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Gamma-probe guided sentinel lymph node extirpation to assess patterns of nodal metastasis in spontaneous head and neck malignancies of the dog: a second step based on previous experiences on mast-cell tumors

VET/09

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Summary

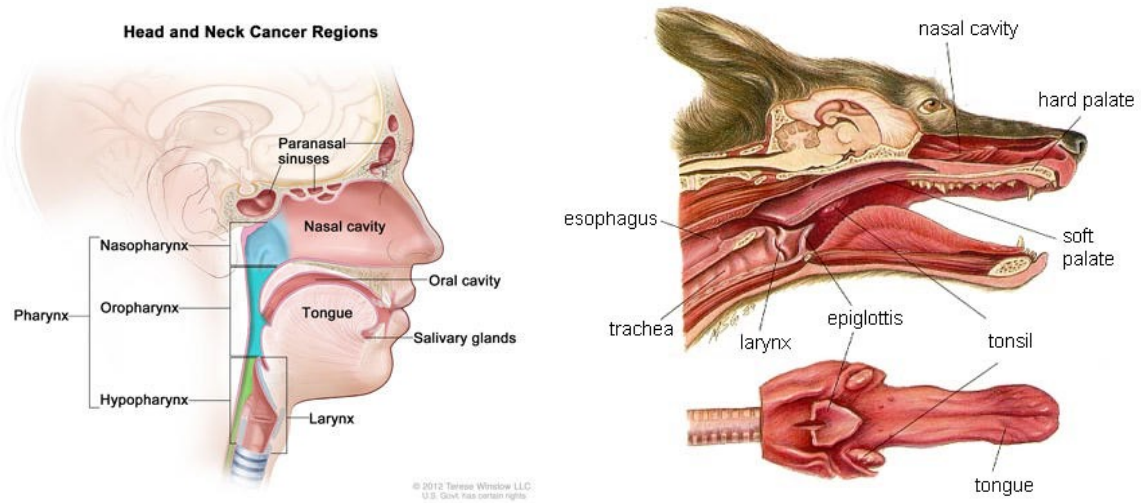
1. Introduction

A companion animal or pet is defined as an animal that shares the household environment and is cared for by humans. After World War II, the increased prosperity and social welfare, alongside individualization, promoted dramatic changes in the human-pet relationship.[1] For instance, the role of companion animals shifted from utilitarian to social and emotional, and nowadays pets are kept for their companionship and regarded as family members.[2] As a result, pet ownership is increasing constantly: in the USA 59% of households owned a pet in 2018, of which 38% owned a dog and 25% owned a cat, leading to an estimated population of 77 million pet dogs and 58 million pet cats;[3] likewise, in 2020 38% of European households owned a pet animal, with a 15% and 22% increase in pet dogs' and cats' populations from 2010 to 2018 and an estimated population of 90 million dogs and 110 million cats.[4,5] The impact of veterinary oncology on canine's welfare is evident when considering that cancer is the most common cause of death in dogs, with an overall estimated incidence of malignant neoplasia ranging from 381 to 852 per 100,000 pet dogs.[6-9] Research and progression in the treatment of cancer dogs is of outmost importance given the increasing prevalence of pets and their companionship role in modern families. Not to be neglected, cancer-bearing dogs are an effective spontaneous model for the study of oncological diseases in humans, given the similarities in clinical features, biological behavior and response to treatment between several canine and human tumors.[10] In this scenario, the research project presented here attempted to improve the current understanding of the biological behavior of spontaneous head and neck malignancies in dogs, in order to promote the improvement of standards of care for pets, while disclosing potential similarities with the human counterpart that may be valuable for future comparative and translational studies.

The term "head and neck tumors" is commonly used in human medicine and refers to tumors arising from the oral cavity, pharynx (nasopharynx, oropharynx, hypopharynx), larynx, paranasal sinuses and nasal cavity and salivary glands (Figure 1A), with squamous cell carcinoma of the mucosal surfaces being most commonly diagnosed; tumors of the brain, eye, esophagus, thyroid gland and skin are not consistently included.[11] The same term was readapted for dogs, to include all tumors arising from the tip of the nose to the thoracic inlet, including those of the superficial tissues (cutis, subcutis and muscles) and those involving the deep structures of the head and neck, including the upper aerodigestive system, nerves, thyroid gland, the eye, the ear and bones of the splanchnocranium; tumors of the neurocranium and central nervous system are excluded.

Malignancies of the head and neck have the tendency to involve the tributary lymph nodes as part of their natural biological behavior, both in humans and in companion animals. In humans, it is estimated that up to 30-80% of patients with head and neck tumors will have nodal metastases at presentation, with variable rates of lymphatic spread reported depending on tumor type and characteristics.[12,13] Likewise, neoplastic involvement of the cervical nodes has been described in 35-45% of dogs with malignant head and neck tumors.[14,15]

Tumor staging, as described in the TNM system (Tumor, Node, Metastases), is a well-established prognostic factor for tumor-bearing dogs and is pivotal in the oncological management of canine head and neck tumors.[16,17] The surgical oncologist should be able to identify nodal metastases to correctly stage animals, in order to predict prognosis and suggest appropriate treatments.[18-27]



(A) (B)

Figure 1. *(A) Head and neck caner regions in humans (From: The National Cancer Institute, credit: Terese Winslow); (B) Head and neck regions in dogs (Form: Hill's Pet Nutrition, Atlas of Veterinary Clinical Anatomy)*

1.1. The lymphatic drainage of the head and neck: comparative surgical anatomy

Proper knowledge of the surgical anatomy of the head and neck is mandatory for the surgical oncologist to correctly identify cervical lymphocenters and lymph nodes during surgical dissection. This holds particularly true when considering the anatomical complexity of the lymphatic drainage in the head and neck, both in humans and dogs, characterized by multiple lymphocenters and nodes, overlapping of drainage pathways between lymphocenters, and frequent individual anatomical variations.[14,28,29]

The lymphatic drainage of the canine head and neck is anatomically based on five lymphocenters: the parotid, mandibular and retropharyngeal lymphocenters located in the head (Figure 2A); superficial and deep cervical lymphocenters in the neck (Figure 2B).[28]

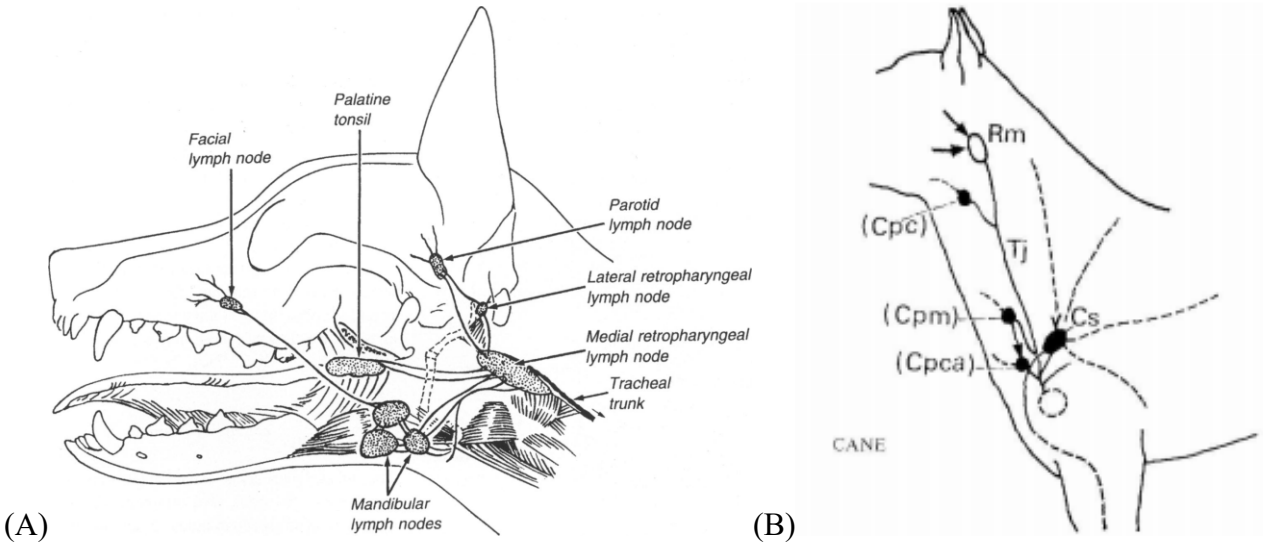


***Figure 2**. Lymph nodes of the canine head (A) (From: Beltz et al, 1995) and neck (B) (From: www.unipi.it). Rm: medial retropharyngeal node; Cpc, Cpm, Cpca: deep cervical nodes – cranial, medial and caudal; Cs: superficial cervical node.*

The parotid lymphocenter is located at the basis of the ear, rostrally to the parotid gland and adjacent to the masseter muscle. This lymphocenter consists of 1-3 small nodes which drains the superficial tissues of the caudal-dorsal muzzle including the eyelids, lacrimal apparatus, external ear, temporomandibular joint and parotid gland, as well as the muscles and bones of the caudal-dorsal muzzle.[30] Efferent vessels from the parotid lymphocenter can drain into the medial or lateral retropharyngeal lymph nodes.[30]

The mandibular lymphocenter comprises the mandibular and buccal lymph nodes. The mandibular nodes are a group of 1-5 nodes located dorsally and ventrally to the linguofacial vein, which drain all parts of the head not drained from the parotid lymphocenter. However, overlapping between the areas of drainage of this two lymphocenters have been consistently described, with the eyelids and superficial tissues of the caudalmost aspect of the muzzle being possibly drained by both lymphocenters.[28,29] Furthermore, random crossing of the lymphatic pathways to the contralateral mandibular lymphocenter has been documented for the floor of the mouth.[29,31] Efferent vessel from the mandibular lymph nodes drain primarily to the ipsilateral retropharyngeal nodes, although to a lesser extent drainage to other nodes of the same lymphocenter and to the contralateral retropharyngeal nodes is also present.[28] The buccal nodes are described in around 9% of dogs, half of which present the node unilaterally [32-34], and can be found in the proximity of the confluence of the facial and superior labial artery.[28] This lymph node was not reported in Suami et al study (2013), although their sample was limited to four canine carcasses.[29]

The retropharyngeal lymphocenter comprise a medial and lateral retropharyngeal node, the latter being present in approximately 30% of dogs underneath the caudal border of the parotid gland and draining into the medial retropharyngeal node.[30]

The medial retropharyngeal node is the biggest node of the head in normal conditions and lays ventral to the wing of the atlas between the digastricus and longus colli muscles dorsally, and the pharynx and larynx ventromedially.[28] Two medial retropharyngeal nodes have been described in 20% of dogs in Baum's anatomical study (1918)[30], although more recent clinical studies consistently reported only one lymph node.[26,27] This node drains all the deep structures of the head, including the tongue, oral and nasal cavity, salivary glands, deep parts of the external ear as well as larynx and esophagus.[28]

The superficial cervical lymphcenter consists of 1-2 or occasionally > 3 nodes located in the adipose tissue on the lateral aspect of the serratus ventralis and scalenus muscles, cranial to the supraspinatus muscle.[28] Up to 3 lymph nodes have been described in recent studies on cancer dogs.[26,27] These nodes drain the skin of the caudal head, including part of the pinna, the superficial lateral neck and the thoracic limb with the exception of a variable area on its medial side.[28,29] Efferent vessels can drain into the right lymphatic duct, left thoracic duct and jugular vein.

The deep cervical lymphcenter are inconsistently present in dogs and can consists of cranial, middle and caudal nodes located along the cervical trachea. They receive lymph from the deep structures of the neck and ultimately drain into the thoracic duct.[28]

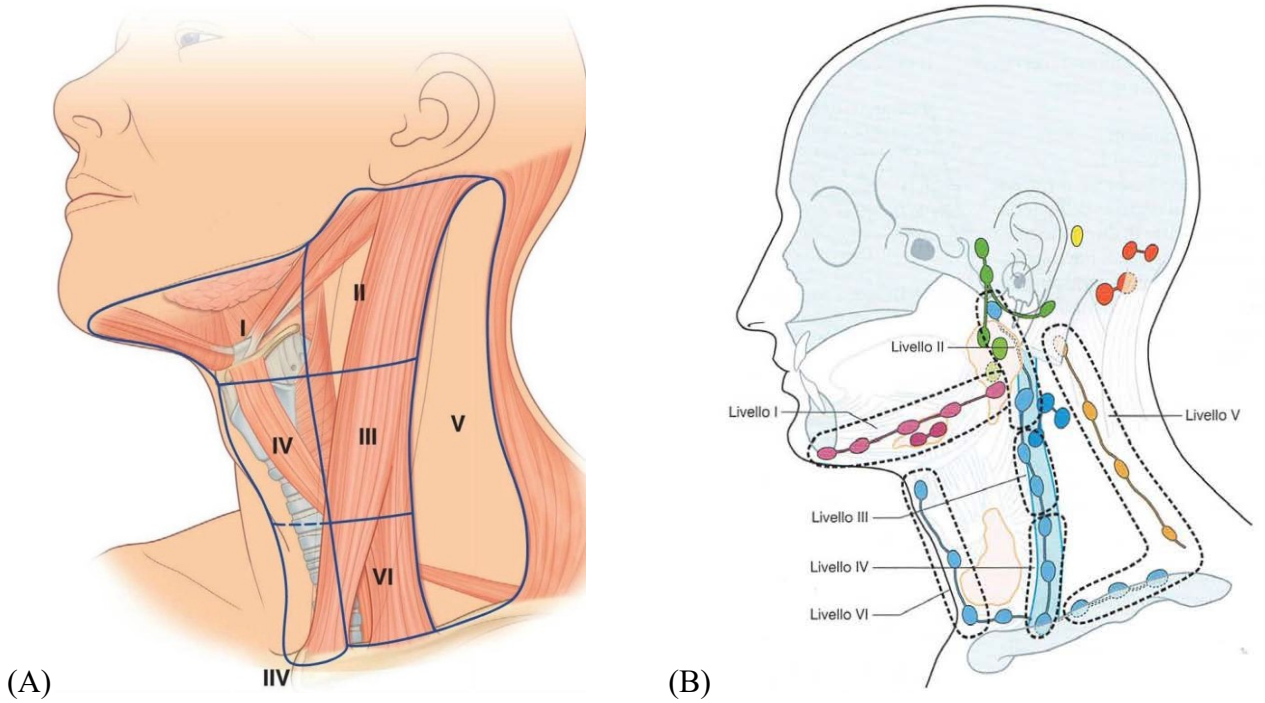
The current knowledge of the surgical anatomy of the lymphatic system of the canine head and neck is largely based on few anatomical studies dating back to the last two to five decades. A sole recent study has been produced on the anatomy of the lymphatic system in healthy dogs, although it is based on dissection of only four cadavers of medium sized female dogs, thus providing questionable evidence and likely unrepeatable results.[29] This gap of knowledge needs to be addressed in further anatomical studies ideally including carcasses of dogs of different breeds, sizes, and sexes in order to accurately identify individual variations of the lymphatic pathways in healthy dogs and to evaluate the impact of skull conformation (brachycephalic, mesocephalic, dolichocephalic breeds), body weight and sexual status on the lymphatic anatomy. It should not be neglected that surgical anatomy describes the lymphatic drainage of healthy patients, although in cancer-bearing dogs (and humans) tumor-induced lymphagiogenesis can result in unpredictable lymphatic drainage pathways.[35] Hence, in tumors-bearing dogs, a thorough knowledge of the surgical anatomy of the lymphatic system, albeit crucial, may not allow to predict the lymphatic drainage of the primary tumor, therefore lymphatic mapping techniques should be applied alongside.

On the human counterpart, the surgical anatomy of the cervical lymphatic system has been thoroughly described, given the complexity of cervical lymphatic paths, to allow the surgeons for precise dissection and accurate nodal staging. It has been estimated that around 40% of nodes in the human body are located in the cervical area.[36] Cervical nodes were firstly classified by Rouviere into a collar of nodes around the upper aerodigestive tract, comprising the submental, facial, submandibular, parotid, mastoid, occipital retropharyngeal nodes, and two vertical rows along the neck including the anterior cervical and postero-lateral cervical nodes (Figure 3B).[37] As soon as neck dissections techniques started to gain popularity among surgical oncologists, the need for a standard and detailed classification of the extent of nodal dissection arose. Hence, the American Joint Committee on Cancer (AJCC) adopted the simplified classification system by Shah et al (1981).[38] This system identifies eight groups or levels of cervical nodes based on their anatomical location and it is the reference for human surgeons when performing neck dissection.[38-40] Nodal levels of Shah's classification and their corresponding locations are detailed in Table 1 and schematically represented in Figure 3.

Level	**Location**
IA	Submental
IB	Submandibular
II	Superior internal jugular (deep cervical) chain, from the base of the skull to the inferior border of the hyoid bone
III	Median internal jugular (deep cervical) chain, from the hyoid bone to the inferior border of the cricoid arch
IV	Inferior internal jugular (deep cervical) chain, between the inferior border of the cricoid arch and the supracavicular fossa
V	Posterior triangle or spinal accessory nodes
VI	Central compartment nodes form the hyoid bone to the suprasternal notch
VII	Nodes inferior to the suprasternal notch in the upper mediastinum

Table 1. *Shah's classification system: levels and location of cervical nodes.*

Since this classification system was created based on the concept of neck dissection, the facial, parotid, mastoid, occipital and retropharyngeal nodes were not included as they are not removed during traditional neck dissection.[12]



(A) (B)

Figure 3. *Anatomical (A) and schematic (B) representation of nodal levels described by Shah et al for elective neck dissection in humans (From: Anastasi et al 2006, Drake et al 2014)*

In 2002, The American Head and Neck Society and American Academy of Otorynolaryngology-Head and Neck Surgery proposed a revision of the neck dissection classification. The rationale in the introduction of a standard classification system for cervical nodes was for surgeons to be able to univocally classify various neck dissection techniques. Thanks to the adoption of a logic, straightforward and easy to remember nodal classification, in 1991 the Committee for Neck Dissection Classification was able to present the first classification system for neck dissection.[41] The Committee, however, supported the introduction of the concept of sublevels into the classification (Figure 4). The sublevels identify areas within the larger levels that carry independent biological significance, as the risk of metastases to each sublevel depends on tumor location. This concept allows for surgical spearing of other subregions when the risk of nodal metastases is confined to a single sublevel.[41]

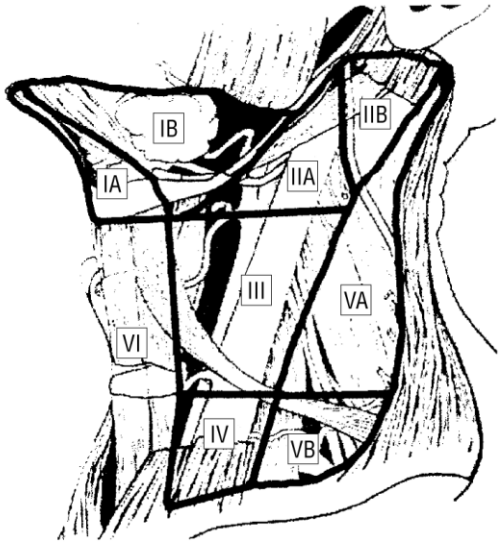


Figure 4. *Sublevels identified by the Committee for Neck Dissection Classification (Form: Robbins 2002).*

This classification system has been further modified to identify more subgroups and further limit the extent of nodal dissection, with identification of ten levels with specific tributary areas.[42]

It is clear that human surgical oncologists are making increasing efforts to develop a detailed and precise, yet straightforward and easy to use system to classify the cervical nodes. Such efforts are dictated by the assumption that a standard anatomical classification is needed to guide the surgeon during nodal removal. Likewise, an effort is needed to identify a standardized and reliable anatomical description and classification of the cervical nodes in dogs, that could guide the surgeon toward a more precise surgical approach to the cervical lymph nodes.

1.2. Incidence and prognostic implications of nodal metastases in canine head and neck tumors

The risk of nodal metastases is tumor-specific, as each tumor type shows a different propensity for lymphatic invasion. For this reason, the available literature is mostly focused on a single tumor type, with only three studies evaluating the risk of nodal metastases for the whole group of head and neck tumors.[14,15,27] Herrings et al (2002) firstly described the incidence of cervical node metastases in a cohort of 52 dogs with various neoplasm of the head and neck.[14] The authors systematically removed the parotid, mandibular and medial retropharyngeal lymphocenters bilaterally at the time of tumor excision and reported a rate of histologically detected nodal metastases of 35%.[14] Skinner et al (2016) reported cervical nodes involvement in 14/31 (45%) dogs with malignancies of the head, detected with histological examination after bilateral excision of mandibular and retropharyngeal nodes.[15] More recently, our research group reported a rate of cervical node metastases of 42% in dogs with head and neck tumors undergoing sentinel lymph node biopsy (SLB) in the absence of clinically evident nodal disease (cN0 neck).[27] These studies provide evidence on the relatively high risk of cervical node metastases, even in dogs with cN0 neck, thus underscoring the importance of lymph node assessment. However, they do not offer an insight on the prognostic impact of identification of cervical metastases due to the inclusion of several tumor types.

Tumor-specific prognostic factors, including cervical node metastases, have been described for several tumor types of the head and neck. The most common malignancies of the head and neck arise from the oral cavity, and include oral melanoma (OM), squamous cell carcinoma (SCC), osteosarcoma (OSA) and fibrosarcoma (FSA).[43-44]

Oral melanoma account for 14-36% of malignant lesions of the canine oral cavity, and it is well-known for its mostly aggressive biological behavior, with high propensity for both local invasiveness and loco-regional/distant metastases.[45] Nodal metastases are encountered in 59-74% of dogs at first presentation [20,46,47], with one study reporting cervical node metastases in 53% of dogs with OM and cN0 neck.[47] Although tumor stage is widely accepted as one of the most important prognostic factors for canine OM, controversies still surrounds the impact of nodal metastases on disease-free interval and overall survival.[17,45] In a cases-series of 140 dogs with OM treated with radiation and combinations of other treatment modalities, pre-treatment metastases to the regional lymph node (RLN) did not correlate with the development of distant metastases, nor were able to predict “time to first event” and overall survival time.[46] Likewise, RLN metastases did not show prognostic significance for remission length or survival time in a previous study on 41 surgically excised OM.[48]

It should be noted, however, that in both studies the assessment of the lymphatic basin was based on cytological or clinical examination of the RLN instead of histopathology on the excised draining nodes, thus potentially leading to underestimation of the actual rate of nodal metastases and to an incorrect evaluation of their impact on patients' outcome.[47,49,50] In two recent studies on canine OM, RLN metastases were detected with histopathology on the excised nodes; although the impact of nodal metastases alone on oncological outcome was not statistically evaluated, both studies reported shorter survival times for dogs with nodal and/or distant metastases at presentation, suggesting a possible prognostic role of loco-regional spread.[19,20] More recently, Turek et al (2020) identified nodal metastases at diagnosis as a negative prognostic indicator in dogs with OM treated multimodally, although lymph node assessment was inconsistently based on cytology/histology or clinical examination in that study population.[51]

Oral SCC and FSA represents around 14% and 8-25% of oral tumors in dogs;[22,43] when considering also feline tumors SCC raises to 27% of oral malignancies and FSA accounts for 13%.[44] Historically, these tumor types have been regarded as locally invasive, with reported rates of local recurrence of 17% for SCC and 54% for FSA, but less prone to nodal and distal metastases than OM.[43,44] Hence, clinicians have been recommending lymphadenectomy for nodal assessment less frequently for canine SCC and FSA than OM.[17] As a consequence, data are lacking on the prognostic impact of nodal metastases for these tumor types, although tumor stage has been recognized as predictive of outcome for both SCC and FSA.[22,23] It is reasonable to assume that the prevalence of nodal metastases for these tumor types has been overlooked in the past decade, due to the tendency to recommend lymph node extirpation only when metastases were clinically suspected.[17] Grimes et al (2019) detected nodal metastases in 29% of dogs with SCC and cN0 neck after surgical excision of mandibular and retropharyngeal nodes[52], and nodal metastases have been reported for FSA as well, although it is not possible to estimate an accurate prevalence based on the current literature.[22,23] These considerations underscore the need for the inclusion of lymph node extirpation and subsequent histological examination in the routine management of canine SCC and FSA, in order to better understand the prevalence of nodal metastases and their impact on disease-free interval and survival.

Osteosarcoma of the oral cavity is less frequent than the appendicular type, and accounts for approximately 14% of canine oral tumors, decreasing to 10% when considering also feline tumors.[43,44] As for oral SCC and FSA, clinicians are unlikely to suggest node extirpation in dogs with oral OSA and cN0 neck.[17] Consequently, information on the actual incidence of nodal metastases is

lacking and their impact on oncological outcome has not been specifically investigated, although the prognostic relevance of tumor stage is well-established.[43]

Likewise, controversies still surround the risk of nodal metastases and their impact on survival for other malignancies of the canine head and neck such as thyroid tumors, nasal tumors and salivary gland tumors. Nodal metastases have been described in up to 45% of dogs with thyroid carcinoma[52,54], although their prognostic impact has not been assessed yet. Hammer et al (2001) described nodal metastases in 17% of dogs with salivary gland tumors, and found a prognostic impact on clinical stage, although the effect of nodal metastases was not specifically assessed.[18] The same considerations hold true for less frequent tumor types that may raise from other anatomical structures of the canine upper aerodigestive system.[55]

Among the cutaneous tumors that can occur on the canine head and neck, mast cell tumor (MCT) is surely the most common, representing 11-27% of cutaneous malignant tumors in dogs.[56,57] The evolving approach to nodal staging in canine MCT well represents the paradigm of lymphatic assessment in companion animal oncology and highlights the increasing interest towards the early detection of nodal metastases. It is well-established that MCT have a tendency to spread to the lymph nodes as the first metastatic site and presence of nodal metastases has been correlated with contracted survival times.[58-62] Hence, detection of nodal metastases is pivotal in correct staging, prognostication and treatment recommendations.[63-67] As for oral tumors described above, nodal extirpation had been recommended more often for clinically abnormal nodes or in presence of other negative prognostic indicators for several years, leading to an estimated risk of nodal metastases at presentation of 18-61%.[67-69] However, the interest of the scientific literature has soon shifted towards the identification of early nodal metastases in clinically normal lymph nodes.[68] It is accepted that palpation is not able to accurately predict nodal metastases, and false negative results are frequent after cytological sampling, leading to the logic conclusion that lymph nodes should be excised and submitted for histopathological evaluation even in the absence of clinically evident nodal disease.[49,67,70,71] In 2014, Weishaar et al proposed a classification system for the histological diagnosis of MCT nodal metastases and identified 4 categories of neoplastic involvement based on the number and distribution of mast cells within the lymph node, namely HN0, HN1, HN2 and HN3.[72] The diseases-free interval was significantly longer for dogs with HN0-1 nodes than those with HN2-3 nodes, underscoring the importance of prompt identification of nodal involvement.[72] In 2018, our research group published the first study on the extirpation of normal-sized RLN, and identified nodal metastases in nearly half of cases, with no statistical correlation between nodal metastases and other prognostic factors except

from tumor size.[68] Thereafter, the literature showed a growing attention towards clinically normal nodes in dogs with MCT, especially after the observation that removal of HN1-HN2 nodes may have therapeutic effects even in the absence of adjuvant treatment.[73] Traditionally, the anatomically closest node, namely RLN, was assessed during nodal staging.[67,69,68,72] In 2014, Worley firstly published a study on the evaluation of the actual draining node, namely the sentinel lymph node (SLN), and reported a high level of discrepancy between the RLN and SLN in 20 dogs with MCT.[74] Thereafter, three studies have been conducted on SLN biopsy in dogs with MCT and reported a non-correspondence between the RLN and SLN in 28-63% of dogs.[26,75,76] These results highlight the need for a personalized nodal staging in dogs with MCT, although the impact of SLN biopsy on the oncological outcome of dogs with MCT is yet to be understood.

While several papers are available on the impact of nodal metastases in dogs with MCT, none of these studies is specifically focused on MCT of the head and neck. Historically, the head and neck has been regarded as a site associated with less favorable prognosis for canine MCT, although tumor site was lacking correlation with the risk of nodal metastases in a recent study.[68] Although the actual risk of nodal metastases for MCT arising on specific anatomical regions has not been fully elucidated yet, it is clear that nodal evaluation is crucial for a correct oncological management of these tumors.

Cutaneous soft-tissue sarcomas (STS) are other integumentary tumors that may involve the canine head and neck region, although they are more frequently reported on the trunk and limbs. Based on a recent review of the literature, around 60% of canine STS are located on the limbs, 35% on the trunk and only 5% on the head and neck.[77] Local recurrence is the main concern when treating canine STS, with rates of recurrence after surgical resection as high as 75% and contracted survival times for dogs with recurrent STS.[78-81] Conversely, loco-regional and distant metastases are historically less frequently reported, with rates of 0-15% and 0-44%, respectively.[82-87] Hence, the available literature is focused on the identification of prognostic factors predictive of local recurrence, while information is lacking on the actual rate of nodal metastases and their prognostic implications.

Although several studies have been conducted to identify potential prognostic markers for various malignancies of the head and neck, none of them is specifically focused on the impact of cervical node metastases. Furthermore, in the current literature there is significant inconsistency in nodal staging techniques and follow-up collection, and results from different studies are thus difficult to compare. Despite the well-establish role of histopathology evaluation of clinically normal lymph nodes for the correct nodal staging of head and neck malignancies[47,50], this procedure is still not routinely performed in the clinical practice for most of the above-mentioned malignancies.[17] A recent

survey study reported a significant variability in current recommendations for nodal staging in dogs with oral neoplasia, with clinicians performing lymphadenectomy and histopathology more commonly in case of clinically evident nodal disease (cN+ neck) and extirpation of clinically normal nodes being more frequently suggested for oral melanoma than squamous cell carcinoma and fibrosarcoma.[17] Such results are surprising, given the strong evidence on the unreliability of lymph nodes size as a predictor of nodal metastases and the high rates of false negatives after cytological sampling.[47,49,76] It is thus clear that a standardization of cervical node staging is needed to accurately estimate the impact of nodal metastases on prognostication for the different tumor types of the canine head and neck.

1.3. Sentinel lymph node concept and mapping techniques in veterinary oncology

The sentinel lymph node (SLN) is the first node within the lymphatic basing that drains a primary tumor. Hence, it is expected to be the first site to harbor metastatic cells and its status is theoretically predictive – or "sentinel", of the metastatic status of the whole lymphatic basin.[88]

The concept of the "sentinel lymph node" was firstly introduced by Gould et al in 1960 to designate the draining lymph node in people with parotid gland cancer.[89] In 1977 Cabanas et al applied the same concept to penile cancer and demonstrated that cancer cells tend to involve the lymphatic system in an orderly manner.[90] At that time however, radical node dissection was still routine. Almost twenty years later the use of vital dyes for intraoperative identification was described in humans with early-stage melanoma in a milestone study that prompted the adoption of the SLN procedure as standard of care for cancer patients.[91] The rationale of developing a targeted approach to the lymphatic basin was to avoid radical lymphadenectomy and the related morbidity in those patients that would ultimately not have nodal metastases.[91] With the introduction of the SLN, surgeons would routinely excise just the draining node for immediate intraoperative histopathological examination and perform further lymphadenectomies only if occult metastases are detected in the SLN, while sparing patients with a negative SLN from the procedure.[90,91] Shortly thereafter, SLN biopsy became routine in the management of several malignancies in human medicine, given its reported accuracy and low morbidity.[92-94] The role of SLN biopsy in humans with cutaneous melanoma has been investigated by large, prospective, randomized trials, and improvements in 10-yers disease-free interval of 71.3% versus 64.7% were reported for patients receiving SLN biopsy versus observation groups.[95] Furthermore, SLN status was a strong predictor of recurrence and tumor-related mortality, and disease-free interval was statistically longer for patients with nodal metastases detected with SLN biopsy versus those who developed clinically obvious disease, underscoring the importance of detection of occult nodal metastases and the accuracy of SLN biopsy.[95] While allowing for accurate staging, the morbidity is significantly reduced with SLN biopsy compared to patients receiving completion or radical lymph node dissection, with surgical complications such as wound infection, seroma, hematoma, lymphedema, pain and numbness reported in around 10% and 40% of patients, respectively.[92] Likewise, SLB biopsy became a cornerstone for nodal staging in women with breast cancer and clinically node-negative axilla, given the high diagnostic accuracy and low morbidity.[96,97] The introduction of SLN biopsy allowed for a significant improvement in 10-years survival rates of node-negative women with breast cancer, shifting from 70% in the 1970s-1980s when completion axillary node dissection was routine, to 90% thereafter when SLN biopsy was introduced for nodal

staging.[94] Furthermore, quality of life outcomes is significantly improved in women undergoing SLN versus completion axillary node dissection, with rates of lymphedema lowering down to 1-3% with a targeted approach to the SLN.[94] Although SLN biopsy has been historically more frequently reported for humans with melanoma and breast cancer, the technique shown beneficial for other malignancies as well, including squamous cell cancer of the head and neck[98,99], colon-rectal cancer,[100-101] Merkel cell carcinoma[102-104], thyroid cancer[105,106], gynecologic tumors[107-109] and lung tumors.[110]

In Veterinary Oncology, the interest towards the "SLN concept" emerged in the last decade, and its incorporation in clinical practice is still ongoing. With the increase of knowledge on the biological behavior of canine malignancies, it became clear that accurate assessment of the lymphatic basin is crucial for prognostication and treatment recommendations of cancer-bearing dogs.[19,20,21,35,66,68,111] Historically, the assessment of the lymphatic basin in cancer dogs was based on palpation and cytological sampling of superficial lymph nodes located closest to the primary tumor.[65] However, several veterinary studies have demonstrated that these techniques yield a considerable rate of false negative results, potentially leading to patients' understaging.[49,47,76,112-114] One study on the diagnostic accuracy of different methods of assessing RLN in dogs and cats with solid neoplasm reported sensitivity and specificity of 60% and 72% for palpation, which compared unfavorably with values of sensitivity and specificity of 100% and 96% for cytology and 64% and 94% for histological biopsy.[49] Likewise, microscopic evidence of nodal metastases was demonstrated in 40% of dogs with malignant oral melanoma and normal-sized RLN[47] and in 64.5% of dogs with cutaneous MCT and clinically normal RLN.[68] Cytological sampling of RLN yields higher diagnostic accuracy than clinical examination, although the risk of false negative results remains significant, especially for specific tumor types, with one study reporting low sensitivity for canine sarcoma, melanoma and mast cell tumor.[76,113] In a recent study, nodal metastases were detected with RLN fine-needle aspirated only in 1 out of 9 lymph nodes that were ultimately metastatic at histopathological examination.[113] The utility of advanced imaging modalities such as ultrasound and computed tomography (CT) for the detection of nodal metastases is also debatable. Skinner et al (2018) evaluated the diagnostic accuracy of contrast-enhanced CT to detect mandibular and retropharyngeal metastases in dogs with oral and nasal tumors and reported a low sensitivity both for micro- and macro-metastases, especially when retropharyngeal nodes were assessed.[50] Hence, histopathological examination on the excised lymph nodes, regardless of their size and appearance, remains ideal for accurate tumor staging in dogs.[68,76,113]

To complicate things further, lymphatic drainage patterns in dogs can be unpredictable, due to individual variations and overlapping of draining areas between different lymphocenters, as well as aberrant drainage pathways induced by the tumor itself.[29,111,115] Hence, the first draining node (the SLN) may not always correspond to the anatomically closest lymph node (the RLN), and with assessment of the RLN there is potential of missing nodal metastases. Unpredictable patterns of nodal metastases have been extensively documented in dogs with head and neck tumors, with metastases occurring to nodes other than the mandibular in 45.5% of dogs in one study[14] and contralateral dissemination identified in 62% of dogs in another study.[15] In a retrospective evaluation of the prevalence of nodal metastases in dogs with oral melanoma and squamous cell carcinoma undergoing completion neck dissection, nodal metastases were histologically detected only in the retropharyngeal node in 6% of animals, and to both the mandibular and retropharyngeal node in 21%, while contralateral metastases occurred in 24% of cases.[52] Our research group recently investigated the impact of SLN biopsy in dogs with head and neck tumors and found that SLN occurred at unpredictable sites in 52% of animals and multiple nodes were excised in 61% of cases with the guidance of the intraoperative mapping technique.[27] Likewise, unpredictable patterns of lymphatic drainage were reported in several recent studies describing different techniques for SLN mapping and biopsy in dogs with MCT, with SLN not corresponding to the RLN in 42-63%.[26,74,75] Similar findings are reported in bitches with mammary tumors.[117,118] On the basis of these observations, the veterinary literature has reported interest in the localization of the draining node that should be excised for histopathological examination, shifting the debate from *how* to assess nodal metastases to *which* node should be assessed. The introduction of the "sentinel lymph node" concept dates back to 2002, when Balogh et al (2002) described the use of radiopharmaceutical and blue dye for SLN detection in 24 oncological dogs and demonstrated its applicability in veterinary medicine.[119] Of 35 metastatic SLN, 34 were identified with the intraoperative mapping procedure and would have been missed with traditional RLN excision.[119] A few years later Tuohy and collogues (2009) firstly advocated the incorporation of SLN biopsy in the staging of canine malignancies given the promising experiences reported in human oncology during the previous 20 years.[35] Afterwards, clinical studies have been conducted on tumor-bearing dogs and different techniques have been described for SLN mapping and biopsy in various canine malignancies.

While several mapping techniques are available, radiopharmaceutical is regarded as the gold standards in human oncology, with detection rates as high as 99% reported for cutaneous melanoma, breast cancer, oral squamous cell carcinoma.[120,121] The technique, as firstly described in people by Krag and Alex, consists of a peritumoral injection of a radionuclide, typically technetium-99m labelled colloids, followed by preoperative lymphoscintigraphic imaging and intraoperative handheld

gamma probe to identify the SLN.[122] Furthermore, vital dyes can be combined to lymphoscintigraphy to aid the surgeon in intraoperative visualization of the SLN. Radiopharmaceutical alone or in combination with blue dyes has been described for SLN mapping both in tumor-bearing and healthy dogs, with promising results. In 2014, Worley described the use of preoperative lymphoscintigraphy combined with intraoperative gamma probing and blue dye in 20 consecutive canine MCT.[74] The combined technique allowed for identification and excision of the SLN in all included dogs, leading to a detection rate of 100%; in 8 animals the SLN differed from the RLN, underscoring the accuracy of the mapping technique.[74] Similar to Worley's experience, our research group recently described the use of radiopharmaceutical and blue dyes for SLN mapping and biopsy in 34 MCT without clinical evidence of RLN enlargement and reported a detection rate of 91% with SLN occurring at unpredictable sites in 63% of cases.[26] Furthermore, with intraoperative gamma-probing surgeons were able to excise a different number of nodes per lymphocenter in different dogs, thus allowing for a non-standardized identification of animal-specific lymphatic networks.[26] Likewise, radiopharmaceutical and blue dye allowed for accurate nodal staging in 23 dogs with various malignancies of the head and neck and cN0 neck, with a detection rate of 83% and a non-correspondence between the SLN and RLN of 52%; intraoperative gamma-probing allowed for excision of multiple in 61% of dogs.[27] When looking at diagnostic performances of the technique, sensitivity was 88.9% and specificity 100%, underscoring the accuracy of lymphoscintigraphy for staging of head and neck tumors.[27] These results were consistent with two previous studies on canine head and neck cancer, that reported 100% detection rates with use of radiopharmaceutical and blue dye.[123,124] Results of preoperative planar lymphoscintigraphy for SLN mapping in 51 dogs with solid malignancies, including MCT, head and neck tumors and mammary tumors, have also been recently published by our research group.[125] The detection rate reported in that study was 95%, with the SLN not corresponding to the RLN in 61.4% of dogs.[125] Radiopharmaceutical was successfully used to map the mammary gland, anal sacs and various cutaneous regions in healthy dogs.[117,126,127] Although specific studies on the safety of radiopharmaceutical and blue dyes in dogs have not been conducted yet, complications related to the administration of the radiotracer or vital dyes have not been reported in the previous studies[74,124], nor have been recorded in three recent studies conducted by our research group[26,27,125], suggesting a high tolerability of lymphoscintigraphy and blue dye in dogs.

The main drawbacks of radiopharmaceutical for SLN mapping are the relatively high costs and the restrictions to the use of radioactive materials.[111,115] Hence, other techniques have been proposed for SLN mapping in dogs, including indirect lymphography with radiography[128] or CT[118,124,129-132], contrast-enhanced ultrasound (CEUS)[75,123] and near-infrared imaging (NIR).[133] Indirect lymphography is a preoperative mapping technique based on the injection of a radiopaque contrast

medium peritumorally and the subsequent visualization of the drainage pathway(s) to the SLN. Brissot et al (2016) performed indirect lymphography using iodized oil and radiographic imaging in 30 dogs with solid tumors and reported a detection rate of 96.6%; despite the high detection rate and accessibility of the technology required, this technique was not reported thereafter and its use in veterinary practice is still limited due to the highly variable timing for contrast migration to the SLN and risk of adverse reactions.[115,128,134] Conversely, indirect lymphography with aqueous contrasts and CT are gaining popularity in veterinary oncology. The technique consists of preoperative CT scans immediately after a peritumoral injection of the contrast medium until the lymphatic pathways and SLN are visualized.[111,115] Indirect CT lymphography has proven successful for SLN mapping in dogs with mammary tumors[118,129], anal sac gland adenocarcinoma[130], tumors of the head[124,131], integumentary mast cell tumors[76] solid tumors of various sites.[132] Despite the high detection rates reported in studies describing the preoperative identification of SLN on CT scans, this technique is lacking an intraoperative mapping of the SLN, thus potentially raising the risk of missing SLNs during surgical dissection.[115,131,132] In Randall's study, indirect CT lymphography was able to identify a SLN only in 11/20 dogs, while a SLN was identified in all dogs with preoperative lymphoscintigraphy and intraoperative gamma-probing and blue dye.[124] Hence, the combination with vital dyes such as methylene blue and indocyanine green have been advocated to improve diagnostic performances of indirect CT lymphography. In a recent study on SLN mapping in dogs with oral tumors, detection rate with indirect CT lymphography alone was as low as 42.1%, although it raised to 100% when intraoperative indocyanine green NIR or methylene blue were combined.[133]

Similar to indirect CT lymphography, CEUS is based on a peritumoral injection of microbubble contrast medium and preoperative identification of the draining lymph node or nodes with ultrasound, although the technique does not allow for intraoperative localization of the SLN.[115] Two clinical reports are available on the use of CEUS for SLN mapping in tumor-bearing dogs. In a first study on head and neck tumors, CEUS revealed the SLN in 8/10 dogs, comparing unfavorably with 10/10 SLN detected when the same animals underwent lymphoscintigraphy.[123] More recently, Fourier et al (2020) used CEUS for SLN mapping in canine MCT, and reported a preoperative detection rate of 95.5%, although only 56.7% of SLN were ultimately excised.[75]

NIR is an emerging mapping technique in veterinary medicine, and involves a peritumoral injection of a fluorescent agent, most commonly indocyanine green, that emits a light in the near infrared spectrum which is detected by a dedicated NIR camera and visualized in real-time by the surgeon.[115] Most of the available literature on NIR for SLN mapping in dogs consists of pre-clinical studies for its application in human patients or feasibility studies on healthy dogs, providing evidence on the safety and efficacy of this modality.[135-137] Use of NIR in a clinical setting has so far been reported in

a single study, with promising results: NIR with indocyanine green allowed for the identification of SLN in 14/14 dogs, leading to a detection rate of 100%.[133] Conversely from indirect CT lymphography and CEUS, NIR imaging is thus a merely intraoperative mapping method, although the fluorescence of superficial lymph nodes can also be seen through the skin.[137,138]

The mapping techniques described in veterinary medicine are summarized in Table 2.

Mapping Technique	***Phases***	***Performances***	***Advantages***	***Disadvantages***	***References***
Radiopharmaceutical +/- methylene blue	Preoperative + intraoperative	Detection rate: 83-100% Sensitivity: 88.9% Specificity: 100%	Accuracy, intraoperative guidance, safety	High costs, restrictions for radioactive materials	Lurie 2009, Worley 2014, Ferrari 2020, Randall 2020, Chiti 2021
Indirect radiographic lymphangiography	Preoperative	Detection rate: 96% Sensitivity: NA Specificity: NA	Availability, low costs	Unpredictable timing for contrast migration, no intraoperative guidance, risk of adverse reactions	Brissot 2016, Mayer 2016
Indirect CT lymphangiography	Preoperative	Detection rate: 42%-89% Sensitivity: NA Specificity: NA	Availability, safety	Variable detection rates, no intraoperative guidance	Soultani 2016, Grimes 2017, Majeski 2017, Rossi 2017, Randall 2020, Colivignarelli 2021
Indirect CT lymphangiography + methylene blue/NIR	Preoperative + intraoperative	Detection rate: 100% Sensitivity: NA Specificity: NA	Availability, safety	Need for further validation	Wann 2021
CEUS	Preoperative	Detection rate: 80-95% Sensitivity: NA Specificity: NA	Availability, Safety, low costs	No intraoperative guidance	Lurie 2009, Fourier 2020
NIR (Indocyanine green)	Intraoperative	Not reported as single technique on cancer dogs	Availability, safety	No preoperative mapping	On healthy dogs: Knapp 2007, Cabon 2016, Townsed 2018
Methylene blue	Intraoperative	Not reported as single technique	Availability, safety	No preoperative mapping	In combination with radiopharmaceutical: Worley 2014, Ferrari 2020, Chiti 2021; in combination with NIR: Wann 2021

Table 2. *Diagnostic performance, phases (preoperative and/or intraoperative), advantages and disadvantages and references for existing SLN mapping technique in veterinary oncology*

1.4. Sentinel lymph node biopsy in head and neck cancer: the path from human to canine oncology

Identification of the SLN in head and neck tumors is particularly challenging. Indeed, the head and neck feature a complex network of lymphatics with multiple overlapping drainage pathways and individual variations reported in healthy dogs and humans.[29,139] To complicate things further, solid tumors are able to induce neolymphangiogenesis, thus potentially stimulating the creation of novel and unpredictable drainage pathways.[35] In dogs with tumors of the head and neck, metastases to lymph nodes other than the mandibular are reported in nearly half of cases,[14] and contralateral dissemination occurs in up to 62% of animals with oral tumors.[15]

In humans, the early detection of cervical node metastases is pivotal for correct treatment recommendation and prognostication. In people with oral squamous cell carcinoma, the presence of cervical lymph node metastases is the most adverse independent prognostic indicator with the exception of distant metastases[140], and survival is reduced by half in cases of clinically apparent cervical metastases.[141] Likewise, a prognostic and therapeutic role of the extirpation of early metastatic nodes has been demonstrated for canine MCT.[72,73] Conversely, robust evidence on the impact of the early detection of nodal metastases in canine head and neck malignancies is lacking, due to inconsistent recommendations for the management of cN0 neck in the veterinary practice.[17] To complicate things further, several tumor types with various metastatic patterns and biological behavior are classified as head and neck tumors, hence the impact of extirpation of metastatic nodes should be address in studies focusing on specific tumor types. Nonetheless, given the prognostic relevance of tumor stage, a prompt identification of metastatic spread to the lymphatic basin could allow for accurate staging, prognostication and treatment choices.[18-25] Furthermore, consistent incorporation of accurate nodal staging in the management of canine head and neck tumors is crucial to collect prognostic information and assess the potential therapeutic role of metastatic nodal removal.

Historically, two strategies are available for accurate nodal staging of head and neck tumors in humans: elective neck dissection (END) and sentinel lymph node biopsy (SLB). Elective neck dissection was introduced in human medicine in 1905 with a landmark publication describing the personal experience of Dr. George Washington Crile.[142] The “Crile operation” consisted of an en-bloc resection of the cervical lymph-node bearing tissue between the superficial and deep cervical fascia, that soon became standard of care for the staging of cervical nodes in humans after a second publication.[142] Since then, the literature has made great efforts to standardize the procedure and

established specific indications for END.[143] In 2002, a modified classification for END techniques was presented by the American Head and Neck Society and American Academy of Otorynolaryngology-Head and Neck Surgery, which prompted a more selective approach with the excision of specific sublevels that are at higher risk of metastases.[41,143]

Traditionally, END is recommended to manage tumors with a risk of occult nodal metastases > 20%, given the improved survival times reported for patients who underwent END compared to watchful waiting approach, even in case of salvage intervention after neck failure.[144,145] Most commonly, END is performed for SCC of the upper aerodigestive system given their high propensity for cervical nodes metastases, with reported rated of occult nodal metastases of 20-30% even in early stages;[144,146,147] other indications include malignant thyroid tumors, tumors of the parotid gland and skin tumors such as melanoma.[148] The main disadvantage of END is the well-established aesthetic and functional morbidity, namely scar formation, shoulder pain, limitations of abduction and scapular wining, that can occur in almost all patients treated with traditional END, although less frequently reported with modified techniques.[147] Such a high morbidity may be unacceptable when considering that 70-80% of patients will ultimately not have occult nodal metastases.[147]

SLB has gained increasing acceptance in the last decade, as a less invasive yet accurate technique for nodal staging in human patients with head and neck cancer.[121] Although the reintroduction of SLN for early-stage melanoma dates back to 1992,[91] thereafter END has been routinely performed for staging of people with head and neck tumors, especially those at high risk of occult nodal metastases such as oral SCC; it has been recently estimated that SLB is used in less than <5% of suitable cases in the United States of America.[150] However, SLB has proven successful for the detection of occult nodal metastases in early-stage squamous cell carcinoma, with reported sensitivity of 89% and a negative predictive value of 94%.[151] In a recent study, the diagnostic accuracy of SLB for nodal staging in people with oral SCC and cN0 neck was compared with END.[121] The overall sensitivity and negative predictive value were comparable between the two techniques, with SLB having 81% sensitivity and negative predictive value of 93%.[121] A disadvantage of SLB compared to END is the lower diagnostic accuracy reported for floor-of-mouth tumors, caused by the so-called “shine through phenomenon”, which refers to the impossibility of detection of the SLN when it is within the flare of radiation by the tumor.[121,152] A super-selective level I neck dissection or the use of hybrid tracers with fluorescence label may be added to the traditional SLB procedure in case of floor-of-mouth tumors to improve the sensitivity, although it should be noted that the lower accuracy did not result in shorter disease-specific survival in a recent study.[121]

Although a targeted approached with SLB offers significant advantages in terms of less invasiveness and lower costs, the debate on which techniques is superior for staging of cN0 neck is still

unsolved.[121,149] While both techniques allow for accurate nodal staging, randomized trials are warranted to provide evidence on the superiority of one of the two. To date, in the decision process several factors are taken into account to optimize the management of people with head and neck cancer and cN0 neck, among others: patients' personal preferences and institutional factors.[149]

The path to an accurate staging of cN0 neck in veterinary oncology was similar the human counterpart, although robust evidence and standardization are still lacking on our side. The first description of END for staging of head and neck tumors dates back to 1995, when Smith et al described a technique for ipsilateral or bilateral excision of retropharyngeal, parotid and mandibular nodes in 6 carcasses and 3 dogs.[153] The same technique was reapplied a few years later in a cohort of dogs and cats with spontaneous head and neck malignancies, allowing for the detection of metastases not involving the mandibular nodes in nearly half of dogs.[14] A surgical approach for bilateral removal of mandibular and retropharyngeal nodes through a single ventral midline incision was then described by Green et al in 2017.[154] Skinner et al (2016) performed the technique on 31 tumor-bearing dogs and founded nodal metastases in 11 retropharyngeal nodes without concurrent mandibular node involvement, and contralateral dissemination in 61% of dogs.[15] More recently, END was reported in 27 dogs with oral melanoma and 21 dogs with squamous cell carcinoma and allowed for detection of nodal metastases in 37% and 29% of cases, respectively.[52]

Contrary to the human counterpart, complications reported with these END techniques are rare and self-limiting in dogs, mainly consisting of transitory edema of the muzzle or lip.[15,17,52,153] However, some drawbacks exist to the application of END also in dogs. None of the described approaches for END in dogs allows for extirpation of all the potentially metastatic cervical nodes. The unilateral approached described by Smith may indeed miss contralateral nodal metastases, and although the technique can be performed on both sides, this approached is rarely performed. Conversely, other approaches that are more commonly used do not include the excision of the parotid nodes, which can harbor metastases in 9-16% of dogs.[14,27] SLB, on the contrary, allows for a target nodal approach potentially minimizing the risk of missing nodal metastases. A few studies are available on SLB in dogs with malignancies of the head and neck and results have been so far promising, with high detection rates and diagnostic accuracy.[27,123,14,131] In 2009, Luire et al firstly described SLB in dogs with spontaneous tumors of the head and neck and reported a detection rate of 100% with radiopharmaceutical and of 80% with CEUS.[123] Indirect CT lymphography was also used to map the cN0 neck in cancer dogs, allowing for detection of contralateral dissemination in 8% of animals in one study.[131] The technique was compared with lymphoscintigraphy in a more recent study, with detection rates of 55% and 100% respectively.[124] The diagnostic accuracy of SLB with radiopharmaceutical was confirmed by a recent study of our research group, conducted in the contest

of the present research project.[27] In that study, the cN0 neck was mapped in 23 dogs with naturally occurring malignancies of the head and neck, with a detection rate of 83%, sensitivity of 88.9% and specificity of 100%.[27] Lastly, Wan et al (2021) recently described the combined use of indirect CT lymphography and vital dyes for SLB in dogs with oral tumors and reported a detection rate of 100% with the combined technique.[133]

SLB in head and neck cancer is still at its infancy in veterinary oncology, and although a few studies have demonstrated the feasibility of different mapping techniques, the actual impact of a target nodal approach and its benefits compared to END warrant further investigations.

1.5. General aim

In the last 20 years, canine oncology has reported increasing interest in the detection of occult nodal metastases for several tumor types, and efforts have been made to identify the best strategy for accurate nodal staging to promote prompt treatment recommendations. In this scenario, our research group at University of Milan have contributed to the current knowledge on nodal staging in cancer dogs. Our commitment was firstly raised by the observation that nodal metastases were often histologically detected in dogs with MCT when performing RLN excision, even in the absence of clinically evident nodal disease; such observation resulted in the publication of a first paper demonstrating occult metastases in nearly half of dogs with cutaneous MCT of various sites and normal-sized RLN.[68] However, the identification of the RLN based on the anatomical location of the primary tumor is not always straightforward, and with longer follow-ups available we observed that some patients would eventually develop clinically evident nodal disease to another lymph node. These observations were consistent with the current literature, with a few papers reporting unpredictable patterns of drainage for various tumor types, especially for head and neck malignancies.[14,15] Hence, we decided to start exploring the potential role of SLN mapping and extirpation in the management of cancer dogs. Despite the presence of several mapping methods, lymphoscintigraphy is a cornerstone of SLN mapping in human medicine for several malignancies, including tumors of the head and neck.[155] Given the availability of the required technology (gamma camera and gamma probe) and permission for storage of radiopharmaceuticals at our veterinary teaching hospital, we decided to use lymphoscintigraphy in combination with vital blue dye to serve the purpose.

Firstly, the procedure was implemented in the management of dogs with integumentary MCT, given the high incidence of this tumor. In the same context, it was decided to investigate the application of SLN mapping with radiopharmaceutical and blue dye in dogs with spontaneous malignancies of the head and neck and cN0 neck, which was the main research topic of the present PhD project. The main research hypothesis was that incorporation of SLB, defined as the surgical extirpation of the whole SLN(s) as per excisional biopsy, with radiopharmaceutical and blue dye would improve the management of dogs with malignant head and neck tumors and cN0 neck, by allowing for a more accurate staging than traditional RLN excision. Given that the inclusion of SLB in dogs with MCT and head and neck tumor share the same principles, and considering that the two projects were conducted simultaneously, with reciprocals contaminations, in the present Thesis results of both projects will be presented.

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2. Biopsy of sentinel lymph nodes after injection of methylene blue and lymphoscintigraphic guidance in 30 dogs with mast cell tumors

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*Ferrari R, Manfredi M, **Chiti LE**, Zani DD, De Zani D, Rabbogliatti V, Giudice C, Stefanello D. Sentinel lymph node biopsy guided by combination technique (lymphoscintigraphy and blue dye) for mast cell tumor in dogs: results in 31 cases*

The sentinel lymph node (SLN) is the first lymph node (LN) receiving drainage from a primary tumor and is expected to be the first site of metastasis.[1] Since its first description in 1992, identification of the SLN with radionuclides, followed by its histopathological evaluation, has become routine for oncologic staging in human cancer patients.[1-4]

Interest in the prognostic role of lymph node status in canine cancer patients has increased during the past decade. Several studies reported the evaluation of non-palpable/normal-sized lymph nodes as possible sites of early metastasis in the last 5 years.[5-13] To date, however, LN biopsy is most commonly performed on the regional lymph node (RLN, i.e., the LN anatomically closer to the mass) rather than the SLN in veterinary oncology. Due to the weakness of data on the lymphatic network in dogs and to the hypothesized variability of lymph drainage between healthy and cancer tissue, the evaluation of RLN might lead to incorrect clinical staging, as this node may not always correspond to the draining node. [6-10, 12-16]

As a consequence, recent veterinary publications have reported the interest in the identification of the SLN in dogs.[17-25] Epithelial and round cell tumors generally spread via lymphatic vessels and primarily metastasize to lymph nodes; they accordingly represent an excellent model to test SLN mapping techniques in cancer-bearing dogs.[23,26] Among round cell neoplasms, mast cell tumors (MCTs) is a prevalent skin malignancy in dogs that are known to spread first to LNs.[27]

In 2014, Worley described her experience regarding 20 canine MCTs and demonstrated the utility of lymphoscintigraphy for identification of the SLN, underlining the high level of discrepancy between the SLN and the clinically identified RLN.[20] However, due to its explorative nature, Worley's study

still left a gap in the knowledge of SLN mapping and extirpations in dogs with MCT: the absence of reported clinical status (normal or abnormal) of the RLN; the SLN mapping was performed even in the presence of positive cytological node; the absence of Patnaik grade-1 tumors and Kiupel grading system; Weishaar categorical classification for MCT nodal metastases was not available yet and was thus not applied for lymph node histological evaluation, potentially leading to a less objective identification of nodal metastasis.[20] Additional studies are thus warranted to better determine the impact of SLN extirpation in dogs with MCTs.

The present prospective case series study aims to report the outcomes associated with sentinel lymph nodes (SLNs) detection and extirpation guided by radionuclide and methylene blue injections in dogs with cutaneous and subcutaneous mast cell tumors (MCTs). The impact of SLN biopsy on oncologic staging was evaluated using histopathology data and anatomic correspondence with the clinically expected RLN. We hypothesized that lymphoscintigraphy combined with methylene blue injection would allow detection of at least one SLN in dogs with cutaneous or subcutaneous MCT, leading to a high detection rate and that these SLNs would not correspond to clinically expected RLNs in most tumors. Furthermore, we assumed that the SLN would harbor occult early or overt metastasis (HN2 and HN3, respectively; in according to Weishaar et al., 2014)[12] in at least 30% of SLNs biopsied, pointing out the utility of SLN mapping and extirpation for a correct lymph node staging in canine MCT, even in the presence of low-grade tumors.

2.1. Materials and Methods

This observational study was conducted from January 2017 to December 2018 at the Veterinary Teaching Hospital of the Università degli Studi di Milano. Client-owned dogs with a cytological diagnosis of one or more gross MCTs amenable to curative-intent surgery in the presence of a non-palpable/normal-sized RLN were prospectively included.[13] Dogs eligible for inclusion should not have distant metastasis excluded by ultrasonographic-guided cytology of the spleen and liver.[28,29]

All owners signed written informed consent to SLN mapping as well as the surgical procedure. Exclusion criteria were dogs with T0 (i.e., a scar from previous surgery with infiltrated margins) cutaneous and subcutaneous MCT and pregnant dogs.

General anesthesia was induced in all dogs with different protocols based on the pre-operative anesthesiologic evaluation of each dog. Pre-operative and intraoperative SLN identification and the surgical procedure were performed on the same day. A dose of 6–30 MBq/0.5 ml technetium-99 metastable (99mTc) labeled nano-sized human serum albumin (Nanoalbumon, Radiopharmacy Laboratory Ltd, Budaörs, Hungary) was injected peritumorally in four sites at a distance of 1-2 mm

from the gross margins of the tumor.[30] The injection was subcutaneous. Regional dynamic (2-minute, one frame per second) and planar static images (2 minutes) were acquired using a single-head gamma camera (Picker Prism 2000XP). The injection sites were masked with 2-mm lead foil to achieve better visualization of the draining path when necessary. The first lymph node station (also called lymphocentrum) along the draining path was reported as the SLN station. Every first LN station in each path was considered as the SLN station if more than one lymphatic path originated from the primary tumor. Dogs were aseptically prepared for surgery at the end of the nuclear medicine procedure, and 0.4 ml of 5 mg/ml sterile methylene blue (SALF S.p.A, Cenate Sotto, Bergamo, Italy) was injected peritumorally in four sites before MCT excision.

All tumors were excised with curative intent surgery (2–3 cm lateral margins and at least one deep fascial plane). Surgeons changed surgical instruments and gloves for SLN extirpation, after MCT removal. Intraoperatively, a hand-held gamma probe (Crystal probe SG04, Crystal Photonic GmbH, Berlin, Germany) detected radioactive tissues and guided the soft tissue dissection to the lymphocentrum identified by the pre-operative lymphoscintigraphy. Surgeons excised each LN belonging to that lymphocentrum with a radioactive count (RC) of at least twice the RC of a distant body region (background count) and any visible blue LN. These LNs were considered SLNs. Surgeons checked the ex-vivo RC of the first SLN removed and extirpated further non-colored LNs belonging to the same lymph node station if the RC was equal to or greater than 10% of the RC of the hottest SLN removed.[31] Excised primary tumor and SLNs were placed in hermetic boxes with a 10% formalin neutral-buffered solution and left in the nuclear medicine room. Boxes were sent to the histopathology laboratory when the count rate was lower than the background count. Surgical instruments and disposable materials were monitored and, if contamination was present, held in the nuclear medicine room for decay in storage. Staff members who were pregnant or suspected of being pregnant were not allowed to participate at any point in the procedure.

Dogs were hospitalized for at least 24 hours and then discharged upon the decision of the clinician responsible for the case. Radiologists also checked dogs for residual radioactive activity before discharge: dogs were discharged with an RC at 1 meter from the patient equal to or lower than the background count.

The histopathology report included evaluation of (a) the MCT according to both the Kiupel and the Patnaik grading system, (b) the surgical margin status [trimmed according to the tangential (en face) sectioning method and defined as infiltrated versus not infiltrated], and (c) the SLN metastatic status according to Weishaar et al. (Table 1).[32-34] Each lymph node was cut longitudinally at the level of hilus. Additional multiple slices (1.5 mm thick) were obtained from each half for lymph nodes thicker than 3 mm (minor axis). All obtained slices were processed for histology and paraffin-embedded.

Serial microtomic sections were cut for each slice and stained with hematoxylin and eosin and with Giemsa stain.

Recorded data for each dog included: dog signalment; MCT dimension, site (divided into the trunk; distal limb – below the elbow and stifle joint; head and neck; genital – including vulvar, scrotum, prepuce; tail; and digit), and presentation (first vs. recurrence); RLN, clinically identified as the node anatomically closest to the MCT; SLN identified by lymphoscintigraphy; SLN identified by methylene blue; histopathological data; and any possible surgical complications.

Classification	***Histopathological criteria***	***Proposed interpretation***
HN0	None to rare (0-3), scattered, individualized (isolated) mast cells in sinuses (subcapsular, paracortical, or medullary) and/or parenchyma per X400 field (0-3 mast cells per X400 field), or does not meet criteria for any other classification below.	Non-metastatic
HN1	Greater than three individualized (isolated) mast cells in sinuses (subcapsular, paracortical or medullary) and/or parenchyma in a minimum of four X400 fields (unless otherwise stated, at least four X400 fields each, which contain more than 3 mast cells)	Pre-metastatic
HN2	Aggregates (clusters) of mast cells (≥3 associated cells) in sinuses (subcapsular, paracortical or medullary) and/or parenchymal, or sinusoidal sheets of mast cells	Early metastasis
HN3	Disruption or effacement of normal nodal architecture by discrete foci, nodules, sheets, or overt masses composed of mast cells	Overt metastasis

Table 1. *The classification system for histopathological evaluation of node metastasis proposed by Weishaar et al., 2014.*

2.2. Results

Thirty-four MCTs in 30 dogs were included in the study. The dogs comprised seven Labrador retrievers, five mixed breeds, four Golden retrievers, two Dogo argentinos, and 12 dogs belonging to one of the following breeds: Beagle, Italian hound, American staffordshire terrier, Greater swiss mountain dog, Tosa inu, Boxer, Pug, Weimaraner, Yorkshire terrier, Dachshund, English setter, and Maltese. Twelve dogs were female (11 spayed), and 18 dogs were male (4 neutered). The mean and median age was 7.5 and 7 years, respectively (range 1–14 years), and the mean and median body weight was 28 and 31 kg, respectively (range 3.5–67 kg).

The mean and median dimensions of MCTs were 2.1 and 2 cm, respectively (range 0.6–6 cm). Two tumors were recurrences (one after surgery alone and one after surgery plus radiation therapy). Tumors' locations were: the trunk (16/34), distant limb (9/34), genitals (4/34), head and neck (3/34), tail (1/34), and digits (1/34).

Lymphoscintigraphy permitted identification of at least one SLN in 31 out of 34 tumors, with an identification rate of 91%. The procedure failed to identify the SLN station in two dogs with one MCT each (both recurrences). Methylene blue injection and lymphoscintigraphy identified a subcutaneous inguinal structure not classified as lymphoid tissue on histopathology in another dog (histopathology reported eosinophils and no neoplastic mast cells within scar tissue). This dog had undergone ipsilateral unilateral mastectomy for mammary epithelial tumor one year previously.

In one dog with one MCT located on the trunk in which pre-operative scintigraphy identified a sentinel axillary lymph node station, the owner refused the surgery. The other 26 dogs (30 tumors) with SLN identification underwent gamma-probe guided SLN extirpation, with the removal of a total of 57 SLNs (Table 2).

Lymph node station	*Total number of SLNs removed*	*Number of SLNs removed in each lymph node station*
Mandibular	4	2*
Prescapular	8	1
Axillary	6	2
Accessory axillary	5	1 or 2†
Inguinal	25	1 or 2
Popliteal	8	1 or 2
Internal iliac	1	1
Total	57	

Table 2. *Description of the SLNs removed. Legend: SLNs: sentinel lymph nodes. * A third mandibular node was wrongly removed in both side of the dog (dog 22 in table 3). † A third accessory axillary node was wrongly removed in one dog*

All SLNs removed were also blue-stained, and surgeons did not find any blue lymph nodes without radioactivity, leading to a 100% correlation between "hot" and "blue" nodes. Surgeons wrongly removed 3 additional lymph nodes due to their contiguity with the SLN (two located at the mandibular station and one at the accessory axillary station). These three additional lymph nodes were not blue-stained and, had an ex vivo RC of zero when separated from the SLN.

Among the 30 tumors mentioned above, the SLN corresponded to the clinically expected RLN in 11/30, the SLN did not correspond to the clinically expected RLN at all in 13/30, and the SLN only partially corresponded to the clinically expected RLN in 6/30 (Table 3). Specifically, pre-operative lymphoscintigraphy identified more than one draining path, and an additional lymph node station different from the RLN was identified as the SLN in these six MCTs (Table 3).

No side effects were recorded during SLN mapping. Postoperatively, an abscess occurred at the site of SLN removal in one dog (which resolved with antibiotics), and seroma in two dogs. Mild, temporary edema of the region drained by the SLNs occurred in three dogs during the first 5 days after surgery. Partial dehiscence at the site of MCT excision occurred in four dogs, all with a surgical wound reconstructed with a linear pattern and healed by second intention. The surgical defect resulting from MCT excision was reconstructed with a genicular flap in one dog, and a postsurgical seroma occurred, requiring the use of active drain suction. Finally, a free skin graft completely failed, and the defect was left to heal by second intention in one dog.

Histological examination of the 57 SLNs reported 21 HN0, 4 HN1, 26 HN2, and 6 HN3. Twenty-four tumors were cutaneous MCTs, of which 20 were Patnaik grade II–Kiupel low grade and 4 were Patnaik grade I–Kiupel low grade. The primary tumor was a subcutaneous MCT in the remaining six (the SLNs in these tumors were as follows: 6 HN0, 1 HN1, 2 HN2, and 5 HN3). Evaluation of the histological margins revealed 28 complete excisions and two tumors excised with infiltrated margins.

Dog	*Weight (kg)*	*MCT dimension (cm)*	*MCT location*	*Lymphocentrum of RLN*	*Lymphocentrum of SLN (number of SLN removed)*
1	16	1.5	Ventral neck – R	Prescapular – R	Prescapular – R (1)
2	18	4	Ischiatic tuberosity region – R	Inguinal – R	Inguinal – R (2)
3	7.3	3	Scapular region – R	Axillary* – R	–
4	37	1	Lateral thorax 13th rib – L	Accessory axillary or Inguinal – L	Accessory axillary – L (2)
5	31	4	Lateral thorax 13th rib – L	Accessory axillary or Inguinal – L	Accessory axillary – L (1)
6	41.5	2	Stifle – R	Popliteal – R	Inguinal – R (2)
		2	Flank – L	Inguinal – L	Inguinal – L (2)
		0.7	Ventral thorax – R	Axillary – R	Prescapular – R (1)
7	31	1	Popliteal region – R	Popliteal – R	Popliteal – R (1) Inguinal – R (1)
8	34	1	Preputial – L	Inguinal – L	Inguinal – L (1)
9	27	2.5	Stifle – R	Popliteal – R	Popliteal – L (2) Inguinal – L (1)
10	51	6	Lateral thorax – R	Accessory axillary – R	Axillary – R†
11	33	2	Scrotal – R	Inguinal – R	Inguinal – R (1)
12	33	1	Shoulder – L	Prescapular – L	Prescapular – L (1)
13	33	3	Between 3rd and 4th mammary gland – L	Inguinal – L	Inguinal – L (2)
		1	3rd digit, hindfoot – R	Popliteal – R	Popliteal – R (2)
14	34	2.5	Forearm – R	Axillary – R	Axillary – R (2) Prescapular – R (1)
15	34	0.6	Lateral thorax – R	Accessory axillary – R	Axillary – R (2)
16	62	3	Para-preputial – L	Inguinal – L	Inguinal – L (2)

17	38	2.2	Popliteal region – R	Popliteal – R	Popliteal – R (1) Inguinal – R (2)
18	9.7	1	Temporomandibular joint – L	Mandibular – L	Prescapular – L (1)
		0.6	Scapular region – R	Prescapular – R	Prescapular – R (1)
19	31	3	Flank – L	Inguinal – L	–
20	35	2	Forearm – R	Axillary – R	Prescapular – R (1)
21	31	3.5	Ventral thorax – L	Axillary – L	Accessory axillary – L (1) Axillary – L (2)
22	32	1	Nose – middle	Zygomatic – R and L	Mandibular – R (2) Mandibular – L (2)
23	5	1	Stifle – R	Popliteal – R	–
24	26	0.6	Leg – L	Popliteal – L	Inguinal – L (2)
25	5.7	0.8	Base of the tail – R	Inguinal – R	Internal iliac – R (1)
26	35	0.6	Preputial – R	Inguinal – R	Inguinal – R (2) Inguinal – L (1)
27	3.5	2.5	Ventral thorax – L	Axillary – L	Prescapular – L (1)
28	20	4.7	Leg – R	Popliteal – R	Popliteal – R (2) Inguinal – R (2)
29	23	1	3rd mammary gland – L	Inguinal – L	Accessory axillary – L (1)
30	22	5.5	Thigh – R	Inguinal – R	Inguinal – R (2)

Table 3. *Correspondence between clinically detected RLNs and SLNs. Legend: MCT: Mast cell tumor; RLN: Regional lymph node; SLN: Sentinel lymph node. * Dog with recurrence MCT and prescapular node already removed during the first surgery. † The owner did not allow the removal of an axillary node in this dog. R: right side of the body; L: left side of the body*

2.3. Discussion

SLN mapping and extirpation with radionuclide and injection of methylene blue led to the detection of at least one SLN in 31/34 dogs of this study, without increasing the morbidity related to traditional MCT excision and regional lymphadenectomy. SLNs differed from clinically expected RLNs in 19/30 tumors and were histologically classified as metastatic (HN2-HN3) in 32/57.

The assessment of neoplastic LN invasion in veterinary oncology has undergone essential changes during the last 20 years, shifting from acknowledgment of the inaccuracy of physical examination alone to the constant application of cytology and histopathology to define metastatic status and, most recently, discussion of which lymph node the clinicians should sample.[35-37] SLN mapping is the cornerstone in the staging of different tumors in human medicine,[38] while application of the procedure in veterinary medicine is still in its infancy. The combined technique using lymphoscintigraphy and methylene blue injection is a feasible and safe procedure for the detection of SLNs in dogs with cutaneous and subcutaneous MCTs without RLN alteration in according to the results of the present paper. This finding is consistent with a study by Worley in 2014 even if this previous paper also included T0 tumors and dogs with cytological positive regional lymph node, without reporting the clinical status (normal or abnormal) of the RLN.[20] Surprisingly, despite the reported benefits of SLN mapping in tumor staging, a no further published paper focusing on lymph node staging in dogs with MCT referred to SLN. Considering this, the authors hope that the confirmation of these results reported in the present study could highlight the role of SLN mapping also in canine oncology.

Lymphoscintigraphy allowed the detection of the SLN in 31/34 of tumors in our study. Considering the low correspondence between RLN and SLN, if surgeons would have removed the RLN, the actual draining nodes would not otherwise be excised totally or partially. The benefit of lymphoscintigraphy holds particularly true for MCTs in dogs because a standard anatomic location of the draining lymph node cannot be identified, as with other skin neoplasms such as human melanoma.[39] Particularly, the benefit of lymphoscintigraphy increases when the neoplasm is localized on the trunk or in the head and neck region, where the lymphatic drainage is complex and unpredictable, possibly involving more than one lymphatic path.[37] A study using a canine model reported 10 lymphatic regions (lymphosomes) for each half of the body, respectively drained by 10 different lymphocentrum. This lymphatic topography, although helping in having an idea of which lymphocentrum could drain cutaneous tumor in dogs, also highlighted how the edges of these lymphatic regions are not so clearly distinguishable in the body surface. The location of a cutaneous tumor could belong to different lymphatic regions, allowing for simultaneous drainage from different lymphatic path.[40] This could be

an explanation for the presence of a tumor drained by SLNs belonging to two different lymphocentrum, one of them being not the anatomical closest to the tumor.

Besides, the use of an intraoperative gamma probe permitted correct evaluation of the single lymphocentrum and the removal of a different number of SLNs belonging to the same anatomical lymph node station in different dogs. In this optic, the mapping and extirpation of SLNs represent a non-standardized, single patient-based procedure, even when SLN corresponds to the expected RLN. Some authors have suggested possible variability in the number of lymph nodes belonging to the same lymphocentrum in different dogs, although studies focusing on the anatomy of the lymphatic system in dogs are lacking.[40] In this context, radio-guided extirpation of SLNs permits the identification of any remaining "hot" lymph nodes not directly visible on the surgical bed after removal of the first node, thus allowing for complete extirpation of all the draining nodes (Table 2).

On the other hand, not all the lymph nodes forming the lymphocentrum identified preoperatively corresponded to the first draining node in the present study: the proximity of two lymph nodes led to incorrect extirpation of an ex vivo no-"hot" LN in three tumors (Table 2). In none of these dogs did the supplementary LN biopsy causes any additional complications; however, surgeons should take care to ensure the correct orientation of the gamma probe during the intraoperative RC evaluation on the surgical field of the lymphadenectomy when two nodes are close to each other. Particularly, surgeons should pay attention if one of the LN is not blue using the combination technique in consideration of the 100% correspondence between radiotracer and methylene blue.

In humans with breast cancer and cutaneous melanoma, combining a radiotracer and methylene blue injection maximizes the rate of SLN identification while decreasing the risk of false-negative results.[31,41] However, side effects such as allergic reactions, temporary skin tattooing, blue discoloration of the operating field, and a factitious drop in intraoperative oxygen saturation, have prompted some clinicians to discontinue the use of methylene blue.[42-48] The increase in the SLN identification rate achieved with the sole use of radiocolloid during the past 20 years, likely due to increased experience among surgeons, corroborated the omission of methylene blue.[49] The authors of the present paper observed a high correspondence between the detection of SLN with methylene blue and with scintigraphy in the absence of acute or chronic side effects, as reported previously by Worley.[20] In the authors' opinion, methylene blue injection is particularly useful after SLN detection with the gamma probe to delineate the lymph node margins respect to the surrounding tissues, especially in fatty dogs or at particular sites, such as the inguinal, axillary, and abdominal regions, where gentle dissection is required to avoid accidental damage to neurovascular structures. On the other hand, the injection of methylene blue around the primary tumor could decrease visualization of the deep fascial plane, especially during the dissection of small masses in areas with reduced

subcutaneous tissue (e.g., distal extremities). Because of the learning curve for SLN detection by lymphoscintigraphy and the likely time-related improvement in detection, it is likely that, as in human medicine, veterinary surgeons abandoned the injection of methylene blue in due course. However, currently, the combined technique may be helpful for surgeons at the beginning of their learning curve.

A SLN was identified in 31/34 of MCTs in this study, which is comparable to the rate reported in human breast cancers (90% to 100%).[50,51] The procedure failed to identify an SLN in dogs with scar tissue either at the primary tumor site or in the expected region of the draining lymph node in our study. In the 4 dogs with a T0 tumor included in the study of Worley (2014), surgical scars were shorter than 3.5 cm, suggesting the prior execution of an excisional biopsy rather than a curative-intent surgery, as instead was the case of the two dogs included in our study with a recurrent tumor.[20] SLN biopsy in human medicine is usually performed in tumors at first presentation because the surgical scar probably disrupts lymphatic drainage, resulting in a significant SLN detection failure rate.[52] However, even in the case of breast cancer in women, where SLN biopsy is a well-established procedure, there is no consensus on the management of cases with previous ipsilateral tumors and negative SLN. Additionally, even if most surgeons consider previous surgery to be a contraindication for a new SLN mapping procedure, no data either support or refute this concept. The use of lymphoscintigraphy for SLN mapping in recurrent tumors has been investigated only in a few studies, reporting a low identification rate and abnormal radioactive colloid uptake with anomalous lymphocentrum detection in comparison to what expected in the case of an untreated neoplasm.[52-54]

Limitations of the described technique include the low availability of veterinary facilities with permission for radiotracer storage and the risk of staff exposure, even if the cumulative doses are minimal compared with the exposure allowed by legislation. Other SLN mapping techniques without scintigraphy overcome the latter limitation.[23] However, scintigraphy is the gold standard method in human medicine,[55] and no comparative data on the feasibility and cost of different techniques have been reported in veterinary medicine. This diagnostic procedure has an additional cost, but clinicians must advise the owner about the high percentage of occult metastatic SLN, and that lymphadenectomy seems to have not only a staging purpose but also a therapeutic value.[16,56] Another limiting aspect is the prolongation of anesthesiologic time due to the pre-operative lymphoscintigraphy, particularly in dogs with multiple tumors that have to be mapped and excised on the same day. After this case series, our surgical team decided to perform the pre-operative lymphoscintigraphy the day before surgery, to reduce the anesthesiologic time. In the absence of any complication, surgeons discharged the dog in on the third day. The radiotracer is injected on the day

of the pre-operative lymphoscintigraphy, and radioactivity is checked the second day, just before surgery. If radioactivity is not present, the radiotracer is re-injected again.

The RLN was not excised and submitted to histology to verify the absence of metastasis in the anatomical closest lymph node in 13 MCTs in which the SLN differed from the RLN. Indeed, there was no evidence that the RLN was a draining node in these dogs, and surgeons decided to excise only the SLN to reduce the surgical dose. The utility of SLN detection and biopsy should also be evaluated based on patient outcomes and the false-negative rate (how frequently a patient with a negative SLN develops a lymph node metastasis). In the present study, the authors assessed only the SLN metastatic rate. This rate was 32/57 (56%), considering SLNs with early (HN2) or overt (HN3) metastasis from MCT. This rate was collected even if the neoplasms were characterized by a low histologic grade or a subcutaneous location and associated clinically normal regional lymph nodes, all variables suggestive of benign clinical behavior.

A non-metastatic lymph node was removed in the remaining 25/57 SLNs. Nowadays, no data are available on the effect and contraindication of removing normal, non-metastatic lymph nodes. Based on the paper of Suami et al. (2016), after lymphadenectomy in dogs, the lymphatic vessels of the obstructed area connected to the lymph nodes in an adjacent region within 3 weeks from surgery. These collaterals probably act as bypasses to prevent the manifestation of lymphedema, but they could also operate as new metastatic pathways of residual cancer.[40] This canine population should be followed in the future to acquire further outcome data.

Sentinel lymph node mapping and extirpation with radionuclide and injection of methylene blue was associated with low morbidity and allowed detection of SLNs in all dogs with MCT at first presentation and absence of scar tissue. A SLN mapping technique followed by extirpation and histologic examination is advocated in every case of subcutaneous and cutaneous MCT in consideration of the discrepancy between RLNs and SLNs, and the relatively high number of positive SLNs. The differing number of SLNs at the same site among dogs achieved in the present study highlighted the importance of intra-operative radio-guided examination, even if the draining node belongs to a RLN station. Additional studies should clarify if the removal of SLNs with occult metastasis were considered therapeutic in dogs with low-grade MCTs, thus obviating the need for adjuvant treatments. The presence of scar tissue, both for a recurrent tumor or along the lymphatic pathway, seemed to be a limitation for SLN mapping with radionuclide and methylene blue injection. Further studies are warranted to assess the applicability of this mapping technique in the presence of scar tissue.

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3. To map or not to map the cN0 neck: impact of sentinel lymph node biopsy in canine head and neck tumors

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Tumor staging is of outmost importance in the oncological management of canine malignant head and neck tumors (MHNT) and failure to identify metastatic spread to the lymphatic basin may result in patient understaging and incorrect treatment recommendations, potentially worsening the prognosis.[1-9] Despite a lack of standardization in lymph node assessment, especially in the absence of clinically evident nodal disease (cN0 neck), it is well established that detection of nodal metastases should rely on histopathological examination of the excised draining lymph nodes.[10-15] However, identification of the draining node(s) in the head and neck is not straightforward due to individual variation in the lymphatic pathways of the canine head and neck as well as a variable number of lymphocenters and/or lymph nodes per lymphocenter.[9,14,16-18] Furthermore, unpredictable patterns of nodal metastasis for MHNT have been documented, with contralateral dissemination in up to 24-62% of dogs, and involvement of mandibular nodes, which are most commonly sampled due to their superficial location, reported in only 54-87% of cases.[17,18,20,21]

Elective node dissection (END) with extirpation of multiple lymphocenters of the head has been advocated both in human and small animal oncology for accurate staging of the cN0 neck. Although the procedure seems to be well tolerated in the canine patient, with only minor complication being reported and a lower morbidity than in humans, it involves extensive dissection and potentially unnecessary extirpation of non-draining nodes.[16,21-23] Hence, lymphatic mapping techniques and sentinel lymph node biopsy (SLNB) are gaining acceptance owing their potential to accurately detect the presence of lymphatic metastases by harvesting the first node that drains a tumor (the sentinel lymph node, SLN), while limiting extensive dissection.[23-25] Despite the development of different SLN mapping methods, preoperative lymphoscintigraphy with intraoperative gamma probing has cemented its role in human oncology and is regarded as the gold standard for SLN mapping of several malignancies, including MHNT.[26] This technique has been validated in dogs with naturally occurring tumors, including those of the head and neck.[9,19,27] Reported detection rates as high as 100% compare favorably with other mapping techniques and suggest that lymphoscintigraphy may be considered the

gold standard for SLN mapping in canine patients as well.[19,27] Hence, we prospectively applied this mapping technique to determine its diagnostic accuracy for SLNB in canine MHNT, and to investigate the relationship between location of primary tumor and SLN identified with this technique. The impact of SLNB on tumor staging was also assessed in a cohort of dogs with naturally occurring MHNT. We hypothesized that lymphoscintigraphy and blue dye is an accurate technique for guiding SLNB in MHNT and that it would allow for extirpation of clinically unpredictable nodes that my harbor occult nodal metastases, thus causing an upstaging in some dogs.

3.1. Materials and methods

This prospective observational study was conducted in a single institution from May 2017 to July 2020. We included consecutive client-owned dogs with cytologically or histologically confirmed spontaneous MHNT at first presentation or T0, amenable to surgical excision, and absence of clinical/tomographic evidence of nodal metastases (cN0 neck). We excluded dogs with RLN lymphadenomegaly (subjectively defined based on shape, consistency, comparison with contralateral nodes and bodyweight) detected during clinical examination (palpable nodes) or CT (non-palpable nodes) and cytologically confirmed nodal metastases. Prior to enrollment, all dogs staged negative for distant metastases according to the current recommendations for each tumor type. Owners had to sign a written informed consent to diagnostic and surgical procedures as well as data recording. All the procedures were performed following Institutional guidelines for animal welfare under the control of the National Ministry of Public Health.

Signalment data, location of MHNT and of the clinically expected RLN were collected. The RLN was identified following previous anatomical studies.[28-29] For preoperative SLN mapping, dogs were induced under general anesthesia and regional lymphoscintigraphy was performed: Technetium-99 metastable labeled nano-sized human serum albumin (Nanoalbumon; Radiopharmacy Laboratory Ltd, Budaors, Hungary) was injected subcutaneously/submucosally in four quadrants at 1-2 mm from the margins of the tumor at a dose of 7.8 – 31 MBq/0.5 mL (median: 23.31MBq); a single-head gamma-camera (Prism 2000 XP; Picker International, Highland Heights, OH, USA) was used to acquire static and dynamic planar images until the first draining lymphocenter(s) were visualized.[9]

Sentinel lymph node biopsy was performed concurrently with MHNT excision. Depending on the estimated anesthetic risk of each dog and duration of the surgical procedure, preoperative SLN mapping and surgical excision of MHNT and SLN(s) were performed either on the same day or on two consecutive days to reduce the duration of anesthesia. Anesthetized dogs were aseptically prepared for surgery and sterile methylene blue 1% (SALF S.p.A.; Cenate Sotto, Bergamo, Italy) was

injected peritumorally in four quadrants at a dose of 0.4 mL just before moving the patient to the operating theater, approximately 10-15 minutes before surgical incision. Lymphadenectomy was performed before tumor excision to minimize cross contamination of the surgical field with tumor cells. A hand-held gamma probe (Crystal probe SG04, Crystal Photonic GmbH, Berlin, Deutschland) was used to measure the radioactive counts (RC) through the skin in order to direct the surgical incision over the lymphocenter and to guide the extirpation of the radiolabeled hot node(s). A node was defined as "hot" if it had RC two times greater than background counts.[9,30] Surgeons excised every hot and/or blue node(s) belonging to the lymphocenter(s) identified by preoperative lymphoscintigraphy and measured RC again ex-vivo. The gamma-probe was then used to measure residual RC in the lymphocenter and additional lymph nodes having counts 10% or greater than the first SLN were also extirpated.[9,30] Furthermore, every visible node belonging to the draining lymphocenter(s) was excised, even if not hot and not blue, in order to reduce the risk of residual disease.[14] Primary MHNT was then excised according to current recommendations for tumor type and location.

The additional surgical time for SLNB, surgical complications, and any adverse reaction to methylene blue or radiopharmaceutical were recorded. Correspondence between SLN(s) identified by preoperative lymphoscintigraphy, SLN(s) identified intraoperatively with the gamma probe and blue dye, and clinically expected RLN was assessed. The number of excised lymphocenters and lymph nodes per lymphocenter was also recorded.

If the procedures were performed on consecutive days, dogs were hospitalized overnight and peritumoral injection of radiopharmaceutical was not repeated before the second procedure. Dogs were hospitalized postoperatively for at least 24 hours and discharged at the discretion of the attending clinician.

The primary tumor and all the excised nodes were processed for histopathology after 24 hours for radioactive decay. Sentinel lymph nodes were cut longitudinally at the level of the hilus and additionally sliced every 1.5 mm if thicker than 3 mm at their minor axis; all sections thus obtained were processed for histopathology. Histopathological reports included MHNT histological diagnosis (tumor type), the surgical margin status (infiltrated or tumor-free), and presence or absence of SLN metastasis (SLN+ versus SLN-)

Dogs were rechecked 7 and 14 days after surgery and further rechecks were scheduled if deemed necessary by the attending clinician or if required by the owner. Thereafter, in the absence of clinical signs related to suspected tumor progression, periodic rechecks (including clinical examination, thoracic radiographs, and abdominal ultrasound when deemed necessary) were scheduled every 3 months to monitor tumor progression. Descriptive statistics were used to summarize obtained data.

Sensitivity and specificity evaluated the performance of SLNB with radiopharmaceutical and blue dye followed by histopathology. Positive and negative likelihood ratios (LR+; LR-) were calculated to assess the propensity of SLNB to rule in (LR+) or rule out (RL-) nodal metastases.[31] The strength of evidence of the likelihood ratios to rule in/out the presence of nodal metastases was classified according to Hayden and colleagues.[32] Likelihood ratios also indicate the magnitude of change from pretest assessment of the probability of SLN+ to the likelihood of SLN+ neck after knowing the result of the test. Positive and negative predictive values (PPV; NPV) were calculated considering a 35%-45% prevalence of nodal metastases in MHNT, estimated on the basis of the available literature.[17,18] The 95% confidence interval (C.I.) for sensitivity and specificity were calculated with Clopper and Pearson's method.[33] For likelihood ratios, 95% C.I. were calculated with Marill's method.[34]

3.2. Results

Twenty-three MHNT in 23 dogs were included in the study. Signalment data, location of the MHNT, clinically expected RLN and SLN(s) are detailed in Table 1.

The combined use of radiopharmaceutical and blue dye identified at least one SLN in 19/23 animals, leading to an estimated detection rate of 83%. A SLN was not detected in 4 dogs, all affected by thyroid tumors. Preoperative lymphoscintigraphy identified more than one draining lymphocenter in 4/23 (17%) dogs. Fifty-one lymph nodes were excised, with more than one lymph node removed in 14/23 animals (61%) and multiple nodes excised from the mandibular lymphocenter (from 1 to 4 nodes) and from the superficial cervical lymphocenter (from 1 to 3 nodes). Conversely, only 1 retropharyngeal node was identified in the study population. Fifteen non-hot, non-blue nodes were visualized during surgical dissection of the SLN and extirpated in 7 dogs; all these nodes belonged to the mandibular lymphocenter.

In 12 dogs (52%), lymphoscintigraphy identified at least 1 lymphocenter that did not correspond to the clinically expected RLN. Of these, in 5 cases the SLN was completely different from the RLN; in 5 cases lymphoscintigraphy identified multiple SLNs, one that corresponded to the RLN and one or more at unpredictable sites; in 3 cases identification of the RLN was not possible based on the location of the primary tumor, and lymphoscintigraphy permitted to excise at least one SLN (Table 1). In 2 dogs with MCT of the conjunctiva (case #16) and eyelid (case #20) the SLN was the parotid node, which corresponded to the RLN based on the study by Suami et al (2013) but did not fully correspond based on previous anatomical studies that suggested an overlapping of lymphatic drainage of this area between the parotid and mandibular lymphocenters (Table 1).[28,29]

Based on ex-vivo RC count, 34 (67%) of the 51 excised nodes were hot, with at least one hot node being extirpated from the sentinel lymphocenter in every dog; 27/34 (79%) hot nodes were also blue stained, while 7 (21%) nodes in 6 dogs were hot but not blue stained. Seventeen nodes (33%) were excised despite having a RC of 0, of which 2 in 2 dogs with an oral melanoma (OMM) and squamous cell carcinoma (OSCC) were blue stained. At least one blue node was excised in all but 3 dogs with an adenocarcinoma of the parotid gland, an oral fibrosarcoma and an MCT (Table 1). Median dimensions of the excised nodes at the longest diameter was 15 mm, ranging from 2 to 30 mm.

Median surgical time for SLNB was 20 minutes, and no major complications related to this procedure were recorded in the included cases. Transitory self-limiting edema of the muzzle and seroma formation at the lymphadenectomy site occurred in 3 and 2 dogs, respectively, and remitted spontaneously within 7-15 days from surgery.

At histopathology, tumor types included: MCT (n=8), OMM (n=5), thyroid carcinoma (n=4), oral fibrosarcoma (FSA) (n=2), oral osteosarcoma (OSA) (n=1), soft-tissue sarcoma (n=1), parotid gland adenocarcinoma (n=1), OSCC (n=1). Nodal metastases were detected in 8 animals (42%), of which 4 (21%) had an unpredictable SLN and 1 had a parotid node excised and would have thus been under-staged without the mapping procedure (Table 1).

During the follow-up period (median 250 days, range 25 – 1185 days), 1 dog with OMM experienced locoregional recurrence (histologically confirmed metastases to another node located medially to the second third of the left jaw at 180 days), 2 dogs with oral FSA and OMM had local and distant tumor progression (120 days and 180 days respectively), and 1 dog with an adenocarcinoma of the salivary gland had distant progression (800 days) (Table 1). The 4 dogs that developed progressive disease had SLN-. (Table 1).

Sensitivity and specificity were 88.9% (95% C.I. 51.8 – 99.7%) and 100% (95% C.I. 69.2 – 100%), respectively. As specificity was 100%, LR+ tended to infinity and only the lower limit of the 95% was calculated (3.34); LR- was 0.11 (95% CI 0 – 0.424) indicating a moderate increase in the likelihood of having SLN- in case of a negative test (Hayden 1999). PPV and NPV were 100% (95% C.I. 57.9% - 100%) and 94.4% (95% C.I. 63.7% - 99.8%), respectively, when considering a prevalence of nodal metastases of 35%; they were 100% (95% C.I. 57.9% - 100%) and 91.7% (95% C.I. 63.7% - 99.8%) with an estimated prevalence of nodal metastases of 45%.

Case	*Signalment*	*Tumor location*	*RLN (Suami et al 2013; Bezuidenhout 2013)*	*SLN*	*SLN hot*	*SLN blue*	*SLN Status*	*Tumor type*	*Progressive Disease*	*Outcome*
#1	Pug, M, 3.5y, 9.7kg	Left parotid region - cutaneous	Left mandibular vs left parotid	Left superficial cervical	yes	yes	HN2	Cutaneous MCT, grade 2 Patnaik, Low Kiupel	no	Alive at 1185 days
#2	Labrador R., M, 1y, 32kg	Nose	Left mandibular	Right mandibular	no	no	HN0	Cutaneous MCT, grade 1 Patnaik, Low Kiupel	no	Alive at 760 days
				Right mandibular	yes	yes	HN0			
				Right mandibular	yes	yes	HN0			
				Left mandibular	no	no	HN0			
				Left mandibular	yes	yes	HN0			
				Lt. mandibular	yes	yes	HN0			
#3	Dachshund, Fn, 11y, 5kg	Sublingual thyroid	ND	ND	ND	ND	ND	Follicular carcinoma	no	Alive at 685 days
#4	Golden R., M, 12y, 37kg	Rostral mandibula	ND	Left mandibular	no	no	-	Melanoma	LRR at 180 days DM at 525 days	Euthanasia at 525 days
				Left mandibular	no	yes	-			
				Left mandibular	yes	yes	-			
#5	Mixed-breed, M, 12y, 47kg	Left parotid gland	Left medial retropharyngeal, left parotid	Left mandibular	no	no	-	Parotid adenocarcinoma	DM at 800 days	Death at 854 days
				Left mandibular	no	no	-			
				Left mandibular	no	no	-			
				Left mandibular	no	no	-			
				Left parotid	yes	no	-			
#6	Mixed-breed, Fs, 10y, 23kg	Right thyroid	Right medial retropharyngeal	ND	ND	ND	ND	Papillary carcinoma solid variant	no	Alive at 630 days
#7	Pinscher, M, 15y, 6.4kg	Tongue	Right medial retropharyngeal	Left mandibular	no	yes	+	Squamous cell carcinoma	no	Death due to uncontrolled epilepsy at 25 days
				Left mandibular	no	no	-			
				Left mandibular	no	no	-			
				Left medial retropharyngeal	yes	yes	-			

				Right mandibular	yes	no	-			
				Right mandibular	no	no	-			
				Right medial retropharyngeal	yes	yes	-			
#8	Mixed-breed, Mn, 12y, 22.3kg	Left temporal region - cutaneous	Left mandibular vs left parotid	Left superficial cervical	yes	yes	HN2	Subcutaneous MC	no	Death at 150 days due to LR of another MCT (hindlimb)
				Left superficial cervical	yes	yes	HN0			
				Left superficial cervical	yes	yes	HN0			
#9	Italian Hund, M, 9y, 19.4kg	Right thyroid	Right medial retropharyngeal	ND	ND	ND	ND	Follicular carcinoma	no	Alive at 515 days
#10	Boxer, M, 9y, 42kg	Left neck - cutaneous	Left superficial cervical	Left superficial cervical	yes	no	HN0	Subcutaneous MCT	no	Alive at 485 days
				Left superficial cervical	yes	no	HN0			
#11	Rhodesian, Mn, 8y, 45.8kg	Caudal right mandibula	Right mandibular	Right mandibular	yes	yes	-	Amelanotic melanoma	no	Death at 90 days due to appendicular OSA
				Right mandibular	yes	yes	+			
#12	Chow-chow, Fn, 1y, 20.7kg	Caudal left mandibula	Left mandibular	Left mandibular	no	no	-	Fibrosarcoma grade 1	no	Alive at 485 days
				Left mandibular	yes	yes	-			
				Left mandibular	no	no	-			
#13	Am-staff, Mn, 3y, 25kg	Left auricular region - cutaneous	Left mandibular vs left parotid	Left prescapular	yes	yes	HN1	Cutaneous MCT, grade 2 Patnaik, Low Kiupel	no	Alive at 395 days
				Left prescapular	yes	yes	HN1			
#14	Labrador R., Fn, 11y, 29.4kg	Middle right mandibula	Right mandibular	Right mandibular	no	no	-	Pleomorphic sarcoma grade 3	LR and DM at 120 days	Euthanasia at 120 days
				Right medial retropharyngeal	yes	no	-			
#15	Fox terrier, Fn, 9y, 8kg	Left temporal region - cutaneous	Right mandibular vs right parotid	Right superficial cervical	yes	yes	HN2	Cutaneous MCT, grade 2 Patnaik, Low Kiupel	no	Alive at 260 days
#16	Tibetan terrier, M, 2y, 10kg	Upper left conjunctiva (T0)	Left parotid vs left mandibular	Left parotid	yes	no	HN0	MCT	no	Alive at 250 days
#17	Flat-coated R., Fn, 12y, 28kg	Left thyroid	Left medial retropharyngeal	ND	ND	ND	ND	Medullary carcinoma	no	Alive at 240 days
#18	Labrador R., M,	Right labial vestibulum	Right mandibular	Right mandibular	yes	yes	-	Melanoma	DM at 180 days	

				Right mandibular	yes	yes	-		LR at 210	Euthanasia at 210 days
#19	Mixed-breed, Fn, 11y, 10.25kg	Rostral-right mandibula	Left mandibular	Right mandibular	yes	yes	-	Osteosarcoma	no	Death at 192 days due to uncontrolled diabetes mellitus
				Left mandibular	yes	yes	-			
				Right medial retropharyngeal	yes	yes	-			
#20	English setter, F, 6m, 15kg	Right third eyelid	Right parotid vs right mandibular	Right parotid	yes	yes	HN3	MCT	no	Alive at 160 days
#21	Mixed-breed, M, 8y, 35kg	Left upper lip	Left mandibular	Left mandibular	yes	yes	-	Melanoma	no	Alive at 90 days
				Left mandibular	no	no	-			
				Left mandibular	yes	yes	+			
				Left mandibular	no	no	-			
#22	Bernese, Fn, 4.5y, 38.5kg	Right neck - subcutaneous	Right superficial cervical	Right superficial cervical	yes	yes	-	STS	no	Alive at 90 days
#23	Bernese, M, 6y, 42.5kg	Left upper lip	Left mandibular	Left mandibular	yes	no	-	Melanoma	no	Alive at 60 days
				Left mandibular	yes	yes	+			

Table 1. *Signalment, location of mHNT, location of RLN, location of SLN identified by preoperative lymphoscintigraphy, gamma probe and blue guidance, tumour type and outcome of the 23 dogs included in the study. M: male; Mn: neutered male; F: female; Fn: neutered female; ND: not determined; MCT: mast cell tumour; +: histologically positive for metastases; -: histologically negative for metastases; LRR: loco-regional recurrence; LR: local recurrence; DM: distant metastases.*

3.3. Discussion

In this study, preoperative lymphoscintigraphy and intraoperative gamma probing and blue dye allowed for extirpation of at least one SLN in 19/23 dogs with MHNT, leading to a detection rate of 83%, with minimal morbidity. Arguably, identification of the draining lymphocenter based on the anatomical location of the primary tumor may be misleading, due to individual variations of drainage pathways and tumor-induced lymphangiogenesis.[17,18,20,14] Although Suami and colleagues (2013) described three lymphatic basins in the canine head and neck (mandibular, parotid, dorsal superficial cervical), an overlapping of the lymphatic vessels in this area was observed; furthermore, the inclusion of only 2 live dogs and the mapping of the superficial areas only are two main limitations of that study that may have hampered the accuracy and repeatability of their results, owing the wide range of anatomical variations between dog breeds and of possible different locations of MHNT.[28] Moreover, consistency is often lacking among anatomical studies and lymphosomes do not always correspond.[28,29] Hence, some authors recommend the extirpation of multiple lymphocenters of the head for nodal staging in dogs with MHNT and cN0 neck.[16-19,22] With this technique, contralateral dissemination was identified in 24-62% of dogs with lateralized tumors,[14,18] and in 21-45% of cases metastases were detected in lymph nodes other than the mandibular.[14,17,18,20] Similarly, in the sample population intraoperative radiopharmaceutical plus blue dye guided the extirpation of at least one SLN other than the clinically expected RLN in 52% of dogs and corresponding to the ipsilateral mandibular lymphocenter in only 22% of dogs, underscoring the unpredictability of the lymphatic drainage of the canine head and neck.

In human medicine END is suggested in patients with OSCC and OMM that have an estimated risk of occult nodal metastases > 20%, which means that 70-80% of patients potentially undergo unnecessary extensive dissection with increased morbidity and costs.[23] Lately, SLNB has been proposed as an alternative to END in humans with OSCC and OMM to reduce the surgical extent and morbidity associated with END while still allowing to detect occult nodal metastases.[23,35] Reported results are encouraging, with sensitivity as high as 87% and NPV of 94% for early stage OSCC.[35] Likewise, in canine surgical oncology interest has been drawn on SLNB, with several mapping techniques being described for various tumor types including MHNT, and detection rates as high as 100% reported for lymphoscintigraphy.[19,21,27] In the sample population, we report a detection rate of 83%, which is lower than in previous studies, due to the inclusion of 4 dogs with thyroid tumors in which a SLN was not identified.[19,27] Both hematogenous and lymphatic spread have been reported for thyroid tumors in dogs, and the impossibility to detect a SLN in these 4 dogs may suggest a lack of tumor-associated lymphatic drainage in early-stage thyroid carcinomas rather than a failure of

SLNB. Reported rates of nodal metastases for thyroid carcinomas varies significantly among studies (7-38%), probably due to the lack of standardization in staging modalities and inclusion of early to advance stage tumors.[36-38] Recently, metastases to surgically excised deep cervical lymph nodes have been described in 45% of dogs with thyroid carcinoma, several of which had negative prognostic factors such has bilateral tumors, lymphatic or vascular invasion and capsular invasion.[39] Conversely, the 4 thyroid tumors included in the present study were early-stage and no negative prognostic factors were recognized, except for the ectopic location of 1 thyroid carcinoma.[36] Although the impact of recognized prognostic variables and tumor stage on the likelihood of lymphatic spread has not been assessed yet for canine thyroid carcinoma, it is reasonable to assume that early stage tumors without negative prognostic factors may have a lower risk of lymphatic dissemination. The lack of tumor progression that we report without adjuvant therapies after a median follow-up of 572 days further corroborates the hypothesis of the absence of lymphatic network in these early-stage thyroid carcinomas. This consideration is consistent with the prolonged survival times previously reported for surgically resected early-stage thyroid tumors and with metastases being more frequently detected during necroscopy (60-80%) than at the time of treatment (7-38%).[37,38]

In the study population, at least one "hot" node was extirpated in each dog, and only 7/34 hot nodes were not blue stained, confirming the correspondence between RC and blue dye.[9,30] In human medicine adverse reactions to methylene blue injection are not rare, and some authors suggest the omission of blue dye owing the high detection rates reported with use of the radiopharmaceutical alone.[40] In dogs, however, side effects to vital dyes have not been observed neither in previous studies nor in the sample population. [9,30] Hence, it is the authors' opinion that blue dye should be used in association with radiopharmaceutical to improve intraoperative visualization of the target lymph nodes and avoid inadvertent damage to surrounding neurovascular structures. Conversely, two excised nodes belonging to the sentinel lymphocenter detected preoperatively were blue but not "hot", and more interestingly one of these nodes was metastatic at histopathological examination. In the latter case, the dog had a OSCC of the tongue and the histological architecture of the lymph node was completely effaced by metastatic cells; thus, it may be hypothesized that this lymph node did not drain enough radiopharmaceutical to be detected intraoperatively due to neoplastic occlusion of the lymphatic network, as previously reported.[42,43] Additional nodes without RC and not blue-stained were extirpated from the mandibular lymphocenter in 7 dogs in order to reduce the risk of residual nodal disease.[14] This decision was mainly dictated by the explorative nature of this study, although it is worth mentioning that intraoperative identification of the SLN in the mandibular lymphocenter may not always be straightforward, given the close proximity of multiple nodes in this location, and non-hot nodes may thus be accidentally removed during dissection.[9] However, it should be noted that

none of these nodes had occult metastases at histopathology, underscoring the accuracy of the combined technique and confirming that non-hot/non-blue nodes may not need to be removed.

Intraoperative gamma probing guided the extirpation of multiple SLNs in 10 dogs, owing the ability of this technology to accurately detect residual RC in the surgical field.[15,19] Furthermore, in 2 dogs with MCT of the conjunctiva and eyelid, the technique guided the extirpation of the parotid lymph, which is not often excised in clinical practice given the difficulty to identify lymphatic structures in that area.[14,30] The latter dog (case #20) had an overt metastatic parotid node (HN3[41]) and received adjuvant chemotherapy based on the SLNB findings. The possibility to detect multiple nodes and to evaluate the parotid lymphocenter represent some advantages of intraoperative guided SLNB compared to preoperative mapping alone or END. Indeed, although Smith and colleauges (1995) originally described a technique for END that allowed for extirpation of mandibular, retropharyngeal, and parotid lymphocenters,[16] more recently published studies reported the excision of bilateral mandibular and retropharyngeal nodes only, while the parotid nodes were not assessed.[14,18] Hence, with the latter form of END there is potential to miss nodal metastases to the parotid lymphocenter.

According to our results, another potential advantage of SLNB is the reduced surgical extent compared to END. In the sample population, only 4 animals underwent the excision of multiple lymphocenters, while for the majority of the dogs, lymphoscintigraphy guided the dissection towards a single lymphocenter, thus reducing the surgical extent. Although END seems to be well tolerated in the canine patient, with only minor complications reported, studies assessing the actual complication rate and morbidity of the procedure are still lacking.[15,18] With SLNB we recorded only self-limiting complications occurring in 26% of dogs, underscoring the tolerability of this procedure.

To validate our results, we statistically evaluated the diagnostic accuracy of SLNB with radiopharmaceutical and blue dye. The fact that specificity was higher than sensitivity suggests that the procedure is more useful to confirm than to rule out nodal metastases. However, the expected rate of false negatives based on these results is low (approximately 10%) and the high NPV (91.7 – 94.4 %) indicate that in the sample population the technique was useful in ruling out nodal metastases as well. These results are encouraging and underscore the accuracy of the technique, although they should be confirmed on a larger sample and considering the prevalence of nodal metastases for each tumor type.

In one dog with an OMM of the rostral mandible, SLNs (left mandibular nodes) were negative for metastases but this dog developed locoregional relapse to a lymph node located rostral to the mandibular nodes at 180 days (Table 1). This case was considered a possible false negative based on previous experience in human medicine.[23] In humans, the ability to identify the draining lymph node is site-specific, and SLNB is reportedly less sensitive for floor of the mouth tumors due to the “shine-

through effect".[23] In these cases, the high radioactivity of the area around the tumor may obscure the visualization of the SLN. Similarly, it may be hypothesized that in our case the actual SLN was not identified because it was in the flare of the OMM, and an upper echelon lymph node, that was not metastatic already, was excised instead. It should be noted that the gamma probe used in the present study has an integrated tungsten 40° collimator, which enables detection of radiation from a specific direction and is considered optimal for breast cancer in humans. However, interchangeable collimators between 20-90° are commercially available to improve the gamma sensitivity, and the potential benefits of the use of different collimators to discriminate between tumor and SLN signal in specific anatomical location, such as the floor of the mouth, warrant further investigations.

Three dogs with an oral FSA, an OMM and a parotid gland adenocarcinoma developed distant metastases despite having SLN-, thus suggesting that the metastatic process occurred through an hematogenous rather than lymphatic way. While nodal metastases from oral FSA and salivary gland tumors reportedly occur in 3-10% and 11% of dogs, respectively, locoregional spread is commonly observed in OMM, with reported rates of nodal metastases as high as 59-74% .[1,3,44-46] However, both in dogs and humans OMM locoregional metastases are not always correlated with distant spread; this observation has led to the "marker hypothesis" which states that removal of a metastatic node has merely staging significance and loco-regional control but does not improve outcome, although extirpation of positive SLNs in canine OMM has not been previously assessed for improved overall survival.[46-48] In the future, the significance of SLN+ versus SLN- should be evaluated taking into account the biological behavior of each tumor type.

In our study, occult nodal metastases were detected in 42% of dogs with cN0 neck, and of those, 5 (22%) had a SLN that would have not been excised without the mapping procedure due to a lack of correspondence between the SLN and the RLN (n=4) or to its location in the parotid lymphocenter (n=1), thus underscoring the importance of the correct identification of the draining lymph node for tumor staging in the cN0 neck. Histopathological identification of occult nodal metastasis is a crucial step of SLNB, and in human medicine it is well established that serial sectioning and immunohistochemistry on excised nodes increase the ability to identify occult nodal metastases.[23,48] Likewise, at our institution SLNs are routinely processed with serial sectioning potentially increasing the sensitivity of the technique, although in canine oncology studies assessing the potential advantages of serial sectioning for the detection of occult nodal metastases are still lacking.[14,15]

Another question that is yet to be answered, is how to treat dogs with SLN+ following SLNB. In human medicine, patients that have occult nodal metastasis to the SLN undergo adjunctive surgery to remove the remainder of the lymphatic basin.[23,50] In canine oncology there is still a gap of knowledge on the potential benefits of additional lymph node dissection in dogs with metastatic SLN.

Excision of early metastatic lymph nodes (HN2[41]) in dogs with low-grade MCT resulted in a good long-term survival even without adjuvant treatment, suggesting a potential therapeutic effect of metastatic nodal removal.[51] However, there is still no evidence of the therapeutic effects of lymphadenectomy in dogs with MHNT and SLN+/SLN-, and SLNB or END are currently performed for staging purposes only. These issues should be addressed in future studies comparing the tumor-specific outcome of dogs with a positive SLN that undergo additional lymphadenectomies versus medical treatment or watchful waiting after SLNB. Despite its high diagnostic accuracy, lymphoscintigraphy is not readily available for most veterinary facilities due to the costs of the technology required and the need for specific permissions to store the radiotracer; thus, more accessible intraoperative SLN mapping techniques should be validated by assessing their diagnostic accuracy in comparison with lymphoscintigraphy to allow for a greater dissemination of SLNB in veterinary oncology.

The decision to include various tumor types was dictated by the explorative nature of our study and did not preclude the evaluation of the diagnostic performances of radiopharmaceutical and blue dye for SLNB and of its impact on tumor staging. However, to better understand the role of SLNB in the oncological management of MHNT the impact of this procedure on prognostication should be assessed. Furthermore, the accuracy and morbidity of SLNB and END should be compared in order to confirm the potential benefits of a targeted lymph node approach.

Given the unpredictability of the lymphatic drainage of the canine head and neck, with SLN not corresponding to the clinically expected RLN in 63% of cases in the present study, lymph node staging of dogs with MHNT and cN0 neck should rely on SLNB. The use of a mapping technique with intraoperative guidance has the potential to raise the diagnostic accuracy of the procedure by allowing for detection of multiple SLNs and extirpation of target lymph nodes that may be missed with END, thus reducing the risk of false negatives. Despite the impact of a targeted lymph node approach on correct staging of MHNT, the actual prognostic implications of the detection of occult nodal metastases in highly aggressive tumors such as OMM as well as the possible therapeutic role of the excision of cN0 and SLN+ in less aggressive MHNT are yet to be clarified.

3.4. References

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4. Assessing the risk of nodal metastases in canine integumentary mast cell tumors: is sentinel lymph node biopsy always necessary?

Ferrari R, Boracchi P, **Chiti LE**, Manfredi M, Giudice C, De Zani D, Spediacci C, Recordati C, Grieco V, Gariboldi EM, Stefanello D. *Animals (Basel).* 2021 Aug 11;11(8):2373. doi: 10.3390/ani11082373

Partial results presented at 74° Convegno SISVET, 23rd-25th June 2021, Online meeting:

***Chiti LE**, Boracchi P, Ferrari R, Giudice C, Grieco V, Zani DD, Manfredi M, Stefanello D. Factors correlated with sentinel lymph node metastasis in canine mast tumors: when could surgeons avoid lymphadenectomy?*

The identification of nodal metastasis in canine mast cell tumor (MCT) has undergone several changes during the last decade. Initially, regional lymph node (RLN) biopsy was suggested as the standard of care in dogs undergoing excision of integumentary mast cell tumor, given the low sensitivity and specificity of lymph node cytological sampling.[1–4] The attention for lymph node assessment was further supported by the observation of early and overt histological nodal metastases even in non-palpable/normal sized lymph nodes and in low-grade MCT. [3–6]

More recently, the changes in nodal evaluation have led to questioning which lymph node should be assessed in the absence of clinically evident pre-operative lymph node alterations. Indeed, different mapping procedures have been applied to identify the sentinel lymph node (SLN) in dogs with cutaneous and subcutaneous MCT. [4–7] The disagreement between the anatomically closest RLNs and the actual draining SLNs is a common result: 42% of dogs had SLNs differing from the RLN in Worley's study (2014); SLNs did not correspond to the RLN in 63% of cases in the study by Ferrari et al. (2020); clinicians would have incorrectly predicted the draining lymph node in 45.8% of MCT included in a recent study by Fournier et al. (2020); 28% of SLN differed from the RLN in the Lapsley et al. study (2021). Moreover, a high variability in the number of SLNs excised in each dog has been consistently reported.[4–7] These results highlight the importance of SLN mapping and biopsy to achieve an accurate and personalized nodal staging and undermine the prognostic results reported in the previous studies focused on RLN evaluation. Conversely, SLN mapping and biopsy include additional anesthetics, diagnostic and surgical procedures, and additional costs for the owner, although to date there are no data available on the clinical benefits of these procedures, especially in

case of non-high metastatic risk MCT.[8–10] Furthermore, information is lacking on the potential morbidity related to the removal of non-metastatic SLN.

Hence, the present study aims to explore the association between clinicopathological features and SLN metastases in dogs with integumentary MCT in order to determine if it is possible to correctly identify dogs at lower risk of SLN metastases. We hypothesize that no prognostic factors could help in predicting the risk of SLN metastasis and that surgical biopsy of the SLN should always be suggested.

4.1. Materials and Methods

Sample Population

Client-owned dogs with cytologically confirmed integumentary MCT undergoing curative intent surgical resection of the tumor and SLN(s) were prospectively included from January 2017 to December 2020. To be eligible for SLN mapping, dogs must have staged negative for distant metastasis at preoperative ultrasound-guided spleen and liver cytology and have no clinical/ultrasonographic evidence of RLN metastases (based on Suami et al., 2013).[11] This study did not include experimental animals and it was performed on client-owned dogs affected by integumentary mast cell tumor referred to our institutions for therapeutic purpose. All owners gave us consent for staging and treatment procedure, as well for data recording.

Sentinel Lymph Node Mapping, Biopsy, and Histological Evaluation

The SLN was identified by pre-operative lymphoscintigraphy and excised under intraoperative gamma-probe guidance as previously described.[6,7,12] Briefly, planar lymphoscintigraphy was performed the day before or the same day of surgery and consisted of a peritumoral injection of Technetium-99m-human serum albumin colloid and identification of one or more draining lymphocenters that included the SLN/SLNs using a gamma camera. The day of surgery, methylene blue was also injected peritumorally in four sites. Dogs were then moved to the operating theater. All tumors underwent wide-margin surgery (2–3 cm lateral margins and at least one deep fascial plane). For the intraoperative SLN detection, a hand-held gamma probe (Crystal probe SG04, Crystal Photonic GmbH, Berlin, Germany) was used to guide the excision of nodes with a radioactive count (RC) of at least twice the RC of a distant body region (background count). The SLN RC was checked ex vivo and further nodes belonging to the same lymphocenter were excised if the RC was equal to

or greater than 10% of the RC of the hottest SLN removed. Any visible blue nodes were also excised and were identified as SLNs regardless of the RC.

After resection, MCT and SLNs were fixed in 10% neutral buffered formalin. All samples were routinely processed for histology and microtomic sections were stained with hematoxylin and eosin. SLNs were additionally stained with Giemsa. The primary tumor was histologically evaluated to obtain diagnosis of cutaneous or subcutaneous MCT, Patnaik and Kiupel's histological gradings (for cutaneous MCT),[13,14] and margins status. To standardize the histological evaluation of the SLN, each lymph node was cut longitudinally at the level of hilus and multiple slices (1.5 mm thick) were obtained from each half of the lymph node when thicker than 3 mm (minor axis).

Recording Data

Recorded data were signalment (breed, age, sex, weight), MCT anatomic site (divided as previously reported in head and neck, trunk, limb, inguinal region, digit, and tail)[3], MCT size (maximum diameter measured with caliper), MCT presentation (first vs. recurrence), MCT ulceration, number of SLNs removed, number of lymphocenters evaluated, MCT location (cutaneous vs. subcutaneous), cutaneous MCT grade (based on both Patnaik et al., 1984 and Kiupel et al., 2011)[13,14], histological margin evaluation (tumor-free vs. infiltrated), histologic node status (HN) of the SLN/SLNs removed (according to Weishaar et al., 2014: HN0—non metastatic; HN1—pre metastatic; HN2—early metastasis; HN3—overt metastasis).[15] Histologic growth pattern (circumscribed, infiltrative, or combined), mitotic index (0, ≤4, and >4), and presence or absence of multinucleation were also reported for subcutaneous MCT.[16]

Statistical Methods

In case of MCT drained by multiple SLNs, the higher HN was assigned for statistical purposes.

- *Association between HN and MCT Clinicopathological Variables*

The association between HN and MCT clinical and histopathological variables was evaluated by generalized linear models with binomial error. Since the prevalence of HN was the clinically useful measure to evaluate the strength of association, the log-binomial model was preferred to logistic model for categorical variables.[17] For variables measured on continuous scale, log-binomial model fails to converge, thus estimated regression coefficients cannot be considered. For this reason, a generalized linear model with Poisson error with a robust variance–covariance matrix estimation was used.[18]

Two separate analyses were performed: the first for HN0-1 vs. HN2-3 and the second for HN0-HN1-HN2 vs. HN3. Model response was the HN category, coded as 0 if HN0-1 and 1 if HN2-3 for the first

analysis, and coded as 0 if HN0-HN1-HN2 and 1 if HN3 for the second analysis. Explanatory variables were both categorical and continuous. Continuous variables (size, number of SLN, number of lymphocenters) were included into the model in their original measurement scale. Categorical variables (anatomic site, ulceration, Patnaik grade, and cutaneous/subcutaneous location) were codified as dummy variables, thus for a categorical variable with K categories, K-1 dummy variables were included into the regression model and one of the categories was considered as the reference one. The variable "anatomic site" was categorized in 2 groups: sites historically associated with worse prognosis (head and neck, genital (including inguinal, scrotal, perivulvar and perineal) and digit) vs. sites historically associated with better prognosis (lateral thorax and abdomen, and limb, excluding digits).[19] Longest tumor diameter was evaluated in its original continuous measurement scale (1 cm increase) and also considered as a categorical variable, coded as 0 if < 3 cm and 1 if ≥ 3 cm.[8,20] Firstly, univariate analysis was performed for each of the above-mentioned variables. Results of the regression model were reported as prevalence ratio (PR) with corresponding 95% confidence intervals. The prevalence is the proportion of subjects with HN2-3 (or HN3). For each categorical variable with K categories, K-1 prevalence ratios are reported, each one representing the ratio between the prevalence for the category and the prevalence for the reference category. For continuous variables, PR is the ratio between the prevalence of HN2-3 (or HN3) for a unit increase of the variable. In the absence of association between a variable and HN, PR is expected to be 1. The null hypothesis of PR = 1 versus alternative hypothesis PR $\neq$ 1 was tested by Wald statistics.
Multivariable analysis was performed following the "events per variable" (EPV) rule.[21] Accordingly, it was possible to consider a model with 3 variables for HN0-1 vs. HN2-3, and no multivariable analysis was possible for HN0-1-2 vs. HN3.

- *Cluster Analysis and Association with HN*

To investigate the joint association between the seven clinico-pathological variables (explanatory variables) and HN (model response), the appropriate method should have been a multivariable analysis including all the seven variables as predictors. To allow the mentioned analysis, a case series with at least 70 HN3 should have been available. Hence, a dimension reduction technique was applied as an alternative to summarize the patterns of association of the variables in a novel single variable having a small number of categories. The novel variable was obtained by processing all the explanatory variables (except MCT location, because of the high correlation with MCT histological grade) by cluster analysis (agglomerative hierarchical clustering on results from a factor analysis)[22] in such a way that dogs classified in the same category (cluster) were more similar for overall explanatory variables than dogs classified in other categories. The overall association between clusters and HN was evaluated by Fisher exact test. When the number of HN2-3 (or HN3) was

adequate, the association was also evaluated by including clusters as explanatory variable in a generalized regression model with HN as the response variable.

To provide a clinical interpretation of the association between clusters and HN, a concise description of the explanatory variables which mainly characterized each cluster was obtained with a classification tree procedure.[23] The aim was to build a criterion which classified the clusters as a function of the six explanatory variables. The procedure consisted of partitioning data repeatedly in groups which are increasingly homogeneous for cluster classification. Each partition was determined by the explanatory variable which best separated two groups to increase homogeneity within each group. The partition was stopped when each final group was composed by individuals classified in the same cluster. A further advantage of the tree classification procedure is to visualize the hierarchical contribution of each variable to the clusters.

- *Predictive Impact of Variables on HN*

A statistically significant prevalence ratio for a clinical pathological variable provides only partial information about the predictive impact of the variable. In fact, statistical significance does not imply a significant discriminant ability, the latter being related to the probability of correctly classifying the HN of each patient as a function of the values of the variable. The ability of the variables to discriminate between HN0-1 and HN2-3 (or between HN0-1-2 and HN3) was evaluated by calculating the area under ROC curve (AUC). For putative prognostic factors AUC values range from 0.5 to 1, where 1 indicates perfect discrimination ability (i.e., when each MCT with HN2-3 (or HN3) has the worse category of the clinico-pathological variable than each MCT with HN0-1 (or HN0-1-2), and 0.5 indicates no discriminant ability (i.e., to use variable category like a flip coin). AUC values from −1 to <0.5 can be obtained only if MCT with HN2-3 (or HN3) have the better category of the clinico-pathological variable than patients with HN0-1 (or HN0-1-2). The null hypothesis AUC = 0.5 versus alternative hypothesis AUC >0.5 was tested by Mann–Whitney U-test.[24] As an aid to evaluate the strength of discrimination ability, the following criterion was used: below 0.55 negligible; 0.55–0.63 small; 0.64–0.70 moderate; 0.71 and above strong.[25]

All analyses were performed with a software package (R-Software; www.r-project.org, accessed on 10.05.2021), packages logbin (https://cran.r-project.org/web/packages/logbin/), sandwich (https://cran.r-project.org/web/packages/sandwich/), FactoMineR (https://cran.r-project.org/web/packages/FactoMineR/), ROCit (https://cran.r-project.org/web/packages/ROCit), and Verification (https://cran.r-project.org/web/packages/verification). A $p \leq 0.05$ was considered significant.

4.2. Results

Sample Population

Fifty-three dogs were included in the study for a total of 66 integumentary MCT excised with their respective SLN/SLNs. Forty-four dogs had a single MCT, and 9 dogs had multiple MCT (simultaneous or not). Breeds were distributed as follows: 12 (22.7%) mixed breeds, 10 (18.8%) Labrador Retrievers, 5 (9.4%) English Setter, 4 (7.5%) Golden Retrievers, 4 (7.5%) American Staffordshire, 3 (5.7%) Pug, 3 (5.7%) Boxers, 2 (3.7%) Maltese, 1 (1.9%) each of Dogo Argentine, Greater Swiss Mountain Dog, Bull Terrier, Beagle, Bracco Italiano, Tosa, Dachshund, Chihuahua, Weimaraner and Wire Fox Terrier. There were 22 (41.5%) intact males, 21 (39.6%) spayed females, 7 (13.2%) neutered males, and 3 (5.7%) intact females. Mean and median age at presentation were 7.6 and 8 years (range, 1–14 years), respectively. Mean and median weight were 25.8 and 27.5 kg (range, 3–62 kg), respectively.

The 66 tumors were located as follows: 28 on the trunk, 18 on limbs, 9 on the head and neck, 8 on the inguinal region, 2 on the digits and 1 on the tail. Based on previous literature[19], 47 MCT occurred at a favorable prognostic site, while 19 occurred at a site associated with negative prognosis. All but 1 MCT were at first presentation. Tumor mean and median maximum diameter were, respectively 1.9 and 1.5 cm (range, 0.3–7.60 cm). Forty-nine MCT were smaller than 3 cm, and 17 were larger or equal to 3 cm. Ten out of 66 (15.1%) MCT were ulcerated.

Histologically, 50 MCT were cutaneous, and 16 MCT were subcutaneous. All cutaneous MCT were Kiupel-low-grade, of which 12 were Patnaik-grade-I and 38 were Patnaik-grade-II. Eight subcutaneous MCT had an infiltrative growth pattern, 6 had a combined growth pattern and 2 were circumscribed. The mitotic index was 0 in 11 subcutaneous MCT, ≤4 in 4 subcutaneous MCT, and >4 in 1 subcutaneous MCT. Two subcutaneous MCT presented multinucleation, in the others the multinucleation was absent. Excisional margins were infiltrated in 4 cases.

A total of 115 SLNs were removed. A single SLN was found in 35 cases, 2 SLNs were removed in 17 cases, 3 SLNs were removed in 10 cases, and 4 SLNs were removed in 4 cases. The SLNs belonged to the same lymphocenter in 53 MCTs, and to two different lymphocenters in 13 MCTs. Histologically, 47 SLNs were classified as HN0, 16 as HN1, 42 as HN2 and 10 as HN3. Considering the maximum HN value for each MCT, 21 MCT had a HN0 SLN, 9 MCT had HN1 SLN, 29 MCT had a HN2 SLN, and 7 MCT had a HN3 SLN.

Association between HN Category and MCT Clinicopathological Variables

- *HN0-1 vs. HN2-3*

Results of univariate analysis are summarized in Table 1. Only size of the MCT and number of SNL were associated with SLN status: dogs with MCT larger than or equal to 3 cm had a higher prevalence of HN2-3 SLN compared to dogs with smaller tumors (PR = 1.629, $p = 0.016$). In addition, even when size was evaluated in its continuous scale the prevalence of HN2-3 SLN increased proportionally with each cm increase in tumor diameter (PR = 1.161, $p = 0.002$). The prevalence of HN2-3 also increased with the increasing number of SNL (PR = 1.240, $p = 0.031$). Although the AUC was significantly greater than 0.5 for tumor size ($p = 0.019$, $p = 0.044$), number of SNL ($p = 0.021$) and Patnaik grading systems ($p = 0.036$), the ability of the three variables to discriminate MCT with HN0-1 SLN from MCT HN 2-3 SLN was small (AUC < 0.64).

Variable	***PR***	***95% C.I.***	***P***	***AUC***	***95% C.I.***	***P****
Size						
≥ 3 cm vs < 3cm	1.63	1.05-2.44	**0.016**	0.61	0.48-0.75	**0.019**
Increasing of 1 cm	1.16	1.06-1.28	**0.002**	0.62	0.49-0.76	**0.044**
Site						
Favorable vs negative prognosis	0.83	0.44-1.33	0.479	0.54	0.40-0.68	0.232
Ulceration						
Yes vs no	1.12	0.54-1.79	0.693	0.52	0.38-0.66	0.358
SLN number						
Increasing of 1 SLN	1.24	1.02-1.51	**0.031**	0.63	0.50-0.77	**0.021**
SLN lymphocenter						
Increasing of 1 lymphocenter	1.36	0.87-2.13	0.180	0.56	0.42-0.70	0.121
MCT location						
Cutaneous vs subcutaneous	1.38	0.84-2.08	0.148	0.57	0.43-0.71	0.098
MCT histological grade						
Patnaik II vs I	1.66	0.82-4.83	0.244			
subcutaneous vs Patnaik I	2.06	0.97-6.05	0.101	0.62	0.48-0.75	**0.036**

Table 1. *Association between HN (0-1 vs 2-3) and MCT clinicopathological variables. Results of univariate analysis with log-binomial model (discrete variables) and Poisson model with robust variance (continuous variables). Legend: PR=prevalence ratio, the ratio between the proportion of HN 2-3 in the two categories of the variable; C.I. 95% confidence interval; P= p-value of Wald test: null hypothesis PR=1 vs alternative hypothesis PR ≠1; AUC= area under ROC curve to measure discriminant ability of the variable (0.5=no discrimination >0.5 and below 0.55- negligible; 0.55–0.63- small; 0.64–0.70- moderate; and 0.71 and above- strong), P* =p-value for Mann–Whitney U test: null hypothesis AUC=0.5 versus alternative hypothesis AUC>0.5.*

Since 36 HN2-3 SLN were available, a maximum of 3 variables could be included in the multivariable regression model. According to clinical relevance, the following variables were selected to evaluate their joint association with HN0-1 vs. HN2-3: size (continuous scale), number of SNL (continuous scale) and MCT location (cutaneous vs. subcutaneous) (Table 2). The ability of the model to discriminate between HN0-1 and HN2-3 patients was statistically significant ($p = 0.004$) but moderate (AUC = 0.69). When location of MCT was removed from the model, the other two variables retained the statistical significance, and a negligible decrease was obtained from the model discriminant ability (AUC = 0.68).

Variable	***PR***	***95% C.I.***	***P***	***AUC***	***95% C.I.***	***P****
Model with the three variables				0.69	0.57-0.82	**0.004**
Size **Increasing of 1 cm**	1.14	1.02-1.28	0.023			
SLN number **Increasing of 1 SLN**	1.22	1.01-1.47	0.044			
MCT location **Cutaneous vs subcutaneous**	1.09	0.67-1.78	0.717			

Table 2. *Association between HN (0-1 vs 2-3) and MCT clinicopathological variables. Results of multivariable analysis: Poisson model with robust variance. Legend: PR=prevalence ratio, the ratio between the proportion of HN 2-3 in the two categories of the variable; C.I. 95% confidence interval; P= p-value of Wald test: null hypothesis PR=1 versus alternative hypotesis PR ≠1; AUC= are under ROC curve to measure discriminant ability of the model (0.5=no discrimination >0.5 and below 0.55- negligible; 0.56–0.63- small; 0.64–0.70- moderate; and 0.71 and above- strong); P* p-value of Mann–Whitney U test: null hypothesis AUC=0.5 versus alternative hypothesis AUC>0.5.*

- *HN0-1-2 vs. HN3*

Results of univariate analysis are summarized in Table 3. Tumor size was confirmed as a prognostic variable related to the risk of a HN3 SLN both for the 3 cm cut-off and for 1 cm increase (PR = 7.206 $p = 0.012$ and PR = 1.648 $p < 0.001$). The ability of these variables to discriminate between HN0-1-2 and HN3 SLNs was significant ($p = 0.002$ and 0.006) and strong (AUC > 0.71). In addition to size, subcutaneous MCTs were also associated with the presence of at least one HN3 SLN (PR = 4.167 $p = 0.044$), with a significant ($p = 0.018$) and moderate discriminant ability (AUC = 0.68).

A significant strong discrimination ability was also found for Patnaik grading systems (AUC = 0.70 $p = 0.026$).

Variable	*PR*	*95% C.I.*	*P*	*AUC*	*95% C.I:*	*P**
Size						
≥ 3 cm vs < 3cm	7.21	1.73- 47.21	**0.012**	0.76	0.54-0.97	**0.002**
Increasing of 1 cm	1.65	1.35-2.01	**<0.001**	0.74	0.58-0.99	**0.006**
Site						
Favourable vs negative prognosis	0.99	0.15-4.18	0.989	0.5	0.27-0.73	0.500
Ulceration						
Yes vs no	0.93	0.05-4.74	0.946	0.5	0.28-0.73	0.480
SLN number						
Increasing of 1 SLN	1.69	0.84-3.38	0.14	0.62	0.39-0.85	0.129
SLN lymphocentrum						
Increasing of 1 lymphocentrum	3.06	0.78-12.02	0.109	0.63	0.40-0.86	0.055
MCT location						
Cutaneous vs subcutaneous	4.17	1.02-19.42	**0.044**	0.68	0.46-0.91	**0.018**
MCT histological grade						
Patnaik II vs I	0.63	0.07-12.89	0.697	0.70	0.47-0.93	**0.026**
Subcutaneous vs Patnaik I	3.00	0.52-54.76	0.296			

Table 3. *Association between HN (0-1-2 vs HN 3) and MCT clinicopathological variables. Results of univariate analysis with log-binomial model (discrete variables) and Poisson model with robust variance (continuous variables). Legend: PR=prevalence ratio, the ratio between the proportion of HN 3 in the two categories of the variable; C.I. 95% confidence interval for prevalence ratio; P= p-value of Wald test: null hypothesis PR=1 versus alternative hypotesis PR ≠1; AUC= are under ROC curve to measure discriminant ability of the variable (0.5: no discriminant ability >0.5 and below 0.55- negligible; 0.55–0.63- small; 0.64–0.70- moderate; and 0.71 and above- strong); P*=p value of Mann–Whitney U –test: null hypothesis AUC=0.5 versus alternative hypothesis AUC > 0.5.*

Cluster Analysis and Association with HN

To evaluate the association between HN and the whole set of clinical and pathological variables reported in Tables 1 and 3, dogs were grouped in clusters determined by the similarity of their clinical-pathological characteristics according to site (favorable vs. negative prognosis), size (≥3 cm vs. <3 cm), ulceration (yes vs. no), SLN number (1 vs. >1), SLN lymphocenter (1 vs. 2), MCT histological grade (subcutaneous, Patnaik I, Patnaik II).

Four clusters were identified (cluster 1:11 patients, cluster 2:17 patients, cluster 3:25 patients, cluster 4:13 patients). The tree classification procedure of clusters is reported in Figure 1.

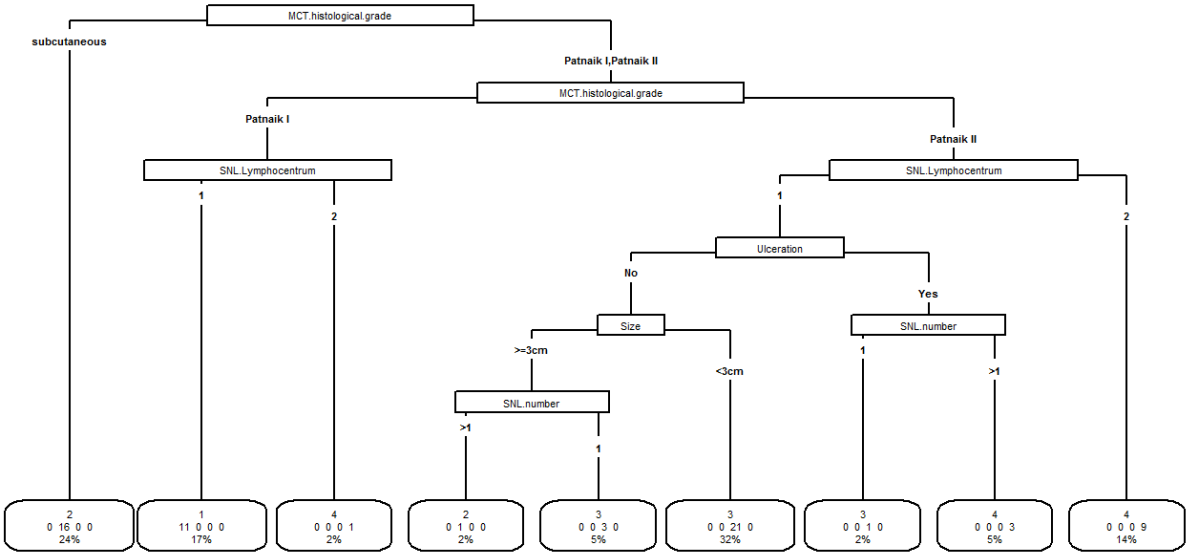

Figure 1. *Results of the tree procedure to classify clusters according to the variables site (favorable vs. negative prognosis), size (≥3 cm vs. <3 cm), ulceration (yes vs. no), SLN number (1 vs. >1), SLN lymphocenter (1 vs. 2), MCT histological grade (subcutaneous, Patnaik I, Patnaik II).*

The importance of the variables to identify clusters is represented in hierarchical order. The most important was MCT histological grade. Each main partition was further subdivided in sub-partitions having each one a more homogeneous cluster composition. The procedure ended with the final classification in which each group is made by subjects classified in the same cluster. All variables were used except tumor site which is not identified as useful to identify clusters after the previous variables were included.

In the boxes of final classification is reported the group cluster, the number of patients in the group, and the percentage of the patient in the group over the total of patients.

The variable which mainly characterized the clusters was MCT histological grade, followed by SNL lymphocentrum, ulceration, size and number of SNL. The variable "tumor site" did not appear to be useful in classifying clusters after entering the above-mentioned variables in the model.

All the 11 dogs in cluster 1 were mainly characterized by Patnaik I MCT and one SNL lymphocenter. Dogs in cluster 2 were mainly characterized (16/17 dogs) by subcutaneous MCT. Dogs in cluster 3 were mainly characterized (21/25 patients) by Patnaik II MCT and one SLN lymphocenter, absence of ulceration, and tumor size <3 cm. In cluster 4, dogs were mainly characterized (9/13 dogs) by Patnaik II MCT and two SLN lymphocenters.

The overall association between clusters and HN (classified as HN0-1 vs. HN2-3) was statistically significant (Fisher exact test p-value = 0.0049) as well as the ability of clusters to discriminate

subjects with HN0-1 and HN2-3 (AUC = 0.68, 95% confidence interval 0.56–0.8, *p*-value = 0.004) (Table 4).

Cluster (n° MCT)	***Prevalence of HN2-3***	***PR***	***95% C.I.***	***z***	**P**
1 (n=11)	0.364	0.91	0.36-2.28	-0.20	0.84
2 (n=17)	0.706	1.77	0.99-3.12	1.96	0.05
3 (n=25)	0.400				
4 (n=13)	0.769	1.92	1.09-3.38	2.27	0.02

Table 4*. Association between clusters and HN 2-3. Results of log-binomial model. Prevalence is the proportion of HN 2-3 within cluster, PR is the ratio between the prevalence of HN 2-3 in each cluster and prevalence in cluster 3 (considered as reference because of the most represented cluster). Legend: C.I. confidence interval; Z= wald statistics; P is the p-value of Wald test for the hypothesis PR=1 versus PR ≠ 1.*

The highest prevalence of HN3 SLNs was observed in cluster 2 (4/17, 25.8%). In cluster 4 the prevalence was 15.4% (2/13) and in cluster 1 it was 9.1% (1/11). None of the 25 dogs in cluster 3 had a HN3 SLN. Overall, the association between HN (classified as HN0-1-2 vs. HN3) and clusters was statistically significant (Fisher exact test p = 0.047). Due to the low number of HN3 SLN and the absence of HN3 SLN in cluster 3, it was not possible to estimate PR (prevalence ratio; the ratio between the prevalence of HN3 in each cluster and prevalence in cluster 3) by a regression model. The ability of cluster classification to discriminate between HN0-1-2 and HN3 was strong (AUC = 0.78), suggesting a potential usefulness of clusters to identify dogs with HN3 SLN.

4.3. Discussion

The increase of knowledge in small animal oncology has drawn interest towards the early detection of metastatic disease even in the absence of clinically relevant alterations. This concept has been applied, for instance, in dogs and cats admitted to second-level diagnostic imaging and to ultrasound-guided cytological sampling of macroscopically normal liver and spleen.[8,10,26–28] Unfortunately, patterns of imaging features and fine needle aspiration are insufficient to determine the status of the lymphatic basin in dogs with MCT with high repeatability.[1,2,5] Thus, histology on the excised lymph nodes remains a cornerstone to assess their metastatic involvement. Although this strategy enjoys the advantages of an early diagnosis of lymph node metastasis and allows for correct prognostication and therapeutic choices in a portion of cases, it could involve an unnecessary surgical procedure for those

dogs with non-metastatic SLNs, and additional costs for the owners. These considerations have led to question whether SLN mapping and biopsy should always be performed or could be avoided in selected dogs.

Firstly, our study confirms the presence of early (HN2) and overt (HN3) metastasis in 54% of the sample population, even in case of subcutaneous and low-grade cutaneous MCT in dogs. In contrast, the remaining 46% of dogs were admitted to the surgical procedure even if SLNs were ultimately non metastatic.

Based on our results, tumor size was associated with SLN metastasis. In 2015, a study conducted on more than 300 dogs with cutaneous MCT found a correlation between tumor size and nodal metastasis at admission, although RLN biopsy was performed in 50 cases only.[20] The overall rate of nodal metastasis in that study was lower than in the present study (18.1%) and nodal metastases were not categorized following Weishaar's system.[20] In another study evaluating the RLN, tumors larger than 3 cm had a significantly higher risk of HN1-2-3 RLN; although tumor size lost its significance when HN2-3 RLN were considered jointly, the risk ratio was 1.40.[3] This contradictory result may have been due to a discrepancy between the RLN and the actual draining SLN, according to the reportedly low correspondence between clinically expected RLN and SLN.[4–7] More recently, a significant correlation between MCT size (1 cm increase) and HN2-HN3 SLN was found in 35 cases, with SLN metastases occurring only in MCT greater than 26 mm.[5] In the present study, MCT size was associated with early and overt metastasis (HN2-3) and, by a greater extent, with overt metastases (HN3) analyzed alone. The risk of having HN2-HN3 (or HN3) increased for each cm increase in MCT diameter and for MCT larger than 3 cm. It may thus be argued that for tumors smaller than 3 cm, SLN mapping and biopsy could be avoided; however, it should be underscored that the discriminant ability of tumor size remains moderate. According to the current veterinary literature, the removal of HN2 lymph nodes has a potential therapeutic effect and should thus be performed anyway.[29,30] Further studies comparing the outcome of dogs with tumors smaller than 3 cm admitted or not to SLN biopsy are needed to confirm the unnecessary nature of the procedure in these cases. Conversely, the strong association that we report between tumor dimension and HN3 SLN when considering the HN3 category alone suggests that SLN mapping and biopsy should always be performed for MCT bigger than 3 cm without clinically evident nodal alterations.

The number of SLN detected for each MCT was also associated with HN2-3. In human breast cancer the detection of a greater number of SLNs is often correlated with larger tumor size.[31] However, in the present sample population of canine MCT, the prevalence ratio of this variable remained statistically significant even in multivariate analysis when adjusted for tumor size, leading to hypothesize an absence of correlation between the number of SLN and size. A hypothesis is that the

initiation of the metastatic process and nodal involvement could activate additional lymphatic networks, thus increasing the number of SLN detected. Further studies should elucidate the real significance of this finding, taking into account the difference in the number of SLN detected with different mapping techniques as well.[32] Moreover, the exact number of SLN to be removed is a post-operative variable not available before admitting the dog to the entire mapping and biopsy procedure. However, this data, if further confirmed, could help clinicians to prepare owners to a possible nodal metastatic status of their dog while waiting for the confirmation of the histopathology.

Surprisingly, subcutaneous location was significantly associated with the presence of overt metastasis (HN3). Subcutaneous MCT represents a unique subset of tumors to which the histological grading systems do not apply. Few studies have focused on subcutaneous MCT and reported an overall good prognosis with metastatic rate ranging from 2 to 6% and prolonged survival (>1000 days).[16,33,34] In those studies, data on nodal metastasis at admission were absent or biased by the absence of lymph node samples (either cytology or histology) in many of the included cases.[16,33,34] In two recent papers, 60% of subcutaneous MCT presented a ≥ HN2 SLN, although no association between cutaneous versus subcutaneous location and presence of nodal metastases was found.[4,5] In the present study, 68% of the 16 subcutaneous MCT had an early (HN2) or overt (HN3) SLN metastasis, confirming the high prevalence of nodal metastasis in this specific MCT subset. A moderate discriminant ability for the risk of having an HN3 SLN was found for subcutaneous versus cutaneous MCT, and the highest prevalence of HN3 SLN occurred in cluster 2 that included all dogs with subcutaneous MCT. Based on these results, the mapping and biopsy of SLNs should be strongly suggested in presence of subcutaneous MCT. However, it should be noted that the clinical identification of subcutaneous versus cutaneous MCT is not always straightforward, and some subcutaneous tumor could thus be overlooked if considered cutaneous during the pre-operative clinical examination.[4] Other histological characteristics have been reported to impact the prognosis of subcutaneous MCT, such as mitotic index, multinucleation, infiltrative pattern, Ki-67, and AgNOR.[16,33,34] In the present paper, mitotic index, multinucleation, and growth pattern were available, but the small number of subcutaneous cases did not permit to include this data in the statistical models. Further studies focusing on subcutaneous MCT only are thus needed to define a possible nodal metastatic risk stratification, after complete staging and SLN biopsy, by histological and immunohistochemical evaluation.

For cutaneous tumors, the Patnaik grading system did not associate with the SLN status, as reported in a recent study.[5] This result is probably due to the absence of Patnaik grade III–Kiupel high grade and Patnaik grade II–Kiupel high grade tumors in the present study. In the retrospective paper by Stefanello et al. (2015), 46.5% of Patnaik grade III and 29.7% of Kiupel high grade MCT had nodal metastasis at admission. In that study, the RLN was evaluated cytologically and/or histologically only

in 18% of the dogs, although the clinical evaluation of the RLN was reported in all animals.[20] Therefore, the cytological and histological samples were probably reserved to clinically abnormal RLNs in many cases.[20] In Fournier's study (2020), 10% of the SLN evaluated were enlarged at palpation, although it is not reported if they drained the four included high-grade tumors.[5] On the contrary, only dogs with non-palpable/normal sized RLN were admitted to the SLN mapping and biopsy in the present paper, thus probably influencing the absence of high-grade tumors. In both the above-mentioned papers as well as and in the present study, Patnaik grade II and grade I MCT did not have a different risk of nodal metastasis.[5,20] These results bring to the conclusion that variables other than Patnaik histological grade should be taken into account to define such risk in dogs with low-grade MCT. In addition, this result suggests that dogs who underwent excision of a low-grade MCT without SLN lymphadenectomy should be as well followed-up for nodal alteration.

A multivariate model including all the variables as predictors would have been the appropriate method to evaluate the joint interaction between multiple variables. As reported in the material and methods section, a case series with at least 70 MCT with HN3 SLN should have been available to allow the inclusion in the aforementioned analysis of all the seven clinicopathological variables examined in the present study. As an alternative, patterns of association of the seven variables were summarized in a single variable (cluster) obtained by classifying dogs in a small number of groups, in such a way that dogs classified in the same group were more similar for overall clinicopathological characteristics than dogs classified in other groups. When clusters were associated with HN0-1 vs. HN2-3, the association was significant but only a moderate ability of discrimination was found. However, the ability increased when the association was tested for HN0-1-2 vs. HN3. These results may suggest a potential usefulness of clusters in the identification of dogs with overt metastatic SLN. However, the precision of the estimates for the association of the variables with HN0-1-2 vs. HN3, both in univariate and clusters analyses, was low (wide confidence intervals) because of the low number of dogs with HN3 SLN, and further studies are needed.

The final decision to perform the sentinel lymph node mapping and biopsy should take into account the association with the nodal staging (as tested in the present study) and the benefit of such procedures on the oncological outcome. As previously cited, recent papers on RLN reported a possible therapeutic effect of lymphadenectomy in the presence of early metastasis in canine MCT, although data on SLN are lacking.[29,30] In the study population, only 3 out of 53 dogs had an MCT relapse at a median follow-up time of 455 days (range, 15–1305 days) (data not shown). Hence a prognostic analysis on tumor progression and survival was not performed. Indeed, statistical analysis on such a low number of events would have led to obtaining unreliable statistical results. The low number of relapses could be hypothetically linked to the less aggressive behavior of the included

MCT despite the presence of a high percentage of nodal metastasis; another potential explanation is the possible therapeutic effect of lymphadenectomy for SLN as well, and the need for a longer follow-up time for dogs with a less aggressive tumor in which surgery drastically decreased the burden of the disease. Further studies with a larger number of dogs and a longer follow-up time, and possibly comparing dogs admitted and not admitted to SLN biopsy, should be focused on prognostic data.

4.4. Conclusions

Although literature reported only few and self-limiting side effects of SLN biopsy in dogs[5,6], the mapping and removal of SLN involves additional anesthetic and diagnostic procedure, as well as additional cost for the owner. Based on the results of the present paper, tumor size in low-grade and subcutaneous MCT is associated with the risk of having HN2-HN3 SLN; however, considering the small to moderate ability of discrimination in distinguished HN2-HN3 from HN0-HN1, further studies should confirm this data before excluding dogs with smaller tumors from the nodal staging procedure. The high prevalence and the strong correlation between MCT equal or bigger than 3 cm, subcutaneous MCT and the presence of HN3 forces the solid suggestion to map and biopsy SLN in these cases.

4.5. References

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5. Scientific production unrelated to the project

During the years that lead to the competition of the present PhD project, I was primarily involved in other on-going research topics and in the clinical practice of the Veterinary Teaching Hospital as part of my training as a clinical researcher. Furthermore, I participated to the clinical activity and research projects of research groups outside my department. As a result, besides the publication related to the project that were presented earlier, I actively participated in studies on other surgical and oncological topics, that resulted in publications on international, peer-reviewed Journals. Albeit not directly related to the project, these studies contributed to the development of both practical and soft skills that were crucial to complete my PhD research project. Hence, these studies will be presented in this section in a concise form:

- Manfredi M, De Zani D, **Chiti LE**, Ferrari R, Stefanello D, Giudice C, Pettinato V, Longo M, Di Giancamillo M, Zani DD. Preoperative planar lymphoscintigraphy allows for sentinel lymph node detection in 51 dogs improving staging accuracy: Feasibility and pitfalls. Vet Radiol Ultrasound. 2021 Sep;62(5):602-609. doi: 10.1111/vru.12995

- **Chiti LE**, Ferrari R, Roccabianca P, Boracchi P, Godizzi F, Busca GA, Stefanello D. Surgical Margins in Canine Cutaneous Soft-Tissue Sarcomas: A Dichotomous Classification System Does Not Accurately Predict the Risk of Local Recurrence. Animals (Basel). 2021 Aug 11;11(8):2367. doi: 10.3390/ani11082367. PMID: 34438827

- **Chiti LE**, Ferrari R, Boracchi P, Morello E, Marconato L, Roccabianca P, Avallone G, Iussich S, Giordano A, Ferraris EI, Agnoli C, Dondi F, Giacobino D, Godizzi F, Stefanello D. Prognostic impact of clinical, haematological, and histopathological variables in 102 canine cutaneous perivascular wall tumours. Vet Comp Oncol. 2021 Jun;19(2):275-283. doi: 10.1111/vco.12673

- **Chiti LE**, Mayhew PD, Massari F. Renal venotomy for thrombectomy and kidney preservation in dogs with adrenal tumors and renal vein invasion. Vet Surg. 2021 May;50(4):872-879. doi: 10.1111/vsu.13593

- Montinaro V, **Chiti LE**, Rossi N, Massari F, Romanelli G. Partial Gastrectomy at the Time of Splenectomy in Two Dogs With Splenic Neoplasia and Gastric Involvement. Top Companion Anim Med. 2021 Mar;42:100487. doi: 10.1016/j.tcam.2020.100487

- Zamarian V, Ferrari R, Stefanello D, Ceciliani F, Grieco V, Minozzi G, **Chiti LE**, Arigoni M, Calogero R, Lecchi C. miRNA profiles of canine cutaneous mast cell tumours with early

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- Massari F, **Chiti LE**, Lisi MLP, Drudi D, Montinaro V, Sommaruga P. Lip-to-nose flap for reconstruction of the nasal planum after curative intent excision of squamous cell carcinoma in cats: Description of technique and outcome in seven cases. Vet Surg. 2020 Feb;49(2):339-346. doi: 10.1111/vsu.13359

- Ferrari R, Marconato L, Buracco P, Boracchi P, Giudice C, Iussich S, Grieco V, **Chiti LE**, Favretto E, Stefanello D. The impact of extirpation of non-palpable/normal-sized regional lymph nodes on staging of canine cutaneous mast cell tumours: A multicentric retrospective study. Vet Comp Oncol. 2018 Dec;16(4):505-510. doi: 10.1111/vco.12408.

- **Chiti LE**, Montinaro V, Lisi MLP, Asta AG, Marches S, Sommaruga P, Massari F. Lip-to-nose flap for nasal plane reconstruction in dogs: A cadaveric and in vivo feasibility study. Vet Surg. 2018 Nov;47(8):1101-1105. doi: 10.1111/vsu.12965

6. Conclusions

In the last decade, the veterinary literature has promoted interest towards the biological behavior of canine oncological diseases, including the risk and impact on survival of lymphatic spread, as a result of three main phenomena: the dramatic changes in the pet-owner relationship, with dogs being considered as family members and the number of pet dog constantly increasing in developed countries; the increasing frequency in the diagnosis of neoplasms in pet dogs resulting from the diffusion of advanced diagnostic tools and growing clinical awareness of oncological diseases in veterinary practice; the observation that several canine malignancies share similar features with the human counterpart, thus making pet dogs excellent spontaneous tumor models.

In this scenario, results of the PhD project here presented have contributed to increase knowledge on the feasibility and impact of SLN mapping and biopsy in dogs with spontaneous tumors, including head and neck malignancies. Based on our studies' results, and in accordance with previous literature, the lymphatic drainage of spontaneous tumors in dogs can't be accurately predicted based on anatomical studies, given the high rates of non-correspondence between the clinically expected RLN and actual SLN. This concept is of outmost importance for canine malignancies of the head and neck due to the complexity of the lymphatic drainage of this region and the relatively high incidence of nodal metastases from the most common tumor types, although it holds true also for integumentary malignancies (MCT) of various sites. While the studies presented in this PhD thesis were conducted on tumors of the head and neck and MCT, it is reasonable to assume that other tumor types with a tendency to lymphatic spread could share similar behavior and drain to clinically unpredictable lymphocenters.

Although several techniques for SLN mapping and biopsy are described both in humans and dogs, radiopharmaceutical with or without blue dying is regarded as the gold standard, due to high detection rates and safety. Accordingly, results of this project underscored the tolerability of the procedure, with minimal morbidity recorded both for MCT and head and neck malignancies, and the reliability of radiopharmaceutical, with high detection rates and low false negative rates. The main drawback of radiopharmaceutical is the need for permission to store radioactive materials, hence this technique is not readily available in veterinary oncology. To overcome such limitations, alternative mapping techniques should be validated in order to allow for SLN mapping and biopsy to become routine in veterinary surgical oncology.

The benefits of extirpation of a metastatic SLN are easily understood, and consist of patient upstaging, correct prognostication and adequate treatment recommendations; such benefits have been thoroughly documented in the present PhD thesis and in the previous literature. However, the

potential drawback of the removal of negative SLN are yet to be understood and should be investigated in future research. As part of our research on the topic, we published a study in 2021 to answer the following clinical question: are there any clinical or pathological characteristics that could allow for identification of dogs with MCT at lower risk of SLN metastases, to be excluded *a priori* from the SLN mapping and extirpation? Surprisingly, the only variables that correlated, in univariate model, with nodal metastases were tumor size, number or SLN and subcutaneous MCT. Conversely, tumor grade was not able to predict the risk of having HN2-3 or HN3 SLN. Despite the correlation of increasing tumor size and MCT > 3 cm with SLN metastases (both HN2-3 and HN3 alone), the discriminant ability was low, thus not providing enough evidence to recommend the exclusion of dogs with MCT < 3 cm from SLN biopsy. Similarly, the observation that subcutaneous MCT have a higher tendency to cause HN3 SLN metastases leads to the strong recommendation of performing SLN biopsy on subcutaneous MCT, although cutaneous MCT should not be excluded from the procedure giving the moderate discriminative ability of the model. Despite the inclusion of 66 MCT, the low prevalence of HN3 in our study population did not allow for a multivariate model including all the prognostic clinical and pathological variables, as at least 70 HN3 would have been necessary to allow for such analysis. Although results of our study underscored the utility of SLN biopsy in dogs with MCT > 3 cm and subcutaneous MCT, it was not possible to identify variables that could safely determine the exclusion of patients from the procedure. Future studies should clarify the indication of the SLN biopsy for MCT, ideally by including a higher number of HN3, and other tumor types.

Lastly, while most of the literature on SLN mapping and biopsy is focused on canine tumors, the same oncological principles hold true for pet cats. At the Veterinary Teaching Hospital of University of Milan, we are currently performing SLN mapping in cats with tumor types that have propensity for nodal spread, although results are not presented here given that we have not yet collected enough cases to draw reliable conclusions on the impact of the procedure. In the future, the impact of SLN mapping and biopsy should be investigated for tumor-bearing cats, in order to promote the introduction of higher standards of care also for feline patients.