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# Current Insights Into Canine Cutaneous Melanocytic Tumours Diagnosis

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Additional information is available at the end of the chapter

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## 1. Introduction

Skin melanoma is a devastating disease, frequently diagnosed in human and dogs, accounting for 0,8% - 2% of all skin tumors in latter species [1].

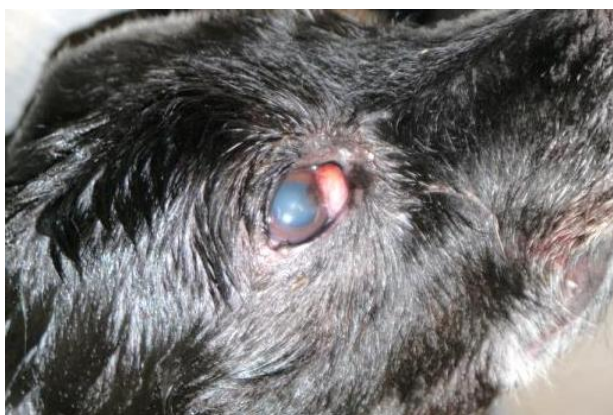
Melanocytic tumours are common neoplasms in dogs, accounting for 4 to 7% of neoplastic lesions in general, and up to 7% of all malignant tumours [2,3]. They generally arise on the oral cavity (Figure 1), lip, skin (Figure 2) and digit, amongst other locations (Figure 3) [4].



**Figure 1.** Canine oral melanoma (courtesy of Dr. Abel Fernandes).



**Figure 2.** Canine cutaneous melanoma.



**Figure 3.** Canine ocular melanoma.

Biologic behaviour of these tumours is often related to its location. Over 85% of melanocytic lesions located on haired skin are described as of benign behaviour. The majority of oral and mucocutaneous junction melanomas, with the exception of the eyelid, and 50% of digital melanomas originated from the nail bed are reported as malignant [5,6].

Cutaneous canine melanocytic lesions are usually benign [7,8]. They are generally detected at a late stage, when excision is rarely curative and metastasis is often detectable in regional lymph nodes [7]. Malignant tumors are found most frequently on the head, ventral abdomen, and scrotum [7]; in the last one, they represent 3,1% of all cutaneous malignant melanomas and 4,7% of scrotal tumours [9]. Amelanotic lesions can occur as cutaneous neoplasms, but are more frequent in the oral cavity, and tend to be behaviorally malignant [10].

Metastasis are often found on regional lymph nodes and lungs, but organs as brain, heart and spleen are also commonly affected [11].

Veterinary nomenclature of canine melanocytic tumours has been subjected to many controversies and changes over time. Through this article, in accordance with the revised World

Health Organization classification system, and in order to simplify and avoid confusion, the authors describe benign lesions as melanocytoma, whereas malignant lesions are referred to as melanoma [12].

Etiology of this neoplasms is still uncertain, and several factors may be related, such as consanguinity, trauma, chemical exposure, hormones and genetic susceptibility [10]. Unlike humans, ionizing solar radiation exposure doesn't seem to be related to canine melanoma initiation [4].

Canine melanocytic tumour diagnosis often represents a challenge for the pathologist, since a high number of neoplastic lesions are quite similar in terms of its clinical and histologic appearance, including carcinoma, sarcoma, lymphoma and plasmocitoma, amongst others [10]. Indeed, cutaneous neoplasms with malignant behavior are more difficult to distinguish histologically from benign neoplasms than oral or lip neoplasms [13].

Diagnosis is usually based on fine-needle aspiration cytology, but biopsy for histopathological examination is essential to determine its malignant potential [4,14]. The most reliable histologic criteria is the mitotic index, defined as the total number of mitotic figures observed per ten high-power light microscopic fields, which is known to be 90% accurate [5,6,15].

Immunocytochemistry has arisen as an extremely useful tool, for both diagnostic and prognostic purposes. A positive diagnosis for melanocytic neoplasms is obtained with a positive labelling of S100 protein, vimentin, Neuron Specific Enolase (*NSE*) and a simultaneous negative labelling with cytokeratin [16].

The treatment of choice for local cutaneous melanomas is surgical excision; tumours with benign histopathology criteria have an excellent prognosis after this surgery. However, for malignant tumors, the prognosis is guarded, since metastatic rates of 30-75% have been reported [5,6].

Alternative therapy methods described in the literature include systemic chemotherapy, radiotherapy [17], photodynamic therapy [18], local hyperthermia [19,20] and intralésional injection of cisplatin or carboplatin.

The poor responses to the conventional therapy are leading to a development of new immunotherapy procedures – including intralésional adenoviral vector-mediated transfer of CD40L, a tumor necrosis factor gene [21], therapy with a plasmid DNA encoding staphylococcal enterotoxin B [22], and systemic treatment with liposome-encapsulated muramyl tripeptide [23].

At last, the most promising therapy appears to be a xenogenetic human tyrosinase DNA vaccine, with minimal local reaction and no systemic toxicity signs, and a great clinical response with significant increasing of the survival time [24].

In this paper, the authors aim to contribute to the understanding of melanocytic tumours in the dog, making a critical review of the literature and discussing the parameters currently considered valid for diagnosis use in canine melanocytic neoplasms.

## 2. Signalment

Cutaneous melanocytic tumours are most common in older dogs (mean age of 9 years old) [25], with a higher mean age for dogs with malignant melanocytic tumours (12 years). However, age has not been related with the patient clinical outcome and survival time [26].

Although all breeds of dog (and crossbred animals) may be affected, some breeds are reported as predisposed, including Schnauzer, Doberman, Scottish Terrier, Irish Setter, Golden Retriever, Chow Chow, Cocker Spaniel, German Shepherd and Rottweiler [4,27]. Breed predisposition is thought to be related to an underlying genetic risk and/or increased pigmentation in the described above breeds [4].

One study [6] has also established a relationship between patient breed and tumour behavior, likely due to genetic susceptibility, as prior described. In the referred work, melanocytic lesions tended to be behaviorally benign in Doberman pinschers and miniature schnauzers, while miniature poodles were the mostly affected breed with malignant melanoma. However, it must be noted that oral neoplasms were also included in that study.

An early report described a higher frequency of these lesions in male dogs [28], but recent literature denies gender predisposition [6,27,29-31].

## 3. Pathogenesis

Melanin is a dark-brown pigment synthesized by melanocytes, dendritic cells found within the basal layer of the epidermis. These cells are dispersed from each other, located between basal keratinocytes, forming adherent and regulatory junctions mediated by epithelial cadherin (E-cadherin) molecules. After its synthesis, melanin is retained in melanosomes and transferred to the adjacent keratinocytes [32].

Conversion of normal melanocytes to clusters of neoplastic melanocytes is a process composed by a series of events: initiation, promotion, transformation and metastasis [4].

Little is known about initiation on most animal melanomas, but ionizing solar radiation exposure – the main initiator factor in Human melanomas [33] - doesn't seem to be related to canine melanomas [4]. A higher incidence of spontaneously mutated cells due to familiar clustering through inbreeding may be a critical initiation factor in domestic animals.

Malignant transformation of canine cutaneous melanocytomas is very uncommon. Regarding cutaneous melanomas, there are a few published case reports, including one by Valentine and team [34], which have described a single case of malignant transformation of a congenital melanocytic nevus in a Golden retriever. Conroy [35] described two cases of melanoma originated from junctional or dermal hamartomas and a single case of a primary melanomas originated from a subcutaneous melanocytoma have also been reported [36]. In summary, canine cutaneous melanomas are thought to arise *de novo* from epidermal and dermal melanocytes.



Promotion phase is related to mutated cells proliferation, with subsequent amplification of cell population and origin of additional mutations [4]. Melanoma promoters include chronic trauma, chemical exposure, drugs and hormones [10], and its action results in reactive hyperplasia of the epithelium, with disruption of regular keratinocyte-melanocyte interactions and proliferation of initiated cells.

The next step in carcinogenesis involves a series of transformation events. Recent developments in genetic and molecular study techniques have identified the role of a few tumour suppressors in melanoma cell lines, giving new insights on the importance of these molecules in canine melanoma development. A reduction or loss of *p16* expression was one of the most commonly found changes, in both benign and malignant tumours, suggesting that inactivation of this pathway is a critical step in the pathogenesis of melanoma [37]. Altered expression of *PTEN*, *TP53*, *Rb* and *p21* have also been related to its progression [38], as well as the presence of various oncogenes (as a result of proto-oncogenes mutation), such as *c-myc*, *c-erb-B-2*, *c-yes*, *c-kit* and *ras* [4].

After local proliferation phase, neoplastic cells may acquire a malignant behavior, and disseminate through hematic or lymphatic vessels to various other organs, originating secondary neoplasms known as metastasis. This complex process has its start with loss of adhesion and detachment of neoplastic cells from the primary mass, hematic and lymphatic vessels intravasation and attachment and proliferation within a secondary location [4].

Metastasis process is dependent of various adhesion molecules regulation by neoplastic cells. Several studies have shown an association between decreased and altered expression of E-cadherin, a calcium-dependent adhesion molecule responsible for melanocyte-keratinocyte interaction, and canine cutaneous melanoma progression [39,40]. CD44, a second transmembrane glycoprotein which facilitates metastasis, is required for several processes, including hyaluronate degradation, cell aggregation and migration, angiogenesis and hematopoiesis [4]. Down-regulation of regular CD44 plus up-regulation of CD44v5 has also been associated with melanoma metastasis, particularly with lymph node metastasis [41].

Autonomous growth is a key requirement for both primary and secondary neoplastic development. The most important autocrine growth factors in animal melanoma include basic fibroblast growth factor (*bFGF*), melanoma growth stimulatory activity or growth regulated proteins, platelet-derive growth factor-A,  $\alpha$ -melanocyte stimulating hormone, and a series of interleukins (*IL-8*, *IL-10* and *IL-18*) [4].

#### **4. Gross morphologic features**

Macroscopically, canine malignant melanoma cannot be differentiated from melanocytoma [42]. Melanomas in dogs tend to be dermal in location, unlike Human melanomas – which are intraepidermal with some degree of dermal invasion. Prognostic schemes, such as Clark's level or Breslow thickness, built based on depth of dermal invasion, are not applicable on canine lesions [43].

Canine melanocytomas share some aspects with Human benign melanocytic lesions, in terms of clinical evolution most common metastatic locations [42], and genetic alterations [44].

Cutaneous melanocytomas are usually symmetrical, circumscribed, but encapsulated [43], solitary, black, brown, or gray cutaneous alopecic nodules [43,45] with a variable size with range of 1-4 cm in diameter (Figure 4 and Figure 5) [43]. Epidermis is usually intact, and alopecia is frequent. Epidermal cells may be hyperpigmented, and the majority of dermal cells are replaced by the tumoral ones, which in larger masses might also extends into the subcutaneous tissue. The tumors may have a variegated appearance, with areas of pigmentation intermingled with no pigmented regions [42].

Canine cutaneous malignant melanomas can vary considerably in appearance, regardless of the location. Melanomas tend to be asymmetrical. The asymmetry may be most readily recognizable in the epidermal component of junctional tumors [43]. Melanomas size vary from some millimeters to as large as 10 centimeters in diameter (mean range being 1 to 3 cm in diameter) [43], but this is not a reliable indicator of malignancy [13,42]. The color is variable, ranging from gray or brown to black, red, or even dark blue [7]. Cutaneous melanoma presentation includes smooth domes, sessile nodules, polypoid, plaquelike [7,43], or even lobulated masses [7]. The larger ones are often ulcerated [7,43]. The tumors may invade deeply into the subcutaneous tissue and along fascial planes [42].



**Figure 4.** Canine cutaneous melanoma.



**Figure 5.** Canine cutaneous melanoma.

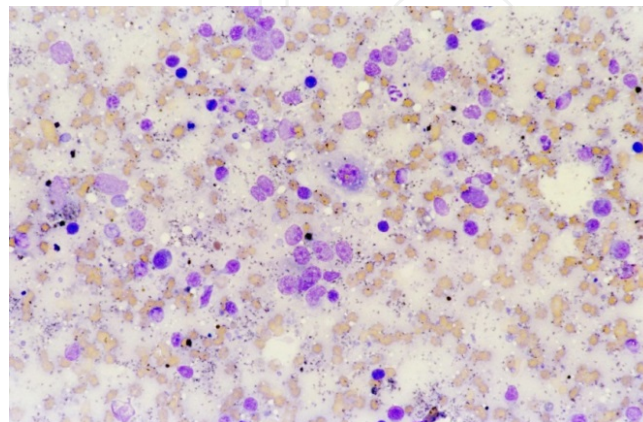
## 5. Cytological diagnosis

Microscopic examination of a cytological specimen obtained through fine needle aspiration has become a valuable technique to obtain a preliminary, and often definitive, diagnosis [46]. Being a quick, non-invasive and inexpensive procedure, it can also provide information on the stage, prognosis and metastasis evidence. Its main limitation reside on the fact that non-pigmented melanomas may strongly resemble other neoplastic lesions, and the amount of cytological sample might be very small and not fully representative of the lesion [4,46].

Several studies in Human cancer have described a strong accuracy in cytological examination in comparison with histopathological findings [47,48], but there are only few studies on the subject in Veterinary Medicine [49]. For instance, Ghisleni and others [50] evaluated a series of cutaneous and subcutaneous masses from dogs and cats through histopathology and cytology, describing an agreement between both techniques in 90.9% of the samples.

Melanocytic tumours are characterized by the presence of cells with abundant cytoplasmic melanin granules. Neoplastic cells may appear with an epithelial (cohesive cells), mesenchymal (single oval or spindle-shaped cells) or round cell morphology. Nuclei may present a central or eccentric location, and is often solitary, though multinucleated forms are occasionally found. Nucleoli tends to be very prominent, with variable shapes such as round, oval or angular. These cells have a light basophilic cytoplasm, with a moderate to high nuclear-cytoplasmic ratio. Varying degrees of pigmentation might be found within the same tumour smear [4,46], (Figure 6 and Figure 7).

Malignant criteria consist, most importantly, of marked anisokaryosis and nuclear pleomorphism, but also of the presence of large and atypical nucleoli [46]. Mitotic index, the most reliable criteria in histopathological evaluation, has no use in cytology. Regional lymph nodes are the most commonly evaluated site while monitoring for metastasis [4].



**Figure 6.** Canine cutaneous melanoma (Wright, 100x).



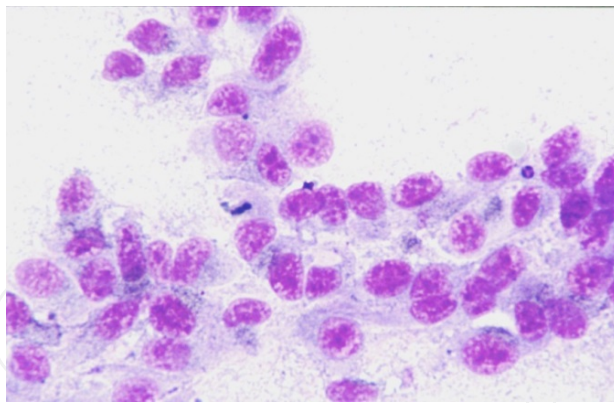


Figure 7. Canine cutaneous melanoma (Wright, 400x).

## 6. Histological diagnosis

Histological characteristics of canine melanocytic neoplasms were defined by World Health Organization, in *International Histological Classification of Tumours of Domestic Animals*, back in 1974 (Figure 8 and Figure 9) [13].

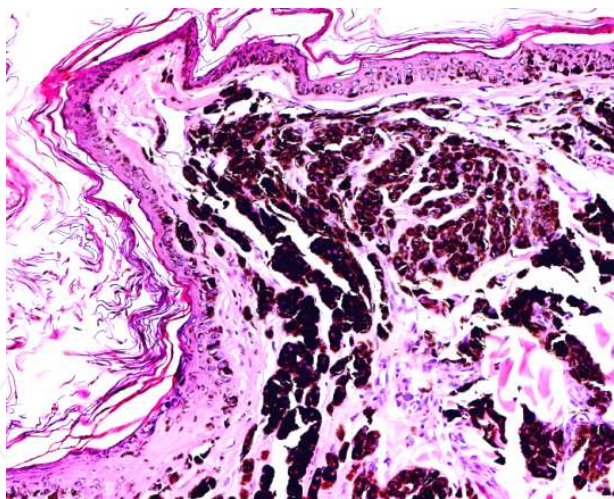
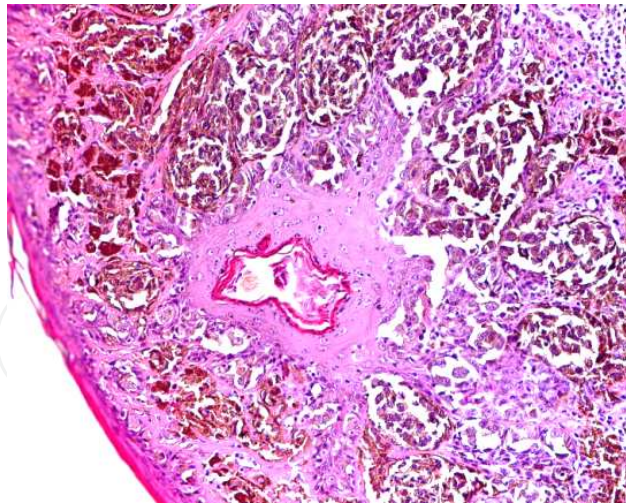


Figure 8. Canine cutaneous melanocytoma (H&E, 200x).

Histological appearance does not always correlate well with biological behavior [8]. More recent studies have provided a numerical “tumor score” taking into consideration mitotic index, nuclear atypia, inflammation, necrosis and volume which appears to have improved correlation between histology and behavior [8].

The term nevus, commonly used in describing pigmented melanocytic lesions of the epidermis and dermis in humans, is not used in veterinary dermatopathology [7].



**Figure 9.** Canine cutaneous melanoma (H&E, 200x).

In this chapter we review several histological parameters on their ability to diagnose and predict prognosis (i.e., prediction of mortality) of canine melanocytic neoplasms. Malignancy cellular features include a characteristic large nucleous, nuclear atypia, hyperchromasia, abnormal chromatin clumping and anomalous mitotic figures [51].

### **6.1. Predominant cell type**

Melanocytic neoplasms are generally composed of one of the following cell types: epithelioid (Figure 10), spindle, mixed (Figure 11), dendritic [51,52], and round cells [43]. Other less commonly described cell types include signet ring and balloon cells [51]. All these cell types may occur either alone or in combination [43]. In melanomas, ganglion cell and multinucleated giant cell forms also may be observed [43]. The epithelioid cell type is the most common type in all locations, whereas a mixture of cell types is seen with less frequency [51].

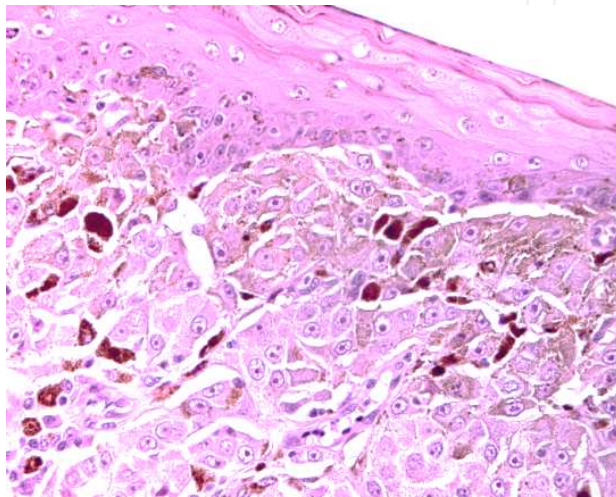
The epithelioid cells are round, with discrete cell borders, abundant glassy cytoplasm, appearing arranged in sheets and larger nests [43]. Similar to their spindle-shaped counterparts melanomas may exhibit large ovoid nuclei and prominent nucleoli [7,43], marked anisokaryosis and variable chromatin patterns [43].

The spindle cell tumors are arranged in streams and interweaving bundles, resembling fibrosarcoma or neurofibrosarcoma presentation. In malignant lesions, the nuclei are large and fusiform with prominent nucleoli [7,43], and moderate to marked nuclear pleomorphism is seen [43]. Spindle cell predominant morphology was statistically associated with benignity in one study (in 71% melanocytomas in contrast to 29% melanomas) [43]. The mixed type consists of both cell morphologies and patterns [7].

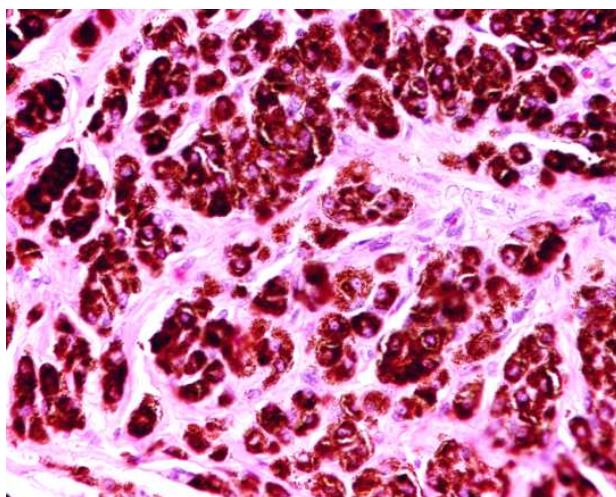
Dendritic melanocytes have a highly angular shape, sometimes with long cytoplasmic processes, and are usually arranged in small nests [43] or organized in tightly swirling streams, often with a fingerprint pattern [7]. The dendritic or whorled forms occurs only in the skin [53].

Round cells tumors have round to polygonal cells arranged in sheets as dense packets of cells, the packets being separated by a scant stroma. The nuclei is large and round in melanomas [43].

The signet-ring cell tumors consist of compact neoplastic clusters, with round to ovoid cells presenting a faintly-eosinophilic cytoplasm and an intensely-stained periphery. A vesicular nuclei, crescent-shaped, and with a peripheral location, gives the cell a signet-ring appearance [52].



**Figure 10.** Epithelioid cell canine cutaneous melanoma (H&E, 400x).



**Figure 11.** Mixed cell canine cutaneous melanoma (H&E, 400x).



Balloon cells are found organized in groups, separated by collagenous septa [52]. The cells are round to polyhedral and have clear or faintly eosinophilic cytoplasm [43,52]. In melanomas, the nuclei are round situated mainly at the periphery of the tumor cells [52]. Usually contains one central prominent nucleolus [7], which sometimes is difficult to detect. Heterochromatin, which is sparse, is dispersed throughout the nuclei [52].

Benign melanocytic cells present an enlarged vesicular nuclei with small nucleoli. Nuclear shape vary on according to the predominant tumour cell type. Mitotic figures are rare, and mitotic atypia is rarely observed. [43].

Although cell type appears to play some role in the prognosis of ocular melanocytic neoplasms, this feature lacked significance in prognosis of melanocytic tumors occurring in the mouth, feet, buccal mucosa and skin of dogs in several studies [8]. On the contrary, the epithelioid shape was associated with an unfavorable course, for 8/15 cases. The difference seems to be significant ( $p = 0,03$ ) [12].

## 6.2. Nuclear atypia

One of the major criteria of malignancy in melanocytic neoplasms arising at any pigmented anatomic site is nuclear atypia. This feature is more valuable in epithelioid tumours than in spindle, whorled type or signet-ring cells, due to the insufficient nuclear detail associated with the later neoplasms [8,44,54]. However, not all studies are consensual [12].

Well-differentiated tumoral melanocytes have a small nucleus with one central nucleolus [8]. In contrary, undifferentiated tumours generally present cells with multiple, large, irregular and eccentrically nucleoli [8].

Several criteria are used to estimate nuclear atypia, including the percentage of nuclei involved [8]. Figures 12 and 13 (below) present moderate and severe nuclear atypia, respectively.

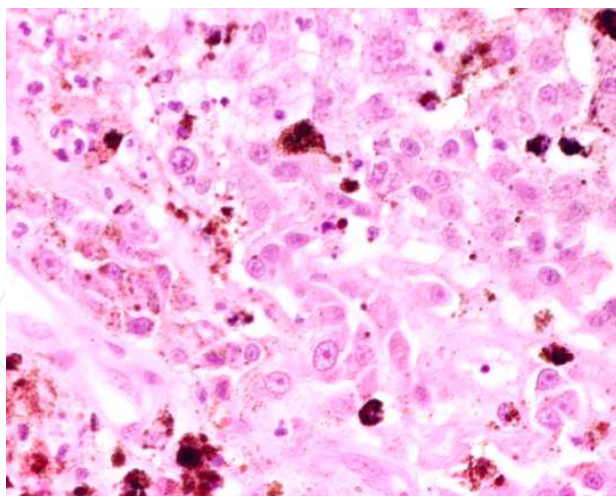
## 6.3. Mitotic index

In animal cutaneous and eye melanocytic neoplasms, mitotic index (MI) is the most reliable histological feature for distinguishing malignant from benign tumors [44,51], and also in predicting the clinical course of the disease [8]. In cutaneous melanoma, an MI of  $\geq 3/10$  hpf is significantly correlated with decreased survival [55].

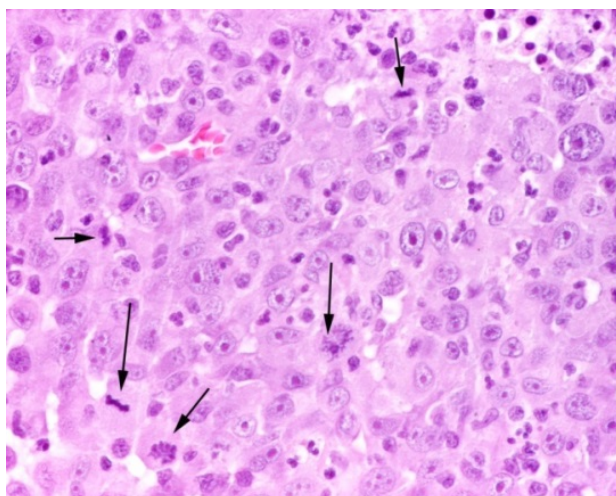
The number of mitoses is usually lower in melanocytomas [ $< 3$  mitotic figures per 10 high power fields (hpf)] than melanomas [42,44,53].

In one study, the mitotic index was strongly correlated with the clinical outcome of tumors. For tumors with a favorable outcome, the mean value of the number of mitosis (on 10 randomly selected high power fields) was 1,98 (from 0 to 27). For tumors with a malignant behavior, it was 18,53 (from 0 to 75) [12].

The evaluation of the MI in conjunction with nuclear atypia classification (Figure 13) offers a more precise value the histological diagnosis [8].



**Figure 12.** Canine cutaneous melanoma with moderate differentiation (H&E, 400x).



**Figure 13.** Canine cutaneous melanoma with evident nuclear atypia and numerous mitosis (arrow), (H&E, 400x).

#### **6.4. Cellular pleomorphism**

Cellular pleomorphism criteria include several features, such as cell size and shape, pigmentation degree and nuclear features (including prominence of nucleoli and chromatin pattern). The usefulness of these parameters as individual prognostic factors is doubtful; however, it increases when these are used together [8,27].

#### **6.5. Degree of pigmentation**

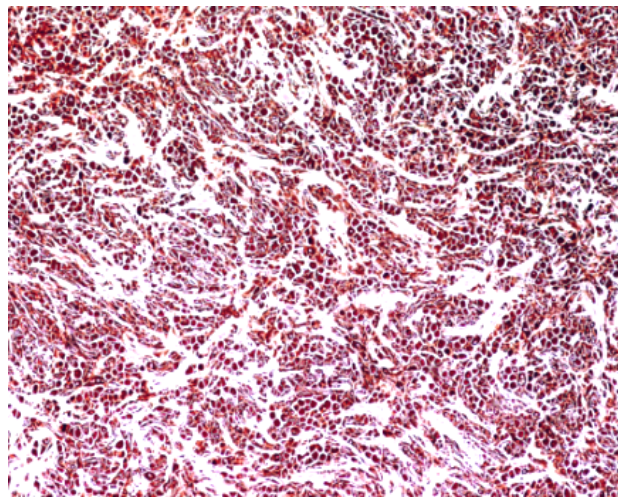
Degree of pigmentation is highly variable, even within a single smear (Figures 14, 15 and 16). Even on histological evaluation of amelanotic-classified tumours is common to detect a few very fine pigment granules in some cells. These are generally punctuate, spherical or elongated,



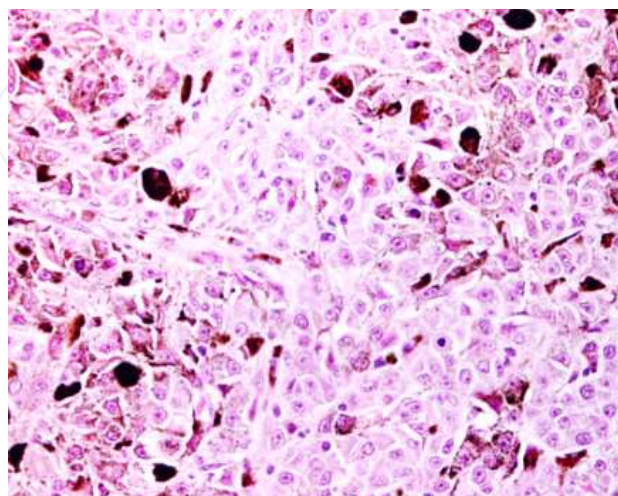
as observed in pigmented keratinocytes. Cells with a very fine pigment may present a dusty gray appearance, instead of the typical granulation image [7]. In summary, it can be difficult to accurately diagnose an amelanotic melanocytic neoplasm and to define the degree of pigmentation [13].

Individual tumoral cells possess a different amount of melanin, which granules are generally small and uniform in size within the same cell. Spindle, round to polygonal, and balloon cells have sparsely-distributed melanin granules, while large epithelioid and dendritic cells are known to have a higher degree of pigmentation [43].

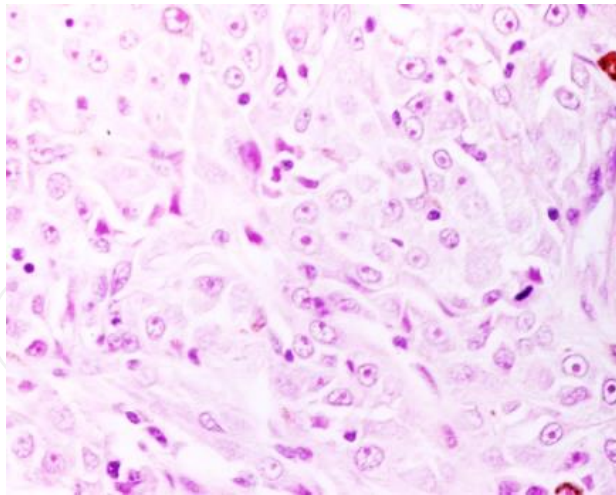
The majority of canine melanocytomas (except for balloon cell ones) have marked to moderate melanin pigmentation overall, especially in the superficial aspect of the tumors [43]. Similarly to cellular morphology, the degree of pigmentation of neoplastic cells was not an indicator of prognosis [12].



**Figure 14.** Canine cutaneous melanoma with marked pigmentation (H&E, 400x).



**Figure 15.** Canine cutaneous melanoma with moderate pigmentation (H&E, 400x).



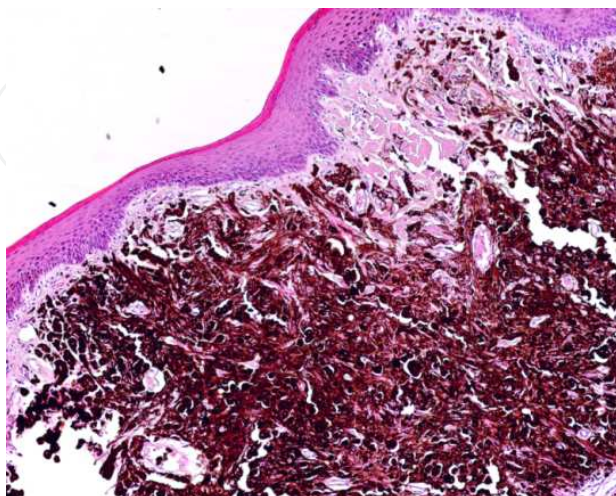
**Figure 16.** Amelanotic melanoma (H&E, 400x).

### 6.6. Junctional activity

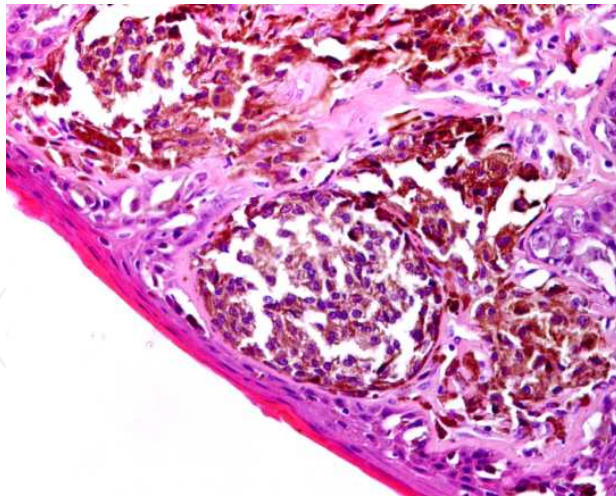
Junctional activity refers to the proliferation of neoplastic melanocytes at the interface between the epidermis and dermis or epithelium and submucosa [53].

The presence or absence of junctional activity is not specific to melanoma and often occurs in melanocytomas [7], however, malignant melanomas arising in the skin often show marked junctional activity, (Figure 17 and Figure 18) [42].

Junctional activity was not statistically associated with survival for skin neoplasms in one study [8]. In contrast, another work considered junctional activity as an independent prognostic factor ( $p = 0,0239$ ) for cutaneous melanocytic neoplasms, and found that its occurrence was associated with a longer survival time ( $p = 0,0046$ ) [55].



**Figure 17.** Canine cutaneous melanoma without junctional activity (H&E, 200x).



**Figure 18.** Canine cutaneous melanoma with junctional activity (H&E, 400x).

### **6.7. Intraepithelial neoplastic cells**

The presence of intraepidermal tumoural cells can be graded as absent, slight (25% of neoplastic melanocytes in epidermis), moderate or prominent (more than 50% of tumour cells are present in the epidermis) [44]. However, this feature appears not to be of prognostic significance [8].

In a recent study was found that the samples of canine melanomas presented medium to prominent scatter of intraepidermal melanocytes, lower pigmentation, and higher nesting of intraepidermal melanocytes, in comparison with melanocytomas [44].

In most melanocytomas, the overlying epidermis is hyperpigmented, regardless of the presence or absence of intraepidermal clusters of neoplastic melanocytes [43].

### **6.8. Ulceration**

Canine melanomas are most frequently ulcerated [44] and particularly larger masses [43] than melanocytomas.

According to Laprie and team [55], ulceration might be taken as a prognostic marker for this neoplasms. In the referred work, the presence of an ulcerated epidermis was associated with a shorter survival time ( $p = 0,0023$ ) and shown to be an independent prognostic factor ( $p = 0,0065$ ) [55]. However, two other studies found no correlation between ulceration and clinical evolution of lip, nail bed [27] or cutaneous melanocytic neoplasms [12,27].

### **6.9. Level of infiltration/invasion**

Melanocytic tumours strictly limited to the dermis, with a shallow depth, are associated with a greater survival time ( $p < 0,0001$ ), and deep level of infiltration has been shown to be a significant prognostic factor ( $p = 0,0012$ ) [55]. One other study that evaluated the level of invasion (in cutaneous and subcutaneous tissues) concluded that tumors confined to the



superficial dermis were associated with a benign course in 94% of cases. On the other hand, tumors reaching the deep dermis and the subcutis showed a malignant behavior; however, the sample number were too low to allow for a conclusion [12].

### 6.10. Necrosis

Necrosis is a common feature, particularly in larger masses [43]. The presence of necrosis was correlated with malignancy and with a short survival time in a study set of 389 melanocytic neoplasms containing both benign and malignant lesions from various locations (mouth, feet and lip, skin) [8]. In other report with a set of 38 malignant melanomas from various locations, no correlation was found with survival time [54]. In summary, necrosis is considered of limited prognostic value in animals [7].

### 6.11. Morphologic classification

Melanocytomas include dermal, compound, and balloon cell tumors, as well as multiple dysplastic melanocytoma syndrome in dogs [43].

Dermal melanocytoma are strictly intradermal in location and larger tumours may extend into the subcutis [43]. Melanocytomas are generally composed by spindle cells disposed in bundles, nests and whorles, with a moderate cellular concentration and a lack of stromal collagen [43,45]. Melanophages might be dispersed throughout the tumoral nodule or in aggregates [43]. Mitotic figures are occasionally seen (inferior to 1 per 10 high power fields), and mitotic atypia is not observed [43].

Some dermal melanocytomas are composed of epithelioid or dendritic cells that are heavily pigmented [43]. Nuclear morphology may be obscured by the large amount of pigment [43]. Although nuclei may be large, there is minimal nuclear pleomorphism [43].

A compound melanocytoma has a wedge-shaped configuration, and its description refers to the fact of including both junctional and dermal components. A numerous and densely packed tumor cell population is present in the dermis, while a variable amount of tumor cells accumulate in clusters and nests within the epidermis, along the dermal–epidermal junction and in the outer follicular wall – this pattern is referred to as ‘junctional activity’ [43].g

Balloon cell melanocytoma have a dermal location, and are predominantly composed of large round cells [43,45], although some fusiform or polygonal melanocytes might be present [42], with an abundant, pale eosinophilic and finely granular cytoplasm [42,43,45]. These lesions often lack readily visible pigmentation; however, dust-like melanin granules may be detected in small numbers [42,43]. Nuclei are small, uniform, and ovoid [43,45] and mitotic figures are rarely observed [42,43].

Multiple dysplastic melanocytoma syndrome mostly resemble compound melanocytomas on low magnification. However, their incidence increases in larger lesions. Also, cytologic atypia and mitotic figures are present and some of the proliferating melanocytes have irregularly shaped and enlarged hyperchromatic nuclei [43].

Canine melanocytoma–acanthomas are mixed tumors that are composed of a benign melanocytic proliferation, resembling compound melanocytoma, and a benign epithelial proliferation [43,45]. The epithelial component usually appears follicular and resembles an isthmus-type tricholemmoma or infundibular keratinizing acanthoma [43]. The epithelial population forms a mass in the dermis composed of cords and nests with occasional small cystic structures containing keratin [45]. Melanocytic cells form nests in the epidermis and sometimes in the cords of epithelial cells within the dermal mass; melanocytic spindle cells can form whorls and bundles between the epithelial cords and nests [45]. A dermal melanocytoma–acanthoma has been immunophenotyped in a German Shepherd dog identifying the presence of keratinocytes and melanocytes [56].

Dermal melanomas have no junctional activity, but in some cases the tumour might extend deeply into the subcutaneous tissue. There might be a predominance of a certain cell type, but most tumors reveal a mixture of spindle cells, round to polygonal cells, and/or epithelioid cells. While spindle cells are poorly pigmented, more round and epithelioid cells tend to have a moderate to abundant amount of melanin granule [43]. Other important features in this kind of neoplastic lesions include a marked nuclear pleomorphism, nucleolar prominence, a moderate mitotic rate (3 or greater per 10 high power fields), atypical mitotic figures, asymmetry of the tumor nodule and a lymphoplasmacytic cell population [43].

Melanomas have an obvious ‘junctional activity’ pattern, with tumor cells distributed through the dermal–epidermal junction, as well as at higher levels of the epidermis, particularly in those of the nail bed and lip [43]. The intraepidermal element is mainly composed of epithelioid melanocytes, disposed individually or arranged in nests and clusters [43]. Tumours with numerous melanocytes distributed through all levels of the epidermis are referred to as lesions with a ‘pagetoid’ pattern [43]. Other melanomas present numerous individual neoplastic melanocytes present within the basal cell layer only, which is also referred to as an ‘atypical lentiginous infiltrate’ [43].

Spindle cell and desmoplastic melanomas, a subgroup of dermal melanomas, is composed predominantly of spindled melanocytes densely packed, or arranged loosely within abundant pale stroma [43]. Occasionally, there is a prominent fibroblastic component associated with the spindle shaped melanocytes, and collagen may become more abundant than the tumor cells [43]. The vast majority are amelanotic, thus a Fontana–Masson stain usually is necessary to detect the presence of melanin granules; cells are usually arranged in bundles or palisades, mimicking tumors of neural origin [43].

Balloon cell melanoma (clear cell melanoma), possess large cells with a clear eosinophilic cytoplasm [43]. The majority of balloon cell melanomas are amelanotic [43]. Some dust-like melanin granules may be present in a few tumor cells, and Fontana–Masson staining may be required for their demonstration [43,45]. These cells have a large vesicular nuclei with a prominent nucleoli [7,43]. Mitotic activity is generally low [43,45] and these dermal masses exhibit no junctional activity [45].

Signet-ring melanomas are composed of round to polygonal cells [45,52], with a pale eosinophilic cytoplasm and a darker periphery [52]. The nuclei are vesicular, crescent shaped and



located at the periphery, giving the cells the appearance of signet-rings [52]. Nucleoli are prominent and occasional multinucleated cells may be present [45].

## 7. Histochemical diagnosis

The histological diagnosis of melanoma can be a challenge for the pathologist, especially in amelanotic tumours. On the other hand, in tumours heavily pigmented the observation of cellular features could be very difficult, requiring the use of bleaching, a histochemical method where melanin is extracted [7].

In cases where the diagnosis of a melanocytic tumor is not evident, histochemical methods specific for the cells producing melanin, such as DOPA (dihydroxyphenylalanine) reaction can be used [7,43].

## 8. Molecular diagnostic methods

A diagnosis of melanocytic tumors in dogs is not always easy to obtain only by conventional histological methods. Melanoma is often similar to other tumours types and has a highly variable histologic pattern which implies an accurate differential diagnosis. Furthermore, the distinction between benign and malignant tumors is not always easy [7]. Thus, it is essential the research of additional tools that can be used in melanocytic tumours diagnosis and to achieve a more accurate prognosis [57].

### 8.1. Classical diagnostic markers

Several markers are used to evaluate the presence of proteins normally found in melanocytes or in cells of neuroectoderm origin. The most common antibodies used are S-100 protein, *melanoma-associated antigen* (Melan-A)/MART-1, HMB-45, MEL-1, vimentin, *Neuron Specific Enolase* (NSE), microphthalmia transcription factor (MiTF), PNL2, tyrosinase, and tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2). Tumor cells are usually positive for vimentin, S100, neuron-specific enolase, and Melan-A, and negative for cytokeratin [7]. However, there is a heterogeneity of antigen expression. The majority of melanomas are S100 positive, but other tumour types are also positive to this protein. NSE is also positive in smooth muscle and neuroendocrine tumours. Melan-A and HMB-45 expression is not a constant in every canine melanocytic tumours. Vimentin is expressed in other tumours as sarcomas [7,58-61]. HMB-45, tyrosinase, and tyrosine hydroxylase showed 100% specific but low sensitivities. One study refers that PNL2, TRP-1, and TRP-2 seems to be highly sensitive and specific for the diagnosis of canine amelanotic tumours, but it was only performed in oral melanomas [62].

In the absence of an ideal marker that excludes definitively other tumour types, confirms a diagnosis of canine melanoma and positively reacts with tumour cells in all melanomas, a diagnostic panel must be performed, including different antibodies [58-61].

## 8.2. Growth fraction

The proliferative activity has provided valuable information on cell growth kinetics and consequently tumour behavior in melanocytic tumours [63]. There are some markers that estimate tumor proliferation, by identifying steps associated with cell cycle [64].

MIB-1 is the monoclonal antibody which is reactive against Ki-67 nuclear antigen, a protein present in all active phases of the cell cycle (G1, S, G2 and M) while being absent in resting phase (G0) [65,66].

In canine melanocytic tumours, Ki-67 may be useful in distinction between benign and malignant tumors: melanocytomas seem to have a significant lower growth fraction than malignant melanomas [67]. Furthermore, KI-67 could be an important prognosis factor in canine cutaneous melanocytic tumours [64,68].

## 8.3. DNA ploidy

Changes in DNA content may reflect chromosomal alterations and represent tumour genetic instability [69-72]. Changes in the DNA ploidy constitute an early event in carcinogenesis [73,74] and detection of aneuploid cell population of pre-neoplastic lesions can be considered a factor risk of the emergence of malignant tumours [75]. Although usually benign tumors were diploid [76] and aneuploidy were malignant [77], diploidy is not synonymous of a benign behavior [78]. Moreover, not all malignant tumors are aneuploid [79,80].

There are few studies about the ploidy assessment in canine melanocytic tumors [81,82] and its usefulness is still discussed. DNA index and ploidy balance seem to provide an additional tool to evaluate melanocytic tumors, being useful in the distinction of benign and malignant melanocytic tumours, mainly in amelanotic lesions [82]. Flow cytometry apparently has a limited utility for predicting the biological behavior of pigmented canine melanomas. DNA content and nuclear morphometric variables have little value in predicting survival time [83].

## 8.4. c-kit expression

The c-Kit protein (CD117), a transmembrane receptor that belongs to RTK III family, is a growth factor for melanocyte migration and proliferation. A loss-of-function KIT mutation are usually related with human melanocytic tumors [84,85].

In canine cutaneous melanocytic tumours, c-kit immunolabeling (both extension and intensity) were generally higher in melanocytomas than in malignant melanomas. The lack of c-kit expression in canine cutaneous malignant melanomas might be used as a criteria of tumor aggressiveness, helping to achieve a proper diagnosis [86].

## 8.5. Matrix metalloproteinase 2 and 9 expression

MMPs are zinc- and calcium-dependent proteases that promote not only the disruption and remodeling of structural barriers [87-89] but also a response to signaling molecules, acting as ligand for cellular adhesion receptors [90-92].

MMP-2 is widely distributed and constitutively expressed by most cells [93,94]. This protease has major roles reducing cell adhesion, stimulating cell migration and differentiation, and acting as an anti-inflammatory factor [95]. MMP-9 expression is normally induced, while almost MMPs are constitutively secreted after their translation [93,96], and may act as anti- or pro-inflammatory factors [95].

MMPs play a pivotal role in cancer development and progression [92,94,96-98] contributing to tumour proliferation, invasion, intravasation into circulation, extravasation, migration to metastatic sites and angiogenesis [90,94,99-101], deregulate the balance between growth and antigrowth signals in the tumour microenvironment [97,102,103], orchestrate inflammation [94,102,104], and evade apoptosis [89,91,102].

In canine cutaneous melanocytic tumours, MMP-2 and MMP-9 may be taken as a complement to histology in tumour diagnosis, especially in borderline lesions. Both MMP-2 and MMP-9 were expressed in the majority of canine cutaneous melanocytic tumours. MMP-2 is most commonly expressed in melanocytomas than in melanomas [105]. MMP-9 was overexpressed in malignant melanomas, compared with its expression in melanocytomas [106].

Additionally, in canine malignant melanomas a switch may occur in the MMP expression profile during tumour progression; meaning that the aggressiveness, evaluated by nuclear grade, seems to be associated with a decrease of MMP-9 and an increase of MMP-2 expression [105].

## **8.6. Inflammatory cells associated to tumour**

The tumour associated inflammatory infiltrate may be modulate and determine tumor behavior [107]. The most studied cells in CCMT are macrophages and T-lymphocyte, however, studies on the matter are scarce.

### *8.6.1. Tumour Associated Macrophages (TAMs)*

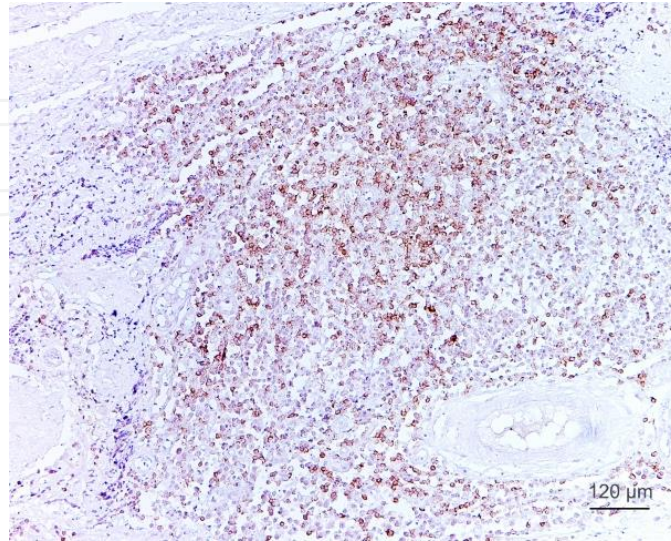
Macrophages constitute the most abundant leukocytes in the tumor environment, recruited by a number of chemoattractants that are produced by the tumor cells and tumor-associated stroma [108,109]. TAMs play a critical role in tumour progression and invasion by inducing neovascularization, suppressing immunocompetent cells and supporting cancer stem cells [110-113].

One study published by our group found that canine cutaneous melanocytomas present a lower number of TAMs than malignant melanomas. TAMs could constitute an important marker of canine melanocytic aggressiveness, being implicated in the progression of melanocytic precursor lesions to malignant melanoma [114].

### *8.6.2. T-Lymphocytic Infiltrate (TLI)*

In spite of the fact that the role of the T-lymphocytic infiltrate in cancer tumorigenesis remains controversial, several studies showed that the presence of TILs are related to tumoural behavior in different tumour types [115,116].

Preliminary studies of our group showed that there is a difference between TILs in benign and malignant melanocytic neoplasms, whereas all melanocytomas presented little or even absence of TILs and melanomas had a more intense TILs, (Figure 19), [117].



**Figure 19.** Abundant CD3+TILs in canine cutaneous melanoma (IHC, 100x), courtesy of Dr. Patricia Monteiro.

### 8.7. E-cadherin/ $\beta$ -catenin

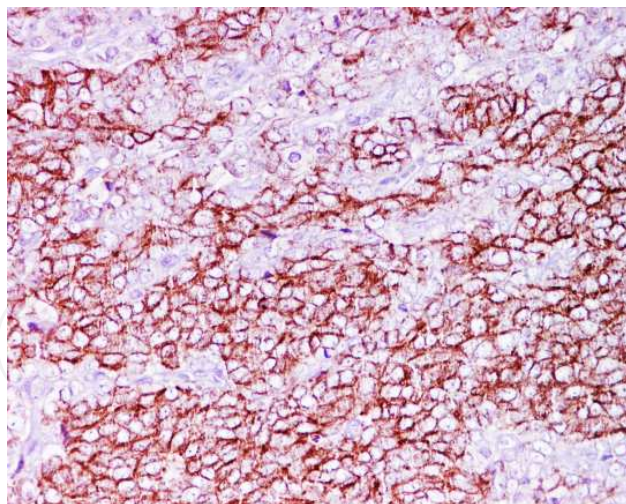
Epithelial cadherin (E-cadherin) is a transmembranar glycoprotein which belongs to a family of cell to cell adhesion molecules dependent of the present of calcium molecules to bind cytoskeleton proteins through catenins. A decreased or altered expression of E-cadherin molecules often represents an increased invasiveness of tumour cells and ultimately malignancy in animal and human epithelial tumours [118,119].

In canine melanocytic tumours, the benign lesions present a membranous labelling (Figure 20) and the malignant ones an erroneous labelling, with a cytoplasmatic predominant immunostaining. Additionally, a loss of E-cadherin expression is noted in melanoma [120]. A loss of membrane E-cadherin/ $\beta$ -catenin complex is also detected in canine melanoma showing that a disruption of E-cadherin/ $\beta$ -catenin complexes and a increase of  $\beta$ -catenin may be associated with canine melanocytic tumours progression and aggressiveness [121].

### 8.8. Cox-1 and Cox-2 expression

Cyclooxygenase (COX), also known as the prostaglandin H-synthase, is an enzyme involved on prostanoids biosynthesis. Cox-1 and Cox-2 are the two cyclooxygenase isoforms identified to date, similar in its structure but produced by different genes. The biological functions are also different: Cox-1, constitutively expressed in many tissues, plays an important role in the regulation of normal physiological, while Cox-2 is usually absent from normal cells but induced by growth factors, inflammatory reactions, tumour promoters and oncogenes [122-125].





**Figure 20.** Strong membranar E-cadherin expression (IHC, 200x), courtesy of Dr. Mariana Santos.

Cox-1 and Cox-2 expression was recently described in canine melanocytic tumours [126-128]. Cox-1 is expressed in almost every tumours, both benign and malignant melanocytic skin lesions. Regarding Cox-2, melanocytomas did not present a positive immunolabelling, but in melanomas Cox-2 expression was present in more than 50% of the tumours [114,127]. The differences observed suggest that Cox-2 expression could be a useful tool in canine melanoma diagnosis, particularly in borderline lesions.

COX-2 expression was also observed in tumours with epithelium ulceration, necrosis, high mitotic index and nuclear grade and in less pigmented neoplasms, which could represent the higher aggressiveness of Cox-2 positive melanocytic tumours. In canine malignant melanomas, Cox-2 is associated with a higher cellular proliferation [114]. Besides the relation with tumour behavior [127], Cox-2 over-expression relates with a poor overall survival [129].

## 8.9. Angiogenesis

Vascular system is essential in oxygen and nutrients supply, elimination of metabolism products and promoting efficient access of leukocytes [130].

Angiogenesis is a complex process by which new blood vessels develop from pre-existing vasculature [131-133]. It is a fundamental requirement for organs development. Angiogenesis is also implicated in the pathogenesis of different pathological alterations, such as cancer and inflammation [134-136].

The tumor-associated neovascularization, by establishing continuity with the systemic circulation, allows tumor cells expressing their critical advantage in growth and facilitates metastization [137-139].

### 8.9.1. Vascular endothelial growth factor

Angiogenesis is a delicate process, tightly regulated by the balance of pro- and anti-angiogenic factors [140]. Among the angiogenic factors, VEGF is the more powerful and ubiquitous

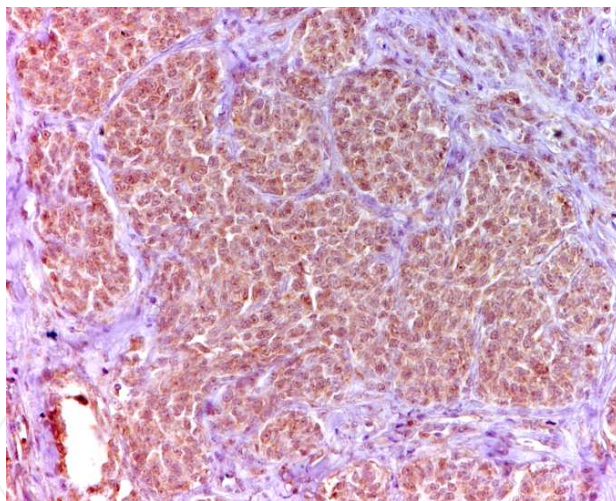


vascular endothelial growth factor, capable of inducing proliferation, migration, specialization and survival of endothelial cells [141,142]. Functions of VEGF family members related to neoplastic pathogenesis are linked not only to its angiogenic capacity [143], but also with the lymphangiogenesis [144,145], immunosuppression [146-148], stimulation and recruitment of endothelial and hematopoietic precursors of the bone marrow [149,150] and anti-apoptotic activity [150].

In canine melanomas, the only study published on VEGF expression is in oral melanomas. High blood concentrations of VEGF were correlated to a shorter survival time in dogs receiving definitive therapy and were associated with tumour stage [151].

This is a promising area, since VEGF may be a good indicator of preneoplastic change in melanocytic lesions [137,152]. VEGF plays a role in human melanoma progression [153,154] with a strong involvement in the switch from the radial to the vertical growth phase and the metastatic phase. So, anti-angiogenic agents might even interfere with or block melanoma progression [155].

Preliminary studies performed by Gomes J and Pires I (data unpublished) show that VEGF may be useful as a discriminating factor between malignant melanoma and benign, since it is more intensely expressed in melanomas (Figure 21) than in melanocytomas.

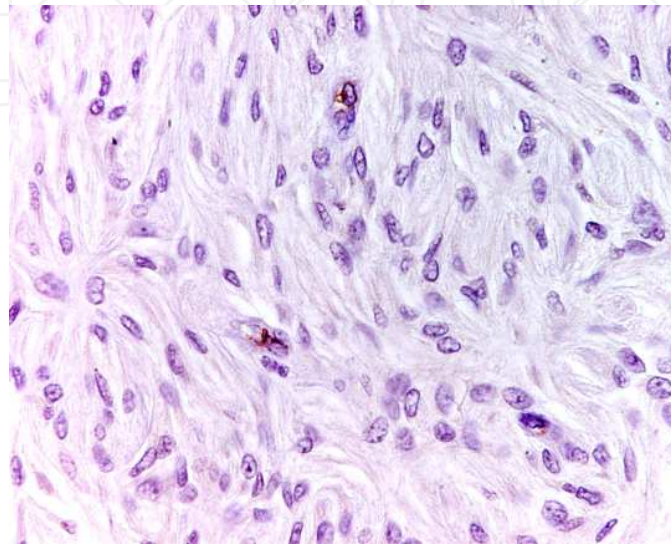


**Figure 21.** Canine cutaneous melanoma with a strong and diffuse VEGF expression (IHC, 200x), courtesy of Dr. Joana Gomes.

#### 8.9.2. *Microvessel Density (MVD)*

Tumor angiogenesis can be estimated through a quantification of microvessel density (MVD). The most widely used method is the immunohistochemical methods, in which specific markers for endothelial cells are employed, as von Willebrand factor (Figure 22), CD34 and CD31 [156-158].

MVD seems to be important for diagnostic purposes in canine MT's. MVD is significantly higher in melanoma than in melanocytomas [159] and its expression has been associated with a high mitotic index, necrosis and ulceration (study performed by Gomes J and Pires I, data unpublished). However, its prognostic significance is still discussable [159,160].



**Figure 22.** Tumoural neovessels positive to von Willebrand factor (IHC, 400x), courtesy of Dr. Joana Gomes.

## 9. Conclusions

The incidence of melanoma is increasing annually both in man and in dog. Given that dogs and humans share the same environment and similarities between human and canine melanoma it is urgent to discuss common mechanisms in melanoma development in both species.

Melanoma diagnosis in dogs can be challenging due to the variety of its histological appearances, especially when pathologists are facing amelanotic or metastatic lesions. Although the definitive diagnosis of a melanoma is often difficult by the lack of specific markers that can distinguish these lesions, immunohistochemistry plays a key role in the differential diagnosis with other neoplasms.

Additionally, the distinction between benign and malignant melanocytic tumours is not always easy, especially in borderline lesions, thus the importance of a strong knowledge of new markers of malignancy for the establishment of a definitive diagnosis and a correct therapy management and prognosis establishment.

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