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ADJUVANT CSPG4 ANTIGEN ELECTROVACCINATION IN CANINE ORAL MALIGNANT MELANOMA

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ABBREVIATIONS

18F-FDG = 18Fluorine-fluorodeoxyglucose
ADCC = antibody-dependent cellular cytotoxicity
ALCAM = activated leukocyte cell adhesion molecule
APC = antigen presenting cell
bFGF = basic fibroblast growth factor
CAMs = cell adhesion molecules
CEACAM1 = carcinoembryonic antigen-related cell adhesion molecule 1
CEP = circulating endothelial progenitor cell
CMM = canine malignant melanoma
CR = complete response
CS = chondroitin sulfate
CSC = cancer stem cell
CSPG4 = chondroitin sulfate proteoglycan 4
CT = computed tomography
CTLA-4 = T-lymphocyte-associated protein
DC = dendritic cells
DFI = disease free interval
ECM = extracellular matrix
EM = electron microscopy
END = elective neck dissection
EP = electroporation
FDA = Food and Drug Administration
FNA = fine needle aspiration
GD = disialoganglioside
GET = gene electrotransfer
GM-CSF = colony-stimulating factor
HIF-1α = hypoxia-inducible factor-1α
HMB-45 = human melanoma black-45
ICAM-1 = intercellular cell adhesion molecule 1
ICC = immunocytochemistry
ID = intradermal
IHC = immunohistochemistry
IL-12 = interleukin-12
IM = intramuscular injection
ISH = *in situ* hybridization
L-MTP-PE = liposome-encapsulated lipophilic derivative of muramyl dipeptide
LAK = lymphokine-activated killer
mAb = monoclonal antibody
MAPK = mitogen-activated protein kinases
MC = metronomic chemotherapy
MCC = mast cell count

MCSP = melanoma chondroitin sulfate proteoglycan
Mel-CAM = melanoma cell adhesion molecule
MHC = major histocompatibility complex
MC = mitotic count
MiTF = microphthalmia transcription factor
MRI = magnetic resonance imaging
MST = median survival time
MTD = maximum tolerated dose
MVD = microvessel density
NF-κB = nuclear factor-κB
NK = natural killer
NLRs = nucleotide-binding domain like receptors
NOD = nucleotide-binding oligomerization domain
OMM = canine oral malignant melanoma
OMM = oral malignant melanoma
OR = objective response
OS = overall survival
PAMPs = called pathogen-associated molecular patterns
PD-1 = programmed cell death 1
PD-L1 = programmed cell death ligand 1
PDGFR-α = platelet-derived growth factors receptor α
PDGFR-β = platelet-derived growth factors receptor β
PFI = progression free interval
PFS = progression free survival
PR = partial response
PRRs = pattern recognition receptors
RT = radiotherapy
RTK = tyrosinase kinase receptors
SD = stable disease
SLN = sentinel lymph node
T-reg = regulatory T cells
TAA = tumor associated antigen
TGF-β = transforming growth factor-β
Trc = T cell receptor
TRLs = Toll-like receptors
TRP-1 = tyrosinase-related proteins 1
TRP-2 = tyrosinase-related proteins 2
TSP-1 = thrombospondin-1
VCAM-1 = vascular cell adhesion molecule 1
VEGF = vascular endothelial growth factor
WHO = World Health Organization

INTRODUCTION

Canine melanocytic tumors

Spontaneously occurring melanoma is one of the most diagnosed tumors in dogs worldwide, accounting for 7 % of malignant tumors and representing the most common malignant neoplasm of the oral cavity. According to data collected from the Cani-DNA biobank on 2350-melanocytic tumors, 70 % were histologically diagnosed as malignant, 30 % as benign tumor i.e., the so-called melanocyotmas based on WHO classification of melanocyte-derived skin tumors (Smith et al., 2002, Gillard et al., 2014;).

Melanocytomas appear as a slowly growing pigmented nodules, mostly localized in the skin. Surgical resection is curative and associated with a good prognosis (Ramos-Vara et al., 2000).

In this dataset, the anatomical site distribution revealed that canine oral melanoma is the most common form (62%), followed by cutaneous (27%), digital and ocular melanoma as shown in Figure 1. Melanoma in dogs can have extremely different biologic behaviors depending on a large variety of factors, one of these being the anatomical localization. In fact, 97% of oral canine melanocytic tumors are malignant with high metastatic rate. In contrast, cutaneous melanocytic tumors appeared less aggressive, with only a 43% malignancy rate. Interestingly, canine digital and ungual localizations, are 84% and 100% malignant, respectively, and display more aggressive than cutaneous tumors (Gillard et al., 2014; Laver et al., 2018).

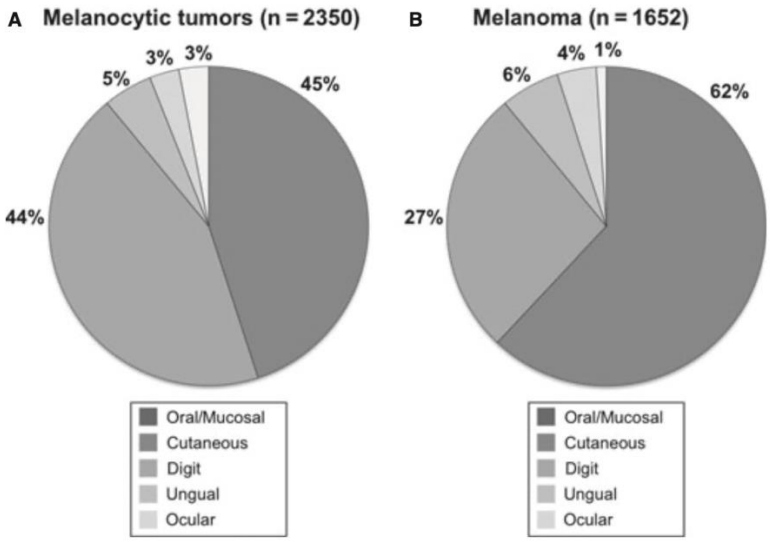


Figure 1 *Distribution of the canine melanocytic tumors depending on the anatomical site. (A) Percentages of the 2350 melanocytic tumors, including benign tumors (*A, *30%; n = 698) and malignant tumors (*B, *70%; n = 1652), depending on the anatomical site. (B) Percentage of the 1652 melanomas, depending on the anatomical site. Adapted from Gillard et al., 2014.*

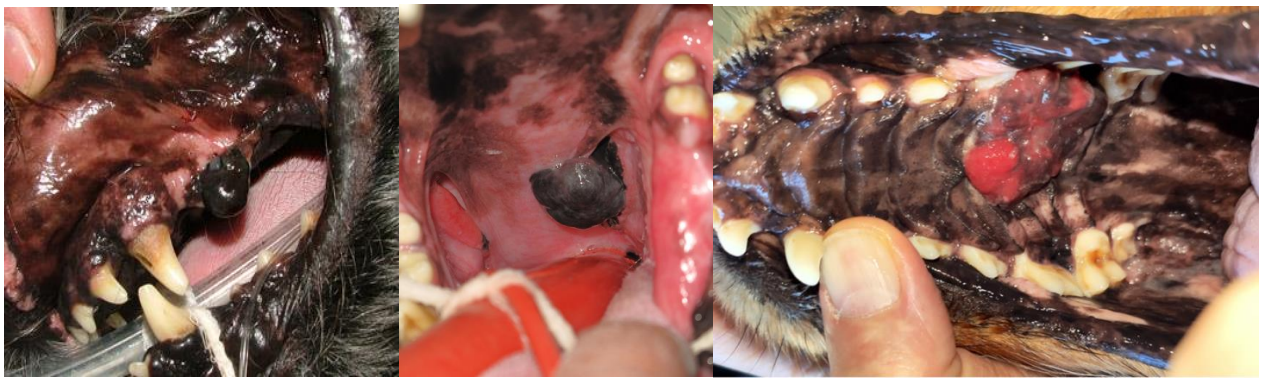
Malignant melanoma typically occurs in middle-aged to older dogs, with no gender predilection. The relative risk of developing oral melanocytic neoplasia appears to grow with age in comparison with other oral malignancies. Dogs with less malignant oral melanomas are reported to have a mean age of approximately 8 years, whereas dogs with highly malignant oral melanoma have a mean age of approximately 12 years (Smith et al., 2002; Bergman, 2007).

Some breeds are considered at risk to develop melanocytic tumors, these include: Airedale Terrier, Boston Terrier, Boxer, Chihuahua, Chow Chow, Cocker Spaniel, Doberman Pinscher, English Springer Spaniel, Golden Retriever, Irish Setter, Miniature Schnauzer, Scottish Terrier, Poodles, Beauce Shepherds, Rottweilers, and Labrador Retrievers. Schnauzer dogs (miniature and standard), Scottish Terriers, and Irish Setters have been suggested to have a higher incidence of digital malignant melanomas (Gillard et al., 2014; Nishiya et al., 2016).

CANINE ORAL MALIGNANT MELANOMA

Epidemiology

Malignant melanoma is a relatively common tumor in dogs and it is the primary oral malignancy, accounting for 14.4% to 45.5% of all oral tumors (Nishiya et al., 2016; Sarowitz et al., 2017).
Oral malignant melanoma (OMM) develops in dogs older than 10 years and is more commonly diagnosed in golden and Labrador Retrievers, Scottish and Boston Terrier, Cocker Spaniel, Poodle, Daschund and Chow Chow (Ramos-Vara et al., 2000; Gillard et al., 2014). However, in comparison with other malignant tumor, oral malignant melanoma tends to occur in dogs of lower body weight (Liptak, 2020).
Despite previous studies have reported that male dogs have a higher incidence of melanoma than female dogs, several more recent studies have found no gender predisposition (Ramos-Vara et al., 2000, Bergman, 2007; Teixeira et al., 2010).
Canine oral melanoma arises in the following locations given in order of decreasing frequency: gingiva (42%), lips (38%), hard palate (11%) and tongue (7%), rarely tonsils and pharynx (Todoroff & Brodey, 1997; Bergman, 2007).



2a *2b* *2c*

Figure 2 *Melanotic malignant melanoma arising from the maxilla 2a and tonsil 2b and partially melanotic from the hard palate 2c.*

Differential diagnosis for oral masses should include squamous cell carcinoma, fibrosarcoma, osteosarcoma, acanthomatous ameloblastoma, and peripheral odontogenic tumors (Todoroff & Brodey, 1997; Bergman, 2007).

Pathology and etiology

Melanoma arises from melanocytes which are dendritic cells derived from the neuroectoderm and melanoblasts of the neural crest that during embryogenesis process migrate to the dermis and epidermis, mucous membranes, and eyes. Melanocytes are found within the basal layer of the epidermis interspersed between basal keratinocytes. They normally do not form attachments with or even touch each other but rather form adherent and regulatory junctions with five to eight neighboring keratinocytes via epithelial cadherin (E-cadherin) molecules. Melanocytes are cell specialized in pigment production through the melanosome by a number of melanosomal glycoprotein (Smith et al., 2002; Nishiya et al., 2016; Bergman et al., 2020).

Melanin is not retained within the normal melanocyte. It is packaged in melanosomes and transferred through their dendritic processes to keratinocytes. Conversion of normal melanocytes, that are nonpigmented and isolated from other melanocytes, to pigmented and clustered neoplastic melanocytes is a multistep process, with initiation as the first event, then followed by promotion, transformation, and metastasis (Smith et al., 2002).

The next step in carcinogenesis requires one or more promoting factor(s). Promoters, possibly acting in an epigenetic fashion by disrupting gap-junctional intercellular communication, stimulate proliferation of the mutated cell, allowing for amplification of the cell population, persistence of the mutation, and opportunities for additional mutations. Promoters can include chronic trauma, chemical exposure, burns, hormones, infections, drugs, and other causes for reactive hyperplasia. It can be supposed that mucosal melanoma arises as a result of chronic injury (either mechanical or inflammatory) that results in reactive hyperplasia of the epithelium, disruption of normal keratinocyte-melanocyte interactions, and amplification of cells initiated spontaneously or by unidentified environmental factors (Smith et al., 2002).

Genetic factors play a critical role in the pathogenesis of human melanoma as well as in canine melanoma. Four critical molecular phases in the development and progression of malignant melanoma have been identified: (1) onset of genetic instability, (2) enhanced and inappropriate cellular proliferation, (3) acquisition of invasive and metastatic traits, and (4) promotion of tumor angiogenesis (Modiano et al., 1999).

Melanocyte growth is controlled by the surrounding keratinocytes by several mechanisms as extracellular communication through paracrine growth factors, intracellular communication through second messengers and signal transduction and intercellular communication through cell adhesion molecules, cell–matrix adhesion, and gap junctional intercellular communication (Haass et al., 2005).

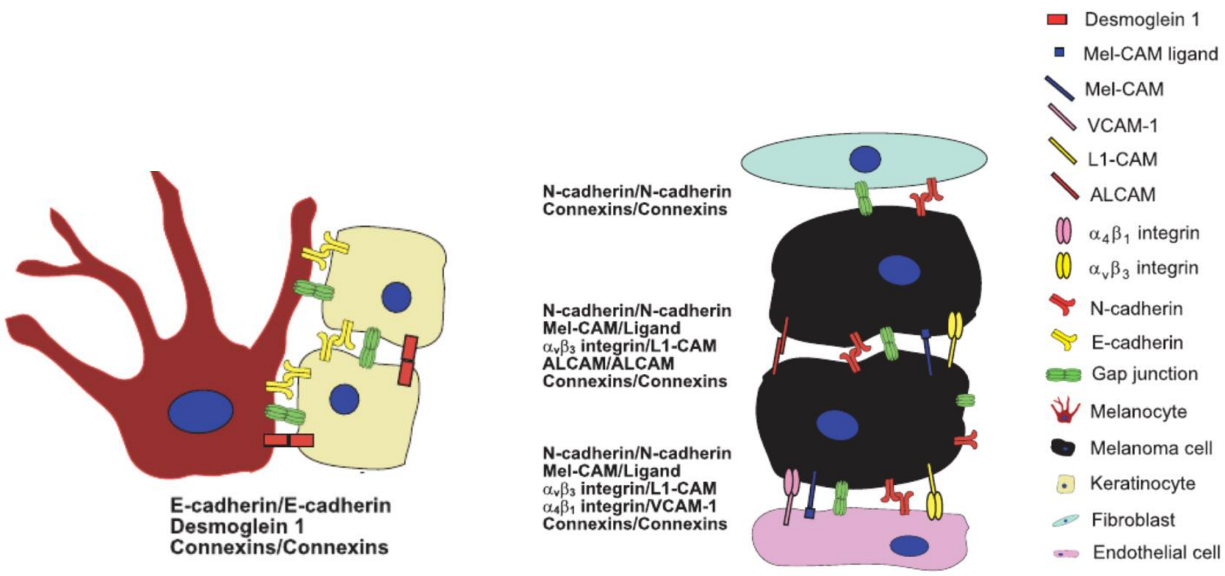
Under normal conditions, homeostasis determines whether a cell remains quiescent, proliferates, differentiates, or undergoes apoptosis. Dysregulation of the homeostasis control may disrupt the balance of the epidermal melanin unit and trigger a constant proliferative activity of melanocytes, which may lead to the development of melanoma. Hanahan and Weinberg (2011) have proposed that most of human cancer cells' genotypes manifests six essential alterations in cell physiology that dictate malignancy. These 6 hallmarks of cancer include (1) self-sufficiency in growth signals, (2) insensitivity to antigrowth signals, (3) ability to evade apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis (Hanahan & Weinberg, 2011).

The exact molecular mechanisms of this dysregulation in melanoma are still unknown. However, it has been suggested that melanoma cells escape from control through different pathways as:

1- down-regulation of receptors important for communication between and adhesion to keratinocytes (e.g.; E-cadherin);
2- up-regulation of receptors and signaling molecules not found on melanocytes but important for melanoma–melanoma and melanoma–fibroblast interactions [e.g.; N-cadherin, melanoma cell adhesion molecule (Mel-CAM)];
3- loss of anchorage to the basement membrane because of an altered expression of the extracellular-matrix binding integrin family (Haass et al., 2005).

The changes in cell–cell adhesion receptor composition often reflect aggressive properties of melanoma cells. One of these changes is a shift from E-cadherins found on normal melanocytes for attachment to keratinocytes, to N-cadherin on melanoma cells, which allows coupling to fibroblasts and endothelial cells in the tumor stroma. Additionally, even if melanoma cells express E-cadherin, it does not seem functional. Recently it has been suggested that the evaluation of E-cadherin, particularly in amelanotic tumors, could be helpful in distinguishing the most aggressive oral melanomas, and the use of E-cadherin expression could represent one further tool for the prognostication of canine melanocytic tumors (Silvestri et al., 2020).
Although normal melanocytes express few cell–cell adhesion receptors of the immunoglobulin gene superfamily of cell adhesion molecules (CAMs), melanoma cells show an increase in expression of several melanoma cell adhesion molecules such as Mel-CAM, L1 cell adhesion molecule (L1-CAM), activated leukocyte cell adhesion molecule (ALCAM), vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule 1 (ICAM-1), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). For instance, in melanoma-melanoma interactions, Mel-CAM and its ligand appear to play a role as co-receptor for N-cadherin because its down-modulation disrupts gap junctional intercellular communication. VCAM-1 is a cytokine inducible cell adhesion molecule primarily expressed on vascular endothelial cells. As a receptor for $\alpha_4\beta_1$integrin, expressed by malignant melanoma, it facilitates melanoma cell attachment to the vascular endothelium prior to extravasation. ICAM-1 in melanoma is correlated with melanoma progression and increased risk of metastasis and can be induced in a cell-specific manner by several cytokines, e.g., tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interferon-gamma (IFN-δ). CEACAM1 is involved in intercellular adhesion and subsequent signal transduction events. Interaction of CEACAM1 with the β3 integrin covers an important role in melanoma cell migration and invasion and therefore its expression is associated with increased potential of progression and metastasis (Haass et al., 2005). Once malignant melanomas have escaped the keratinocytes control, they became able to invade the tissue by degradation of extracellular matrix (ECM) mediated by metalloproteinases (Nishiya et al., 2016).
An important aspect in the metastatic spread of melanoma is its interaction with the various components of the ECM. It seems that only transformed melanocytes can survive in the altered environment of the dermis, and this survival seems to be dependent upon expression of the cell–

ECM adhesion molecules such as the integrins. In particular, β_3 integrin down-expression is linked with increased metastatic potential (Haass et al., 2005).



3a *3b*

Figure 3 *Cell–cell adhesion of melanocytes and melanoma cells. Melanocytes adhere to keratinocytes via E-cadherin and desmoglein, which enables them to communicate with each other through gap junctions (3a). In melanoma cells E-cadherin is down-regulated. They interact with each other through N-cadherin, Mel-CAM/Mel-CAM ligand, avb3integrin/L1-CAM, ALCAM and connexins, with fibroblasts through N-cadherin and connexins, and with endothelial cells through N-cadherin, Mel-CAM/Mel-CAM ligand, $\alpha_v\beta_3$integrin/L1-CAM, $\alpha_4\beta_1$integrin/VCAM-1 and connexins (3b) (Haass et al., 2005).*

Environmental factors

Several etiological factors seem to be associated with the development of canine malignant melanomas including consanguinity, trauma, chemical exposure, hormones and genetic susceptibility. In contrast to human cutaneous melanomas, for which ultraviolet (UV) light exposure is a significant etiopathogenetic factor, oral melanoma is a highly aggressive non-UV-driven disease in human and dogs. Sunlight exposure may cover a role in the development of the disease on the sun-exposed areas of the body as face and pinna, but it is unlikely that UV-light could be involved in oral mucosal melanoma. Other factors, such as inflammation, presence of pigmented cells, trauma, chemical agents and oral microbiota may contribute to tumors' growth (Dzutsev et al., 2015; Nishiya et al., 2016).

Genetic factors and Molecular aspects

A genetic base should be considered based on the higher incidence of melanoma in some purebred dogs such as Standard and Miniature Schnauzers, Doberman Pinschers, Scottish Terriers, Irish and Gordon Setters, and Golden Retriever (Modiano et al., 1999; Smith et al., 2002).

The malignant melanoma evolution is based on the succession of different molecular phases that does not arise from a single defect, but rather, as a result of dysregulation of two broadly defined gene categories: (1) tumor suppressor genes whose functions are down-regulated; and (2) dominant-acting oncogenes whose functions are inappropriately up-regulated or enhanced. Proto-oncogenes are genes whose protein products are essential to normal cellular growth and proliferation. Oncogenes are defined as altered proto-oncogenes that promote abnormal cell proliferation. Alterations of proto-oncogenes occur through point mutations, gene amplification, and translocation (Sulaimon & Kitchell, 2003).

Several studies in canine melanoma have investigated recurrent mutations with possible effect in specific locations and specific breeds, reflecting different genetic backgrounds. Comparison through genome-wide gene expression profiles of 18 primary metastatic OMMs and 10 primary non metastatic OMMs has identified in the first ones a mechanism of 'up-regulation' of pro-metastatic gene expression similar to human melanoma metastatic lymph nodes (Bowlt et al., 2019).

Human and canine melanoma share some similar somatic mutations suggesting common pathways. TP53 is a tumor suppressor gene encoding for the p53 protein involved in cell cycle progression, DNA repair, cellular senescence and apoptosis. Normal p53 protein products have an indirect effect on tumor growth, either by inhibiting proliferation or by promoting cell death in the event of DNA injury. The ability of p53 to inhibit tumor growth is achieved by activating downstream effector genes that then modulate cell cycle arrest, DNA repair, and apoptosis. Mechanisms of inactivating TP53 include a combination of gene aberrations such as point mutations, base deletions, and insertions into the gene that lead to the production of truncated proteins or proteins with altered aminoacidic sequences (Sulaimon & Kitchell, 2003).

Mutations in TP53, with loss of functions, have been reported in human mucosal melanoma. Similarly, TP53 gene mutations have been reported in 8-13 % of canine oral malignant melanoma (Wong et al., 2019).

MDM2 is a proto-oncogene and a negative regulator action on the p53 tumor suppressor. Amplification of MDM2 has been demonstrated in both human and canine mucosal tumors. Consequently, it is possible that in both canine and human oral melanoma the lack of the protective action of p53 pathway against tumor development is gained by MDM2 gene amplification rather than through TP53 mutation or loss-of-function (Palma et al., 2021).

Somatic mutations of NRAS have been reported in canine oral malignant melanoma, at position Q61, matching the most frequent somatic alteration found in NRAS-mutated melanomas in humans (Gillard et al., 2014).

PTEN is a tumor suppressor gene encoding a protein (PTEN) that acts as a brake on the PI3K/AKT pathway with the consequent regulation of cell growth, survival, and migration. Both human and canine mucosal melanomas have disruption of PTEN by nonsense or frameshift mutations, causing a loss of gene function followed by uncontrolled neoplastic cell proliferation (Wei et al., 2016; Palma et al., 2021).

The proto-oncogene *KIT* that encodes a receptor tyrosine kinase, has been shown to be expressed in both human and canine melanoma (Murakami et al., 2011; Newman et al., 2012). Mutations in c-Kit frequently occur in human oral melanoma and may occur in 16% of metastatic canine oral mucosal melanomas. The KIT mutations can result in the activation of KIT signaling, leading to

growth and anti-apoptotic signals, including MAPK and PI3K/AKT, known to promote cancer development (Palma et al., 2021).

In humans, a significant proportion of cutaneous melanoma harbor a recurring hot spot mutations in different genes including BRAF (50%), RAS (20%) and/or NF1 (25%). All these gene mutations, missing in mucosal melanoma, could lead to constitutive activation of the MAPK signaling pathway (Hernandez et al., 2018). In canine patients, mutations in exon 15 of BRAF have been not found, similar to human mucosal malignant melanoma. BRAF V600E mutations are rarely present in canine oral melanoma accounting for <4% (Mochizuki & Breen, 2015).

The rarity of BRAF mutations, together with a lack of UV mutational signatures in NRAS or PTEN, in addition to the anatomical location of human and canine oral melanoma, suggest non-UV- driven pathways for their development and progression (Gillard et al., 2014).

Despite the relative rarity of BRAF and NRAS mutations, MAPK pathway activation appears to be a common feature detected in both canine and human malignant melanoma. In fact, alterations in orthologous chromosome regions encoding MAPK pathway genes occur. For example, Canis familiaris chromosome (CFA) 30 harbors some MAPK-related genes; a variety of genomic rearrangements of these genes could promote MAPK pathway activation in malignant melanoma (Fowles et al., 2015). Furthermore, a recent paper has highlighted the presence in canine oral melanoma of recurrent focal amplification on CFA 10 and 30 being associated with poor outcome and negative prognostic factors such as high MI (mitotic index) and amelanotic phenotype (Prouteau et al., 2020). A striking parallel is that both canine and human malignant melanoma frequently exhibit RAS/ MAPK and/or PI3K/AKT/mTOR signaling pathway activation, which occurs in the absence of some recognized highly recurrent genomic aberrations (Fowles et al., 2015; Hernandez et al., 2018).

Gene	Human	Canine
MDM2	19% CNV	23% CNV
NRAS	12% SNV	7% SNV
PTEN	21% CNV	7% SNV
TP53	12% SNV	13% SNV

Table 1 *Incidence of Mutations in Driver Genes Shared by Primary Canine and Human Oral Melanomas. Abbreviations: CNV, copy number variant; SNV, single-nucleotide variant (Palma et al., 2021).*

BIOLOGIC BEHAVIOR

Canine oral malignant melanoma (OMM) is an aggressive tumor with high metastatic rate and with metastasis occurring early in the disease process. Oral melanoma displays an extremely variable behavior that can be predicted by evaluating several clinical factors such as site of growth, tumor size and clinical stage, histological and immunohistochemical factors (Smedley et al., 2011a; Bergman et al., 2020; Liptak, 2020).
Metastatic sites are several, including mostly lymph nodes and lungs but also liver, meninges, and adrenal glands (Smith et al., 2002). The reported metastatic rate to regional lymph nodes and distant sites such as lungs and other organs ranges from 41% to 74% and from 14% to 92%, respectively (Liptak, 2020).

Site

In most cases, the anatomic site of melanoma is highly predictive of local invasiveness and metastatic propensity. Melanomas involving the haired-skin, distant from mucosal margins tend to behave in a less malignant manner and can be cured with surgical resection in many cases (Laver et al., 2018). In contrast, oral and/or mucosal melanoma, similar to human mucosal melanoma, have been usually considered an extremely malignant tumor, with a high degree of local invasiveness and high possibility to spread to draining lymph nodes and distant sites (Bergman, 2007). Malignant melanoma may arise in any part of the oral cavity, the gingival mucosa represents the most common site. Ramos-Vara et al. reported that mandibular labial mucosa was the most affected area (Ramos-Vara et al., 2000).
Some breeds as Chow Chow and Shar Pei seem to be predisposed to develop lingual melanoma, although lingual melanoma is more rarely seen (Dennis et al., 2006).

Size and Stage

Tumor size is an important prognostic factor for oral tumors, especially in melanoma. The staging system for oral melanoma according to the World Health Organization (WHO) is based on the size of the tumor and the presence of regional and distant metastasis. Dogs with stage I melanomas have tumors smaller than 2 cm in diameter, while dogs with stage II and stage III have tumors measuring between 2 to 4 cm, and more than 4 cm, respectively. Dogs with metastasis at the level of draining lymph nodes and with distant metastatic disease are classified as stage III and stage IV, respectively (Owen, 1980) (Table 2).

T	*Primary Tumor*	N	*Regional lymph nodes*	M	*Distant metastasis*
T1	Tumor < 2 cm in diameter	N0	No evidence of regional node involvement	M0	No evidence of distant metastasis
T2	Tumor 2–4 cm in diameter	N1	Histologic/Cytologic evidence of regional node involvement	M1	Evidence of distant metastasis
T3	Tumor >4 cm in diameter	N2	Fixed nodes		

Stage I	T1 N0 M0	*Stage III*	T2 N1 M0 or T3 N0 M0
Stage II	T2 N0 M0	*StageIV*	AnyT, AnyN andM1

Table 2 *Traditional World Health Organization TNM-based staging scheme for dogs with oral melanoma. Adapted from Owen LN. TNM Classification of Tumors in Domestic Animals. 1st ed. Geneva, Switzerland; 1980.*

Nevertheless, it should be mentioned that the WHO staging scheme has some limitations which may lead to an imprecise staging. Firstly, the size of the tumor is not standardized to the size of the patient, therefore a melanoma of 1.7 cm without lymph node metastasis is a stage I melanoma in a large breed dog, as well as in a small breed one. Besides, in this system the histological parameters are not considered. For these reasons, various investigators have pursued other prognostic factors in canine oral melanoma to possibly develop alternative staging systems. These investigations have continued to find size to be strongly prognostic but have also evidenced the following negative prognostic factors: incomplete surgical margins; location (caudal mandibular and rostral maxillary do more poorly); tumor mitotic index >3, and detection of bone invasion/lysis. Specifically, Hahn et al. (1994) proposed an alternative system for staging canine oral malignant melanoma considering features such as tumor size, location and mitotic index for a better understanding of the disease extent and patient classification (Hanh et al., 1994; Proulx et al., 2003). In a recent paper, other authors have evaluated potential associations between oral melanoma size and several histologic parameters suggesting that tumor size could also predict lymphatic invasion. Results indicated that lymphatic invasion can confidently be ruled out for tumors <6.5 mm in diameter (100% sensitivity) and ruled in for tumors ≥24.5 mm in diameter (100% specificity) (Carroll et al., 2020).

CLINICAL STAGING

The diagnosis of dogs with oral melanoma is relatively straightforward and include several procedures finalized to diagnose and stage the tumor according the TNM system. This system is considered for all oral tumors (Bergman, 2007; Liptak, 2020) and, as mentioned earlier is based on:

- *Tumor T*: size of the primary tumor and invasion into adjacent structures in particular bone.
- *Nodes N*: size, mobility and presence of metastatic disease within regional lymph nodes including mandibular, parotid, and medial retropharyngeal bilaterally.
- *Metastasis M*: presence of distant metastatic disease to the lungs and/or elsewhere

Complete staging of the dog is imperative before performing surgical excision of the primary mass and draining lymph nodes. Before undergoing any staging and/or diagnostic procedures, a minimum database including history, physical examination, complete blood count, biochemical profile, and urinalysis should be collected.

History, macroscopic appearance and clinical signs

Oral melanoma within the rostral part of maxilla and mandible is more easily noted by the owner in contrast with tumors growing in the caudal part of the oral cavity, including pharynx and tonsil. The majority of melanoma grow as a sessile mass. Occasionally, asymptomatic nodules less than 1 cm are found during scaling. Most of smaller neoplasms are raised, heavily pigmented, sometimes pedunculated with a low malignant potential and histologically described as well differentiated melanocytic neoplasm. Despite this, it is still uncertain if this outcome is due to the biologic behavior or simple surgical excision. Larger lesions (>3 cm, T3) are sessile, often with ulcerated surface, and usually associated with clinical signs of oral disease such as halitosis, increased salivation, dysphagia, bloody discharge, loose teeth, pain on mouth manipulation and regional lymphadenomegaly. Gingival melanoma tends to be irregularly oval, immobile and shaped by the anatomy of the jaw and teeth. The surface may be homogenously black but, in some cases, there are less pigmented areas as in amelanotic melanoma, that may induce pigment secondaries. Amelanotic neoplasms may be white or pink or covered with red granulation tissue secondary to ulceration. The tumor consistency is firm unless necrosis and secondary infection causes necrosis and secondary infection have caused irregular softening (Munday et al., 2017).
As part of the clinical staging, regional lymph nodes should be assessed. The regional lymph nodes include mandibular, parotid and medial retropharyngeal. Not all regional lymph nodes can be assessed through palpation including the parotid and the medial retropharyngeal ones. Metastatic disease has been detected in nearly 70% of enlarged lymph nodes, but notably in 40% of normal sized lymph nodes (Williams & Packer, 2003).
Based on these findings, it is recommended to perform at least cytology of the draining local lymph nodes, regardless lymph node size. Additionally, fine needle aspiration (FNA) of regional lymph node is suggested, considering that lymphatic pathways, particularly in the canine head, have been shown to be complex with multiple lymphocenters receiving drainage (Bergman et al., 2020).

Most importantly metastatic disease can be detected in the medial retropharyngeal or parotid lymph node but not in the mandibular one (Skinner et al., 2017). In contrast with previous studies advising a concordance between cytology and histology (Herring et al., 2002), a recent study has highlighted that the interpretation of cytology and histopathology of lymph nodes in dogs with melanocytic neoplasms is highly variable with low correlation between cytology and histopathology reports (Skinner et al., 2017; Grimes et al., 2017). In 2014 Boston et al. found that the sensitivity, specificity, positive predictive value, and negative predictive value of lymph node palpation, compared with histologic evaluation, for detection of metastatic disease, were 65.6%, 77.8%, 84.0%, and 56.0%, respectively. The sensitivity, specificity, positive predictive value, and negative predictive value of lymph node cytologic evaluation, compared with histologic evaluation, for detection of metastatic disease were 78.1%, 64.3%, 83.3%, and 56.3%, respectively (Boston et al., 2014). Consequently, given the low sensitivity of both lymph node palpation and cytologic evaluation, biopsy of the draining lymph node, or even better of the sentinel lymph node, may be a more accurate predictor of metastatic status of the patient (Bergman et al., 2020). In line with this aspect, a recent paper has underlined that, in OMM as for other oral tumors, bilateral retropharyngeal and mandibular lymphadenectomy is recommended. The results reported in this study have revealed that assessment of ipsilateral mandibular lymph node does not exclude the absence of nodal metastatic disease (Grimes et al., 2019).

DIAGNOSTIC CYTOLOGY

Fine-needle aspiration (FNA) cytology is the standard procedure providing reliable diagnostic information in many cases of oral malignant melanoma. Fine needle aspirate cytology is cost-effective, widely available, easy to perform, and a rapid method of diagnosis. In most cases, it can be carried out without the need for general anesthesia or even sedation. Quick Romanowsky stains (Diff-Quick, Wright-Giemsa) are used for melanoma cytology specimen examination providing good nuclear and cellular details (Friedrichs & Young, 2020).

In some cases, especially if the tumor is pigmented, the diagnosis of OMM may be confirmed with cytology. Cytology of benign melanocytic tumors is generally rewarding because they tend to exfoliate high number of cells that usually show minimal size variation and are well differentiated with numerous dark green-black cytoplasmic melanin granules. Nuclei are small and cytoplasm is abundant (Monti & Cian, 2016).

However, in some cases, the results of FNA cytology are unrewarding or unsatisfactory because the tumor is associated with high degree of inflammation and necrosis, therefore additional, sometimes more invasive methods are required for a definitive diagnosis. The diagnosis of oral melanoma involves multiple stages and cytology is often the initial step (Liptak, 2020).

In malignant melanoma, the cells can adopt the appearance of epithelial (sheets of cohesive cells), mesenchymal (individualized oval or spindle cells), or discrete round cell tumors, and all three cytologic appearance may be evident in the same tumor. Individual melanoblasts are round, oval, or spindle in shape with moderately high N:C ratios, lightly basophilic cytoplasm, and round or oval nuclei with fine chromatin and distinct nucleoli (Friedrichs & Young, 2020).

Malignant morphological features frequently seen are marked anisokaryosis and anisocytosis, variable N:C ratio, large nuclei, multiple and pleomorphic nucleoli and coarse chromatin (Grimes et al., 2017; Smith et al., 2002). In some cases, neoplastic cells may appear similar to those of benign melanoma, not displaying criteria of malignancy; other than being pleomorphic, varying from round to spindle shaped or epithelioid cells (Monti & Cian, 2016).

Highly pigmented tumors do not present a diagnostic challenge, and fine black melanin granules may be so numerous that they obscure all cellular details. Cells with varying degrees of pigmentation are typically found within the same tumor, and melanin granules may be sparse in some cells. Amelanotic tumors present a greater diagnostic challenge. Usually, a faint scattering of fine gray-black melanin granules is found in a few cells to support a diagnosis, but cells may be completely devoid of pigmentation. Amelanotic melanoma with marked anaplasia and polymorphism can be challenging to differentiate from soft tissue sarcoma and carcinoma requiring additional tests (Friedrichs & Young, 2020).

In previous studies, it has been reported that the anti-Melan A antibody usually results in cytoplasmic positivity in >92% of canine oral melanoma. In 2015 Przeździecki assessed the reliability of routine cytology and immunocytochemistry (ICC) in diagnosis of amelanotic melanoma, suggesting that ICC, using anti-cytokeratin, anti-vimentin, and anti-Melan A antibodies, is an excellent supporting method for presurgical diagnosis of poorly differentiated oral malignancies in dogs. Specifically, the combined use of cytology with immunocytochemistry for diagnosis of canine amelanotic melanomas was found to have 100% of sensitivity and specificity compared with only routine cytology that revealed a sensitivity of 66.7% and specificity of 85.7% in diagnosing amelanotic melanoma (Przeździecki et al., 2015).

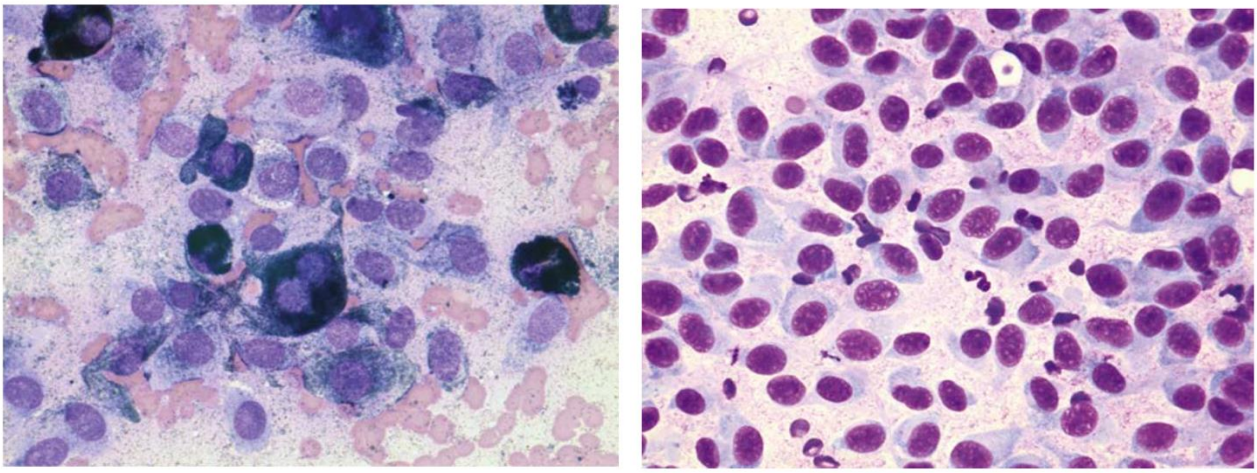


Figure *Fine-needle aspirate of a melanoma in a dog. The fine melanin granules in the tumor cells and in the background (left picture) Fine-needle aspirate of a melanoma in which melanin granules are not visible (amelanotic melanoma) (right picture) (Friedrichs & Young, 2020).*

An additional cytologic challenge is the identification of melanoma metastatic lymph nodes. This is due to the varied appearance of metastatic melanocytes and the presence of melanophages, which are macrophages containing abundant melanin. Physiologically melanophages carry melanin to draining lymph node for removal but this process occurs often in dogs with inflammation. Dogs with melanocytic tumors are susceptible to pigmentary incontinence from the tumor and inflammation

associated with tumor. Sometimes the melanophages may be erroneously classified as metastatic cells despite the fact they contain coarse collection of melanin within phagolysosome rather than fine granulation as in neoplastic melanoblasts (Grimes et al., 2017; Friedrichs & Young, 2020).
The cytological diagnosis of a metastatic lymph nodes is based on number of melanin-containing cells, presence of cohesive cells, morphology and features of malignancy:

- **non-metastatic lymph node:** absence of melanin-containing cells or rare (0-5 cells per smear), absence of no nonpigmented cells exhibiting cohesion, mesenchymal morphology, or features of malignancy;
- **metastatic lymph node**: presence of increased numbers (> 5 cells per smear, but actual numbers could be often much higher) of melanin-containing cells, cells are spindle shaped, exhibiting cohesiveness, and/or had distinct features of malignancy;
- **equivocal lymph node**: increased numbers of melanin-containing cells (> 5 cells per smear), individualized and round with no significant features of malignancy, including anisocytosis, anisokaryosis, variation and/or increase in nuclear-cytoplasmic ratio, multinucleation, and increased mitotic activity (Grimes et al., 2017).

The sensitivity of FNA cytology in diagnosis of metastatic lymph nodes has been reported as 63% for melanomas, with specificity ranging between 83% and 96% (Fournier et al., 2018).
Although FNA cytology remains a noninvasive and affordable test, additional histopathologic assessments should be recommended for more robust staging information and considering the frequency of metastatic disease to multiple lymph nodes. Furthermore, histology in conjunction with immunohistochemistry (IHC) is needed for most nonpigmented oral tumors, as amelanotic melanoma (Bergman et al., 2020).

DIAGNOSTIC HISTOLOGY

Despite the fact cytology can be reliable in some cases, histologic evaluation is often necessary to achieve a definitive diagnosis. In order to obtain a representative specimen, it is fundamental to perform a deep wedge or punch biopsy, avoiding the central part of the tumor which is often necrotic and likely to result not diagnostic, avoiding the superficial hyperplastic epithelium. Cautery is not suggested since it may alter the biopsy specimen. Biopsies should always be performed within the oral cavity and not through the lip to avoid seeding tumor cells in the normal skin, thus compromising the following curative-intent surgical resection (Liptak, 2020).

Microscopic features

Some of the terms used to define melanocytic tumors in humans have been adopted in veterinary medicine, in particular this terminology is referred to cells type, invasion of neoplastic melanocytic cells to epithelium and submucosa, junctional activity, lentiginous spread, and pagetoid growth. Histologically, canine melanoma exhibits several similarities with the human counterpart, with tumor growing in sheets, nests or bundles, frequently with an associated in situ/intraepithelial

component. Melanocytic neoplasms are most commonly composed of one of the following three cell types:

- *epithelioid or polygonal type* (20% of the cases) consists of closely pack round or polyhedral cells with abundant cytoplasm, well defined borders, and large central nuclei with one or more prominent nucleoli;
- *spindloid or fibromatous type* (35% of the cases) displays nuclei that are ovoid or elongate with small nucleoli;
- *mixed type* (40 % of the cases) is characterized by both epithelioid and spindloid. Other less commonly described cell types include: whorled type, cellular, balloon cell signet ring, and clear cell, and an adenomatous/ papillary type has also been mentioned in one report (Ramos-Vara et al., 2000; Spangler & Kaas, 2006; Munday et al., 2017).

Several studies have examined the predominant cell type as a potential prognostic parameter for canine cutaneous and oral or lip melanocytic neoplasms, but none has found a statistically significant association with survival (Millanta et al., 2002).

Melanocytic neoplasms may present both an epidermal-epithelial and dermal-submucosal component to the neoplasm, or dermal and submucosal with no detectable epidermal or epithelial component. Several features are described in oral melanocytic neoplasms including extension of neoplastic cells into the junctions submucosa-mucosa, junctional activity, lentiginous spread and pagetoid growth.

- *Extension of the neoplastic cells* to the junction submucosa-mucosa is defined as the presence of neoplastic melanocytes within the superficial layer of submucosa with no involvement of basal membrane.
- *Junctional activity* refers to the proliferation of neoplastic melanocytes in the basal layer at the interface between the epidermis and dermis or epithelium and submucosa.
- *Lentiginous spread* is defined as intraepidermal proliferation of migrating melanocytes residing within basal layer; it is possible to differentiate junctional activity from lentiginous spread based on the cohesive nature of the neoplastic melanocytes that prevent lateral spread. Since it is not possible to evaluate migration in a histology specimen both terms are used interchangeably in human medicine. Lentiginous spread is also an important feature for evaluation of surgical margins.
- *Pagetoid* refers to the presence of either individual or small aggregates of neoplastic cells within the upper levels of the epidermis or epithelium from the stratum basale and it was originally used to describe the carcinoma cells (Paget cells) that infiltrate the epidermis overlying mammary ductal carcinomas in situ (Smith et al., 2002; Munday et al., 2017).

Highly malignant melanomas are often invasive, with junctional activity and deep nodular expansion into the underlying tissue. Histology of these neoplasm reveals intraepithelial proliferation of nests of heavily pigmented neoplastic cells spreading laterally although these tumors macroscopically

appear as well circumscribed nodules. Intraepithelial neoplastic cells tend to be better differentiated compared with the submucosal neoplastic melanocytes and they show often immunolabelling for Melan-A and PLN-2. Therefore, is it advisable to submit a surgical biopsy sample from the edge of the lesion including also intact mucosa epithelium to achieve a final diagnosis (Smedley et al., 2011a).

Histopathology is considered the gold standard for diagnosis of metastatic lymph nodes. As previously mentioned, cytology has some limitations and may not correlate with histology report leading to an inaccurate staging of the patient. The presence of neoplastic melanocytic cells at the level of lymph nodes can be reported as macrometastasis or micrometastasis. Nodes are considered to have macrometastasis when the nodal architecture is effaced or distorted by large group of neoplastic cells. Nodes are considered micrometastatic if the sinuses or perisinusoidal parenchyma contain low numbers (5-15) of individualized or clustered cells that had morphology consistent with melanocytes (Grimes et al., 2017).

Additional diagnostic tests

Amelanotic and poorly differentiated melanomas represent a diagnostic challenge for the pathologist since a number of neoplasms may mimic melanoma microscopically. The combined lack of pigmentation and the indistinct cellular morphology of many oral malignant melanomas elude a definitive diagnosis. In light of this, several diagnostic methods have been developed in an effort to reach a more precise diagnosis. More traditional histochemical techniques are taking second place to immunohistochemistry (IHC), incorporating the use of monoclonal and polyclonal antibodies. Other methods include electron microscopy (EM) and, more recently*, in situ* hybridization (ISH) (Smith et al., 2002).

Histochemistry

Bleaching melanomas with hypochlorite is the simplest traditional technique; melanin pigment is extracted since it can obscure cell details and mitotic activity. The Masson-Fontana silver stain, is based on argentaffin reaction, that allows an easier identification of melanin granules especially in melanoma barely pigmented. The main disadvantage of this technique is the lack of specificity-substances such as lipofuscin and argentaffin granules can cross-react with these stains (Munday et al., 2017).

The DOPA (dihydroxyphenylalanine oxidase) test is more specific. This tyrosinase enzyme converts tyrosine to melanin. For practical purposes, this test is more applicable to nonpigmented melanomas, where the above chemical reaction results in the formation of brown/black pigment in the cytoplasm of neoplastic cells, confirming the presence of DOPA oxidase (tyrosinase) in those cells. The major disadvantage of this test is that it requires fresh tissue. These traditional methods are now being superseded by IHC (Smith et al., 2002).

Electron microscopy and in situ hybridization

Electron miscroscopy is based on the ultra-structurally identification of melanosomes and premelansomes in cells of amelanotic melanoma. Melanosomes indicate that the cells in which they occur are actively producing melanin pigment. The main drawback of this technique is that melanosomes and premelansomes are not entirely specific to melanomas because they are also found in other tumors. In situ hybridation (ISH) is a highly specific and sensitive tool and can be performed on formalin-fixed, paraffin-embedded tissues using a complementary DNA probe for tyrosinase-specific mRNA. Because amelanotic and pigmented melanomas contain similar levels of tyrosinase-specific mRNA, ISH is potentially a valuable tool for the diagnosis of poorly differentiated melanomas. Both the above-mentioned techniques require more technical skills and equipment; therefore they are rarely applied in veterinary practice (Smith et al., 2002).

Immunohistochemistry

Immunohistochemistry (IHC) is a staining procedure that uses antibodies to recognize specific markers or molecules defined as *tissue antigens*. Several antibodies are used for immunohistochemistry labelling in detection of melanocityc tumors, especially of the amelanotic phenotype. These include S-100, Melan-A, HMB-45 human melanoma black-45 (HMB-45), microphthalmia transcription factor (MiTF), PNL2, tyrosinase, and tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2) (Smedley et al., 2011b; Kamstock et al., 2020).

- *S-100*: is an intracellular and intranuclear acidic, calcium-binding protein. Most melanomas are reported to be S-100 positive, but the same is true for many other neurogenic and nonneurogenic tumors, including carcinoids. S-100 shows nuclear and cytoplasmic expression (Smedley et al., 2011b; Grandi et al., 2014).
- *Melan-A* is a protein that elicits a cytotoxic T-cell response, with narrow tissue distribution and generally strongly positive staining in melanocyte cytoplasm. Melan-A is a product of the MART-1 gene, which is recognized as an antigen on neoplastic melanocytes by autologous cytotoxic T lymphocytes. An immunohistochemical review on 122 canine oral melanomas found a positivity of 92 % among 122 canine oral melanomas (Ramos-Vara et al., 2000; Smith et al., 2002).
- Monoclonal antibodies against HMB-45 recognize the melanosomal glycoprotein gp-100, which specifically defines a premelanosomal antigen in fetal melanocytes and melanocytic neoplasms.
- *MiTF* is a nuclear transcription factor protein necessary for embryonic development and postnatal viability of melanocytes; however, the specificity of this marker is controversial. It has nuclear expression.
- *PLN2* is a generated monoclonal antibody (mAb) that recognizes normal and neoplastic melanocytes. Although the antigen recognized by PNL2 remains unknown, some studies have confirmed its usefulness as a diagnostic marker (Giudice et al., 2010; Smedley et al., 2011b; Kamstock et al., 2020).

- *Tyrosinase:* catalyzes the conversion of tyrosine to levodopa (L-dopa), which provides the substrate for the production of the pigment melanin. Tyrosine hydroxylase can be detected within human epidermal cells, specifically within the cytosol of melanosomes (Smedley et al., 2011b).
- *TRP-1 and TRP-2* are glycoproteins that cover a critical role in melanogenesis, and TRP-1 has been found to be largely restricted to the early unmelanized melanogenic compartment of melanocytes. Both show high specificity and sensitivity.

Other molecules involved in immunohistochemistry assay are vimentin and cytokeratin filaments. Melanoma cells should have a positive result for vimentin with a cytoplasmic expression, and negative for cytokeratin. Smedley et al. explored the sensitivity and specificity of S-100, Melan A, HMB-45, TRP-1, TRP-2, MiTF, PNL2, and tyrosine hydroxylase IHC in canine oral amelanotic melanocytic neoplasms as compared to canine subcutaneous soft tissue spindle cell sarcomas.
The authors found that Melan-A, PLN-2, and tyrosine reactive protein 1 and 2 have a sensibility of 81%, 89%, 55% and 79%, respectively. A cocktail containing all four antibodies has revealed 100% of specificity in diagnosis of oral melanocytic tumors (Smedley et al., 2011b). In contrast, antibodies against S-100 and MiTF have shown high sensitivity but low specificity. Antibodies commonly used in human melanoma diagnosis such as HMB-45 and tyrosinase have displayed high sensitivity but low sensitivity for oral melanoma (Ramos-Vara et al., 2000; Smedley et al., 2011b).

DIAGNOSTIC IMAGING

Different diagnostic imaging procedures can be performed for staging to evaluate the local extent of the tumor and/or the presence of local and distant metastasis. This information is essential for treatment planning and prognosis.

An initial assessment of the oral primary mass can be obtained with regional radiographs including open mouth, intraoral oblique, lateral, ventrodorsal, dorsoventral projections. In a few cases radiographs can reveal the presence of bone lysis, indeed this aspect is radiographically detectable only when 40 % or more of the cortex is destroyed. Therefore, normal radiographs do not exclude involvement of the bone. Bone invasion, as detected by advanced imaging and/or histologically, has been reported to occur in up to 57.0% of cases (Nishiya et al., 2016; Liptak, 2020).

Additionally, a recent paper has underlined the role of bone lysis as negative prognostic factor in dogs with oral melanoma (Camerino et al., 2021).

Advanced diagnostic imaging such as computed tomography (CT) and magnetic resonance imaging (MRI) are usually chosen over radiographs; CT is superior in revealing bone details and MRI would provide accurate information on invasion of adjacent soft tissue structures (Kafka et al., 2004; Ghirelli et al., 2013).

CT represents the standard screening test in human medicine and it has been found to be more accurate than thoracic radiography for detecting pulmonary metastasis. The higher sensitivity of CT compared with thoracic radiography is due primarily to the superior contrast resolution of CT and the lack of anatomic superimposition on pulmonary parenchyma due to thin-section collimation of CT images. CT can detect pulmonary nodule of 1 mm, while with radiographs the minimum size for a reliable pulmonary nodule detection approached 7-9 mm; superior sensitivity of CT is reported to be even more evident when large breed dogs have been considered (Nemanic et al., 2006; Armbrust et al., 2012).

Bearing in mind that oral melanoma may also spread to abdominal organs, an abdominal ultrasonography is also recommended if advanced imaging is not elected (Bergman et al., 2020).

In all oral tumors including OMM, lymph node metastasis can influence treatment recommendations and prognosis, therefore nodal staging results of outmost importance. Despite a lack of standardization in lymph node assessment, as above mentioned, detection of nodal metastases should rely on histopathological examination of the excised draining and of the sentinel lymph node (Grimes et al., 2017).

Lymph node mapping is a technique that allows identification of the “sentinel lymph node’’ (SLN) defined as the first lymph node within the lymphatic basin that drains the primary tumor and therefore examination of the SLN is indicated to detect the presence of loco-regional nodal metastasis. The use of indirect computed tomography lymphangiography with different protocols has been reported for detection of sentinel lymph node in head and neck cancer. This contrast-based technique, through lymphatic uptake, allows a direct visualization of SLN’s afferent lymphatic vessels. This technique has been compared with lymphoscintigraphy, the latter has been shown to be markedly more reliable in detection of sentinel lymph nodes (Beer et al., 2018; Randall et al., 2020, Chiti et al., 2021).

No correlation has been found between the use of indirect CT lymphangiography in identifying SLN in dogs with a pre-operative diagnosis of melanoma and mast cell tumors and histopathology findings supporting the idea that CT lymphangiography alone is not so accurate to diagnosis SLN metastasis (Grimes et al., 2019).

Other SNL mapping techniques reported for head and neck that include intraoperative dyes, contrast enhanced ultrasonography and use of lipid and water-soluble contrast agent (Liptak, 2020).

The use of radioactive tracers (technetium-99m) for SLN mapping is a standard procedure in human cancer therapy, in particular, in human melanoma. Radio-guided SLN mapping includes preoperative planar imaging and the intraoperative application of a hand-held gamma probe. Despite high detection rates this technique is not applicable on large scale in veterinary medicine due to radioactive materials (Beer et al., 2018).

Although not widely available, a recent paper has reported the use of positron emission tomography with 18Fluorine-fluorodeoxyglucose (18F-FDG) in dogs with OMM. This imaging test, commonly used for staging purposes in human cancers patients, reveals the biological variability of tumor tissue through injection of sensitive marker (i.e. 18F-FDG), tracer of tumor metabolism. Though this technique has seemed to yield high sensitivity, the lack of standardization FDG uptake values of normal and tumor tissue has led to low specificity (Willcox et al., 2021).

GRADING AND PROGNOSTIC FACTORS

Canine melanoma exhibits an extremely variable behavior but several prognostic factors would orientate the clinician toward the appropriate staging, treatment, and prognosis. Factors as size, site, clinical tumor stage and histological parameters should always be considered for a better understanding of melanoma biologic behavior (Bergman et al., 2020).
The histological grade is commonly predictive of survival, metastasis and biologic behavior and relies mostly on the evaluation of mitotic count, nuclear atypia and Ki-67 (Bergin et al., 2011; Smedley et al., 2011b).

Mitotic count (MC)

MC is determined by counting the total number of mitosis in 10 consecutive hpf (total area 2.73 mm^2) commencing in the area of highest mitotic activity. According to this method, oral and lip melanocytic neoplasms with $\geq$4 mitoses per 10 hpf have been associated with an increased risk of patient death within 1 year of diagnosis. This threshold value had a sensitivity of 90% and a specificity of 84%. Although this parameter is easily measurable, it has some limitations. Firstly, MC may be of difficult quantification in ulcerated areas and with high level of pigmentation or in spindloid tumor lacking of nuclear details. Apoptotic cells and cells undergoing mitosis display similar morphologic features leading to mistakes. Lastly, standardization in the size of field (2.37 mm^2) would provide a more accurate value of mitotic count (Smedley et al., 2011a; Munday et al., 2017).

Nuclear atypia

Nuclear atypia indicates the relative degree of cellular maturation. Well-differentiated or typical melanocytic tumor cells are characterized by a small nucleus with a single, centrally oriented nucleolus and minimal clumping of chromatin material. In contrast, highly malignant melanoma contains undifferentiated cells characterized by fine and evenly dispersed chromatin at the periphery of the nucleus, large and irregularly shaped nucleoli eccentrically located. Nuclear atypia is evaluated with a scale from 1 to 10, representing the subjectively estimated percentage of nuclei involved (i.e., 0 = no nuclear atypia, 1 = 1–9% involved nuclei, 2 = 10–19% involved nuclei, 3 = 20–29% involved nuclei, etc.). The threshold of $\geq$ 30% (> 4) atypical nuclei per 100 cells is adopted for oral melanocytic neoplasm. Histological evaluation of nuclear atypia has a high positive predictive value for epithelioid-predominant melanocytic neoplasms and for spindloid neoplasms, with sufficiently observable nuclear details, in terms of prognosis. Significant nuclear atypia is associated with a poor prognosis, and minimal or no atypia is associated with a favorable prognosis (Spangler & Kass, 2006; Bergin et al., 2011; Smedley et al., 2011a).

Ki-67

Ki67 is marker of the growth fraction of neoplastic cells and it predicts cell proliferation more accurately than a phase index such as mitotic index. It is expressed in all cell phases except in G_0. Ki67 immunolabeling is easily assessable in heavily pigmented neoplasm and Ki67 immunolabelling provide an objective measurement. Several methods have been reported for its measurement, corroborating the prognostic significance of this value in canine melanocytic neoplasm. In one study in which the average number of positively labeled neoplastic cell nuclei within 5 optical grid reticle 1 mm^2 areas was determined at 400x, dogs with Ki67 index above the threshold value of 19.5 was associated with worst outcome. The sensitivity and specificity of this threshold was 87.1% and 85.7% (Bergin et al., 2011).

Others prognostic indicators

Additional parameters have been explored for prognostic use in canine melanocytic neoplasia. Several studies have investigated the *degree of pigmentation* as potential prognostic marker with though a threshold value has not been identified. Despite high level of pigmentation seemed to be correlated with better survival, low or no pigmentation did not reliably associate with poor outcome. Therefore, pigmentation should not be evaluated as an individual prognostic factor. When considered with nuclear atypia, degree of pigmentation has revealed greater prognostic significance for oral melanocytic neoplasms (Bergin et al., 2011; Smedley et al., 2011a).

Even though *vascular/lymphatic invasion* has not been assessed as prognostic indicator, its recognition is a criterium of malignancy.

The presence of *inflammation* and/or *necrosis* should not be used to determine prognosis and no cut-off value have been determined.

The presence of *ulceration* has been associated with poor prognosis in one study on digital melanoma but its role remains uncertain in oral melanoma.

The level of invasion did not appear to be related to survival time. However, in one study absence of bone lysis was statistically associated with improved outcome and recently in another survey the presence of bone invasion has been reported as a negative prognostic factor (Proulx et al., 2003; Smedley et al., 2011a; Camerino et al., 2021).

One study examined microvessel density (MVD) and the number of mast cells within the neoplasms (mast cell count, MCC) founding that MCC and MVD were significantly correlated, and that high MCC and MVD in canine melanocytic neoplasms were associated with a poor prognosis (Mukaratirwa et al., 2006; Smedley et al., 2011a).

Another study evaluated platelet-derived growth factors receptors (PDGFR)-α and -β expression in stage II and III CMMs. Immunolabelling for both PDGFR-α and -β receptors was observed at cytoplasmic level and co-expression of both PDGFRs-α and -β was detected in 37.5% of the samples. Dogs with oral CMM expressing both PDGFR-α and -β had a statistically significant lower disease-free interval (DFI) and a lower overall survival (OS) compared with dogs with CMM not co-expressing these receptors (Iussich et al., 2017).

In human melanoma, COX-2 expression has been linked to malignant behavior and accumulating evidence suggests that cyclooxygenase-2 (COX-2) may play a critical role in canine melanoma. COX-2 is overexpressed in both oral and cutaneous melanomas, which seems to be related to the degree of pigmentation, MI, Ki-67 proliferation index and survival rate indicating that COX-2 is related to the acquisition of malignancy and negatively correlated with prognosis in canine patients with melanoma (Silveira et al., 2021).

Location	Oral/Lip Melanocytic Neoplasms		Cutaneous/Digit Melanocytic Neoplasms
Distant metastasis	Poor prognosis		Poor prognosis
Lymphatic invasion	Poor prognosis		Poor prognosis[a]
Mitotic index	10 consecutive fields starting in area w/highest mitotic activity		10 random fields
	Avoid areas of ulceration for both methods		
	< 4/10 hpf	Favorable prognosis	< 3/10 hpf
	≥ 4/10 hpf	Poor prognosis	≥ 3/10 hpf
Nuclear atypia[b]	% atypical nuclei in 200 cells counted		Subjective assessment
	< 30%	Favorable prognosis	< 20%
	≥ 30%	Poor prognosis	≥ 20%
Degree of pigmentation	Subjective assessment		
	% Pigmented cells		Scale 0 (no pigment) to 2 (high pigment)
	≥ 50%	Favorable prognosis	2
	< 50%	Uncertain prognosis	0 to 1
Presence of ulceration	No prognostic significance		Poor prognosis
Level of infiltration/invasion	Shallow w/no bone lysis	Favorable prognosis	Limited to dermis
	Deep w/possible bone lysis	Poor prognosis	Extends beyond dermis
Ki67 index	Average number of positive nuclei per grid (5 hpf grid areas counted)		% of positive nuclei in 500 cells counted
	Avoid areas of ulceration and inflammation and assess highest staining areas for both methods		
	< 19.5	Favorable prognosis	< 15%
	≥ 19.5	Poor prognosis	≥ 15%

Table 3 *Recommended parameters for prognostication in oral melanocytic neoplasms from Smedley et al., 2011.*

TREATMENT OF CANINE ORAL MALIGNANT MELANOMA

Multimodality treatment including surgery, radiotherapy, chemotherapy and/or immunotherapy is usually elected for OMM. Surgery and radiotherapy, used alone or in combination, are effective for local control. More recently electrochemotherapy has been used as an alternative option for local treatment and has been gaining ground. Chemotherapy and immunotherapy are utilized to control systemic disease, which is a common cause of death.

Surgery

Surgery represents the most effective local treatment option for management of oral melanoma. The dose of surgery is generally based on the anatomic site of the melanoma. In most cases, partial mandibulectomy or maxillectomy, cheek/lip *en bloc* excisions with or without mucosal reconstruction or skin flap reconstructions are required as 2-3 cm of margin are recommended, with resection of the underlying bone, especially in case of gingival OMM. For mucosal melanoma originating from lip, tongue or tonsil not in proximity of the bone, surgical excision of only soft tissues may be adequate. Different factors may influence the prognosis of dogs treated with surgery alone or combined with other treatments including age, tumor size, clinical stage, the ability of the first treatment to achieve local control, and histologic and IHC criteria (Bergman et al., 2020; Liptak, 2020).

In a study performed by Boston et al., among oral melanoma surgeries performed with wide surgical margins, 79.3% were completely excised based on histologic evaluation, and the recurrence rate was 8.3% in this group. The median survival time (MST) of these dogs was 354 days, with 30 % of dogs alive at 1-year (Boston et al., 2014).

In another study 70 dogs with OMM were treated with curative-intent surgery, including 2 to 3 cm of bone margins and 1 cm of soft tissue margins. Clean margins were achieved in 73% of the patients with 10 % of those developing local recurrence. Dogs treated with surgery as sole treatment had a progression-free interval (PFI) of 567 days, and an MST of 874 days. However, more than 80 % of patients in this study had OMM classified as stage I and II (Tuohy et al., 2014).

Achievement of complete surgical margins deeply influences the recurrence rate and consequently the prognosis. Although surgical excisions are often aggressive, cosmetic and functional results as well as owner satisfaction are optimal (Liptak, 2020).

Elective neck lymph nodes dissection has been reported in dogs with oral tumors. In one study Grimes et al. suggested, based on histologic evaluation, that removal of only 1 mandibular lymph node was insufficient to definitively rule out lymph node metastasis in dogs with OMM, thus they recommended bilateral lymphadenectomy of the mandibular and medial retropharyngeal lymph node (Grimes et al., 2019).

A survey has underlined the wide variability regarding the indication for routine lymph node extirpation and pathologic evaluation during staging of canine oral tumors; despite elective neck dissection (END) decision making was influenced by tumor type and size, OMM turned out to be the tumor where END was most commonly recommended, even more than oral squamous cell carcinoma (Congiusta et al., 2020).

Nevertheless, this approach presents some drawbacks, for some cases overtreatment with the potential for increased post-operative complications (Green & Boston, 2017).
Sensitive methods for SLN mapping and biopsy have been explored in veterinary medicine with encouraging results (Beer et al., 2018; Randall et al., 2020). These procedures may provide a less invasive alternative approach for some patients while still conferring the benefit of accurate staging with pathologic evaluation of the lymph nodes through selective lymphadenectomy, as in human melanoma (Leong et al., 2006).

Radiation therapy

Radiation therapy (RT) plays an important role in the management of OMM and it can be used as adjuvant/post-operative or palliative therapy. Although melanoma has been traditionally considered as ''radio-insensitive", requiring a higher dose for each fraction, this notion remains controversial (Khan et al., 2011). It has been suggested that melanoma cells have a large capacity to accumulate and repair sublethal radiation damage and this explains the resistance of melanomas to conventional radiotherapy protocols. It has been hypothesized that the response of melanomas to radiation might be improved by the use of high-dose fractions (Bateman et al., 1994).
The main radiation protocols for melanoma are based on hypofractionation (3–6 RT fractions once or twice weekly), due the alpha/beta ratio range of 0.5–2.5 Gy. These ratios are similar to those of late-responding normal tissues, which are preferentially damaged by radiation delivered in large doses per fraction (Bentzen et al., 1989). On one hand hypofractionation schedule requires few anesthetic episodes, low cost and less commitment, on the other hand the biological equivalent dose can result in lower overall and biologic equivalent dose leading to reduced response rate and increased risk of late side effects. Therefore, for patient with better prognosis decreased dose per fractions is advised (Bergman et al., 2020).
Common side effects are usually confined to radiation field, and may include alopecia, skin hypo- or hyperpigmentation, dry radiodermatitis, and oral mucositis. Ideally, radiation field should include the regional lymph nodes, regardless of whether they have macroscopic alterations suggestive of regional metastasis (Williams & Packer, 2003; Bergman et al., 2020; Larue & Gordon, 2020).
The high rate of distant metastasis remains the significant limiting factor for curative-intent treatment of canine oral melanoma (Blackwood & Dobson, 1996; Theon et al., 1997).
The percentage of partial and complete response rate to RT ranged from 25% to 67% and 19% to 69%, respectively, yielding an overall response rate of 82% to 94% (Bateman et al., 1994; Blackwood & Dobson, 1996; Proulx et al., 2003; Kawabe et al., 2015; Tollett et al., 2016).
According to several studies, the reported PFS (progression free-interval) ranges from 3.6 to 8.6 months with a median survival time varying from 5.3 to 11.9 months (Bateman et al., 1994; Theon et al., 1997; Freeman et al., 2003; Proulx et al., 2003; Murphy et al., 2005; Cancedda et al., 2016).
As for surgical treatment, the size of irradiated melanoma seems to have a prognostic role. In one study on 105 oral tumors, 38 of which were melanoma dogs with T1 had better survivals compared with dogs with T2 and T3 lesions (Theon et al., 1997).

Another study found that dogs with oral melanomas treated with a 9 Gy per fraction, once weekly for 4 weeks, and with tumors less than 5 cm^3 had significantly longer MST (Blackwood & Dobson, 1996).

Chemotherapy as sole treatment has generally provided little benefit in terms of clinical outcome for the local control of canine melanomas; however, a few published studies have revealed that the association of radiation with chemotherapy may potentially contribute to local tumor control and/or improve overall survival. In one study, dogs with incompletely resected oral melanoma treated with either cisplatin (10–30 mg/m2 IV) or carboplatin (90 mg/m2 IV) given once weekly 1 h before receiving radiation therapy (6 x 6 Gy) had a median survival time of 11.9 months, representing the longest survival time when compared to the survival time previously reported for dogs with incompletely resected oral malignant melanoma (Freeman et al., 2003).

Cancedda et al. compared the efficacy of radiation therapy (5 x 6 Gy) alone and radiation therapy with post-radiation temozolomide (60 mg/m2 PO for five consecutive days every 28 days) in dogs with measurable malignant melanoma. Dogs treated with temozolomide had a significantly longer median time to progression (6.8 months) than dogs only irradiated (3.6 months) (Cancedda et al., 2016).

Other studies combining radiation with chemotherapy agents, such as carboplatin, cisplatin, or melphalan, did not have a significant impact on either time to progression or overall survival time (Rassnick et al., 2001; Proulx et al., 2003; Murphy et al., 2005).

Chemotherapy

Despite tumor control of oral melanoma with local therapies, such as surgery and/or radiation therapy, most of the patients succumb to distant metastatic disease. Therefore, the use of systemic therapies is typically recommended. Several chemotherapy protocols have been published in the veterinary literature with minimal improvement in survival times when compared to local treatment alone.

Many protocols, mainly based on platinum agents, have been studied. Boria et al. reported an overall response rate of 18% and a MST of 119 days in oral melanoma using cisplatin (50 to 55 mg/m^2/IV/ every three weeks) in combination with piroxicam (Boria et al., 2004)

In another study Rassnick et al. reported an overall response rate of 28% using carboplatin as a single agent (300 to 350mg/m^2 IV every 21 days) prior to surgery in dogs with MM (Rassnick et al., 2001).

Brockley et al. evaluated the effect of carboplatin on the survival of 63 canine patients diagnosed with oral, cutaneous, or digital malignant melanomas. The addition of carboplatin, administered at 300 mg/m^2 IV every 21 days for a total of 4–6 cycles, after local therapy, did not lead to a significant increase in survival times. Specifically, the overall median survival time for patients with oral melanoma was 389 days (Brockley et al., 2013).

In a comparative study reported by Tuohy et al., surgery with or without any adjuvant therapy (carboplatin, cyclophosphamide as a metronomic therapy, radiotherapy, or xenogeneic canine melanoma vaccine) did not improve overall survival time in dogs with oral melanoma. In this study, only 19 % and 1.5 % of cases were classified as stage III and IV, respectively. The MST reported was

874 and 396 days for dogs that had surgery or surgery plus adjuvant therapy, respectively (Tuohy et al., 2014).
In another survey, Dank et al. compared the outcome of 17 dogs treated with surgery, adjuvant carboplatin (150 to 300 mg/m^2/IV every three weeks), with or without radiation therapy. The MST was 387 days for the group treated with radiotherapy and 440 days for the group without the addition of radiotherapy (Dank et al., 2014).
Boston et al. evaluated the efficacy of systemic adjuvant therapies in 151 dogs with oral melanoma after surgery. Carboplatin, lomustine, dacarbazine, doxorubicin, metronomic chemotherapy, or OnceptTM (Pfizer) melanoma vaccine were the adjuvant therapies administered. No benefits in survival times among dogs that had surgery alone (MST of 335 days for 98 dogs) versus dogs that had surgery followed by systemic adjuvant therapy (MST of 352 for 53 dogs) was obtained (Boston et al., 2014).
The role of COX-2 inhibitors in canine melanoma (in vitro and in vivo) has been explored in several studies, suggesting its involvement in many promoting tumor pathways as angiogenesis, inflammation, melanoma cell proliferation and migration. Despite a wider knowledge of COX-2 in human medicine, in the context of canine melanoma their exact role as potential therapeutic target has not been fully elucidated (Gregório et al., 2016; Yoshitake et al., 2020).

Metronomic chemotherapy

Considering the disappointing results achieved with the use of maximum tolerated dose (MTD) chemotherapy and COX-2 expression in melanoma cells, clinical trials have been directed toward the use of metronomic chemotherapy (MC), usually in combination with immunotherapy, especially in humans (Borch et al., 2016; Kareva, 2017).
MC is defined as the oral administration of chemotherapy at low, minimally toxic doses, on a frequent or continuous schedule of treatment, with no extended drug-free breaks. MC exerts its action through several mechanisms with effect on neovascularization, on the immune system, on cancer cells and cancer stem cells (CSCs) and inducing tumor dormancy (Gaspar et al., 2018).
Angiogenesis and vasculogenesis are fundamental steps for tumor growth. Angiogenesis consists of the generation of new blood vessels from mature and rapidly dividing endothelial cells present in pre-existing capillaries, while vasculogenesis is the formation of new blood vessels from bone marrow-derived circulating endothelial progenitor cells (CEPs), involved in ‘’tumor repopulation‘’ during the drug free period of MTD chemotherapy. Endothelial cells, under the effect of MC are susceptible to apoptosis and selective inhibition of proliferation and migration; in parallel MC targets CEPs, blocking their mobilization and decreasing their viability. Additionally, MC exerts a down regulation effect on pro-angiogenic factors (e.g. vascular endothelial growth factor [VEGF], basic fibroblast growth factor [bFGF], hypoxia-inducible factor-1α [HIF-1α] and angiopoietin family) and upregulation effect on anti-angiogenic factors (e.g. endostatin and thrombospondin-1 [TSP-1]) (Gaspar et al., 2018). MC is also capable to reverse the immune tolerance state by depleting the number and impairing the function of T-regs, those suppress anticancer immune responses and promote the establishment of neoplastic tolerance and malignity. Furthermore, MC may be

potentially skilled to disrupt, via its anti-angiogenic, CSCs niche which is considered the major source of tumor resistance to conventional chemotherapy and radiation therapy.
Finally, MC induces a state of tumor dormancy, possibly inducing a long-term asymptomatic control of the disease (Gaspar et al., 2018). In veterinary medicine most of protocol used in metronomic regimens see the combination of low dose cyclophosphamide or chlorambucil, non-steroidal anti-inflammatory drug (usually piroxicam), and thalidomide (Biller, 2014; Cancedda et al., 2016; Finotello et al., 2017; Polton et al., 2018).
The association of MC with immunotherapeutic strategies has shown some promising results in the treatment of melanoma, suggesting a possible synergic action able to enhance an antitumor response (Borch et al., 2016; Kareva, 2017; Petrizzo et al., 2018). The use of metronomic chemotherapy in melanoma canine patients is not precisely defined, however in two studies patients with OMM treated with anti-CSPG4 vaccine received MC when distant metastasis occurred, leading in some cases to prolonged disease control (Piras et al., 2014, Giacobino et al., 2021). Nevertheless, the multimodality treatment adopted in case of malignant melanoma may not help to clearly define the precise impact of MC in terms of outcome in melanoma patients.

Electrochemotherapy

Electroporation is a method that uses electric field pulses to induce an electrically mediated reorganization of the plasma membrane of the cell, increasing its permeability. Electrochemotherapy (ECT) combines local or systemic administration of chemotherapeutic drugs such as bleomycin or cisplatin that have poor membrane permeability, followed by the delivery of permeabilizing electrical pulses within the tumor. The principal mechanism of electrochemotherapy is electropermeabilization of the cells in the tumors, which enables the drug to reach intracellular targets. In veterinary medicine, electrochemotherapy has been widely used because of its high efficacy in all solid tumors including melanoma (Spugnini & Baldi, 2019).
One of this first application of ECT in canine oral melanoma was described by Spugnini et al. that treated a cohort of 10 dogs with OMM, proposing ECT as single therapy or after surgery following recurrence. One week after completion of the treatment (four ECT sessions one week apart), a CR was obtained in seven dogs, and the rest had either SD or a PR. The MST of the dogs was 6 months (mean survival of 16 months). All dogs with either SD or PR eventually developed progressive disease, but four dogs with an initial CR remained in remission for 16–36 months. No major local or systemic side effects were observed, except local vitiligo-like discoloration in three dogs, which could potentially indicate recruitment of the immune system by the therapy (Spugnini et al., 2006).
Gene electrotransfer (GET) is a method in which plasmid DNA, encoding a therapeutic gene, is transported into cells by reversible electroporation. In this way, increased production of the desired protein and its release into the extracellular matrix or bloodstream are achieved. One of the most widely investigated GET methods in oncology is GET of a plasmid encoding interleukin-12 (IL-12); GET can be combined with ECT due to their synergistic action on neoplastic cells; intratumoral ECT directly destroys neoplastic cells, while GET into surrounding tissues transfects healthy cells potentially enhancing the antitumor immune response. IL-12 has different antitumor effects with direct activation of acquired and innate immunity. It promotes the activation of T cells, enhances T

cell survival and the effector functions of T cells and natural killer cells, and promotes interferon gamma (IFN-γ) secretion (Nemec et al., 2020).

Milevoj et al. evaluated the safety and efficacy of the combination of ECT with bleomycin and GET of plasmid encoding canine interleukin 12 (IL-12) in 9 dogs with OMM. Patients were treated with a combination of cytoreductive surgery and several cycles ECT and IL-12 GET. One month after treatment, the objective response (OR) rate was 67% with a median survival time of 6 months, despite this 40 % of patients died for unrelated causes. Interestingly a reduction of regulatory T cells (T-reg) percentage was detected over the treatment protocol, possibly attributable to systemic antitumor response to IL-12 GET (Milevoj et al., 2019).

In a recent study 67 dogs with primary OMM, non-candidates for first-line therapy, were treated with ECT. Based on RECIST criteria, the overall response rate was 100%, 89.5%, 57.7%, and 36.4%, in stage I, II, III and IV, respectively. Only patients with OMMs stage I, II and III with partial or complete response achieved benefit in terms of quality of life. The median time to progression was 11, 7, 4 and 4 months, and median survival time after the treatment was 16.5, 9.0, 7.5 and 4.5 months, for patients in stage I, II, III and IV, respectively. Noteworthy local response was significantly improved in dogs with stage I and II and without bone involvement (Tellado et al., 2020).

Electrochemotherapy may be an effective local treatment for canine OMM when no alternative treatment is available. Better response is expected in stage I and II patients with tumors without bone involvement. Results obtained so far are encouraging, ECT may represents an effective and safe treatment with tolerable toxicity profile, especially for selected patients where first line options as surgery and/or radiotherapy cannot be elected. Nevertheless, dogs with considerable macroscopic disease should be selected carefully in order to avoid undesirable complications and toxicosis related to the massive necrosis of tumor tissue (Spugnini & Baldi, 2019).

IMMUNOTHERAPY

Classical clinical management of OMM is based on local control achieved by surgery and/or radiotherapy, and treatment of microscopic metastatic disease. Bearing in mind the high metastatic potential of OMM and the minimal reported benefit obtained with chemotherapy, the research has been oriented towards the development of new therapeutic strategies and the immunotherapy is one of the most promising options.

"It is when oncology meets immunology that cancer immunotherapy begins" (Rolih et al., 2017).

It is widely recognized that the immune system plays a critical role in the development and progression of cancer. There are several mechanisms behind cancer-immune system interactions, but a recent greater understanding of them has revealed that innate and adaptive immune effectors are involved in tumor recognition and control (Swann & Smyth, 2007).
In people immunotherapy has dramatically changed the treatment landscape in patients with advanced melanoma (Albertini, 2018).
Additionally, melanoma is a highly immunogenic tumor that lend itself to immunotherapy (Modiano et al., 1999). In light of this, a wide array of pathways of the immune system have been identified as attractive targets in promoting anti-tumor responses in cancer melanoma patients.

Tumor immunology

The immune system is generally divided into 2 primary components: the innate immune response and adaptive or acquired immune response.

- **Innate immunity** is rapidly acting but usually not very specific and includes physicochemical barriers (e.g., skin and mucosa), blood proteins such as complement, phagocytic cells (macrophages, neutrophils, dendritic cells [DCs], and natural killer [NK] cells), and cytokines that coordinate and regulate the cells involved in innate immunity.
- **Adaptive immunity** represents the acquired arm of immune system that allows for exquisite specificity. It is able to remember the previous existence of the pathogen (i.e. ''memory''), responding actively on repeated exposure to the pathogen and differentiating self from non-self. Adaptive immunity consists of T and B lymphocytes. The T cells are further divided into CD8 (cluster of differentiation) and major histocompatibility complex (MHC) class I cytotoxic helper T cells (CD4 and MHC class II), NK cells, and regulatory T cells (Tregs). B lymphocytes produce antibodies (humoral system) that may activate complement, enhance phagocytosis of opsonized target cells, and induce antibody-dependent cellular cytotoxicity.

The innate and adaptive arms of immunity are not mutually exclusive; they are linked by the innate response's ability to stimulate and influence the nature of the adaptive response and the sharing of effector mechanisms between innate and adaptive immune responses (Bergman, 2019).
Immune responses can be further separated by whether they are induced by exposure to a foreign antigen (an "active" response) or if they are transferred through serum or lymphocytes from an

immunized individual (a “passive” response). Although both approaches have the ability to be extremely specific for an antigen of interest, one important difference is the inability of passive approaches to generally confer memory. The principal components of the active/adaptive immune system are lymphocytes, antigen-presenting cells, and effector cells. Furthermore, responses can be subdivided by whether they are specific for a certain antigen or a nonspecific response whereby immunity is attempted to be conferred by upregulating the immune system without a specific target (Bergman, 2019).

The principal components of the active/adaptive immune system are lymphocytes, antigen-presenting cells, and effector cells. Specifically, B lymphocyte (CD19+) are covered by several antigen receptors that can bind and respond to a single antigen. As a result of this antigen binding, through appropriate stimulation, B cells will divide repeatedly and differentiate into two populations. One population, antibody-producing plasma cells, are the major sources of antibodies. The other B cell population becomes memory B cells that can persist within an animal for months or years. When an animal encounters an antigen for a second time, these memory B cells respond rapidly, producing large numbers of plasma cells, and additional memory cells.

The T-cell receptor (Trc) exposed on T cell, or T lymphocytes surface are responsible for recognizing fragments of antigen as peptides bound to MHC molecules. These are divided into three classes: MHC1 span the membrane of almost every cell in an organism, while MHC2 are restricted to cells of the immune system as APC (antigen-presenting cell) as B lymphocytes, macrophages, dendritic cells and Langherans cells. A third MHC class binds the complex system (Poli et al., 1998).

The T helper lymphocytes (CD4+, CD8-) are crucial in achieving and regulating effective immune response. These cells recognize antigen-complex MHC2 processed by APC, while T cytotoxic lymphocytes (CD8+ CD4-) recognize antigen-complex MHC1 exposed on different cells.

Once activated CD4 T cells carry out several functions as stimulation of B cells antibodies production, activation of T cytotoxic lymphocytes, macrophages and production of interferon-γ, that in turn active NK cells (natural killer). CD8 T cells kill directly cancer cells, infected or damaged cells or triggering cytokine release APCs. Regulatory T cells (CD4+ CD25+) control immune response blocking the activities of B lymphocytes, macrophages and T cytotoxic lymphocytes. Natural killer cells (CD4-CD8-) or also known as large granular lymphocytes (LGL) are critical for innate immune system response. Typically, immune cells detect the MHC presented on infected cell surfaces and by several mechanisms cause cellular apoptosis. NKs miss the self-markers of MHCI and display the unique ability to recognize and destroy stressed cells in the absence of antibodies and MHC stimulation, allowing a quicker immune reaction (Poli et al., 1998 A-B).

Cancer immunoediting from immune surveillance to immune escape

In the interaction of host and tumor cells, three essential phases have been proposed as part of immunoediting process: elimination, equilibrium and escape, which are designated the 'three E's'. These phases can lead to the complete elimination of the tumor, to an equilibrium in which the immune system maintains residual tumor cells that survived the first phase in a functional state of dormancy allowing them to reside in patients for decades before eventually giving rise to recurrent primary tumors or distant metastases, or finally, to the uncontrolled growth of tumor cells (Dunn et al., 2002).

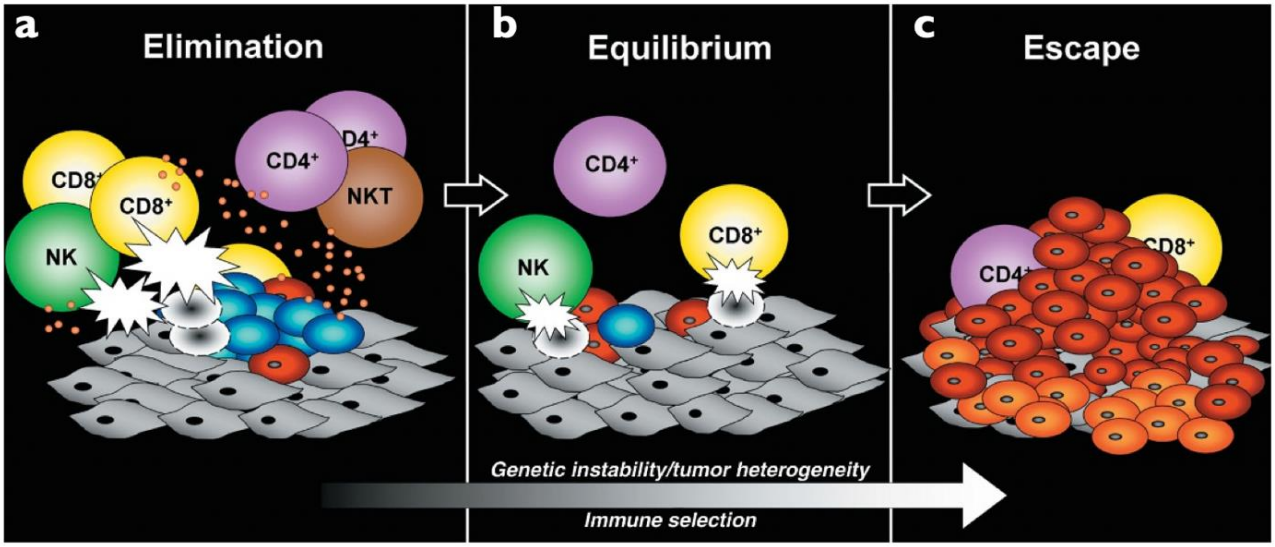


Figure 5 *The three Es of cancer immunoediting. Cancer immunoediting encompasses three process. (a) Elimination corresponds to immunosurveillance. (b) Equilibrium represents the process by which the immune system iteratively selects and/or promotes the generation of tumor cell variants with increasing capacities to survive immune attack. (c) Escape is the process wherein the immunologically sculpted tumor expands in an uncontrolled manner in the immunocompetent host. In a and b, developing tumor cells (blue), tumor cell variants (red) and underlying stroma and non-transformed cells (gray) are shown; in c, additional tumor variants (orange) that have formed as a result of the equilibrium process are shown. Different lymphocyte populations are as marked. The small orange circles represent cytokines and the white flashes represent cytotoxic activity of lymphocytes against tumor cells* (Dunn et al., 2002).

The concept of the immunosurveillance implies that immune system can recognize and destroy most precursors of cancer inhibiting carcinogenesis and maintaining cellular homeostasis. Nevertheless, immunosurveillance is only one aspect of the complex relationship between the immune system and cancer. In fact, the notion that the immune system protects the host against tumor formation on one hand and shapes the tumor during its development on the other, gives rise to the cancer immunoediting hypothesis. The cancer immunoediting theory is based on the immune system's ability to select tumor variants that are better suited to survive in an immunologically intact environment with the final generation of tumors that are better able to withstand the tumor-suppressing action of the immune system (Dunn et al., 2002; Schreiber et al., 2011).

Tumors reduce antineoplastic immune responses (immunoevasion) through several pathways. Firstly, cytokines as interleukin-10 (IL-10) and transforming growth factor-β (TGF-) β produced by tumor cells and regulatory T-cells (Tregs) suppress anti-tumor immunity, secondly decreasing

antigen presentation and modified antigen presenting cell (APC) function, also impeding the development of protective immunity (Atherton et al., 2016). Other mechanisms include impaired DC function via inactivation ("anergy") and/or poor dendritic cells (DC) maturation through changes in IL-6/IL-10/vascular endothelial growth factor/granulocyte/macrophage colony-stimulating factor (GM-CSF)10; further mechanisms are MHC-I loss through structural defects, changes in B2-microglobulin synthesis, defects in transporter-associated antigen processing, or actual MHC-I gene loss, and MHC-I antigen presentation loss through B7-1 attenuation. B7-1 is an important costimulatory molecule for CD28-mediated T-cell receptor and MHC engagement act (Bergman, 2019).

The goal of tumor immunology has been to better understand the immune system components in order to use immunotherapeutic approaches to potentiate patients' own immune response against cancer and overcome or eliminate the existing tumor tolerance and mechanisms of immune evasion.

Immunotherapeutic strategies in canine malignant melanoma

Immunotherapy encompasses a wide range of different treatment modalities and it has been exploring extensively in several cancer types including melanoma. The most relevant therapeutic strategies reported for malignant melanoma include: monoclonal antibodies, nonspecific immunotherapy activated by bacteria, oncolytic virotherapy, lymphokine-activated killer cells, gene therapy, and vaccines.

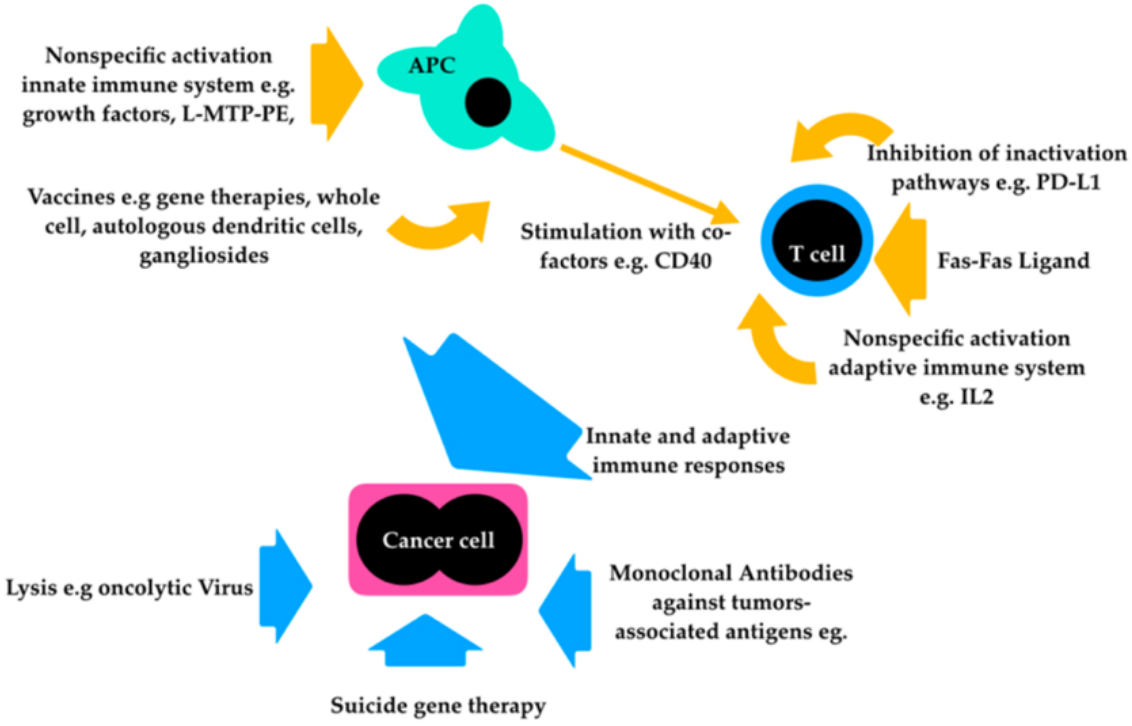


Figure 6 *Summary of the different strategies for immunotherapy in canine oral malignant melanoma (Almela & Ansón, 2019).*

Monoclonal antibodies

Checkpoint inhibitors

Tumors have developed numerous mechanisms for suppressing the antitumor immune response, including production of inhibitory cytokines, recruitment of immunosuppressive immune cells, and upregulation of coinhibitory receptors, known as immune checkpoints. The primary effector cells of the adaptive immune response against cancer are the T lymphocytes. Importantly, T-cell activation is strictly regulated by costimulatory or coinhibitory signals known as immune checkpoints that provide a regulatory feedback mechanism to limit the effector phase of T-cell expansion and function. Immune checkpoints play key part in the tolerance for self-antigens and expansion of T cell response. These inhibitory pathways are upregulated in many cancers, and immune checkpoints play a key role in cancer-associated immune suppression and immune evasion. Increased understanding of these mechanisms has led to the development of immunotherapies targeting cancer-associated immunosuppression explored in several canine spontaneous tumors (La-Beck et al., 2015; Borgatti et al., 2020; Mason et al., 2021).

Immune checkpoints are targeted with the use of monoclonal antibodies (mAbs). Checkpoints are proteins that programmed cell death 1 (PD-1), its ligand, programmed cell death ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Both PD-1 and CTLA-4 are proteins expressed on the surface of cytotoxic T-lymphocytes. Inhibitors of immune checkpoint have been developed as a mean to ‘’take the breaks off’’ of an otherwise impeded anti-cancer immune response. At present, three engineered humanized immune-checkpoint inhibitors targeting either PD-1 (Pembrolizumab, Nivolumab) or CTLA-4 (Ipilimumab) have been approved by the Food and Drug Administration (FDA) for human patients with advanced melanoma (Rotte, 2019).

Despite the impressive benefits of the immune-checkpoint blockade, its use can be hampered by the occurrence of serious adverse events, mostly aberrant immune activation leading to undesirable off-target inflammation and autoimmunity which can affect multiple organs (Abdel-Wahab et al., 2016).

In a recent pilot clinical study, seven dogs with OMM were treated with a rat–dog chimeric anti-PD-L1 mAb. The objective response rate was 14.3% and an 81% reduction of the tumor burden was observed. In four dogs with confirmed pulmonary metastasis, the estimated MST was superior (93.5 days) compared with a historical group (54 days) (Maekawa et al., 2017).

Malignant melanoma expresses high levels of disialogangliosides GD2 and GD3, making these antigens ideal targets for mAbs. In a study, the expression of disialogangliosides GD2 and GD3 on canine OMM and their in vitro ability to mediate antibody-dependent cellular cytotoxicity (ADCC) using murine anti-GD2 and -GD3 mAbs was assessed, revealing that mAbs reacted with these antigens and could target and trigger tumor killing by multiple canine effector populations (Helfand et al., 1994).

Nonspecific Immunotherapy Activated by Bacteria

Bacteria released signature molecules called pathogen-associated molecular patterns (PAMPs) that activate innate immune responses (nonspecific) through the activation of so-called pattern recognition receptors (PRRs) that include Toll-like receptors (TLRs) and a cytoplasmic receptor known as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Both TLRs and NLRs activate downstream pro-inflammatory signaling through nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK) pathways (Hill & Imai, 2016).
A heat-killed suspension of Corynebacterium parvum was administered intratumorally in dogs with OMM after surgery. A potential antitumor activity was implied based on longer survival time observed in treated dogs with stage II and III disease (MacEwen et al., 1986).
In a randomized, double-blinded clinical trial, a liposome-encapsulated lipophilic derivative of muramyl dipeptide (L-MTP-PE) was used to treat 98 dogs with spontaneous OMM. It was administered alone or in combination with recombinant canine granulocyte macrophage colony-stimulating factor (GM-CSF) adjunctly with surgery. L-MTP-PE resulted to prolong the survival of dogs with early (stage I) OMM (MacEwen et al., 1999).

Oncolytic Virotherapy

Despite being in its very early stage, oncolytic virotherapy is an emerging approach to treat cancer. Oncolytic viruses selectively infect, replicate and kill cancer cells, while leaving healthy cells intact, thus aside from displaying selective cytotoxicity toward cancer cells, may favor the restoration of the immune anti-cancer function. Different viruses have been tested for canine cancer therapy in murine models and canine tumor cell cultures, for example, adenovirus, poxvirus, reovirus, vesicular stomatitis virus, and paramyxovirus (Sánchez et al., 2018).
Most viruses are genetically modified to selectively lyse tumor cells without affecting normal cells, while some viruses have a natural phenotype which allows them to replicate only in cancer cells (Igase et al., 2015).
The main drawbacks of this strategy include the selective targeting of the oncolytic viruses to tumor tissue, relatively poor virus dissemination throughout solid tumor tissue, inefficient viral replication in immune-competent hosts, and a disadvantageous ratio between anti-viral and anti-tumoral immunity (Sánchez et al., 2018).
Two studies have assessed the vitro e oncolytic potential of selected viruses against canine cancer cells in cell cultures. Cytotoxicity of genetically manipulated Lister strain of vaccinia virus (LIVP6.1.1) against four different canine cancer cells in cell culture was 83 %; in the other study reovirus induced more than 50% cell death in three canine mammary gland tumors and one canine malignant melanoma cell line (Gentschev et al., 2013).
In another study, three models (dog, mice and cells) of malignant melanoma were treated with a modified version of the oncolytic vesicular stomatitis virus VSV-GP that efficiently lysed more than 50% of cells, prolonged the survival of mice reducing the number of lung metastasis (Kimpel et al., 2018).

Lymphokine-activated Killer (LAK) Cell Therapy

T lymphocytes have a driving role in anti-tumor immune responses. Passive immunotherapy, indicated as lymphokine-activated killer (LAK) cell therapy, implicates the administration of autologous activated lymphocytes without cancer specificity. These lymphocytes have been previously stimulated and expanded with certain cytokines. This form of immunotherapy is expected to trigger the cytotoxic activity of the administered lymphocytes against target tissues, and indirectly induce cell-mediated immunity by activation of T lymphocytes and natural killer cells (Hoshino et al., 2008).

LAK therapy has been evaluated in 15 tumor-bearing dogs, seven of them with malignant melanoma, combined with palliative surgery. Therapy was administered intravenously at two to four-week intervals and resulted in an increase of CD8+ T-cells and a decreased ratio of CD4+ to CD8+ T-cells. Although promising results, the sample size was small, only three phenotypes were evaluated (CD3+, CD4+, and CD8+), and no clinical follow-up was performed (Mie et al., 2016).

Gene therapy

Gene therapy relies on the delivery of foreign DNA into cells also known as transfection. Non-viral vectors utilizing liposome delivery, DNA protein complexes, and viral vectors are used to transfer the DNA. Several gene products that can be delivered, such as cytokines, suicide genes, tumor or bacterial antigens, and proapoptotic genes. Cytokines are able to promote adaptive as well as innate immune responses. Intratumoral delivery of cytokines may induce antitumor responses without the adverse effects often associated with systemic delivery (Rosenberg et al., 1994; Glikin & Finocchiaro, 2014).

The efficacy of several cytokines has been explored in canine OMM, with particular attention to IL-2 and IL-12 (Rosenberg et al., 1994; Quintin-Colonna et al., 1996).

Superantigens are bacterial proteins capable of activating a large number of T-cells. Their local expression via gene delivery may be able to effectively activate tumor-infiltrating immune effector cells, while avoiding the adverse effects associated with systemic exposure. This approach has been used in canine OMM by means of lipid-complexed plasmid DNA encoding staphylococcal enterotoxin B combined with either growth factor GM-CSF or IL-2 (Dow et al., 1998; Thamm et al., 2003).

Melanoma cells exhibit some pathways that lead to decreased apoptosis. The induction of apoptosis has been shown to enhance immune responses against tumor antigens. Intratumoral administration of the human Fas-ligand gene was evaluated in a phase I clinical trial in four dogs with stage III disease with OMM. In three dogs, a 12.5–58% reduction of tumor burden was reported, and no adverse effects were observed in all dogs (Bianco et al., 2003).

Suicide gene therapy relies on the capacity of the gene product to convert a non-toxic prodrug into a toxic compound. This approach has been evaluated by Finocchiaro and colleagues for CMM using intratumoral injections of lipid-complexed plasmid DNA encoding the herpes simplex thymidine kinase suicide gene that sensitizes transfected cells to ganciclovir. The suicide gene therapy was combined with surgery by means of irradiated xenogeneic cells genetically modified to secrete

human IL-2 and GM-CSF, and with an autologous or allogeneic tumor cell vaccine plus interferon-β (Finocchiaro et al., 2008; Finocchiaro & Glikin, 2008; Finocchiaro et al., 2015).

The patients treated with the combination had significantly longer overall survival than the controls (untreated or surgery alone or suicide gene therapy alone) with minimal toxicity. Based on the results obtained from these studies it could be deduced that this approach was safe and effective with >50% of those dogs dying from non-melanoma-related causes. Despite this, it is difficult to draw conclusions since suicide gene therapy was proposed in an adjuvant setting combined with more than one intervention without control groups (Finocchiaro & Glikin, 2012).

Active immunotherapy in the form of vaccines represents one potential therapeutic strategy for melanoma. DNA is relatively inexpensive and simple to purify in large quantity. The antigen of interest is cloned into a bacterial expression plasmid with a constitutively active promoter. The plasmid is introduced into the skin or muscle with an intradermal or i.m. injection. Once in the skin or muscle, professional antigen-presenting cells, particularly dendritic cells, are able to present the transcribed and translated antigen in the proper context of MHC and costimulatory molecules. The bacterial and plasmid DNA itself contains immunostimulatory sequences that may act as a potent immunological adjuvant in the immune response. In February 2010, OnceptTM (Pfizer) was the first cancer vaccine to receive full approval from the US Department of Agriculture. OnceptTM is a bacterial plasmid DNA vaccine encoding the human tyrosinase gene, a xenogeneic antigen. Tyrosinase is a melanosomal glycoprotein, essential in melanin synthesis. The rationale behind OnceptTM vaccine is that the xenogeneic tyrosinase could break the tolerance against a self-tumor differentiation antigen, inducing antibody, T-cell, and antitumor responses. Sequence identity between partially sequenced canine tyrosinase and human tyrosinase is 91% (Bergman, 2003).

The vaccine is administered with a needle-free device, intramuscularly and is licensed for the adjuvant treatment of stage II and III OMM after loco-regional control (Zuleger et al., 2017).

After approval, several studies have reported efficacy with contradictory results (Grosenbaugh et al., 2011; Ottnod et al., 2013; Boston et al., 2014; Treggiari et al., 2016; Verganti et al., 2017).

Some papers reported the vaccine to significantly prolong survival in CMM (Grosenbaugh et al., 2011), whereas others did not (Ottnod et al., 2013; Boston et al., 2014; McLean & Lobetti, 2015 Treggiari et al., 2016; Verganti et al., 2017).

Inclusion criteria, tumor localization and staging, and treatment protocols were heterogeneous across the studies. MSTs from these studies ranged from 335 to 477 days (Ottnod et al., 2013; Treggiari et al., 2016). The quality of evidence in these studies is low due to either low number of dogs included, absence of control group, the retrospective nature and level of censoring (more than 50% of vaccinated dogs were censored from the analysis (Almela & Ansón, 2019).

In a recent multinstitutional retrospective study, a large series of dogs were treated with multimodality treatment including surgery and /or radiotherapy and OnceptTM. One hundred thirty-one dogs with oral melanoma were enrolled in the study; median time to progression, median progression-free survival, and median tumor-specific overall survival were 304, 260, and 510 days, respectively. Authors observed a safe and low toxicity profile with relatively favorable survival outcomes in dogs with OMM treated with a multimodality approach that included OnceptTM (Turek et al., 2020).

Cluster of differentiation 40 (CD40) is a co-stimulatory molecule expressed on the surface of B-cells and APCs. The binding of CD40 to its ligands (CD40L) expressed on T-cells triggers humoral as well as cell-mediated immunity. CD40 also induces apoptosis in cancer cells. Treatment of canine OMM with a replicate-deficient adenovirus as a vector, expressing the immunostimulatory gene CD40L (AdCD40L), administered intra- and peritumorally after either incisional or excisional surgery has been reported in some studies. These studies demonstrated that local adenovector immunogene therapy using CD40L was safe and could have beneficial effects on dogs (von Euler et al., 2008; Westberg et al., 2013).

Vaccine

Vaccines against cancer are built on the concept that cancer cells express a genetic panel, that codify for proteins, significantly different from their natural counterparts. Vaccination aims to educate the immune system to recognize these components, trigger an efficient and long-last immunity able to eliminate cancer cells. Tumor associated antigen (TAA) are proteins that derive their immunogenicity from their distinct expression by tumor cells. Although TAA are associated with a malignant cell phenotype, they can also be expressed by normal cells. However, the expression of TAA by the malignant cell has unique features that contribute to their immunogenicity (Cavallo et al., 2007).
Multiple vaccination strategies have been devised to induce an antitumor immune response, including allogeneic whole cell tumor vaccines, DC vaccination and DNA vaccination.

- **Whole-cell tumor vaccine**: tumor cells are collected directly from cancer tissue (autologous vaccine) or similar cell tumoral lines (allogeneic vaccine), inactivated or irradiated and then administered to patient. In a phase II clinical trial using allogeneic whole-cell vaccination, a canine melanoma cell line was transfected with xenogeneic human gp100 (a melanocyte specific trans-membrane protein i.e. a melanoma TAA), killed by irradiation and administered intradermally to 34 dogs with spontaneous malignant melanoma (stages II to IV) in an attempt to break tolerance with a combination of self and xenogeneic antigens. The vaccine was well tolerated in dogs. Objective evidence of tumor regression (one complete response and five partial responses) was observed in six of the 34 dogs (17.6% objective response rate) (Alexander et al., 2006).
- **DC vaccine** involves harvesting DCs and then loading these professional APCs with relevant TAAs as peptides, proteins, tumor lysates, and viral vectors (Cavallo et al., 2014). Using this strategy, autologous DCs derived from bone marrow were ex vivo expanded and transfected with an adenovirus expressing the human melanoma antigen gp100. The vaccine was administered subcutaneously in three dogs: one healthy, and the other two with stage I and III oral melanoma after surgical excision and radiotherapy. No adverse effects were observed and two of the vaccinated dogs did not experience recurrent disease at the site of the initial lesion, nor at distant sites 48 and 22 months after the DC vaccination, respectively (Gyorffy et al., 2005). In another study T-cell immunity with recruitment of CD8 and CD4 T-cells was detected in the positively responding sites of three healthy dogs injected with autologous

DC cells that were pulsed with lysates from a canine malignant melanoma cell line (CMM-2) (Tamura et al., 2008).

- **DNA vaccine** implies the use of a naked plasmid DNA vaccine to elicit the immune response against disease providing a variety of practical benefits for large-scale vaccine production that are not as easily manageable in other vaccine forms, including whole tumor cells, recombinant protein or viral vectors. Indeed, DNA vaccines are simple vehicles for in vivo transfection and antigen production. The simplicity of their manipulation, their high flexibility, their manufacturing and storage stability make DNA plasmids a very attractive cancer immunotherapy option (Fioretti, 2010; Cavallo et al., 2014).

DNA Vaccine in treatment of OMM

DNA vaccines are not replicating and the vaccine products are expressed within the host cells. They can be constructed to mimic the specificity and safety of subunit vaccines. Due to the production and processing of immunogenic proteins within host cells, DNA vaccines are likely to induce immune responses in a manner similar to live-attenuated vaccine types, while causing no pathogenic infection in vivo. By directly administering DNA vaccines into the host, the host cells express the antigenic protein. This process has been known to induce both Ag-specific antibody and cellular responses (Lee et al., 2015).

A DNA vaccine is composed of plasmid DNA, which includes the specific sequence of the antigen of interest under the control of a mammalian promoter (commonly the cytomegalovirus immediate early promoter and its adjacent intron A sequence), a transcription termination signal and a prokaryotic antibiotic resistance and can be easily produced in bacteria (Glenting & Wessels, 2005).

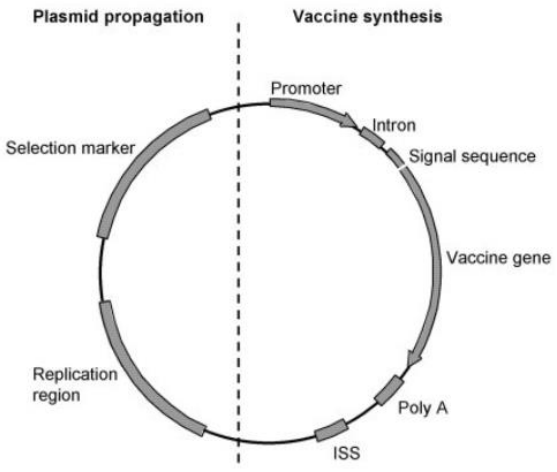


Figure 7 *Genetic elements of a plasmid DNA vaccine. Plasmid DNA vaccines consist of a unit for propagation in the microbial host and a unit that drives vaccine synthesis in the eukaryotic cells. For plasmid DNA production a replication region and a selection marker are employed. The eukaryotic expression unit includes an enhancer/promoter region, intron, signal sequence, vaccine gene and a transcriptional terminator (poly A). Immune stimulatory sequences (ISS) add adjuvanticity and may be localized in both units (Glenting & Wessels, 2005).*

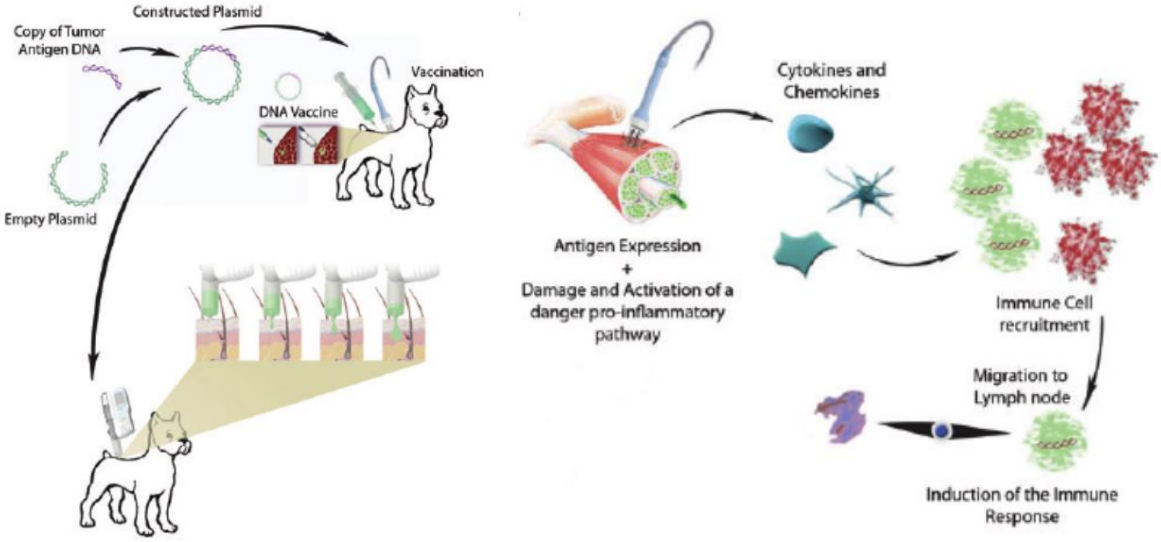
Mechanism of action

For delivery of DNA vaccines, the majority of DNA vaccine studies have utilized muscle or skin as an immunization target. In intramuscular (IM) injection, DNA vaccines are taken up and expressed by muscle cells and local antigen presenting cells (APCs). The local APCs then migrate to the draining lymph nodes for the induction of adaptive immune responses. In case of intramuscular injection, muscle cells can upregulate the expression of MHC class I and co-stimulatory molecules. However, myocytes lack MHC class II expression and thus would not be expected to prime CD4+T lymphocytes, which have a driving role in the immune response and are crucial for the induction and maintenance of immune memory (Rice et al., 2008).

The APCs, not muscle cells are believed to present antigen to CD4+ and CD8+ T cells by cross-presentation of secreted antigen or by direct transfection of DNA vaccines. Indeed, antigens must be transferred to a professional APC, usually a DC that will likely migrate to the site of DNA inoculation in response to inflammatory or chemotactic signals following vaccination. This indirect process of antigenic material transfer, possibly as apopotic vesicles, is termed cross-presentation. In this case, APCs can provide co-stimulatory signals and cytokines necessary for stimulation of naive T-cells. In addition, antigenic proteins expressed and/or secreted by APCs or non-hematogenous target tissues (e.g., muscle cells in the case of IM injection and keratinocytes in the case of intradermal injection) are presumably recognized by B cells for subsequent antibody production in association with helper functions of Ag-specific CD4+ T cells. In intradermal (ID) delivery, DNA can be taken up by Langerhans cells and/or dermal dendritic cells, which migrate to the draining lymph nodes for induction of adaptive immune responses. In particular, DNA vaccines, which are generated from bacteria, contain un-methylated CpG motifs and stimulate the innate immune responses by interacting with Toll-like receptor 9 expressed on the surface of APCs. This non-specific activation of APCs likely influences Ag-specific immune responses to DNA vaccines (Lee et al., 2015).

Electroporation as DNA vaccine delivery method

The appropriate delivery systems and/or vaccination routes should be considered carefully since it is correlated to DNA vaccine immunity. Most DNA vaccines are delivered subcutaneously or intradermally with or without the use of devices that increase their distribution. However, it is increasingly apparent that the immunogenicity of DNA vaccines greatly depends upon the delivery methods used for immunization and numerous clinical trials have confirmed that standard needle and syringe delivery is not sufficient (Impellizeri et al., 2014).



a *b*

***Figure 8**. High-pressure devices and EGT and possible adjuvant mechanisms. A plasmid DNA vaccine encoding a tumor antigen is generated by molecular biology techniques and delivered to canine patients. (a) Needleless device. DNA vaccine is forced through a tiny orifice that is held against the skin. This creates a very fine,high-pressure stream of medication that penetrates the skin, depositing the DNA in the tissue beneath where it is uptaken by fibroblasts and resident dendritic cells/APCs. Right: EGT.A nucleic acid (e.g. a plasmid DNA) encoding a tumor antigen is injected intramuscularly or intradermally. Then brief electrical pulses are delivered through a suitable device, electroporate cell membrane and induce enhanced local antigen expression. (B) Adjuvant mechanism of action. The EGT causes a minimal tissue damage and concomitant inflammation. The induced cytokines and chemokines mediate local immune cell recruitment (larger cells: APCs;smaller cells: T lymphocytes) and trafficking to the draining lymph nodes that ultimately leads to T cell expansion and tumor cell killing (shrinking cell) (Impellizeri et al., 2014).*

In particular, there are two main limitations to effective immunization following normal plasmid DNA injection: 1) plasmid DNA entry in the cells and subsequent nuclear localization is very limited; 2) resident APC lack the expression of co-stimulatory molecules needed as part of the T-cell activation process in the absence of an underlying inflammatory condition. Even if the reasons why DNA vaccines fail to induce potent and effective immune responses in human clinical trials have not yet been fully understood, the combination of inefficient DNA plasmid delivery methods, the consequent low levels of antigen expression and, most importantly, the lack of stimulation of the innate immune system are all together responsible for the low potency of naked DNA vaccines (Fioretti et al., 2010).

For these reasons, numerous strategies have been explored for optimizing the immunological response after a DNA vaccination. One approach to enhance plasmid DNA uptake has been the use of intradermal delivery via high-pressure devices, currently used to deliver OnceptTM (Bergman et al., 2006).

Development of needle-free injection systems, such as Bioinjector 2000®and Vitajet® use high pressure to force the dissolved DNA vaccine into the dermis or muscle, leading to a better immune response than the traditional IM or ID route of administration. Needle-free jet injectors use metal springs to power the injection. The device's nozzle is held against the patient's skin at the injection site, and once activated, the device injects a fine stream of the vaccine at a high pressure, penetrating into the skin and transducing local APCs (Goubier et al., 2008; Impellizeri et al., 2014).

One of the most promising strategies is electroporation (EP). The principle behind EP is the use of brief electrical pulses that create transient 'pores' in the cell membranes, large molecules such as DNA or RNA easily enter cell cytoplasm. Immediately following cessation of the electrical field, the pores close without causing cell death. DNA EP has been shown to be a safe methodology and one which results in dramatically enhanced DNA vaccine cellular uptake, enhanced protein expression and a concomitant increase in longer term immune responses against the target antigen in a variety of species. Indeed, EP itself works as an adjuvant to enhance the necessary "danger signals" which better activate the immune system: the insertion of needles and the electrical current produces some tissue damage (generation of cellular debris and apoptotic bodies), which causes secretion of inflammatory chemokines and cytokines, recruitment of APCs, macrophages and lymphocytes to the injection site (Impellizeri et al., 2014).
Several devices have been introduced for EP-based DNA vaccination. One of this is the CliniporatorTM (IGEA Srl, Carpi, Italy) used in the clinical practice for electrochemotherapy and electro-gene transfer.

Figure 9 *CliniporatorTM IGEA Srl, Carpi, Italy.*

A number of studies have been performed with gene electrotransfer to various tissues, but mainly to muscle, skin and tumors. In particular, the muscle is the most frequently used organ for clinical trials involving DNA vaccination protocols since it is a natural "protein-factory" and is able to produce a large amount of protein after transfection with a small amount of plasmid (Hojman et al., 2007; Iezzi et al., 2012; Impelizzeri et al., 2014).

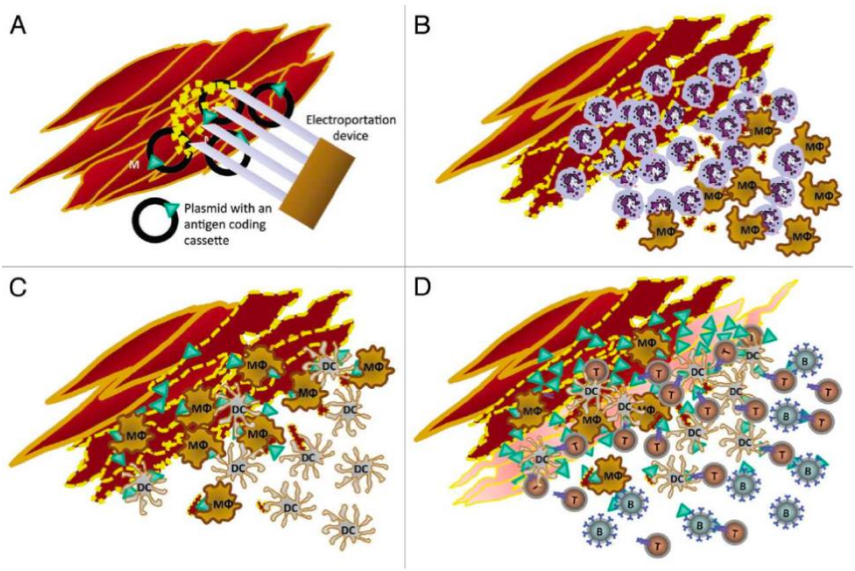


Figure 10 *Cellular events following intramuscular electroporation of 50 mg of plasmid in 20 ml of saline through Cliniporator (Igea, Carpi, Italy). Insertion of the electroporator needles into the quadriceps muscle of the mouse and delivery of two low voltage pulses of 150V of 25 ms with a 300 ms interval (A). One-6 h later the damaged myofibers and cell debris (dotted yellow) are surrounded by polymorphonuclear (N) and mononuclear leukocytes (MW) (B). One or two days later mature and differentiated tissue macrophages (MW) and dendritic cells (DC) progressively become prominent among inflammatory cells infiltrating the numerous necrotic myofibers (C). On the third-fourth day from the electroporation, intact and regenerating muscular fibers (pale red) are overexpressing the protein encoded by the plasmid (green triangles), while the area is being infiltrated by B (B), T (T) and dendritic cells (DC). Interestingly, CD11b+ macrophages and CD11c+ DC and, later, CD4+ T cells are often in direct contact with antigen expressing muscle cells or antigen expressing fragments and each other (D).*

Tumor associated antigens and oncoantigens

The immune system detects tumors through specific TAAs that are potentially recognized by both cytotoxic T lymphocytes and Abs. TAAs may be common to a particular tumor type, be unique to an individual tumor or may arise from mutated gene products such as ras, p53, p21, and/or others, and this aspect has enabled a more rational design and more sophisticated strategies for targeted anti-tumor vaccination. Theoretically any mutated, abnormally expressed or over-expressed TAA could be exploited as a target to design a specific anti-cancer DNA vaccine, however it is important to underlined that being a TAA does not necessarily mean to be a good target for immunotherapy (Iezzi et al., 2012; Rolih et al., 2017; Bergman, 2018).

Lollini et al. have expressed the view that an ideal oncoantigen should display a low level of expression in normal cells and tissues, and a high level in transformed cells and their microenvironment (Lollini et al., 2006).

Cavallo et al. coined the term “oncoantigens” for TAA as antigens that have a pivotal role in the promotion of tumor progression. They can be expressed by cells undergoing neoplastic transformation, or by cells in the tumor microenvironment, such as stromal, endothelial and inflammatory cells, or by both (Cavallo et al., 2007).

Two steps are required in the transition from tumor antigen to become a verified oncoantigen. The first is the acquisition of a functional role of the molecule in fostering the transformed phenotype of a given neoplasm, i.e. the “onco” portion of the oncoantigen definition; the second step is the

evidence that the immune system recognizes the molecule and mounts an effective response affecting tumor progression, i.e. the “antigen” part.

There are three different classes of oncoantigens based on the localization:

-*Class I oncoantigens* are expressed on cell surface, they act as receptors and adhesion molecules and are susceptible to T-cell cytotoxicity and to direct and indirect antibody-mediated immune response.

-*Class II oncoantigens* are present in tumor microenvironment, being mostly growth factors, angiogenic factors and component of extracellular matrix. These oncoantigens are targeted by direct and indirect antibody-mediated immune response.

-*Class III oncoantigens* are intracellular proteins working as non-receptors tyrosine kinases, transcription factors and cell cyle molecules. They are inhibited by T-cell cytotoxicity.

Class I oncoantigens are considered the ideal targets for effective anti-cancer immunotherapeutic strategies being susceptible to the attack of both T cells and antibodies, not impaired by the downregulation of MHC glycoproteins on the surface of tumor cells (Iezzi et al., 2012; Rolih et al., 2017).
When a vaccine-elicited immune response is directed against antigens with a significant role in driving the progression of a neoplastic lesion, the likelihood of immune selection of escaping antigen-loss variants is sensibly decreased. The immune mechanisms could develop a negative selection pressure on cells expressing oncoantigen, and this could, in turn, result in a reduction of the expression of the oncoantigen, which might also lead to cell death. Following vaccination, oncoantigens expressed by tumor cells and the stromal cells would be the target of both the cell-mediated and the antibody-mediated immune response (Lollini et al., 2006; Cavallo et al., 2007).
Antibodies do not involve MHC molecules in order to recognize and bind to their target, so oncoantigens, accessible to antibodies, are the target of direct and indirect antibody-mediated reactions and are not impaired by the downregulation of MHC glycoproteins on the surface of tumor cells. In some cases, the binding antibodies with oncoantigens suppress oncogenic signalling pathways. The absence or downregulation of an oncoantigen from the tumor cell membrane or the tumor microenvironment as a result of an antibody response might directly impair the progression of transformed cells. Additionally, indirect antibody-mediated immune reactions against oncoantigens, such as antibody-dependent cellular and complement-mediated cytotoxicity, also have a major role in the control and prevention of tumor growth. Indirect presentation of oncoantigens by dendritic cells and other professional antigen presenting cells can activate a CD4+ T-cell helper response, which is a crucial trigger for significant antibody production (Cavallo et al., 2007).
However, the majority of TAAs are self-antigens since they are derived from naturally occurring proteins. Therefore, to induce an effective reaction a vaccine must be able to sneak through central and peripheral tolerance mechanisms that normally impede autoimmune reactions to self-antigens. Overcoming this tolerance is, therefore, a major challenge. A strategy that is believed to be helpful

in this direction is the use of orthologous proteins or peptides from a different species (xenoantigens) as immunogens. Xenoantigens are believed to act as 'altered self' proteins, that is, proteins bearing amino acid changes in one or more epitopes and thus capable of breaking tolerance by means of T-cell responses cross-reactive (see below) against the endogenous nonmutated TAA (Cavallo et al., 2007; Iezzi et al., 2012; Cavallo et al., 2014).

The role of CSPG4 as oncoantigen

Chondroitin sulfate proteoglycan 4 (CSPG4), originally referred to as high molecular weight-melanoma-associated antigen (HMW-MAA) or melanoma chondroitin sulfate proteoglycan (MCSP), was first identified on human melanoma cells thirty years ago. Further research at that time also identified the murine ortholog of CSPG4, named nerve/glial antigen 2 (NG2) (Campoli et al., 2010). The CSPG4 is a transmembrane protein constituted by three main structural domains (Figure 10): a large extracellular domain, a transmembrane region and a short cytoplasmic domain. The extracellular domain of CSPG4 consists of three subdomains (D1-3) and contains the sequences for the glycosylation with the chondroitin sulfate (CS) chains that can influence the distribution of the proteins on the cell surface and also expresses binding sites for extracellular matrix (ECM) proteins, tyrosinase kinase receptors (RTK) growth factors, integrins, matrix metalloproteinases and lectins. The transmembrane region represents the structural connections between the between the outside and the inside parts. The cytoplasmic domain contains some recognizable sites that appear to be important for proteoglycan function, as the PDZ-binding domain (Rolih et al., 2017).

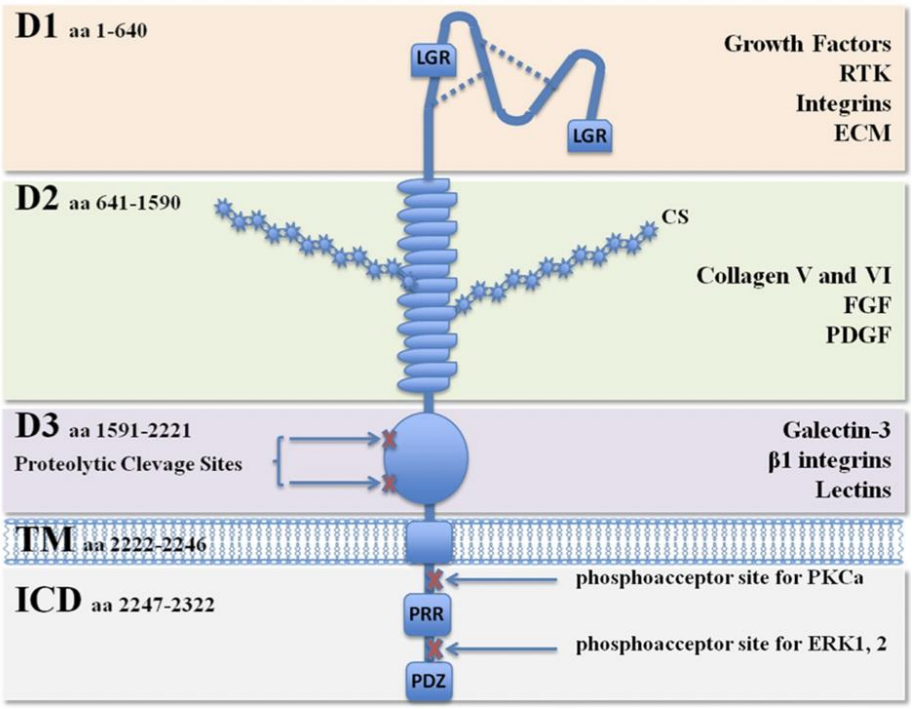


Figure 11 *Schematic drawing of CSPG4 protein. CS chondroitin sulfate, D1, D2 and D3 subdomains of the extracellular portion, TM transmembrane domain, ICD intracellular domain, LGR laminin G-type regions, PRR proline-rich region, PDZ PDZ binding domain. The most important molecules interacting with each subdomain of CSPG4 are indicated on the right. RTK receptor tyrosine kinase, ECM extracellular matrix, FGF fibroblast growth factors, PDGF platelet-derived growth factor, PKC protein kinase C, ERK extracellular signal-regulated kinases (Rolih et al., 2017).*

Studies on the signaling mechanisms of CSPG4 indicate that it coordinates several intracellular pathways, therefore is involved in tumorigenesis at multiple levels. Specifically, CSPG4 is involved, as co-receptor, in two types of signaling pathways: receptor tyrosine kinase (RTK) signaling through the MAPK cascade and integrin signaling through (FAK) activation. Through the activation of these oncogenic pathways, the over-expression of CSPG4 could sustain selective growth, survival advantage, migration, invasion and spreading of tumor cells (Rolih et al., 2017).
CSPG4 has been associated with the progression of several cancers, especially in human melanoma. The link between CSPG4 and melanoma progression was first appreciated as a result of its widespread expression in the majority (70% or greater) of superficial spreading and nodular human melanomas (Campoli et al., 2010; Price et al., 2011).
In particular, CSPG4 plays a fundamental role in melanoma cell proliferation, migration and metastasis, facilitating the growth and survival of malignant melanoma and controlling the consequences of microenvironment on melanoma progression. CSPG4 is highly expressed on malignant cells, but shows restricted distribution in normal tissues (Rolih et al., 2017).
Additionally, CSPG4 displays higher expression on activated pericytes in the tumor microenvironment making reasonable a possibility of inducing antiangiogenic effects mediated by CSPG4 targeting in the tumor microenvironment. In light of its localization on the cell membrane of tumor cells, CSPG4 can be susceptible to attack from both the humoral and cellular responses. Since the down-regulation of MHC class I molecules is a common mechanism by which melanoma cells avoid cytolytic attack from fully activated antigen-specific T cells, CSPG4 expression allows the immune system to target cancer cells independently of the MHC class I status of each patient. Moreover, CSPG4 is expressed on cancer-initiating cells and is responsible for recurrence and metastasis (Maciag et al., 2008; Wang et al., 2010).
Considering all these aspects CSPG4 can be considered as an ideal oncoantigen to be targeted in tumor immunotherapy strategies.
In 2011 Mayayo et al. were the first to investigate CSPG4 expression in canine melanoma. The CSPG4 staining was mostly restricted to the tumor cell membrane with a different grade (or score) of expression in the different subtypes, being higher in epithelioid and in the more aggressive amelanotic phenotype. These results obtained labeled CSPG4 as a new potential marker for canine malignant melanoma diagnosis and as a promising candidate antigen for translational immunotherapy studies in dogs (Mayayo et al., 2011; Rolih et al., 2017).

CSPG4 antigen electrovaccination as treatment in canine oral malignant melanoma

Several canine tumors are “under the microscope” of comparative oncology for their translational relevance, and canine melanoma is one of these. Canine tumors mimic the progression of human malignancies better than any other preclinical model available so far, indeed they grow over long periods of time following the natural evolution of human tumors, developed metastasis and recurrence, and provide similar response to conventional therapies. Therefore, the study of spontaneous tumors developing in dogs as models for human malignancies is a priceless translational tool for accelerating the development of novel immunotherapeutic strategies with a

substantial impact on the management of both canine and human oncological patients (Rolih et al., 2017).

The discovery of TAA has permitted a more focused development of immunotherapeutic strategies as DNA vaccines. Vaccinations against cancer relies on the concept that cancer cells display a genetic panel that codifies for proteins (as TAA) notably different from the normal cells and capable of stimulation an immune response. Although DNA vaccines have induced immune responses to viral proteins, vaccinating against tissue specific self-proteins on cancer cells represents a hurdle to overcome. One way to induce immunity against a tissue specific differentiation antigen on cancer cells is to vaccinate with xenogeneic antigen or DNA that is homologous to the cancer antigen (Cavallo et al., 2014).

The antigen of interest is cloned into a bacterial expression plasmid with a constitutively active promoter. The plasmid is introduced into the skin or muscle with an intradermal or intramuscular injection and afterward professional antigen presenting cells, mainly dendritic cells, present the transcribed and translated antigen in the proper context of MHC and costimulatory molecules. The bacterial and plasmid DNA itself contains immunostimulatory sequences that may act as a potent immunological adjuvant in the immune response. As mentioned previously an important breakthrough in the field of tumor vaccination and in the treatment of canine melanoma was achieved with a DNA vaccine encoding the human tyrosinase commercialized it under the name OnceptTM and currently the only veterinary therapeutic tumor vaccine licensed by the United States Department of Agriculture (USDA) for the treatment of oral and digital melanoma (Bergman et al., 2006). However, the use of OnceptTM has not been approved in Europe.

In 2014 Riccardo et al. have proposed new xenogeneic DNA vaccine for the treatment of dogs with oral malignant melanoma expressing as TAA the CSPG4.

CSPG4 is an early cell-surface progression marker involved in tumor cell proliferation, migration and invasion. Interestingly human CSPG4 (HuCSPG4) displays 82% homology and 88% similarity to its canine counterpart in its amino-acidic sequence and the frequency of CSPG4 expression in canine and human melanoma lesions has been reported about 60% and 80%, respectively (Campoli et al., 2010; Mayayo et al., 2011).

In the study of Riccardo et al., fourteen dogs with stage II–III surgically resected CSPG4 positive OMM were administered monthly, through electroporation under anesthesia, a vaccine containing a pcDNA3.1 plasmid coding for the human CSPG4 sequence (Riccardo et al., 2014). Electrovaccination was elected since it combines the advantages of DNA vaccination and electroporation. Specifically, the former is easy to handle, applicable to a broad population, safe, and induces both cellular and humoral immune responses, whereas the latter enhances the expression of the protein encoded by the immunizing DNA and prolongs the duration of the immune response (Impellizeri et al., 2014). Only dogs with an oral MM characterized by a CSPG4 score ≥3/8 were considered as suitable vaccination candidates and compared with the control group in terms of immune-mediated response and outcome. The huCSPG4 plasmid (500 mg in 200 mL of 0.03% NaCl) was injected into the muscles of the caudal thigh. Two minutes after plasmid injection, nine electric pulses (1 high voltage, amplitude 450 V, length 50 ms, frequency 3 HZ; 1 second pause; eight low-voltage amplitude 110 V, length 20 ms, pause 300 ms) were applied to the injection site using

the CLINIPORATOR (Igea). Dogs were monitored for acute, late local or systemic side effects (Riccardo et al., 2014).

Vaccination-induced immune response was evaluated in PBMC and sera of CSPG4 positive vaccinated dogs. A strong HuCSPG4- and cCSPG4-specific antibody response was detected in all vaccinated dogs after the third vaccination. Additionally, post-vaccination sera from all vaccinated dogs (except in two dogs) resulted capable to considerably inhibit SK-MEL-28 cell proliferation (human melanoma cell lines), as compared with pre-vaccination sera. In contrast, the evaluation of PBMC collected pre and post the third vaccination did not reveal significant increase in IFNδ-secreting cells suggesting that the vaccination induced a low frequency, if any, of circulating T-cells that were cCSPG4 reactive. The antibody immune response was translated into an improved outcome of vaccinated dogs compared with control group. In particular, MST and DFI in vaccinated dogs were significantly longer than in non-vaccinated CSPG4-positive dogs (group II) and MST of group I was significantly longer even when compared with the entire nonvaccinated control population (CSPG4 positive and negative dogs). Based on these results, the authors concluded that electroporation with DNA-based vaccine against chondroitin sulfate proteoglycan-4 (CSPG4) in dogs with stage II–III surgically resected CSPG4-positive oral malignant melanoma was effective, had therapeutic efficacy and was able to overcome the host unresponsiveness to the self-antigen (Piras et al., 2017; Riccardo et al., 2014).

Later, a prospective study has evaluated the median survival time and the disease-free interval of 23 dogs with resected II/III-staged CSPG4-positive oral cMM treated with adjuvant HuCSPG4 electrovaccination that were compared with 19 dogs II/III-staged CSPG4-positive oral cMM treated with *en-bloc* surgery alone. The plasmid and the device used as well as the vaccination protocol were identical as reported in Riccardo at al., starting the electroporation the 3rd–4th post-operative weeks and it was repeated after 2 weeks and then monthly; dogs surviving over 2 years were then vaccinated every 6 months (Riccardo et al., 2014). The MST and DFI of vaccinated group and in control group were 684 and 477 days, and 220 and 180 days, respectively. The percentage of local recurrence and lung metastasis were 35% and 39% in the vaccinated dogs and 42% and 79% in the control group, respectively. The immune response induced by the vaccine was assessed by using a recombinant HuCSPG4 ELISA assay and the level of antibodies binding the HuCSPG4 was significantly higher in the post-vaccination than in pre-immune sera. Interestingly, survival times of vaccinated dogs with body weight <20 kg and with a CSPG4 score $\geq$5 was significantly longer than both the entire population and the control group, the was true for the DFI. With the reference to body weight, this aspect highlights the importance of the level of the antibody titer induced by the vaccine as the humoral response could have role in prolonging both the DFI and survival time of vaccinated dogs. Additionally, authors have suggested a potential impact of CSPG4 positivity on the outcome. Indeed, despite CSPG4 score did not correlate with the survival of non-vaccinated dogs, the improved outcome of dogs with CSPG4 $\geq$5 compared with vaccinated dogs with a CSPG4 <5 could be explained with a greater prevalence of CSPG4-negative tumor clones in the latter. This aspect has possibly facilitated the evasion of anti-CSPG4 immunity induced by the vaccine and, ultimately allowing the progression of the disease. Collectively these results, as in the first study by Riccardo, corroborate the role of adjuvant HuCSPG4 electrovaccination as potential effective, immunogenic and safe treatment in canine OMM (Piras et al., 2017).

Experimental section

Project 1

Evaluation of outcomes in dogs treated with novel chimeric HuDoCSPG4 vaccine as part of a multimodality treatment for canine oral malignant melanoma.

INTRODUCTION

Malignant melanoma (MM) is considered the most common oral malignancy in dogs, accounting for 14.4% to 45.5% of oral tumors (Bergman, 2007; Nishiya et al., 2016). Oral malignant melanoma exhibits aggressive behaviour with rapid growth, local invasiveness and high propensity to metastatise, especially to regional lymph nodes and lungs (Bostock, 1979; Nishiya et al., 2016; Liptak, 2020).

Current recommended treatment is wide surgical excision of the primary tumor resulting in a variable survival time (Boston et al., 2014; Tuohy et al., 2014). Radiotherapy can be used as primary treatment when aggressive surgery is not feasible or as an adjunct to surgery, inducing an overall response rate of 82% to 94% (Proulx et al., 2003; Khan et al., 2011; Cancedda et al., 2016; Liptak, 2020).

Systemic treatments, mostly platinum based agents, have been used against macroscopic disease and associated with surgery and radiotherapy, still leading to no particular clinical and survival benefit (Rassnick et al., 2001; Boria et al., 2004; Murphy et al., 2005; Borckley et al., 2013; Boston et al., 2014; Dank et al., 2014).

Despite local tumor control is achieved by means of *en bloc* surgery alone or in conjunction with radiotherapy, many patients succumb to metastatic disease (Freeman et al., 2003; Proulx et al., 2003; Boria et al., 2004; Murphy et al., 2005; Boston et al., 2014; Kawabe et al., 2015; Cancedda et al., 2016; Turek et al., 2020).

In light of this and considering the high immunogenicity of melanoma (Modiano et al., 1999) immunotherapy has been representing an attractive strategy to address conventional treatments for management of canine malignant melanoma. Different approaches have been used ranging from vaccines (allogeneic, autologous, xenogeneic), gene therapy, monoclonal antibodies, nonspecific immunotherapy activated by bacteria and lymphokine-activated killer cell therapy (Dow et al., 1998; Soergel et al., 1999; MacEwen et al., 1999; Alexander et al., 2006; Finocchiaro & Glikin, 2012; Ottnod et al., 2013; Finocchiaro et al., 2015; Maekawa et al., 2017; Almela & Anson, 2019).

Chondroitin sulfate proteoglycan-4 (CSPG4) is a class 1 oncoantigen involved in several oncogenic pathways as melanoma tumor cell progression, survival and metastatisation, being poorly expressed in healthy tissue. Therefore, CSPG4 has gained value in terms of optimal immunotherapeutic target (Yang et al., 2004; Mayayo et al., 2011; Rolih et al., 2017).

Previous studies have demonstrated that xenogeneic electrovaccination against CSPG4 was immunogenic, safe and able to prolong survival time in dogs affected by oral malignant melanoma

(OMM). Specifically, dogs were vaccinated with a plasmid coding for the human CSPG4 (HuCSPG4) in order to break immune tolerance. The xenogeneic HuCSPG4 DNA vaccine was able to trigger a consistent humoral response detected in the post-vaccination sera, resulting in improved outcome (Riccardo et al., 2014; Piras et al., 2017).

To overcome unresponsiveness against a self-antigen, a hybrid DNA vaccine that codes for a chimeric CSPG4 molecule has been generated, which is partly derived from the human (Hu) and partly from the canine (Do) sequence (HuDoCSPG4). It can be supposed that electrovaccination with a hybrid plasmid coding for a chimeric protein containing both xenogeneic (HuCSPG4) and homologous (DoCSPG4) domains could be effective on one hand in breaking the host immune tolerance and on the other hand able to induce a high affinity immune response.

The aim of this prospective bicentric study was to assess survival time, in clients owned dogs with OMM, stage II-III, CSPG4 positive that underwent *en bloc* surgery and/or radiotherapy followed by adjuvant intramuscular electrovaccination with chimeric hybrid human/dog (HuDo)CSPG4 DNA vaccine. In order to investigate the efficacy of the vaccine, these data have been compared with a group of dogs affected by OMM, stage II-III, CSPG4 positive, treated with surgery and adjuvant human CSPG4-antigen electrovaccination and with a control group of dogs that received surgery alone (historical data, including also those already included in Boston et al., 2014).

MATERIALS AND METHODS

Patients enrolment

Dogs of the study were prospectively enrolled at the Veterinary Teaching Hospital of Grugliasco (Turin) and Clinic Veterinary Tyrus of Terni from 2014 to 2020. The study was approved by the Ethical Committee of the University of Turin (Italy) and the dogs were treated according to the Good Clinical Practice guidelines for animal clinical studies. Both the Ethics Committee of the University of Turin and the Italian Ministry of Health approved the trials (0004230-20/02/2018-DGSAF-MDS-P and 0015537-28/06/2017-DGSAF-MDS-P); a written consent form was signed by the owners before dogs' enrolment in the study. Pre-treatment work up consisted of physical examination, blood count, serum biochemistry and urinalysis. Complete tumor staging included pre-operative total body CT scan (computed tomography); alternatively, according to the owners' decision, skull, three views chest radiographs and abdominal ultrasound were performed. For the majority of the cases a preliminary diagnosis was obtained by incisional biopsy of the primary tumor, in few cases by cytology. Fine needle aspirate of draining lymph nodes was available in some cases, if not complete staging was achieved by regional lymphadenectomy at the time of primary mass *en bloc* resection followed by histologic evaluation. Patients were staged according to the World Health Organisation tumor, node, metastases (TNM) guidelines (Table 1).

Inclusion criteria were a definitive histological and/or immunohistochemical diagnosis of oral malignant melanoma, absence of any concurrent life-threatening disease, oral melanoma staged II-III with achieved local control by surgery and/or radiotherapy followed by chimeric (HuDo) anti-CSPG4 electrovaccination, and minimum follow up of 1 year on 31st December 2021. For each dog, the following data were collected: signalment, age, gender, neuter status, breed, weight, tumor size

and site within the oral cavity. The type of surgery (maxillectomy, mandibulectomy lip/cheek excision) and radiotherapy protocols were recorded. A regional lymphadenectomy involved ipsilateral or bilateral mandibular and retropharyngeal lymph nodes excision.
For the evaluation of surgical excision margins, melanoma sample surface was stained with a specific dye (Tissue Marking Dye [TMD], Triangle Biomedical Sciences, Durham, NC, USA), then fixed in 10% formalin (Ramos-Vara & Borst, 2017). Surgical margins were considered complete if the narrowest histologic margin was >2 mm. The following information were retrieved from the histopathology reports: Ki67 expression (polyclonal Ki67 antibody A-047; DAKO; cut-off of 19.5), mitotic count (<4/10 high-power fields [hpf] or ≥4/10 hpf), nuclear atypia (% atypical nuclei in 200 cells counted, < or ≥30%), surgical margins and CSPG4 immunohistochemical score (Bergin et al., 2011; Kamstock et al., 2011, Mayayo et al., 2011; Smedley et al., 2011).
Only dogs bearing an OMM with a CSPG4 score ≥3/8 were included in the electrovaccination schedule. Type of vaccination protocol, number of vaccine doses, adverse effects and other treatments apart from the vaccine were also recorded. The study was conducted on three groups: group A involving dogs with CSPG4-positive OMM treated with surgery (and/or RT) plus adjuvant anti HuDoCSPG4 DNA vaccination, group B involving dogs with CSPG4-positive OMM treated with surgery (and/or RT) plus adjuvant anti HuCSPG4 DNA vaccination and group C involving dogs with CSPG4-positive OMM treated with surgery alone.

Table 1 Traditional World Health Organization TNM-based staging scheme for dogs with oral melanoma. Adapted from Owen LN. TNM Classification of Tumors in Domestic Animals. 1st ed. Geneva, Switzerland; 1980.

T	*Primary Tumor*	N	*Regional lymph nodes*	M	*Distant metastasis*
T1	Tumor < 2 cm in diameter	N0	No evidence of regional node involvement	M0	No evidence of distant metastasis
T2	Tumor 2–4 cm in diameter	N1	Histologic/Cytologic evidence of regional node involvement	M1	Evidence of distant metastasis
T3	Tumor >4 cm in diameter	N2	Fixed nodes		

Stage I	T1 N0 M0	*Stage III*	T2 N1 M0 or T3 N0 M0
Stage II	T2 N0 M0	*Stage IV*	Any T, Any N andM1

In vivo electrovaccination

The different types of DNA vaccines, chimeric hybrid HuDoCSPG4 and HuCSPG4 were administered according to two vaccination protocols: *ministerial* and *monthly*.
In the *ministerial*, vaccination started at the 3rd-4th post-operative week and was repeated after 2 weeks and then monthly for four times. Then dogs were restaged every three months by performing CT scan of the thorax. Following the sixth vaccination, a semestral booster was administered. In the *monthly* protocol, vaccination started at the 3rd-4th post-operative week and was repeated after 2

weeks and then monthly. Patients surviving over 2 years were then vaccinated every 6 months with boosters.
Dogs of group A were electrovaccinated with the hybrid HuDoCSPG4 plasmid coding for a chimeric protein including the N-terminal portion of the HuCSPG4 protein and the C-terminal portion of the canine DoCSPG4 protein (Riccardo et al., 2019; paper submitted under revision [2021/2022]) while dogs of group B received a pcDNA3.1 plasmid coding for the HuCSPG4 generated as already previously described (Riccardo et al., 2014; Piras et al., 2017).
If during the vaccination protocol dogs developed distant metastatic disease metronomic chemotherapy was introduced with the association of cyclophosphamide (alternatively chlorambucil), piroxicam and thalidomide.
The following procedures were the same for both protocols. Patients underwent short general anaesthesia, then the plasmid (chimeric or human, 500 μg in 200 μL of 0.03% NaCl solution) was injected in the muscles of the caudal thighs. Two minutes after plasmid injection, nine electric pulses (1 high voltage, amplitude 450 V, length 50 ms, frequency 3 HZ; 1 s pause; 8 low-voltage amplitude 110 V, length 20 ms, pause 300 ms) were delivered within the injection site using the CLINIPORATOR (Igea, Carpi, Italy).
Patients recovered quickly from anaesthesia and were discharged within 30-40 min. Patients were strictly monitored for acute, late local or systemic side effects. At each vaccination, clinical examination, blood-work, and restaging with CT of the thorax or three-view chest radiographs were performed. Blood samples for sera and peripheral blood mononuclear cells (PBMC) were also collected, aliquoted and cryopreserved at −80◦C until use.

Follow-up data

During the adjuvant vaccination protocol all dogs were re-staged with radiographs or computed tomography. Follow-up information was collected from medical records or by phone conversation with referring veterinarians and dog's owners.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 9.0.0 for Windows, GraphPad Software, San Diego, California, www.graphpad. com), with statistical significance set at a $P<.05$.
The data were summarized using descriptive statistics, and were indicated as mean, median and range. Survival time was estimated using Kaplan Meier method. The overall survival times (OST) was defined as the interval from the day of surgery to the date of death or euthanasia, reporting if it was or not melanoma related. Within the group, Kaplan-Meier survival analyses were used to investigate the effect of disease stage (stage II and stage III vs. control) and metronomic chemotherapy on survival time. Animals alive at the end of the study or lost to follow-up were censored at the date they were last known to be alive.
A sample size calculation was performed to estimate the number of dogs needed to detect difference in survival time. According to the literature, dogs treated with surgery and/or radiotherapy have a MST ranging from 220 to 335 days (Boston et al., 2014; Baja et al., 2022). In this

study it was evaluated that 25 dogs were adequate for control and vaccinated groups assuming to double the MST with 70 % power and a significance level of 0.05.

RESULTS

Patient characteristics, groups and protocols

Medical record identified 53 dogs diagnosed with OMM that fulfilled the inclusion criteria and were prospectively enrolled in *Group A* (surgery and/or RT plus HuDoCSPG4 elettrovaccination). Of these 27 were males (8 castrated, 19 intact), 26 females (24 spayed, 2 intact). Age ranged from 7 to 15 years (mean and median 11 years) and weight from 4 to 36 kg (mean 20.2 kg, median 20 kg). Represented breeds were 15 mongrels, 4 Pinschers, 4 Golden Retrievers, 3 German Shepherds, 2 Setters, 2 English bulldogs, 5 Cockers English Spaniels, 2 of each of Daschund, Pekingese, Jack Russel, Poodles, Shar Pei and one each of French Hound, Labrador Retriever, Hovawart, Amstaff, Alaskan Malamute, Rottweiler, Shi-tzu and Yorkshire. Thirty-two out of 53 dogs received the ministerial protocol, the remaining dogs underwent the monthly scheme.
Twenty-five dogs were included in *Group B* (surgery plus HuCSPG4 electrovaccination). There were 18 males (9 castrated, 9 intact), 7 females (4 spayed, 3 intact), age ranged from 4 to 14 years (mean 11.6 years, median 12 years). Mean and median weight were 18 kg and 13 kg (range 5-32 kg), respectively. In group B twenty-two dogs received the ministerial protocol and 3 the monthly protocol. Three out of 25 dogs received the ministerial protocol, the remaining dogs underwent the monthly scheme.
Twenty-seven dogs were included in the control *Group C* (surgery alone). In group C 18 dogs were males (3 castrated, 15 intact), 9 females (7 spayed, 2 intact). Mean and median age was 12 years (range 7-16 years), mean and median weight were 22.4 kg and 16 kg (range 5-55kg), respectively. No statistical differences regarding age and weight distribution were evident within the 3 groups.

Table 2 Patients' groups and electrovaccination protocols.

	Enrolled patients	Ministerial protocol	Monthly protocol
***Group A* (HuDo)**	53	32	21
***Group B* (Hu)**	25	3	22
***Group C* (control)**	27		

Clinical staging and histology

Clinical staging was performed by total body CT in 46 dogs in group A, 20 in group B and 16 in group C; the remaining dogs of each group were staged with chest radiographs and abdominal ultrasound. Staging procedures identified 30 OMM staged II and 23 staged III in group A, 10 OMM staged II and 15 OMM staged III in group B, 11 oral MM stage II and 16 stage III in group C. Margin status was available for 92 out 105 dogs of the entire population, except in 13 dogs, including 3 patients that

received adjuvant RT in which this information was not retrievable. Fifty-two dogs out of 105 were identified with metastatic lymph nodes.
Histopathological data for MC, nuclear atypia, immunohistochemical expression for Ki67, margin status and score for CSPG4 are summarised in Table 3 and Table 4.
In group A mitotic count was ≥4/10 hpf in 44 OMM (mean 15, median 10, range 1-60), in group B and C it was ≥4/10 hpf in 24 (mean 25.6, median 22.5, range 3-68) and 22 MM (mean 17, median 15, range 1-47), respectively.
Nuclear atypia was ≥30 % in 25, 16 and 16 OMM of group A, group B and group C, respectively. It was not available in 9 cases of group A and 4 case of group B.
In group A, Ki67 expression was ≥19.5 in 35 cases (mean 38, median 39.5, range 6-83), in group B, it was ≥ 19.5 in 22 cases, in the remaining 3 this data was not detectable (mean 34.8, median 30, range 21-50). In group C Ki67 was ≥19.5 in 23 cases, <19.5 in 3 cases and undetectable in 1 case (mean 34, median 25, range 11-80).

Treatment

Different treatments for every group including type of surgery, radiotherapy protocol, lymphadenectomy (bilateral or ipsilateral) and metronomic chemotherapy are summarised in Table 5. Among the 53 dogs of group A, 12 had an excisional biopsy performed by the referring veterinarians followed in 3 cases by revision surgery and ipsilateral lymphadenectomy before starting immunotherapy. Two out of 25 dogs of group B had excisional biopsy. For the remaining patients of group A, a surgical revision was not performed since no macroscopic disease was detected at presentation. All dogs that received palliative radiotherapy (from 4 to 6 fractions weekly per cycle) had bilateral lymph nodes irradiated prophylactically. Four out of 7 dogs underwent RT for recurrence followed to marginal excision; the remaining three were treated in the naïve setting for macroscopic disease.
In group A and in group B the mean numbers of electrovaccinations were 9 (range 4-28) and 15 (range 4-37), respectively. In group A the vaccination protocol was well tolerated, no dogs developed systemic reactions or toxicities that required veterinary intervention.

Table 3 Histological and immunohistochemical parameters of OMMs in each group

Parameters	Threshold	Group A (=53)	Group B (=25)	Group C (=27)
Nuclear atypia	<30%	19 (30%)[a]	5 (20%)[a]	9 (33%)[a]
	≥30%	25 (70%)[a]	16 (64%)[a]	18 (67%)[a]
Mitotic count (MC)	<4/10 hpf	9 (17%)[a]	1 (4%)[a]	3 (11%)[a]
	≥4/10 hpf	44 (83%)[a]	24 (96%)[a]	24 (89%)[a]
Ki67	<19.5%	18 (34%)[a]	0 (0%)[a]	3 (11%)[a]
	≥19.5%	35 (66%)[a]	22 (88%)[a]	23 (85%)[a]
Margins status	clear	31. (58.5%)[a]	18 (72%)[a]	21 (78%)[a]
	infiltrated	14. (26.5%)[a]	5 (20%)[a]	3(11%)[a]
	unknown	8 (15 %)[a]	2 (8%)[a]	3(11%)[a]

[a] % in brackets

Table 4 Immunohistochemical CSPG4 score of OMMs from dogs included in the study

CSPG4 score	Group A (=53)	Group B (=25)	Group C (=25)
3/8	4	3	2
4/8	6	5	4
5/8	9	5	5
6/8	18	3	5
7/8	10	7	8
8/8	6	2	3

Table 5 Type of treatment of OMMs from dogs included in the study

Type of treatment	Group A (=53)	Group B (=25)	Group C (=27)
Mandibulectomy	12 (26%)[a]	13 (52%)[a]	14(52%)[a]
Maxillectomy	11 (21%)[a]	4 (16%)[a]	5 (18.5%)[a]
En-bloc excision lip cheek	11 (21%)[a] 6 5	6 (24%)[a] 2 4	8(30%)[a] 5 3
Lymphadenectomy bilateral ipsilateral	38 (72%)[a] 12 26	22 (88%)[a] 6 16	25 (92.5%)[a] 8 17
Radiotherapy	7 (13%)[a]	0	0
Metronomic chemotherapy	19 (35%)[a]	4 (16%)[a]	3 (11%)[a]

[a] % in brackets

Clinical outcome and response to CSPG4 electrovaccination

The median survival time (MST) of group A, B and C are summarized in Table 6. In the HuDoCSPG4 vaccinated dogs (*group A*) the 6-, 12-, 18- and 24-month survival rates were 96, 69, 47 and 32 %, respectively. At the end of the observation period (31st December 2021), 6 dogs of the group A (11%) were still alive and 47 (89%) were dead, 28 (60%) because of the OMM and 19 (40%) for unrelated causes; two dogs were lost to follow up.

In the HuCSPG4 vaccinated dogs (*group B*) the 6-, 12-, 18- and 24-month survival rates are 96, 76, 44 and 32%, respectively. At the end of the study, all patients (96%) were dead except one. Fourteen (56%) succumbed because of the tumor, 9 (37.5%) for unrelated causes (1 lymphoma, 1 kidney disease, 2 prostatic carcinoma, 1 hepatoid glands carcinoma, 1 cardiac hemangiosarcoma, 1 neuro-orthopaedic disease, 2 others for tumor unrelated causes).

In the control group (*group C*) the 6-, 12-, 18- and 24-month survival rates are 74, 37, 29 and 18%, respectively. Twenty-six (96%) out of 27 were dead. One dog was still alive while 20 patients (77%) died because of melanoma, 1 for cardiomyopathy, 2 for neuro-orthopaedic disease, 1 for GDV, 1 for aspiration pneumonia, and 1 for unrelated cause.

Table 6. Median survival times and percentages of survival in group A, B and C

	MST (days)	% MST			
		6 months	12 months	18 months	24 months
Group A	653	96	69	47	32
Group B	567	96	76	44	32
Group C	310	74	37	29	18

Median survival times in group A and group B were 653 days (range 171-1504 days) and 567 days (78-2784 days), respectively. Dogs of both groups (group A, p = 0.009; group B, p = 0.034) lived significantly longer compared with control group (MST of 310 days, range 54-1325 days). Considering the MST of vaccinated dogs of groups, A and B, despite the prolonged survival of dogs of group A, statistical analysis did not reveal any significant difference (p = 0.87) (Figure 1).

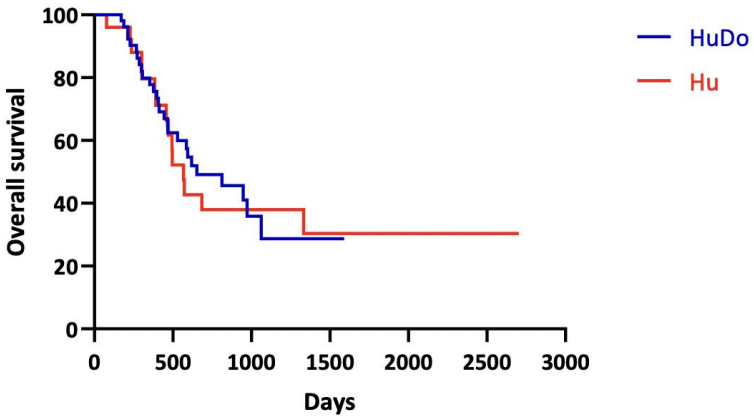
The clinical stage of the tumors in group A was not significantly (p = 0.94) associated with survival time (MST, 948 and 653 days for dogs with stage II and stage III disease, respectively).

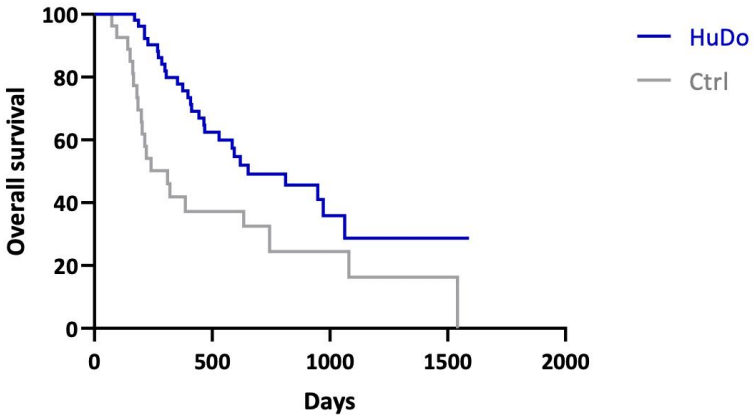
Thirty dogs enrolled in group A were classified as having stage II and 23 as having stage III OMM. Evaluating MST according to the clinical stage of group A and group C, vaccinated dogs with stage II OMM experienced a longer survival (948 days, range 187-1575) compared with control group dogs staged II (427 days, range 95-1081); vaccinated dogs with stage III OMM (30 dogs) lived longer (653 days, range 171-1183) compared with control group dogs (16 dogs) with stage III OMM (310 days, range 74-1542).

Kaplan-Meier survival probabilities for death attributable to OMM were not significantly different between dogs with OMM in stage II (p = 0.07) and stage III (p = 0.06) of group A and group C, despite being close to statistical significance. Kaplan Meier curves referring to stage division are shown in Figure 2.

In group A, 19 dogs (35%) received metronomic chemotherapy during the vaccination protocol showing a MST of 543 days compared with MST of 812 days of those not treated with chemotherapy. No statistical significance was found (p = 0.27). The same statistical analysis was not performed for groups B and C because of the very small size sample of dogs receiving adjuvant metronomic chemotherapy.

When survival times were estimated evaluating the ministerial and the monthly vaccination protocol, dogs of the ministerial protocol experienced a longer MST (812 days, range 172- 1286 days), than those undergoing the monthly protocol (585 days, range 171-1504 days). Nevertheless, statistical analysis did not reveal any statistical difference (p = 0.56, Figure 3). This statistical evaluation was carried out only for group A considering the limited number of vaccinated dogs in group B that received the ministerial protocol (3 ministerial vs. 22 monthly), (Figure 3).





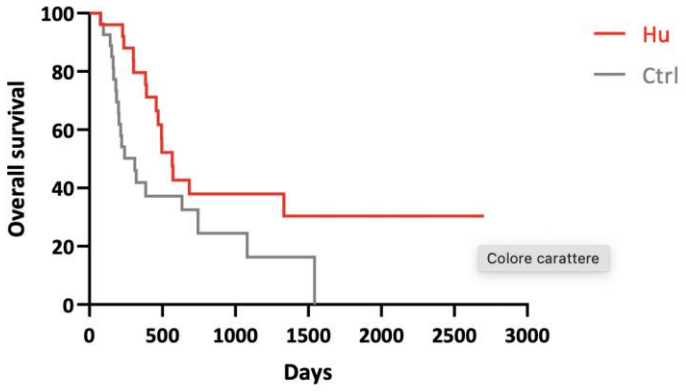


Figure 1 Kaplan-Meier curves comparing the MST of group A and B, group A and C, group B and C. Survivals in days of dogs vaccinated with HuDoCSPG4 (group A, blue line) and dogs vaccinated with HuCSPG4 (group B, red line, p = 0.87). Survivals in days of dogs vaccinated with HuDoCSPG4 (group A, blue line,) and control group (group C, grey line p = 0.009). Survivals in days of dogs vaccinated with HuCSPG4 (group A, red line,) and control group (group C, grey line p = 0.034).

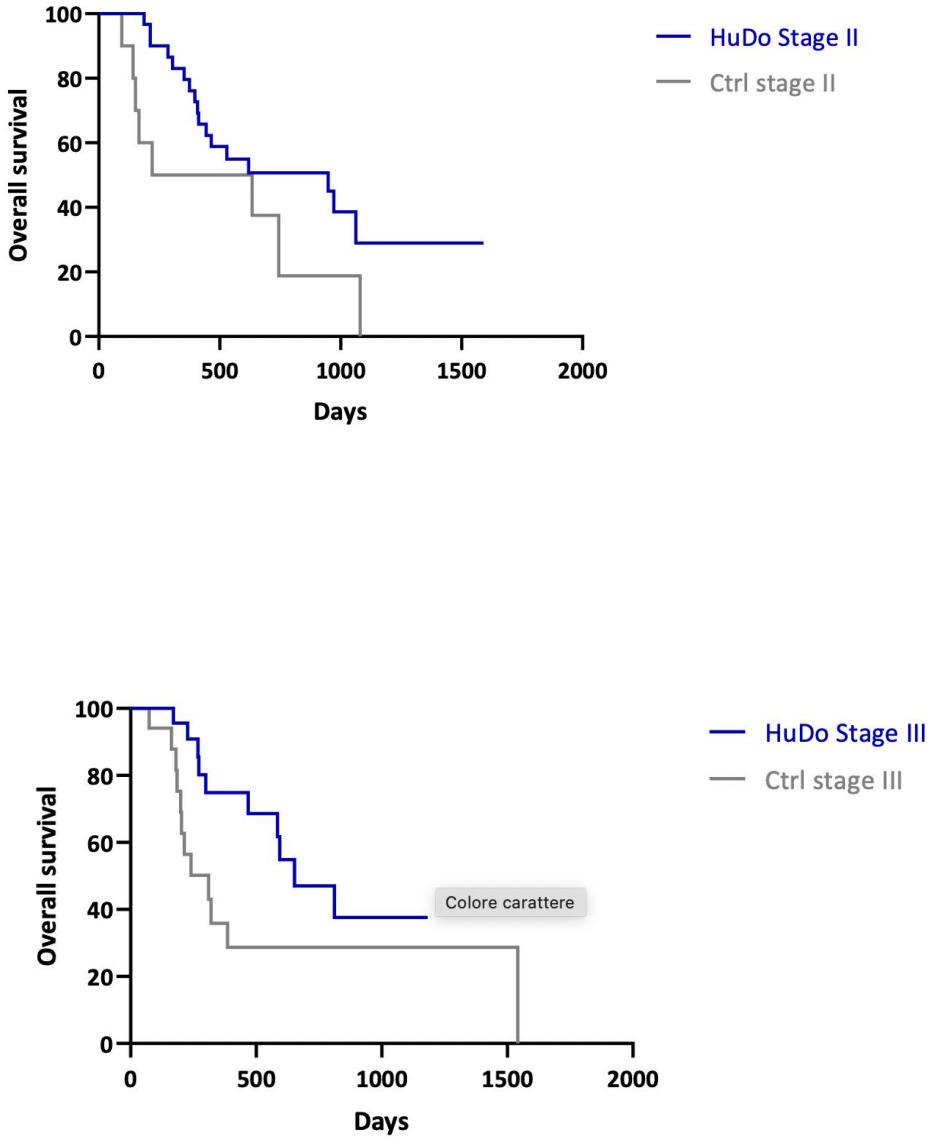


Figure 2 Kaplan-Meier curves comparing the MST of stage II and stage III dogs in group A (HuDoCSPG4) and group C (control group). Survivals in days of stage II dogs vaccinated with HuDoCSPG4 (group A, blue line) and stage II dogs of control group (group C, grey line, p = 0.07). Survivals in days of stage III dogs vaccinated with HuDoCSPG4 (group A, blue line,) and stage III dogs of control group (group C, grey line p = 0.06).

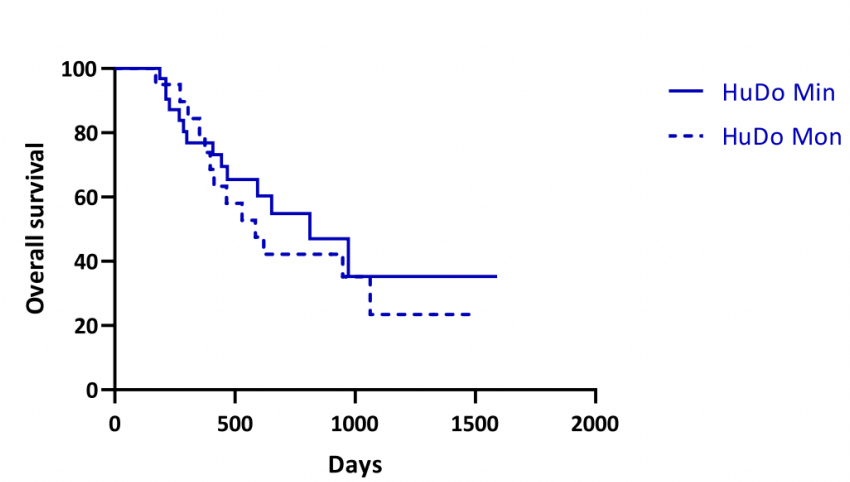


Figure 3 Kaplan-Meier curves comparing the MST of dogs in group A (HuDoCSPG4) that received the ministerial protocol (blue line) and dogs that received the monthly protocol (blue dotted line, p = 0.56).

DISCUSSION

Currently, the therapy of choice for OMM is surgery consisting of resection of the primary tumor and regional lymphadenectomy, with radiotherapy used in combination (adjuvant) or as a sole treatment for macroscopic disease control (Boston et al., 2014; Tuohy et al., 2014; Cancedda et al., 2016; Grimes at al., 2019,).

Immunotherapy is changing the prospect of canine OMM treatment, focusing on molecules with a pivotal role in melanoma progression, such as CSPG4. This oncoantigen appears an ideal target, in terms of DNA vaccination, since it is characterised by restricted distribution in normal healthy tissue and high expression in both human and canine melanoma cells (Rolih et al., 2017).

This clinical prospective study has been designated to investigate the efficacy and safety of electrovaccination with a hybrid plasmid coding for a chimeric protein (HuDoCSPG4) in dogs with OMM staged II and III. To underline the results obtained in this study, the survival time of dogs treated adiuvantly with anti-CSPG4 electrovaccination was compared with an historical control group of dogs CSPG4-positive treated with surgery only. Additionally, dogs electrovaccinated with HuDoCSPG4 were also compared with those vaccinated with HuCSPG4.

Based on this study it is possible to conclude that anti HuDoCSPG4 electrovaccination is safe and potentially able to prolong the survival time. The same authors have performed two previous trials where immunogenicity, efficacy, and safety of a DNA vaccine coding for HuCSPG4, have been demonstrated and proposed as an adjuvant treatment in dogs with OMM locally controlled by means of surgery and/or radiotherapy (Riccardo et al 2014; Piras et al. 2017).

The rationale behind the use of a chimeric DNA vaccination in dogs with OMM is supported by several reasons, even in light of outcomes reported in previous studies (Riccardo et al., 2014; Piras et al., 2017). Firstly, it is widely recognized that DNA vaccines are easy to produce, stable and characterized by a low cost of manufacturing; additionally, they can induce both humoral and cellular response (Fioretti et al., 2010). Secondly, being the hybrid plasmid encoding the chimeric CSPG4 protein partially derived from the human (Hu) and partially from the dog (Do) CSPG4 sequence (HuDoCSPG4), the heterologous and autologous domains could, on one hand, break the

host immunotolerance overcoming the unresponsiveness to self-CSPG4 and, on the other hand, be capable to induce a highly affine immune response, possibly protecting dogs from local recurrence and metastatic disease. Dogs affected by a CSPG4-negative melanoma were not enrolled in this study, since it is supposed not to benefit immunologically from CSPG4-immune-targeting; however, they were included in a previous paper displaying an intermediate behavior in terms of prognosis (Piras et al. 2017). Another aspect in favour of a chimeric vaccine is the potential possibility of its translation to human oncology.

In this study the survival time of dogs vaccinated with the chimeric plasmid was significantly longer ($p = 0.009$) compared with the control group dogs. When survival time of dogs of group A and B were compared, dogs vaccinated with HuDoCSPG4 lived longer compared with those vaccinated with HuCSPG4, nevertheless this result was not statistically significant ($p = 0.87$).

Authors are aware that the disease-free interval would be a superior endpoint for evaluating the efficacy of the electrovaccination since is not influenced by the owner's decision. In this study the disease-free interval was not considered as an accurate end point for two essential aspects. Firstly, this endpoint could be deeply influenced by the diagnostic imaging accuracy used for re-staging during the vaccination protocol. In fact, more than 50% of dogs of group B were restaged by thoracic radiographs, and this was different from group A dogs that were restaged with CT-scan. This is due to in part to the evolution of the restaging procedure offered to owners and in part to equipment availability. Regarding this, it should also be noted that dogs of group C (control group) were rechecked much less frequently compared with vaccinated dogs. Both these aspects could have potentially led to a later detection of progressive disease (local recurrence and/or metastatic disease) with consequent potential undervaluation of dogs of both dogs of group B (metastasis) and C (both local recurrence and metastasis) compared with those of group A. Bearing in mind these aspects, a further limitation of the study is the comparison of treated groups with historical control not vaccinated groups. Dogs receiving the vaccine and fulfilling the inclusion criteria may represent potential better responders leading to an overestimation of the vaccine's effect. Methodological differences in outcome assessment, staging method and timing of staging may results in selection and detection bias.

No significant difference in outcome were evident when the two different vaccination protocols (ministerial vs. monthly) were compared, even though dogs that received the ministerial protocol appeared to live longer in comparison with those undergoing the monthly one. Previous studies on anti-CSPG4 electrovaccination have demonstrated that the vaccine is able to produce an effective level of antibodies against HuCSPG4 starting from the 3^{rd}-4^{th} vaccination, being capable of binding the antigen and likely inducing its down-modulation, thus impairing the tumorigenic properties (Riccardo et al., 2014; Piras et al., 2017); this has appeared also true for HuDoCSPG4 (paper submitted under revision [2021/2022]). Additionally, the level of antibodies remained high and stable for months despite dogs did not undergo a monthly vaccination (paper submitted under revision [2021/2022]). Considering this prolonged humoral protection, the *ministerial* protocol is preferred since it implies less anesthesia sessions, being more convenient both under a financial (as the owners have to cover the expenses of the vaccination as no specific funds are available) and practical points of view.

Dogs administered with metronomic chemotherapy showed shorter survivals compared with dogs not receiving it, but longer than control group. Despite this result was not statistically significant it reflects the more advanced stage of OMM (distant metastasis) that has justified its use. Understanding if metronomic chemotherapy may help to improve the outcome in stage IV OMM may be the next goal even though the precise impact of metronomic chemotherapy cannot be clearly delineated because of the combination with electrovaccination. Additionally, there is no equivalent group of dogs in the control arm. Nevertheless, after that the anti-CSPG4 electrovaccination has delayed the appearance of the metastatic spread, the feeling is that metronomic chemotherapy may play a role in delaying, in many cases, a further and rapid tumor progression.

Disease clinical stage obtained according to World Health Organization criteria has been recognized as a prognostic indicator for survival time in dogs with OMM (Harvey et al., 1981; Bostock, 1979). Although there was no significant association between disease stage II and III survival times of vaccinated dogs of group A and control group dogs, vaccinated dogs with stage II-III OMM displayed a longer survival time than dogs with stage II-III OMM of control group. Considering the p value close to a significance level ($p = 0.07$ for stages II, $p = 0.06$ for stages III) one possible explanation of these not significant statistical results may be the limited number of dogs in group A (30 stage II and 23 stage III) and group C (11 stage II and 16 stage III). Despite this, these data may support the perception that the HuDoCSPG4 vaccine efficiency, and the consequent associated immune response, is strengthened in early-stage patients, i.e., in those dogs having the local disease controlled and thus displaying a longer survival time. Additionally, as also mentioned in the previous paper (Piras et al., 2017), the different procedure of lymphadenectomy (bilateral vs ipsilateral; only mandibular, mandibular and retropharyngeal) may have modified the exact definition of the N status of the TNM staging, thus impacting the final clinical stage. This limitation is due to the progressive changes of the standard care treatment over the time.

None of the dogs of group A developed local and/or systemical adverse reaction after the vaccine injection. These data were collected at the day of electrovaccination and from owners' reports.

One important limit of this study is that dogs of group A were enrolled prospectively but without any randomization, therefore a possible selection bias should be considered. However, as for most veterinary studies, the proposal of treatments different from the standard of care, are deeply influenced by the owners' compliance, especially if expenses are not covered by dedicated funds. Similarly, regarding the comparison between the control group and the vaccinated groups of dogs, it should be noted a possible further bias consisting of the advantage in favour of vaccinated patients (especially dogs of group A) in terms of diagnostic imaging during the follow-up. In fact, different imaging procedures were often used for tumor restaging, undergoing the vaccinated dogs a closer and often more efficient monitoring than dogs of the control group; this may have led to an underestimation of tumor progression in dogs of the control group and partially in dogs of group B; in the latter, in fact, CT was used less frequently than dogs of group A.

Nevertheless; even considering the above-described limitations concerning the study design, the results and in particular the longer survival of dogs of group A compared with the group C presented here support and confirm the safety and efficacy of the anti-HuDoCSPG4 adjuvant vaccination in dogs with OMM. Additionally, the results obtained here are in line with those obtained with the

anti-HuCSPG4 adjuvant vaccination in a similar cohort of dogs (Riccardo et al., 2014; Piras et al., 2017). Finally, these data should be still considered cautiously as the number of dogs included is still limited.

Project 2

Evaluation of prognostic impact of pre-treatment neutrophil to lymphocyte and lymphocyte to monocyte ratios in dogs with oral malignant melanoma treated with surgery and adjuvant CSPG4-antigen electro vaccination: an explorative study

INTRODUCTION

Malignant melanoma (MM) represents an aggressive disease in both human and canine patients. The oral cavity is the most common localization in dogs (Bergman, 2007; Simpson et al., 2014). Canine oral malignant melanoma (COMM) invades locally and is characterized by a high and early metastatic potential to regional lymph nodes and lungs, although other sites may also be affected. The clinical-biological behavior of MM in dogs may be predicted based on prognostic factors including anatomic site, size, and clinical stage (Smith, 2002; Bergman et al., 2020; Harvey et al., 1981). Other prognosticators include Ki67, mitotic index, nuclear atypia, degree of pigmentation, and platelet-derived growth factor receptor (PDGFR)-α/−β co-expression (Smedley et al., 2011a, Bergin et al., 2011, Iussich et al., 2017).

Conventional therapies such as surgery and/or radiation are finalized to local tumor control. Nevertheless, metastatic disease still remains the major cause of death (Tuohy et al., 2014; Boston et al., 2014) with adjuvant dose-intense chemotherapy appearing to be not determinant in prolonging survival (Rassnick et al., 2001; Boria et al., 2004; Brockley et al., 2012; Dank et al., 2014). For these reasons, and also considering the high immunogenicity of MM (Modinao et al., 1999), immunotherapy with different types of vaccine has been proposed as an adjuvant, systemic therapeutic tool, with encouraging results over the last few years (Piras et al., 2017; Grosenbaugh et al., 2011; Treggiari et al., 2016).

It is recognized that inflammation plays a central role in cancer development and progression (Hanahan & Weinberg, 2011). Systemic inflammation is associated with alterations in peripheral blood leukocytes that can be captured by the neutrophil to lymphocyte ratio (NLR) (Zahorec, 2001). The prognostic impact of NLR has been reported in various human cancers including melanoma, for which an increased NLR has been associated with a shorter survival (Templeton et al., 2014; Zaragoza et al., 2016). Specifically, lymphocytes are crucial for building an effective humoral and cellular response directed against the tumor; on the other side monocytes recruited within the tumor produce growth factors and cytokines leading to immunosuppression and angiogenesis. All this creates an optimal tumor microenvironment. In addition, according to a recent human meta-analysis, lymphocyte to monocyte ratio (LMR) seems to be an independent predictor of survival in several solid (nasopharyngeal, gastrointestinal, urinary and lung cancers) and haematological (lymphoma) malignancies, with low LMR rep-resenting a negative prognostic factor (Nishijima et al., 2015, Porrata et al., 2012).

In veterinary medicine, leukocyte counts and ratios have also been investigated as a potential prognostic and diagnostic biomarker for several malignancies including lymphoma, soft tissue sarcoma, osteosarcoma and mast cell tumors (Chiti et al., 2020; Sottnik et al., 2010; Macfarlane et al., 2016; Skor et al., 2017; Macfarlane et al., 2016), but no information is available regarding COMM. In this explorative retrospective study, we aim to evaluate the potential prognostic impact of pre-treatment NLR and LMR in COMM, assuming that a greater value of NLR and lower value of LMR would be associated with a decreased survival. We also aim to establish an NLR and LMR cut off associated with the outcome of MM canine patients.

MATERIALS AND METHODS

Case selection

Medical records of client-owned dogs referred to the Veterinary Teaching Hospital of the University of Torino from September 2009 to December 2019 were retrospectively reviewed searching for animals affected by COMM. All the patients enrolled in this study received adjuvant CSPG4-antigen electrovaccination, according to different protocols. Clinical staging was performed by total body CT scan, alternatively three view chest radiographs and abdominal ultrasound were obtained. Local treatment consisted of *en-bloc* surgical resection (maxillectomy, mandibulectomy, lip/cheek excision followed by reconstruction). The inclusion criteria were histological diagnosis of COMM surgically resected, availability of pre-treatment haematology with leukocyte differential count performed within 60 days before surgery, absence of distant metastatic disease (M1 of the TNM clinical staging system) and follow-up data of least 6 months. Dogs were excluded if, within 2 months before the definitive tumor treatment, were affected by a concomitant pathological condition able to compromise the minimum follow-up of 6 months and were administered with antibiotics and/or corticosteroids that could have interfered with NLR and LMR. For each patient the following parameters were recorded: signalment, tumor location, type of local treatment, TNM (Owen, 1980), stage and outcome (cause of death and overall survival time [OST]). OST was defined from the day of surgery to the date of death or euthanasia, reporting if it was or not COMM-related. During the adjuvant vaccination protocol all the dogs were re-staged with radiographs or computed tomography. Given the different diagnostic accuracy of the two techniques in identifying pulmonary metastasis, only the OST was chosen as end point in our statistical analysis. Follow-up information was collected from medical records or by phone conversation with referring veterinarians and dog's owners. Animals alive at the end of the study or lost to follow-up were censored at the date they were last known to be alive. A written consent was obtained from the owners for the anaesthetic, diagnostic, histological and surgical procedures. Regarding the adjuvant anti-CSPG4 DNA electrovaccination (started on 2009), dogs were treated according to the Good Clinical Practice guidelines for animal clinical studies. Both the Ethical Committee of the University of Turin and the Italian Ministry of Health approved the study; before entering the study, a written consent was always obtained by the dog’s owners.

Histopathology, blood collection and calculation of leukocyte ratios

For each COMM specimen, degree of pigmentation (<50% or ≥ 50%of pigmented cells), immunohistochemical analysis for Ki67 expression (using polyclonal Ki67 antibodyA-047, DAKO; <19.5% or ≥ 19.5%), mitotic count (MC, <4/10 high-power fields [hpf] or ≥ 4/10 [hpf]), nuclear atypia (<30 or ≥ 30% atypical nuclei in 200 cells counted), were evaluated (Bergin et al., 2011; Smedley et al., 2011). CSPG4 immunohistochemical score (from 0 to 8) was also available for all COMM samples and obtained as previously reported (Mayayo et al., 2011). Histologic evaluation of excision margins, bone invasion and presence of ulceration in the postsurgical samples were also considered (Kamstock et al., 2011).

An additional immunohistochemistry for CD3+ lymphocytes was also performed on 10 samples randomly selected among COMM samples. Pre-treatment haematological analysis on whole blood in ethylenediaminetetraacetic acid (EDTA) anti-coagulant, including complete blood count and leukocyte differentials, were performed by different laboratories. To verify instrumental results, May-Grunwald-Giemsa stain blood smears were assessed by clinical pathologists. Since different analysers were used, we considered universally accepted reference intervals (Rizzi et al., 2011) as reported in Table 1. NLR and LMR were calculated as the ratio of the absolute count of neutrophils to lymphocytes and absolute count of lymphocytes to monocytes, respectively. Regarding the clinical stage based on the TNM system (Bergman, 2007; Owen, 1980) authors evaluated its impact on leukocyte ratios considering stage I to II ($T_{1,2}$, N0, M0) vs stage III to IV (Any T, any N, M0). This stage division allowed to investigate lymph nodes involvement as a prognostic factor since none of our cases was classified as T3N0M0 (stage III without lymph nodes involvement), consequently all dogs with COMM staged I to II were free of lymph node metastasis unlike dogs with COMM staged III to IV. In addition, the impact of the T variable on NLR and LMR was carried out considering it individually (tumor size T1if ≤2 cm, T2if between 2 and 4 cm, and T3if >4 cm).

Table 1 Hemogram reference intervals

Haematological value	References Intervals
White blood cell count (WBCC)	6.0–17×10^3/μL
Neutrophil count (NC)	3.0–11.5×10^3/μL
Lymphocyte count (LC)	1.0–4.8×10^3/μL
Monocyte count (MC)	0.15–1.35×10^3/μL

STATISTICAL ANALYSIS

Statistical analysis was applied to explore correlations between NLR and LMR with age, tumor pigmentation, T variable (tumor size), nuclear atypia, mitotic index, Ki67, CSPG4 expression, ulceration, bone invasion, excision margin status, and clinical stage. Statistical analyses were performed with R, a free software environment for statistical computing and graphics (version 3.6.3) and a P-value ≤ .05 was considered significant. Wilcoxon-Mann-Whitney rank sum test was used to evaluate NLR and LMR differences in classes based on stage/lymph nodes involvement (stage I-II vs stage III-IV), nuclear atypia, pigmentation, ulceration (presence/absence), margins (infiltrated vs not infiltrated) and bone invasion (presence/absence); Kruskal-Wallis rank sum test was applied to highlight differences in NLR and LMR values across subgroups based on the T variable ($T_{1,2,3}$ based on the TNM system);correlations between NLR and LMR with MI, Ki67, CSPG4, T and age(continuous variables) were evaluated by Spearman's rank correlation rho. The median OST was estimated by Kaplan-Meier method and a log-rank test was used to compare the survival distribution of samples. Patients that died for causes different from COMM were censored from survival statistical analysis. Median time to follow-up for the entire population was calculated from the day of surgery. The best cutoff for NLR and LMR in terms of potential prognostic impact was estimated by cut pointr package (Thiele, 2019). After having obtained the best cut off for NLR and LMR, patients were divided into two classes above or under the cut off ratio in order to investigate a possible prognostic impact on survival. In addition, Kaplan-Meier curves were performed and settled considering three different end points of blood sample collection, in particular at 15, 30 and 60 pre-operative days.

RESULTS

Patients characteristic

A total of 86 dogs were retrieved from medical records, but only 39 dogs met all the inclusion criteria. The main reason of exclusion was unavailability of pre-treatment haematology with manual count within 2 months before definitive treatment. Median age was 11 years (range 8-14 years) and median weight was 25 kg (range 3-46 kg). Clinical staging identified six COMMs staged I, 17 staged II, 14 staged III and two staged IV (M0). According to the T variable of the TNM system, 20 COMMS were T1, 16 were T2 and three were T3. Thirty-five dogs underwent definitive *en-bloc* excision of the primary tumor and regional lymphadenectomy, whilst four dogs had an excisional biopsy performed by the referring veterinarians. At the end of the of study, 9 (23%) dogs were still alive and 30 (77%) were dead. Out of 30 dogs, 19 were dead because of COMM, in particular four for local recurrence, 13 for distant metastasis (lungs, brain) whilst two dogs were euthanized because of both. Eleven dogs succumbed for other causes: car accident (n = 1), chronic kidney disease (n = 3), neuro-orthopaedic disease (n = 1), prostatic transitional cell carcinoma (n = 1), laryngeal carcinoma (n = 1), laryngeal paralysis (n = 1), gastric ulcer (n = 1) and 2 dogs for unknown rea-sons. Median OST was 943 days (range 68-2603 days, Figure 1). Median survival time for dogs that died for COMM was 412 days (range 68-972), median survival time for dogs that died for causes other

than COMM was 776 days (range 173-1768). Median time to follow-up for the entire population was 494 days (range 68-2603).

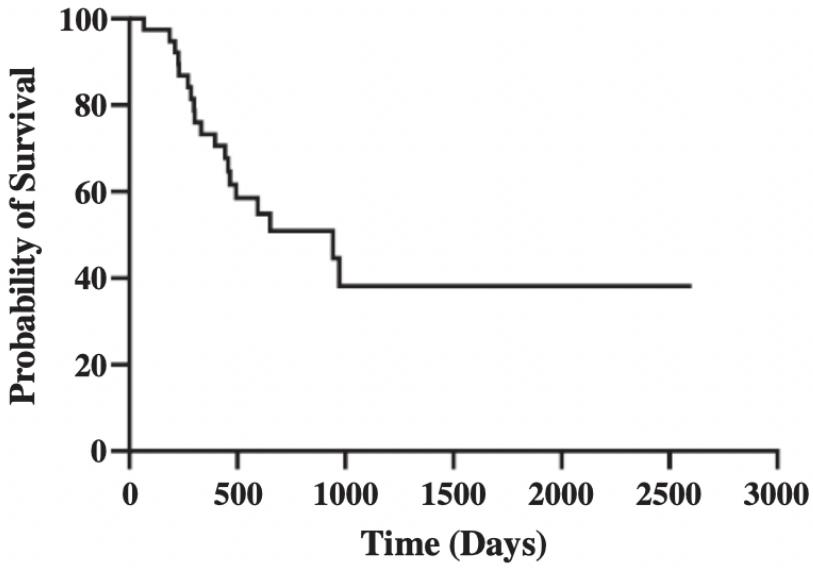


Figure 1

Kaplan-Meier estimated survival probability of the entire population of dogs with COMM. COMM, canine oral malignant melanoma

Histology and haematology

Histopathological data for pigmentation, nuclear atypia, MI and immunohistochemical expression scores for Ki67 and CSPG4 are summarized in Tables 2 and 3. Ki67 expression resulted undetectable in one case. Median MI was 12 (range 0-55), median tumor size was 2.5 cm (range 0.5-6). Twenty-one COMMs out of 39 presented ulceration. In two cases this information was not available. Excision surgical margins not infiltrated histologically in 28 cases, infiltrated in 7 and not known in 4 cases. Histologically, non infiltrated margins exceeded 2 mm in all samples. On histology report bone invasion was detected in 22 out 39 COMMs. CD3+ immunohistochemical analysis detected only mild multifocal lymphocyte infiltration and inflammation in the 10 randomly chosen out of our 39 COMM samples; lymphocytes appeared more numerous in the superficial corion (not associated with the tumor), being mostly localized within ulcerated areas rather than intratumoral. Pre-treatment haematology analyses were performed after a median of 12 days (range 0-60 days) before the definitive treatment. Four dogs showed leukocytosis with neutrophilia, two further dogs revealed only neutrophilia, three dogs had lymphopenia, of which one with monocytosis and one with monocytopenia. One dog displayed monocytosis. All these changes were classified as mild, except in one case in which the neutrophilic leukocytosis was marked. The hemograms of these patients are shown in Table 4. All the others 29 (74%) dogs exhibited values within normal limits. One dog was on medication for idiopathic epilepsy whilst three further dogs had concurrent chronic renal concurrent disease.

Table 2 Histological and immunohistochemical parameters of COMM from dogs included in the study

Parameters	Threshold	Overall population
Degree of pigmentation	<50%	35 (90%)[a]
	≥50%	4 (10%)[a]
Nuclear atypia	<30%	12 (30%) [a]
	≥30%	27 (70%)[a]
Mitotic index (MI)	<4/10 hpf	8 (20%)[a]
	≥4/10 hpf	31 (80%)[a]
Ki67	<19.5%	11 (29%)[a]
	≥19.5%	27 (71%)[a]

[a] % in brackets

Table 3 Immunohistochemical CSPG4 score of COMM from dogs included in the study

CSPG4 score	patients n=39
3/8	4
4/8	7
5/8	5
6/8	7
7/8	11
8/8	5

Table 4 Patients with hemogram that underlined alterations

	WBCC (103/µL)	NC (103/µL)	LC (103/µL)	MC (103/µL)
Patient 1	15.3	**11.8***	1.5	0.9
Patient 2	16.2	**11.7***	3.1	0.6
Patient 3	11.5	9.4	**0.8***	**1.5***
Patient 4	6.8	5.5	**0.8***	**0.1***
Patient 5	**33.1***	**28.1***	2.0	1.0
Patient 6	**18.4***	**14.5***	1.5	0.7
Patient 7	**20.6***	**16.2***	2.9	0.9
Patient 8	**18.7***	**16.3***	1.5	0.8
Patient 9	13.5	8.6	2.6	**1.8***
Patient 10	8.5	6.5	**0.9***	0.7

* in bold values outside reference ranges.

Leukocyte ratios and cut off

Median NLR and LMR for all patients was 3.5 (range 1-14.2) and 3.8 (0.6-16), respectively. NLR did not display association with the stage I to II (i.e., COMMs without lymph node involvement) vs stage III to IV (i.e., COMMs with lymph node involvement) ($p = 0.30$) and this occurred also for LMR ($p = 0.21$). No significant correlation was found between NLR and LMR with the histological/immunohistochemical parameters (pigmentation, nuclear atypia, ulceration, bone invasion, Ki67, CSPG4 and MI), excision margin status (infiltrated vs not infiltrated) and age. Bivariate models with all correlation coefficients between histological parameters and NLR and LMR are illustrated in Tables 5 and 6, respectively. No statistically significant differences among groups based on the T variable (tumor size) for NLR ($p = 0.97$), LMR ($p = 0.19$) and T1,2,3 (based on TNM) were found. Through statistical software (cutpointr), an optimal NLR and LMR cut off were identified in 2.792 and 3.622, respectively. Dividing all the patients into 4 groups with NLR >2.792, NLR <2.792, LMR >3.622, LMR <3.622, and comparing groups for OST, the estimated cut off was not associated with survival (Figure 2). No statistical correlation was found considering the entire population and survival time regardless of NLR ($p = 0.22$) and LMR ($p = 0.92$) cut off values, having blood samples collected within 60 days. Lastly, no statistical correlation was found between leukocyte ratio OST of dogs whose blood sampling occurred at 15 days (NLR $p = 0.67$, LMR $p = 0.38$) and 30 days (NLR $p = 0.66$, LMR $p = 0.61$).

Table 5 Association between neutrophil to lymphocyte ratios (NLR) with stages, histological parameters and age in bivariate analysis

Variable	P-value	Rho
NLR vs stage I-II/ stage III-IV	.30[b]	-
NLR vs pigmentation	.55[b]	-
NLR vs nuclear atypia	.54[b]	-
NLR vs ulceration	.71[b]	-
NLR vs margins (infiltrated/not infiltrated)	.29[b]	-
NLR vs bone invasion	.09[b]	-
NLR vs Ki67	.17	-0.22[c]
NLR vs CSPG4	.29	0.17[c]
NLR vs MI	.76	0.04[c]
NLR vs age	.48	-0.11[c]
NLR vs T (tumor size)	.97	0.05[c]
NLR vs $T_{1,2,3}$	.27[b]	-

[b] Wilcoxon-Mann-Whitney rank sum test, Kruskall-Wallis rank sum test

[c] Spearman's correlation coefficient

Table 6 Association between lymphocyte to monocyte ratio (LMR) with stages, histological parameters and age in bivariate analysis

Variable	P-value	Rho
LMR vs stage I-II/ stage III-IV	.21[b]	-
LMR vs pigmentation	.76[b]	-
LMR vs nuclear atypia	.30[b]	-
LMR vs ulceration	.68[b]	-
LMR vs margins (infiltrated/not infiltrated)	.41[b]	-
LMR vs bone invasion	.09[b]	-
LMR vs Ki67	.26	0.18[c]
LMR vs CSPG4	.16	-0.22[c]
LMR vs MI	.77	-0.04[c]
LMR vs age	.38	0.14[c]
LMR vs T	.19	0.20[c]
LMR vs $T_{1,2,3}$	.11[b]	-

[b] Wilcoxon-Mann-Whitney rank sum test, Kruskall-Wallis rank sum test

[c] Spearman's correlation coefficient

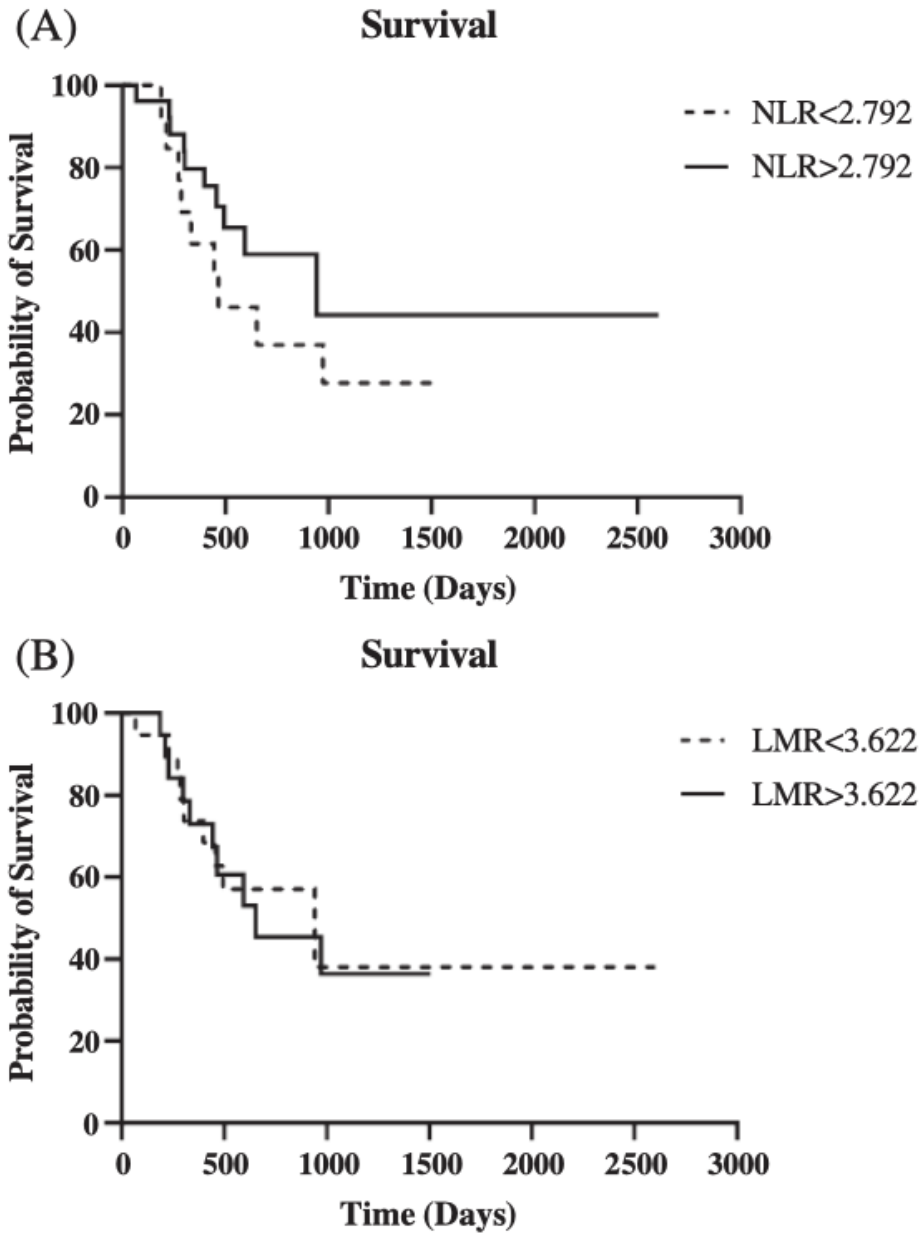


Figure 2

Kaplan-Meier curves considering NLR and LMR cutoff. A, Survival in days of dogs with COMM and a NLR cut off <2.792(dotted lines) and NLR >2.792 (continuous line). B, Survival in days of dogs with COMM and a LMR cut off <3.622 (dotted lines) and LMR>3.622 (continuous line). COMM, canine oral malignant melanoma; LMR, lymphocyte to monocyte; NLR, neutrophil to lymphocyte.

DISCUSSION

This is the first report assessing the prognostic impact of pre-treatment NLR and LMR in dogs with COMM and the first attempt to explore a possible correlation between NLR and LMR with CSPG4 and well-known prognostic factors for COMM such as nuclear atypia, pigmentation, Ki67, MI, clinical stage and lymph node metastasis. Considering the explorative nature of the study also age, tumor ulceration, excision margin status and T variable (tumor size) were included in the analysis. In this sample population of dogs with COMM, pre-treatment NLR did not display a prognostic impact on OST. This finding is in apparent contrast with previous studies conducted on human melanoma in which the prognostic and predictive role of NLR has been investigated over the last years (Templeton et al., 2014; Koh et al., 2015).

In a recent systematic review, an elevated NLR has been recognized as a poor prognostic indicator in various types of cancer. Specifically, NLR was elevated in patient with advanced disease evidenced by increased tumor stage, nodal status and extent of the disease (Guthrie et al., 2013).

In the present study, the authors decided to consider the NLR and LMR rather than the absolute neutrophils, lymphocytes and monocytes count in order to reduce the possible variations of the single parameters. In fact, the number of neutrophils may change daily, being not always in line or proportional with lymphocytes. It has also been reported that NLR ratio can better reproduce fluctuations between neutrophils and lymphocytes (Szkandera et al., 2013). The exact mechanism that binds elevated NLR and poor outcome in human melanoma patients remains unclear. Tumor promoting inflammation is one fundamental hallmark of cancer (Hanahan & Weinberg, 2011).

In patients with advanced stage of cancer, the systemic inflammatory response leads to a change in blood cell composition that suggests an expansion of myeloid component (neutrophils and monocytes) caused by demargination, delayed apoptosis opposed to reduction of the lymphoid compartment as a result of margination, and accelerated apoptosis (Zahorec, 2001). It seems that one of the tumorigenesis pathways mediated by neutrophils consist in the release of cytokines by melanoma cells that interface with receptors expressed on both neutrophils and melanoma cells through an autocrine effect (Dhawan & Richmond, 2002). Cytokines such as vascular endothelial growth factor (VEGF), tumor necrosis factor α(TNF-α), interleukin-2 (IL-2), interleukin-6 (IL-6), and interleukin-10 (IL-10) act as inflammatory mediators providing an optimal tumor microenvironment for cancer progression (Qu et al., 2018). Indeed, it has been widely recognized that tumor and its microenvironment direct neutrophils polarisation and recruitment towards tumor tissue (Masucci et al., 2019). Cancers related-neutrophils, circulating and tumors associated (TANs), are characterized by some functional plasticity. Thus, under the effect of cytokines, epigenetic modifications and microenvironment factors, neutrophils can acquire specific anti-tumor and pro-tumor functions and phenotypes.

The first type of neutrophils (N1) exerts anti-tumor activity through direct and indirect cytotoxicity. The second type (N2) induces immunosuppression promoting tumor growth, angiogenesis and metastasis (Masucci et al., 2019; B Shaul & Fridlender, 2019). A direct comparison between TANs and circulating neutrophils has not been assessed yet, probably because of the heterogeneous level of neutrophils' infiltration in different cancers. However, in a study performed in human patients with pancreatic cancer, the group with high NLR showed high density of infiltrated macrophages

and neutrophils compared with the low NLR group (Takakura et al., 2016). At the moment, correlation between NLR and neutrophils tumor infiltration cannot be predicted but worth to be further investigated. In this study, 74% of our patients did not display alterations of their pre-treatment neutrophil and lymphocyte counts and this might explain why NLR did not show any prognostic significance. In human oncology the strongest association between NLR and outcome has been found in cancers such as mesothelioma, where chronic inflammation covers a crucial role in tumor pathogenesis (Pinato et al., 2012). A similar result was found by Chiti et al., in feline injection-site sarcoma, a tumor in which chronic inflammation plays a significant role as well (Chiti at al., 2020). In that article, NLR seemed to be significantly related to OST and disease-free interval in univariate analysis. On the human side, it has also been established that baseline NLR is strongly and independently associated with outcome of melanoma patients treated with ipilimumab. Specifically, it has been found that patients with NLR≥3 had a significant risk of death and disease progression compared with those with lower NLR (Ferrucci et al., 2015; Ferrucci et al., 2016). Furthermore, in line with previous results, it has been found that, in humans with melanoma, a value of NLR ≥4 before starting ipilimumab treatment is associated with poor overall survival (Zaragoza et al., 2016). As for NLR, also the LMR has been widely investigated as prognostic tool in different types of solid cancers including metastatic melanoma corroborating the concept that a low LMR is associated with poor prognosis and shorter survival (Nishijima et al., 2015; Guthrie et al., 2013). Interestingly, a recent paper has examined the dynamics in the immune system in stage IV melanoma patients, obtaining serial concentrations and measurements of cytokines and immune cell subtypes in peripheral blood. It has been found that LMR in many human melanoma patients had an oscillatory pattern and the progression free survival was significantly improved in those patients who received chemotherapy on the day LMR was elevated. Recent data suggest that, in melanoma advanced patients, the systemic immunity undergoes to fluctuations and the immune system is oriented towards a state of inflammation where states of responsiveness are alternated to tolerance states (Leontovich et al., 2017). This aspect highlights that a single time-point measurement might be too limited to represent at the best the complex balance between tumor and immune system. Serial NLR and LMR values obtained from samples collected at different time points, closer to the day of first treatment, would probably better reflect the complex balance between the host's pro-tumor neutrophils and the anti-tumor lymphocytes effect. In order to explore if blood sample time represents a relevant factor in terms of leukocyte variations and prognostic impact, we performed our statistical analysis on leukocyte ratios and OST selecting patients in which blood sample was collected at a different time point, that is, at 15, 30, and 60 days; nevertheless, no correlations were identified.

In the present study, no significant association of NLR and LMR with OST was observed. However, one aspect needs to be considered regarding patient selection. Most of human studies regarding leukocyte ratios have been performed, before and after the era of immune checkpoint inhibitors, in patients with advanced metastatic melanoma. However, some authors also investigated the prognostic relevance of peripheral blood cell count and their ratios in human melanoma patients at any stage, including early stage. They found that peripheral blood counts and ratios were not associated with survival of patients with localized or regionally metastasized melanoma; besides, blood cell counts were also similar among newly diagnosed patients and patients with recurrent or

progressive disease. On disease progression from regional to distant metastatic disease (stage IV), NLR increased, LMR decreased and they were significantly associated with overall survival in multivariate analysis (Gandini et al., 2016). All the dogs included in our study had evidence of metastasis, if any, not beyond the neck (M0), there-fore involving only the draining lymph nodes of the neck (mandibular and/or retropharyngeal) and/or tonsil; this criterium has always been one of our inclusion parameters for immunotherapy. In line with this, it becomes important to remark that the two dogs with COMM stage IV included in the study had only lymphatic bilateral metastasis at the level of the neck, therefore they were both T2N2bM0, according to the WHO staging system (Owen, 1980). In this study, neither the clinical stage nor lymph node involvement revealed an association with NLR and LMR. Itis possible that a prognostic significance of NLR and LMR may be reached as soon as a distant metastatic progression develops, but not in the early stages, that is, when COMM is still confined to the oral cavity and/or regional lymph nodes. Authors are absolutely aware of the limitations that the present WHO TNM system implicates, which may lead to an imprecise staging of oral tumors. Besides, in this system the histological parameters are not taken into account and the tumor size is not proportioned to the size of the patient, thus leading to a lack of standardization. For this reason, several histological parameters as well as the T variable were also included in the statistical analysis, substantiating the explorative aspect of the study.

An additional critical issue in the present TNM system is the potential inaccuracy of the prognostic evaluation of clinical stage IV as this may include both dogs with (M1) or without distant metastasis (M0), including the latter dogs with only bilateral lymphatic metastasis at the level of the neck. Regardless the treatment applied, median OST was 943 days (range 68-2 603 days). This outcome is doubtless prolonged when compared with the previous outcomes reported for dogs with COMM (Boston et al., 2014).

Authors could speculate that this positive outcome might be partially related to normal pre-treatment haematology analysis. However, it is important to consider that this positive outcome may also be relate d to the efficacy of the multimodality treatment (local treatment and immunotherapy) applied in this series of dogs (Piras et al., 2017). No correlation was identified between NLR and LMR with known prognostic factors such nuclear atypia, pigmentation, MI and Ki67. Itis possible that these parameters do not or do minimally influence the leukocyte count in the same way as the outcome. The same consideration can be carried out about bone invasion and excision margin status despite their recognized negative impact on prognosis (Hanh et al., 1994; Proulx et al., 2003).

A further aspect that merits to be addressed is that an elevated T of the TNM system might be associated with a major risk of ulceration because of chronic trauma and possible inflammation; nevertheless, ulceration has not been identified as a prognosticator in oral melanoma and did not show any correlation with leukocyte ratios (Bergin et al., 2011; Hahn et al., 1994).

In this study, those patients with ulcerated COMMs did not display any abnormalities on their haematology pre-treatment analysis (data not shown). The rationale behind performing CD3+ immunohistochemistry was an attempt to explore a possible correlation between local and systemic immune responses, triggered by tumor ulceration and inflammation, by comparing tumor infiltrating lymphocytes (TILs) and peripheral blood hematologic parameters not yet investigated in

COMM. Interestingly, a relevance between TILs and hematologic parameters in breast cancer was demonstrated where LMR revealed significant correlation with CD8+ (Lee et al., 2018).
However, a previous study has shown that, despite tumor infiltrating lymphocytes have been described in most melanocytic tumors, lymphocytic infiltration is usually mild (Porcellato et al., 2020).
These data might be explained with high tumor mutational burden of melanoma and low expression of tumor-associated antigens by neoplastic melanocytes. Both factors could lead to a reduced immune stimulus for lymphocytes within the tumur and weak local anti-tumor immune response (Pitcovski et al., 2017; Wang & Li, 2019).
In this study, no significant correlation was found between age and leukocyte ratios. In humans it is widely accepted that age represents an independent prognostic factor for melanoma specific survival (Weiss et al., 2016). Furthermore, NLR has been found to correlate positively to age; this data is due to an upward trend of granulocyte count and lymphocyte count decline with age (Li et al., 2015). In contrast with this, in our study more than 80% of dogs older than 10 years had leukocyte counts within normal limits, but it should be outlined that this population of dogs may be too small for make any assumption. Finally, regarding CSPG4, a transmembrane glycoprotein expressed on melanoma cells and involved in tumor cell proliferation, migration and invasion (Price et al., 2011), its immunohistochemical positivity ≥3 was not statistically correlated with OST (Piras et al., 2017).
Even in this study, as previously reported (Piras et al., 2017), the CSPG4 score was not correlated with NRL and LMR, neither with OST. This explorative study has several limitations; first of all, the number of patients is small to draw a final conclusion regarding NLR and LMR prognostic significance. Further limitations are the retrospective nature of the study and the lack of serial preoperative haematological evaluations. Therefore, the results obtained here should be considered cautiously. However, in our hands, pre-treatment NLR and LMR in dogs with COMM treated with surgery and immunotherapy were not associated with survivals when the evaluated hemograms preceded the definitive treatment. Nevertheless, as in human melanoma, it would be interesting to explore if there is any prognostic impact or correlation with survivals when patients develop distant metastasis. The true impact of NLR and LMR on the prognosis for oral canine MM needs to be better defined by further prospective studies involving a larger sample population.

Additional information

The present study has recently been published as:

Camerino M, Giacobino D, Iussich S, Ala U, Riccardo F, Cavallo F, Martano M, Morello E, Buracco P. Evaluation of prognostic impact of pre-treatment neutrophil to lymphocyte and lymphocyte to monocyte ratios in dogs with oral malignant melanoma treated with surgery and adjuvant CSPG4-antigen electrovaccination: an explorative study. *Vet Comp Oncol*. 2021 Jun;19(2):353-361. doi: 10.1111/vco.12679. Epub 2021 Feb 15. PMID: 33443307.

Project 3

Prognostic impact of bone invasion in canine oral malignant melanoma treated by surgery and anti-CSPG4 vaccination: a retrospective study on 68 cases (2010-2020)

INTRODUCTION

Malignant melanoma is the most frequent oral malignant tumor in dogs accounting for 30-40% of all canine oral malignancies (Liptak & Lascelles, 2012; Bergman et al., 2020; Liptak, 2020).
Sites of primary growth are gingiva, internal lip/cheek, palate, tongue and tonsil. Canine oral malignant melanoma (OMM) is an aggressive tumor and it has been reported that its behavior can be predicted by evaluating several clinical factors such as site of growth, size and clinical stage, leukocytes ratio (Bergman et al., 2020; Liptak, 2020; Spangler & Kass, 2006, Camerino et al., 2021), and histological and immunohistochemical factors such as Ki67 expression, mitotic count, degree of pigmentation, nuclear atypia and platelet-derived growth factor receptor expression (Bergin et al., 2011; Smedley et al., 2011a; Iussich et al., 2017). The reported metastatic rate to regional lymph nodes and distant sites such as lungs and other organs ranges from 30.3% to 74.0% (Liptak, 2020; Williams & Packer; 2003) and from 14.0% to 92.0%, respectively (Liptak, 2020).
The treatment of choice for local tumor control, if feasible, is wide surgical excision, regardless of whether or not there is bone invasion at presentation; the feasibility of an *en bloc* excision is influenced by both the tumor location and the size of dog, as a minimum of 1.5-2 cm up to 3 cm of macroscopically normal tissue all around the OMM should be excised (Liptak & Lascelles, 2012; Bergman et al., 2020; Liptak, 2020). Local tumor control is then surgically reached by also performing a neck lymphadenectomy (mandibular and medial retropharyngeal lymph nodes), ipsilaterally or bilaterally (Skinner et al., 2017; Grimes et al., 2019). The removal of lymph nodes followed by histological evaluation also allows for complete tumor staging, as lymph nodes with metastatic OMM may appear clinically and cytologically normal (Grimes et al., 2017; Giacobino et al., 2021). Radiotherapy should be considered as an adjuvant treatment for OMMs that are incompletely excised, as a primary treatment in combination with medical treatment for those cases deemed inoperable or when the owners refuse surgery (Freeman et al., 2003; Proulx et al., 2003; Boria et al., 2004; Murphy et al., 2005; Boston et al., 2014; Kawabe et al., 2015; Cancedda et al., 2016; Turek et al., 2020). An alternative to radiotherapy for local tumor control is electrochemotherapy, which may be contraindicated when bone invasion is already evident (Milevoj et al., 2019; Nemec et al., 2020; Tellado et al., 2020). The results derived from the addition of adjuvant chemotherapy (especially platinum-based agents), to control the metastatic spread, has been disappointing if compared to local tumor control only (Boria et al., 2004; Murphy et al., 2005; Boston et al., 2014; Rassnick et al., 2001; Borckley et al., 2013; Dank et al., 2014).
Thanks to the immunogenic features of melanoma, several studies dealing with immunotherapy have been recently carried out. Melanoma-associated antigens have been identified (e.g.,

tyrosinase and chondroitin sulphate proteoglycan 4 [CSPG4]) and utilized in producing vaccines capable of evoking an immune response against canine OMM (Bergman et al., 2003; Grosenbaugh et al., 2011; Riccardo et al., 2014; Piras et al., 2017; Tarone et al., 2019; Ottnod et al., 2013; McLean & Lobetti; 2015; Treggiari et al., 2016). In particular, the authors' focus has been on CSPG4, a cellular membrane antigen, characterized by restricted distribution in normal healthy tissues and high expression on neoplastic cells in both human and canine malignant melanoma. It coordinates several intracellular pathways regulating different cell functions (i.e. proliferation, migration and survival), thus being involved in tumorigenesis at multiple levels (Campoli et al., 2004; Yang et al., 2004; Price et al., 2011; Rolih et al., 2017). In addition, CSPG4 has also been shown to be overexpressed in human melanoma cancer stem cells and has been associated with poorer prognosis (Tarone et al., 2019; Mayayo et al., 2011). All these features make CSPG4 an ideal antigen to safely and effectively target. A recent paper has shown the advantage of the combination of *en bloc* excision plus adjuvant anti-CSPG4 vaccination in dogs with OMM (Giacobino et al., 2021).
Bone invasion, as detected by advanced imaging and/or histologically, has been reported to occur in up to 57.0% of cases (Liptak, 2020; Nishiya, 2016). However, its influence on prognosis remains to be clearly defined. The aim of this study is to retrospectively evaluate the prognostic impact of bone invasion in a population of dogs affected by stage II-III OMMs locally controlled by surgery and treated adjuvantly with anti-CSPG4 DNA electrovaccination.

MATERIALS AND METHODS

Patient selection and data collection

All dogs of this retrospective study were treated at the Teaching Veterinary Hospital of Grugliasco (Turin) from 2010 to 2020. Dogs with confirmed OMM on histopathology, staged II-III, that underwent surgery and adjuvant CSPG4 electrovaccination were eligible for entry into the study. Specifically, only dogs bearing an OMM with an immunohistochemical CSPG4 score ≥3/8 were considered as suitable candidates for vaccination (Mayayo et al., 2011). Good Clinical Practice guidelines for animal clinical studies were observed and both the Ethics Committee of the University of Turin and the Italian Ministry of Health approved the study (0004230-20/02/2018-DGSAF-MDS-P and 0015537-28/0 6/2017-DGSAF-MDS-P). A written consent form was signed by the owners before dogs' recruitment in the study.
Additional criteria for inclusion were a minimum follow-up of 1 year on April 1st 2021, no concurrent life-threatening disease and information on the presence/absence of bone invasion based on imaging and/or histology. Information retrieved from medical records for each dog included age, sex, breed, body weight, tumor localization within the oral cavity, tumor size and type of surgery performed. Pre-treatment work-up consisted of physical examination, blood count, serum biochemistry, cardiologic evaluation and urinalysis. Complete tumor staging was achieved by means of total body computed tomography (CT scan) for the majority of cases. Alternatively, skull, three view chest radiographs and abdominal ultrasound were obtained. The primary tumor was resected by performing an *en-bloc* excision (mandibulectomy, maxillectomy, lip-cheek excision followed by reconstruction) with regional (ipsilateral or bilateral) lymphadenectomy (Liptak & Lascelles, 2012;

Bergman et al., 2020; Liptak, 2020). Dogs were staged according to the World Health Organisation tumor/node/distant metastases (TNM) guidelines as illustrated in Table 1 (Bergman et al., 2020; Bergman, 2007).

Table 1 WHO staging system for canine oral melanoma

Stage I	Stage II	Stage III	Stage IV
≤2 cm diameter	2–4 cm diameter	>4 cm diameter	Any size
Negative lymph nodes	Negative lymph nodes	+/−Metastatic lymph node	Distant metastasis

Histological and immunohistochemical analyses

Formalin-fixed paraffin-embedded (FFPE) samples of OMM were stained with haematoxylin-eosin for diagnosis according to the tumor pathology guidelines (Ramos-Vara & Borst, 2017). The following histological and immunohistochemical data were recorded for all OMM samples: Ki67 expression (polyclonal Ki67 antibody A-047; DAKO; cut-off of 19.5), mitotic count in 10 high-power fields (MC; <4/10 high-power fields [hpf] or ≥4/10 hpf), nuclear atypia (quantification < or ≥ 30%), surgical margins infiltration status, presence of bone invasion, lymph node evaluation and CSPG4 score.

Immunohistochemistry (IHC) was performed on 4 mm thick paraffin sections. After blocking peroxidase activity (0.3% H_2O_2 in deionised water for 30 min) and heat-induced antigen retrieval (30 min with citrate buffer at 98°C, pH 6), sections were incubated with anti-Ki67 polyclonal antibody (Dako A-047; diluted 1:50) and anti-CSPG4 polyclonal antibody (Sigma Aldrich; diluted 1:40). Detection was performed using the Vector VIP Substrate kit for peroxidase (Vector Laboratories). In case of amelanotic neoplasms, immunohistochemistry with both Melan-A and PNL-2 antibodies was also performed. Ki67 index was assessed according to the methods previously reported by Bergin and colleagues. The previously published threshold of 19.5 was used to predict prognosis (Bergin et al., 2011). A total score ranging from 0 to 8 was assigned to each melanoma sample by adding the value that represented the proportion of CSPG4 positively stained tumor cells (score from 0 to 5) and the average staining intensity of CSPG4-positive tumor cells (score from 0 to 3) (Piras et al., 2017; Mayayo et al., 2011).

The presence of bone within the resection margins was not considered as an inclusion criterium as only the detection of bone invasion at imaging and/or histology was evaluated in the study. Specifically, bone infiltration at imaging was defined as minimal cortical disruption up to advanced destruction of cortex involving the medullary bone in some cases. Bone infiltration at histology was reported when tumoural cells were found within the bone, together with a variable degree of bone destruction. Simple periosteal reaction at imaging was not considered as bone invasion unless bone invasion was detected at histological examination.

Patients' groups

First, all dogs were divided into two groups: group 1 (OMMs with bone invasion) and group 2 (OMMs without bone invasion), regardless of localization. Additionally, the total population was divided into two other groups based on the site of growth and statistically evaluated. Patients with OMMs at the level of cheek, lip, tonsil, soft palate and tongue were included in group 3 (soft tissue group); patients with OMMs attached to the gingiva of either the lower or upper dental arcade were included in group 4 (hard tissue group). A subgroup of group 4, called group 5, consisted of OMMs that were localized to the gingiva but did not invade the bone. Thus, group 4 consisted of OMMs of group 1 (OMMs with bone invasion) and OMMs of group 5 (no bone invasion). The DNA electrovaccination procedure was performed in all the dogs of this study as only OMMs characterized by a CSPG4 immunohistochemical expression ≥3/8 (cut-off value chosen for enrollment in the immunization group) were included (Mayayo et al., 2011). Dogs, under brief general anaesthesia, were vaccinated with plasmids coding for the CSPG4 antigen. The vaccination was started 1 to 3 weeks after surgery and was repeated after 2 weeks and then monthly for a minimum of 6 and a maximum of 24 immunizations. The CSPG4-coding plasmids (500 μg in 200 μL of 0.03% NaCl) were injected into the muscles of the caudal thigh and, 2 minutes later, nine electric pulses were applied to the injection site using the CLINIPORATOR (Igea), an instrument approved for veterinary application. The dogs were monitored for acute, late local or systemic side effects (Riccardo et al., 2014; Piras et al., 2017; Tarone et al., 2019).

STASTISTICAL ANALYSIS

The analyses were carried out using GraphPad Prism (version 9.0.0 for Windows, GraphPad Software, San Diego, California, www.graphpad. com), with statistical significance set at a $p < .05$. For statistical purposes, the Shapiro–Wilk test was used to assess normality of distribution of the variables.

The DFI was calculated from the day of surgery to the first tumor recurrence or metastasis while the MST was calculated as the period from the day of surgery to the patient's death. DFI and MST were analysed through generation of Kaplan–Meier curves; log-rank test was used to compare DFI and MST of patients amongst different groups. Dogs which died from non-COMM-related causes, those lost to follow- up and those still alive at the end of the study were censored. Spearman's correlation was used to look for association between MST and MC or Ki67 of patients with and without bone invasion. Fisher's exact test was used to test for possible association between different groups and the probability of local recurrence and/or metastasis.

RESULTS

Signalment

Sixty-eight dogs fulfilled the inclusion criteria. There were 39 males (23 intact, 16 castrated) and 29 females (5 intact, 24 spayed). The mean and median age were 11.1 and 12 years (range, 6–14 years), respectively; mean and median weight were 19.8 and 18 kg (range, 3– 40 kg), respectively. Twenty-one breeds were represented: 5 Cocker Spaniels, 5 Golden Retrievers, 5 German Sheperd dogs, 4 Pinschers, 3 Pekingese, 3 Yorkshire Terriers, 2 Beagles, 2 Labrador Retrievers, 2 English bulldogs, 2 Dwarf Poodles, 2 Setters, 2 Shih Tzu dogs and one of each of Jack Russell Terrier, Hovawart, Alaskan Malamute, Shar Pei, Dwarf Schnauzer, Rottweiler, Spitz, Amstaff and West Highland White Terrier. The remaining 22 dogs were crossbreeds.

Staging and treatment

Clinical staging identified 38 stage II and 30 stage III OMMs; total body CT scan was performed in 54 cases (79.4%) and chest and skull radiographs in conjunction with abdominal ultrasound in 14 cases (20.6%). Tumor localization within the oral cavity of all patients is shown in Table 2.
Twenty-four dogs underwent mandibulectomy (35.3%) and 14 maxillectomy (20.6%); *en-bloc* excision was performed in 14 dogs (20.6%, 7 OMMs of the cheek and 7 OMMs of the lips) with or without mucosal or skin flap reconstruction or a combination of both. Two dogs (2.9%) underwent both mandibulectomy and *en-bloc* excision of the cheek at the same time. One patient (1.5%) underwent tonsillectomy; a simple excision was performed in 13 patients (19.1%) by the referring veterinarian followed in two cases by a revision surgery. In the remaining cases, surgical revision was not done due to the absence of macroscopic and/or residual disease at clinical examination and staging. Ipsilateral medial retropharyngeal lymphadenectomy was per- formed in 55 dogs (80.9%), ipsilaterally in 39 dogs (70.9%) and bilateral lymphadenectomy was performed in 16 dogs (29.1%). Ipsilateral medial retropharyngeal lymph node was removed in 3 (7.7%) of the 39 dogs and bilaterally in 2 (1.2%) of the 16 dogs. In 13 dogs (19.1%) the lymph node status was assessed for staging purposes only cytologically after fine needle aspiration. In addition to surgery and immunotherapy, 5 dogs (7.3%) received electrochemotherapy (with bleomycin intravenous injection) while 26 (38.2%) received metronomic chemotherapy. This treatment consisted of low dose oral administration of cyclophosphamide, piroxicam and thalidomide.

Table 2 Tumor localization of the OMMs included in the study

Localization	Overall population (n= 68)
Mandible	24 (35.3 %)
Maxilla	18 (26.5%)
Cheek	10 (14.7%)
Lip	10 (14.7%)
Soft palate	1 (1.5%)
Tongue	3 (4.4%)
Tonsils	2 (2.9%)

Characterization of the groups of dogs

The characterization of the five groups of dogs is summarized in Tables 3-5.
Local bone invasion was detected in 28 out of 68 OMMs (41.2%, group 1). Of the entire population 34 out of 68 dogs (50%) had lymph node metastasis. The lymph node metastatic rate in dogs of group 1 was 53.6% (15/28) while it was 47.5% (19/40) in group 2. Twenty- six dogs were included in group 3 and 42 in group 4. The lymph node metastatic rate was 53.8% (14/26) and 47.6% (20/42), respectively. Fourteen dogs were included in group 5 and the lymph node meta- static rate was 35.7% (5/14).
Regarding histological and immunohistochemical parameters, Ki67 was <19.5 in 15/68 (22.1%) OMMs, >19.5 in 49/68 (72.1%) OMMs, unknown in 3/68 (4.4%) and not detectable in one case, 1/68 (1.4%) because of the high pigmentation of the sample. The MC was <4/10 hpf in 10/68 (14.7%) OMMs and ≥4/10 hpf in 58/68 (85.3%) OMMs. Nuclear atypia was <30% in 11/68 (16.1%) OMMs, ≥30% in 52/68 (76.5%) OMMs and not available in 5/68 (7.4%) cases. Based on histopathological of surgical margins, 38/68 (55.9%) OMMs were determined to be completely excised, 21/68 (30.9%) were incompletely excised, and the margin status could not be determined in 9/68 (13.2%) OMMs. Histological and immunohistochemical parameters for every group are summarized in Table 6.

Table 3 Characterization of dogs in group 1 and group 2 based on bone invasion

(Overall patients n = 68)	Group 1 n= 28	Group 2 n= 40
Presence BONE INVASION	**28**	**0**
Absence BONE INVASION	**0**	**40**
Lymph nodes metastasis	**15 (15/28) (53.6%)**	**19(19/40) (47.5%)**

Table 4 Characterization of dogs in group 3 and group 4 based on localization of OMMs'

(Overall patients n= 68)	Group 3 n= 26	Group 4 n= 42
Hard tissue OMMs (gingiva of mandible or maxilla)	**0**	**42**
Soft tissue OMMs (lip, cheek, tongue tonsils, soft palate)	**26**	**0**
Lymph nodes metastasis	**14 (14/26) (53.8%)**	**20(20/42) (47.6%)**

Table 5 Characterization of dogs in group 1 and group 5 based on bone invasion

Group 4 (n = 42) Hard tissue OMMs (gingiva of mandible or maxilla)	**Group 1 n= 28**	**Group 5 n= 14**
Presence BONE INVASION	**28**	**0**
Absence BONE INVASION	**0**	**14**
Metastatic lymph nodes	**15 (15/28) (53.6%)**	**5(5/14) (37.5%)**

Table 6 Histological and immunohistochemical parameters of OMMs in each group

	Threshold	Overall population (n=68)	Group 1 (n= 28)	Group 2 (n= 40)	Group 3 (n= 26)	Group 4 (n=42)	Group 5 (n=14)
Mitotic count	< 4/10 HPF	10	5	5	2	8	3
	≥ 4/10 HPF	58	23	45	24	34	11
Ki67	< 19.5	15	4	11	7	8	4
	≥ 19.5	49	23	26	18	31	8
	Unknown	4	1	3	1	3	2
Nuclear atypia	< 30%	11	3	8	6	5	2
	≥ 30%	52	25	27	17	35	10
	Unknown	5	0	5	3	2	2
Margins	Clear	38	17	21	16	22	7
	Infiltrated	21	9	12	7	14	18
	Unknown	9	2	7	3	6	1

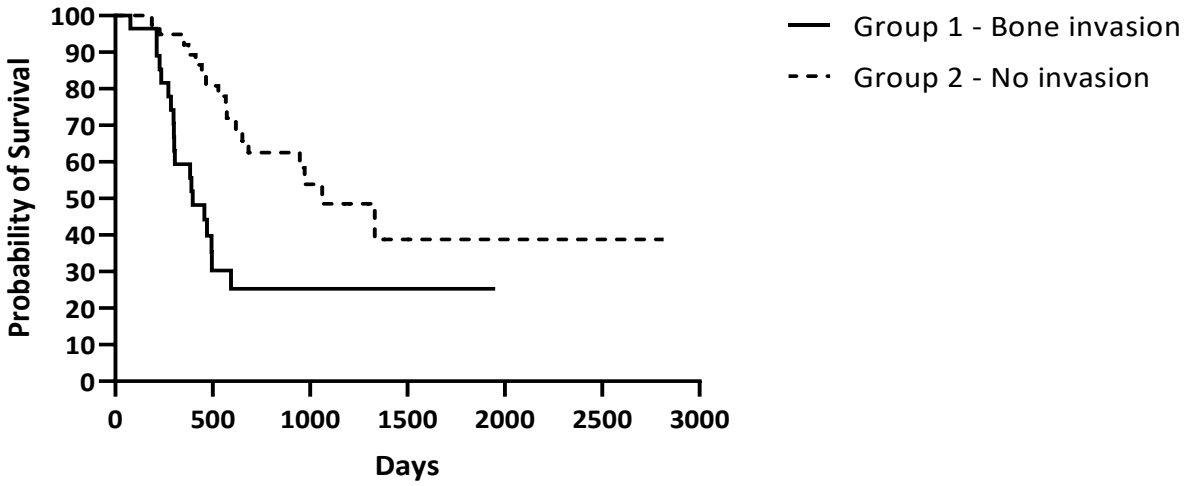
Outcome and prognostic factors

Kaplan Meier curves were analysed for survival times and DFI. At the end of the study, out of the 68 dogs, 7 dogs (10.3%) were still alive (range 544-2815 days) and only one of these dogs (14.3%, 1/7) had an OMM with bone invasion; 60 (88.2%) dogs were dead (range 78-2155 days) and one (1.5%) was lost to follow up at 1178 days. Thirty-seven dogs out of these 60 dogs (61.6%, 37/60) died of OMM-related disease with 19 (51.3%, 19/37) of these displaying bone invasion.

When groups' MSTs were compared, MST of group 1 (OMMs with bone invasion) was 397 days (range 78-1951 days) while it was 1063 days (range 172-2815 days) in group 2 (OMMs without bone invasion), with a significant difference (p = 0.0006) (Figure 1A). The same significant result was evident for DFI; the DFI of group 1 was 193 days (range 29–782 days) and 470 days (range 13– 2815 days) for group 2 (p = 0.004) (Figure 1B). When group 3 (soft tis- sue group) and group 4 (hard tissue group) were compared, MST of group 3 was 1063 days (range 227–2815 days) and was significantly longer (p = 0.004) than that of group 4 (470 days, range 78–2155 days) (Figure 2A). Similar to MST, the DFI of group 3 (470 days, range 13– 2815 days) was longer than in group 4 (202 days, range 21–1681days), although no statistical significance was found (p = 0.115). When comparing dogs with OMMs of group 1 (bone invasion) and group 5 (hard tissue OMMs without bone invasion), dogs of group 5 had longer MSTs (972 days, range 172–2155 days, p = 0.035) and DFIs (261 days, range 21–1681 days, p = 0.058) compared with dogs of group 1 (MST 397 days, DFI 193 days) (Figure 2B). The MST of the entire population was 653 days while DFI was 230 days.

No statistical association was found between groups 1 and 2, 3 and 4 or 1 and 5 in regard to occurrence of metastatic distant disease and the incidence of local recurrence. Additionally, MST and Ki67 ($p = 0.02$, $r = -0.43$) and MC ($p = 0.04$, $r = -0.39$) were only significantly correlated in group 1 (OMMs with bone invasion).

A



B

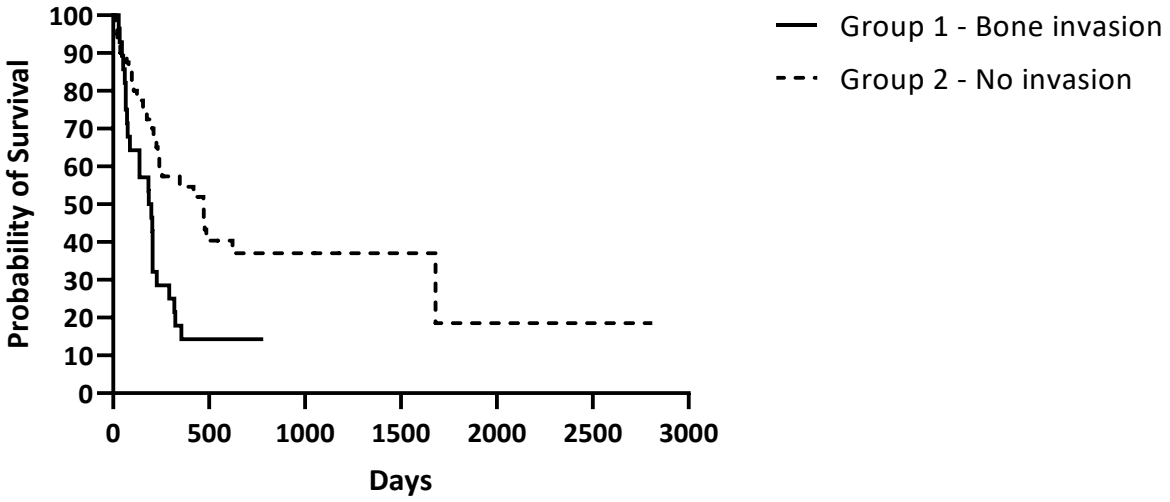
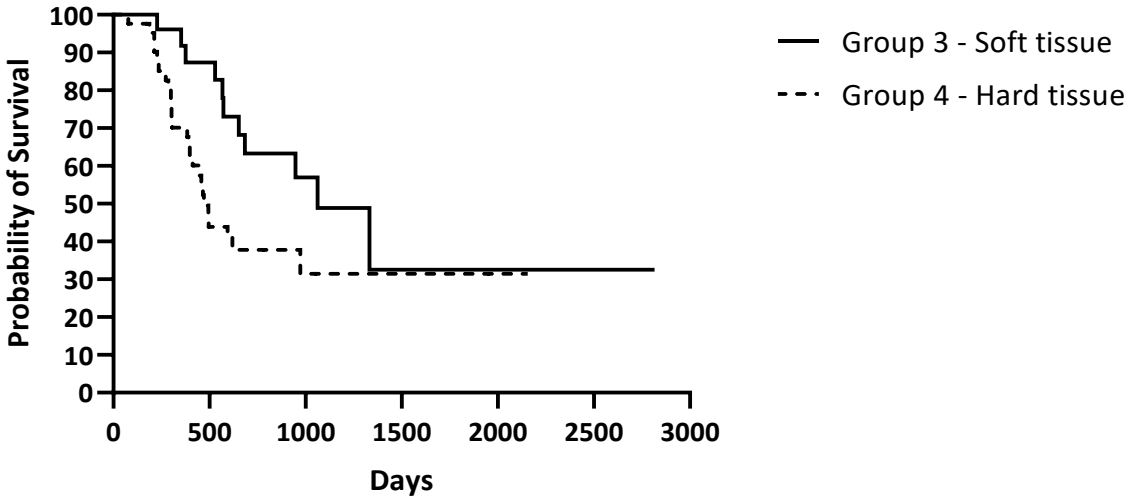


FIGURE 1 Kaplan Meier analysis of (A) median survival time ($p = 0.0006$) and (B) disease free interval ($p = 0.004$) of group 1 and group 2.

A



B

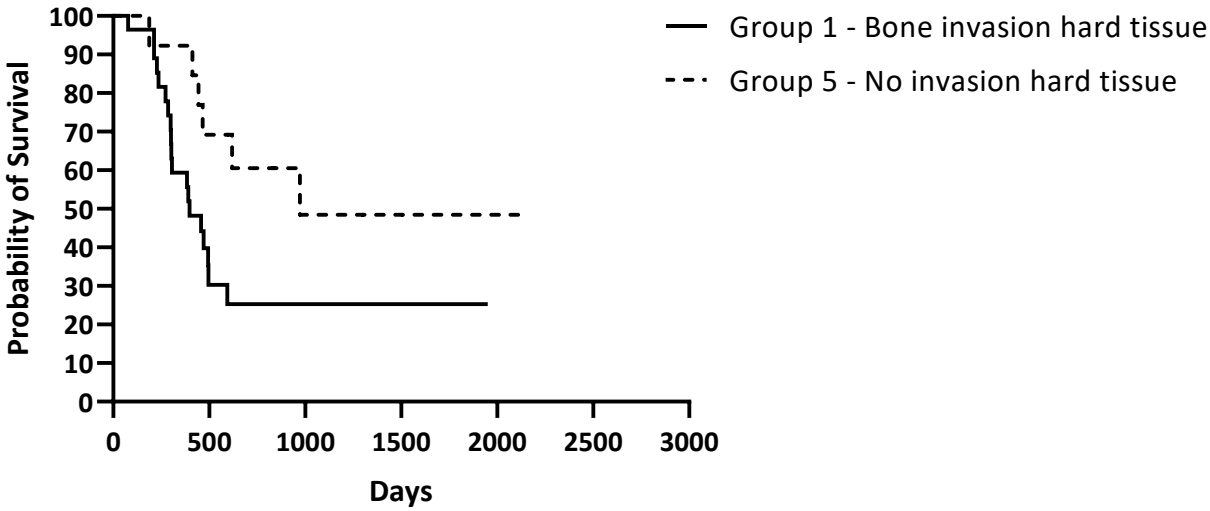


FIGURE 2 Kaplan Meier analysis of (A) median survival time of group 3 and group 4 (p = 0.0433) and (B) median survival time of group 1 and group 5 (p = 0.0357).

DISCUSSION

In this study authors evaluate the impact of bone invasion in dogs with canine OMMs in terms of DFI and MST among different groups based on the presence/absence of bone invasion and OMM's localization (hard vs. soft tissue). The presence/absence of bone invasion was assessed only in dogs of the hard tissue group. All dogs were treated with a multimodal approach by means of surgery and adjuvant anti-CSPG4 vaccination. Some dogs also received metronomic chemotherapy in addition to electrochemotherapy, in few cases. In addition, the prognostic value of bone invasion, MC and Ki67 were evaluated in this study (Smedley et al., 2011).

In recent years, several studies have aimed to identify prognostic markers for melanocytic neoplasms. The prognostic impact of bone invasion in canine OMM has been reported in very few studies and its role remains to be clearly defined. Early studies found that the presence of bone lysis observed on skull radiographs in dogs with oral melanoma did not significantly influence the survival time (Kosovsky et al., 1991; Wallace et al., 1992). In contrast, in another study evidence of bone lysis, recorded in 28% of the cases, was significantly associated with a worse prognosis and time to first event, which included tumor recurrence, regional or distant metastatic spread and death (Proulx et al., 2003). In the Smedley et al. review, several prognostic factors of melanocytic neoplasms were discussed, and the level of invasion was divided into shallow infiltration with absence of bone lysis associated with a favourable prognosis, and deep invasion with bone lysis that negatively affected the patients' outcome (Smedley et al., 2011); the propensity of OMMs to invade locally, involving the bone in some cases, might be due to the high vascular supply and lymphatic network of the oral cavity that provides an optimal microenvironment for tumor growth and metastatic invasion (Mukaratirwa et al., 2006).

Most of the dogs of this study underwent a curative intent surgery to entirely remove the tumor, including at least 1.5 cm up to 3 cm of macroscopically normal bone, soft tissues or both (depending on tumor localization) (Giacobino et al., 2021); OMMs with bone invasion (group 1) were characterized by reduced MST and DFI compared with dogs bearing OMMs without bone invasion (group 2), with a significant difference for both end points. Patients of group 3 (soft tissue) exhibited a significantly longer MST when compared with group 4 (hard tissue); the same was true for the DFI, despite the lack of a significant difference.

The results of this study showed that when only considering dogs with gingival OMMs (group 4, hard tissue), the subgroup that consisted of dogs bearing OMMs without bone invasion (group 5) displayed a significantly prolonged MST compared with those of group 1 (bone invasion). However, there was no significant difference regarding the DFI. The reason behind this group division was to evaluate the impact of bone invasion more precisely in terms of prognosis. It should also be noted that the evaluation of DFI of group 4 and group 5, during the vaccination protocol, may not have been so accurate, considering that these dogs were restaged with radiographs instead of computed tomography. The different diagnostic accuracy of the two techniques could make the DFI a less reliable and precise end point. Additionally, based on previous anti-CSPG4 electrovaccination studies, authors reckon that the immunity induced by the vaccine is more effective in reducing the development of distant metastatic disease rather than local recurrence (Piras et al., 2017).

A significant correlation was observed between Ki67, MC and MST exclusively in dogs of group 1. This result might corroborate the recognized prognostic value of Ki67 and MC, especially in OMMs with bone invasion. As no specific numerical value was attributed to nuclear atypia (which was ≥30% in 76.5% of samples) other than the cut off < or ≥ 30% (Smedley et al., 2011), no statistical correlation was assessed.

In this study, authors decided to include OMMs with evidence of bone lysis detected on CT and/or histology; however, imaging and histology might have some limitations in identifying this feature. On one side, if the lysis process is in the early phase, CT scan might not capture it because of the very superficial and slight remodeling at the level of the bone surface. Additionally, 20% of our patients were staged using radiographs which are less accurate compared with CT in detecting bone invasion; in fact, it has been reported that bone lysis is not evident on radiographs until 40% of the cortex is destroyed. This suggests that CT scan is preferable for oral tumor staging (Liptak, 2020; Gendler et al., 2010; Ghirelli et al., 2013). On the other side, tumors including bone are trimmed for histological evaluation through a cross-sectioning technique. In case surgical margins are wide, as they are in case of mandibulectomy or maxillectomy, the cross-sectioning is performed through the cranial, caudal, and central part of bone specimen (Kamstock et al., 2011). This may miss some areas where bone lysis may be present; this problem should be overcome with further sections, but this is not always feasible. Considering these limitations, the bone invasion may have been underestimated in this study.

Nevertheless, MST was prolonged compared with historically reported survival times (Boston et al., 2014; Tuohy et al., 2014). This result may be partially attributable to the effect of the anti-CSPG4 electrovaccination (Riccardo et al., 2014; Piras et al., 2017). Several clinical trials have been geared towards the use of DNA vaccination as a fundamental step in the management of malignant melanoma (Giacobino et al., Bergman et al.., 2003; Grosenbaugh et al., 2011; Bergman 2006; Impellizeri et al., 2014). Our protocol involves the electrovaccinaion against CSPG4, a class one oncoantigen involved in several oncogenic pathways such as melanoma tumor cell progression, survival and metastasis, and is poorly expressed in healthy tissue. Therefore, CSPG4 has gained value as an ideal immunotherapeutic target (Yang et al., 2004; Price et al., 2011; Mayayo et al., 2011); in recent and ongoing clinical trials, anti-CSPG4 electrovaccination has revealed its potential therapeutic impact, being safe, immunogenic in inducing a significant humoral response and effective in prolonging survival in OMM bearing dogs (Giacobino et al., 2021; Riccardo et al., 2014; Piras et al., 2017). Based on previous and current results, authors reckon that anti-CSPG4 electrovaccination is worthwhile to consider as adjunct treatment considering its positive role in terms of outcome.

The lymph node metastatic rate did not significantly differ between groups 1 and 2, although it was slightly higher in group 1. However, it is difficult to make any conclusions as different procedures were used to stage the lymph node status. Some dogs had their lymph nodes evaluated only cytologically while others underwent ipsilateral lymphadenectomy of mandibular and/or medial retropharyngeal lymph nodes, and only a few had a bilateral neck lymphadenectomy. This limitation, together with the retrospective nature of the study, may have led to underestimation of the actual lymph node status in some patients. Recently, new surgical procedures and methods have

been proposed to improve lymph node staging as part of the clinical staging based on the TNM system (Skinner et al., 2017; Grimes et al., 2019; Green & Boston, 2017; Wainberg et al., 2018).
Regarding the localization of OMMs, the literature reports that rostral tumors may be associated with a longer survival time (Bergman et al., 2020; Hahn et al., 1994; Schwarz et al., 1991). This can be explained by the fact that rostral tumors have a better chance of being completely excised when compared to caudal tumors. Additionally, because of their location, caudal malignant tumors are often detected later in the course of the disease, having already progressed to an advanced stage at the time of diagnosis. Unfortunately, it was not feasible to evaluate OMMs of the present canine population based on this detail because of the incompleteness of data derived from both the retrospective nature of the study and the different diagnostic procedures (CT scan vs radiographs) used to stage and therefore to detect bone invasion. Additionally, another shortcoming of this study is the limited number of dogs included in each group.
According to our data, bone invasion was significantly associated with a shorter MST and a shorter DFI. The negative impact of bone invasion was also evident when authors evaluated the MST and DFI of dogs with OMMs of soft tissue and dogs with OMMs of hard tissue, the latter group having a reduced MST and DFI.
In conclusion, several prognostic markers should be carefully evaluated for prognosis and treatment of OMM and this study further supports that bone invasion is one of these factors. Because of its negative impact on prognosis, bone invasion caused by canine OMM should be assessed, if feasible, through an advanced diagnostic imaging procedure and evaluated via histopathology.

Additional information

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