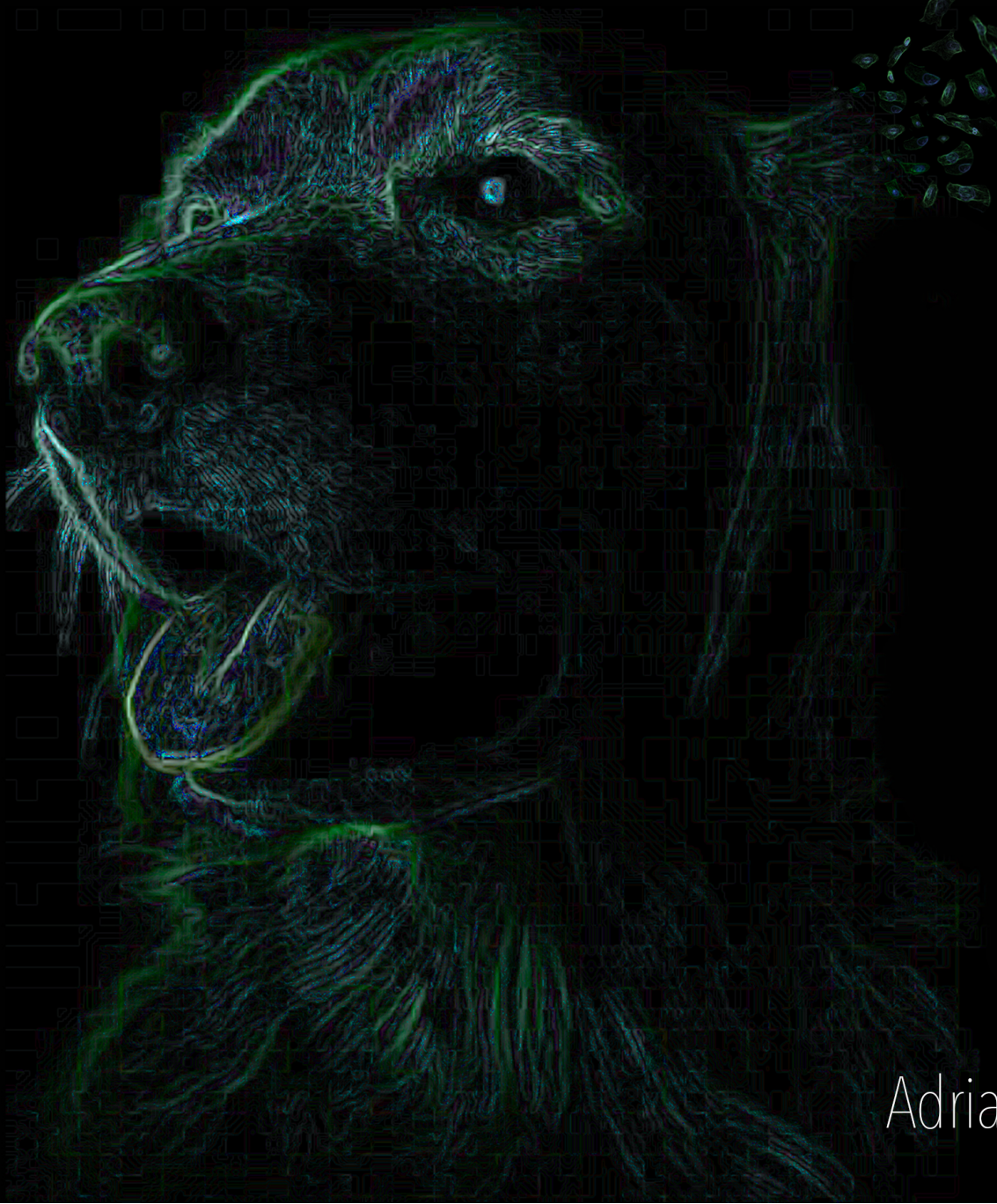
THE CANINE MELANOCYTIC LANDSCAPE:

FROM EPIDEMIOLOGICAL INSIGHTS TO CELLULAR COMPLEXITY IN NORMAL AND NEOPLASTIC MELANOCYTES



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The canine melanocytic landscape: from epidemiological insights to cellular complexity in normal and neoplastic melanocytes

Il mondo dei melanociti canini: dall'epidemiologia alla complessità cellulare nei melanociti normali e tumorali

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A Tatino,

C'è chi sogna di incontrare gli extraterrestri e non ha mai avuto un cane o un gatto e non sa che cosa ha perso, di quanto affetto e intelligenza sono capaci. Non conoscere e non amare gli animali è una grave perdita per la nostra stessa vita e felicità.

Margherita Hack

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Premise and aims of the project

Canine melanocytic tumors have been studied for several decades and are among the most aggressive cancers in this species[107,133]. These tumors tend to invade adjacent tissues and metastasize, often leading to a poor prognosis[132,133]. In dogs, melanoma is the most common malignancy in the oral cavity, accounting for 30% of all oral neoplasms, while cutaneous melanoma represents about 4% of all skin tumors[17,107,135,175]. Moreover, over the years, molecular studies have demonstrated that canine melanomas can serve as a spontaneous model for studying the human equivalent[13,63,154]. This knowledge holds significant promise for the advancement of translational medicine, which aims to bridge the gap between laboratory research and clinical application. Indeed, canine and human melanomas share similar biological behavior and histological characteristics. This is particularly true for a subset of human melanocytic tumors not induced by UV radiation, such as mucosal melanomas because these neoplasms also exhibit genetic and molecular similarities between the two species[113]. As a result, interest in studying canine melanomas has increased. This has led to research focused on understanding tumorigenesis, tumor progression, and metastasis formation with the ultimate goal of identifying effective treatments that could prolong survival times or even achieve complete remission[5,132,139]. In recent years, treatments for melanocytic tumors have shifted away from traditional chemotherapy, which has limited efficacy against this cancer. With the advent of immunotherapy and the growing understanding of aberrantly activated pathways in tumors, research has increasingly focused on investigating the molecular processes of neoplastic cells[139]. The aim is to identify new target molecules that could potentially reverse tumor progression. To achieve this, it is critical to consider not only the neoplastic characteristics of melanocytes but also their physiological behavior to better distinguish between normal and abnormal functions.

This Ph.D. thesis represents a comprehensive study of canine melanocytic tumors, aiming to identify similarities between canine and human melanomas and to exploit these insights for translational research.

Although the individual studies use diverse methodologies and focus on different aspects of melanocytic tumors, they are unified by a common goal: identify and characterize both normal and pathological melanocyte populations across various

anatomical sites to understand the biology of normal and neoplastic melanocytes and to elucidate the mechanisms driving tumorigenesis and metastases formation.
Epidemiology and distribution (Chapter 2). The epidemiological study provided a foundation for understanding the prevalence and geographic distribution of melanocytic tumors in central Italy. This data is essential for risk assessment and targeted prevention strategies. By identifying regions with higher prevalence, we can explore potential underlying environmental, genetic, or biological factors that may influence melanocytic transformation and tumor progression.
Normal melanocyte biology (Chapters 3 and 4). The characterization of normal melanocyte populations and the quantification of normal melanocytes based on the keratinocyte-to-melanocyte ratio across different anatomical sites was crucial to establish a baseline for comparison with neoplastic cells. By examining the expression of melanocytic markers in skin and oral mucosa, we gained insights into the heterogeneity of normal melanocytes and their potential role in tumorigenesis.
Gene expression profiling to understand tumor progression mechanisms (Chapter 5). The single-cell RNA sequencing study aimed to provide a high-resolution view of the transcriptional landscape of normal melanocytes across different body regions. Additionally, by comparing normal and neoplastic cells, this work can reveal key molecular pathways involved in melanocyte differentiation, proliferation, and transformation, which may underlie different propensities for malignancy and metastasis among melanocytes from different anatomical sites. The study is ongoing, but the preliminary results obtained are promising and demonstrate strong technical quality. The high quality of the samples suggests that the findings will be reliable and provide a solid foundation for further analysis.
Advancing tumor models through innovative culturing techniques (Chapter 6). The establishment of two- and three-dimensional cell culture models from fine needle aspiration (FNA) samples represents a significant advancement in the field. By allowing the in vivo replication of the tumor microenvironment, this innovative approach provides a practical and minimally invasive method for studying canine melanocytic tumors. By analyzing the behavior of these cells *in vitro*, we can gain insights into tumor biology and test the efficacy of potential therapeutic agents.
Molecular mechanisms of tumor progression (Chapters 7 and 8). The investigation of specific signaling pathways, such as Rac1 and JAK-STAT3, has shed light on the molecular mechanisms underlying tumor progression and metastasis. By focusing on these shared pathways, the research not only contributes to the understanding

of canine melanocytic tumors but also identifies shared pathways between canine and human melanomas. This study highlights the translational potential of the canine model and its utility in developing novel therapeutic strategies for both species.

Conclusion. In summary, this Ph.D. thesis integrates epidemiology, cellular characterization, molecular profiling, in vitro modeling, and pathway analysis to advance our understanding of canine melanocytic tumors epidemiology, biology, and molecular mechanisms. By revealing the numerous similarities between canine and human melanoma, this Ph.D. studies reinforce the unified vision of the canine model as a valuable tool to inform human medicine with the potential to support the pursuit of novel, cross-species therapeutic strategies.

Introduction

Melanocytes are dendritic cells of neuroectodermal derivation, present in all vertebrates. They are specialized in the production of melanin, a finely granular brown pigment, which contributes to the pigmentation of various tissues, such as the skin, eyes, hair, fur, and feathers of animals[95,100]. Pigmentation plays a critical role in protecting tissues from ultraviolet (UV) radiation damage. In addition to their protective function, melanocytes contribute to animals' visual appearance and camouflage, influencing their survival and reproductive success. Although melanocytes on the skin's surface are far fewer in number compared to epithelial cells, their phenotypic significance is considerable[81]. Additionally, the presence of these cells has been demonstrated in the ear, meninges, heart, and bones[12,91,119,149] where they contribute not only to pigmentation but also to the specific functions of the respective organs (hearing, balance, neuroendocrine functions, vision, etc).

1.1 Origin of melanocytes

Melanocyte development is a complex and intricate process involving the activation of numerous pathways and approximately 200 different genes[95]. The timing of these signaling events is different between species. The evolution of these cells begins in the most dorsal portion of the neural tube from precursors known as neural crest cells, which express SOX10, a marker that remains positive in both melanoblasts and glial cell progenitors. Melanoblasts are intermediate cells that eventually mature into fully functional melanocytes, capable of producing melanin[81]. Key players in their development are the dopachrome tautomerase (Dct) and the microphthalmia-associated transcription factor (MITF). Numerous studies have demonstrated that the absence of MITF prevents melanocyte differentiation[82,138], indeed, it is required for the survival of migrating melanoblasts[81]. The activation of MITF occurs early during the transition from pluripotent neural crest cells to melanoblasts, involving various molecules and pathways. The upregulation of WNT3A gene and downregulation of bone morphogenetic protein 4 (BMP4) and SOX2, for example, appear to activate MITF and induce melanoblasts formation[3,68,142]. Additionally, also factors like PAX3 and SOX10 promote the transcription of MITF[21,54]. Other specific factors necessary for

melanoblast migration and proliferation in the dermis are endothelin receptor type B (EDNRB) and its ligand endothelin B[78]. It is also important to remember that these cells need to change the expression of adhesion molecules during their migration. In fact, in the early stages, before migrating, they express N-cadherin. However, when they start migrating through the dermis, melanoblasts express a high level of E-cadherin and a low level of P-cadherin[97]. Upon reaching the hair follicles, there is a switch from high E-cadherin/low P-cadherin to E-cadherin-negative/medium-high P-cadherin expression[97]. Other pathways activated during these processes include Scar/WAVE and RAC1, which facilitate the cytoskeleton rearrangement and cellular movement through the formation of lamellipodia[77,79]. Another factor identified as playing a role in melanocytic lineage differentiation is FOXD3. Overexpression of this factor in the later stages of migration prevents melanoblast differentiation, while its knockdown increases cells following the melanocyte lineage (Lee et al., 2003). FOXD3 interacts with PAX3 and prevents it from binding to and activating the MITF promoter. This factor is activated in the early stage of melanocyte development by ZIC1 and PAX3[106] and its activity is subsequently maintained by the expression of SNAIL9 and SOX9[99]. Driven by these activated factors, these cells migrate from the neural crest following a dorsolateral and a ventral pathway through the dermis to reach their final destinations. Precursors that leave the neural crest with a certain delay undergo melanoblast differentiation, while those that leave early become neurons and glia[40]. Dorsolateral migration also requires the activity of the receptor tyrosine kinase KIT and its ligand (KITL)[170]. Ventrolateral migration involves a second wave of Schwann cell/melanoblast bipotential precursors that leave the neural crest to generate mature melanocytes in the skin of the trunk and limbs. This subset of cells also expresses MITF and SOX10[2].

At the end of the embryogenesis, there will be two different populations of melanocytes: differentiated, mature melanocyte populations, responsible for the pigmentation of skin, hair, and fur at birth, and melanocyte stem cell populations, responsible for maintaining pigmentation in the adult. The latter are present in the bulge area of hair follicles, and they can migrate from that location to the basal layer and differentiate into mature epidermal melanocytes. melanocyte stem cells niches are also present in other skin structures, such as sweat or sebaceous glands, and the dermis[18].

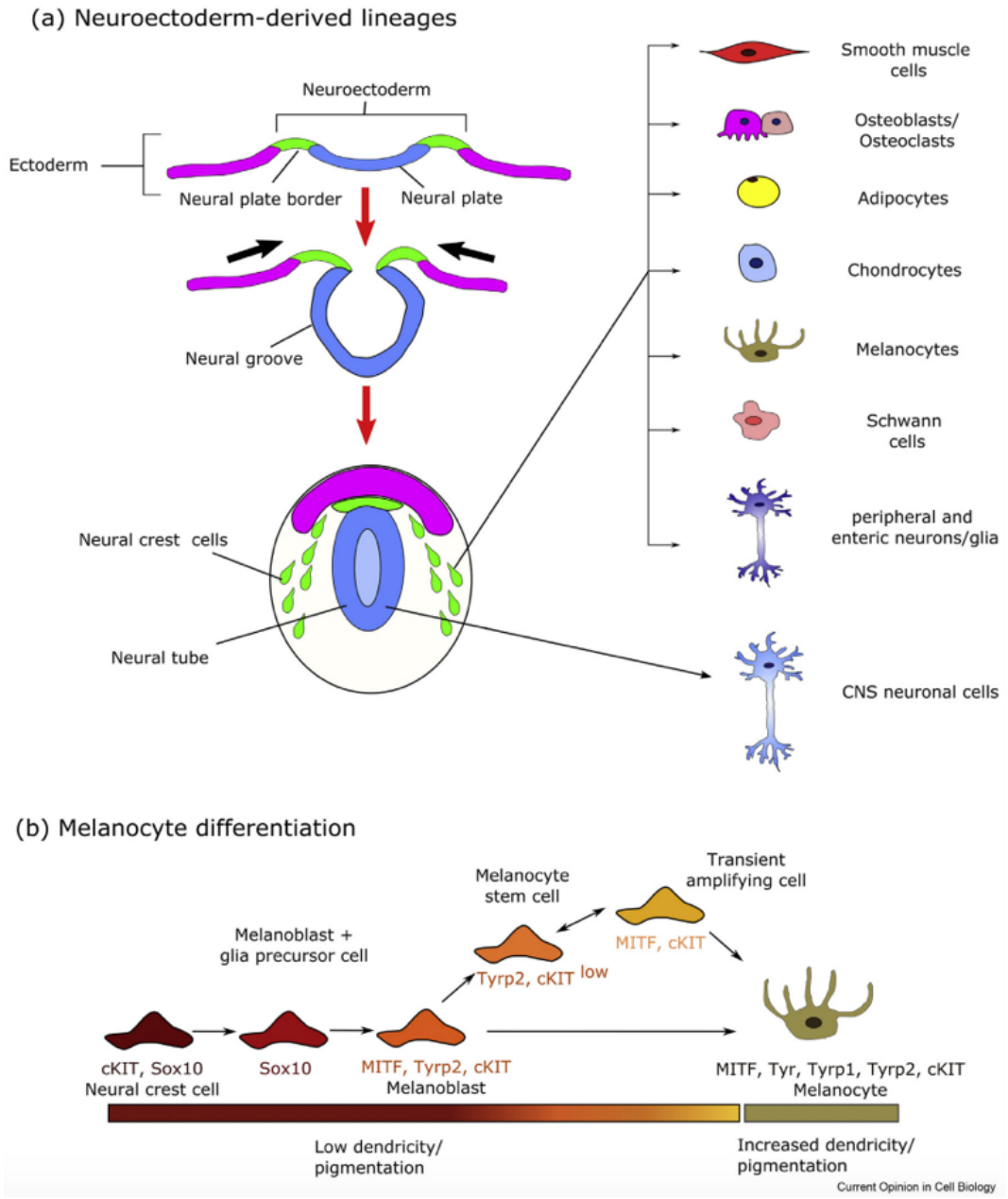

Figure 1. The picture shows the different lineage deriving from neuroectodermal cells (a) and more in detail, the molecular steps of the melanocyte differentiation.
Photo credit: Li M, Knapp SK, Iden S. Mechanisms of melanocyte polarity and differentiation: What can we learn from other neuroectoderm-derived lineages?[80]

1.2 Function of Melanocytes

As previously mentioned, melanocytes are cells specialized in melanin production. This non-protein macromolecule is synthesized through a series of reactions regulated by a specific pathway known as the Raper–Mason pathway, beginning with the precursor amino acid tyrosine[176]. This process, called melanogenesis, occurs in specialized membrane-bound vesicles called melanosomes. Melanosomes are pigment organelles of vertebrates belonging to the lysosome-related organelle (LRO) family, with the crucial function of synthesize melanin while retaining all the toxic intermediates of melanin synthesis within their membranes[95]. Melanogenesis is tightly regulated by factors that facilitate communication between melanocytes and other cells such as keratinocytes and dermal fibroblasts. These interactions regulate the activity, localization, and level of the enzymes involved in the reaction. Some of these factors include melanocyte-stimulating hormone (α-MSH), endothelins (Edn), basic fibroblast growth factor (β-FGF), nerve growth factors (NGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), transforming growth factor beta (TGFβ), and Jagged1/2[64]. The expression of these molecules is regulated by external factors such as UV radiation. These mechanisms lead to the activation of the Raper–Mason pathway which can be divided into two main phases. The first one starts with the precursor tyrosine and is catalyzed by the enzyme tyrosinase, which firstly hydroxylates tyrosine to dihydroxyphenylalanine (DOPA), that is subsequently oxidated to dopaquinone[18]. In mammals and birds, melanin comes in two forms: eumelanin, which is dark brown to black, and phaeomelanin, which is yellow to red. After the dopaquinone formation, the pathway diverges to synthesize one of these two pigments. The subsequent steps depend on the availability of the substrates and the activities of melanogenic enzymes. Crucially, molecular signals influence eumelanin or pheomelanin production. The process begins with the melanocortin 1 receptor (MC1R), expressed on melanocytes, and its ligands, α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotrophin (ACTH)[95]. Activation of MC1R increases tyrosinase activity, shifting from the production of pheomelanin to the synthesis of eumelanin. This second phase starts from the intermediate dopachrome, which, through the enzymes dopachrome tautomerase and tyrosinase-related protein 1, is catalyzed into eumelanin. Pheomelanin production,

on the other hand, begins from dopaquinone and it is dependent on cysteine cysteine or glutathione[18]. In the presence of this amino acid, indeed, the dopaquinone can undergo enzymatic transformation into pheomelanin intermediates. During these processes, melanosomes increment their pigmentation and mature, with the final aim of transferring the melanin granules to the nearby keratinocytes in the skin, hair, or feathers[37,159]. Mature melanosomes are transported along microtubules from the perinuclear area toward the tips of dendrites[141]. At the end of this centrifugal movement, melanosomes are transferred from melanocytes to keratinocytes, where they are located in the superior part of the keratinocytes, protecting the nucleus against UV radiation[124]. Several mechanisms of melanin transfer have been observed: exocytosis-mediated, cytophagocytosis-mediated, tunneling nanotube-mediated, and membrane vesicle-mediated transfer. The melanocyte cell body is located on the basement membrane, while dendrites are in contact with 30–40 keratinocytes. These cells together form an epidermal melanin unit[67].

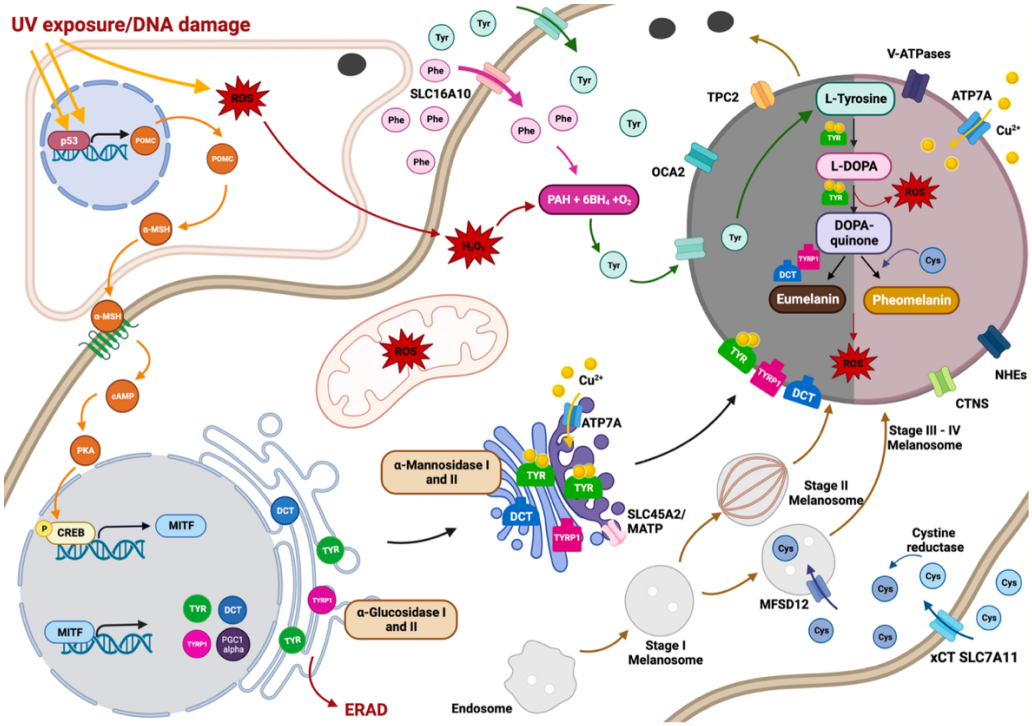

Figure 2. The picture shows the molecular steps involved in the melanogenesis.
Photo credit: Snyman M et al. - The metabolism of melanin synthesis—From melanocytes to melanoma 136

1.3 Canine Melanocytic tumors

Melanocytes can undergo neoplastic transformation, developing into melanocytic tumors[135]. In human medicine, these neoplasms have been studied for a long time, trying to find an effective chemotherapy[27,60]. Indeed, their particularly aggressive nature and ability to metastasize are responsible for a poor prognosis[130]. For this reason, research has continued, leading, in human medicine, to the discovery of driver mutations that allow neoplastic transformation, tumorigenesis, tumor progression, and metastasis formation[34].

In veterinary medicine, progress has been slower, and we are still lagging behind. While epidemiological investigations of these tumors in domestic animals, particularly dogs and cats, began in the 1990s, chemotherapy options were limited at that time[20,88]. Today, we know that melanocytic tumors are prevalent in both domestic and non-domestic animals and share the same biological behavior as in humans[154]. This is particularly true for dogs, companion animals that share habits and lifestyles with their owners and are now even considered potential models for studying these tumors in humans[13,50].

1.3.1 Clinical Features

Melanocytic tumors can be categorized into as benign (melanocytoma) and malignant (melanoma) types. These tumors develop on the skin and mucous membranes[17,56,175]. In dogs, oral melanoma is the most frequent malignant neoplasm in this location, accounting for 30-40% of cases. In contrast, cutaneous melanocytic tumors represent a smaller proportion of skin tumors, comprising around 4%[133,175].

Clinically, this neoplasm often presents as a nodule or a mass lesion, sometimes multilobulated, that can range from a few millimeters to several centimeters in diameter, with a consistency ranging from firm to firm-elastic. Generally, they are sessile, but in some cases, they can be polypoid or plaque-like lesions. If pigmented, the color depends on the quantity of melanin produced: they can range from dark brown or black to soft brown, whereas they appear white in the case of amelanotic tumors[17,133,175]. Large melanocytic tumors, especially those located in susceptible areas, such as the oral mucosa, can develop superficial ulceration. The most common locations for cutaneous melanocytic tumors are the eyelid, lips, and legs (including the nailbed)[56,135,175]. Oral melanoma most commonly develops in the oral

mucosa and the gingiva, but can also arise in the tonsils, oropharynx, and tongue[17]. These tumors are more prevalent in older animals, typically aged 8 to 11 years, but can also affect younger adults[135]. No sex predisposition is reported. Cutaneous melanocytic tumors have been identified in specific dog breeds with highly pigmented skin, such as Cocker Spaniels; Scottish, Boston, and Airedale Terriers; Schnauzers; Doberman Pinschers; Irish Setters; Vizslas; Golden and Chesapeake Bay Retrievers; Irish Setters; Chow Chows; and Boxers[135,175].

Several of these clinical parameters play a crucial role in evaluating the prognosis of melanocytic tumors. Even from the initial signalment, it can be observed that older dogs are more predisposed to developing malignant melanocytic tumors[17,20,133]. Additionally, certain breeds are at a higher risk of developing only the malignant form of the tumor[17,133,175].

Beyond signalment, tumor location is also a significant prognostic factor. Studies have shown that melanocytic tumors of the nail bed and oral cavity tend to have a worse prognosis compared to those located elsewhere[17,56,133,135].

1.3.2 Genetic Features

The genetic features of canine melanocytic tumors have been studied in recent decades. Research has identified genetic mutations and pathways involved in their development and progression, which in some cases mirror those found in human melanomas. Given the biological similarities between canine and human melanocytic neoplasms, studies have focused on identifying the presence of common mutations in human melanoma and in the canine equivalent. These investigations have identified constitutive activation of key pathways, such as PI3K/AKT and MAPK, in both canine and human melanocytic tumors, which are crucial for regulating the cell cycle and tumor growth[25,46]. The PI3K/AKT pathway is regulated by *PTEN,* a tumor suppressor gene that antagonizes PI3K and, therefore, is a negative regulator of the pathway. Loss of function mutations in *PTEN* have been reported in canine melanoma[167]. Similarly, the MAPK pathway, activated by multiple genes, is implicated in human melanoma. While mutations of *BRAF* and *NRAS* have been analyzed in canine melanocytic tumors, they are less common compared to human cases[46,62,125]. However, a small percentage of canine melanomas exhibit $cBRAF^{V595E}$ and $BRAF^{V450E}$ mutations, though not as frequently as in human cases [36,94]. Indeed, $BRAF^{V600E}$, the most common mutation of human cutaneous melanoma, is linked to UV-radiation exposure. Unlike in humans, this

association has not been demonstrated in dogs[93]. In contrast, *RAS* mutations, particularly *KRAS* (codons 12 and 13) and *NRAS* (codons 12, 13, and 61), are more frequently observed in canine melanomas, particularly in digital melanomas[35,62,167]. Interestingly, these *RAS* mutations are mutually exclusive, with no co-occurring mutations in BRAF or c-Kit, suggesting a distinct oncogenic pathway in canine melanomas[35,167].

Recently, research has revealed a high tumor mutational burden in canine oral melanomas, with frequent alterations in the TP53 pathway, which normally exerts tumor-suppressor function[6,62]. Genes involved in this pathway, such as *MDM2*, a proto-oncogene that mainly functions to modulate p53 tumor suppressor activity, and *CDK4*, a regulator of the cell cycle progression, have been found to be frequently amplified in canine oral melanomas, mirroring molecular characteristics of human melanomas[6,112]. While no recurrent mutations were found in the *CCND1* coding sequence, a study demonstrated significant involvement of the Cyclin D1 (CCND1) pathway, suggesting that upstream genetic alterations drive its overexpression[173].

Additionally, the expression of receptor tyrosine kinase protein, known as KIT, has been observed in many canine cutaneous and oral melanocytic tumors. While mutation of *c-Kit*, the associated gene, was linked to deleterious protein effects in some malignant cases, no strong correlation was found between KIT expression and *c-Kit* mutation[26,55,134]. Other genetic alterations, such as mutations in the tumor suppressor gene *PTPRJ*, have been demonstrated in a small number of cases[62,167]. Additionally, a study comparing metastatic and non-metastatic canine oral melanoma identified significant differences in gene expression, with metastatic melanomas showing reduced expression of CXCL12, a gene that encodes for the homonym protein, and increased expression of APOBEC3A, which regulates transcription from interferon-stimulated response elements[24].

Chromosomal analysis has identified recurrent chromosomal alterations in canine melanomas, including gains and losses in specific chromosomes (gains: CFA 10, 13, 17 and 30; losses: CFA 2, 10, 11, 22, and 30)[25,58,111,167].

1.3.3 Histopathology

The diagnosis of melanocytic tumors begins with clinical suspicion, where factors such as pigmentation and tumor location can provide important initial clues. Cytology, followed by histopathology, is typically employed to confirm the

diagnosis, though in certain cases, more advanced diagnostic techniques may be required to reach a definitive conclusion.

As mentioned, melanocytic tumors can be classified into benign and malignant variants in dogs, as in humans and other domestic or non-domestic species. The benign form, known as melanocytoma, is generally easier to diagnose due to its well-differentiated cells, which are often rich in pigment. In contrast, melanoma, the malignant form, can be more challenging to interpret, particularly when pigmentation is absent and cellular atypia is severe[133]. Indeed, in pigmented nodules, cytological examination may suffice for a preliminary diagnosis; however, histopathology is still necessary for a more comprehensive evaluation[48,114]. Amelanotic melanomas, lacking visible pigmentation, pose significant diagnostic challenges, as cellular morphology alone is often insufficient to distinguish these tumors from other neoplasms[133], such as squamous cell carcinomas, lymphomas, fibrosarcomas, or other sarcomas[107]. The characteristics of neoplastic melanocytes vary depending on tumor malignancy and can be appreciated through cytology. In both melanocytomas and melanomas, the cells may exhibit a range of shapes, including polygonal, spindle-shaped, and round. The different cell shapes are associated with varied growth patterns, such as nests or packets, whorls or bundles, and sheets, respectively[175]. The cytoplasm is usually scant, especially in spindle-shaped melanocytic tumors, but can be more abundant in polygonal and round forms. A single neoplasm can contain multiple cellular shapes. The nucleus is typically round, with a nucleolus that is inconsistent in benign forms but prominent in malignant ones. Poorly differentiated tumors show significantly aberrant cellular features change significantly, exhibiting marked anisocytosis and anisokaryosis, and may present binucleated and multinucleated cells[118]. In pigmented tumors, a fine, granular, brown pigment can be observed within the cytoplasm of the cells. If the tumor lacks significant pigmentation, the Fontana-Masson stain can be utilized to highlight even the few melanin granules present. This staining technique is particularly useful in identifying subtle pigmentation that might not be visible with routine histological stains[118].

As previously stated, histopathology plays a crucial role in diagnosing melanocytic tumors by identifying key features. For example, the presence of neoplastic cell proliferation at the dermo-epidermal junction, referred to as "junctional activity," is an important diagnostic clue. Additionally, pagetoid growth, which indicates the

cell migration upward through the epithelium forming intraepithelial nests, further supports the diagnosis[175].

Over the years, research has identified numerous histologic features to assess malignancy and prognosis in both cutaneous and oral melanocytic neoplasms. The mitotic count, defined as the number of mitoses in a field area of 2.37 mm^2, is a crucial parameter for distinguishing between benign and malignant forms[41,76,83]. In oral melanocytic neoplasms has been demonstrated that a mitotic count ≥ 4 correlates with a shorter survival time, while in cutaneous melanocytic tumors, a mitotic count ≥ 3 has been associated with shorter survival times[16,75]. The nuclear atypia is manually assessed by evaluating 100–200 cells at 40× magnification and scoring them as well- or less differentiated. In the literature, it is reported that for cutaneous melanocytic tumors, a threshold of 20% atypical nuclei is linked to more aggressive behavior, while a 30% threshold applies to oral melanocytic neoplasms[16,128,137,155]. Depth of infiltration and vascular invasion are unequivocally key parameters; deeper infiltration is associated with poorer prognosis in both cutaneous and oral melanocytic tumors[28,75,110,121].

Vascular invasion further worsens the prognosis[135]. In cutaneous melanocytic tumors, ulceration is considered a negative prognostic factor, though caution is required in its interpretation, as it could also result from self-trauma or other external causes[132]. Again, in cutaneous melanocytic tumors, the tumor thickness is considered a prognostic factor, assessed by measuring a histologic section of the mass. Indeed, studies have highlighted that a thickness ≥ 0.95 cm is associated with shorter overall survival and a higher mortality risk, while a thickness > 0.75 cm correlates with shorter disease-free survival and an increased risk of recurrence or metastasis. Pigmentation is also a crucial factor[128]. Regarding pigmentation, it was seen that tumors with 50% or more pigmented cells are generally associated with longer survival times, whereas amelanotic tumors tend to have worse outcomes[16,75,128,132].

Taken together, these parameters provide a comprehensive understanding of the tumor's behavior, helping clinicians better predict outcomes and tailor treatments accordingly.

However, there are also borderline cases where a tumor’s classification as benign or malignant is unclear. In such situations, it is highly recommended to perform immunohistochemistry for Ki-67. Indeed, studies have shown that for both cutaneous and oral melanomas, exceeding specific thresholds of Ki-67 positive

nuclei significantly worsens the prognosis[22,28,66]. In cutaneous melanocytic neoplasms, a Ki-67 threshold of ≥ 15% is associated with shorter survival, while in oral melanomas, a threshold of ≥ 19.5 positive nuclei per grid area of a 1 cm^2 optical grid reticle at a magnification of 40x correlates with lower survival times[16,75]. This provides an important tool for distinguishing between benign and malignant forms, allowing for a more accurate prognosis.

Nevertheless, in some challenging cases, a definitive diagnosis to identify the histotype of the poorly differentiated tumor through histology is not possible. In such instances, immunohistochemistry is used as a supportive tool. Various markers are available for diagnosing melanocytic tumors, including Melan-A and PNL-2, which are highly specific[51,117,131]. Additional markers like phosphoribosyl-anthranilate isomerase (TRP)-1, TRP-2, and microphthalmia-associated transcription factor (MITF) are also used but are less frequently expressed in neoplastic cells[133,151]. Other markers, such as human melanoma black (HMB)-45, SRY-Box Transcription Factor 10 (SOX10), and S100 are also common in immunohistochemical panels[33,131].

In highly undifferentiated tumors, immunohistochemistry may not fully clarify the cellular origin, as such tumors may be negative for both melanocytic markers and those for other cell types. In these cases, especially in oral tumors, it has been demonstrated that SOX10 may still be expressed[133]. However, while SOX10 is a sensitive marker, it is not entirely specific to melanocytic cells, which leaves melanoma in the differential diagnosis along with other malignant neoplasms[73,96].

In these challenging scenarios, careful interpretation of histological and immunohistochemical results can help refine the diagnosis, although some cases may still remain inconclusive due to the overlapping expression patterns of certain markers.

1.3.4 Therapeutic approaches

In recent decades, the treatment of melanocytic tumors in veterinary medicine has seen significant advancements. Historically, there was no well-established first-line treatment for melanomas in domestic animals, and traditional chemotherapeutic drugs often showed limited effectiveness, leading to uncertain outcomes[23,52,104]. The primary therapeutic approach of choice for canine melanoma remains **surgical resection**, whenever feasible, ideally with wide margins to ensure the complete removal of the tumor[41,76,104,123]. In addition, the excision of the

sentinel lymph nodes is crucial, as it provides essential information regarding potential metastasis, even in the early stages of tumor spread[104].

Surgery could be complemented by **radiotherapy**, which can also be used as a palliative treatment on its own. Radiotherapy is generally well-tolerated, producing minimal side effects. It is recommended not only for targeting the site of the primary tumor but also for treating the sentinel lymph nodes, regardless of whether they show signs of metastatic involvement[14,104]. While surgery remains the cornerstone of treatment, radiotherapy provides an additional therapeutic benefit, particularly in cases where complete surgical removal is challenging or when the tumor show evidence of metastasis. The combination of these two approaches—surgery and radiotherapy—helps to maximize the chances of controlling the tumor and improving the overall prognosis for the patient[45].

More recently, **electrochemotherapy** has been increasingly utilized, often in combination with surgical resection but also as a standalone treatment for the primary tumor. This technique is a local control technique that involves the application of electrical pulses inducing cell membrane electroporation to enhance the uptake of chemotherapeutic drugs by cancer cells, making the treatment more effective[116]. Electrochemotherapy has gained popularity due to its ability to target tumor cells while minimizing damage to surrounding healthy tissue. It is particularly valuable in cases where surgery may not be feasible or when complete tumor removal is difficult, offering a less invasive yet effective therapeutic option. Its use has been proposed in oral malignant melanomas that are difficult to be resected, either as an adjunct to surgery or as a single treatment[45,146]. A recent study on dogs with stage III-IV inoperable oral melanoma showed that the combination of electrochemotherapy with gene electrotransfer of interleukin-2 (IL-2) peritumorally and interleukin-12 (IL-12) intramuscularly significantly increases progression-free survival, improving the treatment outcomes by slowing down tumoral progression[145].

In addition to these therapeutic alternatives, the advent of **immunotherapy** and the subsequent discovery of novel target molecules have significantly advanced treatment options for various tumors, including melanocytic tumors. While a universally accepted, standard treatment has yet to be established, the introduction of these newer therapeutic approaches has led to marked improvements in prognosis, disease-free intervals, and overall survival times for affected animals. Today, we understand that the immune system actively works to

neutralize cells that have altered behavior due to irreparable genomic damage, such as neoplastic cells. The first line of defense involves T-lymphocytes, which are activated and initiate an immune response against antigens they do not recognize[171]. However, neoplastic cells develop mechanisms to evade this immune surveillance, effectively silencing the body's antitumor immune response[39]. Studies have shown that this immune suppression can be overcome by identifying molecular targets that prevent immune cells from attacking neoplastic cells. To achieve this, it is essential to study the pathways involved in the transformation and progression of neoplastic cells, as they are often interconnected, with no single molecule solely responsible for activation or suppression. Currently, the most commonly used approach involves **the blockage of immune checkpoints that inhibit the antitumoral immune response**[5,104]. To achieve this objective, monoclonal antibodies targeting various checkpoints are used. In melanoma, the studies are concentrated on PD-1 (Programmed Death-1) and its ligand, programmed cell death ligand 1 (PD-L1)[5,104]. PD-1 is an immune checkpoint expressed on the surface of T-lymphocytes. It acts as a brake on the immune response, and by blocking it, immune cells can resume their attack on cancer cells. In veterinary medicine, blocking the PD-1/PD-L1 pathway has been shown to reactivate the antitumor function of T-cells in dogs with melanoma, leading to significant clinical benefits[1,89]. However, this treatment is not without side effects, as it can result in aberrant activation of the immune system, potentially causing autoimmune reactions, as demonstrated in human medicine[1].

Other **monoclonal antibodies have been developed to target tumor-associated antigens (TAAs)** like disialogangliosides GD2 and GD3, which are expressed by melanocytic tumor cells. In vitro studies on dogs have demonstrated that these antibodies can stimulate an antitumor immune response, offering another potential avenue for treatment[61].

Another promising method involves the use **of infectious agents, administered as vaccines**, to stimulate the immune system. Bacteria, for example, produce molecules that activate receptors such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors, triggering the innate immune response[5]. This mechanism has been exploited to enhance the immune response against tumors. In dogs, for instance, after surgical excision of melanomas, intratumoral administration of a heat-killed suspension of *Corynebacterium parvum* significantly extended survival times[87].

Similarly, viral therapies have been explored, using **oncolytic viruses** that replicate within tumor cells, leading to their destruction. This approach, though still experimental in both human and veterinary medicine, has shown promise in canine melanoma cell cultures. Several studies have demonstrated that oncolytic viruses can induce cytolytic effects on melanoma cells[8,47,72,122], and one in vivo study reported that combining oncolytic virus therapy with toceranib (a tyrosine kinase inhibitor) helped maintain stable disease in treated patients[31].

Cancer vaccines have emerged as a promising therapeutic option for canine melanoma, targeting TAAs to stimulate a specific antitumor immune response. These vaccines train the immune system to recognize and attack specific antigens displayed by cancer cells that differ significantly from their normal counterparts[30]. Various vaccine types, including whole cell tumor vaccines, dendritic cell vaccination, DNA vaccination, RNA vaccination, peptide vaccination, and protein vaccination have been explored[9]. **Dendritic cell vaccination** has shown promise in clinical trials. By manipulating autologous dendritic cells to present TAAs, the immune system can be effectively stimulated to mount an antitumor response. A study by Tamura et al. (2008) demonstrated the presence of CD8 and CD4 T-cells in positively responding sites after vaccination[143]. **Whole-tumor vaccines** have also been investigated in canine melanoma, with an in vivo study showing that such vaccines are well tolerated and can prolong survival in affected dogs[4].

Recent advancements in immunogenic therapy include the use of **xenogeneic antigen DNA vaccines**. These vaccines, which utilize bacterial plasmids encoding antigens from different species, have shown potential in stimulating a strong immune response. For example, vaccines targeting chondroitin sulfate proteoglycan-4 (CSPG4) have demonstrated efficacy in treating canine oral melanoma, with increased survival times for the patient and minimal side effects[28,49,105,120].

Oncept™, a vaccine using a bacterial plasmid encoding human tyrosinase, was the first cancer vaccine to receive full approval from the US Department of Agriculture. However, its efficacy is still controversial, as the literature has reported mixed results[152].

Albit costly, **gene therapy** is a cutting-edge treatment option that involves delivering gene products like cytokines, suicide genes, tumor or bacterial antigens, or proapoptotic genes into the tumor, using viral or non-viral vectors. In dogs, cytokines like IL-2, which promotes T-cell growth, have been tested, showing that

its administration after surgery and adjuvant radiotherapy reduced recurrence rates and extended survival times in dogs[69]. Other gene products that have been investigated include superantigens—bacterial proteins capable of activating large numbers of T-cells due to their unique structure. Local expression of superantigens through gene delivery may effectively activate tumor-infiltrating immune effector cells, as demonstrated in studies involving canine melanoma[38,148].

In canine oral melanoma, intratumoral administration of the human Fas-ligand gene has been attempted to enhance tumor cell apoptosis, with studies reporting increased survival times and minimal side effects[19]. Another form of gene therapy, known as gene-directed enzyme prodrug therapy, involves introducing a gene that converts a non-toxic substance into a toxic one, targeting tumor cells[164]. This approach has also been investigated in canine oral melanoma with promising results[43,44].

Another promising target in immunotherapy is CD40, a co-stimulatory molecule expressed on the surface of B-cells and antigen-presenting cells. When introduced via adenovirus-mediated gene delivery, CD40 has been studied in the treatment of melanoma, showing the potential to stimulate the immune response and improve treatment outcomes[156,163].

Unfortunately, in veterinary medicine, the widespread use of immunotherapy as a standard treatment for canine melanoma is still far from being realized. Many studies have only been conducted *in vitro*, meaning their findings have yet to be fully applied in clinical practice.

In addition to immunotherapy, as previously discussed, current research is focused on identifying aberrantly activated pathways in tumors to discover therapeutic targets capable of halting neoplastic progression. By understanding these dysregulated molecular mechanisms, the aim is to develop more precise treatments that can disrupt the growth and spread of cancer cells. This approach represents a significant shift from traditional therapies, emphasizing personalized medicine and targeted interventions that are more effective in controlling tumor progression and improving patient outcomes. For example, research has demonstrated that inhibiting the MEK and mTOR pathways, which are frequently mutated or dysregulated in canine neoplastic melanocytes, leads to a significant increase in apoptosis of cancerous cells[160,161].

Clearly, in melanocytic tumors, multiple pathways are overexpressed or aberrantly activated. Therefore, a thorough study of these mechanisms, along with their

associated molecular cascades, could lead to the development of new therapies that, either alone or in combination with other treatments, can combat tumor progression and metastasis. One such pathway, already identified in human melanoma, is the Rac1 pathway, involved in cellular migration processes, which plays a role in tumor metastasis and is associated with more aggressive forms of melanoma that do not respond to treatment[29]. Another crucial pathway is the Janus kinase (JAK)-STAT3 pathway, which regulates several cellular processes such as apoptosis, immune defense, cell differentiation, proliferation, and angiogenesis, and in human melanoma, it was seen that is associated with metastasis formation and increased cell motility[90,103]. Exploring the expression and role of these pathways in canine melanocytic tumors could help identify new therapeutic targets. These targets could potentially block the mechanisms driving tumor growth and spread, offering a more effective approach to treating canine melanomas and further aligning research with advances in human oncology.

1.3.5 Canine melanocytic tumors as a model

The importance of using dogs, alongside other animals, to study human diseases lies in the ongoing challenge of finding effective therapies for various pathologic conditions. Historically, mice were considered the best model for translational medicine studies. However, this species presents several limitations. First, mice are genetically more distant from humans compared to other animal species[13]. Additionally, because they are raised in controlled environments, mice are not exposed to the wide range of environmental and lifestyle variables that domestic animals, especially dogs, experience. Dogs, in particular, share many habits and living conditions with humans, making them a more relevant model for studying diseases that are influenced by environmental factors[53,144].

Moreover, the use of immunocompromised mice in cancer studies can hinder our understanding of the true pathogenic mechanisms of diseases, particularly the immune response. This underscores the need for more suitable models to accurately evaluate experimental treatments[53]. Finally, the dog, as spontaneous model for human melanocytic tumors, provides a more ethically acceptable alternative to mouse models. This aligns with the 3Rs principles of replacement, reduction, and refinement in animal experimentation.

Regarding melanocytic neoplasms, different species have been identified as models depending on the tumor's location[167]. For example, dogs could be considered a

valuable spontaneous model for studying a specific subset of human melanomas—namely, those in humans not induced by UV radiation, such as mucosal melanomas, particularly oral ones[101]. In contrast, cutaneous melanocytic tumors exhibit significant differences between the two species. Preneoplastic lesions that precede melanoma in humans are not observed in dogs. Additionally, canine cutaneous melanocytic tumors tend to be biologically less aggressive[154]. More importantly, there are notable differences in the mutations present in human versus canine cutaneous melanomas[13,94]. The situation differs when it comes to oral melanocytic tumors, where the similarities between the two species are more pronounced. As mentioned above, canine and human oral melanomas share the same aggressive biological behavior and also exhibit important histopathological similarities[133,134,129,144]. Most critically, they share genetic features, making canine oral melanoma a good model for studying human mucosal melanomas not caused by UV radiation. In the previous paragraphs, key mutations and pathways altered in canine and human oral melanomas were described, such as TP53 pathway[167], linked to the *MDM2* gene[112]; the MAPK and PI3K/AKT pathways, which drive cell proliferation and tumor progression[46,101]; the *PTEN* gene mutations, associated with PI3K/AKT, that disrupt its regulatory function[101,161]; the absence of *BRAF* mutations[93,125]; KIT protein expression[26,55,134]; finally, *NRAS* mutations at conserved hotspots, such as NRASQ61[50].

These shared genetic features underscore the value of canine oral melanoma as a spontaneous model for studying the human equivalent, providing insight into tumor biology and potential therapeutic targets for both species.

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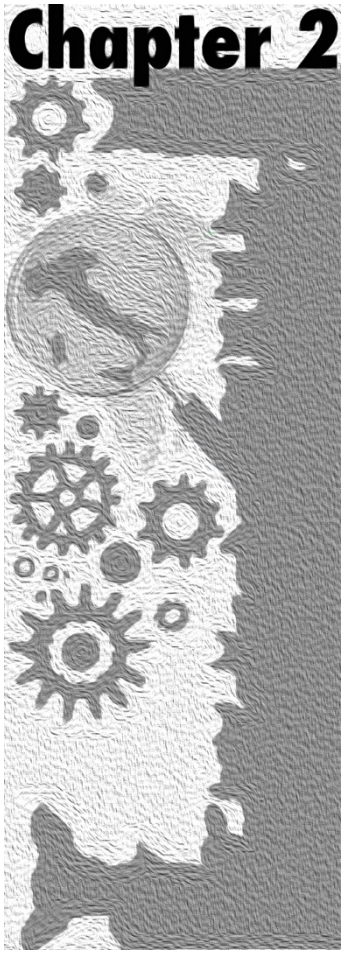

Exploring the epidemiology of melanocytic tumors in canine and feline populations: a comprehensive analysis of diagnostic records from a single institution

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Article

Exploring the Epidemiology of Melanocytic Tumors in Canine and Feline Populations: A Comprehensive Analysis of Diagnostic Records from a Single Pathology Institution in Italy

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Simple Summary: Melanocytic tumors (MTs) are more prevalent in dogs than in cats. In dogs, they are the most frequent malignancy of the oral cavity, whereas in cats, ocular melanomas predominate. This study provides epidemiologic data (2005–2024) on MTs for both dogs and cats. Totals of 21,128 canine and 4808 feline tumors were analyzed by the Veterinary Pathology Service of the Department of Veterinary Medicine (University of Perugia). Among these, 845 canine MTs (329 melanocytomas; 512 melanomas) were diagnosed, 485 from the skin, 193 from the oral mucosa, and 104 from the mucocutaneous junction. Older dogs were more likely to develop melanomas than melanocytomas ($p < 0.001$). In contrast, among feline tumors, only 60 were melanocytic (6 melanocytomas, 53 melanomas). Of these, 29 were cutaneous, 18 were ocular, and 9 were oral. In dogs, melanomas were more common in mucocutaneous locations than in cutaneous locations ($p < 0.05$); moreover, they were more common in the oral cavity compared to all other sites ($p < 0.001$). In cats, ocular melanomas were more common than cutaneous ones ($p < 0.05$). This study provides the prevalence of MTs in dogs and cats, supporting distinct epidemiological patterns of MTs, and confirming the significance of species-specific differences in the tumor prevalence, localization, and age distribution.

Abstract: MTs are prevalent in dogs, representing the most frequent oral malignancy, compared to cats, in which ocular melanomas predominate. This study investigates the canine and feline MT epidemiology (2005–2024) of cases submitted to the Veterinary Pathology Service (University of Perugia). Among the canine neoplasms, 845 (4%) were melanocytic: 329 (39%) melanocytomas; 512 (61%) melanomas. Of these, 485 (57%) were cutaneous (4% of canine cutaneous neoplasms), 193 (23%) were oral (50% of oral canine neoplasms), and 104 (12%) were mucocutaneous. The average age of affected dogs was 10 years. Older dogs were more likely to have melanomas compared to melanocytomas ($p < 0.001$). There were 60 (1%) feline MTs: 6 (10%) melanocytomas; 53 (88%) melanomas. Of these, 29 (48%) were cutaneous (1% of feline cutaneous tumors), 18 (30%) were ocular, and 9 (15%) were oral (22% of feline oral tumors). The average age of affected cats was 11 years. In dogs, mucocutaneous melanomas were more common compared to cutaneous ones ($p < 0.05$); oral melanomas were more common compared to all other sites ($p < 0.001$). In cats, ocular melanomas were more common compared to cutaneous ones ($p < 0.05$). Our study provides the MT prevalence in a selected canine and feline population, revealing MT epidemiological patterns, highlighting species-specific differences in the tumor prevalence, localization, and age distribution.

Keywords: melanocytic tumors; melanoma; canine; feline; epidemiology

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1. Introduction

Melanocytic tumors are described in the human species and in domestic and non-domestic animals [1]. These tumors originate from melanocytes, which are dendritic neuroectodermal cells distributed in many different tissues of the body and specialized in melanin production for protective purposes [2,3]. Across all species, particularly in pets, there exists a distinction between melanocytoma, the benign form, and melanoma, the malignant counterpart [1,2,4–6]. In general, these tumors are typically black, when pigmented, or appear as whitish lesions in the absence of melanin production. These tumors can exhibit an exophytic growth pattern, or they may manifest as sessile nodules regardless of their site of origin [2,5]. The size of melanocytic tumors can range from less than a centimeter, primarily observed in melanocytomas, to several centimeters in the malignant counterpart, which often appear multilobulated and ulcerated [2,5,7,8]. Histologically, these tumors display different growth patterns. The cells can assume round to polygonal to spindle shapes, and they may be arranged in sheets, lobules, or bundles. Typically, benign melanocytic tumors are non-encapsulated but well demarcated, lacking infiltrative growth. Anisocytosis and anisokaryosis are mild, with a low number of mitoses. Conversely, melanomas are more invasive, exhibiting a moderate to marked cellular atypia and a higher number of mitoses [4,6,9–11]. The lack of pigmentation and the marked cellular atypia increase the difficulty of the diagnosis, broadening the spectrum of differential diagnoses. In these challenging cases, immunohistochemistry needs to be performed using specific melanocytic markers, such as Melan-A, PNL2, and Sox10, to confirm the diagnosis [12–14]. Among different species, including humans, melanomas demonstrate biologically aggressive behavior. Indeed, this tumor's tendency towards local invasion and nodal and systemic metastasis leads to a generally unfavorable prognosis [4,5,9,11,14–17]. Due to these biological similarities between domestic animals and humans, and to the growing attention in pet care, in recent decades, the interest in these tumors also in veterinary medicine has increased. This is particularly evident for dogs, and to a lesser extent for cats [1,18–20]. In dogs, it has been highlighted that the majority of melanocytic tumors do not exhibit the same genetic features seen in humans, particularly in the cutaneous and UV-induced forms, which, in the human species, are almost always associated with the $BRAF^{V600E}$ mutation [1,18,20,21]. Indeed, the cutaneous melanocytic tumors in dogs are mostly benign in contrast to their human equivalents, which display aggressive behavior. Additionally, canine digital melanocytic tumors appear to differ from those in humans. Indeed, it has been reported that RAS mutations are more frequent in canine digital melanomas compared to those in humans [22]. However, comparative studies have revealed that certain genes are mutated in both species, especially in oral melanomas, where humans and dogs share TP53 [23], MDM2 [24], NRAS [25], and PTEN [26] mutations, reflective of non-UV-driven initiating molecular events [27]. Therefore, canine oral melanoma is considered a good preclinical model for studying the human equivalent [18,25]. In feline species, genetic studies are few, and the most significant similarities to humans, in terms of tumor biological behavior, are seen in ocular melanomas. This type of melanoma is highly aggressive in both humans and cats, with a poor prognosis due to the lack of effective treatments [28]. Studies have been conducted to evaluate similarities between feline and human ocular melanomas, showing an increased expression of proteins such as KIT, BRAF, GNAQ, and GNA11, which play a crucial role in tumor progression in humans [29–32]. This raises considerations about the potential of the cat as a valuable preclinical model for studying uveal melanoma in humans. Despite this, epidemiological studies in veterinary medicine are few and date back to the early stages of melanoma research in domestic species, with a particular emphasis on dogs [33–39]. These reports indicate that cutaneous melanocytic tumors can arise everywhere on the integument and are relatively common in dogs, accounting for approximately 3–4% of all tumors [2]. This finding has been confirmed by more recent epidemiological studies on canine cutaneous tumors. For example, in Poland [40] and Korea [38], melanomas and melanocytic tumors represented 4.65% and 4.23% of all cutaneous tumors, respectively. In Germany, melanocytic tumors represented 5.2% of all

diagnosed neoplasms in dogs, and the most prevalent location was the integument (92%), where 35.4% of the tumors were malignant [33]. However, incidence rates vary geographically, with lower figures reported in the UK (13 cases per 100,000 dogs annually) [35], and slightly higher rates in regions like Tulsa and Alameda (9% and 7% of neoplastic lesions, respectively) [41]. Overall, melanocytic tumors are a significant health concern for dogs, ranking as the sixth most frequent neoplasm in a Swiss study [42]. Moreover, it is generally described that melanocytomas are more common in the skin [2], as confirmed in an epidemiologic study in Greece [36], but the aforementioned Swiss study revealed that, in their registry, almost two-thirds of melanocytic tumors were malignant [42]. This trend was also observed in Poland, where melanocytomas made up 1.37% compared to 4.65% of melanomas [40]. Within the subgroup of digital neoplasms, melanocytic tumors are the second most frequent type of neoplasia and are often aggressive [43]. Canine oral melanocytic tumors are found in the gingiva, lips, tongue, and hard palate [8]. Oral melanomas are considered the most frequent malignant tumor in dogs, accounting for about 30–40% of oral tumors [8,10,14]. Regarding ocular melanoma, uveal melanocytic neoplasms have been reported to represent the majority of primary intraocular neoplasms (70%), with intraocular melanocytomas being more common than melanomas [44]. While no specific gender predisposition has been demonstrated so far [2,14,39], certain breeds appear to be more prone to the development of melanocytic tumors, particularly those with darker coats and skin. Some studies report that Scottish, Airedale, and Boston Terriers, Miniature and Standard Schnauzers, Doberman Pinschers, Vizslas, Golden and Chesapeake Bay Retrievers, Irish Setters, Chow Chows, Boxers, and Cocker and Springer Spaniels are at higher risk [2,14,33]. More recent analyses have also identified breeds like Rottweilers, Rhodesian Ridgebacks, Labrador Retrievers, and Pekingese/Poodle mixed breeds as being highly represented [33,42,43]. Melanocytic tumors typically arise in older dogs, with an average age ranging from 8 to 11 years, although younger dogs can also be affected [2,39,45]. In cats, melanocytic tumors are less common, with ocular variants, particularly those affecting the iris, being the most frequently observed [46]. Cutaneous forms, often originating from the pinnae, and oral melanocytic tumors are notably rare, with melanocytomas being very uncommon [2]. Dark-haired (black or gray) cats are more susceptible [2]. The reason for the feline species predisposition to ocular melanomas remains largely unknown. Also, in feline species, no gender predisposition is reported, and the average age for the development of a melanocytic tumor ranges between 8 and 12 years [2,9]. Epidemiologic data on melanocytic tumors in Italian dogs and cats are limited. For this reason, a comprehensive epidemiological investigation is needed to understand their distribution and demographic correlations in the canine and feline populations. This study aims to provide epidemiological data on melanocytic tumors in dogs and cats from a single Italian institution.

2. Materials and Methods

2.1. Data Collection

Data were obtained from the electronic archives of the Service of Veterinary Pathology of the Department of Veterinary Medicine at the University of Perugia. The dataset utilized in this study comprised cytologic samples, surgical biopsy cases, and necropsy cases spanning from January 2005 to February 2024. All cases with a diagnosis of neoplasia were included in the study, while hyperplastic and preneoplastic lesions were excluded from the dataset. To export all the diagnoses and the data from each case, we used the keywords “neoplasia”, “tumor”, and “neoformation”. Subsequently, distinct subgroups were delineated for cutaneous and oral tumors, including those originating from the dental and periodontal tissue, tongue, and tonsils. Furthermore, melanocytic tumors in dogs and cats were sorted and extrapolated from the archive using the keywords “melanocytic tumor”, “melanoma”, and “melanocytoma”. The cases with no confirmed diagnosis (e.g., when the melanocytic tumor diagnosis was just included in the differentials) were excluded from the study. Data on melanocytic tumor samples, including sex, age, diagnostic code, species, and breed, were extracted from the database and are summarized

in the Supplementary Tables. Unfortunately, not all cases included in the study had complete anamnesis data available. The diagnosis of melanocytic tumors in our institution is made following the histologic and immunohistochemical guidelines, and the samples are evaluated by board-certified pathologists [5,13].

2.2. Statistical Analyses

Statistical analyses were conducted using Jasp software (version 0.18.3). Initially, descriptive statistics were examined to understand the basic characteristics of the dataset, focusing on metrics. Histograms were generated to assess the normality of the data distribution. The mean age and standard deviation were reported for dogs (normally distributed), while the median age and 95% confidence interval (95% CI) were used for cats (skewed distributed data). To compare age means between tumor types (melanocytoma vs. melanoma), Student *t*-tests (dogs) and Mann–Whitney tests (cats) were employed. The association between the tumor localization and tumor type was assessed using the Chi-square test, with the odds ratios calculated to quantify the likelihood of tumor development in specific sites. Finally, Fisher's exact test was used to examine any association between the tumor types and breeds.

3. Results

Data from our archives included 21128 canine and 4808 feline tumors submitted to our laboratory from January 2005 to February 2024.

3.1. Canine Melanocytic Tumors

Among the canine tumors, 10930 (52%) were cutaneous and 381 (2%) were from the oral cavity. Out of the total canine tumors, 845 were identified as melanocytic tumors (4%), submitted to our laboratories mostly from Umbria and Lazio (Figure 1). A total of 46 cases needed immunohistochemistry using anti Melan-A, PNL2, and Sox10 antibodies to confirm the diagnosis. Specifically, there were 329 (39%) melanocytomas, 505 (61%) primary melanomas, 7 metastatic melanomas (with no primary tumor sampled) (0.8%), and 4 (0.4%) melanocytic tumors diagnosed without further specific indication. In 485 dogs, melanocytic tumors had a cutaneous localization (57%; 4% of all canine cutaneous tumors); specifically, 33 were digital, while in 193 dogs, tumors occurred in the oral cavity (23%; 50% of all canine oral neoplasia). Additionally, among the tumors arising from the mucocutaneous junction, 60 cases (7%) were from the lip and 44 cases (5%) were from the eyelid (Table 1). Furthermore, 49 cases (6%) were ocular, and 4 cases (0.4%) were from other mucosal epithelia (three nasal and one vulvar). Metastases in different sites were observed in 10 cases (1%).

The analysis of the tumor localization and the comparison between the melanocytoma and melanoma groups showed significant differences ($p < 0.001$). In particular, there was a significant association between the tumor type and mucocutaneous and cutaneous sites ($p < 0.05$), indicating that melanomas were more common in mucocutaneous locations compared to cutaneous locations, while melanocytomas were more common on skin rather than on mucocutaneous sites; other associations were found between cutaneous and oral sites ($p < 0.001$), between mucocutaneous and oral sites ($p < 0.001$), and between oral and ocular sites ($p < 0.001$), indicating that melanomas were more common in the oral cavity compared to the mucocutaneous junctions and eyes. Regarding the demographics of the dogs with melanocytic neoplasia, 329 were female (39%) and 471 (56%) were male. In 45 cases (5%), the sex was not reported by the referring vet. The neutering status was not included because only a small portion of the examined population had this information specified in their medical history. Regarding the type of tumor, 196 (23%) males had a diagnosis of melanocytoma, while 272 (32%) males had a diagnosis of melanoma; 120 (14%) females had a diagnosis of melanocytoma, while 209 (24%) females had a diagnosis of melanoma. No significant association was found between the sex and diagnosis (melanoma vs. melanocytoma) ($p > 0.05$). The mean age was 10 years (sd $\pm$ 3.16). The youngest

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dogs diagnosed with a melanocytic tumor were two 1-year-old male and female German Shepherds with a uveal melanocytoma and palpebral melanoma, respectively, while the oldest was a 21-year-old female English Setter, also diagnosed with a uveal melanocytoma. The mean age of dogs with melanocytomas was 9 years (sd ± 3), whereas the mean age of dogs with melanomas was 11 years (sd ± 3) (Figure 2).

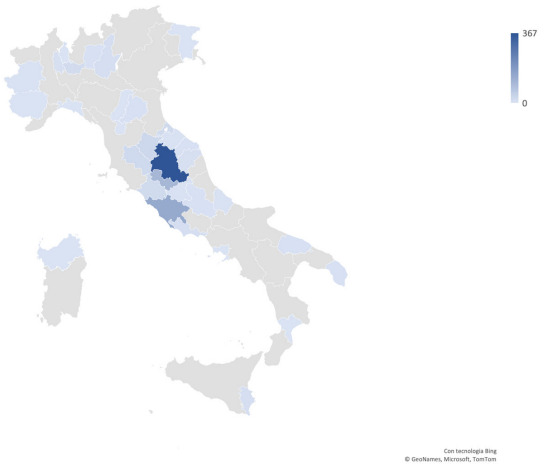

Figure 1. Density map representing the areas from where canine melanocytic tumor samples were submitted to our laboratory. The majority of samples were submitted by practitioners from Umbria and Lazio, and a minority of samples came from other Italian regions.

Table 1. The table shows the numbers of cases of canine melanocytomas and melanomas and their percentages in the most represented tumor locations.

Location	Melanocytomas	Melanomas	Percentage *
Cutaneous	253	228	57%
Mucocutaneous	43	61	12%
Oral	10	182	23%
Ocular	23	26	6%

* The percentage refers to the total number of melanocytic tumors.

The comparison between the age means of the melanocytoma and melanoma groups showed a significant difference ($p < 0.001$), with a higher mean age in dogs with melanomas compared to dogs with melanocytomas. Age data were not available for 93 patients (11%). Of the total dogs diagnosed with melanocytic tumors, 32% were mixed-breed (268 cases). Among the pure breeds, the most common were German Shepherds (41 cases, 4.7%); Rottweilers (36 cases, 4.8%); Labrador Retrievers (35 cases, 4.1%); Golden Retrievers (33 cases, 3.9%); Dachshunds (31 cases, 3.7%); Cocker Spaniels (28 cases, 3.1%); Miniature Pinschers (28 cases, 2.9%); Boxers (24 cases, 2.9%); Yorkshire Terriers (22 cases, 2.6%); Giant Schnauzers (18 cases, 2.1%); Dobermann Pinschers (15 cases, 1.7%); English Setters (15 cases, 1.7%) (Table 2). Breed information was not reported for 66 dogs (7.8%). The comparison between the breeds and the melanocytoma and melanoma groups showed no significant differences ($p > 0.05$).

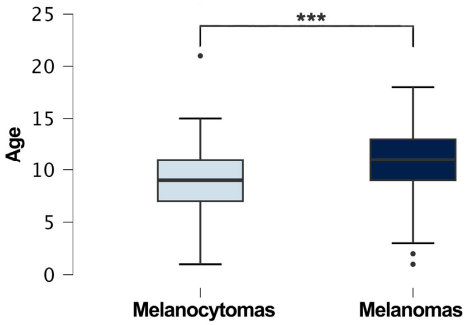

Figure 2. The box plot illustrates the age distribution of dogs diagnosed with melanocytomas and melanomas. Dogs with melanocytomas (287 cases) have a mean age of 9 years, while those with melanomas (462 cases) have a mean age of 11 years. A few outliers are present, with melanocytomas having one older outlier and melanomas showing several younger outliers. The comparison between these two tumor types and the age has a significant difference (*** $p < 0.001$).

Table 2. The table shows the numbers of cases of canine melanocytomas and melanomas and the principal localizations in the most represented breeds in our study.

Breed	**Melanocytomas**					**Melanomas**				
	Cutaneous	Mucocutaneus	Oral	Ocular	Total	Cutaneous	Mucocutaneus	Oral	Ocular	Total
Mixed Breed	81	17	1	6	105	66	23	61	7	157
German Shepherds	11	2	1	5	19	12	4	2	3	21
Rottweilers	7	-	1	-	8	16	2	8	-	26
Labrador Retrievers	8	-	1	1	10	18	2	5	-	25
Golden Retrievers	7	1	-	3	11	6	4	9	1	20
Dachshunds	10	2	2	1	15	9	1	6	-	16
Cocker Spaniels	10	2	-	-	12	2	6	7	-	15
Miniature Pinschers	8	-	-	-	8	15	-	2	-	17
Boxers	8	2	-	1	11	9	-	2	2	13
Yorkshire Terriers	10	-	-	-	10	6	1	4	-	11
Dobermann Pinschers	5	1	-	-	6	7	1	1	-	9
Giant Schnauzers	7	-	-	-	7	9	1	1	-	11
English Setters	1	1	2	1	5	2	-	7	1	10
Other Breeds *	57	14	2	4	77	37	14	47	10	108
Breeds n/a **	23	1		1	25	14	2	20	2	38
Total					329					497

* All breeds with fewer than 10 cases of melanocytic tumors were grouped together under a single category labeled "Other Breeds". ** n/a stands for "not available".

3.2. Feline Melanocytic Tumors

In contrast, considering all the feline tumors, 1828 (38%) were cutaneous and 68 (1%) were from the oral cavity. Out of these, 60 were identified as melanocytic tumors, representing 1% of the total feline tumors, including 6 (10%) melanocytomas and 53 (88%) melanomas, with one case diagnosed as a melanocytic tumor, without further specific

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indication. A total of 14 cases needed immunohistochemistry against Melan-A, PNL2, and Sox10 to confirm the diagnosis. Similar to the dogs, most of the samples were submitted from Umbria and Lazio (Figure 3).

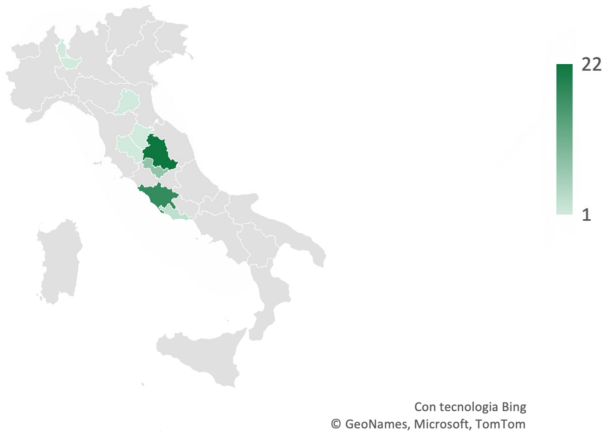

Figure 3. Density map representing the areas from where feline melanocytic tumor samples were submitted to our laboratory. The majority of samples were submitted by practitioners from Umbria and Lazio, and a minority of samples came from other Italian regions.

A total of 29 cases of feline melanocytic tumors were cutaneous (48%; 1% of cutaneous neoplasia), followed by 18 cases of ocular tumors (30%, most of which arose from the iris), 9 cases of tumors arising from the oral cavity (15%; 22% of the oral tumors), and 1 case each from the nasal mucosa and lip (Table 3). Additionally, there were two metastatic melanocytic tumors (3%), one from the spleen and one from the liver, presented without evidence of a primary lesion.

Table 3. The table shows the numbers of cases of feline melanocytomas and melanomas and their percentages in the most represented tumor locations.

Location	Melanocytomas	Melanomas	Percentage *
Cutaneous	6	22	48%
Oral	0	9	30%
Ocular	0	18	15%

* The percentage refers to the total number of melanocytic tumors.

The comparison between the tumor localization and the melanocytoma and melanoma groups showed a significant difference. In particular, melanomas were the most represented in the ocular location compared to the cutaneous one ($p < 0.05$). Cats with melanocytic tumors were 27 (45%) females and 28 (47%) males; for five patients (8%), the sex was not disclosed by the referring vet. The neutering status was not included because only a small portion of the examined population had this information specified in their medical history. Regarding the type of tumor, 3 (5%) males had a diagnosis of melanocytoma, while 24 (32%) males had a diagnosis of melanoma; 2 (3%) females had a diagnosis of melanocytoma, while 24 (32%) females had a diagnosis of melanoma. No significant association was found between the sex and diagnosis (melanoma vs. melanocytoma) ($p > 0.05$). As for the breed, 36 (60%) of the cats were domestic shorthairs, 3 (5%) were Persian cats, and for 21 (35%) of

the patients, the breed information was unavailable (Table 4). The analysis conducted on the possible relation between the breed and a diagnosis of melanocytoma and melanoma did not highlight any significant differences ($p > 0.05$).

Table 4. The table shows the numbers of cases of feline melanocytomas and melanomas in domestic shorthair and Persian cats.

Breed	Melanocytomas				Melanomas			
	Cutaneous	Oral	Ocular	Total	Cutaneous	Oral	Ocular	Total
Domestic Shorthair	4	-	-	4	13	5	11	29
Persian	-	-	-	-	-	-	3	3
Breeds n/a **	2	-	-	2	9	4	4	17
Tot				6				49

** n/a stands for "not available".

The median age was 12 years (95% CI: 10–12.3). The youngest cats diagnosed with a melanocytic tumor were 2 years old, a male and a female, domestic shorthairs with diffuse iris melanomas, while the oldest was a 19-year-old male domestic shorthair with a cutaneous melanoma. Age information was not available for 15 patients (32%). The median age of cats with melanocytomas was 11 years (95% CI: 8–13.9), whereas the median age of cats with melanomas was 12 years (95% CI: 9.9–12.4) (Figure 4).

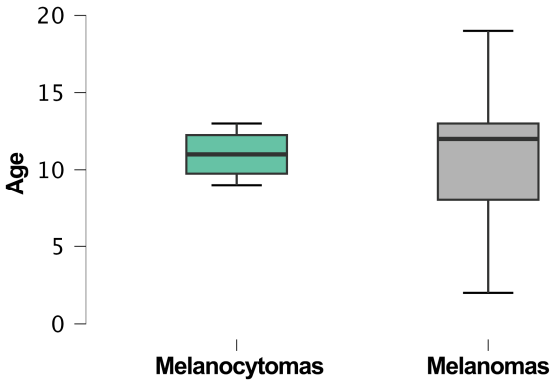

Figure 4. The box plot illustrates the age distribution of cats diagnosed with melanocytomas and melanomas. Cats with melanocytomas (6 cases) had a median age of 11 years, while those with melanomas (49 cases) had a median age of 12 years.

The comparison between the age means of the melanocytoma and melanoma groups did not highlight any significant differences ($p > 0.05$).

4. Discussion

In this study, conducted on samples received in our laboratory from various Italian regions over a 19-year period, we evaluated the frequency and characteristics of melanocytic tumors in dogs and cats. Our findings reveal a higher prevalence of melanomas compared to melanocytomas in both species, in agreement with previously reported data [40]. This was particularly evident in cats, where only 6 melanocytomas were identified, and in canine oral melanomas, where only 10 melanocytomas were diagnosed. Additionally, in

our dataset, the number of canine cutaneous melanomas (228) is slightly lower compared to that of melanocytomas (253). This contrasts with some reports in the scientific literature, which indicate a ratio of melanocytomas to melanomas of 2:1, or even 4:1 in certain studies [2]. In Germany, it has been reported that melanomas on the skin account for 35.4% of all cutaneous melanocytic tumors [33]. However, as previously mentioned, more recent epidemiological analyses have reported different findings. For instance, a study conducted in Switzerland showed that cutaneous melanomas represented about three-quarters of all enrolled melanocytic tumors [42]. Similarly, another study from Poland indicated that cutaneous melanomas accounted for 4.65%, while melanocytomas represented only 1.37%, of all melanocytic tumors in their dataset [40]. In general, it is important to highlight that single-institution studies can be influenced by geographic trends, which may introduce some bias. Despite this, our extensive dataset provides detailed and consistent diagnostic records, thereby offering valuable insights into the epidemiology of these tumors in Italy. Although many of our findings align with those of other studies, as previously mentioned, the unusually high number of cutaneous melanomas in our sample suggests the need for collaboration with other Italian institutions. Expanding this study to include additional cases from different regions would allow for the collection of more comprehensive epidemiological data, helping to refine our understanding of the prevalence and characteristics of these tumors in the broader canine and feline populations. As in other studies, we did not identify a significant association between the tumor type and breed or sex in either species [5].

Furthermore, in both dogs and cats, our data confirm that melanocytic tumors are more common in older animals, with relatively few cases occurring in young patients. This age distribution is in agreement with what is reported in other studies [5,8,47–49]. Specifically, dogs with melanomas tend to be older than those with melanocytomas. The onset of the malignant form of this tumor in older age might suggest that the tumorigenesis and tumor progression are linked to underlying age-related mechanisms. This correlation may indicate that, as animals age, cumulative genetic mutations, diminished immune surveillance, and other age-associated biological processes contribute to the development and aggressiveness of malignant tumors. One more consideration could be that the delay between the tumor onset and its diagnosis can be significant, as owners may not detect tumors early, especially in dogs with long and thick coats or in less visible areas, such as the oral cavity or eyes. This diagnostic delay may have an impact on the correct assessment of the actual age at which these tumors develop, particularly for the slow-growing ones. Moreover, in our study, we did not statistically compare the ages across different breeds because certain breeds are more long-lived than others, and this aspect could have highlighted significant differences. However, our dataset is predominantly composed of mixed-breed dogs, with relatively few purebreds represented. This imbalance could compromise the validity of any analysis based on breed-specific longevity. For this reason, further studies involving more institutions are necessary to investigate this aspect in a more robust manner.

In our cohort, certain breeds, including Rottweilers, Labrador Retrievers, Golden Retrievers, Dobermann Pinschers, and Schnauzers, were overrepresented with melanocytic tumors, consistent with previous reports [50]. Although our study did not reveal a significant association between the breed and tumor type, this finding should be interpreted with caution due to the relatively small sample size within specific breed subgroups, as evidenced by the high proportion of mixed-breed dogs in our cohort. Larger studies with more balanced breed representation are needed to confirm these findings.

Additionally, we found a significant association between the tumor localization and the type of melanocytic tumor. In dogs, there is an association between mucocutaneous and cutaneous sites and the tumor type, indicating that melanomas are less likely to be found in the mucocutaneous location compared to melanocytomas. Other associations between cutaneous and oral sites, mucocutaneous and oral sites, and oral and ocular sites indicate that melanomas are more common in the oral cavity compared to the mucocutaneous and ocular locations. As noted by Esplin et al., this could be because a subset of tumors arising

from the mucous membranes of the lips and oral cavity tend to be well differentiated, exhibiting lower rates of progression compared to their more aggressive counterparts. This finding suggests that some melanocytic tumors in these locations may follow a more indolent course [51]. In contrast, in cats, there is a significative association between the type of tumor and cutaneous and ocular sites, showing that ocular melanomas are the most represented compared to the cutaneous ones. This result is consistent with the scientific literature [28]; however, the reasons why this species is predisposed to ocular melanomas remain largely unknown. While studies have observed this predisposition, the underlying mechanisms, whether genetic, environmental, or linked to anatomical or physiological factors, have yet to be fully elucidated. These associations suggest that the benign or malignant nature of the tumor may depend on its localization, both in dogs and cats. As we also know from the literature, these results may have important implications for the diagnosis, prognosis, and treatment of canine and feline melanocytic tumors.

Regarding the diagnostic approaches, as mentioned before, regular oral examinations in dogs and thorough ocular inspections in both dogs and cats is difficult for owners but could facilitate the earlier detection of melanomas, which could improve the therapeutic approach and, finally, the prognosis.

Further studies should aim to validate these findings across multiple institutions and geographic regions to investigate the impact of potential regional variation, for example, pollutants and environmental differences, and, at the same time, enhance the generalizability of the results.

Furthermore, the relationship between the tumor type, age, and localization suggests that further investigations should be aimed at the understanding of the underlying biological pathomechanisms. Research into genetic, environmental, and lifestyle factors that influence the development and progression of melanocytic tumors in pets could lead to more effective prevention and management strategies.

5. Conclusions

This study provides further data on the epidemiology of melanocytic tumors in dogs and cats, highlighting the predominance of melanomas, the association with age, and the significance of developing a benign or malignant melanocytic tumor based on its localization. These findings could have important implications for veterinary practice and serve as a preliminary step for future research aimed at improving the outcomes of dogs and cats affected by these tumors.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vetsci11090435/s1, Data on melanocytic tumor samples.

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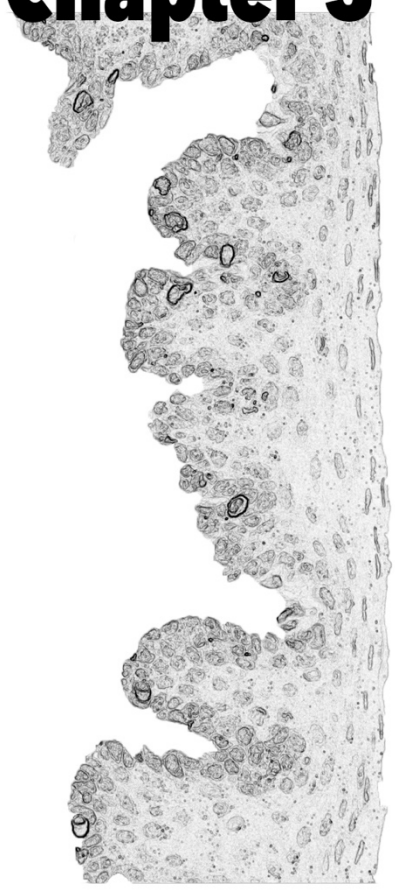
Chapter 3



Canine melanocytes: immunohistochemical expression of melanocytic markers in different somatic areas

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ORIGINAL ARTICLE

Veterinary Dermatology

Canine melanocytes: Immunohistochemical expression of melanocytic markers in different somatic areas

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Abstract

Background: Melanoblasts originate in the neural crest from where they migrate to peripheral tissues and differentiate into melanocytes. Alteration during melanocyte development and life can cause different diseases, ranging from pigmentary disorders and decreased visual and auditory functions, to tumours such as melanoma. Location and phenotypical features of melanocytes have been characterised in different species, yet data on dogs are lacking.

Objective: This study investigates the expression of melanocytic markers Melan A, PNL2, TRP1, TRP2, SOX-10 and MITF in melanocytes of selected cutaneous and mucosal surfaces of dogs.

Animals: At necropsy, samples from five dogs were harvested from oral mucosa, mucocutaneous junction, eyelid, nose and haired skin (abdomen, back, pinna, head).

Materials and Methods: Immunohistochemical and immunofluorescence analyses were performed to assess marker expression.

Results: Results showed variable expression of melanocytic markers in different anatomical sites, particularly within epidermis of haired skin and dermal melanocytes. Melan A and SOX-10 were the most specific and sensitive melanocytic markers. PNL2 was less sensitive, while TRP1 and TRP2 were seldomly expressed by intraepidermal melanocytes in haired skin. MITF had a good sensitivity, yet the expression often was weak.

Conclusions and Clinical Relevance: Our results indicate a variable expression of melanocytic markers in different sites, suggesting the presence of subpopulations of melanocytes. These preliminary results pave the way to understanding the pathogenetic mechanisms involved in degenerative melanocytic disorders and melanoma. Furthermore, the possible different expression of melanocyte markers in different anatomical sites could influence their sensitivity and specificity when used for diagnostic purposes.

KEYWORDS
canine, Melan A, melanocytes, MITF, SOX-10, TRP1

INTRODUCTION

Melanocytes are neural crest-derived cells, which migrate to peripheral tissues (e.g. epidermis, hair follicle, mucosa, cochlea, iris, mesencephalon), where they synthesise melanin pigment.[1] Melanin is important in skin and hair pigmentation, protects skin and uvea from solar radiation and contributes to endolymph formation in the cochlea.[2] Therefore, melanocytic disorders can manifest as syndromic diseases whereby hypopigmentation, blindness and deafness coexist.[3] Also, melanocytic tumours comprise multiple biologically distinct categories, which differ in terms of site of origin (cutaneous versus mucosal), clinical and histological

presentation, biological behaviour, causative role of UV radiation and genetic mutations, in both humans and animals.

Much of the information on melanocytes is gathered from studies conducted on humans, rabbits and mice.[4–6] However, the biology of canine melanocytes remains largely unknown. With the exception of rare reports, the veterinary scientific literature does not provide extensive studies focused on the characterisation of these cells, with some rare exceptions.[7]

In humans, the melanocyte density in the epidermis (expressed as melanocyte: keratinocyte ratio)[8,9] is 1:10, with about 1200 melanocytes/mm^2 of human skin, independently of ethnicity.[9] Nevertheless, the number of melanocytes varies in different anatomical districts. It, therefore, has been hypothesised that an unequal distribution of melanocytes may partially explain the site-specific incidence of some pathological conditions, such as melanoma, offering perspectives not only on the aetiology of this tumour,[10] but also on hyper/hypopigmentation disorders.[11]

Recently, different canine diseases have been proposed as possible spontaneous models for human counterparts, such as vitiligo, Vogt–Koyanagi–Harada (VKH) syndrome and melanoma.[12–16]

Nevertheless, given that knowledge on canine melanocytes remains unexplored or, at least, sparse, further studies to unveil their biology are recommended both for a better characterisation of canine diseases and for an accurate comparison among dogs and humans. Additionally, canine mucosal melanoma has been identified as a possible valuable model for the rare human mucosal melanoma, which, in both species, is a very aggressive disease with a high metastatic rate, no efficient therapies and poor prognosis.[15–17] However, data are still lacking and further studies are required to confirm the strength of this model.[14] To the best of the authors' knowledge, the presence of melanocytes in different somatic areas of dogs has never been assessed systematically in large studies and different breeds; moreover, the expression of different melanocytic markers in different anatomic regions has never been reported.

The aim of the present study was to investigate the expression of different markers, namely Melan A, PNL2, TRP1, TRP2, SOX-10 and MITF, in melanocytes within cutaneous and mucosal surfaces, evaluating their co-expression and the distribution of melanocytic populations in different anatomical sites.

MATERIALS AND METHODS

Case selection

Samples were harvested from five dogs, with no clinically reported dermatological or mucosal lesions, submitted for necropsy after owner's consent. Samples (two to three) were collected from different cutaneous and mucosal sites, less than 3 h after the death of the dog. Sampling was performed harvesting an 8 mm punch biopsy (measured parallel to the epithelial surface) to guarantee a proper evaluation. In the present study samples were collected from: oral mucosa (internal aspect of the cheek), oral mucocutaneous junction (lateral upper lip the canine tooth level), eyelid (lower right eyelid), nose, pinna (tip), ventral abdomen (cranial to the umbilical scar, on the median line), back (lumbar area, on the median line) and head (between the pinnae, on the median line).

Pigmentation evaluation

The presence of pigmentation was evaluated macroscopically on epithelium and hair both before and after inclusion in paraffin and sectioning. Subgrossly, the degree of pigmentation was semiquantitatively evaluated on the epidermis/mucosal surface and assigned one of the following values: 0, pink/whitish skin with no spots; 1, light brown skin with no spots; 2, dark brown skin with no spots; 3, black skin with no spots; P, patchy, because of the presence of spots or irregular distribution of pigment. The same grades were assigned for the evaluation of hair coat pigmentation: 0, white hair; 1, light brown hair; 2, dark brown hair; 3, black hair; P, patchy, because of the presence of spots or different coloration of hair.

Histological and immunohistochemical evaluation

From all samples a routinely stained haematoxylin & eosin slide was prepared. Five micrometre sections were cut from formalin-fixed and paraffin-embedded samples and mounted on poly-L-lysine-coated slides, which then were dewaxed and dehydrated. Immunohistochemical evaluation was performed on serial sections with antibodies reported to be cross-reactive in canine species, raised against Melan A,[18] PNL2,[18] TRP1, TRP2, SOX-10 and MITF. Positive controls were obtained from canine melanomas for Melan A, PNL2, TRP1, TRP2 and MITF, while mammary gland was used as control for SOX-10.[19] AEC was used as a chromogen for its red colour, to differentiate from melanin. Bleaching was not performed. Negative controls were run omitting the primary antibody and incubating control sections with TBS. IBA1 immunolabelling was performed, following protocols reported previously, in selected cases to differentiate melanocytes from melanin-laden macrophages.[20] Immunohistochemical protocols and antibodies used in this study are summarised in Table 1.

For each antibody, the presence of positive cells was assessed within the cutaneous/mucosal epithelium, in the hair bulb and follicular wall and in the other cutaneous/mucosal structures (e.g. glands, nerves, dermis).

There is no gold standard for recognising melanocytes, so we evaluated, together with immunolabelling, the morphology of labelled cells considering:

1. The location within epidermis (basal/suprabasal);
2. The shape (bright round, oval, fusiform or dendritic cells);
3. The presence of intracytoplasmic melanin pigment.

TABLE 1 Protocols of the antibodies used in this study.

Antigen	Clone	Antigen retrieval	Dilution	Distributor
Melan A	A103-M27C10-M29E3 (mouse)	TRIS-Edta buffer, pH 9.0	1:150	Abcam
PNL2	PNL2 (mouse)	Citrate buffer, pH 6.0	1:150	Santa Cruz
TRP1 (TYRP1/gp75)	Polyclonal rabbit	TRIS-Edta buffer, pH 9.0	1:100	LSbio
TRP2	Polyclonal rabbit	Citrate buffer, pH 6.0	1:250	Abcam
SOX-10	A-2 (mouse)	TRIS-Edta buffer, pH 9.0	1:200	Santa Cruz
MITF	C5/D5 (mouse)	TRIS-Edta buffer, pH 9.0	1:75	Merck

Immunofluorescence

Double immunofluorescence was performed on selected samples from nose, oral mucosal and haired skin. The co-expression of melanocytic markers was assessed pairing antibodies with similar antigen retrieval methods. For this purpose, primary antibodies previously described for immunohistochemical evaluation (TRP1, SOX-10 and MITF) were co-localised with anti-Melan A conjugated with Alexa Fluor 594, while TRP2 was co-localised with anti-PNL2 conjugated with Alexa Fluor 488 antibodies (Santa Cruz). SOX10 and TRP1 also could be co-localised, yet co-localisation of TRP1 and TRP2 was not possible, both being primary antibodies raised in rabbit. After dewaxing and dehydrating, the slides were incubated with a 3% hydrogen peroxide methanol solution. Antigen retrieval was performed as reported for immunohistochemical evaluation and primary antibodies were incubated overnight at 4°C with the appropriate concentration. After washing with tris-buffered saline, a secondary goat anti-mouse conjugated with a red fluorochrome (Goat Anti-Mouse IgG H&L-Alexa Fluor 647) and with a 1:200 dilution, was applied for 2 h at room temperature (RT). After a careful wash, a protein block step was performed before incubation with Melan A and PNL2 fluorochrome-conjugated antibodies. To co-localise SOX-10 and TRP1, after incubation of TRP1, a secondary anti-rabbit antibody conjugated with a fluorochrome (Goat Anti-Rabbit IgG H&L-Alexa Fluor 594; Abcam) was used. Finally, a drop of aqueous mounting medium containing DAPI (Abcam) was added and slides were incubated for 5 min at RT, before coverslip mounting. Negative controls were run omitting the primary antibodies and incubating the sections with phosphate-buffered saline.

Samples were evaluated using an Olympus BX51 fluorescent microscope equipped with a Nikon DS-Qi2Mc camera. NIS-ELEMENTS D software was used for image acquisition and analysis.

RESULTS

Case selection and macroscopical evaluation

The five dogs selected for this preliminary investigation were a chow chow, a Labrador retriever, a Siberian husky, a mixed English setter and a mixed breed dog. The five animals showed a variable degree of pigmentation of both skin/mucosa and of hair coat in the sampled areas. The results of macroscopical evaluation are reported in Tables 2 and 3, respectively.

Melan A

Melan A immunolabelling was observed as a granular cytoplasmic reaction. The reaction was strong and specific, and no signs of background stain were present. Melan A positive cells were observed in the basal and suprabasal layers of the epithelium in mucosal, junctional, nasal and cutaneous samples (Table 2; Figure 1a–e). One of the dogs (Siberian husky, Case 5) showed mucosa with a macroscopical patchy pigmentation. In this case, corresponding to the macroscopically evident patcahy pigmentation (P), there were areas with numerous Melan A^+ melanocytes alternating with areas where no positive cells could be observed. A strong expression of this marker also was seen in the hair bulbs and in the infundibula of hair follicles (Table 3; Figure 2a). In hair bulbs, where pigmentation was prominent with H&E visualisation, the expression of Melan A was more intense and located in a higher number of cells. Nevertheless, the expression of Melan A also could be observed in scattered cells in those samples where hair pigmentation was not histologically evident. In one of the samples from the abdominal skin (Case 4), a mild hyperplasia of the epidermis was seen, associated with multifocal, mild lymphocytic and plasmacellular infiltrates in the superficial dermis; in this case, the number of melanocytes appeared to be increased. Generally, no melanocyctes were observed in sebaceous glands, except for Meibomian glands and periocular sebaceous glands, where occasional Melan A^+ cells were observed among the basal cells (Figure 2b). Only in one case (Case 1) were Melan A^+ cells present near apocrine glands in the dermis and these were interpreted as dermal melanocytes (Figure 2c).

PNL2

The expression of PNL2 was seen as a finely granular, cytoplasmic immunolabelling. The expression of this marker was variable and sometimes very faint, with an occasional uneven distribution of the immunoreactivity in the cytoplasm. Positive cells were seen in the basal and suprabasal layers of the epithelium (Table 2; Figure 1f–j). In a similar way to Melan A, patchy-pigmented oral mucosa showed areas with the presence of $PNL2^+$ melanocytes alternating with areas with

TABLE 2 Intraepidermal expression of melanocytic markers in dogs in different cutaneous and mucosal sites.

	N.	Breed	Pigm.	Melan A	PNL2	TRP1	TRP2	SOX-10	MITF
Oral Mucosa	1	Chow chow	2	+	+	+	+	+	+
	2	Mixed breed	2	+	±	±	±	+	+
	3	Labrador retriever	2	+	+	+	+	+	+
	4	English setter	P	–	–	±	–	–	–
	5	Siberian husky	P	±	–	±	±	±	±
Oral MCJ	1	Chow chow	3	+	+	+	+	+	+
	2	Mixed breed	3	+	+	+	+	+	+
	3	Labrador retriever	3	+	+	+	+	+	+
	4	English setter	2	+	+	+	+	+	+
	5	Siberian husky	3	+	+	+	+	+	+
Eyelid	1	Chow chow	2	+	+	+	+	+	+
	2	Mixed breed	3	+	+	+	+	+	+
	3	Labrador retriever	3	+	+	+	+	+	+
	4	English setter	2	+	+	+	+	+	+
	5	Siberian husky	3	+	+	+	+	+	+
Nose	1	Chow chow	2	+	±	+	±	+	+
	2	Mixed breed	3	+	±	+	±	+	+
	3	Labrador retriever	2	+	±	+	±	+	+
	4	English setter	2	+	±	+	±	+	+
	5	Siberian husky	3	+	+	+	±	+	+
Abdomen	1	Chow chow	1	–	–	–	–	+	–
	2	Mixed breed	2	+	–	–	–	+	+
	3	Labrador retriever	1	+	+	–	+	+	+
	4	English setter	2	+	+	–	+	+	+
	5	Siberian husky	2	+	+	+	+	+	+
Back	1	Chow chow	1	+	–	–	–	+	+
	2	Mixed breed	1	+	–	–	–	+	+
	3	Labrador retriever	1	+	–	–	–	+	+
	4	English setter	P	±	–	+	–	–	+
	5	Siberian husky	P	–	–	–	–	±	–
Pinna	1	Chow chow	1	+	+	+	–	+	+
	2	Mixed breed	2	+	+	–	–	+	+
	3	Labrador retriever	1	+	+	–	–	+	+
	4	English setter	2	+	+	+	–	+	–
	5	Siberian husky	3	+	+	+	–	+	–
Head	1	Chow chow	1	+	–	–	–	+	–
	2	Mixed breed	1	–	–	–	–	+	–
	3	Labrador retriever	1	+	±	+	–	+	±
	4	English setter	2	+	–	+	–	+	±
	5	Siberian husky	2	–	–	–	–	+	–

Note: Pigm., degree of pigmentation evaluated macroscopically on the epithelial surface (0/1/2/3 and P, patchy); expression of melanocytic markers is expressed as present (+), absent (–) or patchy (±).

no detectable cells. Occasionally, in homogeneously pigmented epithelia such as the nose and the mucosal junctions, PNL2$^+$ melanocytes were present with a multifocal distribution, unlike the regular presence of immunoreactive melanocytes seen for other markers, particularly Melan A and SOX-10. The immunolabelling was stronger in follicular melanocytes, in both hair bulb (Table 3; Figure 2d) and follicular wall. Scattered PNL2$^+$ cells were present in the basal compartment of Meibomian glands (Figure 2e), yet, in a similar way to Melan A, not observed in sebaceous glands of the other cutaneous areas sampled for this study. Dermal melanocytes were negative in all samples examined, including for Case 1 (Figure 2f).

TRP1

The immunolabelling for TRP1 was cytoplasmic and coarsely granular; the intensity of the labelling was

TABLE 3 Follicular expression of melanocytic markers in hair follicles of dogs, evaluated on different cutaneous sites.

				Melan A		PNL2		TRP1		TRP2		SOX-10		MITF	
	N.	Breed	Pigm.	B	W	B	W	B	W	B	W	B	W	B	W
Perioral	1	Chow chow	1	–	+	–	–	–	–	–	–	–	+	–	–
	2	Mixed breed	3	+	+	+	+	+	+	+	+	+	+	+	+
	3	Labrador retriever	1	+	+	+	+	–	+	–	+	–	+	–	+
	4	English setter	P	–	–	–	+	–	+	–	+	–	+	–	+
	5	Siberian husky	0	–	+	–	+	–	+	–	–	–	+	–	+
Periocular	1	Chow chow	1	+	+	–	+	–	+	–	–	–	+	–	+
	2	Mixed breed	3	+	+	+	+	+	+	+	+	+	+	+	+
	3	Labrador retriever	1	–	+	+	+	–	+	–	+	–	+	–	+
	4	English setter	P	+	+	+	+	+	+	+	+	+	+	+	+
	5	Siberian husky	P	+	+	+	+	–	+	–	+	+	+	–	+
Abdomen	1	Chow chow	1	–	–	+	–	–	–	–	–	+	+	–	–
	2	Mixed breed	1	–	–	–	–	–	–	–	–	+	+	–	+
	3	Labrador retriever	1	+	+	–	–	–	+	–	–	+	+	–	+
	4	English setter	P	–	+	–	–	–	–	–	–	+	–	+	+
	5	Siberian husky	0	+	+	+	+	–	–	+	–	+	+	+	+
Back	1	Chow chow	1	+	+	–	+	–	+	–	+	+	+	–	+
	2	Mixed breed	2	+	+	+	+	–	+	+	+	+	+	–	+
	3	Labrador retriever	1	+	+	–	+	–	+	–	+	+	+	–	+
	4	English setter	P	–	+	–	+	–	–	–	+	–	+	–	+
	5	Siberian husky	3	+	+	+	+	+	–	+	+	+	+	–	+
Pinna	1	Chow chow	1	+	+	+	–	+	–	–	–	+	+	–	+
	2	Mixed breed	3	+	–	+	–	+	–	–	–	+	+	+	+
	3	Labrador retriever	1	+	–	+	–	+	–	+	–	+	+	+	+
	4	English setter	P	+	+	+	–	+	+	+	–	+	+	+	–
	5	Siberian husky	3	+	+	+	–	+	–	+	–	+	+	+	–
Head	1	Chow chow	1	+	+	+	–	+	–	–	–	+	+	–	+
	2	Mixed breed	2	+	–	–	–	+	–	–	–	+	+	–	+
	3	Labrador retriever	1	+	+	+	–	+	–	+	–	+	+	+	+
	4	English setter	P	+	–	+	–	+	–	+	–	+	+	+	+
	5	Siberian husky	P	+	+	+	–	+	–	+	–	+	+	+	+

Note: Pigm, degree of pigmentation of hair coat (0/1/2/3 and P, patchy); expression of melanocytic markers is expressed as present (+), absent (–).
Abbreviations: B, hair bulb; W, follicular wall.

variable, from moderate to strong. In eyelid and oral mucocutaneous junctions (Figure 1k–o), the marker's expression was strong and similar to what observed with Melan A, while no TRP1$^+$ intraepithelial melanocytes were present in most of the samples harvested from the cutaneous sites (Table 2). As for hair bulbs (Figure 2g) and follicular walls (Table 3), the immunolabelling was strong and specific, and apparently increased with the quantity of melanin-containing cells. In all of the samples from haired skin, sparse TRP1$^+$ positive round cells, characterised by moderate amounts of cytoplasm and rounded nucleus, were observed in particular in the superficial dermis. Rare immunolabelled cells were observed in the basal compartment of Meibomian and periocular sebaceous glands (Figure 2h). Dermal melanocytes did not label with TRP1, yet some positive cells were seen among negative melanocytes (Figure 2i) and were interpreted as mast cells.

TRP2

Immunolabelling for TRP2 was strong and coarsely granular, with a cytoplasmic distribution. The intensity of the staining was unpredictably variable, being strong and neat in some cases, in particular on the nose, oral mucocutaneous junction and eyelid junction (Table 2; Figure 1q,rt, respectively), while in haired skin and oral mucosa (Figure 1p,s), the immunolabelling often was faint. Generally, immunolabelling was stronger in hair bulbs (Table 3; Figure 2j) than in intraepithelial melanocytes. In a similar way to PNL2, the immunolabelling of this marker showed a multifocal distribution in all cases in some macroscopically heavily pigmented samples, such as the nose (Table 2). Rare positive cells were observed in the basal compartment of Meibomian glands (Figure 2k). Dermal melanocytes did not label with TRP2, yet in some samples, TRP2$^+$ cells were seen in the dermis (Figure 2l). Morphologically these cells were

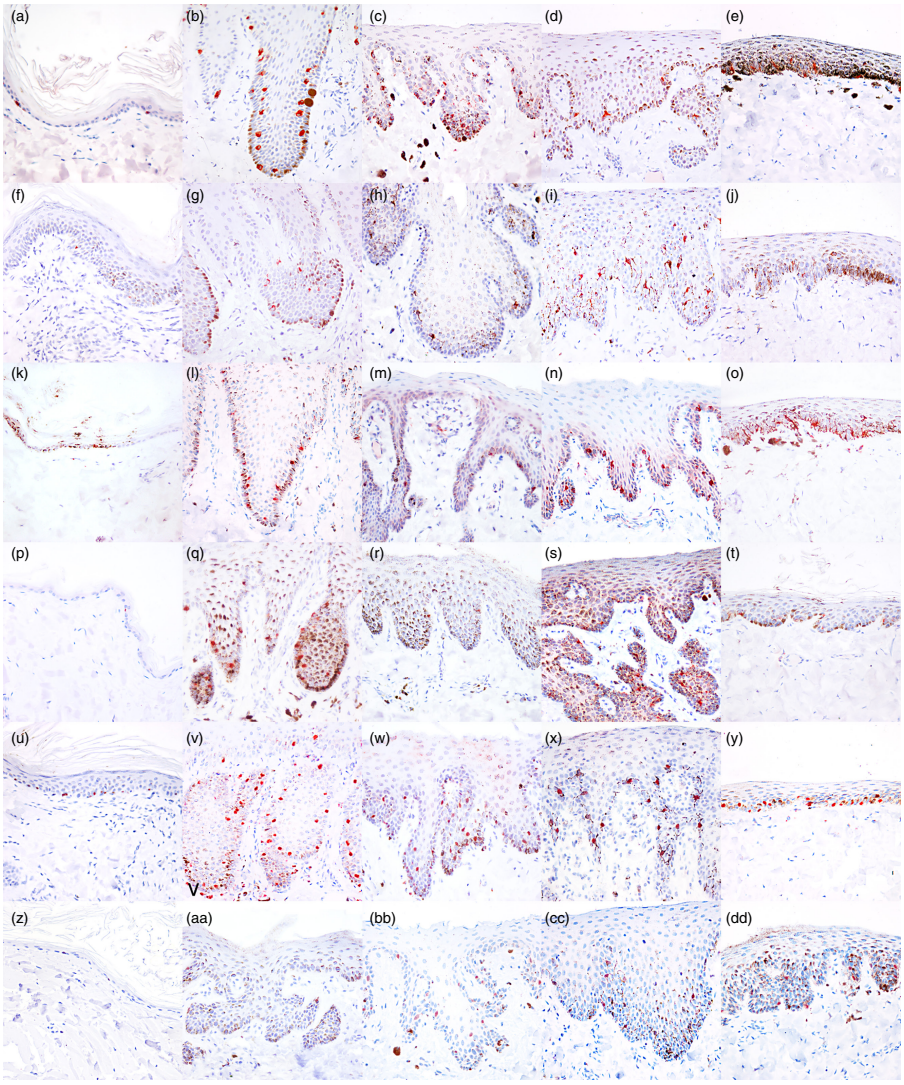
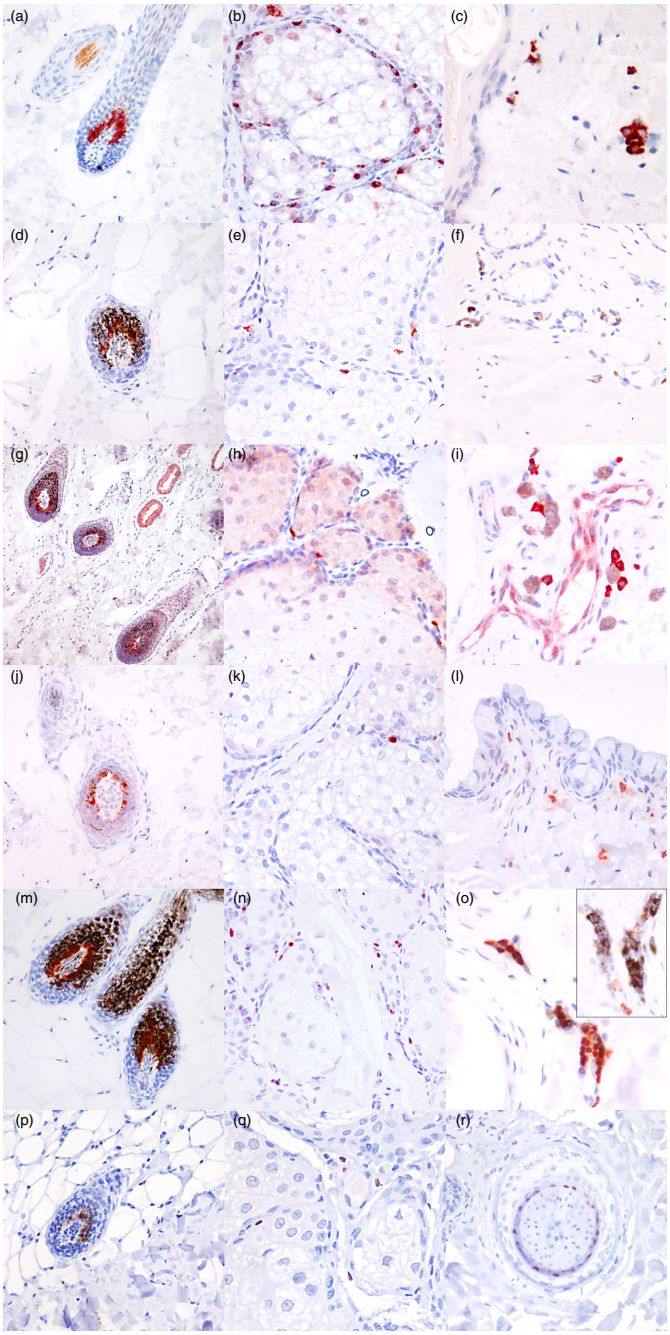


FIGURE 1 Expression of Melan A within epithelia of (a) haired skin, (b) nose, (c) oral mucocutaneous junction, (d) buccal mucosa and (e) eyelid mucocutaneous junction. Expression of PNL2 within epithelia of (f) haired skin, (g) nose, (h) oral mucocutaneous junction, (i) buccal mucosa and (j) eyelid mucocutaneous junction. Expression of TRP1 within epithelia of (k) haired skin, (l) nose, (m) oral mucocutaneous junction, (n) buccal mucosa and (o) eyelid mucocutaneous junction. Expression of TRP2 within epithelia of (p) haired skin, (q) nose, (r) oral mucocutaneous junction, (s) buccal mucosa and (t) eyelid mucocutaneous junction. Expression of SOX-10 within epithelia of (u) haired skin, (v) nose, (w) oral mucocutaneous junction, (x) buccal mucosa and (y) eyelid mucocutaneous junction. Expression of MITF within epithelia of (z) haired skin, (aa) nose, (bb) oral mucocutaneous junction, (cc) buccal mucosa and (dd) eyelid mucocutaneous junction.

FIGURE 2 Expression of Melan A within (a) hair bulb, (b) Meibomian glands and (c) dermal melanocytes. Expression of PNL2 within (d) hair bulb, (e) Meibomian glands and (f) dermal melanocytes (negative). Expression of TRP1 within (g) hair bulb, (h) Meibomian glands and (i) dermal cells, interpreted as mast cells. Expression of TRP2 within (j) hair bulb, (k) Meibomian glands and (l) dermal cells, interpreted as histiocytes/resident macrophages. Expression of SOX-10 within (m) hair bulb, (n) Meibomian glands and (o) dermal melanocytes. The expression of IBA1 was tested on the same sample and dermal melanin-containing cells were negative (inset). Expression of MITF within (p) hair bulb, (q) Meibomian glands and (r) cells of the inner root sheath.



characterised by a moderate quantity of cytoplasm and interpreted as histiocytes.

SOX-10

SOX-10 expression appeared as a strong and diffuse nuclear immunolabelling, regularly expressed in both haired skin, mucocutaneous junctions and oral mucosa (Figure 1u–y). The immunolabelling of the nucleus was sharp and strong and allowed the rapid and solid identification of positive cells. Besides, this neat immunolabelling granted a better identification of melanocytes also in highly pigmented sites, such as the hair bulbs (Table 3; Figure 2m). All positive cells within the epithelia and in hair follicles were morphologically compatible with melanocytes, being characterised by scant clear cytoplasm or filled with melanin pigment and showing dendritic prolongations of the cytoplasm. Positive cells among the basal component of Meibomian glands (Figure 2n) were frequently observed; on other samples from haired skin, no positivity was assessed yet, differently from the other markers, rare positive nuclei were observed in the cutaneous samples from the head of Case 1. Dermal melanocytes were positive (Figure 2o). The same cells were negative for the macrophagic marker IBA1 (inset in Figure 2o). (Positivity also was observed in dermal nerves and in myoepithelial cells of apocrine glands and of mammary gland, that were sampled together with the abdominal skin.)

MITF

Immunolabelling of MITF was seen as a finely granular nuclear reactivity. Often, the immunoreactivity was faint when compared to the other markers, yet nuclear expression was helpful particularly in highly pigmented samples. In a similar way to PNL2, TRP1 and TRP2, melanocyte immunolabelling with MITF was more rarely observed within haired epithelia (Table 2; Figure 1z), while it was more prominent in samples harvested from mucocutaneous junctions (Figure 1bb, dd), oral mucosa (Figure 1cc) and, even if weaker, from the nose (Figure 1aa).

The labelling of melanocytes in hair bulbs was more intense and clearer when compared to the intraepithelial samples (Table 3; Figure 2p). Rare positive cells were observed in the basal compartment of Meibomian glands (Figure 2q). Moreover, the inner root sheet showed a moderate-to-intense immunolabelling in hair follicles (Figure 2r). Dermal melanocytes showed a variable positivity. $MITF^{+}$ cells were scattered in the superficial to mid-dermis; these cells were characterised by rounded shape, a moderate amount of cytoplasm and a central rounded nucleus, morphologically similar to mast cells.

Melanocytic marker co-expression

Melan A showed a clear and moderate-to-strong expression and an almost total co-localisation with TRP1 on oral mucosa and nose (Figure 3a). PNL2 confirmed a lower sensitivity in detecting melanocytes and the signal often was weaker. The co-localisation was possible only with TRP2, because both antibodies required antigen retrieval with citrate buffer. TRP2 was more sensitive than PNL2 in detecting melanocytes (Figure 3b). Melan A and SOX-10 showed an almost complete co-localisation (Figure 3c), with SOX-10 being more sensitive and, as a consequence of the nuclear staining, easier for operators to recognise. On the nose and mucosal sites, SOX-10 showed a sensitivity, which was similar to TRP1 (Figure 3d). Co-localisation of Melan A and MITF confirmed that the latter was less sensitive; in a similar way to immunohistochemical findings, the signal was weak (Figure 3e).

DISCUSSION

The expression of melanocytic markers in canine species has never been assessed in a dedicated study, despite numerous markers already being used in diagnostic environments, particularly for neoplastic lesions, such as amelanotic melanomas.[21–24]

Melan A is considered one of the best and most specific markers for melanocytes in different species, dogs included.[7,25–27] Results from our study confirm the specificity and the usefulness of Melan A for the recognition of intraepidermic melanocytes, both in immunohistochemical and immunofluorescence evaluation. Also, Melan A proved useful for the recognition of dermal melanocytes, particularly in the dermis of the chow chow, where the histiocytic/macrophagic origin of the cells also was ruled out using IBA1. Being a cytoplasmic antigen, Melan A may not be the best immunohistochemical marker for a quantitative evaluation of melanocytes, as a result of its possible interference with cytoplasmic melanin-pigmented granules. In these cases, in fact, a nuclear marker, such as SOX-10 should be preferred as observed in other studies.[28] Melan A also was confirmed as a good melanocytic marker when tested with immunofluorescence and is considered, together with SOX-10, the most sensitive marker for the detection of melanocytes in our samples. These two markers showed an almost complete co-localisation, with SOX-10 being more sensitive and, as a consequence of the nuclear staining, associated with easier recognition by the operator.

Despite being one of the most commonly used markers for melanocytic tumours,[22 27] PNL2 did not show satisfactory results in our study, showing less intense immunolabelling and revealing fewer melanocytes compared to other markers. This result could be the result of a lower expression of the protein itself, which could result in a weaker immunostaining reactivity, less easily recognisable both with immunohistochemical and immunofluorescence evaluation. Moreover, PNL2 appears not to be sensitive in the detection of dermal melanocytes.

The expression of TRP1 in dermal cells could identify dermal melanocytes, yet in our study positive cells were morphologically compatible with mast cells and

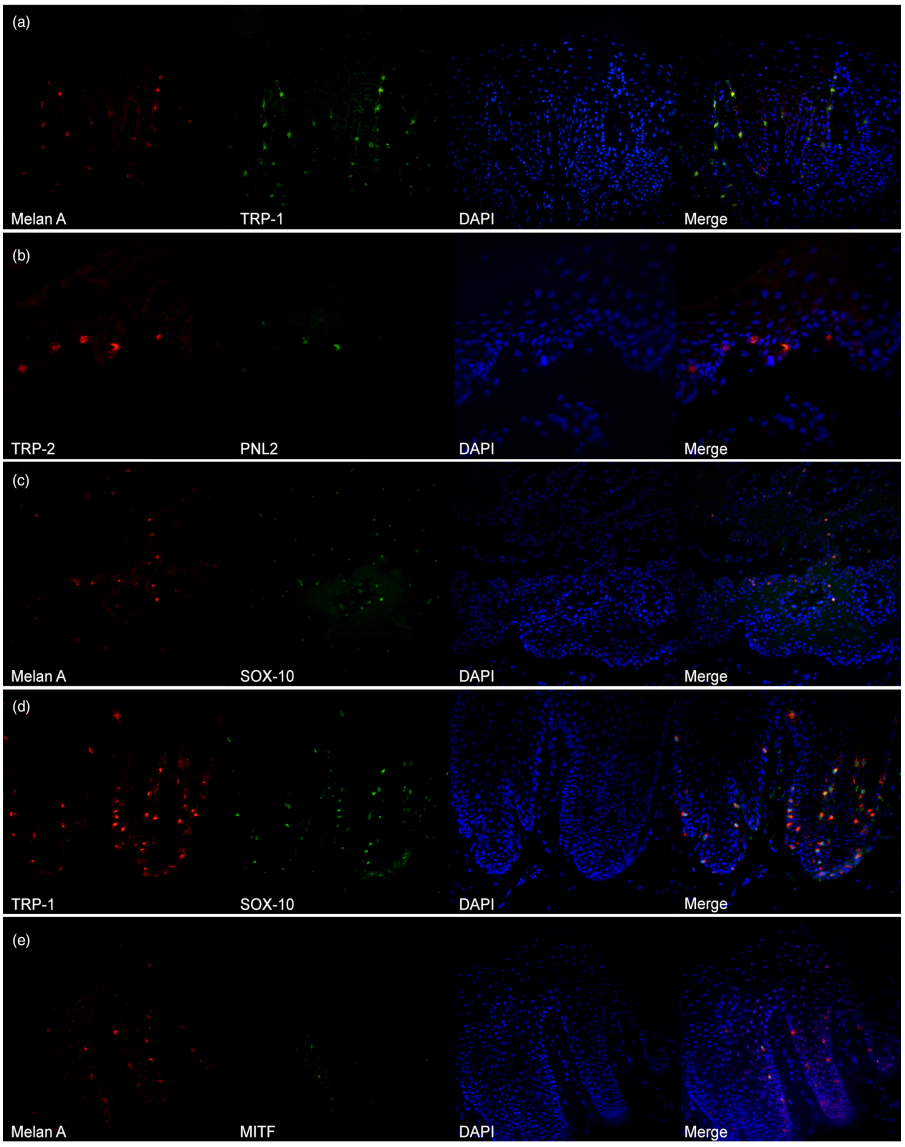


FIGURE 3 Co-localisation of melanocytic markers. (a) Melan A and TRP1 showed an almost total co-localisation on the nose; (b) PNL-2 confirmed a lower sensitivity also when in co-localisation with TRP2; (c) Melan A and SOX-10 showed an almost complete co-localisation for intraepithelial melanocytes; SOX-10 immunolabelling also was co-localised with TRP2 on the nose (d) and mucosal sites; Melan A and MITF did co-localise, yet, as for IHC, MITF signal was weak (e). DAPI was used as a nuclear counterstaining.

TRP1 positivity was not co-expressed with other melanocytic markers in this cellular population. To the best of the authors' knowledge, TRP1 is not expressed in mast cells, and therefore, this result could be interpreted as a nonspecific reaction of the antibody used in this study, likely to have been caused by its polyclonal origin. Currently there are not many monoclonal antibodies available and specific for canine species, and hence, this result should be reassessed with more specific antibodies, preferably targeting specific canine antigens.

Scattered TRP2^{+}cells were observed in the dermis, yet the expression of this marker did not appear to

co-localise with TRP1. Also in this case a nonspecific cross-reaction can be hypothesised. Because of the shape of the TRP1$^+$ dermal cells, the complete lack of pigmentation and lack of cross-reaction with the other antibodies, they could be interpreted as histiocytes/resident macrophages, scattered in the dermis and positive for IBA1 immunolabelling.

SOX-10 is a common marker used to highlight melanocytes in both neoplastic and non-neoplastic lesions in humans,[28–30] and it is recommended for sentinel lymph node assessment in melanoma cases.[31] In humans, no difference has been noted, quantitatively, between SOX-10 and immunostaining for Melan A,[32] in agreement with the findings herein. To the best of the authors' knowledge, this is the first study assessing the normal expression of SOX-10 in canine epithelia and dermis, although some recent data are available regarding its expression in tumours.[24,33] The expression of SOX-10 in mammary gland, also used in the present study as a positive control, previously has been reported in both mouse and human,[19,34] where it plays important regulatory roles in promoting both stem- and EMT-like properties in mammary stem cells.[34]

MITF is another commonly used marker in human medicine and it has shown to be more effective in identifying melanocytes in tumour lesions, rather than in chronic sun-damaged skin, when compared to Melan A.[35] Moreover, it has been suggested as an adjunctive marker that could help in the diagnosis of metastatic tumours that are suspicious for melanoma, yet negative for other melanocytic markers.[36] In dogs, the expression of this marker has been demonstrated in melanomas, but data on MITF expression in this species is scarse.[23] It is worthy of note that in our study, MITF expression was observed in the nuclei of cells from the inner root sheath (IRS), as was reported in mice.[37]

The presence of melanocytes, confirmed by immunohistochemical evaluation, within the basal compartment of Meibomian glands, may be the reason for the presence of a frequent strong melanisation of tumours arising from these structures, when compared to tumours arising from sebaceous glands.[38] Accordingly, the presence of SOX-10$^+$ cells was observed only seldomly in sebaceous glands in occasional samples from the head of one of our cases. It could be postulated that the presence of melanin in Meibomian tumours could be due to a proliferation of a non-neoplastic population of melanocytes, as has been reported in human basal cell tumours, in which the proliferation of melanocytes has been confirmed in different studies.[39]

The presence, particularly in the dermis, of melanocytes expressing different sets of proteins, may indicate the presence of melanocyte subpopulations; these may be at the origin of different melanocytic tumours or melanocytic disorders. Moreover, results from our study suggest the presence of a strong variability among subjects, probably breed-related. For example, dermal melanocytes were confirmed only in a chow chow, which could justify the predisposition towards the development of particular degenerative and neoplastic diseases in some breeds. Nevertheless, the dermal melanocyte subpopulation hypothesis needs to be substantiated by further studies with larger study groups.

One of the limitations of this study is the restricted number of dogs and breeds tested. Although a supposed interbreed variability is supported by our results, nevertheless, it should be tested and supported on a larger scale, possibly by comparing black-coated dogs, which are known to be more predisposed to the development of melanocytic tumours,[16,17] with light-coated breeds. Another point that should be addressed is the implementation of antibodies, with the development of monoclonal antibodies specific for canine species. This probably would reduce the possibility of suspected nonspecific immunolabelling as observed with TRP1 and TRP2.

CONCLUSIONS

Melan A and SOX-10 appear to be the most sensitive and specific markers for melanocytes in canine samples. Moreover, the immunostaining appears sharp and more easily identifiable when compared to the other markers. These data could help in the characterisation of melanocytes in dogs and lay the foundations for a better understanding of the pathogenesis of melanocyte-related diseases.

AUTHOR CONTRIBUTIONS

Ilaria Porcellato: Conceptualization; investigation; funding acquisition; writing – original draft; methodology; visualization; data curation. **Margherita Orlandi:** Methodology; validation; visualization; writing – review and editing; investigation; formal analysis; data curation. **Adriana Lo Giudice:** Investigation; methodology; data curation; validation; writing – review and editing. **Monica Sforna:** Validation; writing – review and editing; visualization. **Luca Mechelli:** Supervision; project administration; resources; writing – review and editing. **Chiara Brachelente:** Funding acquisition; conceptualization; investigation; methodology; validation; formal analysis; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

No conflicts of interest have been declared.

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Résumé

Contexte: Les mélanoblastes proviennent de la crête neurale, d'où ils migrent vers les tissus périphériques et se différencient en mélanocytes. L'altération des ménanocytes durant leur développement et leur vie peut induire différentes maladies : des troubles pigmentaires et des fonctions visuelles et auditives, ainsi que tumeurs telles que le mélanome. La localisation et les caractéristiques phénotypiques des mélanocytes ont été précisées dans différentes espèces, mais ces données manquent chez le chien.

Objectif: L'expression des marqueurs mélanocytaires Melan A, PNL2, TRP1, TRP2, SOX-10 et MITF est étudiée dans des échantillons de peau et de muqueuses de chiens.

Matériels et méthodes: Durant l'autopsie de cinq chiens, des échantillons de muqueuse buccale, de jonction cutanéo-muqueuse, de paupière, de nez et de peau velue (abdomen, dos, pavillon, tête) ont été prélevés ; des analyses immunohistochimiques et d'immunofluorescence ont été réalisées pour évaluer l'expression des différents marqueurs.

Résultats: Les marqueurs mélanocytaires sont exprimés de façon variable selon les différents sites anatomiques, en particulier au sein de l'épiderme de la peau velue et des mélanocytes dermiques. Melan A et SOX-10 sont les marqueurs mélanocytaires les plus spécifiques et les plus sensibles. PNL2 apparait moins sensible, tandis que TRP1 et TRP2 sont rarement exprimés par les mélanocytes intraépidermiques de la peau velue. MITF avait une bonne sensibilité, mais l'expression était souvent faible.

Conclusions et pertinence clinique: Les marqueurs mélanocytaires sont exprimés de façon variable dans différents sites anatomiques, suggérant la présence de sous-populations mélanocytaires. Ces résultats préliminaires ouvrent la voie à la compréhension des mécanismes pathogéniques impliqués dans les maladies mélanocytaires dégénératives et le mélanome. De plus, ces éventuelles différences d'expression des marqueurs mélanocytaires selon le site anatomique pourraient influencer la sensibilité et la spécificité des analyses utilisant ces marqueurs à visée diagnostique.

Resumen

Introducción: los melanoblastos se originan en la cresta neural desde donde migran a los tejidos periféricos y se diferencian en melanocitos. La alteración durante el desarrollo y la vida de los melanocitos puede causar diferentes enfermedades, que van desde trastornos pigmentarios y disminución de las funciones visuales y auditivas, hasta tumores como el melanoma. La ubicación y las características fenotípicas de los melanocitos se han caracterizado en diferentes especies, pero faltan datos sobre perros.

Objetivo: Este estudio investiga la expresión de los marcadores melanocíticos Melan A, PNL2, TRP1, TRP2, SOX-10 y MITF en melanocitos de superficies cutáneas y mucosas seleccionadas de perros.

Materiales y Métodos: durante las necropsias, se obtuvieron muestras de cinco perros de mucosa oral, unión mucocutánea, párpado, nariz y piel con pelo (abdomen, espalda, pabellón auricular, cabeza); Se realizaron análisis inmunohistoquímicos y de inmunofluorescencia para evaluar la expresión de los marcadores.

Resultados: Los resultados mostraron una expresión variable de marcadores melanocíticos en diferentes sitios anatómicos, particularmente en la epidermis de la piel con pelos y en melanocitos dérmicos. Melan A y SOX-10 fueron los marcadores melanocíticos más específicos y sensibles. PNL2 fue menos sensible, mientras que TRP1 y TRP2 rara vez se expresaron en melanocitos intraepidérmicos en la piel con cabello. MITF tenía una buena sensibilidad, pero la expresión a menudo era débil.

Conclusiones y relevancia clínica: Nuestros resultados indican una expresión variable de marcadores melanocíticos en diferentes sitios, lo que sugiere la presencia de subpoblaciones de melanocitos. Estos resultados preliminares inician el camino para comprender los mecanismos patogénicos implicados en los trastornos melanocíticos degenerativos y el melanoma. Además, la posible expresión diferente de los marcadores de melanocitos en diferentes sitios anatómicos podría influir en su sensibilidad y especificidad cuando se utilizan con fines de diagnóstico.

Zusammenfassung

Hintergrund: Melanoblasten stammen aus der Neuralleiste, von der sie ins periphere Gewebe auswandern und zu Melanozyten ausdifferenzieren. Veränderungen während der Entwicklung der Melanozyten und ihres Lebenslaufs können verschiedene Krankheiten bedingen, von Pigmentunregelmäßigkeiten und verminderten visuellen und

auditorischen Funktionen bis hin zu Tumoren wie Melanome. Die Lokalisation und die phänotypischen Merkmale wurden bei verschiedenen Spezies beschrieben, beim Hund gibt es jedoch bisher keine Daten.
Ziel: Diese Studie untersucht die Exprimierung von Melanozytenmarkern Melan A, PNL2, TRP1, TRP2, SOX-10 und MITF an Melanozyten von selektiven kutanen und mucokutanen Oberflächen von Hunden.
Materialien und Methoden: Bei der Nekropsie wurden Proben aus der oralen Mukosa, mucokutanen Übergängen, Augenlid, Nase und behaarter Haut (Bauch, Rücken, Pinna, Kopf) von fünf Hunden genommen; immunhistochemische und Immunfluoreszenz-Analyse wurde durchgeführt, um eine Markerexprimierung zu erfassen.
Ergebnisse: Die Ergebnisse zeigten eine unterschiedliche Exprimierung der Melanozytenmarker an verschiedenen anatomischen Stellen, vor allem innerhalb der Epidermis der behaarten Haut und der dermalen Melanozyten. Melan A und SOX-10 waren die spezifischsten und sensitivsten Melanozytenmarker. PNL2 waren weniger sensitiv, während TRP1 und TRP2 selten an intraepidermalen Melanozyten der behaarten Haut exprimiert wurden. MITF zeigte eine gute Sensibilität, obwohl die Exprimierung oft nur schwach war.
Schlussfolgerungen und klinische Relevanz: unsere Ergebnisse zeigten eine variable Exprimierung der Melanozytenmarker an verschiedenen Stellen, was auf das Auftreten unterschiedlicher Subpopulationen der Melanozyten hinweist. Diese vorläufigen Ergebnisse ebnen den Weg für ein Verständnis der pathogenen Mechanismen, die bei degenerativen Melanozyten-Veränderungen und beim Melanom eine Rolle spielen. Weiters könnte die möglicherweise unterschiedliche Exprimierung der Melanozytenmarker an verschiedenen anatomischen Stellen ihre Sensibilität und Spezifität beeinflussen, wenn sie für diagnostische Zwecke eingesetzt werden.

要約
背景: メラノブラストは神経堤で発生し、そこから末梢組織へ移動してメラノサイトに分化する。メラノサイトの発生・生育過程における変化は、色素障害、視覚・聴覚機能の低下からメラノーマなどの腫瘍に至るまで、様々な疾患の原因となる可能性がある。メラノサイトの位置や表現型は、様々な動物種で明らかにされているが、犬に関するデータは不足している。
目的: 本研究の目的は、犬の皮膚・粘膜のメラノサイトにおけるメラノサイトマーカーMelan A, PNL2, TRP1, TRP2, SOX-10, MITFの発現を検討することであった。
材料と方法: 剖検時に、5頭の犬から口腔粘膜、粘膜皮膚接合部、眼瞼、鼻および有毛皮膚(腹部、背部、耳介、頭部)のサンプルを採取し、免疫組織化学および免疫蛍光解析を行い、マーカーの発現を評価した。
結果　その結果、解剖学的な部位によってメラノサイトマーカーの発現が異なり、特に有毛皮膚表皮内および真皮のメラノサイトの発現にばらつきがあることが判明した。Melan AおよびSOX-10は、最も特異的で感度の高いメラノサイトマーカーであった。PNL2は感度が低く、TRP1およびTRP2は有毛皮膚内の表皮内メラノサイトでほとんど発現していなかった。MITFは感度が高いが、発現が弱い場合が多い。
結論と臨床的意義: 今回の結果は、部位によってメラノサイトマーカーの発現が異なることを示し、メラノサイトの亜集団の存在を示唆するものであった。これらの予備的な結果は、変性メラノサイト障害やメラノーマに関わる発症メカニズムの理解に道を開くものである。さらに、解剖学的部位によってメラノサイトマーカーの発現が異なる可能性があることから、診断目的で使用する場合には、その感度や特異性に影響を与える可能性がある。

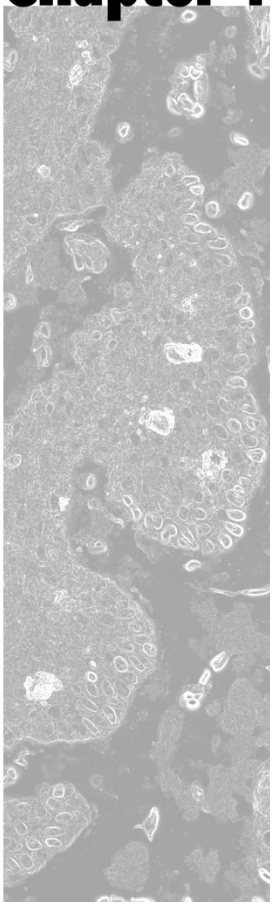
摘要
背景: 黑素细胞起源于神经嵴，从那里迁移到周围组织并分化为黑素细胞。黑素细胞发育和生活过程中的变化会导致不同的疾病，从色素性疾病、视觉和听觉功能下降，到黑色素瘤等肿瘤。黑色素细胞的位置和表型特征已经在不同的物种中得到了表征，但关于犬的数据仍然缺乏。
目的: 本研究研究黑素细胞标志物黑素A、PNL2、TRP1、TRP2、SOX-10和MITF在犬皮肤和粘膜表面黑素细胞中的表达。
材料和方法
尸检时，从五只犬的口腔粘膜、粘膜皮肤连接处、眼睑、鼻子和毛发皮肤(腹部、背部、耳廓、头部)采集样本；进行免疫组织化学和免疫荧光分析以评估标记物的表达。
结果: 结果显示黑素细胞标记物在不同解剖部位的表达不同，尤其是在毛发皮肤的表皮和真皮黑素细胞内。黑素A和SOX-10是最特异和敏感的黑素细胞标志物。PNL2较不敏感，而TRP1和TRP2仅在毛发皮肤表皮内黑素细胞中表达。MITF具有良好的敏感性，但表达往往较弱。
结论和临床相关性: 我们的结果表明，不同部位的黑素细胞标志物表达不同，表明存在黑素细胞亚群。这些初步结果为理解退行性黑素细胞疾病和黑色素瘤的发病机制铺平了道路。此外，当用于诊断目的时，黑素细胞标记物在不同解剖部位的可能不同表达可能会影响其敏感性和特异性。

Resumo
Contexto: Os melanoblastos se originam da crista neural, de onde eles migram para os tecidos periféricos e se diferenciam em melanócitos. Alterações durante o desenvolvimento e ao longo da vida dos melanócitos pode causar diferentes doenças, desde alterações pigmentares e redução das funções auditivas e visuais, a tumores como o melanoma. Localização e características fenotípicas dos melanócitos têm sido detalhadas em várias espécies, mas em cães os dados ainda são escassos.
Objetivo: Este estudo investiga a expressão dos marcadores melanocíticos Melan A, PNL2, TRP1, TRP2, SOX-10 e MITF nos melanócitos de determinadas superfícies cutâneas e mucosas em cães.
Materiais e métodos: À necrópsia, foram coletadas amostras de mucosa oral, junção mucocutânea, pálpebra, nariz e pele pilosa (abdômen, dorso, orelha, cabeça); para se avaliar a expressão dos marcadores, realizou-se imuno-histoquímica e imunofluorescência.

Resultados: Os resultados demonstraram expressão de marcadores melanocíticos em diferentes sítios anatômicos, particularmente na epiderme da pele pilosa e nos melanócitos dérmicos. Melan A e SOX-10 foram os marcadores melanocíticos mais específicos e sensíveis. PNL2 foi menos sensível, enquanto TRP1 e TRP2 foram raramente expressados por melanócitos intraepidérmicos na pele pilosa. MITF apresentou boa sensibilidade, apesar de a sua expressão ser frequentemente fraca.

Conclusões e relevância clínica: Nossos resultados indicam uma expressão variável de marcadores melanocíticos em diferentes regiões, sugerindo a presença de subpopulações de melanócitos. Estes resultados preliminares embasam a compreensão dos mecanismos patogenéticos envolvidos nas doenças melanocíticas degenerativas e no melanoma. Além disso, a possível diferença na expressão de marcadores melanocíticos em diferentes sítios anatômicos pode influenciar a sua sensibilidade e especificidade quando utilizado para diagnóstico.

Chapter 4



Quantification of intraepithelial canine melanocytes in different somatic areas

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Abstract:	Background: In humans, the presence of an even distribution of melanocytes within the skin basal layer allows for uniform pigmentation in healthy and young individuals. Moreover, despite high variability in skin colors and tones, interindividual melanocyte density variability is low. On the other hand, dogs display a high intraindividual pigmentary variability in different anatomic areas. Hypothesis/Objective: Data on canine melanocytes, including their distribution and density, are limited. This study aims to assess melanocyte density across different anatomical areas in dogs of various breeds. Materials and methods: Samples were harvested post-mortem from 22 dogs of different breeds and ages. Samples were collected from: haired skin (back, ventral abdomen, head, pinna), oral and conjunctival mucocutaneous junctions, oral mucosa (buccal mucosa, gingiva, palate) and nose. Immunohistochemical investigation using a cocktail containing Melan-A and SOX-10 antibodies was performed to evaluate melanocytes:keratinocytes ratio (M:K ratio). Results: Variable melanocyte density was recorded in different anatomic areas, with a higher M:K ratio on the eyelid (Mdn=1:4; IQR 1:3.8-1:5.1) and on the nose (Mdn:1:6.5; IQR=1:5.2-1:9.6). Lower ratios were observed on the haired skin, particularly on the head (Mdn=1:113.6; IQR: 1:37.8-1:255.1). Conclusions and clinical significance: Together with different melanocyte densities in different anatomic areas, dogs showed a high interindividual variability, particularly on haired skin. This finding could be associated with color phenotype, sun-exposure, and breed. Variable densities of melanocytes might also justify different incidence of melanocytic tumors in hyperpigmented breeds and in different somatic areas, as well as provide an increased protective effect in chronically sun-exposed areas.

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and in different somatic areas, as well as provide an increased protective effect in chronically sun-exposed areas.

Keywords: melanocyte, Melan-A, SOX-10, canine,

INTRODUCTION

The production of melanin and hence the pigmentation of skin, hair, eyes, and, in some mammals, oral mucosa, depends on the function of the residing melanocytes and can be influenced by different factors, such as age and sun exposure [1–3]. Most of the studies on melanocyte distribution, density, and, in general, biology, have been conducted on humans and experimental animals, but data on canine species are still few [4–8].

In humans, the density of melanocytes varies among anatomical sites with the highest densities observed on the back/shoulders (900 melanocytes/mm^2) and the lowest on the genital area (1500 melanocytes/mm^2) [2,9]. Nevertheless, the presence of an even distribution within the basal layer, allows for a uniform pigmentation in healthy and young individuals.

Melanocyte density has a remarkably small interindividual variability, even when comparing different skin colors and tones in different demographic groups. [10,11]. Different skin phenotypes in fact mainly depend on the amount of melanin and the relative ratio of eumelanin (black to brown pigment) and pheomelanin (red to yellow pigment), together with the size, quantity, and distribution of melanosomes within the epithelia [7,12–14].

The density of melanocytes in humans has also been evaluated in different pathologic conditions, where variations in the number of melanocytes have been observed in association with different cutaneous lesions [1,15,16]. Moreover, it has been postulated that the different melanocyte density,

recorded in different anatomic areas, could be one of the factors involved in the site-specific incidence of melanoma. [2]

To the best of our knowledge, the intraepithelial melanocyte density in different anatomic sites of the dog has never been assessed. A better knowledge of the anatomic distribution of melanocytes on skin and mucosa could be useful in elucidating site-specific lesions' pathogenesis, such as canine uveodermatologic syndrome (Vogt-Koyanagi-Harada-like) syndrome or vitiligo, and also to justify the increased risk of melanoma development in specific areas, such as the oral cavity, and in some canine breeds.

Our group recently assessed the expression of commonly reported melanocytic markers in normal canine skin, finding that Melan A and SOX-10 were the most specific and sensitive markers for the identification of intraepithelial melanocytes in different somatic areas, both on the oral mucosa and haired skin [17].

The aim of the present study is to quantify, by immunohistochemistry with the two aforementioned antibodies, the melanocyte density in different canine anatomic areas, particularly on the haired skin, oral mucosa, mucocutaneous junctions, and nose and to preliminarily evaluate interindividual variability.

MATERIALS AND METHODS

Case selection

Samples were harvested from twenty-two dogs, with no clinically reported dermatological or mucosal lesions, from different breed, sex, and age groups, submitted for necropsy at the Department of Veterinary Medicine, after owner's consent. Samples were collected from different cutaneous, mucocutaneous and oral mucosal sites, less than 6 hours after death. Harvested samples had to be at least 0.8 cm in diameter (measured parallel to the epithelial surface) to

perform macroscopic and histological evaluation. Samples were collected from: oral mucosa (inner aspect of the cheek, just caudal to the labial commissure; cranial part of the hard palate, caudally to the incisive papilla; and gingiva, near the canine teeth), mucocutaneous junction (upper lip on the lateral side, near the canine tooth; lower right eyelid), nose, pinna (tip, where inner aspect was evaluated), ventral abdomen (cranial to the umbilical scar, on the median line), back (lumbar area, on the median line), and head (between the pinnae, on the median line).

Histology and immunohistochemistry

Hematoxylin and eosin (HE) stain was routinely performed, then ten serial 5-µm sections were cut from formalin-fixed and paraffin-embedded samples and mounted on poly-L-lysine-coated slides, which were then dewaxed and dehydrated. Immunohistochemistry was performed with a cocktail of Melan A and SOX-10, [17] following standard procedures. Positive controls were obtained from canine melanomas that had been previously tested positive for the two antibodies. Negative controls were run omitting the cocktail and incubating control sections with TBS.

Pigmentation evaluation

The presence of pigmentation was evaluated macroscopically on the interfollicular and mucosal epithelium after inclusion in paraffin and sectioning, using a magnifying lens. Briefly, the degree of pigmentation of epithelial surfaces was semiquantitatively graded (0: pink/whitish skin with no spots; 1: light brown skin with no spots; 2: dark brown skin with no spots; 3: black skin with no spots; P: patchy) , as previously reported [17].

On HE stained slides, the quantity of melanin present in the epithelium was evaluated and assigned a semiquantitative value (0: no melanin visible; 1: mildly pigmented; 2: moderately pigmented; 3: heavily pigmented).

97

98 **Assessment of canine intraepithelial melanocyte density**

99 Five digital pictures were captured starting from hot spots using a 40x magnification. Cases were
100 included when >250 basal keratinocytes could be counted in the sections. Then, positive
101 melanocytes and basal keratinocytes were manually counted on the acquired pictures. Cases were
102 included when >250 basal keratinocytes could be counted in the sections. Melanocytes where
103 counted only if the nucleus was visible in the section and keratinocytes were considered basal if in
104 direct contact with the epidermal basal membrane [1]. The mean melanocyte:keratinocytes ratio (M:K
105 ratio) from the five pictures was then calculated.

106

107 **Statistical analysis**

108 Normality was assessed with a Shapiro–Wilk test for all continuous variables. Descriptive statistics
109 were used to describe data; values are expressed as medians (Mdn) and interquartile range (IQR).
110 Non-parametric tests were used to test hypotheses. The Kruskal-Wallis test and Mann–Whitney U
111 test were performed to assess differences among groups. Correlation analysis was performed
112 using Spearman's test (ρ). Statistical analyses were performed using IBM SPSS (version 21).

113

114 **RESULTS**

115 **Case selection**

116 The twenty-two selected dogs had a mean age of 8,75 years (3 months-15 years); 15 were male
117 and 7 females. Among the selected cases, 9 were mixed breed dogs, 2 Labrador Retriever, 2
118 German Shepherd, 2 Segugio Italiano, 1 English Setter, 1 Chow Chow, 1 Boxer, 1 Border Collie, 1
119 Cocker Spaniel, 1 Maremma Sheepdog, and 1 West Highland White Terrier. The dogs included in
120 the study are listed in Table 1.

Pigmentation evaluation

Results of the pigmentation evaluation on both macroscopic and histologic samples for each case and somatic area are reported in Table 2. Examples of grading are reported for both macroscopic (Figure 1) and histological grading (Figure 2). The highest mean pigmentation was observed on samples from the nose, both at macroscopic (mean: 2.91) and histological evaluation (mean: 3). Instead, the area that showed a lower pigmentation was the back and, also in this case, the lower grade was recorded both for the macroscopic (mean: 1.14) and the histological samples (mean: 0.7).

Assessment of intraepithelial canine melanocyte density and association with pigmentation

Haired skin

The M:K ratio was very variable on haired skin areas that were examined in the present study. The median melanocyte density was lower on the head (1:113.6; IQR: 1:37.8-1:255.1) and on the back (1:88.5; IQR:1:63.3-1:141), whereas higher densities were recorded on the abdomen and the pinna (1:69.8; IQR:1:37.4-1.181:5 and 1:27.1; IQR: 17.8-1:51.1, respectively). A very high interindividual variability was observed, which was lower on the pinna (Figure 3a). Generally, in cutaneous sites, the number of keratinocytes per melanocyte was inversely correlated with the degree of histological and macroscopic pigmentation ($p<0.05$), apart from macroscopic pigmentation of pinna and abdomen, that did not show statistical significance.

Oral mucosa

The oral site where M:K ratio was higher was the hard palate (median: 1:20.7); samples from this site were also the ones that showed a lower variability in terms of melanocyte density (IQR:

1:12.3-1:31.1). A high median M:K ratio was also observed on the buccal mucosa (1:27.1), but the variability was very high (IQR:1:14.4-1:126.4), with two cases reaching very low melanocyte ratios (<1:500). In this case, both macroscopic and histologic pigmentation grade were inversely associated with the number of keratinocytes/melanocyte ($p<0.05$, $\rho=-.730$, $p<0.05$, $\rho=-.727$ respectively). Gingiva was the oral site showing lower median melanocyte density (1:78.7) and the highest variability (1:28.1-1:236.7) in oral samples (Figure 3b).

Mucocutaneous junctions and nose

Melanocyte density was higher on mucocutaneous junctions and nose (Figure 3c). On the lip mucocutaneous junction the median M:K ratio was 1:8.1 with a low interindividual variability (IQR: 1:6.4-1:11.2). On the eyelid mucocutaneous junction, the median M:K ratio was 1:4 and, also in this case, the variability was low (IQR 1:3.8-1:5.1).

The nose was the area that showed a median M:K ratio, with 1:6.5 (IQR 1:5.2-1:9.6). No correlation was observed between melanocyte density and degree of macroscopic and histologic pigmentation in both mucocutaneous junctions and nose, except between lip and macroscopic degree of pigmentation ($p<0.001$, $\rho=-.670$).

Discussion

The distribution and the and density of canine melanocytes on haired skin, oral mucosa and mucocutaneous junctions has never been assessed in a dedicated study. Dogs are currently considered a good translational model for melanocytic tumors, particularly for the mucosal ones, with the oral melanoma being the most common and, for some aspects, similar to the human counterpart [18–22]. Nevertheless, information on the cell of origin, the normal canine melanocyte, is limited and most of the studies rely on data obtained from humans or other animal species.

Evaluation of the genotypic, metabolic, and phenotypic characteristics of normal melanocytes would be useful not only to better understand neoplastic melanocytes, but would also shed light on the pathogenesis of some pigmentary disorders, such as uveodermatologic syndrome and vitiligo patterns[23,24]. The aim of the present study is to deepen the knowledge on canine melanocytes, focusing on their distribution and density, in a population of dogs of different age, sex, and breed.

Focusing on distribution of melanocytes, we observed that melanocytes are almost absent from the basal layers of the skin in areas of non-pigmented epithelium (Figure 4). This seems to be different from human skin where pigmentation of the skin is uniform, due to a regular distribution of melanocytes in the basal layer of the epidermis [9,25]. In humans, melanocytes are present in similar numbers in different ethnic groups also in the oral mucosa, where they may or may not produce melanin. Pigmentation is attributed to variations in the activity of melanocytes in the basal cell layer of the oral epithelium [26], on the contrary, in dogs we observed that the number of melanocytes was highly variable when comparing pigmented and non-pigmented mucosa, with non-pigmented mucosa occasionally completely negative for melanocytic markers (Figure 4b).

We are aware that the macroscopic and histological evaluation of epithelial pigmentation conducted in our study entails significant subjectivity. Additionally, tracking pheomelanin can pose challenges. However, we cross-referenced with images taken from the dog and confirmed the non-pigmented states of the areas, supporting our observation.

Considering melanocyte density, we observed that, despite being located on the dorsal area, hence more sun-exposed, the head and the back showed a lower median density of melanocytes, when compared to the inner aspect of the pinna and abdomen. This finding may be due to the presence of a thicker hair coat on chronically UV-exposed dorsal areas, being protected from light. On the contrary, melanocyte density was higher in areas where hair coat is thinner or not present

193 (pinna and abdomen), likely providing for a higher protection through melanin production. This
194 finding is be supported also by the histological quantification of melanin, that showed a higher
195 mean value on the abdomen and pinna. Nevertheless, this result has been obtained from a semi-
196 quantitative evaluation and takes in account only one of the variables that contribute to the final
197 pigmentation phenotype. More sensitive methods to evaluate the production of melanin, quantity
198 of melanosomes, and types of melanin (eumelanin VS pheomelanin) should be applied.
199 In oral mucosa, the presence of pigmentation was correlated with melanocyte density only on
200 buccal mucosa, whereas no statistical significance was reached for pigmentation and melanocyte
201 density correlation in palate and gingiva. M:K density was less variable among different dogs on
202 the palate, whereas it was higher on the other sites, presumably because of a higher pigmentation
203 variability observed on buccal mucosa and gingiva.
204 Mucocutaneous junctions and the nose were the sites where melanocyte density was higher and
205 that showed a lower variability among dogs of the different age, sex, and breed. Interestingly,
206 these sites are invariably highly pigmented in healthy dogs and usually do not show the presence
207 of patches non-pigmented areas. This is likely due to the fact that these non-haired areas need for
208 additional UV-light protection.
209 The conspicuous interindividual variability in pigmentation and, hence, melanocyte density, in oral
210 mucosa and on skin, could account for the higher risk of developing melanocytic tumors reported
211 for highly pigmented canine breeds [27]. Despite this, melanomas arising on the non-haired skin of
212 the nose are rare, even though this is the anatomic site with a higher melanocyte concentration.
213 We can postulate that the hyperpigmentation is likely protective from UV-light damage and,
214 differently from the oral cavity which can be easily affected by inflammation secondary to trauma
215 (also caused by chewing or playing) or stomatitis, the nose is an uncommon site for non-UV
216 damage to occur. Indeed, in dogs several copy number alterations and low numbers of single-

nucleotide variations with non-UV-associated mutations were identified in melanomas, postulating the existence of different pathogenetic mechanisms[28].

We can conclude that, differently from humans, dogs have a high intraindividual and interindividual variability in terms of melanocyte density. The interindividual variability could account for the higher risk, in highly pigmented breeds, of developing melanocytic tumors both on cutaneous and oral sites.

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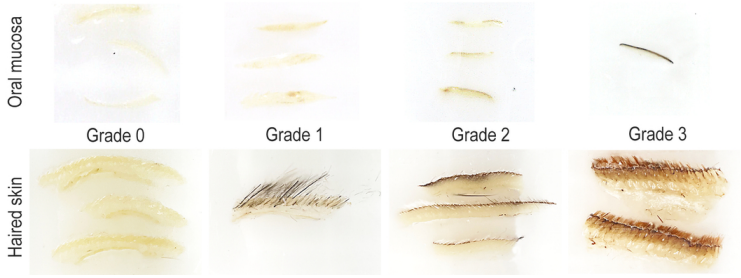
FIGURE LEGENDS:

FIGURE 1 Macroscopical appearance of samples from oral mucosa (gingiva) and haired skin (head) with an example of the pigmentation evaluation grading.

297 **FIGURE 2** Histological appearance of samples from oral mucosa (gingiva) and haired skin (pinna)
298 with an example of the pigmentation evaluation grading.
299
300 **FIGURE 3** Melanocyte density (expressed as number of keratinocytes/melanocyte for a better
301 graphical visualization), is highly variable in different anatomical areas, with mucocutaneous
302 junctions and nose displaying the lower interindividual variability and the highest melanocyte
303 density.
304
305 **FIGURE 4** Comparison of different macroscopical and immunohistochemical results on haired skin
306 (a and d)(M:K ratio = 1:60.2, Case 18), non-pigmented oral mucosa (b and e)(M:K ratio = 1:513,
307 Case 12), and moderately pigmented lip mucocutaneous junction (c and f)(M:K ratio = 1:30.1, Case
308 18).
309
310



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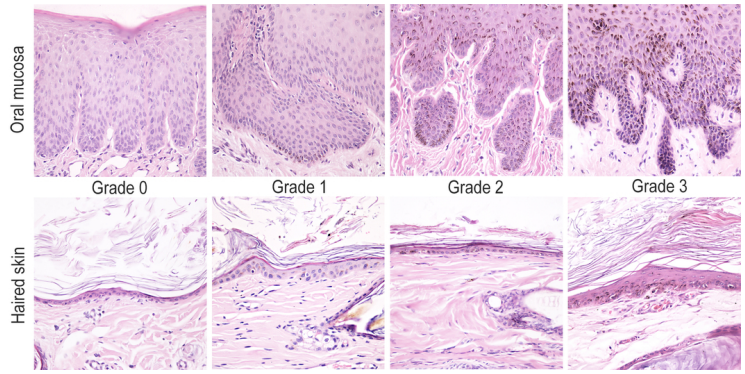

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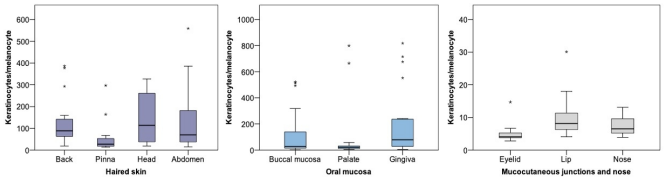

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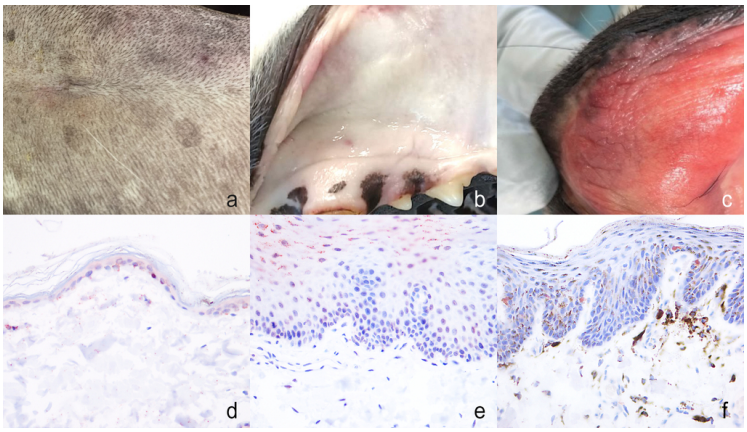

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Table 1. Dogs of the study.

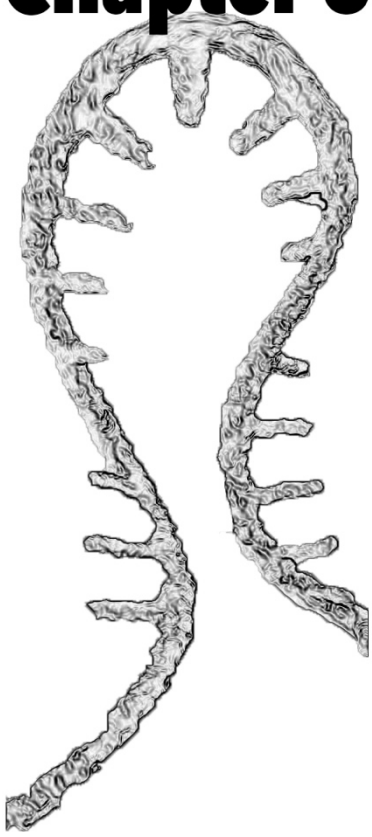
N.	Breed	Age	Sex
1	Mixed breed (German Shepherd cross)	1	M
2	English Setter	1.5	F
3	Mixed breed (Deutsch Kurzhaar cross)	8.5	M
4	Chow Chow	15	F
5	Mixed breed	adult	M
6	Boxer	9	M
7	Labrador Retriever	14	M
8	Labrador Retriever	11	F
9	Mixed breed	adult	M
10	Mixed breed (English Setter cross)	15	M
11	Mixed breed (Siberian Husky cross)	18	F
12	Border Collie	3	M
13	Segugio Italiano	adult	M
14	Cocker Spaniel	15	M
15	Mixed breed	7 mm	F
16	Mixed breed	7.5	F
17	German Shepherd	6	M
18	Segugio Italiano	8	M
19	Maremma Sheepdog	3 mm	F
20	West Highland White terrier	14	M
21	Mixed breed	9	M
22	German Shepherd	10	M

TABLE X

N.	Back			Pinna			Head			Abdomen			Buccal mucosa			Palate			Gingiva			Eyelid			Lip			Nose		
	M	HE	Mean M:K ratio	M	HE	Mean M:K ratio	M	HE	Mean M:K ratio	M	HE	Mean M:K ratio	M	HE	Mean M:K ratio	M	HE	Mean M:K ratio	M	HE	Mean M:K ratio	M	HE	Mean M:K ratio	M	HE	Mean M:K ratio	M	HE	Mean M:K ratio
1	1	1	113,3	2	2	37,8	0	0	135,0	0	0	559,0	2	1	15,9	2	1	24,3	0	0	241,5	2	2	5,5	2	2	8,1	3	2	9,7
2	1	1	62,8	3	2	20,8	3	3	25,6	1	1	79,4	1	1	159,5	3	3	9,3	3	3	31,7	3	2	14,7	3	2	13,1	3	3	11,6
3	2	1	159,5	2	2	13,9	2	1	24,9	2	1	158,5	2	1	20,3	2	2	16,2	2	2	35,0	3	3	3,8	1	0	11,3	2	2	5,1
4	1	0	109,5	1	1	32,6	0	0	238,0	0	0	242,0	2	2	16,1	2	2	17,4	1	1	10,2	2	3	3,8	2	3	8,2	2	1	5,3
5	1	0	141,5	3	1	49,2	2	1	37,7	2	1	118,0	2	2	14,4	2	2	4,9	1	1	30,3	3	3	4,1	2	3	8,9	3	2	6,5
6	3	3	18,7	3	3	13,7	2	2	41,2	2	3	14,7	3	3	2,8	3	2	32,0	1	0	676,0	3	3	3,0	3	3	6,3	3	3	3,9
7	0	0	84,0	1	2	13,4	1	0	232,0	0	0	21,6	1	2	39,8	2	2	16,0	1	0	5,6	3	3	4,0	3	3	6,0	3	3	5,5
8	0	0	92,8	0	1	n/a	2	2	145,3	2	2	21,2	3	3	9,4	3	3	23,4	1	2	n/a	3	3	3,8	3	3	5,6	3	2	5,5
9	1	1	84,8	3	3	18,7	2	2	29,6	2	3	37,4	2	1	139,0	3	3	13,0	2	1	139,3	3	3	5,6	3	2	4,1	3	3	5,4
10	2	2	50,8	2	2	15,1	2	2	46,7	2	3	18,6	1	1	88,7	2	2	11,6	3	2	9,5	3	3	3,9	3	3	6,6	3	3	4,3
11	2	1	92,3	3	2	n/a	1	0	327,0	2	2	39,4	2	2	14,5	2	3	29,0	1	2	25,9	3	3	3,9	3	2	10,4	3	3	5,2
12	1	0	146,5	2	2	67,0	1	0	320,0	2	2	181,5	1	0	513,0	3	3	25,3	3	2	232,0	3	3	5,0	3	3	9,3	3	3	7,8
13	2	1	76,6	3	2	18,1	3	3	18,6	1	0	386,0	1	1	493,0	3	3	18,0	0	0	715,0	3	3	4,3	3	3	6,2	3	3	9,4
14	1	1	43,9	2	3	26,4	2	2	92,3	n/a	3	n/a	1	2	9,1	2	2	6,6	0	0	n/a	3	3	2,8	3	2	6,3	3	3	n/a
15	2	2	65,0	2	1	297,0	2	2	38,0	2	2	67,5	1	0	318,0	2	1	58,3	0	0	78,8	n/a	n/a	n/a	2	2	10,8	3	3	8,8
16	0	0	386,0	2	1	164,0	1	0	161,0	1	1	37,0	3	3	33,7	2	2	31,8	3	3	552,0	3	3	6,7	2	2	11,7	3	3	11,9
17	1	0	139,5	2	2	16,7	0	0	288,0	1	1	281,0	2	2	28,1	3	3	24,2	2	3	818,0	3	3	4,5	3	3	7,4	3	3	9,6
18	2	1	36,0	3	3	27,7	2	2	41,9	2	1	60,2	1	0	522,0	3	3	40,4	3	3	39,5	3	3	3,8	2	1	30,1	3	3	7,4
19	0	0	293,0	1	0	59,0	2	2	36,3	2	1	123,0	2	2	25,9	1	0	665,0	1	0	163,3	3	3	4,3	2	3	13,5	3	2	9,6
20	0	0	377,0	1	1	39,0	1	0	291,0	1	1	303,0	1	2	38,3	2	1	799,0	n/a	n/a	n/a	2	3	6,1	2	3	18,0	3	3	13,1
21	1	0	65,0	2	0	56,8	0	0	271,0	0	1	69,8	3	2	6,2	3	2	6,5	2	2	7,9	3	2	n/a	3	2	6,7	3	2	4,8
22	1	1	43,5	1	1	24,3	1	0	260,8	1	2	44,4	3	2	9,0	3	3	12,1	2	0	176,5	3	3	3,6	3	3	6,8	3	3	5,2

n/a samples were due to: small samples after recut, not sufficient for the analysis, samples where the immunostaining showed background or artifacts, and samples with inflammatory lesions. These samples were impossible to analyze or excluded from the analysis.

Chapter 5



Unravelling canine melanocyte diversity: a single-cell sequencing exploration across body regions

In preparation

This study was the subject of a research proposal for a ESVD (European Society of Veterinary Dermatology) major grant, which was successfully funded. Funding commenced in September 2024, and the results presented here are preliminary and in an early stage of development. These results are currently being prepared for publication

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Abstract

In recent years, the characterization of melanocyte populations in skin and mucosal sites has significantly contributed to our understanding of melanocyte biology and pathology, particularly in human medicine. While similar efforts have been made in veterinary medicine, particularly in dogs, there are still numerous unanswered questions regarding the biology of canine melanocytes. Melanocytic diseases remain a significant concern in veterinary medicine and are characterized by distinct phenotypic presentation. Additionally, the site-specific incidence of canine melanomas in the skin and oral mucosa presents an intriguing area of investigation, given the aggressive behavior of oral melanomas compared to the typically benign behavior of cutaneous ones. Understanding the transcriptomic landscape of canine skin and mucosa is crucial for deciphering tissue-specific gene expression patterns and their implications in health and disease.

This study aims to employ single-cell RNA sequencing (scRNA-seq) techniques to comprehensively profile the transcriptomes of normal melanocytes and adjacent cell populations from oral mucosa and haired skin in dogs.

For this study, samples were collected from seven dogs that underwent surgery or have decedead shortly before sample collection. Both skin and oral mucosa were obtained from five dogs, while oral melanomas were obtained from three dogs. The samples were subsequently minced and digested to obtain single-cell suspensions. Single-cell RNA sequencing was then performed by comparing isolated melanocytes and adjacent cells from skin, oral mucosa, and oral melanomas using state-of-the-art sequencing platforms. Bioinformatics analysis was employed to identify differentially expressed genes, pathways, and regulatory networks associated with these two specific body areas.

Subsequently, the cell type identity of each cluster of cells will be determined by the expression of differentially expressed genes. Comparative analysis between skin and oral mucosal melanocytes and adjacent cell populations will be conducted to delineate tissue-specific gene expression patterns. We hypothesize that distinct canine body areas, namely skin and oral mucosa, harbor unique melanocyte transcriptomic profiles, reflecting tissue-specific functions and microenvironmental cues. The objective of the study is to conduct an initial characterization of the transcriptome of canine healthy melanocytes from oral mucosal and, by revealing the transcriptional heterogeneity of melanocytes, to identify tissue-specific gene expression patterns and potential regulatory mechanisms modulating melanocyte function. By comparing gene expression profiles of cutaneous and mucosal melanocytes, alongside considering the anatomical and immunological differences of their respective host tissues, the long-term objective of this research is to expand our understanding of the role of melanocyte heterogeneity in maintaining skin and mucosal homeostasis. Furthermore, we expect that our study will clarify what is the relationship between melanocyte diversity and the diverse phenotypic and

biological manifestations of melanocytic diseases involving these sites, potentially identifying targets for therapeutic interventions.

Introduction

Melanocytes are pigment-producing cells, derived from the neural crest, and distributed throughout the skin, mucosa and other tissues in the body where they play essential roles in skin pigmentation, protection from solar radiation, and endolymph formation (Bertrand et al., 2020; Brombin and Patton, 2024; Hirobe, 2011). Disorders affecting melanocytes can therefore manifest as syndromic diseases with signs such as hypopigmentation, blindness, and deafness, both in humans and dogs (Cheli et al., 2010; Haase et al., 2022; Hayward et al., 2020; Kim et al., 2014; Strain, 2015; Udagawa et al., 2024; Wen et al., 2024). Melanocytic tumors are also frequent neoplasia in human and veterinary medicine (Barutello et al., 2018; Kim et al., 2021; Smedley et al., 2022; Smith et al., 2002; Van Der Weyden et al., 2020). While much is known about human and laboratory animal melanocytes, canine melanocyte biology remains largely unexplored. Canine diseases like vitiligo and Vogt-Koyanaghi-Harada (VKH)-like syndrome offer potential models for human counterparts (Essien and Harris, 2014; Tham et al., 2019; Yamaki and Ohono, 2008), while canine mucosal melanoma serves as a model for the aggressive human counterpart. In addition, canine melanomas present a very different biological behavior depending on their location, with oral melanomas showing a very aggressive behavior (similar to human oral melanoma), while cutaneous melanomas typically have a benign biological behavior (contrary to human cutaneous melanoma) (Bergman, 2007; Nishiya et al., 2016; Smedley et al., 2011; Smith et al., 2002; Stevenson et al., 2023; Wong et al., 2019). The reason why melanomas in dogs behave so differently depending on the site of origin is still widely discussed, underlining the need for studies that better characterize the biology of canine melanocytes, and attempting to highlight the differences related to their tissue origin. Our group has recently systematically assessed the density of melanocytes in different oral mucosal and cutaneous areas of dogs, providing a demonstration of an uneven distribution of melanocytes in different body regions with different expression profiles of melanocytic markers (Porcellato et al., 2023). These differences may partially explain the site-specific incidence and behavior of some pathological conditions, such as hyper/hypopigmentation disorders and melanocytic tumors. It is still unknown, however, whether these differences reflect different biological properties of melanocytes. In humans, the DNA sequencing has provided effective insights into melanocyte biology, and thanks to the rapidly decreasing costs, a large amount of data has been recently generated, elucidating the pathogenesis of neoplastic and non-neoplastic melanocytic diseases (Wong et al., 2023; Zaman et al., 2024; Zeng et al., 2020). However, the subtle heterogeneity

within cell populations cannot be revealed with these approaches. Single-cell RNA sequencing techniques (scRNA-seq), although more technically challenging, offer many advantages compared to bulk-based transcriptomic measurements. The latter provide an average gene expression for an entire cell population being analyzed but they cannot distinguish between individual cells and are, therefore non suitable for identifying cell types, characterizing cell states, and understanding cellular dynamics within complex tissues or heterogeneous populations (Balderson et al., 2024). In contrast, single-cell sequencing offers unprecedented resolution, enabling the identification of genetic, epigenetic, and transcriptomic differences in single cells of a given cell population. This technique has provided high-resolution maps of melanocyte subpopulations in human hair follicles (Palmer et al., 2023) and epidermis from different anatomical sites (Belote et al., 2021) and has allowed the identification of a transcriptional heterogeneity occurring during melanoma progression (Shi et al., 2023; Tirosh et al., 2016) partially responsible of drug resistance (Belote et al., 2021; Rambow et al., 2018). A scRNA-seq approach elucidated the pathogenesis of vitiligo in human patients, by investigating the immune cell infiltration in and around lesion areas as well and immune cells on peripheral blood (Liu et al., 2024; Yang et al., 2023) and allowed to discover networks of communication between cells, identifying specific states of keratinocytes that sustain inflammation and prevent the process of repigmentation (Shiu et al., n.d.). ScRNA-seq has been seldomly used in canine cancers (Ammons et al., 2023; Ayers et al., 2021) and, to our knowledge, no studies have been conducted in veterinary medicine to elucidate the distinct melanocyte populations and their unique functional characteristics across different body areas in dogs. Our study seeks to enhance the understanding of canine melanocytic biology by combining, for the first time, a single-cell approach to explore normal melanocyte populations between skin and oral mucosa. We contemplate this research as a crucial foundational step in investigating canine melanocytic diseases, such as vitiligo and melanocytic tumors affecting different body regions. Advancing knowledge in this area will be beneficial to veterinary medicine and may offer insights relevant to human health, based on the utility of the dog as a valuable model for analogous human diseases.

To achieve our project's objectives, we have opted for single-cell RNA sequencing (scRNA-seq) using the Chromium platform by 10x Genomics (Wang et al., 2021) for its numerous advantages in exploring melanocyte diversity:

1. Extraordinary resolution granted by the examination of individual cell transcriptomes. This high-resolution approach offers an advantage on bulk sequencing methods.
2. Identification and characterization of rare cell populations: Melanocytes are relatively rare compared to other cells in the epidermis and dermis. Single-cell

sequencing facilitates the identification and characterization of these rare cell subpopulations.
3. Tissue-specific cellular responses: Comparing the same transcriptomes of cells across various body regions (skin versus mucosa), including melanocytes, keratinocytes, and immune cells, can reveal functional differences shaped by unique microenvironments, enriching our comprehension of tissue-specific cellular responses.

Materials and Methods

Sample collection

Tissue samples from healthy skin and oral cavity were collected from seven patients at the University of Perugia, Department of Veterinary Medicine and Institute of Animal Pathology, Vetsuisse Faculty, University of Bern (see Table 1). The samples were derived from dogs undergoing surgery or dogs dead from causes not related to melanocytic diseases. After collection, the samples were immediately stored in complete RPMI with 10% FCS and processed within 2 hours to obtain a single-cell suspension for the gel droplet needed for the single-cell analysis. Firstly, the fragments were cleaned from fat tissue and debris and minced into small pieces in a Petri dish. The removal of fat tissue is crucial for the analysis, as the process relies on viable cells, and adipocytes typically do not survive the tissue digestion procedure. Afterward, they were washed in a tube with PBS and centrifuged. The pellet obtained was then digested for 1 hour with a dispase solution (2.4 U/ml) and then for 20 minutes with collagenase (2 mg/ml). After the digestion arrest, the tissue was filtered through a 70µm strainer first and then through a 40µm strainer. Finally, this cell suspension was centrifuged again, the supernatant was discarded, and the cells were cryo-preserved until the analysis.

Viability test

According to the requirements of 10x Genomics, the optimal viability of cells to be processed for subsequent experimental steps is at least 80%. This cut-off was established to account for the noise generated by the RNA released from damaged cells, which can compromise data quality and introduce variability into downstream analyses. A typical experiment requires 16.5uL of a 1000 cells/uL single-cell suspension to target a cell recovery of 10.000 cells. To achieve this threshold and prevent the inclusion of RNA fragments from deceased cells, fluorescent-activated cell sorting (FACS) was used to eliminate non-viable cells and cell clumps. This strategy ensures a balance between obtaining a sufficient number of single cells in suspension and minimizing the risk of pipeline overcrowding/clumping within the instrument. Non-fixed, separated single cells were incubated with DRAQ5, which selectively binds to double-stranded DNA and DAPI, which penetrates inside the

nucleus of damaged and dead cells. The viable cell population was gated and collected. Following this step, the live/dead staining kit was applied. The cells were incubated with nucleic acid-binding dyes (i.e. propidium iodide (PI) and 7-ADD), which selectively bind to double-stranded DNA. Non-viable cells are visualized as fluorescent cells, whereas live cells remained unstained, as the stains did not penetrate live cells.

Single-cell RNA sequencing and data analysis

In the study, samples for sequencing were taken from both cutaneous and oral mucosal sites in four dogs. For one dog, samples included skin, oral mucosa, and oral melanoma, while in two other dogs, only oral melanoma was sampled. The technology adopted in this experiment, the Chromium (10x Genomics), is a droplet-based system, which includes the following sequential steps:

1- Cell partitioning, labeling, and pooling: the sample containing the cells of interest was loaded onto the Chromium console, where each cell was individually partitioned into nanoliter-scale Gel Beads-In-Emulsion (GEMs). Each GEM contains a gel bead with unique barcoded oligonucleotide and a single cell.
2- Cell lysis and barcoding: within each GEM, the cell was lysed, and its RNA molecules were released. The barcoded bead enabling the identification of the RNA transcripts originating from each cell.
3- cDNA synthesis and amplification: the released RNA molecules within each droplet were reverse transcribed to generate complementary DNA (cDNA) and amplified. Each cDNA molecule was tagged with the same barcode sequence as the bead so that each transcript corresponded to its originating cells.
4- Library preparation: the amplified cDNA molecules from the previous step were pooled together, and sequencing libraries were prepared using standard molecular biology techniques. These libraries contain the barcoded cDNA fragments representing the transcriptome of each individual cell.
5- Sequencing: the prepared libraries were then subjected to next-generation sequencing (NGS) to determine the sequence of the cDNA fragments and quantify the gene expression levels in each cell.
6- Data analysis: Sequencing data was aligned to the UU_Cfam_GSD_1.0 reference transcriptome (NCBI RefSeq assembly GCF_011100685.1) using Cell Ranger (v8.0.1, 10x Genomics). Following initial quality control, analysis will utilize Python libraries including Scanpy (v1.9.5), Pandas (v1.5.3), Matplotlib(v3.7.2), NumPy (v1.25.0), and Seaborn (v0.12.2) for normalization, data integration, dimensionality reduction, clustering, and

cell type annotation 1–5 . These analyses will allow the construction of a transcriptomic atlas capturing gene expression profiles across different cell types (Hunter, 2007; McKinney, 2010; Walt et al., 2011; Wolf et al., 2018).

Preliminary Results

Sample collection

Samples were collected from seven dogs (Table 1). Both cutaneous and oral tissue were obtained from five patients, while melanomas were obtained from the oral cavity of three dogs. Samples of the healthy skin were taken from the abdomen. However, due to the impossibility of taking a sample from this site, the skin sample derived from the patient named Ozzy was collected from the intermandibulary region. Samples from the oral mucosa were obtained from the internal side of the lip, close to the gingiva and inferior labial frenulum.

Table 1. List of fresh tissue samples collected at the Universities of Perugia and Bern. In green all the samples used for the sequencing.

	Oral tumor	normal oral mucosa	normal skin
Czechoslovakian Wolf, F	no	yes	yes
German Shepherd, F	no	yes	yes
Mixbreed, M	no	yes	yes
Maltese, M	no	yes	yes
Ozzy_Mixbreed, M	yes	yes	yes
Shot_Pittbull, M	yes	no	no
Rudi_Mixbreed, M	yes	no	no

Sequencing

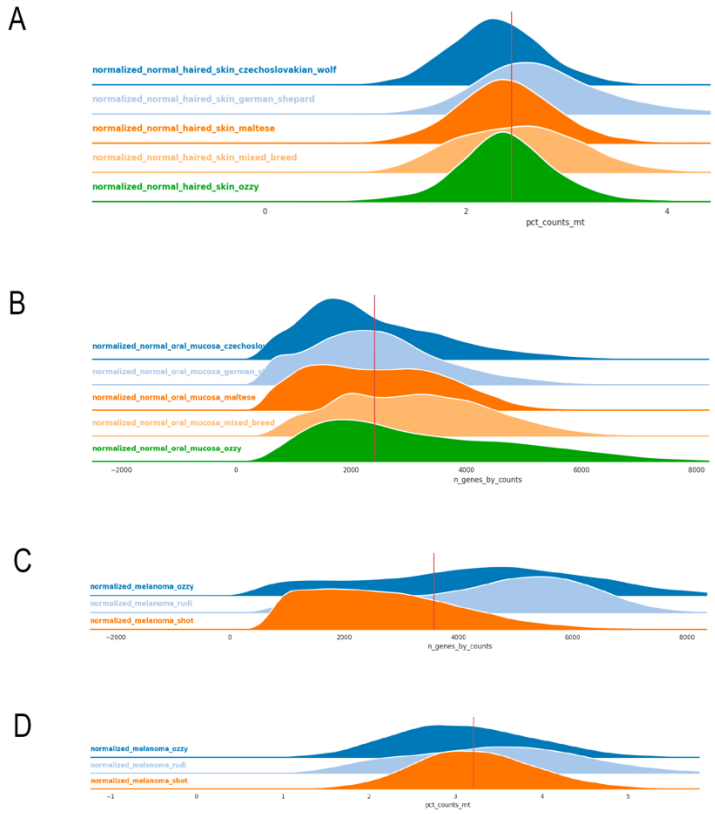
To ensure the reliability and accuracy of the single-cell RNA sequencing data, a comprehensive quality check (QC) was conducted to identify and exclude low-quality cells or reads that might introduce technical artifacts and bias downstream analyses. The QC process revealed a favorable range of median total gene counts per cell across all samples, with values falling between 2,000 and 3,000 genes per cell, as shown in Table 2 and in Figure 1. This range is considered optimal for capturing a robust transcriptomic profile and suggests that the proportion of damaged or stressed cells in the dataset is minimal, ensuring data quality.
In the normal tissues, a comparison of the skin and oral mucosa samples indicated minimal differences in gene counts, although oral mucosa samples exhibited slightly higher gene counts. The overall quality of these samples remained high,

with a mitochondrial gene count always below a cut-off of 10% as previously reported in literature (Ammons et al., 2023), signifying that the cells are healthy and well-preserved. This indicates that the cell sorting process was highly effective in enriching for viable, healthy cells across both skin and oral mucosa samples.
In the melanoma samples, a notable difference was observed between the sample "Shot" and the other two samples, "Ozzy" and "Rudi". The latter exhibited higher gene counts, which may suggest a greater degree of cellular heterogeneity in these samples. This could reflect a more diverse population of cell types, resulting in higher gene expression variability. Although biological factors could contribute to these differences, the influence of technical variability is unlikely, as all samples were processed within the same batch, ensuring consistency in experimental conditions. However, despite these variations, the overall quality of all the melanoma samples remains high, with a target of approximately 10,000 cells per sample and an average read depth of 50,000 reads per cell. These metrics confirm that the sequencing data for melanoma samples are robust and suitable for downstream analysis, enabling detailed investigation of gene expression patterns within the tumor microenvironment.
In summary, the quality check confirmed that the sequencing data are of high quality across all sample types, with minimal technical artifacts and sufficient depth of coverage to support reliable biological interpretation.

Table 2. The table shows all the samples analyzed. In particular, the total number of cells was counted for each sample, together with their mitochondrial gene count. Moreover, the table reports the number of reads per cell and the number of reds of the whole sample.

ID	Estimated Number of cells	Mean Reads per Cell	Median Genes per Cell	Number of Reads
Czechoslovakian Wolf_Skin	11172	54708	2684	611,192,239
Czechoslovakian Wolf_Oral M	14715	43859	2146	645,384,241
German Shepherd_Skin	15196	46715	2710	709,883,094
German Shepherd Oral M	15040	40827	2338	614,044,515
Mixbreed_Skin	7583	79698	2223	604,351,855
Mixbreed Oral M	5060	102690	2855	519,609,811
Maltese_Skin	14787	35414	2084	523,660,897
Maltese Oral M	17625	36650	2509	645,949,106
OzzyMelanoma	10825	61101	4200	661,414,407
Ozzy_Skin	10282	58197	1917	598,377,474
Ozzy Oral M	11278	53887	2496	607,734,570
RudiMelanoma	8204	81481	4630	668,466,886
ShotMelanoma	13995	40709	2310	569,719,333
average	11982	56610	2700	613,829,879

Figure 1. The graphics display the mitochondrial gene count and gene expression quality metrics for all tissue samples. Each density plot shows the distribution of mitochondrial content in the cells of each sample.



Following the QC process, an initial clustering analysis was performed to group cells based on their gene expression profiles.

In all the samples analyzed, a melanocytic population was identified. In the skin samples, melanocytes were present but accounted for only approximately 1% of the total cells. In the oral mucosa samples, the melanocytic population was slightly more abundant, though still relatively underrepresented compared to other cell types. At the time of writing, only one of the three melanoma sample has been evaluated, and further analysis is ongoing. However, this sample showed a high number of normal and proliferating melanocytes.

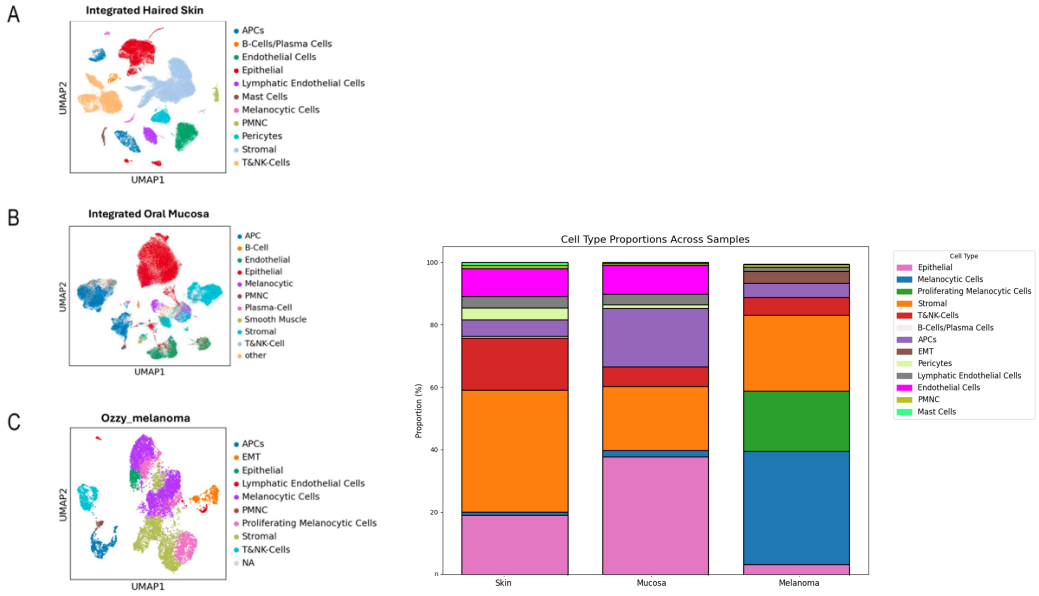
It is important to note that the relatively small amount of tissue sampled may introduce some bias, as it is unclear which specific regions of the tissue were digested and sequenced. However, despite this potential limitation, we observed a consistent distribution of major cell types across all samples. As expected based on the histological characteristics of the normal tissue in these two locations, skin

samples were enriched with stromal cells, while mucosal samples contained a relatively higher proportion of immune cells

.

These findings suggest that, despite the technical complexities involved in studying canine samples, the clustering analysis successfully captured the major cell populations present in both normal and diseased tissues. This provides a solid foundation for further investigation into the molecular characteristics and cellular composition of canine melanomas and their corresponding healthy tissues.

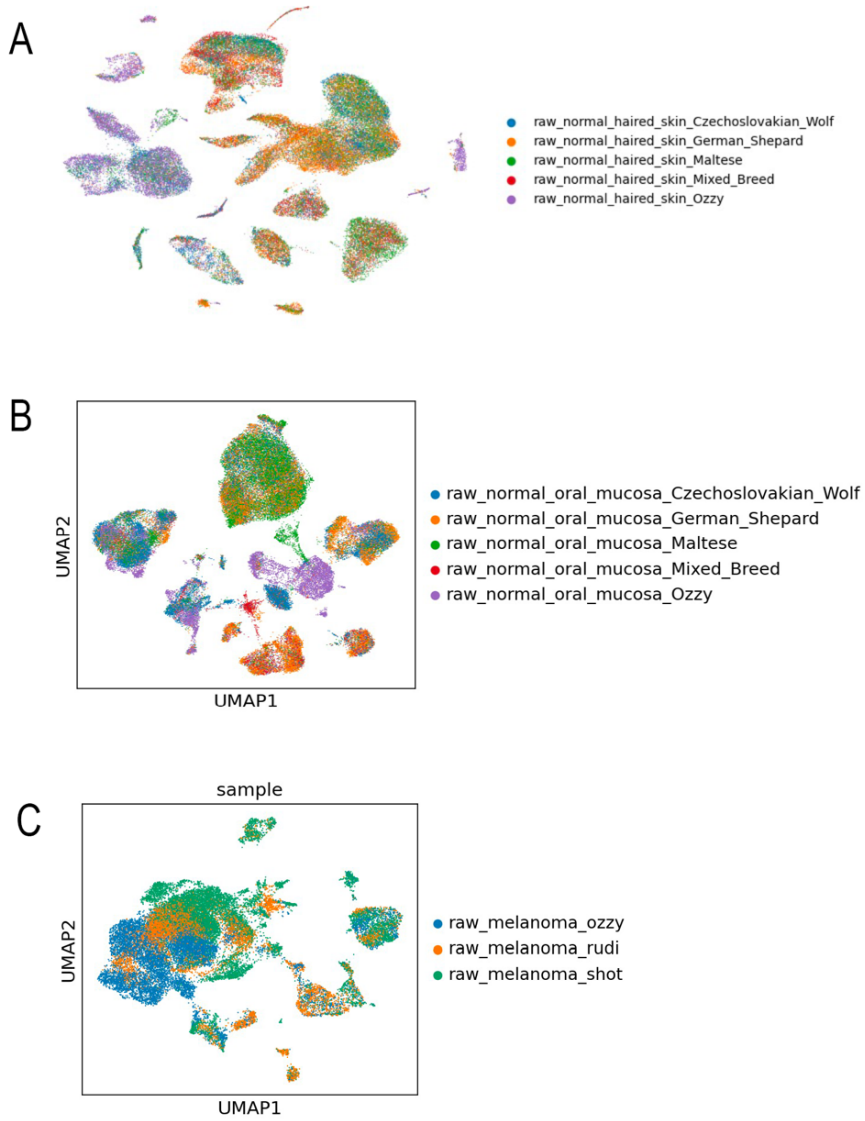
Figure 2. The graphics show the different cell populations in the samples sequenced. **A** and **B** pictures represent skin and oral mucosa integrated samples, while **C** shows the cellular population of one melanoma sample (Ozzy). Melanocytes are present in all three groups, with a low percentage in the cutaneous samples and a high percentage in the neoplastic ones. In the latter melanocytes are represented in two groups, the normal cells and the proliferating ones.



Following the evaluation of the cell populations, the samples were integrated, meaning all skin samples were grouped together, and the same was done for oral mucosa and melanoma samples before clustering was applied. The clustering results offer insights into the similarities and differences between the samples. Each cluster's degree of color mixing indicates how well the samples are integrated. The cutaneous samples exhibited high homogeneity with minimal differences. The oral mucosa samples displayed more variation but still demonstrated a significant overlap, suggesting similarities among the samples. The melanoma samples revealed larger and more distinct sample specific clusters. While the differences observed between the samples do not necessarily imply clonal variation, the lack

of complete overlap suggests that these populations are distinct enough to be analyzed separately. This clustering also explains the initial discrepancies observed in gene counts across the samples, as mentioned before, indicating that certain populations, such as those contained in the melanoma samples, may be more heterogeneous compared to the skin and oral mucosa samples. This heterogeneity provides valuable insight into the cellular diversity of the samples and aids in understanding the complexity of the melanoma subtype.

Figure 3. The graphics represent the clustering of the samples of each group. The cutaneous and melanoma samples (A, C) show different and well-defined cell populations, while oral mucosa samples (B) seem to display a higher heterogeneity of cellular populations.



Preliminary Observations

In summary, the key findings of this study to date include the successful sequencing of all skin, oral mucosa, and oral melanoma samples, all of which exhibited high-quality data, enabling single-cell resolution analysis of diverse cell populations. Preliminary clustering and annotation have been performed, revealing a heterogeneous population representing the whole tissue across all samples.
The analysis is still ongoing, with the most time-consuming task being the manual annotation, because automated tools and well-established genetic references for dogs are still limited and much of the annotation had to be performed manually.
Ultimately, this detailed annotation will provide further insights into the biology of melanoma and the normal melanocytes populations, advancing our understanding of the melanocyte's physiology and pathology. One of the planned analyses will compare the cell populations based on their highly variable genes, alongside trajectory analysis to trace the transition of cells from healthy to cancerous states. An additional and important goal of this study will be the comparison between the canine samples and human datasets. Considering previous studies that identify canine oral melanoma as a promising model for studying its human equivalent, we hope to find significant biological similarities. Ideally, these findings would further validate the dog as a spontaneous model for human melanoma. If confirmed, this could pave the way for extending this research beyond cancer to include autoimmune diseases, which are a key focus in many human studies.

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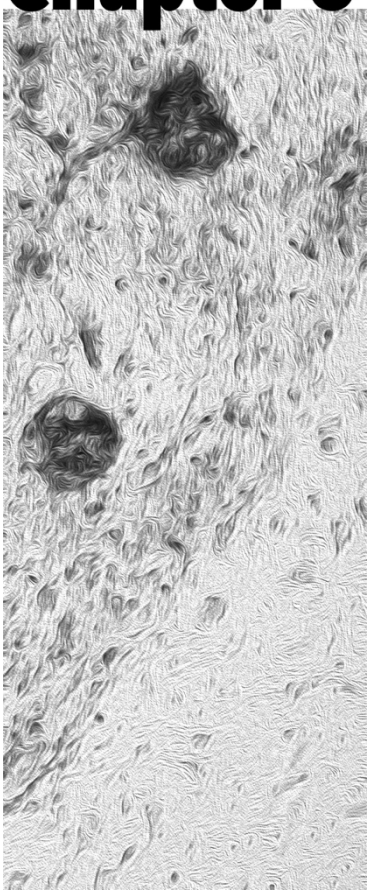
Chapter 6



Establishment of primary cell cultures from canine oral melanomas via fine-needle aspiration: a novel tool for tumorigenesis and cancer progression studies

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animals

MDPI

Article

Establishment of Primary Cell Cultures from Canine Oral Melanomas via Fine-Needle Aspiration: A Novel Tool for Tumorigenesis and Cancer Progression Studies

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Simple Summary: Oral melanomas are the most common oral tumors in dogs. They are usually aggressive, invasive, and bear a poor prognosis. These neoplasms have genetic and biological similarities with human oral melanoma, suggesting the potential use of the dog as a model in comparative studies. Primary two- and three-dimensional cell cultures from spontaneously arising canine oral melanocytic tumors and their nodal metastasis are established in this study, starting from cells sampled by fine-needle aspiration (FNA). This technique could be considered a helpful and less invasive method to collect samples for cell cultures, particularly for cases where surgery cannot be performed or when the owner refuses a surgical approach. This cell culture model contributes to the array of in vitro models, providing valuable tools for characterizing neoplastic cells and investigating the cellular pathways supporting cancer progression and invasion.

Abstract: Oral melanomas are the most common oral malignancies in dogs and are characterized by an aggressive nature, invasiveness, and poor prognosis. With biological and genetic similarities to human oral melanomas, they serve as a valuable spontaneous comparative model. Primary cell cultures are widely used in human medicine and, more recently, in veterinary medicine to study tumorigenesis, cancer progression, and innovative therapeutic approaches. This study aims to establish two- and three-dimensional primary cell lines from oral canine melanomas using fine-needle aspiration as a minimally invasive sampling method. For this study, samples were collected from six dogs, represented by four primary oral melanomas and five lymph nodal metastases. The cells were digested to obtain single-cell suspensions, seeded in flasks, or processed with Matrigel® to form organoids. The cell cultures were characterized through flow cytometry using antibodies against Melan-A, PNL2, and Sox-10. This technique offers a minimally invasive means to obtain cell samples, particularly beneficial for patients that are ineligible for surgical procedures, and enables the establishment of in vitro models crucial for comparative studies in mucosal melanoma oncology. To the best of our knowledge, this is the first work establishing neoplastic primary cell cultures via fine-needle aspiration in dogs.

Keywords: melanocytes; oral melanoma; fine-needle aspiration; primary cell culture; dog

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1. Introduction

Melanocytic tumors are the most common malignant neoplasms of the oral cavity in dogs [1–3]. In particular, canine oral mucosal melanomas (OMMs) typically display aggressive behavior, invade surrounding tissues readily, and have a propensity for metastasis to regional lymph nodes as well as distant sites [3–6]. These neoplasms are often detected at an advanced stage with considerable tumor extension. Their invasive growth often leads to profound penetration—frequently associated with bone lysis—rendering the primary neoplasm frequently inoperable, especially in the presence of distant metastases [5], thereby contributing significantly to the poor prognosis associated with oral melanoma [5,6]. Nowadays, different therapeutic approaches are available to treat this tumor, with surgery remaining the cornerstone [7]; however, in cases where extensive tumor growth hinders surgical intervention, alternative local treatments such as radiotherapy and electrochemotherapy have demonstrated efficacy in reducing tumor size [7,8]. Immunotherapy, which bolsters the immune response against the neoplasm or targets specific molecular pathways associated with tumor progression and metastasis, is showing promising results in the treatment of different tumors in both humans and dogs [9,10]. This therapeutic approach can be pursued through vaccination, electrovaccination, gene therapy, and checkpoint inhibitors [7,11–13]. Nevertheless, the limited availability of commercially available drugs for dogs and the predominant reliance on in vitro models in research present challenges [14].

While in vitro models are extensively employed in human medicine, studies on canine OMM, especially those centered on three-dimensional cell cultures, are limited [15]. This is attributed to the recent realization of dogs' significance as spontaneous models for studying human melanomas, especially oral ones, which share several traits with their human counterparts including responses to similar immunotherapeutic approaches [14,16–20]. In addition, research on the immune environment of canine OMM has expanded, offering valuable insights into these tumors [21–25]. The rarity of mucosal melanoma in humans accounts for fewer possibilities of performing clinical studies in large groups of patients but the availability of a spontaneous animal model could represent a significant advantage. Establishing 3D cell cultures, potentially incorporating specific immune cell co-cultures, would provide an important tool to study interaction mechanisms between neoplastic and immune cells and test new drugs against mucosal melanomas.

Unfortunately, establishing cell lines from surgical excision in canine OMM can pose challenges, often due to the poor clinical condition of the affected dogs or the ineligibility of the tumor for surgery. Similarly, obtaining an incisional biopsy is sometimes challenging because owners opt for minimally invasive procedures. Furthermore, when cytology results along with staging confirm metastatic melanoma, owners may not consent to further procedures, including incisional biopsies. Additionally, patients are often referred to oncology centers after relapse or following surgeries performed by other practitioners. In the former scenario, owners frequently decline further treatments, while in the latter, only the metastasis can be sampled if present. Consequently, FNA emerges as a valuable tool for obtaining sufficient cells to initiate primary cell cultures, facilitating the creation of both 2D and 3D models. FNA sampling is a procedure often performed for diagnostic purposes and it is frequently carried out during collateral diagnostic exams, such as X-rays or CT scans, which are necessary to evaluate the tumor origin and stage. Furthermore, all these procedures necessarily require sedation of the dog, thus allowing for safer manipulation.

This technique has found successful application in human medicine for various tumor types, particularly in the establishment of three-dimensional cell cultures [26–31]. This approach can also offer a means for personalized disease characterization tailored to each patient's condition.

This study aims to establish two-dimensional and three-dimensional cell lines of canine OMM, using FNA as a sampling method. More specifically, three-dimensional cell cultures will include spheroids and organoids formation. The former are spherical cultures established starting from a two-dimensional culture without a scaffold, while the latter

are directly set up with a scaffold from the digested sample and can be considered a more complex unit. The use of FNA could be considered a helpful technique to implement the collection of viable neoplastic cells. Additionally, these in vitro models can serve as invaluable tools for investigating different oncological aspects, spanning from elucidating molecular pathways associated with tumor progression and metastasis formation to unraveling the complexities of the tumor microenvironment.

2. Materials and Methods

2.1. Reagents

DMED/Ham's F12 medium (10-090-CV) and Matrigel® (CLS354230) were obtained from Corning®, New York, NY, USA;

Penicillin/Streptomycin (ECB3001D), Amphotericin B (ECM0009D), Gentamycin (ECM0011B), and Fetal Bovine Serum (FBS) (ECS5000L) were purchased from Euroclone, Pero, Italy;

Collagenase I (C0130) and 0.025% trypsin (C-41012) were ordered from Sigma-Aldrich, St. Louis, MO, USA;

Melanoma-associated antigen (PNL2) (sc-5950306), PNL2-Alexa Fluor 488 antibody (sc-59306), and Sox-10 antibody, (sc-365692) were bought from Santa Cruz Biotechnology, Santa Cruz, CA, USA;

Melan-A (a103-m27c10-m29e3), Melan-A Alexa Fluor 594 antibody (a103-m27c10-m29e3), Mouse and Rabbit Specific HRP Detection IHC Kit (ab93677), and 3-amino-9-ethylcarbazole chromogen (AEC Substrate System) (ab64252) were purchased from Abcam, Cambridge, UK;

Ki-67 antibody (clone MIB-1) was obtained from Dako, Denmark;

Tyrosinase-related protein 1—TRP-1 (ls-b4011) from LSBio, Lifespan Biosciences, Lynwood, Washington, DC, USA;

LEUCOPERM reagents (buf09B) and LYNX Rapid RPE-Cy7 Antibody Conjugation Kit (lnk111pecy7) were bought from Bio-Rad, Hercules, CA, USA.

2.2. Sample Collection

This study was conducted following the ethical approval of the University of Perugia (Ethical approval number n. 18/2022). Samples for this study were collected from OMMs of sedated patients, after owner consent. Inclusion criteria comprised: (a) prior cytological, histological, or immunohistochemical diagnosis of oral melanoma (using Melan A, PNL2, and Sox-10 antibodies) [6,32–37], with or without associated lymph node metastasis; (b) tumor diameter > 1 cm^2; (c) no previous anti-neoplastic therapies (i.e., chemotherapy or electrochemotherapy). The histologic criteria for the inclusion of the tumor as a melanoma in our study were as follows: moderate to marked cellular atypia, more than 4 mitoses in 2.37 mm^2, infiltrative growth, and vascular invasion. If the tumor was poorly differentiated, immunohistochemistry was performed to characterize it and the diagnosis of melanoma was confirmed when the neoplastic cells were positive for Melan-A or PNL2, and Sox-10 [6,34,36].

Samples were collected from hospitals with an oncology service; hence, patients were often referred from other facilities where the surgeries and histological diagnostic evaluations were previously conducted.

Samples were collected using a 25-gauge sterile needle attached to a 5 mL sterile syringe. The needle was inserted perpendicularly into the nodule or the metastatic lymph node, applying backward pressure through the syringe holder, followed by rapid release. This sequence of movements was repeated 10–20 times without removing the needle from the mass but shifting it laterally, thus sampling an area within a few millimeters. Depending on the size and the consistency of the tumor, this process was repeated (up to 5 times), with the needle inserted into different areas. Subsequently, the material was immediately expelled into 2 mL sterile tubes containing complete medium consisting of DMED/Ham's F12 medium supplemented with Penicillin/Streptomycin (100 U/L;

100 μg/mL), Amphotericin B (2.5 μg/mL), Gentamycin (50 μg/mL), and 20% FBS. The collected samples were then processed within 2 h.

2.3. Cell Culture

The collected samples were washed three times in medium without FBS and the collagenous stroma was digested with 2 mg/mL collagenase I for 1 h at 37 °C. The enzymatic activity was halted by adding complete medium and cells were collected via centrifugation (at 340× *g* for 7 min).

To establish two-dimensional cell cultures, cells were seeded at the density of 2×10^4 cells/cm^2 and then cultivated in complete medium at 37 °C in a humidified atmosphere containing 5% CO_2 until reaching 80/90% confluency. The culture medium was changed every 48 h.

To establish organoids, 8×10^4 cells were suspended in 40 μL of complete medium with 70% Matrigel® and were gently seeded in the wells of the pre-warmed 24-well plate, forming a drop. The 24-well plate was incubated at 37 °C for 5 min followed by a plate inversion for an additional 10 min to optimize the drop shape. After that, the plate was reverted again for 20 min, the complete medium was added, and cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 48 h. The organoids were expanded when the drop exhibited a high cellular density.

To establish spheroids, cells obtained from 2D cultures were used, following the method described by Saraiva et al. [38]. Briefly, the 96-well plate round bottom was coated with 50 μL of 1.5% sterile agarose. After cooling, 1×10^3 cells resuspended in 200 μL of complete medium were added to each plate well and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Cell Characterization

Flow cytometry was performed to confirm the melanocytic cellular phenotype in cases 1, 2, 3, and 6. Cases 4 and 5 did not reach a sufficient yield to proceed with the analysis.

The technique was carried out on two-dimensional primary cell cultures. Upon reaching 80/90% confluency, cells were collected and then aliquoted into flow cytometry tubes at the density of 1×10^6 cells/100 μL. Subsequently, these cells were treated with LEUCOPERM reagents for permeabilization, according to the manufacturer's protocol.

Samples were labeled with 10 μL final volume of anti-Melan-A Alexa Fluor 594 antibody, anti-PNL2-Alexa Fluor 488 antibody, and anti-Sox-10 antibody, conjugated in-house with a LYNX Rapid RPE-Cy7 Antibody Conjugation Kit.

Antibodies were diluted at the optimal concentration (according to the manufacturer's instructions) in dilution buffer immediately before use. Following centrifugation, cells were resuspended in 400 μL of sheet fluid for the flow cytometric acquisition using BD FACS Canto II equipped with two lasers (488 nm and 640 nm) and with BD FACSDivaTM Software (version 4.2). Acquisition parameters were set at FSC 429 V, SSC 341 V, AF488 264 V, AF594 330 V, PE-Cy7 401 V. Data were processed with Kaluza analysis software vers. 2.1.

In the organoid culture of case 2, hematoxylin and eosin staining and immunohistochemistry were performed to evaluate the morphological and phenotypical aspects of these cultures.

Organoids were collected gently by disrupting the drop and centrifuged in a 1.5 mL tube (7 min at 340× *g*). The supernatant was discarded and the pellet was processed following the method described by Yoshimoto et al. [39]. Hence, the pellet was fixed for 20 min at room temperature adding 4% paraformaldehyde (pH 7.2), and then dehydrated using a series of increasing concentrations of alcohol. Subsequently, the pellet was embedded in paraffin and the formalin-fixed and paraffin-embedded (FFPE) organoids obtained were processed routinely.

The antibodies used for the phenotypical characterization on immunocytochemistry were against Melan-A, PNL2, Sox-10, and TRP-1. Ki-67 was used to assess the proliferation index. Four-micron sections were cut from each FFPE sample, mounted on polarized slides, and dried. After dewaxing, antigen retrieval was performed according to the antibody manufacturer's instructions. Peroxidase and protein block followed and, then, the diluted antibodies were applied to the sections according to the manufacturer's instructions, followed by a 2-hour incubation period. Following a rinse, a secondary biotinylated goat anti-polyvalent antibody was applied for 10 min. Finally, to reveal the immune complexes, the slides were incubated with peroxidase-labeled streptavidin for 10 min and then with AEC. Carazzi's hematoxylin was used as a counterstain.

3. Results

3.1. Case Study

Six cases were collected (anamnestic data and diagnosis are reported in Table 1). Three of the six cases were purebred (Pug, Shih-Tsu, and Miniature Pinscher), while the others were mixed breed (Figure 1); four dogs were males and two females. All dogs had a confirmed cytologic or histologic diagnosis of OMM with or without lymph nodal metastasis (Figure 1 and Table 1). For cases where the histopathology for diagnostic procedures was performed in our laboratories and, therefore, FFPE blocks were available (cases 2, 3, and 6), immunohistochemistry was performed with antibodies against Melan-A, PNL2, and Sox-10 [32–34] (Figure 2). Regarding sample collection for cell cultures, in three cases (cases 3, 5, and 6), FNA was performed on both the primary tumor and nodal metastasis. In one case (case 4), FNA was obtained only from the primary tumor, as the dog had no signs of nodal metastasis. Lastly, in two cases (cases 1 and 2), FNA samples were collected only from lymph node metastases because the primary tumor was not available due to prior surgical intervention by another practitioner.

Table 1. Anamnestic data, sampling site, and cytologic or histologic diagnosis. Age is reported in years; F indicates female, while M indicates male. Diagnoses marked with an asterisk (*) indicate cases diagnosed in our laboratory. Absence of an asterisk indicates diagnoses performed in another laboratory. The sign ✓ indicates the presence of the sample.

Case	Dog			Tumor				
	Breed	Age	Sex	Sampling Site		Diagnosis		
				Primary Tumor	Lymph Node	Cytology	Histology	Immunohistochemistry
1	Pug	14	F		✓	✓		
2	Miniature Pinscher	12	F		✓	✓*	✓*	✓
3	Mixed breed	12	M	✓	✓	✓*	✓*	✓
4	Shih-Tsu	12	M	✓		✓	✓	
5	Mixed breed	10	M	✓	✓	✓		
6	Mixed breed	12	M	✓	✓	✓*	✓*	✓

Animals **2024**, *14*, 1948

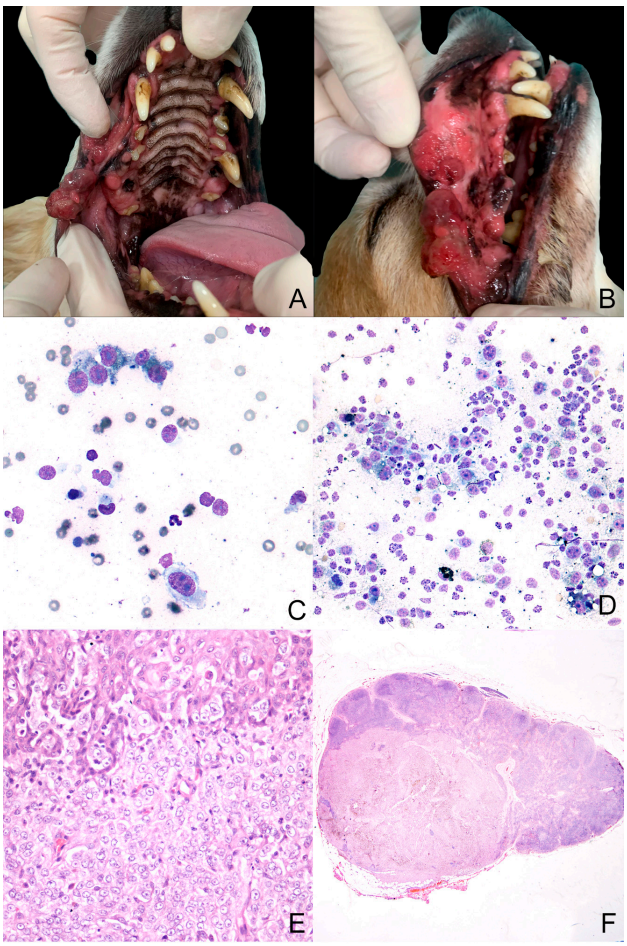

Figure 1. Case 3 (**A**–**F**). (**A**,**B**)—Macroscopic presentation of oral melanoma: The tumor was located on the right side of the superior lip. It was multilobulated, poorly pigmented, and multifocally ulcerated; (**C**,**D**)—cytologic samples (MGG Quik Stain® (04-090805; Bio Optica, Milano, Italy), 40× and 20× for (**C**) and (**D**) pictures, respectively) from the primary tumor and the lymph node, respectively. They show a population of neoplastic cells with marked anisocytosis and anisokaryosis. Sometimes, inside their cytoplasm, there is a finely granular greenish material (melanin); (**E**,**F**)—histology from the primary tumor and lymph node, respectively (hematoxylin–eosin, 40× and 10× for (**E**) and (**F**) pictures, respectively). The primary tumor appears densely cellular. The cells are arranged in lobules, supported by a moderate fibrous stroma. Neoplastic cells show a moderate amount of cytoplasm with poorly defined cell borders, round nuclei with vesicular chromatin, and prominent nucleoli.

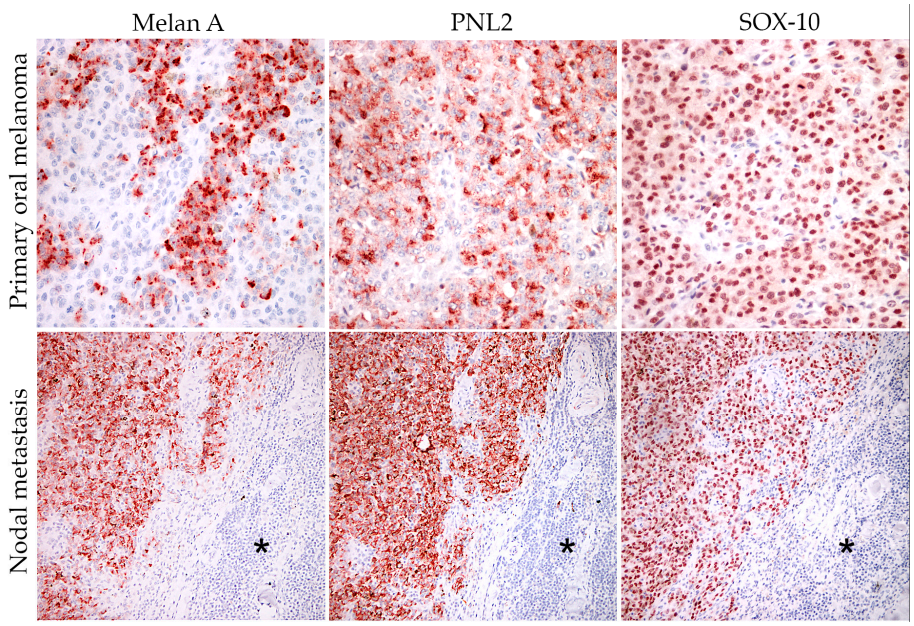

Figure 2. Case 3—Immunohistochemical expression of melanocytic markers (Melan-A, PNL2, and Sox-10) in both primary tumor (20×) and nodal metastasis (10×) (the asterisks represent the normal nodal tissue). The immunolabeling of Melan A and PNL2 was granular and cytoplasmic. Sox-10 was expressed in the nucleus of neoplastic cells. Carazzi's hematoxylin was used as a counterstain.

3.2. Primary Cell Culture

Two-dimensional primary cell cultures were obtained from all patients. Neoplastic cells were adherent after 24–48 h. Initially, neoplastic cells in culture were more polyhedral in shape; however, after the first passage, they achieved a more spindle/dendritic-shaped morphology. Highly pigmented tumor cell cultures showed a finely dark brown granulation (melanin pigment) in the cytoplasm (Figure 3). This pigment could also be observed free in the medium. Neoplastic cells occasionally showed cytoplasmatic optically empty vacuoles of variable size. Two-dimensional cell cultures of primary tumors were cultivated for 1 month, with a change of culture medium every 48 h, and remained vital for the whole period. Two-dimensional cell cultures of nodal metastases (1, 2, 3, and 6) yielded a confluency between 5 and 10 days and were stocked for further experiments.

Three-dimensional cell cultures were obtained in two ways. Spheroids were seeded from case 1 (nodal metastases) forming aggregates of round to polygonal neoplastic cells (Figure 4). Organoids were obtained and subsequently stored from cases 1, 2, 3, and 4 (nodal metastases in cases 1 and 2; primary tumors in cases 3 and 4). They were organized in multiple aggregates of different dimensions embedded in Matrigel® (Figure 5). In some cases, a two-dimensional cell layer started to grow from the scaffold, occasionally reaching confluency (Figure 5D).

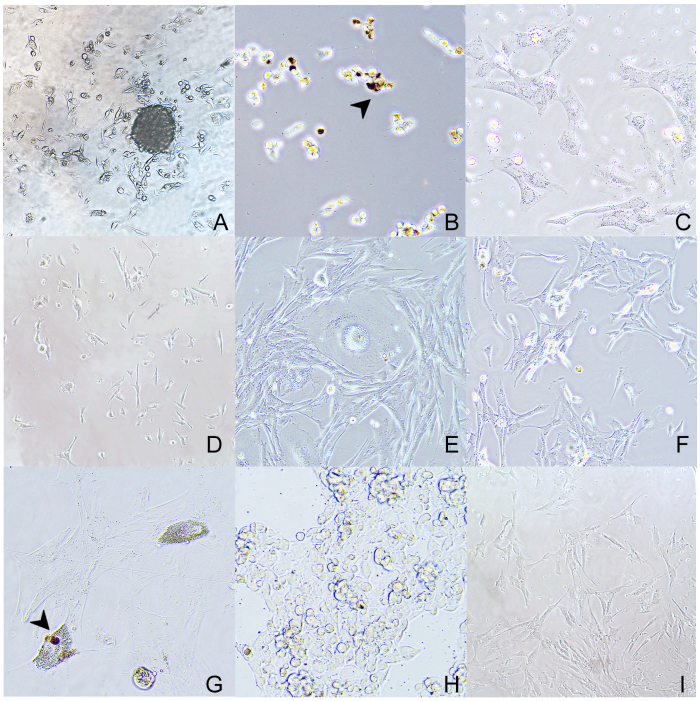

Figure 3. Two-dimensional cell cultures of neoplastic melanocytes obtained from primary oral neoplasia ((**A**) 20×—case 3; (**B**) 20×—case 4; (**C**) 40×—case 5; (**D**) 20×—case 6) and nodal metastasis ((**E**) 40×—case 1; (**F**) 40×—case 2; (**G**) 40×—case 3; (**H**) 40×—case 5, (**I**) 20×—case 6). The cells are often arranged in bundles, they are spindle-shaped with moderate cytoplasm, and well-defined cell borders that show dendritic prolongments. Within the cytoplasm of scattered cells, a dark brown finely granular pigment (melanin) is seen (arrowhead).

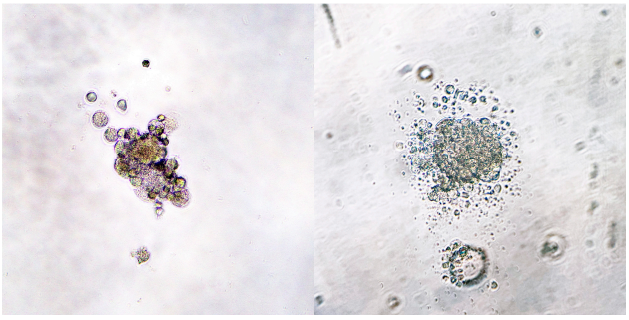

Figure 4. Case 1—Spheroids day 3 (40×). A thousand cells were seeded in a 96-well plate coated with 1.5% agar.

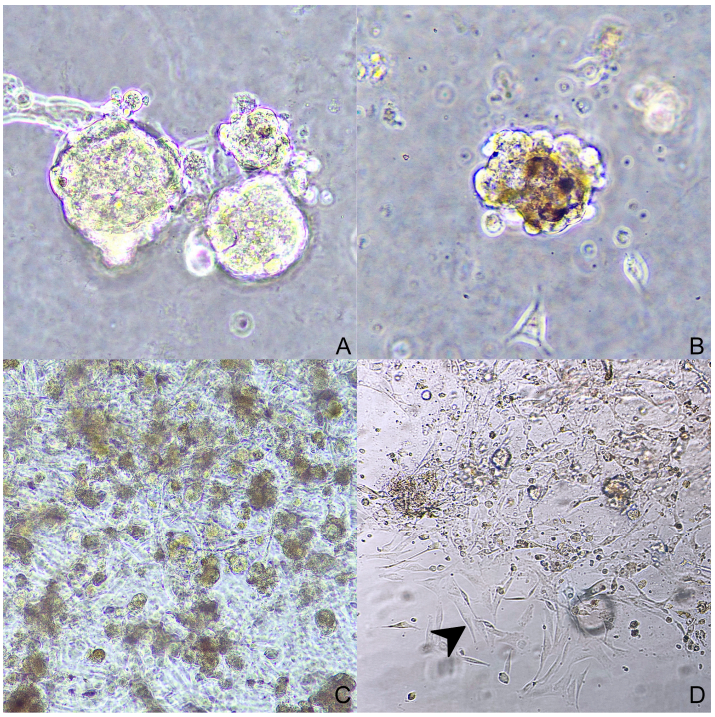

Figure 5. Organoids: ((**A**)—40×) case 1; ((**B**)—40×) case 4; ((**C**)—10×) case 2; ((**D**)—20×) case 3; in picture (**D**) neoplastic melanocytes are growing outside the Matrigel®, creating a two-dimensional culture (arrowhead).

3.3. Cell Culture Characterization

During the flow cytometry analysis, cases 1, 2, 3, and 6 exhibited varying degrees of sensitivity to the permeabilizing treatment: case 1 showed signs of cellular stress visible from the morphological dot plot, while cases 2, 3, and 6 demonstrated satisfactory and normal morphological characteristics. The fluorescence peaks were well-defined, indicating the success of the labeling protocol, with a clear separation between the negative and the positive peaks. The fluorescence peaks, considering the area of the histogram representing positive fluorescence, were used to calculate the percentage of antibody-positive cells corresponding to the fluorescence (Figures 6 and 7).

Case 1 presented consistent percentage values for the fluorescence of all three antibodies used, suggesting that over 70% of the isolated cells showed concurrent expression of Melan-A, PNL2, and Sox-10. Conversely, in case 2, a very high peak was observed for Sox-10, representing approximately 90% of the cells in the sample, contrasting with a discernible fluorescence representing only about 40% of cells for the Melan-A antigen and PNL2. Case 3 had clear and well-marked peaks representing 98% of the cells in the sample for Melan-A and PNL2, while positivity for Sox-10 was detected in only 79% of the cells. Case 6, on the other hand, exhibited a markedly distinct response compared to the previously analyzed cases: despite sharing similar characteristics with the other samples, it yielded positive signals for only a small percentage of cells for the three antibodies tested (Table 2).

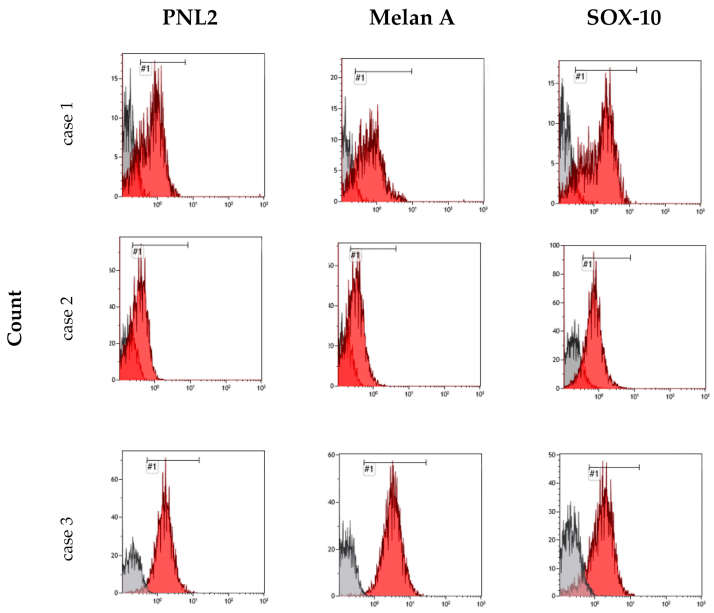

Figure 6. Flow cytometric analysis of cases 1, 2, and 3. The histograms show the different fluorescence intensity patterns for the tested markers: PNL2, Melan-A, and Sox-10. The red peaks represent the positive cells, while the grey peaks represent the negative ones. The sign #1 indicates the name of the gate.

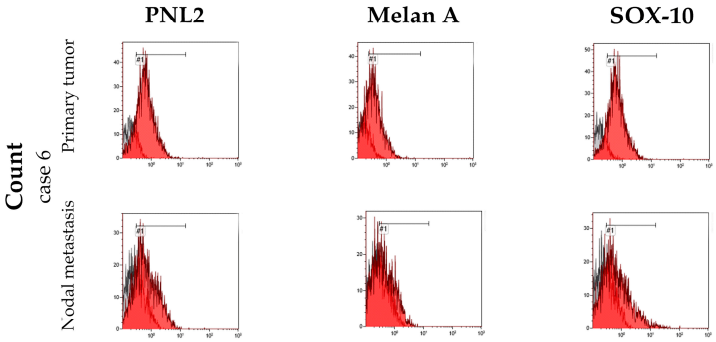

Figure 7. The histograms show the fluorescence intensity patterns for neoplastic melanocytes of primary tumor and lymph node metastasis marked with PNL2, Melan-A, and Sox-10 of case number 6. The red peaks represent the positive cells, while the grey peaks represent the negative ones. The sign #1 indicates the name of the gate.

Table 2. Percentage of positive cells in flow cytometric analysis of cases 1, 2, 3, and 6 ([a] = primary tumor; [b] = nodal metastasis).

Antigen	Percentage of Positive Cells				
	Case 1	Case 2	Case 3	Case 6 [a]	Case 6 [b]
PNL2	66.6	53.4	95.8	74	43.8
Melan-A	73.9	53.4	99	48.2	19.2
Sox-10	85	82	78.5	79.2	39.6

Hematoxylin and eosin staining was performed on organoids of case 2 to evaluate their morphology. They showed variably sized aggregates of polygonal cells. The cells showed a moderate amount of cytoplasm, occasionally filled with finely granular, brown pigment. The nuclei were round to oval with single and prominent nucleoli. Cytomegaly was occasionally observed (Figure 8).

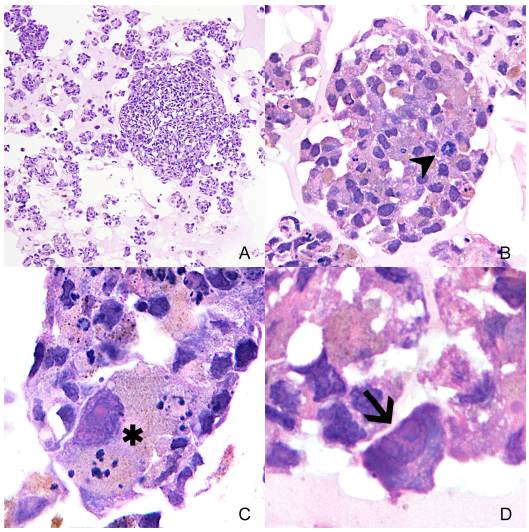

Figure 8. Variably size organoids ((**A**)—10×) of case 2 in hematoxylin and eosin. The cells show marked anisocytosis and anisokaryosis in all organoids. In Subfigure (**B**)—40×, mitotic figures are seen (arrowhead). In Subfigure (**C**)—100×, a cell with marked cytomegaly and karyomegaly is shown (asterisk). Subfigure (**D**)—100×, a high magnification of a cell showing multiple nucleoli (arrow).

Immunohistochemistry was conducted on the same FFPE tissue sample from case 2 to assess the phenotypical characteristics of the cells. Antibodies targeting PNL2, Sox-10, and TRP-1 exhibited widespread positivity (Figure 9), whereas Melan-A antibody positivity was limited. PNL2 and TRP-1 showed a diffuse cytoplasmatic positivity, while Sox-10 demonstrated a diffuse nuclear positivity. Additionally, Ki-67 nuclear immunolabeling was detected in approximately 80% of the cells.

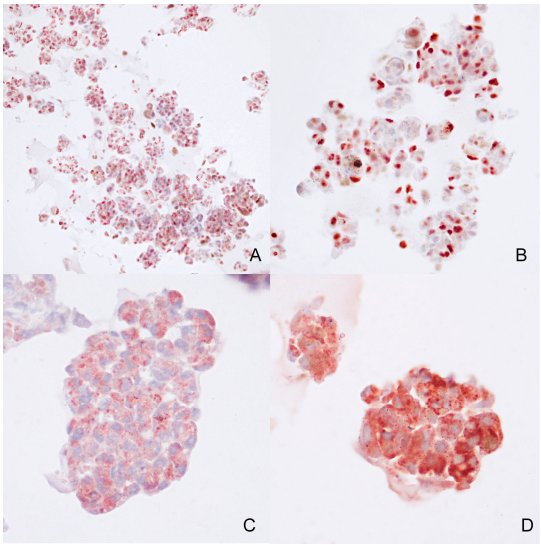

Figure 9. Case 2—Immunohistochemical expression of Sox-10 ((**A**)—10×), Ki-67 ((**B**)—20×), PNL2 ((**C**)—40×), TRP-1 ((**D**)—40×), in melanoma organoids.

4. Discussion

In this study, fine-needle aspiration was used as a method to obtain primary cell cultures from canine OMM and nodal metastases, as already reported in human medicine [28,40,41]. We successfully preserved a total of six distinct cell cultures, comprising two out of four derived from the primary oral tumor and four out of five obtained from lymph nodal metastasis. To the authors' knowledge, this is the first study that reports primary tumor cell culture initiation from fine-needle aspirates in dogs.

Our study aimed to address a technological gap by providing a model for functional studies through the establishment of a method to obtain in vitro cell cultures using a non-invasive sampling technique. The cell lines obtained underwent comprehensive morphological and phenotypical characterization employing immunocytochemistry and flow cytometry, using antibodies against Melan-A, PNL2, and Sox-10. Future studies involving functional assays to assess the proliferative index of neoplastic cells and evaluate their sensitivity to specific agents in order to demonstrate the correlation between the behavior of primary tumor-derived cell cultures and the original tumors are planned by our research group.

Primary melanoma cell lines in dogs have been reported in veterinary medicine [42–44], yet their utilization and establishment are still relatively limited, particularly concerning organoids. As per the authors' knowledge, organoids derived from canine OMM have not previously been established, highlighting the novelty of this study in the field of veterinary oncology.

Primary cultures are important to investigate molecular pathways and genetic mutations specifically involved in neoplastic cell transformation and phenotype definition. These alterations, often lost in immortalized cell lines due to multiple cell passages, are more robustly assessable in primary cultures. Therefore, our study primarily aimed at standardizing an easily accessible and relatively less invasive method for collecting tumor cells from dogs to establish primary cell cultures. FNA technique sampling is a widely used diagnostic tool, easily executable by veterinary practitioners, and minimally invasive for the patient.

This method simplifies sample collection for in vitro model establishment from canine tumors, eliminating the need for immediate tissue processing in a sterile environment.

In particular, canine OMM can be challenging to sample for different reasons; first, oral melanomas are frequently non-eligible for surgery due to local extent or widespread dissemination. Moreover, owners may decline a demolitive oncological surgery due to associated prohibitive costs, poor prognosis, and potential compromise of anatomical functionality, alongside aesthetic concerns [8,45]. As a consequence, the proposed possibility of obtaining an FNA sample during routine diagnostic procedures could facilitate the collection of a larger number of samples without imposing additional risks or procedures on the patient.

Additionally, sampling primary oral tumors through the FNA technique could offer advantages over whole tissue sampling, particularly in these cases where tissue microbiota is abundant (i.e., canine oral mucosa or the gastrointestinal tract). As a matter of fact, canine OMMs are frequently ulcerated and harbor substantial bacterial overgrowth on the surface [46]. In our group's experience, despite extensive curettage of excisional samples and repeated washing with antibiotics, cultures often remain susceptible to contamination. Conversely, direct aspiration with FNA from the inner portion of the tumor seems to mitigate this problem, providing cleaner samples.

However, despite its advantages, FNA has inherent limitations. Achieving an adequate cell yield for culturing often necessitated multiple tumor samplings from different parts of the lesion. Despite this, an adequate number of cells may not be reached consistently. This complicates the attainment of confluence and necessitates more passages to generate an adequate number of cells for experiments. Increased passages heighten the risk of molecular expression changes and accelerate aging of neoplastic cells. Moreover, blood contamination, particularly in samples collected from primary tumors, poses a technical challenge.

Our observations indicated a higher success rate in culturing cells obtained from lymph nodes compared to primary oral melanomas, likely due to the higher cellular concentration in the former. This was particularly evident in cases 1, 2, and 3, where samples from lymph node metastases yielded between 5 and 7 $\times$ 10^6 cells/mL, reaching confluency more readily and in less time compared to primary oral melanoma. Moreover, metastatic tumor cells within lymph nodes might have acquired motility by losing intercellular junctions [47,48], therefore accounting for an easier exfoliation, limiting cell stress and damage

In this study, we sought to explore the feasibility of establishing three-dimensional (3D) cell cultures from FNA-derived samples of canine OMMs. Working with 3D cell cultures better mirrors cell–cell and cell–microenvironment interactions, providing a closer representation of tumor heterogeneity and the microenvironment [49].

Nowadays, cancer therapy and immunotherapy are being increasingly used in veterinary medicine [7,14]; therefore, despite the aforementioned challenges, the proposed method exploiting FNA may represent an innovative tool to expedite and broaden oncology studies in dogs and pets in general, providing accessible 3D models. These models, particularly organoids, represent a more faithful in vitro representation compared to 2D cell cultures and could serve as an essential platform to investigate the interaction between neoplastic and co-cultured immune cells in a 3D setting, providing additional insights into the immune environment. In addition, a broader array of in vitro models might provide more robust comparative data for the rare human mucosa melanoma. Finally, in a perspective application of personalized medicine in veterinary medicine, these methods might be a useful asset.

5. Conclusions

To the best of the authors' knowledge, this research represents the first utilization of fine-needle aspiration to establish both two-dimensional and three-dimensional primary neoplastic cell cultures from dogs. This study provides a reliable method for initiating primary cell culture, significantly expanding the study of canine melanocytic tumors

as a model for their human counterparts. In an era where personalized medicine is becoming increasingly required, the use of primary cell cultures derived from fine-needle aspiration offers a faster and more effective means to test and provide substantial support in cancer therapy.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Perugia (2021-USDPAMM-0041730; 18 February 2021), including an informed consent for collection of patients' samples.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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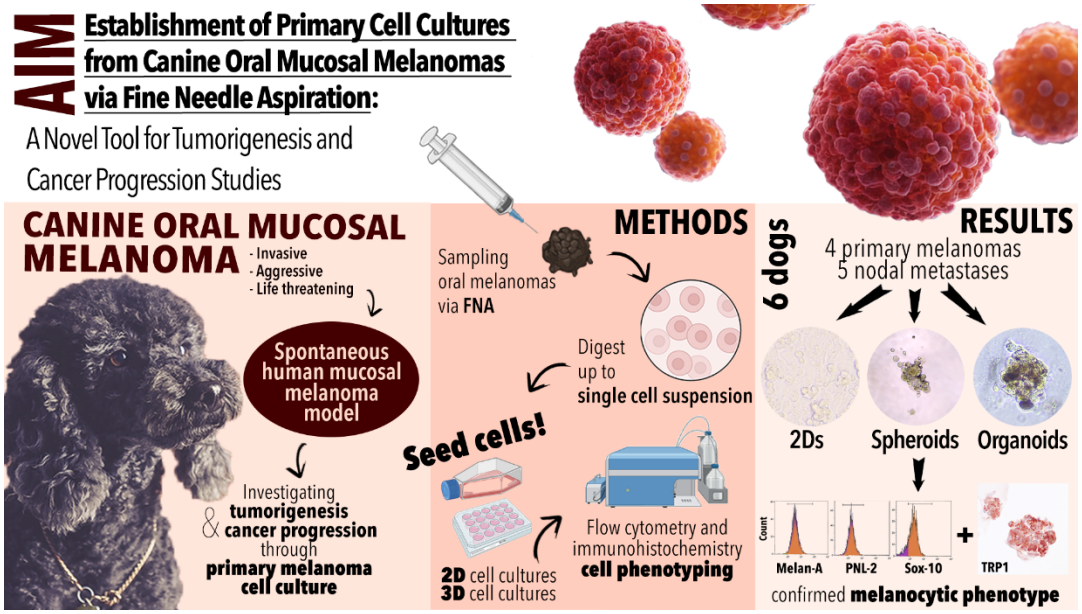
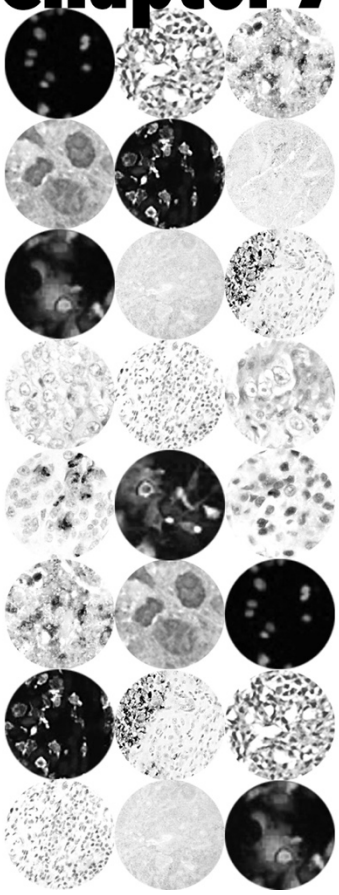
Graphical abstract



Chapter 7



Rac1 signaling-associated genes are upregulated in nodal metastasis of canine oral mucosal melanoma

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Keywords:	melanoma, gene expression profiling, exon microarray, RT-qPCR, Rac1, GTP binding protein, nodal metastasis
Abstract:	Oral mucosal melanomas (OMMs) are the most frequent oral malignancy in dogs, characterized by aggressive local behavior and high metastatic rate. The mechanisms that drive canine OMM metastasis are still largely unknown, providing for limited therapeutic approaches once the disease has spread to metastatic sites. The objective of this investigation was to evaluate the differences in gene expression between canine primary OMMs and their matched nodal metastases. Transcriptional profiling of formalin-fixed, paraffin-embedded biopsies of four canine OMMs and their respective lymph node biopsies was performed using exon microarrays. Confirmation of the differential expression of selected genes was subsequently sought by quantitative RT-PCR (RT-qPCR) on 13 paired samples (primary tumor-metastatic lymph node). Results highlight the activation of pathways associated with actin cytoskeleton organization, cellular motility and migration. In particular, microarray assay indicated increased expression, in lymph node metastases, of genes (including ELMO1, VAV3 and DOCK2) associated with Rac1 signaling-regulated cell migration. The differential expression of several genes was validated by RT-qPCR. Overall, the results of this investigation point to a significant role for Rac1 signaling in the pathogenesis of OMM metastasis to regional lymph

	nodes. The Rac1 signaling-associated genes highlighted herein are indeed involved in the activation of cellular migration and one, or more, may represent a future therapeutic target to prevent OMM-metastatic dissemination.

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ABSTRACT

Oral mucosal melanomas (OMMs) are the most frequent oral malignancy in dogs, characterized by aggressive local behavior and high metastatic rate. The mechanisms that drive canine OMM metastasis are still largely unknown, providing for limited therapeutic approaches once the disease has spread to metastatic sites.

The objective of this investigation was to evaluate the differences in gene expression between canine primary OMMs and their matched nodal metastases.

Transcriptional profiling of formalin-fixed, paraffin-embedded biopsies of four canine OMMs and their respective lymph node biopsies was performed using exon microarrays. Confirmation of the differential expression of selected genes was subsequently sought by quantitative RT-PCR (RT-qPCR) on 13 paired samples (primary tumor-metastatic lymph node). Results highlight the activation of pathways associated with actin cytoskeleton organization, cellular motility and migration. In particular, microarray assay indicated increased expression, in lymph node metastases, of genes (including ELMO1, VAV3 and DOCK2) associated with Rac1 signaling-regulated cell migration. The differential expression of several genes was validated by RT-qPCR.

Overall, the results of this investigation point to a significant role for Rac1 signaling in the pathogenesis of OMM metastasis to regional lymph nodes. The Rac1 signaling-associated genes highlighted herein are indeed involved in the activation of cellular migration and one, or more, may represent a future therapeutic target to prevent OMM-metastatic dissemination.

Keywords: canine oral mucosal melanoma; gene expression profiling; exon microarray; TR-qPCR; Rac1, GTP binding protein; nodal metastasis.

INTRODUCTION

Oral melanocytic tumors are common in dogs, accounting for up to 17% of all oral neoplasms[43] and approximately 30-40% of all oral malignancies.[60] In general, canine oral malignant melanomas (OMMs) are characterized by aggressive biologic behavior and shorter median survival time, when compared to their cutaneous counterpart. For dogs treated with surgery alone, the survival rate is less than 35%, and local recurrence is frequent (about 10% of cases) even when surgical margins are clean.[3] Metastases to regional lymph nodes and distant organs are frequently reported (in up to 74% of cases).[45] Tissue invasion and metastasis are biological hallmarks of malignant tumors. Metastasis is defined by the spread of neoplastic cells to sites physically discontinuous with the primary tumor. Tumor cells can reach these sites by penetrating blood vessels, lymphatics, and body cavities.

In general, metastatic dissemination appears to be facilitated by pro-metastatic genetic and epigenetic changes in the genome of the primary tumor, and interactions between neoplastic cells and other cells (such as immune cells, fibroblasts, and endothelial cells) in both the initial and distant microenvironments.[15]

The identification of pro-metastatic genetic and epigenetic events, in addition to those driving tumor development, and understanding of the pathways underpinning the metastatic cascade in cancer, is critical to discover molecular targets for the prevention and treatment of distant metastases.[5,66] In melanomas, neoplastic cells display plasticity and highly migratory behavior, facilitating dissemination to distant sites; these characteristics are likely attributed to a neural crest-like reprogramming of neoplastic

melanocytes.[25] Indeed, malignant melanoma cells reactivate pro-migratory and pro-invasive pathways, leading to aberrant dissemination.[24] Nevertheless, the pathomechanisms involved in local invasion and metastasis of both human and canine mucosal melanoma remain poorly understood.[5,6]

Recently, gene expression profiling has proven to be a powerful tool for the identification of genes involved in metastasis in canine OMM.[5] It was shown that primary OMMs that metastasized were characterized by reduced expression of CXCL12, a protein that normally stimulates the migration and activation of hematopoietic progenitor cells, endothelial cells, and several leukocytes,[10] and increased expression of APOBEC3A, when compared to non-metastasizing primary OMMs. Similarly, in human head/neck melanomas APOBEC3A overexpression has been identified, supporting its role in the increase of mutational load, and in promoting inter- and intratumoral heterogeneity.[2] However, genomic analysis of the metastatic process in canine OMMs is still in its infancy. In another recent study, podoplanin, a cell-surface protein that modulates signal transductions that regulate cell proliferation, differentiation, migration, invasion, epithelial-to-mesenchymal transition,[62] was also identified as a driver protein for ameboid invasion in both human and canine mucosal melanoma, hence promoting tumor progression and metastasis.[57]

The aim of the present study was to investigate the molecular basis of canine OMM lymph node metastasis, evaluating differences in gene expression between primary OMMs and matched regional lymph nodal metastases by microarray mRNA profiling and quantitative RT-PCR.

MATERIALS AND METHODS

Case selection and sample collection

Formalin-fixed paraffin-embedded (FFPE) samples of canine primary OMMs and regional lymph node metastases from 13 dogs were retrospectively selected from diagnostic histopathology archives [Pathology Department at the Animal Health Trust (UK) and Department of Pathobiology at the University of Utrecht (the Netherlands)]. Tissue samples were surgically excised from dogs (between 2012 and 2018) for treatment and staging purposes. The confirmed diagnosis of both primary OMMs and metastatic OMMs was obtained by microscopic examination of H&E-stained sections by a board-certified pathologist (SDP). Histological evidence of lymph node metastasis was defined as the presence of large groups of neoplastic cells showing a mass effect on the lymphoid tissue, or the presence of small cohesive groups (>5 cells per high power field/400x) of pleomorphic cells containing melanin pigment. One single representative block was selected from each of the primary OMMs and their lymph node metastasis. Each neoplastic area was outlined using a fine-point permanent marker. A piece of Parafilm large enough to cover the region of interest was placed on the H&E-stained slide. Using a fine-point permanent marker, the entire tissue and the region of interest within the tissue were outlined on the parafilm. The marked parafilm was then transferred to the corresponding FFPE tissue block, matching the outline with the shape of the OMM tissue in the block. Using the tip of a permanent marker, shallow but visible indentations were drawn along the outline of the region of interest. Three tissue cores were extracted from the highlighted area of each FFPE block using a 3.0 mm Miltex Biopsy punch with plunger (Agar Scientific, UK).

RNA isolation, purification and quantification

Total RNA was isolated from all three tissue cores obtained for each FFPE primary OMM and matched lymph node metastasis using the RecoverAll Total Nucleic Acid Isolation Kit (ThermoFisher Scientific, Paisley, UK). OMM RNA samples were further purified (i.e. to

remove melanin) by spin column filtration (OneStep PCR Inhibitor Removal Kit; Zymo Research, Freiburg, Germany). Total RNA concentration was measured by spectrophotometry (NanoDrop 1000 Spectrophotometer, ThermoFisher Scientific), followed by fluorometry assay (Quant-iT RiboGreen RNA Assay Kit, ThermoFisher Scientific).

Global gene expression profiling

RNA amplification, labelling and microarray hybridization

Fragmented, biotinylated single-stranded cDNA was prepared from 29 ng of each of 4 FFPE primary OMMs and matched metastasis RNA samples using the GeneChip WT Pico Reagent Kit (ThermoFisher Scientific).

A mixture of 4 unlabeled synthetic RNAs was added (at the beginning of the procedure) to each tumor RNA sample to act as 'labelling controls', and a mixture of 4 labelled bacterial DNAs was added (at the end of the procedure) to each labelled tumor cDNA sample to act as hybridization controls. Each cDNA was individually hybridized to an array in a Canine Gene 1.1 ST Array Strip (ThermoFisher Scientific), in a proprietary hybridization cocktail (ThermoFisher Scientific). Array strip washing and streptavidin- phycoerythrin staining were undertaken by the GeneAtlas System (ThermoFisher Scientific) Fluidics Station, and array scanning by the GeneAtlas System (ThermoFisher Scientific)

Microarray data analysis

Exon-level probe set expression values were generated by quantile normalization, log2 transformation and signal summarization, performed using the RMA algorithm [29], implemented within 'Affymetrix Expression Console Software 1.3' (ThermoFisher Scientific). 'Outlier arrays' were considered to be those that had any single sample quality, labelling quality and hybridization quality metric value ≥2 standard deviations away from

the mean of the metric value for all the arrays.[73] Outlier arrays were excluded, and processing of the raw probe-level signal intensity data was repeated to generate both quantile normalized and log2-transformed exon and gene-level probe set expression values. Gene-level probe sets ('Transcript clusters') with 'crosshyb_type' = 1 (unique hybridization target) and 'category' = 'main' annotations[74], and for which at least 10% of its exons was 'present' (detection above background p-value <0.01[75]) in at least ≥2 of the primary OMMs and ≥2 of the primary OMM lymph node metastases, were considered to be expressed and used for subsequent analyses.

The similarity between the global expression profiles of primary and lymph node OMMs was visualized by hierarchical clustering performed using WebMeV.[19] Transcript clusters displaying statistically significant differential expression between primary OMM and lymph node metastases were identified by moderated T-test (p-value < 0.05) performed using the R Limma package.[37,72] P-values were adjusted by permutation testing[9] to compensate for "chance" p-values of 0.05 obtained due to multiple testing. Transcription clusters demonstrating a >2-fold difference in median expression level between primary OMM and lymph node metastases were subject to further analysis.

Functional annotation enrichment analysis

The biological processes and pathways overrepresented amongst genes with different expression, comparing primary OMMs and OMM lymph node metastases, were identified using DAVID.[17,27] The functional annotations associated with differentially expressed genes were compared with those ascribed to all Transcript clusters ('crosshyb_type' = 1 and 'category' = 'main') for which the expression of at least 10% of its exons was detected above background in ≥2% of the tumors in the primary OMMs and metastatic OMMs cohort, and over-represented biological processes and pathways identified.

Quantitative RT-PCR analysis

cDNA synthesis

Total RNA (80 ng) was reverse transcribed in 20 µL reactions using the iSCRIPT™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.[52] Controls reactions without reverse transcriptase (RT-) were included to enable checking for genomic DNA contamination.

Gene-specific preamplification and quantitative PCR (qPCR)

To increase the sensitivity of qPCR analysis, for each cDNA sample a 20 µL preamplification reaction was performed using 3 µL of cDNA diluted 1:10 (equivalent to 0.4 ng of RNA), 1 µL of TaqMan Gene Expression Assay (Table 1), 10 µL of SsoAdvanced™ Preamp Supermix (Bio-Rad, Hercules, CA, USA). Preamplification reactions were run for 3 min at 95°C, followed by 10 cycles of 15 s at 95°C and 4 min at 58 ◦C.

QPCR amplification was performed using 1 µL of preamplification reaction product, 10 µL of SsoAdvanced Universal Probes Supermix (Bio-Rad, Hercules, CA), 1 µL of TaqMan Gene Expression Assay (Table 1), and RNase-free water to a final volume of 20 µl. QPCR cycling conditions included an initial denaturation step of 15 s at 95°C, followed by 40 cycles at 95°C for 5 s and at 60°C for 30 s. RT- controls were enrolled in the qPCR to check for potential genomic DNA contamination. Both preamplification and amplification reactions were run in 96-well optical plates on a StepOne Plus Real-time PCR instrument (Applied Biosystems, California, USA). Three technical replicate qPCR reactions were performed for each preamplified cDNA sample, and the mean Cq value calculated from values determined using StepOne Software v2.3 (Applied Biosystems, California, USA). QPCR amplification efficiency was assessed as previously described.[40] The 'Livak method' was used to calculate normalized value (2^-ΔCq) of the target genes[38].

Selection of endogenous control reference genes for relative quantification of gene expression

Following the MIQE guidelines[8], qPCR analysis requires data normalization with the most stably expressed endogenous control (EC) genes to minimize data variation that can mask or exaggerate biological changes. Three candidate genes were chosen as a potential EC: ACTB, GAPDH and RPS18. Candidate ECs expression stabilities were examined with four algorithms, comparative ΔCq method[58], BestKeeper[50], NormFinder[1], and GeNorm[67]. The RefFinder tool[71] was used to compare and integrate the output data of the four algorithms, and rank the candidate ECs in the order of the decreasing stability of their expression across all the RNA samples[21].

Statistical analysis

Statistical analysis was performed on normally distributed 2^-ΔCq values. Gene expression data were analyzed using an unpaired t-test to compare the normalized expression value (2^-ΔCq) of selected genes in metastatic OMM *versus* primary OMM cases. Statistical analyses were performed with GraphPad Prism 9 (GraphPad, San Diego, CA, USA). Statistical significance occurred when $P < 0.05$.

Results

Total RNA yields and purity

Total RNAs isolated from OMM FFPE biopsies were of high purity, with OD260/280nm ratios ranging from 1.9 - 2.0, and OD260/230nm ratios from 1.9 to 2.2. Total RNA yields varied between 50 and 100 ng.

Tumors included in differential gene expression analysis

In order to define changes in the gene expression profiles of primary OMMs associated with the process of metastatic dissemination to a regional lymph node, comparative global gene expression analysis of biopsies of canine primary OMMs (4) and their lymph node metastases (4) was performed. The use of matched primary and metastatic tumors intended to minimise potential confounding effects of tumor genetic heterogeneity.

The microarray expression data for one lymph node metastasis sample ('3S') was excluded from the analysis because it was an "outlier array" (see Materials and Methods), having an 'all_probeset_rle_mean' quality metric value 2.24 standard deviations away from the mean of the metric value of all the arrays.

The gene-level probe set expression values obtained for 4 primary and 3 metastatic OMMs were compared for 9,964 Transcript clusters (category = main; crosshyb_type = 1). A Transcript cluster was considered to be present if ≥10% of its exons were present both in ≥2 primary OMMs and ≥2 metastatic OMMs. Unsupervised hierarchical clustering of the 4 primary OMMs and 3 OMM lymph node metastases on the basis of the expression levels of the 9,964 genes suggested a higher degree of similarity between unrelated OMM lymph node metastases, and between different primary OMMs, respectively, than between a primary OMM and its derivative lymph node metastasis (Figure 1).

Genes displaying differential expression between primary and metastatic OMMs

In total, 501 Transcript Clusters (genes) displayed a statistical difference in expression (p-value <0.05) between primary and metastatic OMMs, after permutation testing-adjustment. A >2-fold change (median primary/median metastatic expression value or median metastatic/median primary expression value) was considered to be authentic and more likely to be reproducibly measurable. Greater than 2-fold differences in expression between the primary and OMM metastases were exhibited by 151 genes, with 13

displaying increased expression and 16 decreased expression, respectively, in the lymph node metastases (Figure 2 and Supplementary Table 1).

Functional annotation enrichment analysis:

The frequencies of functional annotations assigned to 148 of the 158 Transcription Clusters that were differentially expressed between the primary and metastatic OMMs, and which had unique Ensembl canine gene IDs, were compared with those associated with all the Transcript Clusters expressed by the OMMs (9,123 of 9,964 which had associated Ensembl canine gene IDs). Over-represented amongst the genes exhibiting differential expression were 4 Gene Ontology Consortium biological processes and 4 KEGG pathways potentially involved in the metastatic process (Table 2).

RT-qPCR assessment of differential gene expression

The relative expression levels of selected genes were derived using the normalized value 2^- (Cq target gene – Cq reference gene) values. ACTB was selected as the best endogenous control, with respect to GAPDH and RPS18, by utilizing four mathematical approaches: comparative ΔCq method, BestKeeper, NormFinder, and GeNorm (Table 3).[21]

RT-qPCR analysis featured 13 pairs of primary OMMs and their lymph node metastases, including the 4 sample pairs subject to global gene expression profiling, and 10 subsequently collected primary tumor: lymph node metastasis sample pairs. The analysis sought to confirm the differential expression of 7 selected genes directly associated with Rac1 activation (Figure 3). The statistically significant >2-fold increased median expression of CORO1A, ITGA4 and VAV3 in the lymph node metastases determined by microarray-based transcriptional profiling was validated by RT-qPCR. RT-qPCR assay also confirmed the 'direction' of the >2-fold difference in the median expression of ELMO1 (increased

expression in OMM lymph node metastases), and SPARC and MMP2 (both decreased expression in OMM lymph node metastases), in accordance with the results of the microarray analysis, although the differences in expression did not reach statistical significance. DOCK2 exhibited statistically significant >2-fold decreased median expression in the lymph node metastases, in contrast to the >2-fold increased median expression observed in the lymph node metastases in the 4 sample-pair microarray investigation.

DISCUSSION

OMM is the most common malignant cancer of the oral cavity in the dog,[22,46] with frequent metastases and poor prognosis. The propensity of OMMs to metastasise is likely to be associated with pro-metastatic changes in gene expression in primary tumor cells.[5] Identification of these alterations holds the key to pinpointing potential targets for the control and treatment of OMM metastatic dissemination. Thus, this study aimed to identify potentially metastasis-associated differences in gene expression between canine primary OMMs and their relative regional lymph nodal metastases.

Several of the genes exhibiting differential expression between primary and metastatic OMMs are associated with cell motility, and are directly and/or indirectly involved in the regulation of the Rac1 signaling-associated genes.[32,35,39,41] Variation in the expression of these genes are also frequently correlated with highly aggressive tumor subtypes in humans.[11,14,35,39]

Rac1 (Ras-related C3 botulinum toxin substrate 1) is the major ubiquitous isoform of Rac expressed in mammalian tissue, as part of the Rho family of small GTPases, which controls assembly of the cellular actin cytoskeleton. Lamellipodia are actin polymerisations at the leading edge of cells, representing the main force for cell migration, and their formation is under the control of Rac1. Indeed, loss of Rac1 in tissues and cell cultures

leads to the loss of lamellipodia and a general reduction of migration speed.[33] The results of our study support the hypothesis that activation of downstream effectors and upstream regulators of Rac1 may facilitate canine OMM cell migration to a regional lymph node.

This study highlighted the increased expression in canine OMM lymph node metastases of a number of genes involved with cell migration-associated Rac1 signaling.

VAV3 is a member of the VAV gene family. VAV proteins activate pathways leading to actin cytoskeletal rearrangements, mainly acting as a guanine nucleotide exchange factor for RhoG, RhoA and Rac1. Elevated VAV3 expression has been linked to prostate cancer progression and post-treatment recurrence,[36] to glioblastoma cell migration, invasion and proliferation and also to a shorter overall survival[42], lymphatic metastasis and perineural invasion in gastric cancer.[64] Microarray and RT-qPCR-determined increased expression in lymph node metastases indicate that this gene may also be involved in canine OMM progression.

Coronin1A (encoded by the *CORO1A* gene) plays a pivotal role in the cytoskeleton in highly motile cells, contributing to the formation of plasma membrane protrusions essential for cell locomotion. Notably, this protein is particularly enriched in haematopoietic tissues.[47] Overexpression of CORO1A prompts the translocation of Rac1 to the plasma membrane, favouring its activation.[12] We hypothesize that the increased expression of CORO1A may be associated with the acquisition of a highly motile phenotype in OMM cells, as previously suggested in human mammary tumors.[31]

Integrin alpha 4 (ITGA4) mediates cell-cell adhesion important in immune function. ITGA4 is also widely expressed in neural crest cells, leukocytes, striated and smooth muscle, and neurons, and mediates their migration, most likely co-localising and activating Rac1[48]. A recent study on human cutaneous melanoma suggests that ITGA4 is closely related to the occurrence and development of melanoma, and that it can promote the metastasis of melanoma by favoring the aggregation of melanoma cells in the lymphatic system.[49]

Moreover, overexpression of ITGA4 has been demonstrated in human gastrointestinal stromal tumors, where it is associated with an unfavourable prognosis[53].

Dock2 (Dedicator Of Cytokinesis 2) is a member of the CDM family of proteins, known to regulate the actin cytoskeleton by functioning upstream of Rac1.[65] DOCK2 activates Rac1 and regulates the actin cytoskeleton through a self-inhibiting interaction with ELMO1.[13,55] Proteins encoded by *ELMO1* interact with dedicator of cytokinesis proteins to promote phagocytosis and cell migration[30]. Mutation-effected aberrant activation of *ELMO1* and *DOCK2* has been reported in human oesophageal adenocarcinoma, a highly invasive tumor prone to early metastasis.[18] RT-qPCR assay did not confirm the >2-fold increase in median DOCK2 expression in OMM lymph node metastases determined by microarray gene expression profiling.

Although several RAC1 signaling-associated genes displayed increased expression in OMM lymph node metastases, RT-qPCR assay suggested that this was not the case for MMP2, and SPARC, respectively. Downregulation of SPARC expression is related to the Rac1 signaling-associated pathways. Secreted protein acidic and cysteine-rich (SPARC, also known as osteonectin) is a matrix-associated protein, usually playing a role in bone mineralisation, regulating cell interaction with the extracellular milieu during development and in response to injury.[7] SPARC expression has been associated with an aggressive, mesenchymal-like phenotype in a variety of human cancers, including melanoma. In particular, its expression in melanoma cells was associated with decreased E-cadherin and increased N-cadherin expression levels suggesting that this protein may regulate epithelial–mesenchymal transition[51]. However, there is evidence that SPARC is a permissive factor for Rac1 that becomes fully active only when SPARC levels are low; indeed, suppression of SPARC expression induces the formation of lamellipodia extension.[54] The potentially decreased SPARC expression in canine OMM lymph node metastases demonstrated herein is conceptually consistent with the latter finding, although

further investigation of a possible role for SPARC in canine OMM metastasis is warranted. Matrix metallopeptidase 2 (MMP-2) is an enzyme able to cleave components of the extracellular matrix, precisely gelatin type I and collagen types IV, V, VII and X. It has been shown that Membrane Type 1 Metalloprotease (MT1-MMP), highly expressed in neural crest cells, mediates human melanoma cell invasion through the activation of its target MMP-2 and, in turn, MMP-2 activation is required to sustain Rac1 activity and promote cell migration.[56]

Rac1 is also implicated in epithelial-mesenchymal transition (EMT), inducing downregulation of E-cadherin and upregulation of N-cadherin, vimentin, and SNAIL1 in human cancers.[70] Melanocytes do not belong to the epithelial lineage and therefore the term EMT cannot be formally attributed to the progression of malignant melanoma. However, various studies have demonstrated that melanoma cells display EMT-like phenotype switching, both in humans and dogs.[34,59,68] Indeed, differentiated melanocytes do express E-cadherin and loss of this protein, which represents a hallmark of EMT in epithelial tumors, is also evident in late-stage malignant melanomas, especially in nodal metastases[44].

RAC1 has been identified as an oncogene in human cutaneous melanoma.[61] In particular, the p.P29S hot spot mutation likely destabilises RAC1 favouring its active GTP-bound state.[26] Intriguingly, both $RAC1^{P29S}$ mutant melanoma cells and RAC1 wild type melanoma cells reduce their proliferation by downregulation of Rac1.[28] However, RAC1 mutations have not been found in human mucosal melanomas, or in primary and metastatic canine OMMs.[69] Beyond the p.P29S missense mutation, Rac1 is activated by other molecular mechanisms in human cutaneous melanoma.[16] Some of these mechanisms may be similar to the putative Rac1 activation-promoting gene expression changes identified in the current study. This study has demonstrated that canine OMM lymph node metastases are characterized by increased expression (relative to primary OMMs) of a set of genes

involved in cell motility, in particular genes involved in Rac1 signaling. The potential key role of Rac1 and its upstream activators and downstream effectors in the modulation of critical elements of metastasis has been highlighted, as it has been done previously in human medicine.[3] It is possible that melanoma cells are able to disseminate to distant sites due to re-acquisition of plastic and highly migratory behaviour characteristic of their embryonic precursors.[23] Rac1 activation could be one of these mechanisms as it is essential for lamellipodia formation. Indeed, lamellipodia formation is also used by dendritic cells to interact with the endothelium, polarise, and actively crawl toward the draining lymph node.[63]

The main limitation of this study is the relatively low number of primary OMMs and lymph node metastasis biopsy pairs analysed. Fine needle aspiration (FNA) remains a mainstay diagnostic technique when evaluating for metastasis to regional lymph nodes in animals, being less invasive compared to surgical excision and frequently preferred to histological examination of the lymph node, despite the latter technique being considered the gold standard for the identification of nodal metastasis[20]. Moreover, dogs with OMM are often inoperable at diagnosis, hence, cytology would be the only viable option to provide a complete staging. Therefore, there are only a limited number of instances where both a primary OMM and a regional lymph node biopsy are available. A further constraint is that it was necessary to use FFPE tissue biopsies. Degradation of RNA by formalin fixation is well known, and probably mainly affects the accurate quantification of genes expressed at low levels.

The role of Rac1 signaling in tumorigenesis, angiogenesis, invasion, and metastasis has been demonstrated for several different human tumor types[3], and it may have a similarly important role in canine OMM development and progression. Targeting Rac1 and its regulatory network would be beneficial for cancer treatment, but this has unfortunately proved to be difficult. Rac1 signaling is involved in many processes of normal cell

physiology, including cellular plasticity, migration and invasion, cellular adhesions, cell proliferation, apoptosis, reactive oxygen species (ROS) production and immune responses.[4] Therefore, targeting Rac1 in a clinical setting might bear undesirable side effects. Investigation of both the downstream effectors and the upstream regulators of Rac1 could indicate the best unimodal/multimodal therapeutic approaches for canine OMMs. At this stage, additional validation in different preclinical cancer models is needed to supplement the striking *in vitro* evidence of the potential benefits of targeting Rac1.[41]

Ethics statement

This study (Project number 59-2016) was approved by the Animal Health Trust (AHT) Ethics Committee. Informed, written consent was obtained from the owners of dogs whose oral melanoma and lymph nodal biopsies were included in this study. A melanoma/lymph node biopsy could be withdrawn from the study at any time. Patient treatment was unaffected by the study.

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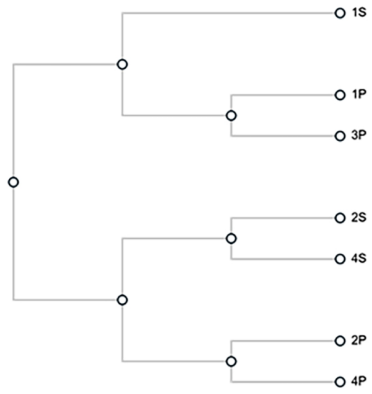
FIGURE 1: Comparison of the global gene expression profiles of primary OMMs and matched lymph node metastases. Hierarchical clustering (average group linkage, distance = 1 - Pearson Correlation Coefficient) of 4 primary OMMs (P) and 3 matched lymph node metastases (M) on the basis of the expression levels of 9,964 genes.

FIGURE 2: Fold differences in the median expression levels (measured by exon microarray) of 501 genes between 4 primary OMMs (P) and 3 matched lymph node metastases (M) are depicted. Red spheres denote 7 genes involved in RAC1 signaling-associated regulation of cell migration, whose differential expression was subsequently assayed by RT-qPCR. The red arrow indicates two genes (CORO1A and DOCK2) that displayed highly similar fold change differences in expression. The dotted line represents a permutation testing-adjusted (PTadj.) t-test p-value of 0.05.

FIGURE 3: PCR normalized expression levels. The figure shows the normalized expression value (2^-ΔCq) of target genes examined in the primary tumor samples (white column) and metastasis samples (gray column). The expression levels of ITGA4, VAV3, and CORO1A genes are significantly higher in metastasis compared to primary tumors. Conversely, DOCK2 shows reduced expression in metastasis compared to primary tumor. ELMO1, SPARC, and MMP2 display no statistically significant difference between the two groups ($p > 0.05$). An unpaired t-test is employed for comparing the two groups of data (metastasis vs primary tumor); *$p < 0.05$; **$p < 0.01$; **$p < 0.001$.



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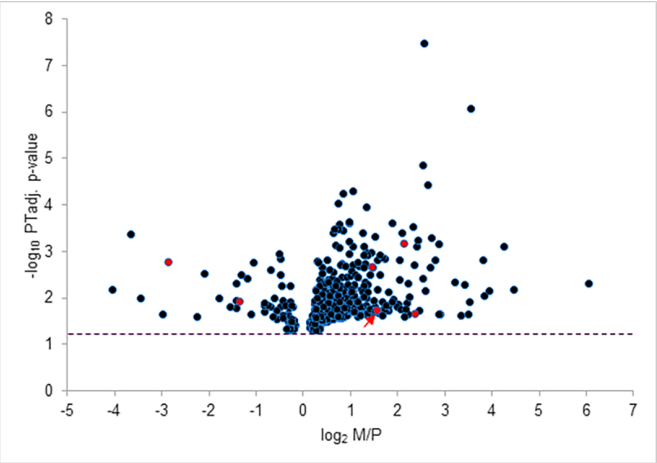

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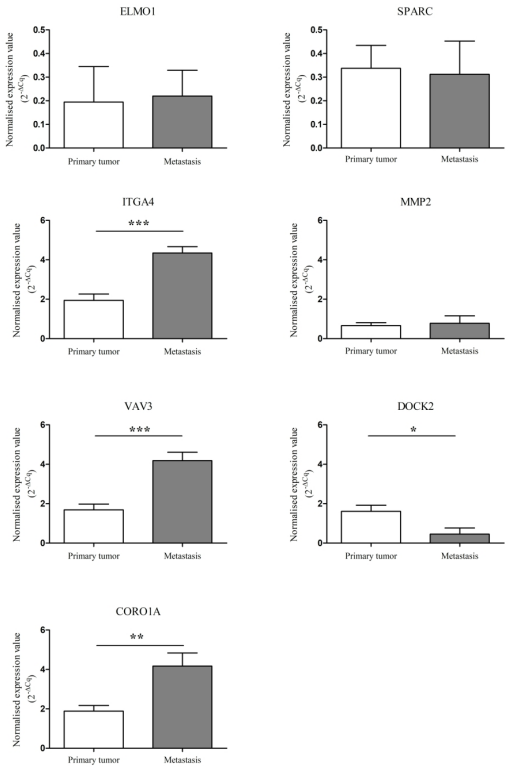

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Gene Symbol	Reference Sequence	TaqMan assay ID	Exon boundary	Amplicon (bp)
ITGA4	XM_003502135.1	Cg04426096_m1	20-21	64
SPARC	XM_005619272.4	Cf02661382_m1	5-6	67
CORO1A	XM_547069.7	Cf02706862_m1	9-10	159
VAV3	XM_022420692.1	Cf02705614_m1	11-12	148
DOCK2	XM_546246.5	Cf02703791_m1	15-16	85
ELMO1	XM_005628727.3	Cf00919416_m1	5-6	89
MMP2	XM_014109407.1	Cf01548727_m1	1-2	65
ACTB	NM_001195845.2	Cf04931159_m1	-	52
RPS18	NM_001048082.1	Cf02624916_g1	5-6	99
GAPDH	NM_001003142.2	Cf04419463_gH	5-6	54

Table 1. TaqMan Gene Expression Assays. The table summarizes the TaqMan assays used in the study to detect target genes (white boxes) and candidate normalization control genes (grey boxes). Gene symbol (first column), interrogated transcript sequence (second column), TaqMan probe ID (third column), base co-ordinates of exon-exon junction spanned by the used PCR assay (fourth column), and amplicon length (last column) are listed. All selected probes hybridise to two different exons (except for ACTB) and are specific for *Canis familiaris* mRNA transcripts. Design of the TaqMan assay for ITGA4 was based on the *Cricetulus griseus* CHO-K1 cell line ITGA4 mRNA transcript, however, an antibody raised against the *Cricetulus griseus* ITGA4 protein demonstrates 100% cross-reactivity with the *Canis familiaris ITGA4 protein*..

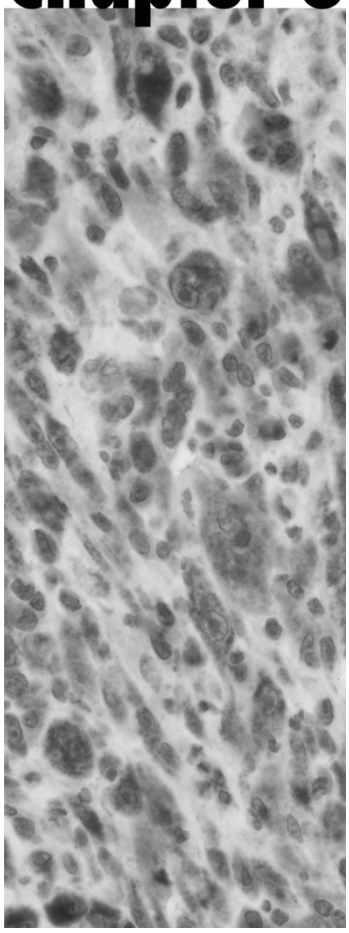
Functional annotation	Fold enrichment	P-value	Gene expression	
			M>P	P>M
GO:0045785~positive regulation of cell adhesion	15.187	0.015	*APBB1IP, VAV3*	*CYTH3*
GO:0030036~actin cytoskeleton organization	7.087	0.017	*ELMO1, CORO1A, DOCK2, FMNL1*	
GO:0001768~establishment of T cell polarity	47.249	0.041	*CCL19, DOCK2*	
GO:0007165~signal transduction	4.588	≤0.001	*TRAF5, ERBIN, SPOCK2, CDC42SE2, APBB1IP, LNPEP, FYB*	*SPARC*
cfa04514: Cell adhesion molecules	7.002	≤0.0001	*PTPRC, CD226, ICOS, PECAM1, DLA-DOB, ITGA4, CD2*	
cfa05200: Pathways in cancer	2.741	0.008	*TRAF5, IGF1, PLCB2, CDKN1B, STK4, PLCG2, JAK1, LEF1*	*ADCY2, MMP2*
cfa04062: Chemokine signaling pathway	4.951	≤0.001	*CCR7, PLCB2, ELMO1, CCL19, CXCL13, DOCK2, VAV3*	*ADCY2*
cfa04670: Leukocyte transendothelial migration	5.001	0.015	*PLCG2, PECAM1, VAV3, ITGA4*	*MMP2*

Table 2. Differentially expressed gene-associated enriched functional annotations. GO: Gene Ontology. M: Metastatic OMMs; P: Primary OMMs.

ALGORITHM	**EC RANK**		
Delta Cq	ACTB	GAPDH	RPS18
BestKeeper	GAPDH	ACTB	RPS18
NormFinder	ACTB	GAPDH	RPS18
GeNorm	GAPDH | ACTB		RPS18
RefFinder comprehensive ranking	ACTB	GAPDH	RPS18

Table 3. RefFinder ranking stability order. The table shows stability values ranking assigned by RefFinder which integrate the data obtained from the comparative ΔCq method, BestKeeper, NormFinder, and GeNorm algorithms. RefFinder tool generates a comprehensive ranking by ordering the candidate endogenous controls from most stable (first column) to least stable (third column).

Chapter 8



JAK-STAT3 pathway in canine oral melanoma: a potential therapeutic target?

Manuscript in preparation

Abstract submitted as Poster at the ECVP 5th cutting edge congress Madrid - 28th to the 31st of August in San Lorenzo de El Escorial and awarded in the category of Poster Flash

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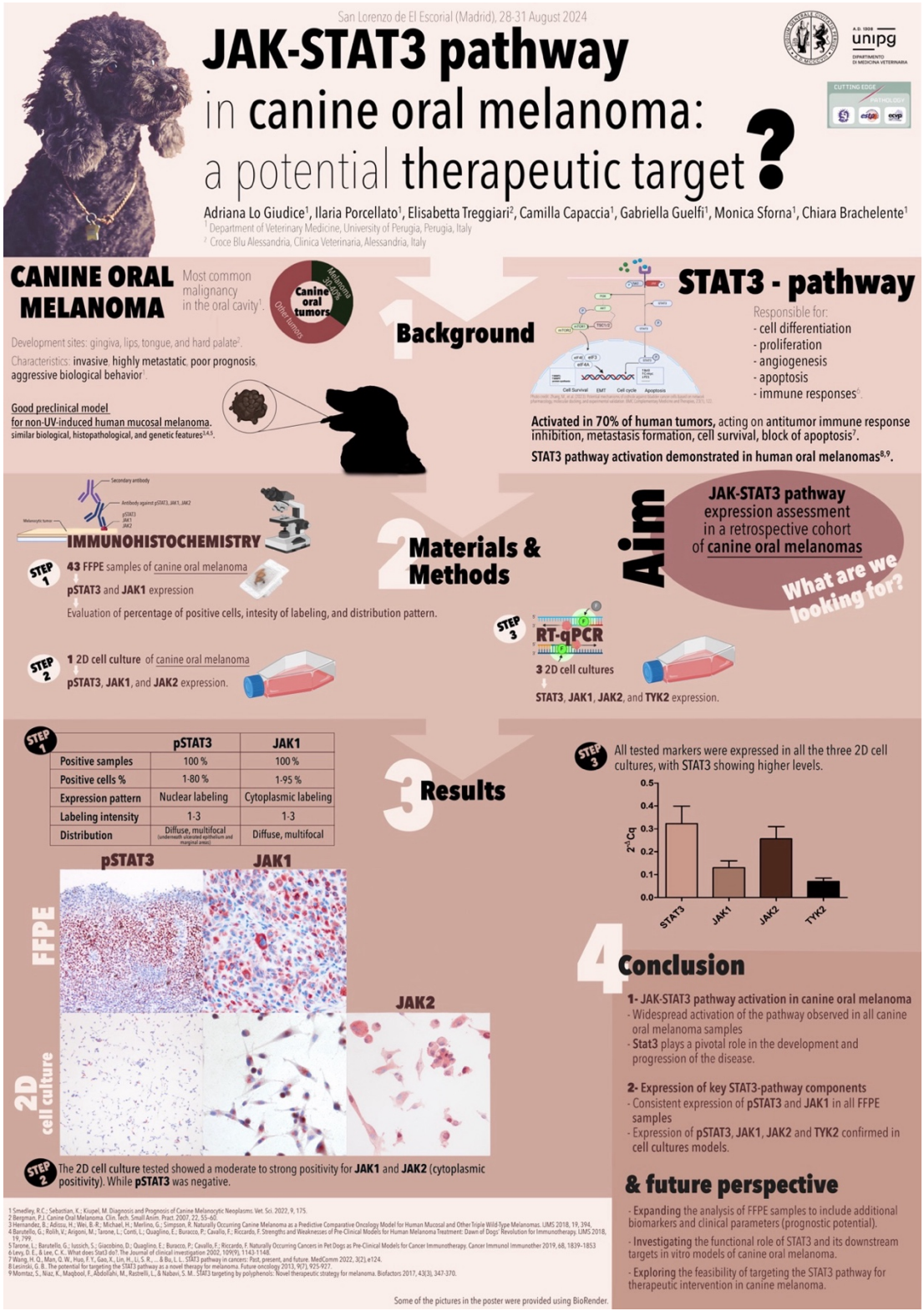
Abstract

Background Canine oral melanoma is an aggressive tumor with limited therapeutic options and short survival times. Signal transducer and activator of transcription (STAT)3 is a regulator of transcription that can be constitutively activated in different types of cancer, but can also be activated by Janus kinases (JAK). Our aim is to assess the expression of pSTAT3 and JAK1 in a retrospective cohort of canine oral melanomas.
Materials & Methods Forty-three cases of canine oral melanomas were retrospectively selected and immunohistochemistry was performed to assess the expression of pSTAT3 and JAK1. The expression of STAT3, JAK1, JAK2, and TYK2 was also assessed by RT-qPCR on three OM cell cultures. On 2D cell culture, the expression of pSTAT3, JAK1, and JAK2 was assessed by IF.
Results Expression of pSTAT3 was observed in the nucleus of neoplastic cells, with variable percentages (1-80%) in all tested melanomas and cell cultures. Instead, JAK1 was observed in the cytoplasm of melanoma cells (50-95%) in all tested cases. Gene expression analysis confirmed highly expressed STAT3 and a higher expression of JAK2 among the tested Janus kinases.
Conclusion Results from the present study show activation of the STAT3 pathway in canine oral melanoma, identifying it as a potential prognostic marker and an interesting therapeutic target.



	pSTAT3	JAK1
Positive samples	100 %	100 %
Positive cells %	1-80 %	1-95 %
Expression pattern	Nuclear labeling	Cytoplasmic labeling
Labeling intensity	1-3	1-3
Distribution	Diffuse, multifocal (underneath ulcerated epithelium and marginal areas)	Diffuse, multifocal

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Final remarks and Future perspectives

Melanocytic tumors, both in humans and dogs, remain highly aggressive neoplasms, often bearing a poor prognosis due to metastatic spread, and, despite the research advancement, a standardized and effective therapeutic approach is still lacking[133,139]. The potential of using dogs as a spontaneous model for studying human mucosal melanomas could offer a promising avenue for advancing research, allowing for deeper investigation into the mechanisms of tumorigenesis, tumor progression, and metastasis[13,63,113,154]. Moreover, it provides an opportunity to test new pharmacological agents that may effectively eliminate neoplastic cells. However, even though studies have identified biological and genetic similarities in this tumor between humans and dogs, the path to fully harnessing this model is still long, as there remain numerous unanswered questions regarding biological behavior and genetic implications of melanocytic neoplasms in dogs.

Chapter 1 of this doctoral project focused on analyzing the epidemiological background of these tumors in both dogs and cats, investigating a population whose samples were processed at the Veterinary Pathology Service of the Department of Veterinary Medicine, University of Perugia. While epidemiological studies on these tumors are somewhat outdated[20,135,137], an updated, albeit small-scale, analysis was necessary. The study confirmed that melanomas are more common in older patients and certain breeds, emphasizing the high frequency of oral melanomas in dogs and ocular melanomas in cats. Interestingly, our dataset revealed a higher number of cutaneous melanomas compared to what is reported in the current literature, highlighting potential variability in regional incidence or case reporting[85]. Hence, future studies should aim to involve multiple institutions to broaden the scope of analysis and incorporate additional parameters for more robust conclusions.

Following this, the project shifted to studying normal canine melanocytes, as scientific literature in this area is still limited. Understanding normal physiology is crucial for identifying pathological alterations. **Chapter 3**, indeed, investigated the expression of different specific markers (Melan-A, PNL2, TRP1, TRP2, SOX-10, MITF) in melanocytes across different body regions using immunohistochemistry and immunofluorescence. The results showed that Melan A and SOX-10 were the most specific and sensitive melanocytic markers. Moreover, their expression is variable,

in terms of sensitivity between the different somatic regions, suggesting that distinct subpopulations of melanocytes may exist, with variable distribution across different body regions[109].

Chapter 4 was similarly involved in the study of normal melanocytes as a continuation of the previous work. The focus was on assessing melanocyte density across different body regions through immunohistochemistry using a cocktail of antibodies, specifically Melan-A and SOX-10, which resulted, as mentioned above, the most specific and sensible. The results revealed that melanocytes are particularly abundant in areas such as the nose and eyelids. This observation could have clinical relevance, potentially explaining the higher frequency of melanocytic tumors or melanocyte-targeting diseases in these areas.

To further investigate these cells, a transcriptomic study using single-cell RNA sequencing (scRNA-seq) was developed (**Chapter 5**). This study evaluated both normal and neoplastic melanocytes, yielding promising preliminary results, with melanocytic populations identified in all examined samples. The project is still ongoing and the ultimate goal is to identify substantial differences between normal and neoplastic melanocytes and to compare these findings with existing human datasets. The could help to establish key similarities that justify the use of the dog as a spontaneous model for human oral and mucosal melanomas.

The collection of fresh melanoma samples has also facilitated the development of a new project described in **Chapter 6**: the establishment of cell cultures using fine needle aspiration as a sampling method for studying these tumors. This study demonstrated that this technique can yield reliable results in generating two- and three-dimensional cell cultures, overcoming the challenge of obtaining sufficient tissue samples for enzymatic digestion, particularly when dog owners decline surgical therapy[86]. This capability is highly valuable for personalized therapy, as it allows the establishment of autologous cell cultures, which help to determine the most effective therapeutic approach for the patient.

Having both fresh samples and a vast archive of formalin-fixed paraffin-embedded (FFPE) samples enables further investigation into one of the most critical aspects of tumor biology: the alteration of cellular pathways. Chapters 7 and 8 focus on exploring pathways known to be dysregulated in human melanoma and particularly involved in tumor metastatization. Specifically, **Chapter 7** examines the overexpression of the RAC1 pathway and its downstream molecules. This pathway is reported to be overexpressed in human melanomas and is associated with a more

aggressive tumor behavior[29]. Our study confirmed that also in dogs the Rac1 pathway and the associated molecules are overexpressed. In **Chapter 8**, the expression of the JAK-STAT3 pathway[103], also active in many different human cancers, was investigated. The study confirmed the expression of JAK1, JAK2, pSTAT3 in FFPE tissue and in melanoma cell cultures *in vitro*. These studies are still ongoing, with future directions focused on validating the expression of the RAC1 pathway *in vitro*. In both studies—regarding the RAC1 and JAK-STAT3 pathways—the ultimate goal is to identify and test therapeutic molecules that specifically target these pathways for clinical use. By doing so, we aim to pave the way for more precise and effective treatments that could potentially improve patient outcomes and advance the therapeutic landscape for melanoma.

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Appendices

Curriculum Vitae and Publication list

europass

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WORK EXPERIENCE

02/01/2018 – 30/04/2018 Vienna, Austria
TRAINEE VETERINÄRMEDIZINISCHE UNIVERSITÄT WIEN

- Internal medicine of Ruminants
- Avian Pathology
- Fish Pathology

25/02/2019 – 09/08/2019 Perugia, Italy
TRAINEE ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELL'UMBRIA E DELLE MARCHE

Work in laboratoy of Histopatology

18/03/2020 – 31/12/2022 Terni, Italy
GENERAL PRACTITIONER CENTRO VETERINARIO SALEMA

- Internal medicine of Companion Animals
- Diagnostic Imaging
- Surgery of Companion Animals

06/02/2023 – 30/04/2023 Bern, Switzerland
VISITING PH.D. STUDENT VETSUISSE-FAKULTÄT (VETERINÄRMEDIZIN)

- Establishment of primary cell culture
- Participation in pathology training activities (supervised histopathology rounds, dermatopathology rounds, neuropathology rounds)

EDUCATION AND TRAINING

09/2007 – 07/2012 Adrano , Italy
HIGH SCHOOL DEGREE - LICEO SCIENTIFICO BILINGUE Liceo Statale Giovanni Verga

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05/03/2014 – 17/10/2018 Perugia, Italy
GRADUATION IN VETERINARY MEDICINE Università degli Studi di Perugia

Address Via San Costanzo, 4, 06121, Perugia, Italy | **Website** https://www.medvet.unipg.it/ | **Final grade** 110/110 e Lode |

Thesis Characterization of immunophenotype in feline melanocytic tumors and investigation of possible histological prognostic factors

19/11/2018 – 19/11/2018 Perugia, Italy
ABILITAZIONE PROFESSIONALE Università degli Studi di Perugia

Address Via San Costanzo, 4, 06121, Perugia, Italy | **Website** https://www.medvet.unipg.it/

19/12/2018 – 12/07/2021 Matelica
SPECIALIZATION IN ANIMAL HEALTH, LIVESTOCK PRODUCTION AND ANIMAL HUSBANDRY Univeristà degli Studi di Camerino

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RESIDENCY - EUROPEAN COLLEGE OF VETERINARY PATHOLOGISTS Università degli Studi di Perugia

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LANGUAGE SKILLS

Mother tongue(s): **ITALIAN**

Other language(s):

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken production	Spoken interaction	
ENGLISH	B2	B2	B2	B2	B2

Levels: A1 and A2: Basic user; B1 and B2: Independent user; C1 and C2: Proficient user

PUBLICATIONS

2021
Tumor-infiltrating lymphocytes (TILs) in feline melanocytic tumors: A preliminary investigation

2022
Tumor-Associated Macrophages in Canine Oral and Cutaneous Melanomas and Melanocytomas: Phenotypic and Prognostic Assessment

2023
Canine melanocytes: Immunohistochemical expression of melanocytic markers in different somatic areas

2024
Establishment of Primary Cell Cultures from Canine Oral Melanomas via Fine-Needle Aspiration: A Novel Tool for Tumorigenesis and Cancer Progression Studies

2024
SOX-10 and TRP-1 expression in feline ocular and nonocular melanomas

2024
Exploring the Epidemiology of Melanocytic Tumors in Canine and Feline Populations: A Comprehensive Analysis of Diagnostic Records from a Single Pathology Institution in Italy

A.Lo Giudice,I.Porcellato,G.Giglia,M.Sforna,E.Lepri,M.T.Mandara,L.Leonardi,L.Mechelli,C.Brachelente

SUBMITTED AND UNDER REVISION PUBLICATIONS

Quantification of intraepithelial canine melanocytes in different somatic areas

Accepted

Rac1 signaling - associated genes are upregulated in nodal metastasis of canine oral 2 mucosal melanoma

Submitted

Modifying Phrases in Veterinary Pathology Diagnostic Reports: The Veterinary Professionals' Perception on the Diagnostic Confidence.

Under revision

Advancing Canine Cutaneous Dermatopathological Diagnosis: Immunohistochemistry to Distinguish Infectious and Noninfectious processes

In preparation

Diagnostic challenge in veterinary pathology: abdominal neoplasia in a cat

In preparation

GRANTS AND FUNDING AWARDED

07/2024 – 07/2025

Unravelling Canine Melanocyte Diversity: A Single-Cell Sequencing Exploration Across Body Regions - Funded by European Society of Veterinary Dermatology (ESVD) - Type of grant: Major Grant (15.000 €)

Recently, characterization of melanocyte populations in skin and mucosal sites has significantly contributed to melanocyte biology and pathology understanding, particularly in human medicine. While, similar efforts have been made in veterinary medicine, particularly in dogs, there are still numerous unanswered questions regarding canine melanocytes biology. Melanocytic diseases indeed remain a concern in veterinary medicine and are characterized by distinct phenotypic presentation. Additionally, the site- specific incidence of cutaneous and oral canine melanomas presents an intriguing investigation area, given the aggressive behaviour of oral melanomas compared to the typically benign behaviour of cutaneous ones. Understanding the transcriptomic landscape of canine skin and mucosa is crucial for deciphering tissue-specific gene expression patterns and their implications in health and disease. The aim is to employ single-cell RNA sequencing to comprehensively profile the transcriptomes of normal melanocytes and adjacent cell populations from oral mucosa and haired skin in dogs. Tissue samples will be collected from skin and oral mucosa of dogs undergoing surgery or who have died from causes not related to melanocytic diseases. Single-cell RNA sequencing will be performed on 3 dogs by comparing isolated melanocytes and adjacent cells from skin and oral mucosa, using state-of-the-art sequencing platforms. Bioinformatics analysis will be employed to identify expressed genes, pathways, and regulatory networks associated with these two specific body areas. The cell type identity of each cluster of cells will be determined by the expression of differentially expressed genes. Comparative analysis between skin and oral mucosal melanocytes and adjacent cell populations
will be conducted to delineate tissue-specific gene expression patterns. We hypothesize that distinct canine body areas, namely skin and oral mucosa, harbour unique melanocyte transcriptomic profiles, reflecting tissue-specific functions and microenvironmental cues. The short-term objective of the study is to conduct an initial characterization of the transcriptome of canine healthy melanocytes from oral mucosal and, by revealing the transcriptional heterogeneity of melanocytes, to identify tissue-specific gene expression patterns and potential regulatory mechanisms modulating melanocyte function. By comparing gene expression profiles of cutaneous and mucosal melanocytes, alongside considering the anatomical and immunological differences of their respective host tissues, the long-term objective of this research is to expand our understanding of the role of melanocyte heterogeneity in maintaining skin and mucosal homeostasis. Furthermore, we expect that our study will clarify what is the relationship between melanocyte diversity and the diverse phenotypic and biological manifestations of melanocytic diseases involving these sites, potentially identifying targets for therapeutic interventions.

Proceedings

Published

Porcellato, I., **Lo Giudice, A**. L., Capaccia, C., Brachelente, C., Guelfi, G., Starkey, M., ... & Di Palma, S. (2024). RAC1 pathway in canine oral melanoma progression and prognosis. *Journal of Comparative Pathology*, *210*, 57.

Orlandi, M., Brachelente, C., Sforna, M., Mechelli, L., **Lo Giudice, A**., & Porcellato, I. (2024). Expression of SOX-10 and TYRP-1 in feline melanoma. *Journal of Comparative Pathology*, *210*, 104.

In press

Lo Giudice A., Porcellato I., Treggiari E., Capaccia C., Guelfi G., Sforna M., Brachelente C. (2024). JAK-STAT3 pathway in canine oral melanoma a potential therapeutic target.

Giglia G., **Lo Giudice A.**, Reginato A., Brachelente C., Leonardi L., Lepri E., Mandara M.T., Mechelli L., Sforna M., Porcellato I. (2024). Multinucleated giant cells (MGCS): a phenotypic assessment in neoplastic and non-neoplastic lesions of dogs and cats**.**

Congress Abstracts

POSTER

EXPRESSION OF SOX-10 AND TYRP-1 IN FELINE MELANOMA

Margherita Orlandi[1], Chiara Brachelente[1], Monica Sforna[1], Luca Mechelli[1], Adriana Lo Giudice[1], Ilaria Porcellato[1]

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Abstract

In felines, ocular and nonocular melanomas are uncommon tumors that represent a diagnostic challenge for pathologists, especially when amelanotic. To date, the immunohistochemical diagnostic panel in cats is based on specific melanocytic markers (Melan-A and PNL2) and a nonspecific but sensitive marker (S100). In human medicine, SOX-10 is reported to be a sensitive antibody for the detection of melanoma micrometastasis in the lymph node. TRP-1, an enzyme involved in melanogenesis, has recently been used in humans and dogs as a specific melanocyte marker. The aim of this study was to evaluate the crossreactivity and the expression of SOX-10 and TRP-1 antibodies in feline normal tissue and melanocytic tumors. Thirty-one cases of ocular, cutaneous, and oral melanomas were retrospectively evaluated and confirmed by histopathological examination and by immunolabeling with Melan-A and/or PNL2. SOX-10 nuclear expression in normal tissues was localized in epidermal, subepidermal, hair bulb, and iridal stromal melanocytes and dermal nerves. In melanomas, nuclear expression of SOX-10 was detected in ocular (11/12; 92%), oral (6/7; 86%), and cutaneous sites (12/12; 100%). TRP-1 cytoplasmic immunolabeling in normal tissue was observed in epidermal and bulbar melanocytes and in the lining pigmented epithelium of the iris and in its stroma. Its expression was positively correlated to the degree of pigmentation in the tumor and was observed in 75% of ocular (9/12), 43% of oral (3/7), and 33% of cutaneous melanomas (4/12). This study demonstrated the cross-

reactivity of SOX-10 and TRP-1 antibodies in feline non-neoplastic melanocytes and their expression in ocular and nonocular melanomas.

ORAL PRESENTATION

RAC1 PATHWAY IN CANINE ORAL MELANOMA PROGRESSION AND PROGNOSIS

Ilaria Porcellato[1], Adriana Lo Giudice[1], Camilla Capaccia[1], Chiara Brachelente[1], Gabriella Guelfi[1], Mike Starkey[2], Monica Sforna[1], Stefano Di Palma[3]

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Abstract

Introduction: In human medicine, the overexpression of genes involved in the RAC1 pathway is postulated to play a role in anti-apoptotic, pro-proliferative, pro angiogenic properties and in tumour metastasis. Moreover, activation of the RAC1 pathway is associated with resistance to both targeted therapy and chemotherapy. The aim of this study was to assess the involvement of RAC1 pathway members in the progression and metastasis of canine oral melanocytic tumours.

Materials and methods: Thirty cases (25 primary oral melanomas, including five with nodal/distant metastases, and five oral melanocytomas) were selected. RT-qPCR was performed on five oral melanomas, five metastases and five melanocytomas to investigate the expression of ELMO1, DOCK2, CDKN1B, CORO1A, VAV3, CCR7 and MMP2. Immunohistochemistry for ELMO1, p27 (CDKN1B), ITGA4, VAV3 and CORO1A expression was performed on all cases.

Results: ELMO1 and VAV3 displayed significantly higher expression in melanomas than in melanocytomas, at both mRNA and protein level ($P < 0.05$). Cytoplasmic expression of p27 was more commonly observed in melanomas than in melanocytomas ($P = 0.001$). The protein expression of ELMO1 and cytoplasmic p27 was higher in cases with metastases when compared with the non-metastatic tumours ($P < 0.05$). ELMO1, cytoplasmic p27 and VAV3 were also directly correlated with well-known histological prognostic features, such as mitotic count and atypia, and inversely correlated with pigmentation.

Conclusions: The RAC1 pathway appears to be activated in the progression and metastasis of canine oral melanomas, especially involving ELMO1, p27 and VAV3. These molecules could be further investigated regarding their relevance as prognostic markers and potential therapeutic targets.

ORAL PRESENTATION

ADVANCING CANINE CUTANEOUS DERMATOPATHOLOGICAL DIAGNOSIS: IMMUNOHISTOCHEMISTRY TO DISTINGUISH INFECTIOUS AND NONINFECTIOUS PROCESSES

Adriana Lo Giudice[1], Serenella Silvestri[2], Elisa Rampacci[1], Alessandra Fondati[3], Luca Luciani[4], Katarzyna Małgorzata Walczak[5], Luca Mechelli[1], Fabrizio Passamonti[1], Chiara Brachelente[1]

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5. Institute of Veterinary Pathology, University of Zurich, Zurich, Switzerland

Abstract

Background: Pyoderma represents one of the most frequent canine dermatologic diseases. While it typically presents with recognizable signs, there are instances where its clinical and histopathologic features overlap with those of noninfectious dermatopathological conditions such as neutrophilic pustular dermatosis, sterile granuloma and pyogranuloma syndrome, and sterile folliculitis and furunculosis. Distinguishing infectious from noninfectious is crucial, as their treatment differs significantly. Despite an appropriate diagnostic approach, uncertainty in diagnosis may persist, leading to a delayed treatment. Methods that allow microorganism visualization in tissues hold promise in discerning between infectious and noninfectious etiologies, thereby accelerating the diagnosis.

Objective: The aim of the study is the evaluation of the applicability of immunohistochemistry (IHC) as a supplementary diagnostic technique to distinguish between sterile and infectious skin diseases.

Methods: To achieve the objective, a retrospective study was conducted using anti-Gram-positive and anti-Gram-negative antibodies on formalin-fixed and paraffin-embedded (FFPE) skin sections from 39 dogs with confirmed diagnosis of infectious or noninfectious dermatitis. A previous step was conducted on positive control tissues containing known bacteria.

Results: The sensitivity and specificity of the technique were 67% and 50%, for Gram-positive and for Gram-negative immunolabeling respectively, while the specificity was 83% for the anti-Gram-positive antibody and 81% for anti-Gram-negative antibody.

Conclusions: Although further studies are needed, IHC could be considered a complementary tool for the histologic diagnosis of canine infectious and noninfectious inflammatory processes, especially when investigating the role of Gram-positive bacteria in pustular diseases.

POSTER FLASH - *Awarded*

JAK-STAT3 PATHWAY IN CANINE ORAL MELANOMA: A POTENTIAL THERAPEUTIC TARGET?

Adriana Lo Giudice[1], Ilaria Porcellato[1], Elisabetta Treggiari[2], Camilla Capaccia[1], Gabriella Guelfi[1], Monica Sforna[1], Chiara Brachelente[1]

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Abstract

Background: Canine oral melanoma is an aggressive tumor with limited therapeutic options and short survival times. Signal transducer and activator of transcription (STAT)3 is a regulator of transcription that can be constitutively activated in different types of cancer, but can also be activated by Janus kinases (JAK). Our aim is to assess the expression of pSTAT3 and JAK1 in a retrospective cohort of canine oral melanomas.
Materials & Methods: Forty-three cases of canine oral melanomas were retrospectively selected and immunohistochemistry was performed to assess the expression of pSTAT3 and JAK1. The expression of STAT3, JAK1, JAK2, and TYK2 was also assessed by RT-qPCR on three OM cell cultures. On 2D cell culture, the expression of pSTAT3, JAK1, and JAK2 was assessed by IF.
Results: Expression of pSTAT3 was observed in the nucleus of neoplastic cells, with variable percentages (1-80%) in all tested melanomas and cell cultures. Instead, JAK1 was observed in the cytoplasm of melanoma cells (50-95%) in all tested cases. Gene expression analysis confirmed highly expressed STAT3 and a higher expression of JAK2 among the tested Janus kinases.
Conclusion: Results from the present study show activation of the STAT3 pathway in canine oral melanoma, identifying it as a potential prognostic marker and an interesting therapeutic target.

POSTER

MULTINUCLEATED GIANT CELLS (MGCS): A PHENOTYPIC ASSESSMENT IN NEOPLASTIC AND NON-NEOPLASTIC LESIONS OF DOGS AND CATS

Giuseppe Giglia[1], Adriana Lo Giudice[1], Alice Reginato[2], Chiara Brachelente[1], Leonardo Leonardi[1], Elvio Lepri[1], Maria Teresa Mandara[1], Luca Mechelli[1], Monica Sforna[1], Ilaria Porcellato[1]

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2.CDVet Laboratorio Analisi Veterinarie, Roma, Italy

Background: Multinucleated giant cells (MGCs) are observed in inflammatory and neoplastic lesions. In granulomatous reactions, macrophages fuse to form large MGCs. In tumors, MGCs may originate from macrophages (tumor-associated inflammation) or neoplastic cells. Despite numerous lesions show these fascinating cells in veterinary medicine, their characterization has been sporadic. This pilot study aimed to investigate the phenotype of MGCs in selected lesions of dogs and cats, encompassing both inflammatory and neoplastic lesions.

Materials & Methods: For this purpose, various lesions were retrospectively selected from FFPE archive material at the Veterinary Pathology Service of the Department of Veterinary Medicine (Perugia, Italy) based on the presence of MGCs. Seven peripheral giant cell granulomas, 6 feline injection-site sarcomas (FISS), 6 osteosarcomas (OSA), 4 oral melanomas, 2 giant cell tumor of tendon sheath, and 1 granulomatous rhinitis were submitted to IHC using a panel of macrophagic-specific antibodies (IBA1, CD204, CD206, CD163, MAC387), in addition to phenotyping markers specific of the investigated lesion.

Results: In most cases, MGCs showed a strong and diffuse positivity for IBA1, and negativity for the other macrophagic markers and specific antibodies (i.e. RUNX2 in osteosarcomas, Melan-A/PNL2/SOX-10 in melanomas). Occasional CD206 and MAC387 expression was observed in FISS. In tumors, some MGCs with cellular atypia were negative for macrophagic markers.

Conclusion: MGCs in different inflammatory and neoplastic lesions seem to have a histiocytic origin, although their specific origin and role within the neoplastic microenvironment remain unknown. Intracellular nuclear atypia and negativity to macrophagic markers might help the pathologist distinguish intratumoral MGCs of neoplastic origin from non-neoplastic histiocytic elements