.14	1.98	Range = 3 - 18	1: 0.75
	Labrador Retriever	13	23.21	4.13	Median = 10	
	Boxer	12	21.43	8.82	Mode = 7/11	
Epidermal tumor (n=60)	Mixed	14	23.33	1.54	Range = 3 - 17	1: 1.22
	Labrador Retriever	12	20.00	3.81	Median = 9	
	Dogo Argentino	6	10.00	85.71	Mode = 8/9/10	
Squamous cell carcinoma (n= 32)	Mixed	8	25.00	0.88	Range = 3 - 15	1: 1.13
	Dogo Argentino	5	15.63	71.43	Median = 910	
	Labrador Retriever	4	12.50	1.27	Mode = 9/10	

(n=4; 11.8%). Males were more affected than females (female to male ratio 1:1.27) while the opposite happen with sebaceous adenoma (female to male ratio 1: 0.72). Both tumors in this group had a higher number of cases in older dogs. Sebaceous adenomas were most common on 13-year-old dogs but had a higher age interval between 11 and 14-year-old. Sebaceous epithelioma had similar number with the most common age being 10 and 12 years-old and a high frequency age interval of 10 and 14 years old.

Vascular tumors comprehended 7.93% of all skin tumor types. This group presented 94 cases distributed by three tumor histotypes, being hemangioma (n=68; 72.34%), lymphangioma (n=3; 3.19%) and hemangiosarcoma (n=23; 24.47%). Within the total, these had a proportion of 5.74%, 0.25% and 1.94%, respectively. Tumors from this group were often found in hindlimb (n=11; 11.70%), followed by multicentric development (n=10; 10.64%) and abdominal region (n=9; 9.57%). The most common breeds in this group were mixed-breed dogs (n=33; 35.11%), Labrador Retrievers (n=15; 15.95), Boxers (n=13; 13.83%) and Dogo argentino (n=5; 5.32%), these breeds had a breed-specific prevalence of 3.63%, 4.76%, 9.56% and 71,43%, respectively. Dogo argentino had a higher prevalence compared with the other common breeds. This group had a higher number of cases in 7- and 8-year-old dogs. Hemangiomas were the most frequent affecting mainly mixed-breed dogs (29.41%), Labrador Retrievers (19.12%), Boxers (17.65%) and Dogo Argentino (4.41%). The most common sites were hindlimb region (13.24%) abdominal region (13.24%), dorsal region and forelimb region (10.29% each). Hemangiosarcomas were often found in the perigenital area (21.74%) followed by multicentric development (17.39%). Dogo Argentino was one of the most common breeds, was overrepresented and had the highest prevalence within breed in hemangiomas and hemangiosarcomas (42.86%; 28.57%, respectively),

Soft tissue sarcomas (STS) consisted of fibrosarcoma (n=2; 0.17% of all skin tumor types), perivascular wall tumors (n=20; 1.69%), peripheral nerve sheath tumor (n=39; 3.29%), liposarcoma (n=2; 0.17%), sarcomas NOS (n=21; 1.77%), synovial sarcoma (n=3; 0.25%) and myxosarcomas (n=3; 0.25%). STS were often diagnosed in mixed-breed dogs (n=41; 45.56% of all affected breeds), Labrador retrievers (n=11; 12.22%) and Boxers (n=9; 10.00%). With a breed-specific prevalence of 4.52%, 3.49% and 6.62%, respectively. Soft tissue sarcomas were mostly found in the hindlimb (n=22; 24.44%) and forelimb region (n=21; 23.33%), followed by a multicentric development (n=9;10.00%). The age interval with a higher number of cases was between 9 and 12 years-old dogs, representing 54.10% of all ages registered. In these group 49 cases were benign (54.4%) and 41 were malignant. Among this group, peripheral nerve sheath tumors (n=39; 43.3% of all STS) were most common, followed by sarcomas NOS (n=21; 23.3%). Peripheral nerve sheath was most common found in mixed-breed dogs

(48.72%), Boxers (10.26%) and German Shepherds (10.26%) and sarcoma NOS had a higher frequency in Labrador Retrievers (33.33%) and mixed-breed dogs (33.33%). These tumors were often found on the extremities (hindlimb, n=9; n=4 and forelimb n=8; n=4, respectively), peripheral nerve sheath tumor had also a higher frequency for multicentric development (n=4; 10.26%) but within sarcoma NOS multicentric development was not observed. Both tumor types had a higher number of cases in older dogs, 12-year-old dog (n=7; 18.42%) and 10-year-old dog (n=5; 25.31%) respectively. A high number of perivascular wall tumor cases was found in comparison to others diagnosis, affecting most commonly mixed breed dogs (55.00%) and Boxers (20.00%), originating in the hindlimb and forelimb region (n=8; 40.00% and n=5; 25.00%, respectively) Older dogs were also most affected (9- to 12- year-old dogs comprised 61.11% of all cases).

Hepatoid gland tumors represent 7.43% of all cutaneous tumors making this tumor type the sixth most common in this survey. These comprised 88 cases, distributed in hepatoid adenoma (n=57; 64.77% of all hepatoid gland tumors; 4.81% of all skin tumors), followed by hepatoid epithelioma (n=14; 15.09%; 1.18%), hepatoid carcinoma (n=10; 11.36%; 0.84%) and hepatoid neoplasm NOS (n=7; 7.95%; 0.59%). The top 3 more common breeds in this group were mixed-breed dog (n=46; 52.27%), Labradors Retriever (n=6; 6.82%) and Estrela Mountain dog (n=4; 4.55%), presenting a breed-specific prevalence of 5.07%; 1.90%, 20.00%, respectively. Common sites of development were in the buttock area (n=67; 76.14%), followed by a multicentric development (n=8; 9.09%) and tail (n=6; 6.82%). These tumors had a higher tumor frequency in male dogs (n=76; 86.40%) compared to female dogs (n=12; 13.60%) (female to male ratio = 1: 6.33) and were most common in dogs within the age interval of 9- to 14-year-old.

In the group of **tumors like lesions**, acrochordons were the only encountered tumor histotype within this group with 71 cases (5.99% of all cases), this group occurred more frequently in the abdominal region (n=10; 14.08%), the forelimb region (n=10; 14.08%), multicentric development (n=9; 12.68%) and hindlimb (n=6; 8.45%). Boxers (n=15; 21,13%), Mixed-breed (n=14; 19.72%), and Labrador Retrievers (n=10; 14.08%) were the most common breeds with a prevalence within breed of 11,03%, 1.54% and 3.17%, respectively. The age interval with an increased number of cases was from 7-years-old to 11 years-old (59.70% of all cases with age registered), with the highest number of cases being on 11-year-old dogs (n=11; 16.41%).

Hair follicle tumors (n=69; 5.82% of all skin tumors) consisted of trichoepithelioma (n=27; 2.28% of all tumor types), trichoblastoma (n=20; 1.69%), infundibular keratinizing acanthoma (n=10; 0.84%), pilomatricoma (n=6; 0.51%),

subungual keratoacanthoma (n=3; 0.25%), tricholemmoma (n=2; 0.17%) and pawpad keratoma (n=1; 0.08%). For this group, the most common breeds were mixed breed (n=27; 41.18%), German shepherds (n=7; 10.29%) and Basset Hounds (n=5; 7.35%). Basset Hounds had a higher breed-specific prevalence in comparison to the other most common breeds, 38.46%, while mixed-breed dogs had 3.08% and German shepherds had 7.78%. These tumors were often found in the hindlimb region (n=11; 15.94%), dorsal region (n=10; 14.49%), neck (n=8; 11.59%) and also a multicentric development (n=9; 13.84%) was among the most common. The number of cases was higher in 6-year-old dogs (n=11; 16.92%) and 9-year-old dogs (n=9; 14.06%). Male dogs (53.60%) were more affected than female dogs (46.40%). Trichoepitheliomas were the most common in this group (39.71%) and had a higher number of cases in the dorsal region (n=5; 17.86%) as well as the hindlimb region (n=4; 14.29%), however multicentric development (n=7; 25.00%) was the most common type of anatomical distribution. This tumor had a higher frequency in dogs within 5- and 11- years-old and Basset-Hound, Boxers and Golden Retrievers (n=2; 7.1% each breed) were the most common breeds. Basset Hounds had a higher breed-specific prevalence (15.38%) in comparison to mixed-breed dogs with only 1.76% and Boxers with 1.47%. Trichoblastoma was the second most commonly found in this group (29.41%) and often was found in the head region (facial, cranial region and ears comprising 60.00% of all the cases) and also in the neck area (n=3; 15.00%). Most common breeds were mixed breed (n=4; 20.00%), German Shepherds (n=3; 15.00%) and Cocker Spaniels (n=2; 10.00%). Cocker Spaniels and German Shepherds had a higher prevalence within breed (4.26% and 3.33%, respectively) while mixed-breed dogs had only 0.44%. In terms of age this tumor was observed mostly in older dogs specially in 9-year-old dogs (16.66%) and 13- year-old dogs (16.66%). The following most common tumor diagnosis in this group was infundibular keratinizing acanthoma and this tumor was most common in mixed-breed dogs (40.00%) and German Shepherds (30.00%). Infundibular keratinizing acanthomas were often found in the dorsal region (n=3; 30.00%) and hindlimb region (n=3; 30.00%).

The nineth most common tumor type group of skin tumor was **histioproliferative disorders** and had 61 cases, corresponding to 5.15% of all cutaneous tumors histotypes. This group consisted of histiocytomas (n=58; 95.08% of histioproliferative disorders) and histiocytic sarcomas (n=3; 4.92%). Within all tumors, these two presented a proportion of 4.89% and 0.25%, respectively. Histiocytoma was the most common tumor type within this group. The three most common breeds for this tumor were mixed-breed dogs (n=20; 34.48%), Boxers (n=9; 15.52%) and French Bulldogs (n=7; 12.07%). French Bulldogs had the higher breed-specific prevalence value of 17.07% in comparison to the first two breeds mentioned which recorded 2.20% and 6.62%,

respectively. This tumor type was commonly found in the ears (n=10; 17.24%), facial region (n=7; 12.07%), pectoral region (n=7; 12.07%) and cranial region (n=5; 8.62%). Histiocytomas had a higher number of cases in dogs at an early age, concentrated in dogs with less than 3-years-old (comprising 70.37% of the cases with age registered) however 1-year-old dogs were most often affected (n=14; 25.92%) followed by dogs with less than 1-year-old (n=10; 18.51%).

Epidermal tumors consist of 5.06% of all cutaneous tumors making them the tenth most common tumor type group. This had 60 cases and 3 different tumor types were diagnosed, such as squamous cell carcinoma (n=32; 53.33%), papilloma (n=22; 36.66%) and basal cell carcinoma (n=6; 10.01% of all epidermal tumors). Mixed breed dogs (n=14, 23.33%), Labradors Retrievers (n=12; 20.00%) and Dogo Argentino (n=6; 10.00%) were the most common breeds. Dogo Argentino had the highest breed-specific prevalence (85.71%), the other two breeds had a prevalence of 1.54% and 3.81% respectively. These tumors had a high proportion of multicentric development (n=12; 20.00%) and were often found in the forelimb region (n=9; 15.00%) and facial region (n=5; 8.33%). Epidermal tumors were most common found in the age interval of 8- to 10-years-old. The most common histotype was squamous cell carcinoma (STC) comprising 2.70% of all cutaneous tumors. The most common breeds were mixed-breed dogs (n=8; 25.00%), Dogo Argentino (n=5; 15.63%) and Labrador Retriever (n=4; 12.50%), with Dogo Argentino with the higher prevalence within breed (71.43%) of all three breeds mentioned (mixed breed dogs = 0.88%; Labrador Retriever =1.27%). SCC had most often multicentric development (31.25%) followed by facial region, forepaw region and hindlimb region (9.38% each region). This tumor type had a higher tumor frequency being recorded in male dogs (female to male ratio = 1: 1.13) and were most common in dogs within the age interval of 9- to 12-year-old, which comprised 52.92% of all age's registered. Three cases of SCC were subungual, all affected male dogs with older ages (9-, 10- and 12-year-old dogs). Papilloma was the second most common tumor in this group and was common observed in Labrador Retrievers (31.82%), mixed-breed dogs (22.73%) and Boxers (13.64%). In this tumor the most often observed anatomical site were forelimb region (27.27%), facial region (9.09%), hindpaw region (9.09%) and multicentric development (9.09%) was also observed. Basal cell carcinoma had a higher frequency of affected male dogs than females (female to male ratio = 1: 5.00)

Hamartomas had 56 cases observed, corresponding to 4,73% of all cutaneous tumors. The most common anatomical sites were forelimb region (n=6; 10.71%), abdominal region and neck (n=5; 8.93%, each location). However, this tumor had the higher frequency in multicentric development (n=9; 16.07%). Mixed breed (n=18; 32,14%), Labrador Retriever (n=13; 23,21%) and Boxer (n=12; 21.43%) were the most

common breeds, with 1,98%, 4,13% and 8,82% corresponding, respectively, to the prevalence with breed. The age interval with an increased number of cases was from 7 years old to 12 years-old (65.38% of all cases with age registered).

Melanocytic tumors were not one of the most 10 common tumors, representing only 3.63% of all cutaneous tumors (n=43). Melanocytomas (n=23) represents 53.48% of all melanocytic tumors and 1.94% of all skin tumors, on the other hand melanoma (n=20) represented 46.51% of melanocytic tumors and 1.69% of all skin tumors. In this group mixed-breed dogs (n=13; 30.23%) and Labradors Retriever (n=10; 23.26%) were the most common breeds and often a multicentric development (n=8; 18.60%), followed by development in abdominal region (n=5; 11.63%) and hindpaw region (n=5; 11.63%) was observed. Older dogs were more frequently affected. Melanocytomas occurred often in mixed-breed dog (n=10; 43.48%), Dogue the Bordeaux (n=2; 8.70%) and Pinscher (n=2; 8.70%) with a breed-specific prevalence of 1.10%, 28.57% and 4.35%, respectively. These tumors had a higher proportion of multicentric development (n=5; 21.74%) and often occur in abdominal regions (n=4; 17.39%). These tumors had a higher frequency in 11-year-old dogs (18.18%), followed by 7-, 9-, 10- and 14-year-old dogs (13.63% each age mentioned). Melanomas had a higher number of cases in Labrador Retrievers (n=9; 45.00%), mixed-breed dogs (n=3; 15.00%) and Golden Retrievers (n=2; 10.00%). Labrador Retrievers and Golden Retrievers had a higher breed-specific prevalence (2.86%, 3.85%) in comparison to mixed-breed dogs (0.33%). This tumor type had a higher frequency of development in hindpaw region (n=4; 20.00%), forepaw region (n=3; 15.00%) and a higher multicentric development was observed (n=3; 15.00%). The age interval with a higher number of cases was from 8-years-old to 13- years-old.

Plasmacytic tumors and **apocrine gland tumor**, both had the same number of cases (n=18; 1.52% of all skin tumors). Plasmocytic tumors consisted of plasmacytomas and one case of multiple myeloma. Plasmacytomas (n=17, 1.43% of all skin tumors), were most common in mixed-breed dogs (n=6; 35.29%), Labrador Retrievers (n=2; 11.76%) and West Highland White Terrier (n=2; 11.76%). West highland white terrier had a higher breed prevalence (15.38%) in comparison to the other two breeds (0.66%; 0.63%, respectively). The most common anatomical sites were hindlimb region (n=5; 29.41%), facial region (n=4; 23.53%) followed by a multicentric development (n=3; 17.65%). These tumors occur more frequently in older dogs with the greatest number of cases occur in 9-year-old dogs (17.64%) and the age interval with the highest frequencies was between 9- and 13-years-old. Plasmacytomas had a higher frequency of male dogs affected (female to male ratio = 1: 1.43). Apocrine gland tumors consisted of apocrine adenoma (n=4; 44.4%), apocrine carcinoma (n=6; 33.3%), apocrine adenocarcinoma, apocrine cistoadenoma, apocrine neoplasm NOS and cutaneous clear

cell adnexal carcinoma, all with only 1 case (accounting for 5.6% each). The most common breeds were mixed breed (n=8; 44.44%). The anatomical sites that were more often represented were abdominal region (n=4; 22.22%), dorsal region (n=4; 22.22%) and costal region (n=2; 11.11%). These tumor types had a higher frequency in older dogs specially on 12-year-old dogs (27.77%) but the age interval of 10- to 14-year-old comprised most of the cases with age registered (72.22%). In apocrine carcinomas Chow-chows had the highest breed-specific prevalence (20.00%).

The less common tumor types were **musculoskeletal tumors** (n=8, 0.68% of all skin tumors), **epithelial tumor NOS** (n=8; 0.68%), **lymphomas** (n=6; 0.51%), **round neoplasm NOS** (n=3; 0.25%), **mesenchymal neoplasm NOS** (n=3; 0.25%), cutaneous neoplasm NOS (n=8; 0.68%) and mixed **mesenchymal neoplasm** (n=1; 0.08%). Musculoskeletal tumors consisted of osteosarcoma (n=4), Chondroma (n=1), Leiomyoma (n=1), Leiomyosarcoma (n=1), Rhabdomyosarcoma (n=1). This tumor type group had a higher number of cases in mixed-breed dogs (n=3; 33.33%), Labrador Retrievers (n=2; 22.22%) and Bullmastiff (n=1, 11.11%). The breed-specific prevalence was higher (12.50%) in Bullmastiffs when compare to the other breeds. The anatomical site with a greater number of cases was shoulder (n=2; 22.22%) and dogs with 10 years-old had a higher frequency (44.44%) of cases reported. Epithelial tumors NOS consisted of carcinoma NOS (n=4; 50.00%), adenocarcinoma NOS (n=1; 12.50%), adenoma NOS (n=1; 12.50%), anaplasic carcinoma (n=1; 12.50%) and carcinosarcoma (n=1; 12.50%). These tumors were found often in mixed-breed dogs (n=2; 25.00%) and had a higher multicentric development (n=2; 25.00%). Dogs with 10-years-old had the greatest number of cases comprehending 50.00% of all ages registered. Lymphomas were reported in mixed-breed dogs comprehending most of the number of cases (33.33%). These tumors were found in the facial region (n=2), dorsal region (n=1), forelimb region (n=1) and shoulder (n=1). This tumor type had a higher frequency in older dogs with 8- and 13- year-old dogs comprehending 66,66% of all ages registered. Round cell neoplasm NOS only had 3 cases, 2 cases were from mixed-breed dogs (n=2; 66.66%) and 1 from a Beagle (n=1; n=1; 33.33%). Tumor development was reported in two sites, abdominal region (n=1, 33.33%) and ear (n=1; 33.33%) and all three cases were reported in female dogs. Mesenchymal neoplasm NOS only had 3 cases and two breeds were reported them being, mixed-breed dogs with the higher frequency (n=2; 66.66%) and Rottweiler (n=1; 33.33%). Two anatomical sites were reported dorsal region (n=1, 33.33%) and forelimb region (n=1; 33.33%). Cutaneous neoplasm NOS were all classified as malignant and undifferentiated, mot common breeds were mixed-breed dogs (37.50%) and Labrador Retrievers (25.00%) and were presented in older dogs from 7- to 12-years-old. Mixed mesenchymal neoplasm only had 1 tumor case of a

Angiolipoleiomyoma (0.08% of all skin tumors) in a mixed-breed female dog of 3-years-old.

Discussion

The skin is regularly subjected to a wide-ranging variety of chemical and physical insults, environmental influences, and exposure to carcinogenic agents (e.g., ultraviolet radiation from sunlight). As a result, it is prone to neoplastic proliferation consisting in a frequent site of different neoplasm histotypes onset in domestic animals.[12,18,27,28,32,40] The skin, the largest organ in the body, accommodate populations of epithelial, mesenchymal, and local immune cells, which play a part in homeostasis and protection against external factors. This study described the epidemiological tendencies for canine cutaneous tumors in Portugal, based on the analysis of 1.185 biopsy cases submitted to the laboratory over the past 7 years (2014–2020). In spite of the fairly small sample size as compared with probes performed in other European countries and in the United States[28,59], this study may elucidate about the frequently diagnosed tumor histotypes, anatomical tendency of canine cutaneous tumors location, preferential ages and gender and common dog breeds at risk in this specific geographic region. To compare current and previous epidemiological studies conducted on canine cutaneous tumors, the most common benign and malignant neoplasms observed in each retrospective analysis is reviewed in **Table 2-F.** Discrepancies in the outcomes may be attributed to methodological variations (e.g., data and inclusion/exclusion criteria) between studies. Thus, a prudent interpretation is mandatory.[28,37,46,59]

Several histologic grading systems were proposed for delineating cutaneous **mast cell tumor** prognostic due to the high incidence and variable biological behavior of MCTs in dogs, issue that it is well documented. [8,18,29,36,46,47] In this study, of all the cases classified with Kiupel's grading system, about 71% (or more than two-thirds) of cutaneous MCT were classified as low grade and, less than one-third as high grade, similarly to previous studies conclusions.[36,51,56] In the Patnaik classification system, grade II (Intermediately differentiated – 64,88%) and grade III (poorly differentiated – 22.22%) displayed higher frequency than grade I (well differentiated – 12.88%) which differs a bit from prior investigations since in the herein study there was a higher frequency of grade III tumors and lower of grade I.[8,44,45] Subcutaneous MCT were less prevalent than the cutaneous variant. Both cutaneous and subcutaneous MCT were analyzed independently since the existing histologic grading scheme is not appliable to subcutaneous mast cell tumors.[36,51] In accordance with several investigations, a high breed-prevalence was also recorded in Labrador Retrievers and Boxers.[5,24,41,44,59,61]

Boxers had a higher number of cases classified as low grade (Kiupel's) and grade I and grade II (Patnaik's) in comparison to high grade and grade III. Labrador Retrievers had the opposite, a higher number of grade II and III lesions than grade I, however with Kiupel's system this was not observed, high grades had lower number of cases, these results support findings from previous studies for Boxers for having more predisposition to low grade MCTs and for Labrador Retrievers usually having predisposition for more aggressive MCT forms.[35,54,61,62]

In previous studies fibromas were most common in the limbs and heads of dogs but herein, most of fibroma diagnoses were observed in the abdominal region, limbs and extremities and dorsal region; head regions were not recorded.[24,31] Affected dogs were mostly middle age to older dogs. Lipomas had a higher frequency in female dogs affected, reinforcing the information that bitches have a predisposition to these tumors.[34] In terms of anatomical distribution, the majority of the cases presented a multicentric development however, pectoral, costal, and dorsal regions as well as limbs were also commonly affected.[24,26,31,42] Some dog breeds have an increased risk to develop lipomas such as Labrador Retrievers and Cocker Spaniels and in our finding this two were common and had a high breed-specific prevalence's (6.67%, 4.26%, respectively) which supports previous studies.[24,53] In our findings, as with similar studies a small portion of lipomas was infiltrative.[7,23,26,38] Infiltrative lipomas were most common observed in female dogs (66.66% of all infiltrative lipomas) which supports the predisposition to females and majority of areas affected was the limbs (66.66%) as well as the dogs affected were middle age to older dogs (9- to 13-year-old dogs).[24,31]

Being one of the most common cutaneous neoplasm, sebaceous tumors were often found on the head region such as facial, cranial and neck region with a high tendency for multicentric growth where majority of the cases had this type of anatomical distribution and there was a noticeable predisposition in Labrador Retrievers and Cocker Spaniels for this type of tumors.[24,26] Sebaceous adenoma had a high frequency age interval (10- to 13-years-old) within the peak incidence described in previous studies.[26] Sebaceous epithelioma had a higher concentration of cases in dogs between 10- and 15-years old. These outcomes agree with the literature.[26]

In hemangioma the most common breeds were Labrador Retrievers, Boxers, Dogo Argentino, German Shepherd and Golden Retrievers and in hemangiosarcomas, the most common purebred dogs were Dogo Argentino, German Shepherd and Pitbull. According to the literature, shorthaired and light skin dogs can have an increased risk in the development of these tumors, and in this study Boxers, Dogo Argentino and Pitbull had a higher breed-specific prevalence and this breeds can be considered shorthaired and also have or can have light skinned which supports previous studies.[31,33,34,52,60,64]

However Golden Retrievers and German Shepherd dogs also were found to have a predisposition to these tumors.[26,31] These tumors also had a higher frequency in older dogs as described in the literature (higher frequency age interval 7- to 11-years old in hemangiomas and 8- to 11-years old in hemangiosarcomas).[24,31,60] The anatomical distribution of hemangiomas majority consisted of limbs, abdominal region and dorsal region and as being stated that hemangiomas have a predilect occurrence for these areas (limbs and abdominal) usually have predilection to solar induced hemangiomas and dorsal regions for non-sun induced hemangiomas.[31,33,42] In terms of anatomical sites predisposition in hemangiosarcomas, similarly to hemangiomas usually if it's a solar induced neoplasm there's a predilection to abdominal regions and limbs and for non-sun neoplasm can appear at any site, in our study there was some cases observed in the abdominal area however the most common was perigenital area and multicentric development.[4,34]

In soft tissue sarcomas, perivascular wall tumor appeared mostly in older dogs specially from 9- to 12-years-old also majority of the cases (65.00%) were found in the limbs followed by the areas in the trunk (abdominal, dorsal, costal, pectoral region) all consistent with the information in precious studies.[2,9,26,30,49,55] Large breed dogs are at increases risk for perivascular wall tumor and the two most common breeds were primarily mixed-breed, that's being already state to be at increased risk and the secondly Boxers.[24,31] Peripheral nerve sheath tumors similarly to perivascular wall tumors, had a higher frequency in older dogs but had a full range of ages going from 2-year-old dogs to 18 year-old dogs and majority of cases were found also in the limbs and trunk area such as costal and pectoral region.[14,17,26,31]

As described in some studies, hepatoid gland tumors are one of the most common, as observed in this study. Hepatoid adenomas had a high frequency age interval between 9- to 13-years-old and there is a predisposition to male dogs to be affected by this tumor and Cocker Spaniels were also commonly affected and with a high prevalence within breed (6.38%) which goes towards the literature, since this breed is known to have a predisposition for these type of tumors.[24,26,63] The anatomical distribution also confirms previous studies as the buttock area and tail are the most affected locations. Hepatoid epitheliomas results in this study such as age incidence (10- to 13-years-old dogs) and sex predisposition (male dogs with an increased risk) were supported by the literature.[26] In terms of hepatoid carcinoma, Siberian Huskies had a higher breed-specific prevalence and, as well as the other diagnosis in this group, sex predisposition (male dogs at increased risk) and older dogs with a higher incidence corroborates previous observations.[26,31]

Table 2-F: Comparison of present and past epidemiological studies on canine cutaneous tumors

EPIDEMIOLOGICAL STUDY	3 most common tumor types				Breed predispositions for cutaneous tumor in purebred dogs
	BENIGN TUMOR	**% OF ALL CASES**	**MALIGNANT TUMOR**	**% OF ALL CASES**	
Portugal N=1.185	Benign soft tissue tumors Benign sebaceous gland tumor Hemangioma	9.70 8.10 7.93	Cutaneous mast cell tumor Peripheral nerve sheath tumor Squamous cell carcinoma	20.59 3.29 2.70	Cutaneous mast cell tumor - Labrador Retriever, Boxer Peripheral nerve sheath tumor – Boxer, German Shepherd Squamous cell carcinoma – Dogo Argentino, Labrador Retriever Hemangioma - Labrador Retriever, Boxer Benign soft tissue tumor - Labrador Retriever, Golden Retriever Benign sebaceous gland tumor - Labrador Retriever, Cocker Spaniel
Japan n=1.435	Lipoma Benign hair follicle tumors Benign sebaceous tumors	9.69 9.34 8.50	Soft tissue sarcoma Mast cell tumor Squamous cell carcinoma	18.40 16.24 4.67	Soft tissue sarcoma−Labrador Retriever, Golden Retriever Mast cell tumor−Pug, Labrador Retriever Benign sebaceous tumor−American Cocker Spaniel Histiocytic sarcoma−Flat Coated Retriever, Bernese Mountain Dog
Korea n=748	Lipoma Histiocytoma Basal cell tumor	11.36 7.49 6.82	Mast cell tumor Apocrine carcinoma Melanoma	8.82 3.07 2.41	NR
Switzerland n=11.740	Lipoma Hair follicle tumor Histiocytoma	12.47 12.34 12.10	Mast cell tumor Soft tissue sarcoma Melanocytic tumor	16.35 10.86 8.63	Mast cell tumor−Boxer, Nova Scotia Duck Tolling Retriever, Rhodesian Ridgeback Histiocytoma−Flat-Coated Retriever Melanocytic tumors−Magyar Vizsla, Airedale Terrier Epidermal tumors−Standard Schnauzer, Giant Schnauzer
North America n=25.996	Lipoma Adenoma Papilloma	27.44 14.08 7.02	Mast cell tumor Hemangiopericytoma Other sarcomasa)	10.98 2.93 2.84	Mast cell tumor−Boxer, Rhodesian Ridgeback, Vizsla, Boston Terrier Soft tissue sarcoma−Rhodesian Ridgeback Melanoma−Vizsla, Miniature Schnauzer, Chesapeake Bay Retriever, Boxer Lymphoma−Scottish Terrier Hemangiosarcoma−Boxer Squamous cell carcinoma−Dalmatian

NR, no record. a) Other sarcomas consisted of myxosarcoma, osteosarcoma, leiomyosarcoma, chondrosarcoma, lymphangiosarcoma, rhabdomyosarcoma.

Acrochordons occur often in the abdominal region and in the limbs which encounters the literature since this lesions are usually truncal or in areas that have

pressure points such as areas in the legs also majority of affected dogs are older as seen in previous studies.[20,31,39]

In hair follicle tumors, trichoblastomas and trichoepitheliomas were often diagnosed, whilst tricholemmomas were infrequently seen. Trichoblastomas were often found on the head (facial region, cranial region and ears) and neck and a higher frequency of male dogs affected was observed as in a previous study.[1,26,31] Cocker Spaniels and mixed-breed dogs were two breeds commonly affected by trichoblastomas corroborating previous data reporting that these breeds present increased frequency of this type of tumors.[26,31] Trichoepitheliomas had a tendency for multicentric growth and for development in the dorsal region. Cases of Basset Hounds observed had multicentric grow which is known for its predisposition to multicentric trichoepithelioma.[24,26,31] In terms of anatomical distribution some of the sites most commonly observed such as dorsal and neck region were in agreement with previous studies.[26] Golden Retrievers also had a higher frequency and this tumor, had a higher frequency in dogs 5- and 11-years-old, all of which is consistent with other studies.[26] Infundibular keratinizing acanthoma were often found in the dorsal, neck region as well as in the hindlimb region and had the majority of cases between 4- to 10-year-old cases, with the highest frequencies being in those extremities which is supported by research.[26,31] German Shepherds were on of the most common breeds that had infundibular keratinizing acanthoma and this breed is one of the breeds that have a marked predisposition to the development of this tumors.[24,31] **Pilomatricomas** had the highest frequency of cases in dogs with 6-years-old, Basset Hounds were the most affected breed and the majority of cases was located in the limbs all of this outcomes supports previous studies.[31]

Majority of histioproliferative disorders were constituted by histiocytomas. In the current study, the higher frequency age interval was found in dogs with less than 3 years old but, specially, in dogs with 1 or less than 1-year-old. However the full range of cases was larger including dogs with a few months till 14-year-old, supporting previous studies data that all ages can be affected but younger dogs have an increased risk.[26,31] Most of the lesions were solitary and concentrated in head regions (facial, cranial regions and ear) and limb extremities which is also consistent with the literature.[26,31] Boxers have a predisposition to the development of histiocytoma and as such, in this study it was one of the most common breeds affected presenting a higher breed-specific prevalence.[43]

Squamous cell carcinomas developed in mature and senior dogs. These tumors occurred primarily with a multicentric development followed by solitary lesions mainly on the head, limbs, limbs extremities and abdominal region, consistent with research.[26,31] Previous studies had reported a high occurrence of digit SCC.[28] This tumor, arising in this precise location has a different prognosis in comparison to other skin regions.[6]

Belluco *et al*.[6] showed that canine digital SCC hardly ever metastasized, but had a predisposition for multicentric growth.[6] In the present study, only one of the digital SCC had multicentric development, all the rest were solitary masses. The age interval with higher frequencies in this study for this tumor type (9- to 12-year-old) is within the peak incidence reported for SCC.[26]

The high frequency age interval observed in apocrine adenomas was between 8- and 12-years-old as already shown in previous studies.[26] A predisposition of Old English sheepdogs for apocrine adenomas is referred in the literature.[26] However, in our series we only have a single case of this specific breed that curiously presented this kind of lesion. Thus, although the breed-specific prevalence is high, we do not have enough data to support this previous evidence. In apocrine carcinomas the age peak was in 13-year-old dogs comprised 50% of all cases with age registered with this diagnosis which is one of the extremities of the interval of peak incidence for apocrine adenomas already documented.[26] Chow-chows had a higher prevalence within breed than the other breeds observed (20.00%) which supports the information from previous studies stating that this breed has an increased risk for this specific diagnosis.

Our findings, jointly with prior studies, suggest a higher predisposition for plasmacytic tumors in male dogs compared to female dogs. Additionally, the majority of plasmacytomas occur in older dogs.[3,10,13,48] Plasmacytomas usually develop as solitary lesions but multiple and numerous masses can occur. The great majority of our cases consisted in solitary lesions often observed in the limbs and in the facial region, which also supports research.[13,50]

Melanocytic tumors in specific melanocytomas occurred most commonly in the abdominal and costal regions as well as in the limbs however most of the cases had multicentric development, according to the literature canine melanocytomas can appear in any part of the body but can appear more often in the truncal area and occasionally on the extremities, in our findings we did observed a big part of the distribution in the trunk and on the extremities which supports the literature however the frequency of multicentric development it's a little higher than expected.[25,31] In terms of age incidence, the range was from 5- to 14-years-old with the frequency peak interval between 9- to 11-years old which is within the peak occurrence described in previous studies. Melanomas had majority of cases located in the limbs or head region which supports the information described in research and older dogs were also commonly affected especially from 8- to 13-years-old.[16,24,34]

Tumors with a low frequency (n<5) in our database (**Table 2-B**) were not sufficient for a correct analysis to compare with other studies and make conclusions about their epidemiological behavior.

In summary, the present paper describes the epidemiology of canine cutaneous tumors in this laboratory. Mast cell tumor, benign soft tissue tumors, sebaceous tumors, vascular tumors, and soft tissue sarcomas were the most presented tumor types. The discrepancy in the outcomes may reflect different etiologies and biological behavior of some skin tumors throughout geographical regions.

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CHAPTER 3

Canine cutaneous mast cell tumors

CHAPTER 3 - Canine cutaneous mast cell tumors

Epidemiological assessment of the risk of canine mast cell tumors based on the Kiupel and Patnaik classification systems.

Introduction

One of the leading causes of mortality in dogs is cancer (accounting from 14 to even 27% of all deaths in previous studies).[1,12] The quantity of diagnosed tumors is continuously rising.[1,12,18,47,44,50] since improved health care for pets currently broadens their life expectancy, allowing for the diagnosis of late-in-life diseases, such as cancer. Statistical data reveal that 50% of dogs reaching 10 years of age perish of neoplastic diseases.[1,7,8,12,49] One of the most frequently diagnosed tumors are skin tumors and between 7% to 21% of all occurrences are mast cell tumors (MCTs).[18,52] MCTs can be presented as an isolated, small and single case or have a multicentric growth. Plus they can infiltrate the neighboring tissues and metastasize to the lymphatic system and internal organs, displaying a wide-ranging clinical course of action.[4,31,33,46,52] There is a great effort to identify factors that can influence the prospective course of this disease however, one of the most important predictors that can determine morphological characteristics, metastatic potential of a tumor and can also determine response to treatment and the prognosis is the histological grade.[3,6,11,15,33,39,40,54,55]

Prior to 2011, the most commonly used histological grading system for mast cell tumors was the three-grade Patnaik system[35] which recognize three different categories: grade I (well differentiated MCTs), grade II (moderately differentiated MCTs), and grade III (poorly differentiated MCTs) based on infiltration depth, cellularity, cellular and nuclear pleomorphism, presence of giant cells, cytoplasmic granules, number of nucleoli, and number of mitotic figures.[35,44] Grade I are well-differentiated tumors which can cover up to 8%-53% of all MCT cases[48] and mostly develop in the dermis and less often in subcutaneous tissue.[44] Tumors classified with this grade do not invade nearby tissues, hardly ever metastasize and generally, after surgical removal with clean margins, they do not recur. The cells look pretty like normal mast cells; they are monomorphic, round, or egg-shaped with cell nucleus in the center where the nucleoli is barely visible. Mitotic figures are very rarely found. There are distinctive and abundant cytoplasmatic granularities. These tumors have a predictably good long-term prognosis and a 12-month survival probability up to 100%.[48] Grade II, or intermediate grade tumors account for

approximately 59%-76% of all mast cell tumors.[48] Cells are organized in threads or sizable clusters. These are larger and pleomorphic and have a small number of granularities in comparison to the previous grade. The nuclei are enlarged, and the nucleolus is visibly noticeable. The mitotic activity can range from minor to moderate and the number of mitotic figures is higher than grade I. There is a higher tendency to invade neighboring surroundings into the deeper layers of the skin and disseminate to other parts of the body in comparison to grade I. When these tumors are surgically removed if incomplete or with narrow margins, they have more chances to recur. Tumors classified as grade II have a 12-month survival probability of 87%–92%.[48] Approximately 5%-26% of all MCT cases are classified as grade III or high grade.[48] They are poorly differentiated, invade deep into the skin and underlying tissue and are extremely aggressive, presenting a metastasis rate of roughly 55% to 95%. Grade III tumors are particularly likely to recur. The cells and cell nuclei are characterized by substantial pleomorphism and granularities are frequently invisible. The cell nuclei are large, round, doubled or more. The nucleoli are clearly visible, and there is a high mitotic cell activity and, atypical mitotic figures are frequently observed.[44] Requires aggressive therapeutic management since patients detain a poor long-term prognosis and about a 12-month survival probability of 16%–46%.[48] In grade II tumors can be difficult to predict a dog's outcome, because in some cases it can behave more like grade I tumors. Nevertheless, others behave more aggressively, as grade III. An additional challenge is the trustworthiness of the grade evaluated and attributed by the pathologist. There is some degree of subjectivity with this system.

As a result of this challenges related with behavior variability and unpredictable clinical course, Kiupel *et al*.[25] proposed a new 2-grade classification system divided only in low-grade and high-grade, based on the morphology of the cell's nucleus and the number of mitotic division figures. Low grade tumors have a higher frequency, usually between 59%-89%, and high-grade tumors a smaller frequency of 11%-41%.[48] Mast cell tumors classified as high-grade tumor need to have at least 3 multinucleated cells, three cells with bizarre nuclei per 10 high-power fields, 7 mitotic division figures, and karyomegaly. All tumors that do not present at least one of these criteria are graded as low-grade. High grade tumors have a more aggressive behavior, a tendency to recur and metastasize, and a reduced survival time. In the case of high-grade mast cell tumor, the median survival time is about 4 months (12-month survival probability of 24%) and for low-grade MCTs is more than 2 years (12-month survival probability of about 95%).[25,48]

The evaluation of an association between the development of MCTs and canine breeds has been performed primarily concentrating on assessing the risk in a specific dog breed in a particular region. The relation of the dog's age, sex, and body weight and

in castrated or sterilized dogs with this tumor has been already demonstrated.[2,10,19,18,27,42,50,51,53] Mast cell tumors can develop in any part of the body, though most frequently in the torso (50–60%), limbs (25–40%), and head and neck (10%).[52] However, some locations are correlated with a worse prognosis such as perineal or perianal area and mucocutaneous junctions.[4,11,31,52] Numerous studies have shown a breed-related predisposition to certain tumor, including skin neoplasia.[15] For example, there is an increased risk of malignant histiocytosis in the Bernese Mountain Dog, malignant melanomas in the Schnauzer and anal sac gland carcinoma in the English cocker spaniel.[12,13,19,38] In terms of mast cell tumors, in Boxers an increased risk has been shown.[27,52] However there has been also described an increased risk for MCTs in breeds such as Labrador Retriever, Bullmastiff, Shar-Pei, Boston Terrier, Staffordshire Bull Terrier, Rhodesian Ridgeback, Pug, Weimaraner, Beagle, Golden Retriever, and Vizsla.[32,42,50,51,53] The breed prevalence of cutaneous tumors frequently reflects the popularity of such breeds in the geographical region where the analysed populations reside. The relationship among the breed and the clinical aspects of the disease is not sufficiently explored. In Boxers and Pugs a milder course of MCTs have been noticed, in Golden Retrievers a multicentric MCT form has been related while more aggressive forms have been noted in young age Shar-Peis.[11,30,32] A comparison of the clinical presentation of MCTs within single breeds will offer more information about the complex biology of these neoplasms and simultaneously, may provide based evidences for the implementation of genetic studies focusing on the aetiology.

Hence, a retrospective analysis assessing the risk of development MCTs and the respective histological grades presented could be extremely helpful for prognosis. There are no epidemiological studies in the veterinary literature based on both the Kiupel and the Patnaik classification system, which results remain the most common used prognostic factors for determining the course of the disease. The aim of this study was to conduct an epidemiological analysis of the risk of MCT development in dogs in relation to other skin tumors. The relationships between the dog's breed, age, sex, tumor anatomical location, and degree of MCT malignancy by the Patnaik's classification system and Kiupel two-grade malignancy scale with the development of cutaneous MCTs were assessed.

Materials and Methods

The study consisted in the analysis of 1.185 canine cutaneous tumors from the archives of the Laboratory of Veterinary Pathology from Institute of Biomedical Sciences Abel Salazar (LPV-ICBAS), University of Porto, sent for histological evaluation and

diagnosed between 2014-2020. Within this group, cases previously diagnosed as cutaneous MCTs were selected and clinicopathological data (breed, age, sex, anatomical location) from each patient were collected. All the cases were revised and subclassified according to the three-grade malignancy scale of Patnaik *et al*[35] and the two-grade malignancy scale of Kiupel *et al*.[25] All 244 MCTs were classified with at least one of this classification systems, when possible both classifications were used. With the clinicopathological data collected, four age groups were distinguished: (1) dogs with less than 3 years-old, (2) 4–6 years, (3) 7–10 years, and (4) 11–18 years. Additionally, nine anatomical locations were established: (1) buttock area, (2) ear, (3) head and neck, (4) limbs, (5) multicentric, (6) perigenital area, (7) tail (8) trunk and (9) Skin not otherwise specified (Skin NOS). A control group was defined comprising dogs diagnosed with cutaneous tumors others than MCTs, with the restriction that subcutaneous MCTs were excluded from the analysis.

All the data recorded was submitted to statistical analysis using the using IBM® SPSS® Statistics version 26 (IBM SPSS, Armonk, NY, U.S.A.). Values of $P<0.05$ were considered significant. The risk of MCT development according to breed, sex, location, and age was determined based on the odds ratio (OR). A univariable analysis was executed for each variable to determine the ORs with 95% confidence intervals (CIs). For each specific breed, ORs were calculated by comparing the MCT incidence in the analyzed breed with that in the other breeds diagnosed with other cutaneous tumors (control group). Analogous calculations were conducted for tumor location. For the calculations of ORs relative to age, the group with older dogs (11-18 years of age) were regarded as the basal group. Males were the basal group in the determination of ORs for sex.

Results

In this study 244 MCTs were retrieved from LPV-ICBAS database, accounting for 21.03% of all cutaneous tumors diagnosed. The analysis involved 244 cases of cutaneous MCTs from 222 dogs, representing a total of 34 breeds (33 purebreeds and mixed breed). Some breeds were represented in the control group but not in the MCTs group due to the low frequency reflected in the later and for that reason, 44 breeds of the control group were replaced into a subgroup, named “other breeds”.

Table 3-A: Frequency of MCTs in various breeds of dogs.

BREEDS	ALL MCTS		CONTROL GROUP#	
	NUMBER	%	NUMBER	%
LABRADOR RETRIEVER	67	27.46	142	15.50
MIXED	64	26.23	335	36.57
BOXER	37	15.16	75	8.19
GOLDEN RETRIEVER	10	4.10	26	2.84
FRENCH BULLDOG	8	3.28	10	1.09
PIT BULL	7	2.87	13	1.41
PUG	5	2.05	2	0.22
GERMAN SHEPHERD	4	1.64	26	2.84
YORKSHIRE TERRIER	4	1.64	15	1.64
BEAGLE	3	1.23	12	1.31
BULL TERRIER	3	1.23	2	0.22
PINSCHER	3	1.23	7	0.76
POODLE	3	1.23	15	1.64
BOUVIER BERNOIS	2	0.82	3	0.33
BULLMASTIFF	2	0.82	5	0.55
CASTRO LABOREIRO DOG	2	0.82	0	0.00
COCKER SPANIEL	2	0.82	39	4.26
ROTTWEILER	2	0.82	12	1.31
BELGIAN SHEPHERD	1	0.41	1	0.11
BOERBOEL	1	0.41	2	0.22
BOSTON TERRIER	1	0.41	0	0.00
BULLDOG	1	0.41	2	0.22
CANE CORSO	1	0.41	2	0.22
COCKER	1	0.41	13	1.42
DOBERMAN	1	0.41	6	0.66
ENGLISH SETTER	1	0.41	1	0.11
ESTRELA MOUNTAIN DOG	1	0.41	9	0.98
FLAT COATED RETRIEVER	1	0.41	2	0.22
JACK RUSSELL TERRIER	1	0.41	3	0.33
POINTER	1	0.41	1	0.11
POMERANIAN DOG	1	0.41	0	0.00
RHODESIAN RIDGEBACK	1	0.41	1	0.11
SPITZ	1	0.41	2	0.22
WEIMARANER	1	0.41	1	0.11
OTHER BREEDS	0	0.00	133	14.52
TOTAL	244	21.03***	916	78.97

*** Percentage of dogs with MCT among all tested dogs
# Total number of dogs with other skin tumors in a specific dog breed

Patnaik and Kiupel classification systems

One hundred and thirty-seven MCT cases were classified with both systems, 88 and 19 cases were classified only with one of them, Patnaik's and Kiupel's system, respectively. Out of the total, we had 225 cases classified with the Patnaik classification system and 156 with Kiupel classification system.

Breeds evaluation

The greatest number of MCTs in purebreds were diagnosed in Labrador Retrievers (27.46% of all cases diagnosed) followed by Boxers, Golden Retrievers, French Bulldogs, Pit Bulls and Pugs (ranging from 15.16% to 2.05% - **Table 3-A**). Mixed breed dogs were the second most common, when considering all breeds. Data on the frequencies of breeds according to Patnaik and Kiupel classification system were similar to the data from all MCTs and are presented in **Tables 3-B and 3-C**, respectively.

The highest predisposition for MCT (**Table 3-D**), in comparison to other cutaneous tumors development, was detected in Labrador Retrievers (OR= 2.063), Boxers (OR= 2.004), French Bulldogs (OR=3.071) and Pugs (OR=9.561). Cocker Spaniels (OR=0.186) also had a significant value thought for a decreased predisposition to MCT development.

Concerning breed predisposition in cases classified with Patnaik's histological grade (**Table 3-E**) or Kiupel's histological grade (**Table 3-F**), some breeds appear with an increased risk for MCT development when in the general analysis of MCTs (without classification discrimination – **Table 3-D**) did not appear at increased risk. Since both classification systems had different frequencies (n=225 for Patnaik's and n=156 for Kiupel's), this could be responsible for some discrepancies in the analysis. In the Patnaik's classification system (**Table 3-E**), Boxers had a higher predisposition to grade I and grade II (OR= 5.902 and OR=1,989, respectively). Two other breeds had an increased risk for grade II MCTs, namely Labrador Retrievers (OR=2.128) and Pug (OR=12.873). Cocker Spaniels had a decrease risk for developing grade II tumors (OR=0.155). French Bulldogs and Pit Bulls have a noted predisposition to grade III MCTs (OR= 7.878 and OR= 4.434, respectively). For Kiupel's (**Table 3-F**), in terms of low-grade MCTs development, Labrador Retrievers (OR=2.306), Boxers (OR=2.616), Pugs (OR=17.084) and Bull terriers (OR=12.694) presented higher risk. Furthermore, a predisposition to high-grade tumors was noted in Pit Bulls (OR=4.962).

Table 3-B: Frequency of MCTs in various breeds of dogs according to the Patnaik grading system.

BREEDS	ALL MCTS		PATNAIK						CONTROL GROUP #	
			GRADE I		GRADE II		GRADE III			
	NUMBER	%	NUMBER	%*	NUMBER	%**	NUMBER	%***	NUMBER	%
LABRADOR RETRIEVER	61	27.11	8	13.11	41	67.21	12	19.67	142	15.50
MIXED	59	26.22	4	6.78	38	64.41	17	28.81	335	36.57
BOXER	35	15.56	10	28.57	22	62.86	3	8.57	75	8.19
FRENCH BULLDOG	8	3.56	0	0.00	4	50.00	4	50.00	10	1.09
GOLDEN RETRIEVER	7	3.11	1	14.29	6	85.71	0	0.00	26	2.84
PIT BULL	7	3.11	1	14.29	3	42.86	3	42.86	13	1.42
PUG	5	2.22	0	0.00	4	80.00	1	20.00	2	0.22
GERMAN SHEPHERD	4	1.78	0	0.00	4	100.00	0	0.00	26	2.84
YORKSHIRE TERRIER	4	1.78	2	50.00	0	0.00	2	50.00	15	1.64
BEAGLE	3	1.33	0	0.00	3	100.00	0	0.00	12	1.31
BULL TERRIER	3	1.33	1	33.33	2	66.67	0	0.00	2	0.22
PINSCHER	3	1.33	0	0.00	3	100.00	0	0.00	7	0.76
POODLE	3	1.33	0	0.00	3	100.00	0	0.00	15	1.64
BOUVIER BERNOIS	2	0.89	0	0.00	1	50.00	1	50.00	3	0.33
BULLMASTIFF	2	0.89	0	0.00	2	100.00	0	0.00	5	0.55
CASTRO LABOREIRO DOG	2	0.89	0	0.00	1	50.00	1	50.00	0	0.00
ROTTWEILER	2	0.89	0	0.00	1	50.00	1	50.00	12	1.31
BELGIAN SHEPHERD	1	0.44	0	0.00	0	0.00	1	100.00	1	0.11
BOERBOEL	1	0.44	0	0.00	1	100.00	0	0.00	2	0.22
CANE CORSO	1	0.44	0	0.00	0	0.00	1	100.00	2	0.22
COCKER	1	0.44	0	0.00	0	0.00	1	100.00	13	1.42
COCKER SPANIEL	1	0.44	0	0.00	1	100.00	0	0.00	39	4.26
DOBERMAN	1	0.44	0	0.00	0	0.00	1	100.00	6	0.66
ENGLISH SETTER	1	0.44	0	0.00	1	100.00	0	0.00	1	0.11
ESTRELA MOUNTAIN DOG	1	0.44	0	0.00	1	100.00	0	0.00	9	0.98
FLAT COATED RETRIEVER	1	0.44	0	0.00	1	100.00	0	0.00	2	0.22
JACK RUSSELL TERRIER	1	0.44	1	100.00	0	0.00	0	0.00	3	0.33
POINTER	1	0.44	0	0.00	1	100.00	0	0.00	1	0.11
POMERANIAN DOG	1	0.44	0	0.00	1	100.00	0	0.00	0	0.00
RHODESIAN RIDGEBACK	1	0.44	0	0.00	0	0.00	1	100.00	1	0.11
SPITZ	1	0.44	0	0.00	1	100.00	0	0.00	2	0.22
WEIMARANER	1	0.44	1	100.00	0	0.00	0	0.00	1	0.11
OTHER BREEDS	0	0.00	-	-	-	-	-	-	133	14.52
TOTAL	225	19.21***	29	12.89	146	64.89	50	22.22	916	78.22

* Percentage of dogs with low-grade MCT in a specific dog breed

** Percentage of dogs with high-grade MCT in a specific dog breed

*** Percentage of dogs with low/high grade MCT among all tested dogs

Total number of dogs with other skin tumors in a specific dog breed

Table 3-C: Frequency of MCTs in various breeds of dogs according to the Kiupel grading system.

BREEDS	ALL MCTS		KIUPEL				CONTROL GROUP#	
			LOW GRADE		HIGH GRADE			
	NUMBER	%	NUMBER	%*	NUMBER	%*	NUMBER	%
LABRADOR RETRIEVER	42	26.92	33	78.57	9	21.43	142	15.50
MIXED	42	26.92	28	66.67	14	33.33	335	36.57
BOXER	25	16.03	21	84.00	4	16.00	75	8.19
FRENCH BULLDOG	5	3.21	3	60.00	2	40.00	10	1.09
PUG	5	3.21	4	80.00	1	20.00	2	0.22
GOLDEN RETRIEVER	4	2.56	3	75.00	2	50.00	26	2.84
BULL TERRIER	3	1.92	3	100.00	0	0.00	2	0.22
GERMAN SHEPHERD	3	1.92	3	100.00	0	0.00	26	2.84
PINSCHER	3	1.92	3	100.00	0	0.00	7	0.76
PIT BULL	3	1.92	0	0.00	3	100.00	13	1.42
POODLE	3	1.92	2	66.67	1	33.33	15	1.64
YORKSHIRE TERRIER	3	1.92	1	33.33	2	66.67	15	1.64
BULLMASTIFF	2	1.28	2	100.00	0	0.00	5	0.55
COCKER SPANIEL	2	1.28	1	50.00	1	50.00	39	4.26
BEAGLE	1	0.64	1	100.00	0	0.00	12	1.31
BOSTON TERRIER	1	0.64	0	0.00	1	100.00	0	0.00
BOUVIER BERNOIS	1	0.64	0	0.00	1	100.00	3	0.33
BULLDOG	1	0.64	0	0.00	1	100.00	2	0.22
DOBERMAN	1	0.64	0	0.00	1	100.00	6	0.66
ESTRELA MOUNTAIN DOG	1	0.64	1	100.00	0	0.00	9	0.98
FLAT COATED RETRIEVER	1	0.64	1	100.00	0	0.00	2	0.22
JACK RUSSELL TERRIER	1	0.64	1	100.00	0	0.00	3	0.33
POINTER	1	0.64	1	100.00	0	0.00	1	0.11
ROTTWEILER	1	0.64	0	0.00	1	100.00	12	1.31
SPITZ	1	0.64	1	100.00	0	0.00	2	0.22
OTHER BREEDS	0	0.00	0	0.00	0	0.00	129	14.08
TOTAL	156	14.55***	111	71.15	45	28.85	916	85.45

* Percentage of dogs with low-grade MCT in a specific dog breed

** Percentage of dogs with high-grade MCT in a specific dog breed

*** Percentage of dogs with low/high grade MCT among all tested dogs

Total number of dogs with other skin tumors in a specific dog breed

Table 3-D: Odds Ratios (ORs) and 95% confidence intervals (CIs) for MCT in various dog breeds. 2-sided fisher exact test

BREED	MAST CELL TUMOR (MCT)		
	ODDS RATIO (OR)	95% (CI)	p-VALUE
LABRADOR RETRIEVER	2.063	(1.479 - 2.879)	0.000[a]
BOXER	2.004	(1.314 - 3.057)	0.002[b]
GOLDEN RETRIEVER	1.463	(0.696 - 3.076)	0.303
FRENCH BULLDOG	3.071	(1.199 - 7.867)	0.035[b]
PIT BULL	2.052	(0.810 - 5.199)	0.161
PUG	9.561	(1.843 - 49.583)	0.006[b]
GERMAN SHEPHERD	0.571	(0.197 - 1.650)	0.369
YORKSHIRE TERRIER	1.001	(0.329 - 3.044)	1.000
BEAGLE	0.938	(0.263 - 3.350)	1.000
BULL TERRIER	5.689	(0.945 - 34.237)	0.066
PINSCHER	1.616	(0.415 - 6.298)	0.447
POODLE	0.748	(0.215 - 2.604)	0.779
BOUVIER BERNOIS	2.515	(0.418 - 15.137)	0.284
BULLMASTIFF	1.506	(0.290 - 7.809)	0.642
CASTRO LABOREIRO DOG			
COCKER SPANIEL	0.186	(0.045 - 0.775)	0.006[b]
ROTTWEILER	0.623	(0.138 - 2.800)	0.746
BELGIAN SHEPHERD	3.765	(0.235 - 60.417	0.377
BOERBOEL	1.881	(0.170 - 20.827)	0.508
BOSTON TERRIER			
BULLDOG	1.881	(0.170 - 20.827)	0.508
CANE CORSO	1.881	(0.170 - 20.827)	0.508
COCKER	0.286	(0.037 - 2.196)	0.323
DOBERMAN	0.624	(0.075 - 5.209)	1.000
ENGLISH SETTER	3.765	(0.235 - 60.417)	0.377
ESTRELA MOUNTAIN DOG	0.415	(0.052 - 3.289)	0.698
FLAT COATED RETRIEVER	1.881	(0.170 - 20.827)	0.508
JACK RUSSELL TERRIER	1.252	(0.130 - 12.093)	1.000
POINTER	3.765	(0.235 - 60.417)	0.377
POMERANIAN DOG			
RHODESIAN RIDGEBACK	3.765	(0.235 - 60.417)	0.377
SPITZ	1.881	(0.170 - 20.827)	0.508
WEIMARANER	3.765	(0.235 - 60.417)	0.377

* When no data is available, the control for that breed was 0 and no statistical analysis was performed.

[a] significant at p<0.001

[b] significant at p<0.05

Table 3-E: Odds Ratios (ORs) and 95% confidence intervals (CIs) for MCT in various dog breeds according to Patnaik's classification system. p-value (p) 2-sided fisher exact test.

BREED	MAST CELL TUMOR (MCT)		PATNAIK					
	OR (95% CI)	p	GRADE I		GRADE II		GRADE III	
			OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
LABRADOR RETRIEVER	2.027 (1.437 - 2.860)	0.000[a]	2.076 (0.902 - 4.780)	0.115	2.128 (1.423 - 3.184)	0.000[a]	1.721 (0.878 - 3.375)	0.114
BOXER	2.066 (1.342 - 3.179)	0.001[b]	5.902 (2.648 - 13.152)	0.000[a]	1.989 (1.193 - 3.317)	0.012[b]	0.716 (0.218 -2.355)	0.791
GOLDEN RETRIEVER	1.099 (0.471 - 2.566)	0.825	1.223 (0.160 - 9.331)	0.574	1.467 (0.593 - 3.628)	0.430		
FRENCH BULLDOG	3.340 (1.303- 8.563)	0.014[b]			2.552 (0.790 - 8.247)	0.114	7.878 (2.381 - 26.072)	0.004[b]
PIT BULL	2.230 (0.879 - 5.657)	0.091	2.481 (0.314 - 19.629)	0.356	1.457 (0.410 - 5.177)	0.473	4.434 (1.221 - 16.093)	0.045[b]
PUG	10.386 (2.002 - 53.888)	0.004[b]			12.873 (2.336 - 70.931)	0.004[b]	9.327 (0.831 - 104.633)	0.148
GERMAN SHEPHERD	0.620 (0.214 - 1.794)	0.489			0.964 (0.332 -2.804)	1.000		
YORKSHIRE TERRIER	1.087 (0.357- 3.308)	0.778	4.449 (0.969 - 20.430)	0.093			2.503 (0.556 - 11.258)	0.218
BEAGLE	1.018 (0.285 - 3.638)	1.000			1.580 (0.441 - 5.669)	0.448		
BULL TERRIER	6.176 (1.026 - 37.182)	0.055	16.321 (1.437 - 185.343)	0.089	6.347 (0.887 - 45.415)	0.093		
PINSCHER	1.755 (0.450 - 6.840)	0.424			2.724 (0.696 - 10.656)	0.148		
POODLE	0.812 (0.233 - 2.828)	1.000			1.260 (0.360 - 4.407)	0.727		
BOUVIER BERNOIS	2.729 (0.453 - 16.433)	0.257			2.099 (0.217 - 20.314)	0.447	6.211 (0.634 - 60.804)	0.192
BULLMASTIFF	1.634 (0.315 - 8.478)	0.630			2.531 (0.486 - 13.166)	0.248		
CASTRO LABOREIRO DOG								
COCKER SPANIEL	0.100 (0.014 - .735)	0.002[b]			0.155 (0.021 - 1.138)	0.033[b]		
ROTTWEILER	0.676 (0.150 - 3.040)	1.000			.520 (0.067 - 4.026)	1.000	1.537 (0.196 - 12.064)	0.501
BELGIAN SHEPHERD	4.085 (0.255 - 65.558)	0.356					18.673 (1.151 - 303.012)	0.101
BOERBOEL	2.040 (0.184 - 22.600)	0.483			3.152 (0.284 - 34.980)	0.359		
BOSTON TERRIER								
BULLDOG								
CANE CORSO	2.040 (0.184 - 22.600)	0.483					9.327 (0.831 - 104.633)	0.148
COCKER	0.310 (0.040 - 2.383)	0.326					1.418 (0.182 - 11.058)	0.527
DOBERMAN	0.677 (0.081 - 5.652)	1.000					3.095 (0.365 - 26.214)	0.311
ENGLISH SETTER	4.085 (0.255 - 65.558)	0.356			6.310 (0.393 - 101.446)	0.256		
ESTRELA MOUNTAIN DOG	0.450 (0.057 - 3.569)	0.697			0.695 (0.087 -5.527)	1.000		
FLAT COATED RETRIEVER	2.040 (0.184 -22.600)	0.483			3.152 (0.284 - 34.980)	0.359		
JACK RUSSELL TERRIER	1.359 (0.141 - 13.123)	0.585	10.869 (1.096 - 107.783)	0.117				
POINTER	4.085 (0.255 - 65.558)	0.356			6.310 (0.393 - 101.446)	0.256		
POMERANIAN DOG								
RHODESIAN RIDGEBACK	4.085 (0.255 - 65.558)	0.356					18.673 (1.151 - 303.012)	0.101
SPITZ	2.040 (0.184 - 22.600)	0.483			3.152 (0.284 - 34.980)	0.359		
WEIMARANER	4.085 (0.255 - 65.558)	0.356	32.679 (1.993 - 535.883)	0.060				

*When no data is available, one of the parcels for the statistical analysis for that breed was 0 and no statistical analysis was performed.

[a] significant at p<0.001
[b] significant at p<0.05

Table 3-F: Odds Ratios (ORs) and 95% confidence intervals (CIs) for MCT in various dog breeds according to Kiupel's classification system. p-value (p) 2-sided fisher exact test.

BREED	MAST CELL TUMOR (MCT)		KIUPEL			
	OR (95% CI)	p	LOW GRADE		HIGH GRADE	
			OR (95% CI)	p	OR (95% CI)	p
LABRADOR RETRIEVER	2.008 (1.351 - 2.985)	0.001[b]	2.306 (1.478 - 3.597)	0.000[a]	1.363 (0.642 - 2.891)	0.403
BOXER	2.140 (1.313 - 3.488)	0.004[b]	2.616 (1.539 - 4.447)	0.001[b]	1.094 (0.381 - 3.137)	0.782
GOLDEN RETRIEVER	0.901 (0.310 - 2.617)	1.000	0.951 (0.283 - 3.194)	1.000	1.592 (0.366 - 6.927)	0.381
FRENCH BULLDOG	3.000 (1.011 - 8.898)	0.054	2.517 (0.682 - 9.286)	0.158	4.214 (0.896 - 19.828)	0.105
PIT BULL	1.362 (0.384 - 4.835)	0.717			4.962 (1.362 - 18.077)	0.035[b]
PUG	15.132 (2.910 - 78.703)	0.001[b]	17.084 (3.093 - 94.377)	0.002[b]	10.386 (0.924 - 116.738)	0.134
GERMAN SHEPHERD	0.671 (0.201 - 2.245)	0.788	0.951 (0.283 - 3.194)	1.000		
YORKSHIRE TERRIER	1.178 (0.337 - 4.117)	0.737	0.546 (0.071 - 4.174)	1.000	2.794 (0.619 - 12.606)	0.187
BEAGLE	0.486 (0.063 - 3.764)	0.705	0.685 (0.088 - 5.318)	1.000		
BULL TERRIER	8.961 (1.485 - 54.067)	0.024[b]	12.694 (2.098 - 76.818)	0.010[b]		
PINSCHER	2.546 (0.651 -9.953)	0.167	2.383 (0.489 - 11.614)	0.253		
POODLE	1.178 (0.337 - 4.117)	0.737	1.102 (0.249 - 4.884)	0.705	1.365 (0.176 - 10.570)	0.539
BOUVIER BERNOIS	1.963 (0.203 - 18.997)	0.467			6.917 (0.705 - 67.846)	0.175
BULLMASTIFF	2.366 (0.455 - 12.305)	0.271	3.343 (0.641 -17.439)	0.170		
COCKER SPANIEL	0.292 (0.070 - 1.222)	0.109	0.204 (0.028 - 1.503)	0.115	0.511 (0.069 - 3.806)	1.000
ROTTWEILER	0.486 (0.063 - 3.764)	0.705			1.712 (0.218 - 13.464)	0.466
BOSTON TERRIER						
BULLDOG	2.948 (0.266 - 32.712)	0.376			10.386 (0.924 - 116.738)	0.134
DOBERMAN	0.978 (0.117 - 8.183)	1.000			3.447 (0.406 - 29.254)	0.286
ESTRELA MOUNTAIN DOG	0.650 (0.082 - 5.168)	1.000	0.916 (0.115 - 7.300)	1.000		
FLAT COATED RETRIEVER	2.948 (0.266 - 32.712)	0.376	4.155 (0.374 - 46.191)	0.291		
JACK RUSSELL TERRIER	1.963 (0.203 - 18.997)	0.467	2.767 (0.285 - 26.828)	0.368		
POINTER	5.903 (0.367 - 94.872)	0.270	8.318 (0.517 - 133.928)	0.205		
SPITZ	2.948 (0.266 - 32.712)	0.376	4.155 (0.374 - 46.191)	0.291		

*When no data is available, one of the parcels for the statistical analysis for that breed was 0 and no statistical analysis was performed.

[a] significant at p<0.001

[b] significant at p<0.05

Anatomical distribution evaluation

In terms of anatomical distribution, the greatest numbers of MCTs were noted in the limbs (29.10%) and they were dominated by Patnaik grade II and Kiupel low grade tumors (74.63% and 68.89%, respectively), followed by trunk (27.05%), multicentric growth (12.30%) and head and neck (10.66%). All these three locations had the highest

frequency on grade II (57.91%, 76.92% and 42.11%, respectively) and low-grade tumors (68.18%,75.00% and 70.00%, respectively) (**Tables 3-G, 3-H** and **3-I**). The highest frequency for the grade III and high-grade tumors were for buttock area (66.67% and 100,00%, respectively), perigenital area (50.00% in grade III) and tail (50.00% in high-grade) (**Tables 3-H** and **3-I**).

The perigenital area was considered of significant highest risk for cutaneous MCT development (OR=2.474) (**Table 3-J**). In contrast, buttock area presented a decreased risk for developing these neoplasms (OR=0.128). In the analysis of the lesions distribution while classified according with Patnaik system, the perigenital area exhibits a substantially greater risk of being affected with a high grading (grade III) lesion (OR=6.615) (**Table 3-K**). Another location with high OR and high risk for grade III was the trunk (OR=1.868). In turn, the limbs were found to be the region with the highest risk for grade II tumor development (OR=1.648). Buttock area, head and neck presented a decrease risk for grade II tumors (OR=0.071 and OR=0.396, respectively). Based on Kiupel classification, MCTs distribution amongst the different anatomical regions did not displayed significant values (**Table 3-L**).

Table 3-G: Frequency of MCT grades by tumor location

Anatomical distribution	All MCTs		Control group	
	N	%	N	%
Limbs	71	29.10	220	24.02
Trunk	66	27.05	212	23.14
Multicentric	30	12.30	140	15.28
Head and Neck	26	10.66	117	12.77
NOS	23	9.43	81	8.84
Perigenital area	14	5.74	22	2.40
Ear	8	3.28	29	3.17
Buttock area	3	1.23	81	8.84
Tail	3	1.23	14	1.53
Total	244	100	916	100

Table 3-H: Frequency of MCT according to Patnaik classification system by tumor location

Anatomical distribution	All MCTs		Patnaik grade						Control group	
			Grade I		Grade II		Grade III			
	N	%	N	%	N	%	N	%	N	%
Limbs	67	28.85	7	10.45	50	74.63	10	14.93	220	24.02
Trunk	64	28.21	9	14.06	37	57.81	18	28.13	212	23.14
Multicentric	26	12.82	2	7.69	20	76.92	4	15.38	140	15.28
NOS	23	12.82	4	17.39	17	73.91	2	8.70	81	8.84
Head and Neck	19	7.69	5	26.32	8	42.11	6	31.58	117	12.77
Perigenital area	14	4.49	0	0.00	7	50.00	7	50.00	22	2.40
Ear	7	2.56	1	14.29	5	71.43	1	14.29	29	3.17
Buttock area	3	1.28	0	0.00	1	33.33	2	66.67	81	8.84
Tail	2	1.28	1	50.00	1	50.00	0	0.00	14	1.53
Total	225	100	29	12.89	146	64.89	50	22.22	916	100

Table 3-I: Frequency of MCT according to Kiupel classification system by tumor location

Anatomical distribution	All MCTs		Kiupel grade				Control group	
			Low grade		High grade			
	N	%	N	%	N	%	N	%
Limbs	45	28.85	31	68.89	14	31.11	220	24.02
Trunk	44	28.21	30	68.18	14	31.82	212	23.14
Head and Neck	20	12.82	14	70.00	6	30.00	117	12.77
Multicentric	20	12.82	15	75.00	5	25.00	140	15.28
NOS	12	7.69	11	91.67	1	8.33	81	8.84
Ear	7	4.49	5	71.43	2	28.57	29	3.17
Perigenital area	4	2.56	4	100.00	0	0.00	22	2.40
Buttock area	2	1.28	0	0.00	2	100.00	81	8.84
Tail	2	1.28	1	50.00	1	50.00	14	1.53
Total	156	100	111	71.15	45	28.85	916	100

Table 3-J: Odds Ratios (ORs) and 95% confidence intervals (CIs) for MCT in various anatomical locations. 2-sided fisher exact test

ANATOMICAL DISTRIBUTION	MAST CELL TUMOR (MCT)	
	OR (95% CI)	p-VALUE
BUTTOCK AREA	0.128 (0.040 - 0.410)	0.000[a]
EAR	1.037 (0.468 - 2.298)	1.000
HEAD AND NECK	0.814 (0.519 - 1.278)	0.443
LIMBS	1.298 (0.947 - 1.779)	0.114
MULTICENTRIC	0.777 (0.509 - 1.185)	0.263
PERIGENITAL AREA	2.474 (1.246 - 4.910)	0.012[b]
TAIL	0.802 (0.229 - 2.813)	1.000
TRUNK	1.231 (0.893 - 1.698)	0.206

[a] significant at p<0.001
[b] significant at p<0.05

Table 3-K: Odds Ratios (ORs) and 95% confidence intervals (CIs) for MCT in various anatomical locations according to Patnaik's classification system. p-value (p). 2-sided fisher exact test.

ANATOMICAL DISTRIBUTION	MAST CELL TUMOR (MCT)		PATNAIK					
	OR (95% CI)	p	Grade I		Grade II		Grade III	
			OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
BUTTOCK AREA	0.139 (0.044 – 0.445)	0.000[a]	-	-	0.071 (0.010 - 0.515)	0.000[a]	0.430 (0.103 1.800)	0.306
EAR	0.982 (0.425 - 2.272)	1.000	1.092 (0.144 - 8.306)	0.613	1.085 (0.413 - 2.849)	0.802	0.624 (0.083 - 4.678)	1.000
HEAD AND NECK	0.630 (0.379 - 1.047)	0.084	1.423 (0.532 - 3.802)	0.409	0.396 (0.189- 0.829)	0.008[b]	0.931 (0.388 - 2.233)	1.000
LIMBS	1.342 (0.971 - 1.854)	0.086	1.007 (0.424 - 2.388)	1.000	1.648 (1.134 - 2.395)	0.010[b]	0.791 (0.389 - 1.608)	0.611
MULTICENTRIC	0.724 (0.463 - 1.132)	0.171	0.411 (0.097 - 1.746)	0.294	0.880 (0.531 - 1.458)	0.709	0.482 (0.171 - 1.360)	0.219
PERIGENITAL AREA	2.696 (1.357 - 5.358)	0.009[b]	-	-	2.046 (0.858 - 4.880)	0.104	6.615 (2.679 - 16.334)	0.000[a]
TAIL	0.578 (0.130 – 2.561)	0.751	2.301 (0.292 - 18.114)	0.376	0.444 (0.058 - 3.405)	0.708	-	-
TRUNK	1.320 (0.951 – 1.832)	0.099	1.494 (0.670 - 3.331)	0.371	1.127 (0.753 - 1.687)	0.599	1.868 (1.028 - 3.395)	0.042[b]

[a] significant at p<0.001
[b] significant at p<0.05

Table 3-L: Odds Ratios (ORs) and 95% confidence intervals (CIs) for MCT in various anatomical locations according to Kiupel classification system. p-value (p). 2-sided fisher exact test

ANATOMICAL DISTRIBUTION	MAST CELL TUMOR (MCT)		KIUPEL			
	OR (95% CI)	p	LOW GRADE		HIGH GRADE	
			OR (95% CI)	p	OR (95% CI)	p
BUTTOCK AREA	0.134 (0.033 - 0.550)	0.000[a]	-	-	0.479 (0.114 - 2.016)	0.420
EAR	1.437 (0.618 - 3.340)	0.467	1.443 (0.547 - 3.807)	0.403	1.601 (0.370 - 6.927)	0.377
HEAD AND NECK	1.004 (0.604 – 1.669)	1.000	0.986 (0.545 - 1.783)	1.000	1.197 (0.496 - 2.887)	0.634
LIMBS	1.283 (0.879 - 1.872)	0.194	1.226 (0.788 - 1.906)	0.352	1.657 (0.866 - 3.168)	0.139
MULTICENTRIC	0.815 (0.493 - 1.348)	0.468	0.866 (0.488 - 1.536)	0.779	0.792 (0.307 - 2.041)	0.824
PERIGENITAL AREA	1.069 (0.363 -3.146)	0.783	1.519 (0.514 - 4.491)	0.515	-	-
TAIL	0.837 (0.188 – 3.718)	1.000	0.586 (0.076 - 4.497)	1.000	1.644 (0.211 - 12.788)	0.477
TRUNK	1.305 (0.891 – 1.910)	0.187	1.230 (0.787 - 1.921)	0.407	1.736 (0.907 - 3.322)	0.095

[a] significant at p<0.001

[b] significant at p<0.05

Sex and age-group evaluation

Data representing the frequency of MCTs in the analysed canine population in relation to the dog's sex and the four age groups established are shown in **Table 3-M**. The frequency of MCTs is higher in the older age groups (7-10 years and 11-18 years, accounting for 40.98% and 26.23%, respectively). In terms of the frequency distribution in the classification systems, for Patnaik's system (**Table 3-N**) in grade I, II and III majority of the frequencies were in the 2 oldest groups (7-10 years and 11-18 years) and in Kiupel's system for low-grade and high-grade the same distribution occurs (**Table 3-O**). All age groups had majority of cases in grade II or low-grade tumors (**Tables 3-N and 3-O**). Females dogs (54.51%) were most affected than males (45.49%). As shown in **Tables 3-N and 3-O** even when analysing female and male dogs' distribution within the histological gradings systems the same tendency was seen.

For the age group risk evaluation, an analysis comparing the groups with the youngest dog group (0-3 years) was performed however, no significant outcome was noted. Though since most neoplastic diseases tend to occur later in life, a risk analysis was made comparing the groups with the oldest dog group in this dog population, hypothesizing that this group already had a risk for developing this tumors but did not correspond to the group with the majority of the diagnose cases in general which is between 7.5 and 9 years old. In our hypothesis the group that had the ages that usually are the most commonly diagnosed would be at increased risk and as already mention that neoplastic diseases ten to occur in older dogs, the younger group would not be at increased risk. In this analysis, our hypothesis was partially confirmed, since an

increased risk of MCT development was seen in the older dogs 4-6 years and 7-10 years compared to that in the oldest group 11-18 years, however the group of dogs with 4 to 6 years had a higher risk than the group of 7 to 10 years old (OR= 2,299 and OR=1,471, respectively) (**Table 3-P**). There was no significant value for the youngest group of less than 3 years old. In the Patnaik distribution for grade II tumors, the older groups (4-6 years and 7-10 years) had the highest risk (OR=2,680 and OR=1,629, respectively) (**Table 3-Q**) For the Kiupel distribution in the low-grade tumors there was a higher risk for the group between 4 and 6 years old (OR=2,647) (**Table 3-R**). There was not noted risk for gender.

Table 3-M: Frequency of MCT grade by age and sex.

Variables		All MCTs		Control group	
		N	%	N	%
Age (years)	0-3	16	6.56	58	6.33
	4-6	46	18.85	106	11.57
	7-10	100	40.98	360	39.30
	11-18	64	26.23	339	37.01
	UN	18	7.38	53	5.79
	M ± SD	8.46 ± 3.141		9.36 ± 3.526	
Total		244	100.00	916	100.00
Sex	Female	133	54.51	460	50.22
	Male	111	45.49	456	49.78
Total		244	100.00	916	100.00

UN, unknown

Table 3-N: Frequency of MCT according to Patnaik classification by age and sex

Variables		All MCTs		Patnaik grade						Control group	
				Grade I		Grade II		Grade III			
		N	%	N	%	N	%	N	%	N	%
Age (years)	0-3	15	6.67	2	6.90	7	4.79	6	12.00	58	6.33
	4-6	42	18.67	4	13.79	31	21.23	7	14.00	106	11.57
	7-10	95	42.22	10	34.48	64	43.84	21	42.00	360	39.30
	11-18	59	26.22	9	31.03	37	25.34	13	26.00	339	37.01
	UN	14	6.22	4	13.79	7	4.79	3	6.00	53	5.79
	M ± SD	8.47 ± 3.132		8.92 ± 3.108		8.38 ± 3.103		8.49 ± 3.276		9.36 ± 3.526	
Total		225	100.00	29	12.89	146	64.89	50	22.22	916	100.00
Sex	Female	126	56.00	15	51.72	85	58.22	26	52.00	460	50.22
	Male	99	44.00	14	48.28	61	41.78	24	48.00	456	49.78
Total		225	100.00	29	12.89	146	64.89	50	22.22	916	100.00

UN, unknown

Table 3-O: Frequency of MCT according to Kiupel classification by age and sex

Variables		All MCTs		Kiupel grade				Control group	
				Low grade		High grade			
		N	%	N	%	N	%	N	%
Age (years)	0-3	7	4.49	4	3.60	3	6.67	58	6.33
	4-6	32	20.51	24	21.62	8	17.78	106	11.57
	7-10	64	41.03	46	41.44	18	40.00	360	39.30
	11-18	41	26.28	29	26.13	12	26.67	339	37.01
	UN	12	7.69	8	7.21	4	8.89	53	5.79
	M ± SD	8.51 ± 3.066		8.47 ± 2.957		8.63 ± 3.360		9.36 ± 3.526	
Total		156	100.00	111	71.15	45	28.85	916	100.00
Sex	Female	85	54.49	62	55.86	23	51.11	460	50.22
	Male	71	45.51	49	44.14	22	48.89	456	49.78
Total		156	100.00	111	71.15	45	28.85	916	100.00

UN, unknown

Table 3-P: Odds Ratios (ORs) and 95% confidence intervals (CIs) for MCT for age and sex. 1-sided fisher exact test used for age and 2-sided fisher exact test for sex.

VARIABLES		MAST CELL TUMOR (MCT)	
		OR (95% CI)	p-VALUE
AGE (YEARS)	0-3	1.461 (0.790 - 2.702)	0.148
	4-6	2.299 (1.485 - 3.559)	0.000[a]
	7-10	1.471 (1.040 - 2.082)	0.018[b]
	11-18	1	-
SEX	Female	1.188 (0.895 - 1.577)	0.249
	Male	1	-

[a] significant at p<0.001

[b] significant at p<0.05

**For the age variable a different p-value was used since we had proposed a hypothesis that the oldest groups, especially the group of 7-11-years old would have a significant higher risk*

Table 3-Q: Odds Ratios (ORs) and 95% confidence intervals (CIs) for MCT for age and sex according to Patnaik classification system. p-value (p). 2-sided fisher exact test used.

VARIABLES		MAST CELL TUMOR (MCT)		PATNAIK					
		OR (95% CI)	p	Grade I		Grade II		Grade III	
				OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
AGE (YEARS)	0-3	1.486 (0.790 - 2.794)	0.223	1.299 (0.274 - 6.164)	0.669	1.106 (0.471 - 2.599)	0.823	2.698 (0.986 - 7.382)	0.094
	4-6	2.277 (1.449 - 3.577)	0.000[a]	1.421 (0.429 - 4.709)	0.522	2.680 (1.586 - 4.528)	0.000[a]	1.722 (0.670 - 4.428)	0.286
	7-10	1.516 (1.061 - 2.167)	0.026[b]	1.046 (0.420 - 2.606)	1.000	1.629 (1.058 - 2.507)	0.033[b]	1.521 (0.750 - 3.086)	0.293
	11-18	1	-	1	-	1	-	1	-
	M ± SD								
SEX	Female	1.262 (0.941 - 1.692)	0.136	1.062 (0.507 - 2.226)	1.000	1.381 (0.970 - 1.967)	0.075	1.074 (0.607 - 1.898)	0.885
	Male	1	-	1	-	1	-	1	-

[a] significant at p<0.001

[b] significant at p<0.05

Table 3-R: Odds Ratios (ORs) and 95% confidence intervals (CIs) for MCT for age and sex according to Kiupel classification system. p-value (p). 2-sided fisher exact test used.

VARIABLES		MAST CELL TUMOR (MCT)		KIUPEL			
		OR (95% CI)	p	LOW GRADE		HIGH GRADE	
				OR (95% CI)	p	OR (95% CI)	p
AGE (YEARS)	0-3	0.998 (0.427 - 2.331)	1.000	0.806 (0.273 - 2.378)	1.000	1.461 (0.400 - 5.337)	0.474
	4-6	2.496 (1.497 - 4.162)	0.001[b]	2.647 (1.477 - 4.742)	0.001[b]	2.132 (0.849 - 5.354)	0.113
	7-10	1.470 (0.967 - 2.235)	0.075	1.494 (0.917 - 2.433)	0.115	1.412 (0.670 - 2.976)	0.456
	11-18	1	-	1	-	1	-
	M ± SD						
SEX	Female	1.187 (0.844 -1.669)	0.341	1.254 (0.844 - 1.864)	0.271	1.036 (0.570 - 1.886)	1.000
	Male	1	-	1	-	1	-

[a] significant at p<0.001

[b] significant at p<0.05

Discussion

Mast cell tumors accounted for 21.03% of all examined skin tumors which corresponds to the frequency found in other studies (7-21%).[14,50,52] The results of this study indicated an increased risk of MCT development in four breeds: Labrador Retriever, Boxer, French Bulldog and Pug (**Table 3-D**). The results were similar to those found in other investigations, namely in the UK (where the highest risk was predicted for Boxers, Labrador Retrievers, Golden Retrievers and Staffordshire Bull Terriers) and in

Poland (Shar-pei, American Staffordshire Terrier, Labrador Retriever, French Bulldog and Boxer).[46,51] Variances in MCTs incidence amongst breeds can be linked to the geographical region where the study was performed, breed preferences or popularity in that geographical area and also to the choice of the control group, since in some investigations it comprised insured populations,[10,51] registration in kennel associations[27,51], or hospitalized dogs.[50,51,53] In this study the control group comprised dogs with skin tumors, free of cutaneous MCTs.

In contrast to previous results, herein Golden Retrievers were not found to have a significant association with MCT occurrence, highlighting possible differences amongst the study populations. However, regardless of all the putative variations factors previously mentioned, an overall increase risk of MCTs development in Boxers has been consistently noted[27,42,50,51,53], and it was also encountered in this study corroborate. Additionally, the statistical analysis also revealed an increases risk in French Bulldogs. In the literature, there is a hypothesis that Boxers and French Bulldogs may be genetically related, sharing a common ancestor in their phylogenetic evolution which can possibly explain these specific breed susceptibilities to the development of this particular tumor histotype. Nevertheless, further studies are needed to unveil this particular association.[12,36] Predisposition for MCTs development in Labrador Retrievers has been shown in previously studies.[51,53] These observations were also confirmed in this study with this breed presenting increased risk for Kiupel low grade tumors and for Patnaik grade II tumors, as already suggested by others[46]. However, some reports indicate a higher risk for more aggressive tumors, which was not shown here (though a side note that grade II tumors have variations in behavior and can behave more like a grade I tumors or behave more aggressively like grade III). Low levels of 25(OH)D_3, serum vitamin D, may be a risk factor for MCTs in this breed, as recently proposed.[20]

Boxers and Pugs are characterized by higher susceptibility for developing low grade MCTs as seen in multiple available published data.[5,12,28,29,32] Our analysis confirmed these findings and demonstrated an increased risk for lower grade tumors, in both classification systems. Boxers depicted an increased risk for Kiupel low grade tumors and for Patnaik grade I and II lesions. In Pugs there was an increased risk to Kiupel's low grade lesions and an increased risk for Patnaik grade II lesions, however there was no data available in our series for grade I analysis in this specific breed. French Bulldogs had an increased risk for developing this specific tumor which goes towards previous observations.[46,45] However, when considering the histologic grading risk analysis, only in Patnaik's classification system an increased risk for overall tumor development and for grade III tumors development was achieved. These findings clearly

contrast with those publicized that claimed that this breed presents an increased risk for low grade MCTs[46,43,45]

Moreover, Bull Terrier and Pit Bull, although did not reveal an increased risk for overall MCT development, in the analysis performed for each classification system presented significant results. Bull Terriers, within Kiupel's classification system displayed an increases risk for MCT development, revealing a higher risk for low grade tumors occurrence; while, within Patnaik system, this breed also had an increased risk but no significant results were seen in the grading distribution. For Pit bulls, in both classification systems, even though with no increased risk noted in overall MCT development, an increased risk for high grade and Grade III tumors was encountered. Both Pit bulls and Bull Terriers are a result of a cross between Bulldog and Terrier[9] thus, probably there are underlying genetic factors that can play a role further increasing the risk of these breeds to this disease, as well as other related breeds such as Staffordshire bull terrier. As indicated in literature, MCTs with a different presentation occur in dog breeds that are phylogenetically related with Bulldog; for instance, Boxers have a predisposition to lower grade tumors as seen in several studies and American Staffordshire Terrier may be at increased risk for high grade tumors[47] and again, both breeds are hypothesized to descend from Bulldogs and Terriers. Thus, further studies are needed for a better comprehension of the possible common genetic background of some breeds and the development of some diseases.[12,32,36] Discrepancies between both classification systems risk results can be partially explained by the frequencies variations of these specific breeds in each system. Other processes adding up to the genetic influence compound may be relevant for MCTs carcinogenesis and can be responsible for the biological behavior of tumors in a specific breed. Investigations of mitochondrial DNA conducted in recent years have demonstrated somatic mutations in the mitochondrial DNA D-loop in MCTs, which may also be associated with neoplastic transformation.[45] The current analysis also demonstrated a decreased risk of mast cell tumor development in one breed, Cocker Spaniel (**Table3-D**), which is coherent with literature.[42,50,51]

Even though mast cell tumors can be found in any segment of the body, several researches hypothesized a potential prognostic significance for several sites, i.e. digit, scrotal, inguinal, or perianal.[11,52] A particular study showed that tumor anatomical location could be correlated to a better or worse prognosis.[37] Nevertheless, MCTs can be frequently found on the trunk (50% to 60%), limbs (25% to 40%), and head and neck (10%).[52] The current investigation demonstrated correlations between the anatomical distribution and the development of MCTs. The statistical analysis demonstrated that the perigenital area had the highest risk of MCTs development of all the locations

considered. In terms of risk associated with histological grading, in the perigenital area, according with the Kiupel's system, all the cases were classified as low grade and no increasing risk was detected. However, with Patnaik's classification, this same location revealed higher risk for developing grade III lesions (OR = 6.615). The analysis of all MCTs demonstrated a decreased risk for the buttock area, although this location presented low number of representative cases. Herein, a high risk for higher grade lesions for the perigenital area was found which goes towards the available literature when report the inguinal, scrotal, and perianal areas, as well as the mucocutaneous junctions, as anatomical sites associated with a worse prognosis.[4,11,31,41,52] However, a less favourable prognosis is also related to the surgical procedure approach which can lead to an incomplete tumor resection.[4,33]

With the Patnaik's system, an increase risk for grade III tumors in the trunk (OR = 1,868) was noted. As described in the literature there is a tendency for the development of high-grade MCTs in the inguinal and axillary regions which in our analysis were comprised in the trunk area. These results are in accordance with some authors that described a predisposition for tumors of higher grade in this location[44,46] However, putative causative factors for this occurrence should be investigated. For Patnaik grade II tumors: an increased risk was detected in the limbs and a decreased risk was found in the buttock area, head and neck. With this regard, the publicized data remains controversial. In some studies[46] the thoracic limb presented a higher risk of developing high grade tumors, while in others, this region presented a higher probability of grade I tumors[44] occurrence. A completely different distribution was shown in a research performed in dogs from Croatia by Grabarević *et al*.[17]: grade-III tumors were found primarily on the pelvic limb and neck. Other findings showed a decreased risk for Kiupel low grade and high grade tumors in the head, and a higher risk for either high grade and low grade tumor in the anus.[46] On the other hand, the head and neck were reported to display a higher probability of being affected by Patnaik grade I tumors[44]. According to the literature, results can be very variable and our findings concerning risk for Patnaik grade II tumors do not fit in those publicated, however this specific histotype can be very unpredictable in terms of behavior thus, being able to exhibit either more aggressive or less aggressive features. These differences in several studies can be explained by the methodological approaches established, like the different anatomical division of the body regions, the simplification in the use of colloquial names or merging some body areas into just one region.[44] For a more thorough and viable analysis of the MCTs anatomical distribution prediction, a universal partition of the animal body should be used.

Mast cell tumors development in dogs can happen at all ages, however the majority of cases are diagnosed amongst 7.5 and 9 years of age.[11,31,34,52] Currently, a higher risk of MCT development in adult dogs aged 4–6 (OR = 2.299) and 7–10 years (OR = 1,471) was identified in comparison with the control group previously defined and comprised of older dogs (11-18 years old) (**Table 3-P**). The analysis revealed that the risk of MCT development increased with age, since in the youngest group there was no relevant findings and for dogs older than 4 years old, the risk increased significatively, reenforcing previous observations. Nevertheless, in this study the highest risk was in the age group of 4 to 6 years-old whereas in other studies, the greatest risk affected dogs older than 7 years. Shoop *et al*.[42] observed a 41-fold higher risk of MCT development in 10-year-old dogs, although the control group used was composed of 2-years old dogs. Additionally, Villamil *et al.*[50] noted a higher incidence of MCTs in dogs older than 7 years. In the present investigation, the statistical analyses revealed a few correlations amongst dog's age and the malignancy grade of MCTs. Similarly to the findings of Śmiech *et al*.[46], dogs aged 4–6 years presented higher risk for Kiupel low-grade tumor occurrence (**Table 3-R**). Yet, concerning the Patnaik classification, the age groups of 4-6 and 7-10 years had an increased risk for grade II tumors, but there was a decreased risk from the 4-6-year-old group to the 7-10-year-old group (**Table 3-Q**). In most previous epidemiological studies, no correlations were exhibited between age and the risk of different histological grade for mast cell tumors. These results can vary since the proportions of the different age groups differ from studies.

Regarding the risk of development of MCTs in females and males there are many discrepancies in the veterinary literature although the great majority claim that there is no association.[11,30,37,42,52] The present results confirmed the absence of this association. However, some published statistics suggest that castration and sterilization increase MCT development risk [26,32,53,56], underlying a possible role of sex hormones in their oncogenesis. As the reproductive status of the target canine population under study was not identified, the conclusions herein obtained cannot be generalized or properly interpreted.

Notwithstanding the advancement in the expansion of MCTs treatment procedures and prognostic aspects, the aetiology of this disease has not been yet entirely clarified.[4,21,22,24,23,43] This study demonstrated a relationships between histological grading system and clinicopathological characteristics, such as age, and location of canine MCT, validating the intricate and complex biological nature of this tumor. Retrospective studies conducted in large animal populations present a valuable contribution to the clinical nature of MCTs knowledge. Information obtained in the present

study can be used for the prediction or to determine the impact of several risk factors in breeds that are predisposed to the development of MCTs. The variations noted in the clinical presentation of MCTs amongst predisposed dog breeds reinforces the relevance of the genetic background in MCTs carcinogenesis. Must be highlighted that modern-day dog breeds were designed via selection of particular phenotypical characteristics and inbreeding decreases genetic variation and ends in the development of numerous hereditary disorders, which may include neoplasia.

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CHAPTER 4

Ki67 index and KIT immunoexpression

Chapter 4 - Ki67 index and KIT immunoexpression

The correlation between Ki67 and KIT immunoexpression with other prognostic factors in canine mast cell tumors.

Introduction

Mast cell tumors (MCTs) are one of the most frequently diagnosed cutaneous tumors in dogs accounting for 7% to 21% of all occurrences.[9,33] The biological behavior of canine MCTs can broadly vary and is frequently challenging to predict.[8] Histological grading[11,19] is commonly applied for MCTs prognosis analysis. Other prognostic components have been proposed, proliferation markers such as Ki67 (MIB-1) nuclear antigen labelling index and molecular marker, KIT immunoexpression.[1,2,13,16,21,23,26]

Ki-67 is a more sensitive proliferation marker in comparison to others since this is a nuclear protein that is expressed in all phases of the cell cycle however, it is not expressed by noncycling cells[6,24] like those that are main in the resting phase (i.e., G0). It is, hence, a marker of the growth fraction. The relative number of Ki67- positive cells in a certain tissue is used to determine the proliferation index or the relative number of cells actively involved in the cell cycle (growth fraction).[5,6,14,17,24] A standardized method for determining the Ki67 index has been based on the use of an ocular grid and, in this process, an average of more than 23 Ki-67–positive cells per grid area is associated with shorter survival times, MCT-related mortality[30] and an increased in disease progression and risk of systemic disease. On the other hand, MCTs with lower ki-67 index indicate that these lesions are highly unlikely to recur, even if their surgical excision has been incomplete. In fact, only a small percentage of them may present local recurrence (about 11%). When using a chromogen to label Ki67-positive cells, microscopic examination of slides allows a much easier identification of the areas with the highest index and, therefore, a higher consistency among pathologists.

KIT is a vital transmembrane receptor tyrosine kynase for several cell types, such as some hematopoietic stem cells, mast cells, melanocytes, and germ cells. Binding of SCF by CD117 leads to receptor dimerization and activation of its tyrosine kynase activity.[22] Several signal transduction pathways, like the PI3-kinase and the RAS/Erk pathways have been connected in facilitating KIT functions in mast cells, including survival, cellular proliferation and differentiation, resistance to apoptosis, migration, mobility and chemotaxis, adhesion to fibronectin and enhancement of serotonin and histamine release.[25,27,29] With immunohistochemistry (IHC) it is possible to detect an

aberrant expression of KIT protein that has been shown to be a negative prognostic indicator for canine cutaneous MCTs.[12] KIT is encoded by the proto-oncogene *c-kit*[22] and a correlation between aberrant KIT localization and some activating mutations in this gene were described.[31]

In terms of the immunoexpression of KIT protein, different subcellular expression patterns can be found, precisely pattern I, II and III.[7,16,21,32,31] Pattern I, namely membrane-associated, consists in perimembranous labelling of 90% of neoplastic cells, commonly found in well differentiated MCTs and it is not related to an aggressive biological behavior.[12] A KIT expression with pattern II (paranuclear or Golgi-like) is characterized by focal perinuclear or stippled cytoplasmic labelling and loss of perimembranous labelling in at least 10% of neoplastic cells. Finally, diffuse cytoplasmic labelling in at least 10% of neoplastic cells is consistent with pattern III. The membrane-associated pattern is observed in normal mast cells and the presence of cytoplasmic c-kit immunoexpression correlates with reduced post-surgical survival and increased incidence of local recurrence, higher histological grade and increased cell proliferation.[3,7,12,20]

This work aims to study the role of Ki-67 index and KIT immunoexpression in canine MCTs by analysing the correlations between one proliferation marker (Ki-67), one molecular marker (KIT immunoexpression patterns), histological grading, and other clinicopathological parameters exhibited by the patients.

Materials and Methods

Sample

This study included 35 cases of canine mast cell tumors admitted to Laboratory of Veterinary Pathology from Institute of Biomedical Sciences Abel Salazar, University of Porto for histopathological evaluation and grading. Within this group, cases previously diagnosed as cutaneous MCTs were selected and clinicopathological data (breed, age, sex, anatomical location) from each patient was registered. All the cases were revised and subclassified according to the three-grade malignancy scale of Patnaik *et al*[19] and the two-grade malignancy scale of Kiupel *et al*.[11] Two age groups were distinguished: (1) dogs with less than 8 years-old, (2) older than 8 years-old. Additionally, three anatomical locations groups were established: (1) head and neck, (2) limbs and (3) trunk.

Immunohistochemistry technique

Immunohistochemistry was performed using the Novolink™ Max-Polymer detection system (Novocastra, Newcastle, UK), according with the manufacturer's instructions and employing the following monoclonal antisera: c-KIT (polyclonal, Dako, Glostrup, Denmark) was diluted 1:450 and KI-67 (MIB-1, Dako, Glostrup, Denmark) was diluted 1:50. Sections were rinsed with TBS between each step of the procedure. Labeling was 'visualized' by incubation of the sections with a prepared solution of 3, 3'-diamino-benzidine (DAB) for up to 5 min at room temperature. Finally, sections were lightly counterstained with hematoxylin, dehydrated and mounted.

For positive controls of c-KIT and KI-67, representative sections of canine gastrointestinal stromal tumor and a high-grade canine lymphoma, respectively, were used. For the negative controls, the primary antibody was omitted and replaced by TBS.

Immunohistochemistry evaluation

KIT and Ki67 immunohistochemical labelling was evaluated independently by three pathologists, using methods previously described.[12,30]

For Ki67, all cell counting was performed manually. Areas with the highest proportion of immunopositive neoplastic mast cells were identified at higher magnification using a light microscope. Upon identification of highly proliferative areas, the number of immunopositive cells present in a 10×10mm grid area was counted. The number of immunopositive cells per grid area was evaluated over 5 high-power fields and subsequently averaged to obtain an average growth fraction. Cells on the margins of the tissue sections were not considered due to possible artefactual staining.

Regarding, KIT protein localization, each case was assigned one pattern as previously described[12,30] for MCTs. Pattern 1 (predominately perimembranous), pattern 2 (focal to stippled cytoplasmic localization) and finally pattern 3 (diffuse cytoplasmic localization).

Statistical analysis

Statistical analyses were performed using the IBM® SPSS® Statistics version 26 (IBM SPSS, Armonk, NY, U.S.A.). Mean and standard deviation (SD) were evaluated for quantitative variables. Frequency and percentage were evaluated for qualitative variables Chi-square test/Fisher's exact tests was used to analyse the significance of association between the expression of Ki67 and KIT with other parameters. To determine

the relationship and strength of correlation between patient's age, sex and tumor histological gradings (Patnaik's[19] and Kiupel's[11] classification systems) and Ki67 index and KIT pattern, Spearman's rho and the Kendall rank correlation coefficient tests were used. The value $P<0.05$ was considered significant.

Results

By the time of diagnosis, the mean age of the dogs was 8,18 years (SD =2,897; range, 3-14 years), 19 patients (55.88%) were younger than 8 years old and 15 (44.12%) were older than 8 years old. Out of the total of canine patients, 60% (n=21) were female and 40.00% (n=14) were male (**Table 4-A**).

Table 4-A: The baseline characteristics of patients with mast cell tumors.

VARIBLES	FREQUENCY N (%)	MEAN ± SD	RANGE
AGE (YEARS) (N=34)		8.18 ± 2.897	3-14
< 8 YEARS	19 (55.88)		
≥ 8 YEARS	15 (44.12)		
GENDER (N=35)			
FEMALE	21 (60.00)		
MALE	14 (40.00)		
ANATOMICAL LOCATION (N=33)			
HEAD AND NECK	6 (18.19)		
LIMBS	11 (33.33)		
TRUNK	16 (48.48)		
BREED (N=35)			
LABRADOR RETRIEVER	12 (34.28)		
MIXED	7 (20.00)		
BOXER	4 (11.43)		
GOLDEN RETRIEVER	3 (8.57)		
FRENCH BULLDOG	2 (5.71)		
GERMAN SHEPHERD	2 (5.71)		
BULL TERRIER	1 (2.86)		
BULLMASTIFF	1 (2.86)		
PINSCHER	1 (2.86)		
PUG	1 (2.86)		
YORKSHIRE TERRIER	1 (2.86)		
PATNAIK'S HISTOLOGICAL GRADING (N=35)			
GRADE I	2 (5.71)		
GRADE II	28 (80.00)		
GRADE III	5 (14.28)		
KIUPEL'S HISTOLOGICAL GRADING (N=35)			
LOW GRADE	10 (28.57)		
HIGH GRADE	25 (71.43)		
KIT (N=33)			
PATTERN I	12 (36.36)		
PATTERN II	6 (18.19)		
PATTERN III	15 (45.45)		
KI-67 (%) (N=35)		10.21 ± 13.43	1-81
LOW (< 10%)	20 (57.14)		
HIGH (≥ 10%)	15 (42.85)		

Regarding the Patnaik histological grade for MCTs, 2 (5.71%), 28 (80.00%), and 5 (14.28%) dogs were classified as grades I, II, and III, respectively (**Table 4-A**). Regarding Kiupel's grading system, 10 patients (28.57%) had low grade lesions and 25 (71.43%) had high grade tumors.

With respect to anatomical distribution, 6 out of the 33 lesions were located in head and neck (18,19%), 11 in the limbs (33.33%) and 16 in the trunk (48.48%).

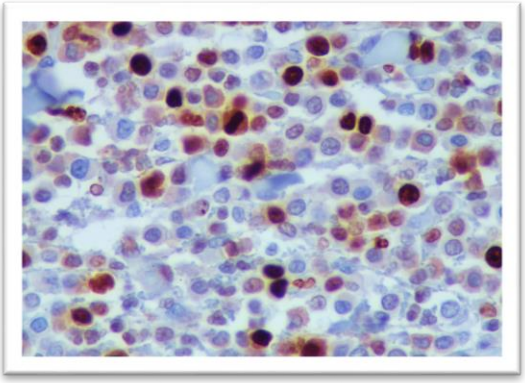

Figure 4-A: Dog, skin, Mast cell tumor (IHC, 400X): Immunoexpression of Ki-67 in neoplastic mast cells. IP=21%

A Ki-67 index mean value was calculated (10,21%) and used as a cut off for the formation of two groups: Low Ki-67 or KI-67<10% group was composed of 20 cases (57.14%) while High-Ki67 or KI-67 ≥10% was composed of 15 lesions (42.85%) – **Figure 4-A.** Twelve out of 33 MCTs displayed KIT pattern I (36.36%) (**Figure 4A-B**); six had pattern II (18.19%) (**Figure 4B-B**) and 15 detain pattern III (45.45%) (**Figure 4C-B**).

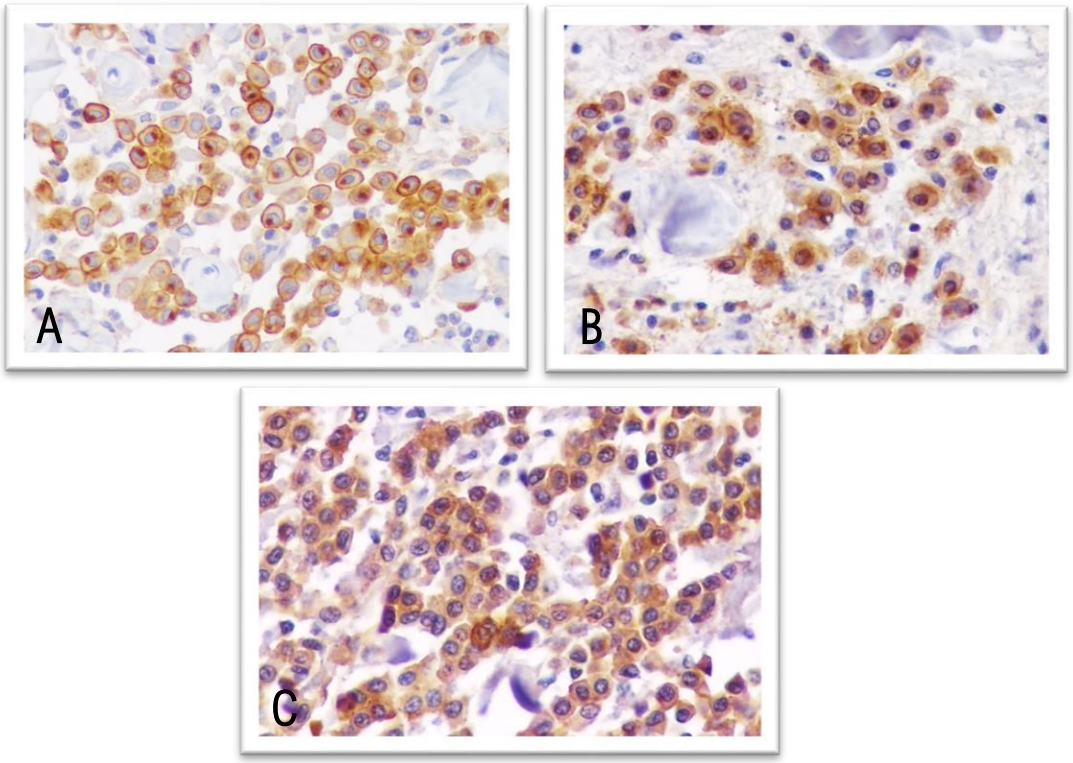

Figure 4-B: Dog, skin, Mast cell tumor (IHC, 400X): Immunohistochemical staining of KIT immunoexpression in mast cell tumors. (A) KIT pattern I (400X). (B) KIT pattern II (400X). (C) KIT pattern III (400X).

Chi-square/Fisher exact test

The association between Ki-67 with other factors in MCT patients has been shown in **Table 4-B**. For the chi-square/Fisher exact test, only for age and Ki-67 index a significant statistical relation was found (p=0.002) showing no independency between variables. For KIT immunopattern, the analysis with chi-square/Fisher Exact test showed an association with the Kiupel's histological grading system (p=0.020) (**Table 4-C**).

Table 4-B: The association between Ki67 with other factors in cutaneous mast cell tumors (Chi-square/Fisher Exact test).

VARIABLES	KI-67 (<10%) N	KI-67 (≥10%) N	P-VALUE
AGE (YEARS)			0.002*
< 8 YEARS	4	11	
≥ 8 YEARS	15	4	
GENDER			0.163
FEMALE	10	11	
MALE	10	4	
ANATOMICAL LOCATION$^{\Delta}$			0.719
HEAD AND TRUNK	12	10	
LIMBS	7	4	
PATNAIK'S HISTOLOGICAL GRADING°			0.141
GRADE I AND II	19	11	
GRADE III	1	4	
KIUPEL'S HISTOLOGICAL GRADING°			0.062
LOW GRADE	17	8	
HIGH GRADE	3	7	
KIT			0.898
PATTERN I AND II	10	8	
PATTERN III	8	7	

°Fisher's exact test
* Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.001 level (2-tailed).
$^{\Delta}$This parameter was regarded as 2 groups in this analysis due to the statistical rules to perform the test.

Table 4-C: The association between KIT with other factors in cutaneous mast cell tumors (Chi-square/Fisher Exact test).

VARIABLES	KIT (PATTERN I) (N)	KIT (PATTERN II) (N)	KIT (PATTERN III) (N)	P-VALUE[#]
AGE (YEARS)				0.982
< 8 YEARS	6	2	7	
≥ 8 YEARS	5	4	8	
GENDER				0.741
FEMALE	6	5	10	
MALE	6	1	5	
ANATOMICAL LOCATION[Δ]				1.00
HEAD AND TRUNK	9	3	10	
LIMBS	2	3	4	
PATNAIK'S HISTOLOGICAL GRADING°				0.152
GRADE I AND II	12	5	11	
GRADE III	0	1	4	
KIUPEL'S HISTOLOGICAL GRADING°				0.020*
LOW GRADE	12	4	7	
HIGH GRADE	0	2	8	
KI 67 INDEX				0.898
LOW (< 10%)	7	3	8	
HIGH (≥ 10%)	5	3	7	

°Fisher's exact test

*Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.001 level (2-tailed).

[Δ]This parameter was regarded as 2 groups in this analysis due to the statistical rules to perform the test.

Spearman's rho and Kendall tau correlation tests

Regarding the Spearman's rho and Kendall Tau tests to analyse the correlations with Ki-67 index (**Table 4-D**), no significant relationship was found between Ki-67 and patient's sex (ρ (33) =-0,236; p= 0,173; τ(33)=-0,236; p= 0,169), Patnaik's histological grading (ρ (33)= 0,111, p=0,526; τ(33)= 0.109 p=0,518) and KIT (ρ(31) =0,042, p= 0,919; τ(31) =0,039, p =0,814). However, in this statistical analysis, age was found to have significant strong negative relationship with Ki-67 index (ρ (32) =-0,523; p= 0,002; τ(32)=-

0,523; p= 0,003), and again Kiupel's histological grading was found to have a moderate positive relationship with ki-67 index (ρ(33) =0,347, p= 0,041; τ(33) =0,347, p =0,043). The correlations between KIT immunopattern and the clinicopathological parameters of the MCT patients, is depicted in **Table 4-E**. In the statistical analysis only Patnaik's and Kiupel's histological grading were found to have correlation with this variable. Patnaik's correlation had a moderate positive relationship with KIT pattern (ρ(31) =0,421, p= 0,015; τ(31) =0,394, p =0,017) and Kiupel's correlation with KIT was a moderate to strong positive relationship (ρ(31) =0,517, p= 0,002; τ(31) =0,492, p =0,003).

Other strong positive association identified was between Kiupel's and Patnaik's histological grading systems (ρ (33) =0,609, p= 0,000; τ(33) =0,595, p =0,000) (**Table 4-F**).

Table 4-D: The correlation between Ki67 with other factors in cutaneous mast cell tumors with Spearman's rho and Kendall's correlation test.

VARIABLES	KI-67 (<10%) N	KI-67 (≥10%) N	SPEARMAN RHO# (ρ) (P-VALUE)	TAU KENDALL# (τ) (P-VALUE)
AGE (YEARS)			-0.523* (0.002)	-0.523* (0.003)
< 8 YEARS	4	11		
≥ 8 YEARS	15	4		
GENDER			-0.236 (0.173)	-0.236 (0.169)
FEMALE	10	11		
MALE	10	4		
PATNAIK'S HISTOLOGICAL GRADING			0.111 (0.526)	0.109 (0.518)
GRADE I	0	2		
GRADE II	19	9		
GRADE III	1	4		
KIUPEL'S HISTOLOGICAL GRADING			0.347* (0.041)	0.347* (0.043)
LOW GRADE	17	8		
HIGH GRADE	3	7		
KIT			0.042 (0.819)	0.039 (0.814)
PATTERN I	7	5		
PATTERN II	3	3		
PATTERN III	8	7		

*Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.001 level (2-tailed).
#Correlation Coefficient; <0.1: poor correlation, 0.1 – 0.3: low correlation, 0.3-0.5: moderate correlation, >0.5 strong correlation.

Table 4-E: The correlation between KIT with other factors in cutaneous mast cell tumors with Spearman's rho and Kendall's correlation test.

VARIABLES	KIT (PATTERN I) (N)	KIT (PATTERN II) (N)	KIT (PATTERN III) (N)	SPEARMAN RHO[#] (ρ) (P-VALUE)	TAU KENDALL[#] (τ) (P-VALUE)
AGE (YEARS)				0.055 (0.764)	0.052 (0.759)
< 8 YEARS	6	2	7		
≥ 8 YEARS	5	4	8		
GENDER				-0.140 (0.438)	-0.133 (0.429)
FEMALE	6	5	10		
MALE	6	1	5		
PATNAIK'S HISTOLOGICAL GRADING				0.421* (0.015)	0.394* (0.017)
GRADE I	2	0	0		
GRADE II	10	5	11		
GRADE III	0	1	4		
KIUPEL'S HISTOLOGICAL GRADING				0.517* (0.002)	0.492* (0.003)
LOW GRADE	12	4	7		
HIGH GRADE	0	2	8		
KI 67 INDEX				0.042 (0.819)	0.039 (0.814)
LOW (< 10%)	7	3	8		
HIGH (≥10%)	5	3	7		

*Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.001 level (2-tailed).

[#]Correlation Coefficient; <0.1: poor correlation, 0.1 – 0.3: low correlation, 0.3-0.5: moderate correlation, >0.5 strong correlation.

Table 4-F: Correlations between Kiupel's histological grading and other parameters (Patnaik's histological grading and c-kit pattern)

VARIABLES	PATNAIK'S GRADE I (N)	PATNAIK'S GRADE II (N)	PATNAIK'S GRADE III (N)	SPEARMAN RHO[##] (ρ) (P-VALUE)	TAU KENDALL[##] (τ) (P-VALUE)
KIUPEL'S HISTOLOGICAL GRADING				0.607** (0.000)	0.595** (0.000)
LOW GRADE	2	23	0		
HIGH GRADE	0	5	5		

*Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.001 level (2-tailed).

[##]Correlation Coefficient; <0.1: poor correlation, 0.1 – 0.3: low correlation, 0.3-0.5: moderate correlation, >0.5 strong correlation.

Discussion

The Labrador Retrievers, Boxers, Golden Retrievers and French Bulldogs predominance among the animal population studied reinforce these well-known breed predispositions for canine MCTs. However, no significant correlations were found between the breed of the animals and any other of the variables studied (Ki-67 and KIT). However, it is important to keep in mind that our population was constituted only by 35 animals thus, more studies including a larger sample are needed to properly investigate these correlations that might help to more accurately, determine the course of the disease for each breed.

Kiupel's histological grading present a moderate positive relationship with Ki-67 index. This can be explained since the cells of the tumor divide more, there are more mitotic figures and according to the Patnaik's and Kiupel's scales, it should get classified as higher grade MCT. Kiupel's and Patnaik's histological grading presented a strong positive relationship, since both systems are similar and created with the same purpose, however in the present investigation Ki-67 index did not elicit a correlation with Patnaik's system. Nonetheless, this can be again a result of the small sample size.

When analysing the correlation of the two immunomarkers with patient's age, we noted a distinct correlation between this variable and Ki-67 index, when using both sets of statistical analyses tests. This suggests a dependent and negative relationship (when one of the variables increases the other one decreases) therefore, in this study, when younger dogs are diagnosed with a MCT, the lesion will probably present a higher Ki-67 index. However, in combination with the correlation between Ki-67 and higher grades, this can suggest that younger dogs diagnosed with MCT could be at risk of developing more aggressive tumors. Further studies are needed to validate this hypothesis.

The KIT receptor is a transmembrane protein,[37] and in dogs, nonneoplastic mast cells have been shown to express KIT exclusively along the cell membrane, while MCTs histologically graded as II or III according to Patnaik *et al*.[21] predominately expressed KIT in their cytoplasm.[24] Results for the correlation between KIT pattern and the clinicopathological parameters have highlighted a moderate to strong correlation between KIT immunoexpression and both Patnaik's and Kiupel's histological grading systems. Since this correlation is define as positive, when one variable "increases" the same happen with the other variable. Since KIT is divided into 3 patterns, type I membrane-associated, type II, paranuclear or Golgi-like and lastly type III, diffuse cytoplasmic, and as mentioned before being a positive relationship this associates an altered cytoplasmic KIT immunoexpression with higher histological grades. These findings confirmed previous studies that associate these parameters.[3,12,21,22,24] The more

aggressive biologic behavior of MCTs presenting an increased KIT expression might be explained by the functional responsibilities of KIT protein and its ligand, SCF, in mast cell physiology. KIT and SCF have been proven to facilitate several functions in mast cell development, including proliferation, maturation, inhibition of apoptosis, adhesion, and migration.[4,17,20,31,37,38] KIT could also be presumed to take part in tumoral progression, as suggested by the correlation of cytoplasmic KIT staining and a higher histological grade, by facilitating neoplastic cell mobility and attaching of fibronectin.[3,4] Also, a correlation between aberrant KIT localization and activating mutations was found.[34] This discovery is probably due to activated KIT molecules being detached from the cell membrane and internalized more quickly than inactivated KIT.[10] However, aberrant KIT protein immunolocation can also occur without a detectable c-kit mutation, suggesting an alternative mean of constitutive activation among which gene duplication or autocrine/paracrine production of KIT's ligand occur.

No statistically significant associations were found concerning animals' gender and other variables. In the majority of epidemiological studies, this parameter did not display an increased risk for development of MCTs. Thus, it is a natural assumption that, if a variable does not present as a risk factor there is a low probability that it's going to have an association with proliferative and molecular markers used as specific complementary test for this disease. Anatomical location also did not display any association with the proliferative and molecular immunomarkers.

Variations within the two sets of statistical tests used, (1) chi-square/Fisher's exact test and (2) spearman's rho and Kendall's tau are observed. However, even though the positive results in the set (1) are in concordance with some of the results of the set (2), some of the results of the second did not match with the one's from the first, however that can be a result of the small sample size in this study since this circumstance can affect the frequencies distribution and the assumptions and guidelines for each test. Another point that can influence the data is the cut-off point used in the ki-67 index analysis, since this was a small sample size and the mean score of ki-67 herein achieved was relatively high, the distribution of frequencies and by consequence the outcome might be influenced. We predict that, increasing the sample size the cut-off score would be different allowing more accurate and solid results. Standardization of the Ki67 positive cells counting method among different pathologists should be helpful to determine the better cut-off value to help the clinician to assess the prognosis of MCTs.

The available data does not allow for conclusions as to consider these parameters as independent elements for prognosis, but it does have results that encourage more research, as at least demonstrates the role as specific complementary

test that could be helpful in determining the course of the disease of the patient, especially in ambiguous cases.

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CHAPTER 5

General Discussion and final considerations

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The three main constitutive parts of this project were to perform an epidemiological study focused on canine cutaneous tumors and mast cell tumors and further investigate putative correlations between MCTs several clinicopathological variables and complementary tests results obtained.

At our laboratory we found 1.185 cases of canine cutaneous tumors were diagnosed between 2014 and 2020, of which 62.86% were classified as benign and 37.13% as malignant. The most common cutaneous tumors found in the database were mast cell tumors (22.70%), followed by benign soft tissue tumors (9.70%) and sebaceous gland tumors (8.10%). Anatomical sites more frequently affected by these were hindlimb (12.07%), forelimb region (8.61%) and the buttock area (7.09%). Additionally, 14.60% cutaneous tumors cases presented multicentric distribution.

As the most common cutaneous tumor in our database, MCTs were the target of thorough investigation. Our data revealed that Labrador Retrievers (26.77%), mixed-breed dogs (25.65) and Boxers (15.24%) were the breeds with higher occurrence of this neoplasm.

Additionally, Boxers (30.15%) and Labrador Retrievers (22.86%) displayed the highest prevalence within purebred dogs. MCTs were often found on the hindlimb region (21.19%), abdominal region (8.92%) and affecting multiple sites (12.27%). Furthermore, MCTs were commonly concentrated in older dogs with ages between 7- and 11-years-old (59.76%).

Approximately 244 canines MCTs cases were identified in LPV databases and, amongst these 225 were subjected to Patnaik classification system, 156 to Kiupel classification system and 137 to both classification systems. Characteristics related with breed, age, sex and anatomical location of MCTs patients, were statistically evaluated in order to determine the risk of developing these lesions. In comparison to other cutaneous tumors, the highest risk for developing MCTs was detected in Labrador Retrievers (OR= 2.063), Boxers (OR= 2.004), French Bulldogs (OR=3.071) and Pugs (OR=9.561). Furthermore, Boxers had a higher predisposition to lower grade tumors (Patnaik's grade I and II and Kiupel's low-grade) (OR= 5.902, OR=1,989 and OR=2.616, respectively). Labrador Retrievers and Pugs presented a high risk for Patnaik's grade II lesions (OR=2.128 OR=12.873, respectively) and Kiupel's low-grade (OR=2.306 and OR=17.084, respectively). French Bulldogs (OR= 7.878) had a high risk for grade III

lesions and Pit Bulls have a noted predisposition to Patnaik's grade III (OR= 4.434) and Kiupel's high-grade tumors (OR=4.962).

The perigenital area and trunk were identified as high-risk regions for grade III tumors development (OR=6.615 and OR=1.868, respectively). In turn, the limbs were found to be the region with the highest risk for grade II tumor development (OR=1.648). Buttock area, head and neck presented a decrease risk for grade II tumors OR=0.071 and OR=0.396, respectively).

The frequency of MCTs is higher in the older age groups (7-10 years and 11-18 years, accounting for 40.98% and 26.23%, respectively). An increased risk of MCT development was seen in the older dogs belonging to 4-6 years and 7-10 years interval groups, when compared to those grouped in 11-18 years range. However, the group of dogs with 4 to 6 years displayed a higher risk than the group of 7 to 10 years old (OR= 2,299 and OR=1,471, respectively). The aged groups of 4-6 years and 7-10 years also depicted higher risk for Patnaik's grade II lesions occurrence (OR=2,680 and OR=1,629, respectively). For the Kiupel distribution in the low-grade tumors there was a higher risk for the group between 4 and 6 years old (OR=2,647). Females dogs (54.51%) were most affected than males (45.49%).

Since specific complementary tests are currently and routinely used in MCTs to identify possible prognostic factors, correlations between two biomarkers, ki67 index and KIT protein immunoexpression with other clinicopathological parameters were also evaluated. Ki67 showed a dependent and negative relationship with age. This correlation suggests that when younger dogs are diagnosed with a MCT, the lesion will probably present a higher Ki-67 index, reinforcing that these younger dogs could be at risk of developing a more aggressive tumor. Nevertheless, more studies are needed to validate this hypothesis.

Moreover, Kiupel's histological grading commonly applied to MCTs presented a moderate positive relationship with ki-67 index. Since that neoplastic cells normally divide more, there are more mitotic figures and according to the Patnaik's and Kiupel's scales, it should get classified as higher grade MCT.

Results for the correlation between KIT pattern and the clinicopathological parameters have highlighted a moderate to strong positive correlation between KIT immunoexpression and both Patnaik's and Kiupel's histological grades. Since KIT protein immunoexpression is divided into 3 patterns: (I) membrane-associated, (II) paranuclear or Golgi-like and lastly (III) diffuse cytoplasmic, these findings relate an modified cytoplasmic KIT immunoexpression with a higher histological grade. MCTs presenting an increased KIT immunoexpression showing a more aggressive biological

behavior could be explained by the functional duties of KIT protein and its ligand, SCF, in mast cell physiology.

As demonstrated, this study described the epidemiological tendencies for canine cutaneous tumors and cutaneous MCTs in Portugal, providing insights about the relevance of specific MCT markers as prognostic factors with potential therapeutically impact. This research elucidates about the frequently diagnosed cutaneous tumor histotypes, anatomical tendency of tumors development, preferential ages and gender and common dog breeds at risk in our specific geographic location. The epidemiological information achieved can assist regional veterinarians, favoring a preliminary diagnosis or suspicion of canine cutaneous tumors and provide more adequate and contextualize prognostic information regarding canine mast cell tumors.