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Clinical and diagnostic implications of sentinel lymph node detection in canine mast cell tumours

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Summary

1. Introduction

The studies focused on canine mast cell tumour (MCT) have exponentially risen in the last ten years, making the MCT a “hot topic” in veterinary oncology and an object of debate in the clinical and research field [1–9]. The histological grade of the tumour is widely recognised as the strongest prognosticator [1,10,11], while the identification of the correct draining LN (so-called “sentinel lymph node”, SLN) and its therapeutic impact has recently gained increased attention in veterinary oncology, representing a revolution in the management of canine MCT [12–16].

The SLN is defined as the first node within the lymphatic basin that directly drains the primary tumour [16–18]. Since MCTs metastasize mainly via the lymphatic route, the rationale behind the SLN mapping is the early detection of the site of metastasis [13,14]. Hence, SLN status is predictive of the status of the whole lymphatic basin [16,18,19].

The staging of MCT practised by many clinicians involves the palpation of superficial regional lymph nodes, abdominal ultrasound, and thoracic radiographs [16,20]. Fine needle aspiration of the regional LN is performed at the discretion of the clinician. Thus, not all lymph nodes are routinely aspirated, especially if small, non-palpable or if ultrasound guidance is required. Until recent years, the surgical treatment consisted of the removal of the primary tumour and the regional lymph node when enlarged, structurally altered, or cytologically metastatic, leading to inaccurate disease staging and treatment. Several studies have demonstrated the lack of correlation between lymph node size and the presence of metastasis [2,21–23]. As micrometastases may take many months to produce palpable lymphadenomegaly, small lymph nodes could hide metastatic disease, therefore, removing a potential tumour-harbouring site could be therapeutic [1,5,24,25]. Moreover, cytological sampling yields a considerable rate of false negative results [26].

In 2002 Balogh and colleagues first described the feasibility of sentinel lymph node detection by injection of technetium-labelled human serum albumin (99mTc-HSA) and subsequent scintigraphy and intraoperative guidance in 24 client-owned tumorous dogs. Since that publication, animals have been used as models for human research [12]. Ten years later, Deanne Worley published a study showing that the use of preoperative lymphoscintigraphy and intraoperative methylene blue dye identified a different draining lymph node from the regional (RLN) in 42% of examined MCTs [9]. These results

pioneered the "mapping era" and the beginning of a new way of tumour staging in veterinary medicine, well-known in the human counterpart. After the publication of this article, numerous studies enriched the literature with different protocols and compounds, both for preoperative and intraoperative use, reporting the detection rate of SNL and the agreement between RLN and SLN [22,26–35]. All these studies highlighted the variable rate of concordance between RLN and SLN in MCTs [26–29,31–34,36,37].

At the same time, Weishaar et al. proposed a new histological classification of lymph nodes in four stages and remarked that the assessment of LN status is a crucial step to establish the extent of cancer disease, to suggest proper adjuvant treatment and to accurately predict prognosis both in dogs and cats [7,21,38–40]. In addition, it has been demonstrated that the extirpation of lymph nodes with early or overt metastasis, in dogs with MCT, improves the outcome [5,41].

The number of lymphadenectomy procedures has enormously increased in the last years in the veterinary practice due to the emerging role of SLN identification, but a limited number of studies have specifically analysed the complications associated with SLN extirpation in dogs [40,42,43].

Several studies demonstrated the prognostic value of the Ki67 index when all histological grades of cMCTs are included [44–52]. However, the literature review provides a plethora of prognostic Ki67 values; thus, the lack of a unique cut-off limits its usefulness in the clinical setting. Moreover, LNs metastatic status has never been considered in these studies. Patnaik Grade 2/Kiupel low grade (G2/LG) represent the majority of diagnosed cMCTs, with a reported incidence of 53.6–57.6% [1,47,53,54]. Even if the histological classification may provide information about the metastatic potential of the MCT, it may not fully characterize its biological behaviour, making it difficult to establish an accurate prognosis [46]. The risk of under- or overtreating dogs with G2/LG and HN2 is real since contrast data are provided in the literature. Some studies suggested treatment with adjuvant systemic chemotherapy after surgical excision, while others proved that dogs have a favourable prognosis when treated by primary tumour excision and lymphadenectomy [5,7,26]. Therefore, to better identify which patients could benefit from adjunctive treatment, the Oncology-Pathology Working Group suggested the use of adjunctive prognostic factors such as Ki67 and mitotic count [1].

In this dissertation, after a brief overview of MCT and SLN detection methods, we investigated the agreement rate between SLN and RLN mapping using both indirect computed tomography lymphangiography (ICTL) and intraoperative methylene-blued dye (MB) peritumoral injection in canine MCT. We also described the agreement between the two techniques, and we focused on all the possible reasons for failure of the mapping techniques. Moreover, we investigated the possible complications associated with sentinel lymphadenectomy comparing three different intraoperative detection techniques and the utility of Ki67 evaluation in low-grade MCTs with early metastatic lymph nodes.

2. Mast cell tumour

2.1 Tumour Biology

Mast cell tumour (MCT) is a hematopoietic neoplasm characterised by uncontrolled proliferation of neoplastic mast cells in one or more organs [8,46,55,56].

MCT can originate from different tissues and is classified as cutaneous, subcutaneous, mucosal, extracutaneous/extra mucosal without skin involvement, and mast cell leukaemia [8]. Cutaneous MCT (cMCT) represents the most common malignancy of the skin in dogs [4,8,20,55,57]. Normally, the majority of MCTs are biologically indolent while a small percentage exhibits a high potential for aggressive biological behaviour [4].

Mast cells are innate immunomodulatory cells that originate from pluripotent hematopoietic stem cells in bone marrow [57,58]. These immature cells circulate in the blood system [58] and differentiate into mature mast cells in all the vascularized tissues. They are normally distributed near the blood vessels, nerves, mucus-producing glands, smooth muscle cells, and hair follicles [57–60]. Mast cell density varies among different organs, increasing in areas normally exposed to environmental antigens, parasites, and pathogens [60]. They are normally distributed in the lungs, skin (dermis and in the dermal-epidermal junction), liver, bronchial smooth muscle of the lung, kidney and all the layers of the gastrointestinal tract [58,60].

Mast cells normally contain cytoplasmic secretory granules filled with different bioactive substances, including heparin, histamine, serotonin, TNF alfa and other factors. A study conducted on 10 experimental dogs, revealed the presence of two types of mast cells: chymase-positive (MCc) and tryptase-positive (MCt), whose density varies among different organs. For example, in the spleen, stomach and rectum the percentage of MCc is higher than MCt, while in the lungs and the intestinal tract more MCt are present. The skin expresses both chymase and tryptase [60].

Mast cells are involved in several biological processes, such as elicitation and development of innate immune responses, type 1 allergy, antiparasitic activity and wound healing, rapidly producing and releasing significant amounts of pro-inflammatory mediators, cytokines and growth factors [57,58,60].

2.2 Incidence and signalment

MCT represents the most commonly diagnosed malignant neoplasia of the skin in dogs, accounting for 8.82-21% of all skin malignancies [55,56,61–64].

Older dogs with a median age of 8-9 years (3.5 months to 15.0 years) are mostly affected, but younger dogs can be affected [47,55,65–68].

No sex predilection has been reported [55,65]. Regardless of the geographic area in which epidemiological studies have been conducted, Boxer, Golden retrievers, Labrador retrievers, Shar-peis, Pugs, Boston terriers, French Bulldogs, Dachshunds, American Staffordshire Terriers, Weimaraners and Pit-Bull terriers are the most reported breeds to be at high risk of developing MCT [65–67,69–71]. Also, mixed breeds could develop MCTs [65,66,68] while German Shepherds, Yorkshire Terriers and Cocker Spaniels are low-risk breeds [67–69].

2.3 Clinical presentation

MCT has been referred to as the “great pretender” because it can mimic more benign lesions. Cutaneous MCTs can be located anywhere on the body and be presented as a solitary mass or in multiple forms (9-21% of cases) [8,57,67,72]. In the latter case, clinicians should distinguish synchronous forms (multiple MCTs growing at the same time in different locations) from recurrence, satellite nodules or asynchronous forms [8]. Subcutaneous MCT are soft like lipomas, and they do not show the characteristic clinical features of skin MCT [57,73], while cutaneous MCT develops in the epidermis and could invade the subcutaneous tissue, subcutaneous (scMCT) arises from subcutis with no epidermal involvement. In rare cases, scMCT could infiltrate hair follicles or underlying musculature [8].

Most patients are asymptomatic. The main clinical local signs associated with MCT are erythema, pruritus or fluctuation in size. Occasionally, manipulation during examination could cause the release of heparin, histamine and other proteolytic enzymes, resulting in swelling, itching or redness. This phenomenon is called Darrier’s sign [8,55,57].

Several clinical prognostic information can be obtained from history and during physical examination that could help clinicians to predict the biological behaviour, even if the definitive diagnosis is histological.

Clinical signs suggestive of aggressive behaviour include:

- **rapidity of growth:** slow-growing, hairless masses present for months are normally less aggressive than rapidly growing ones [4,57];
- **diameter:** a diameter greater than 3 cm is associated with an increased risk of local recurrence and lymph node metastasis [15,66,74,75];
- **ulceration:** undifferentiated MCTs tend to ulcerate and to be more aggressive [4];
- **local infiltration/poor demarcation** from adjacent tissues;
- **satellite nodules** distributed in the lymphatic pathways or around the principal mass;
- **systemic paraneoplastic syndromes** are indicative of metastatic spread: in some cases, dogs can display gastrointestinal signs (vomiting, anorexia, diarrhoea, abdominal pain) because histamine stimulates gastric H2 receptors, increasing hydrochloric acid secretion, hypermotility and causing gastric ulceration. Rarely, collapse or acute anaphylactic reactions, and coagulation abnormalities due to the massive release of histamine have been reported;
- **age**: MCTs rarely occur in young dogs, but if this happens, they are less aggressive [10,67–69,76] except for Shar-Peis. In this breed, MCT occurred at a mean age of 6.1± 2.3 years with a high malignant behaviour [76]. For dogs over 10 years of age, 41 times greater odds of developing MCT are demonstrated than those under 2 years [55,65].
- **breed:** among the predisposed breed, Pug, Bulldog and related breeds tend to develop less aggressive MCT [20,69,77], while Shar-pei normally develops more aggressive MCTs [4,55,57]. According to some authors, French bulldogs can develop aggressive MCT [69,78], while according to others, they are less aggressive [67,68,76]. Golden Retrievers are at increased risk of multiple MCTs [20]
- **recurrence**: The presence of a recurrence 2 cm around the previous location of MCT is associated with a worse outcome [47].
- **anatomic location**: MCT located in the mucocutaneous junction, head and neck, inguinal and perineal region (including prepuce, scrotum and vulva) and subungual are considered more aggressive [4,55,68,69,71], although, some studies did not support this evidence [47,67,77,79,79]. It has been reported that 55-72% of dogs with oral-perioral MCTs had nodal metastases at presentation [80,81] and that MCTs in the inguinal and perineal regions, if appropriately

treated, may have outcomes similar to those located in other anatomic regions [4,8,20,47,57]. Subcutaneous tumours behave in a more benign fashion [73,82].

2.4 Staging

Tumour staging includes all the procedures to determine the extent of the MCT [8]. The staging results guide veterinarians with clinical treatment decisions and prognostic discussions with owners. No one of the clinical factors alone can be a definitive prognostic factor and staging needs to be assessed comprehensively.

Historically, extended tumour staging was recommended in all dogs after a diagnosis of MCT [4,20,47,55,57]. This included palpation of RLN and its cytological assessment, abdominal ultrasound (US), thoracic radiographs, buffy coat smears evaluation and bone marrow aspiration cytology [4,20,47,62]. Thoracic radiographs are indicated to evaluate the sternal lymphadenopathy if potentially corresponding to the draining lymphocenter, since pulmonary metastases are rare, due to the predominantly lymphatic spread of MCT [62,83].

In the last years, several authors have recommended a more case-oriented approach to clinical staging, casting doubt on the effectiveness of full staging in the absence of negative prognostic factors and advocating the appropriate selection of the patient that will benefit from extended or minimal staging [4,8,20,47,57].

The European Consensus recommended to full-stage patients only in case of poorly differentiated tumours, regional LN enlargement, nodal metastases, satellite lesions, local oedema, systemic signs, or in case of extensive treatment planned [57]. In presence of negative prognostic factors, the authors also recommended performing ultrasound-guided aspiration of the normal spleen and liver [57]. Ultrasound (US) evaluation alone has a sensitivity and a specificity for the detection of mast cell metastasis in the spleen of 67% and 68% and of 29% and 93% for the liver, respectively [84]; therefore, their cytological evaluation instead of US evaluation alone has been strongly recommended to detect metastatic disease [85].

While The European Consensus assessed to investigate regional LNs by cytology, the recent “Consensus proposal” published in 2021 by Willmann and colleagues, recommended a cytological assessment of all enlarged LNs (regardless of their location) and the identification of the SLNs (regardless of size) in all cases [8]. Abdominal imaging with ultrasound-guided aspiration of liver and spleen is

recommended in case of clinical or histological criteria indicative of aggressive behaviour [8,47]. To corroborate this finding, Rinaldi and colleagues reported that only 0.7% of 136 dogs with low cMCTs had spleen and liver metastases at diagnosis, confirming that in case of low-grade cMCT, cytology of visceral organs is not an essential step in the clinical staging [86]. Warland and coworkers demonstrated that no one of the 220 dogs experienced distant metastasis without first showing local LN metastasis, highlighting the importance of assessing local LNs [20].

Dogs with cMCT are still staged according to the World Health Organization clinical staging system, published in 1980 by Owen et al. [87] (Table 1). This staging system includes four stages and dogs assigned to stage IV experienced a shorter survival time compared with those with stage II (median 203 vs 15 days) [47]. In all four stages, the assessment of the metastatic status of RLNs is included, implying that cytology/histology is always necessary.

*Table 1 World Health Organisation (WHO) Clinical Staging System for Mast Cell Tumours. * any stage could be classified as substage a (without systemic signs) or substage b (with systemic symptoms)*

Stage	Description
0	One tumour incompletely excised from the dermis, identified histologically, without regional lymph node involvement *
I	One tumour confined to the dermis, without regional lymph node involvement *
II	One tumour confined to the dermis, with regional node involvement *
III	Multiple dermal tumours Large, infiltrating tumours with or without regional lymph node involvement *
IV	Any tumour with distant metastasis *

Due to the nebulous and unclear prognostic significance of stage II and stage III in the TNM, a new clinical staging has been proposed by Horta and colleagues [47]. The authors described an amendment of the WHO staging into five grades that better predicted the prognosis of dogs with MCT (Table 2). In this new system, the impact of

regional LN metastasis became crucial to determine the stage. Moreover, the presence of multiple synchronous MCTs without regional lymph node involvement was demonstrated to be associated with a better outcome (median survival time 381 days) than a single MCT with lymph node involvement (median survival time 203 days) [47]. This new staging system is not commonly used yet.

Table 2 Proposed amendment to the WHO staging system.

Stage	Description
I	Single tumour, without regional lymph node involvement
II	Multiple tumours (≥3) without regional lymph node involvement
III	Single tumour, with regional lymph node involvement
IV	Large and infiltrative tumours, without delimitation, or multiple tumours (≥3), with regional lymph node involvement
V	Any tumour with distant metastasis

2.5 Diagnosis

2.5.1 Cytology

MCTs are easily diagnosed by cytology in 92-96% of cases [8,57,63,88]; in fact, this tumour exfoliates well, thus an adequate number of cells can be easily obtained for diagnostic purposes. The distinctive characteristic is the presence of purple intracytoplasmic granules that stain with methanolic Romanowsky-type stains (including May Grünwald-Giemsa [MGG] and Wright's) and with aqueous-based manual quick stains.

One of the most used stains in clinical practice is the Diff-Quik, which failed to correctly diagnose 5% of MCTs and 7-18% of nodal MCT metastases [89]. In such cases, Toluidine blue, Wright's and Wright-Giemsa should be used to reveal cytoplasmic granules [90]. Cutaneous MCTs are localized in the dermis but may also extend into the subcutis, while subcutaneous MCTs are localized entirely within the subcutis. The distinction between the 2 forms can only be reached by histology, cytology does not allow to differentiate [8].

Different grading systems based on cytology have been developed to provide rapid information about the biological behaviour of MCT before definitive excision [6,88,91–93].

Scarpa and coworkers proposed a new cytologic grading system based on the Kiupel histological grading system [6]. The Kiupel system is based on the cellular morphological features instead of the tissue architecture, as the Patnaik system. Thus, the Kiupel system is easily applicable to the cytologic evaluation. The authors applied the Kiupel grading criteria directly to 50 MCTs stained with May Grünwald-Giemsa. The histological grade was correctly predicted in 94% of cases and the system showed a tendency to underestimate HG MCTs, failing to distinguish HG MCTs in 4% of cases (84.6% sensitivity). The authors explained these results theorizing that nuclear details could be obscured by mast cell granules or nuclei could be poorly stained [6].

In 2016, Hergt and colleagues used the criteria proposed by Kiupel and colleagues, firstly using Giemsa-stained and then de- and restaining the same slides with haematoxylin and eosin (H&E) in 141 samples [88]. The authors speculated that the use of H&E staining allowed a better visualisation of nuclear morphology because H&E does not stain mast cell granules. This cytograding reaches a 97.1% of sensitivity, therefore a risk of underestimation of high-grade MCT is possible also if H&E is used [88].

Another cytologic grading scheme was described by Camus and colleagues [91]. The authors developed a predictive algorithm to distinguish low from high-grade tumours based on outcome assessment. One hundred and fifty-two samples of MCT were stained with a modified Wright's stain. MCTs were classified as HG if few granulations were identified, or if there were 2 of the following 4 cytologic features: presence of any mitotic figures, anisokaryosis >50%, binucleation or multinucleation, nuclear pleomorphism [91]. In contrast with the previous two staging systems [6,88], this cytologic method tends to overestimate cytologic grade. In fact, 31.8% of tumours were cytologically high grade but histologic low grade (false positives) and 1.6% were cytologic low grade but histologic high grade (false negatives) [91].

These 3 studies varied in the type of stains utilized and in the number of microscopic fields evaluated, making them difficult to compare [6,88,91]. Also, any of the previous cytological gradings can differentiate between cutaneous and subcutaneous MCTs, therefore histological examination remains the gold standard for grading and correctly diagnosing the MCT [8,90].

The Oncology Pathology Working Group recommended using the Camus system to acquire preoperative information on MCT behaviour. The group of authors also advised to correlate the cytologically high-grade MCTs with other negative factors, such as the clinical signs, due to the 31.8% of false positives [1].

The cytological evaluation of the lymph nodes is one of the important steps to appropriately stage patients, but accurate detection of metastasis via cytology may be challenging [3,8]. In 2009, Krick and colleagues highlighted the lack of standardised cytological criteria that could be used to differentiate reactive from metastatic lymph nodes [3]. The cytological evaluation of lymph nodes is not straightforward, since they physiologically contain a small number of mast cells [94]. Previous papers have used subjective and different descriptions to diagnose lymph node metastases, which caused considerable confusion in comparing results. The presence of clusters or sheets of mast cells or the increased number of mast cells (in >2 high-power fields or more than 3% of the population), the presence of poorly differentiated mast cells in the lymph node or the presence of at least three of the following characteristics: poor granulation, anisocytosis and anisokaryosis, increased nuclear/cytoplasmic ratio were considered as indicative of lymph node metastasis by different authors [3,20,95,96].

According to the scheme of the aforementioned study, lymph nodes were categorised into five categories with escalating risk of malignancy (normal, reactive lymphoid

hyperplasia, possible metastasis, probable metastasis and certain metastasis) [3]. This system is not universally acknowledged, due to the subjective evaluation of the four classes. Also, pathologists have cast doubt on the prognostic usefulness of possible and probable metastasis categories [55].

2.5.2 Histological examination

Tumour grade is acknowledged as the strongest prognosticator for MCT across multiple studies [1,10,11,46,54,97–99]. After the first histological classification [98], several others have been published [11,97,99].

Since 1984, cMCTs have been graded using a 3-tiered scheme according to different criteria: the extent of tissue involvement, cellularity, cellular and nuclear morphology, mitotic activity, stromal reaction, and oedema/necrosis. According to this system, well-differentiated (grade 1) MCTs are associated with an excellent prognosis, while poorly differentiated (grade 3) have a median survival time of 13 weeks (Table 3) [99].

Nevertheless, according to histological grading criteria, up to 59% of cMCTs were classified as grade 2 (G2), especially when tumours demonstrated borderline histological features [11,100]. G2 tumours showed the widest range of biological behaviour compared to grades 1 and 3. Two studies reported a variable metastatic rate of 5 to 22% even if most of the enrolled animals has not been completely staged and in one study dogs with distant metastasis at the presentation were included in the statistical analyses [52,101–103].

The two-tier grading system proposed by Kiupel in 2011 eliminated the ambiguity of the G2, dichotomizing G2 into low-grade (LG) and high-grade (HG) based on the presence of mitotic figures (mitotic count), multinucleation, bizarre nuclei, and karyomegaly [11]. Also, this system reduced the subjectivity of the Patnaik grading system and its reproducibility problems, since 50–60% of discordance among experienced pathologists was reported with the oldest grading system [1,11,53,62]. This 2-tiered system has high prognostic value and minimal interobserver variability (up to 96.8% agreement).

Recently, the Oncology-Pathology Working Group recommended to use of both Patnaik and Kiupel grading systems, dividing cMCTs into 4 categories (G1/LG, G2/LG, G2/HG, G3/HG) with different prognoses [1]. In fact, the two systems appear to be complementary: the Patnaik system is more sensitive whilst the Kiupel system is more specific in identifying more aggressive cMCTs.

Table 3 Patnaik Histological Grading Criteria.

Tumour grade			
	Low	Intermediate	High
Location	Dermis and interofollicular space	Infiltrate lower dermal and subcutaneous tissue; some extend to skeletal muscles or surrounding tissues	Replace subcutaneous and deep tissues
Cell morphology	Round, monomorphic, ample distinct cytoplasm with medium-sized granules	Round to ovoid, moderately pleomorphic, with scattered spindle and giant cells; distinct cytoplasm with fine granules in most cells, but indistinct cytoplasm and large/hyperchromatic granules in some	Round, ovoid, or spindle-shaped, pleomorphic, medium-sized; indistinct cytoplasm with granules that are fine or not obvious; many giant cells and scattered multinucleated cells
Nuclear morphology	Round, condensed chromatin	Round to indented with scattered chromatin and single nucleoli; some binucleated cells	Indented to round vesiculated, with one or more prominent nucleoli; common binucleated cells
Architecture, cellularity, stromal reaction	Arranged in rows or small groups, separated by mature collagen fibres of the dermis	Moderately to highly cellular, arranged in groups with thin, fibrovascular stroma (sometimes thick and fibro-collagenous with areas of hyalinization)	Cellular, arranged in closely packed sheets; stroma fibrovascular or thick and fibro-collagenous with areas of hyalinization
Mitotic figures	None	Rare (0–2/HPF)	Common (3–6/HPF)

The prognosis for G1/LG is excellent, while G2/LG MCTs are considered less aggressive than G2/HG with a 1-year survival rate of 46% [23,47,89]. The difference in survival between G2/LG and G2/HG seems to be related to the local recurrence rather than to the metastatic spread. The prognosis for dogs with G3/HG grade is poor, with 67-75% tumour-related deaths [1].

Nowadays, both the cytological and histological grading systems do not include the mucosal MCTs and subcutaneous MCTs. Mitotic count (MC) greater than 4 in 10 high-power fields, infiltrative growth, the presence of multinucleate cells and Ki67 ≥23 are recognized as negative prognostic factors in scMCTs [73,82]. Interestingly, a recent prospective multicentric study, reported that 35% of 43 enrolled scMCTs had HN3 regional LNs, which is in contrast with the low metastatic rate reported (4-6%) in previous papers, where the RLNs were not removed and analysed [82,104].

2.5.3 Lymph node histological assessment

The histological classification of lymph nodes published by Weishaar et al. represents a crucial cornerstone in the process of understanding mast cell tumours [7]. Until the publication of the aforementioned classification system, the lack of standardized criteria challenged the diagnosis of LN metastasis and increased the inter-pathologist variability of interpretations of nodal involvement [7,57]. In fact, LNs normally contain a sparse number of mast cells and it is not always easy to distinguish neoplastic from non-neoplastic ones, apart from cases where marked features of cellular/nuclear atypia are present.

In this study, haematoxylin and eosin-stained (H&E) sections of LNs were reviewed and evaluated at low magnification (x20 and x40 total magnification); when no areas with mast cells were identified, a 100x total magnification was used. Lymph nodes were classified into four classes named “HN” (histological node) according to the number, the distribution, and the architectural disruption of nodal mast cells (Table 4).

Moreover, the authors correlated the four HN classes with the disease-free interval (DFI) and the survival (ST). No significant difference for DFI and ST was found when comparing each HN, while when HN0/1 and HN2/3 were grouped, a significantly worse outcome was obtained for the HN2/HN3 group.

This study has some important limitations: all tumours were graded only according to the Patnaik system and surgical treatment, adjuvant chemotherapy and follow-up were not standardized, influencing the outcome. Other important limitations are that the tumour grade was not included in the outcome analyses, biasing the results and that the authors decided arbitrarily to group HN0 with HN1 and HN2 with HN3, while they did not analyse the outcome grouping HN0 plus HN1 and HN2 vs HN3. One more bias in the analysis of the outcome is that the majority of LN were not sentinel lymph nodes [7].

How this four-classes scheme must be interpreted is an ongoing debate. For example, the histological border between HN1 and HN2 is very narrow and is not always easy to distinguish these two classes [23]. In Weishar's study, only eleven lymph node sections were stained with toluidine blue [7]. According to a recent publication, the use of toluidine blue (TB) must be preferred over H&E to detect early metastases and to avoid underestimation or overestimation of the LN status, because of the better visualization of mast cell granules with TB [23]. Moreover, the same author showed an increased accuracy evaluating an additional parallel section at a 200-μm step with a limited cost increment and a minimum impact on the laboratory workload [23].

Table 4 Classification system for histopathological evaluation of node metastasis published by Weishaar [7].

Nodal 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 ×400 field (0-3 mast cells per x 400 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 ×400 fields (unless otherwise stated, at least four ×400 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-metastatic
HN3	Disruption or effacement of normal nodal architecture by discrete foci, nodules, sheets, or overt masses composed of mast cells	Overt metastatic

2.5.4 The Lymph Node Dilemma in Early Metastatic Node Patients (HN2)

The prognostic role of HN2 or early metastatic LNs became a true clinical dilemma since the publication of the histological classification by Weishaar and colleagues [7]. This study intended to prove that dogs with a more extensive nodal involvement (HN2/HN3) had a worse outcome compared to dogs with a less advanced nodal involvement (HN0/HN1) [7]. After that publication, most researchers systematically grouped HN2 and HN3 in the statistical analyses of their studies, suggesting that these patients should both be treated with adjuvant chemotherapy [2,105]. According to Kiupel and Camus also HN1 and HN2 are two different degrees of suspected metastatic disease, underlying the unclear prognostic role of these two categories [90]. Some authors also considered HN1 as metastatic LN [26,42], suggesting adjuvant treatment also in these cases. On the other hand, other authors proved no clinical benefit in the chemotherapy treatment for dogs with low-grade mast cell tumours and HN2 LNs, highlighting the therapeutic impact of lymphadenectomy [5,41].

Patnaik Grade 2/Kiupel low grade MCTs represent the majority of diagnosed cMCTs, with a reported incidence of 53.6–57.6% [10–14]. G2/LG MCTs are considered less aggressive than G2/HG, with a 1-year survival rate of 94% compared to 46%, respectively [10–15]. Anyway, it has been reported that a subgroup of dogs (3–17%) with G2/LG dies of causes related to the tumour; therefore, it could be hypothesized that a subset of G2/LG tumours has some unknown intrinsic characteristics that lead to aggressive behaviour [47,53,54]. Nevertheless, in the cited papers, the histological metastatic pattern of lymph nodes was not graded according to the Weishaar system, and proliferation markers such as Ki67 or mitotic count (MC) have not been evaluated [7,47,54,89]. Even if the histological classification may provide information about the metastatic potential, sometimes it does not fully characterize the biological behaviour, making it difficult to establish an accurate prognosis [46]. Therefore, for a more complete portrayal, the Oncology-Pathology Working Group suggested to evaluate adjunctive proliferation markers such as Ki67 and mitotic count [1].

The clinical relevance of HN2 LNs is therefore still under debate.

Project 1: Ki67 Index in Patnaik Grade 2/Kiupel Low-Grade Canine Cutaneous Mast Cell Tumours with Early Lymph Node Metastasis: A Descriptive Study

The research reported in this PhD thesis has been published in an International Scientific Peer-reviewed international journal: *Ki67 Index in Patnaik Grade 2/Kiupel Low-Grade Canine Cutaneous Mast Cell Tumors with Early Lymph Node Metastasis: A Descriptive Study*. **Cino M***, Gariboldi EM, Stefanello D, Spindler KP, Ferraris EI, Morello EM, Bertola L, Maniscalco L, Martano M. Vet Sci. 2023 Jul 5;10(7):436. doi: 10.3390/vetsci10070436. PMID: 37505841; PMCID: PMC10384441. [41]

Mitotic count (MC) and Ki67 index are the two most investigated proliferation parameters in cMCTs [1,44–52,55,106–108]. Mitotic count is the number of mitotic figures in an area of 2.37 mm^2, avoiding areas of haemorrhage, oedema, necrosis, and cysts [109].

Ki67 is a nuclear antigen expressed during the late G1, S, M and G2 phases of the cell cycle, and its expression decreases after mitosis [110]. Mitotic count is one of the four criteria used to classify cMCTs into HG or LG according to the Kiupel classification system, and different studies have reported a significant difference in survival when a threshold of 5 is applied [44,45,52,55,111]. Several studies have found that the Ki67 index has a prognostic value when all histological grades of cMCTs are included [45,45–52,108,111]. However, the literature reviewed provides a plethora of prognostic Ki67 values; thus, the lack of a unique cut-off limits its usefulness in the clinical setting.

To the authors' knowledge, only four studies investigated the prognostic significance of Ki67 exclusively on patients with G2 cMCTs, but the surgical treatment consisted only of the excision of the cMCTs, and lymphadenectomy was never performed or considered [45,48,49,108]. This represents a gap in the knowledge of the behaviour of G2/LG cMCTs.

Considering the paucity of information on cMCTs with early nodal metastasis and the large variety of Ki67 indices proposed as prognostic cut-off, this study aimed to evaluate

whether the Ki67 index had a predicting value in a homogeneous cohort of G2/LG cMCTs with sentinel/regional HN2 LNs and to describe the clinical outcome of this unique group of animals treated with surgery alone.
The second goal was to explore the correlation between the Ki67 index and MC. Based on the authors' experience, it was hypothesized that G2/LG cMCTs with HN2 LNs have a favourable prognosis regardless of the Ki67 index, and MC is not correlated with Ki67.

Materials and Methods

Selection Criteria

The medical databases of three contributing institutions (University of Parma, University of Milan, and University of Turin) were retrospectively searched for dogs that had undergone surgical treatment for cMCT and lymph node extirpation, with a histological diagnosis of cutaneous mast cell tumour G2/LG with HN2 LNs, either sentinel or regional, between 2017 and 2021. Data of client-owned dogs meeting the inclusion criteria were collected. Due to its non-interventional nature, the study did not require ethical approval; however, all clients consented to surgery as a standard procedure for cMCT treatment. The established inclusion criteria were:

- Both concomitantly curative-intent surgery for cMCT and regional/sentinel lymphadenectomy;
- Availability of stained histological slides of both cMTC and LNs and formalin-fixed paraffin-embedded tissues for review by the pathologists;
- Availability of information about the status of margins, MC and Ki67 index from the original histopathology report;
- Ki67 immunostaining performed with the same antibody and protocol [111];
- No evidence of distant metastasis identified at preoperative thoracic radiographs and ultrasound-guided spleen and liver cytology (stage IV);
- Absence of concurrent illnesses that could significantly reduce the survival time.

Follow-up information was updated up to 15 May 2023. If multiple LNs were excised, the highest HN value diagnosed was considered.
Dogs that presented recurrence of previously excised cMCT, multiple synchronous cMCTs (stage III), subcutaneous MCT, stage 0 disease and HN3 LNs were excluded

from the study. Dogs treated with neoadjuvant and/or adjuvant therapy were also excluded.

Data Collection

Data retrieved included signalment (sex, breed, age and body weight), tumour location (head and neck, trunk (including tail), limbs, digits, inguinal region (including perineal and scrotal), tumour size (the longest diameter measured by a calliper at the time of presentation), presence of tumour ulceration, regional lymph node (RLN) identified according to Suami et al. (2013) if the RLN was enlarged compared to the contralateral, and complete blood count and serum biochemistry [110]. For the assessment of the sentinel lymphocenters, preoperative and intraoperative mapping techniques, as previously described [36,37], were also recorded.

Information about surgery date, histological margins, MC, Ki67 index, date and method of detection of local recurrence, nodal relapse, distant metastasis and de novo cMCT occurrence and date and cause of death were included. Follow-up time was defined as the time from the date of surgery to the date of the last contact with the owner, or last clinical evaluation, or death, or the end of the study. Dogs were followed-up every 3 months by clinical evaluation and abdominal ultrasound for the first year and every 6 months from the second year. If indicated, spleen and liver FNA were performed.

Local relapse was defined as the presence of macroscopic disease at the scar or within 2 cm of the original surgical site [112,113]. Nodal relapse was ascertained by cytological evaluation of the suspected LN [3]; distant relapse was confirmed by cytological diagnosis of the metastatic disease in other visceral organs, such as spleen and liver.

Metastases to RLN/SLN associated with a de novo tumour were not considered as nodal relapse.

Curative-intent surgery was performed with proportional lateral margins in case of tumours smaller than 2 cm, or with 2–3 cm lateral margins for larger nodules. A deep margin of one fascial plane was taken for all tumours.

Histopathological and Immunohistochemical Examination

All cases from the institutions involved were blindly reviewed by two experienced pathologists (L.M., L.B.) to confirm the diagnosis of G2/LG cMCTs and HN2 LNs and to ensure that the evaluation was performed in a uniform manner, in accordance with the most recent and commonly adopted literature for the evaluation of cMCTs [7,11,99,109]. Furthermore, L.M. and L.B., blinded to the clinical and histological data, reviewed MC and Ki67 indices. Margins were defined as “incomplete” if cells were detected at or within <1 mm of lateral and/or deep edges, “narrow” if neoplastic cells were present within ≤3 mm of the lateral/deep edges and “complete” if lateral and deep margins were >3 mm [112]. Early metastatic LNs (HN2) were defined according to Weishaar et al. (2014) on serial longitudinal LN sections stained with metachromatic staining (toluidine blue) and examined at 400 X (Figure 1) [7].

Figure 1 Photomicrograph showing an HN2 lymph node. Giemsa with inset.

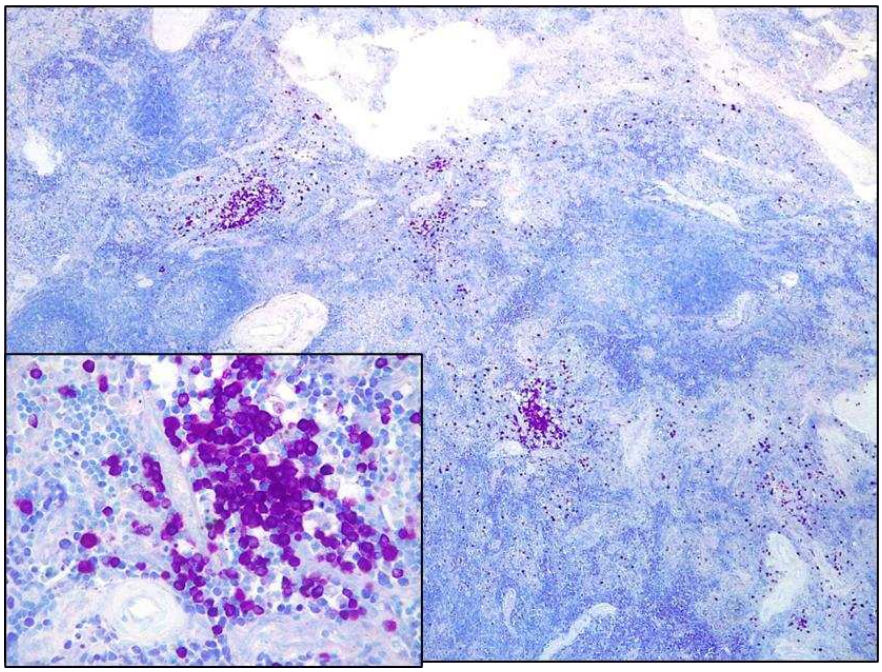
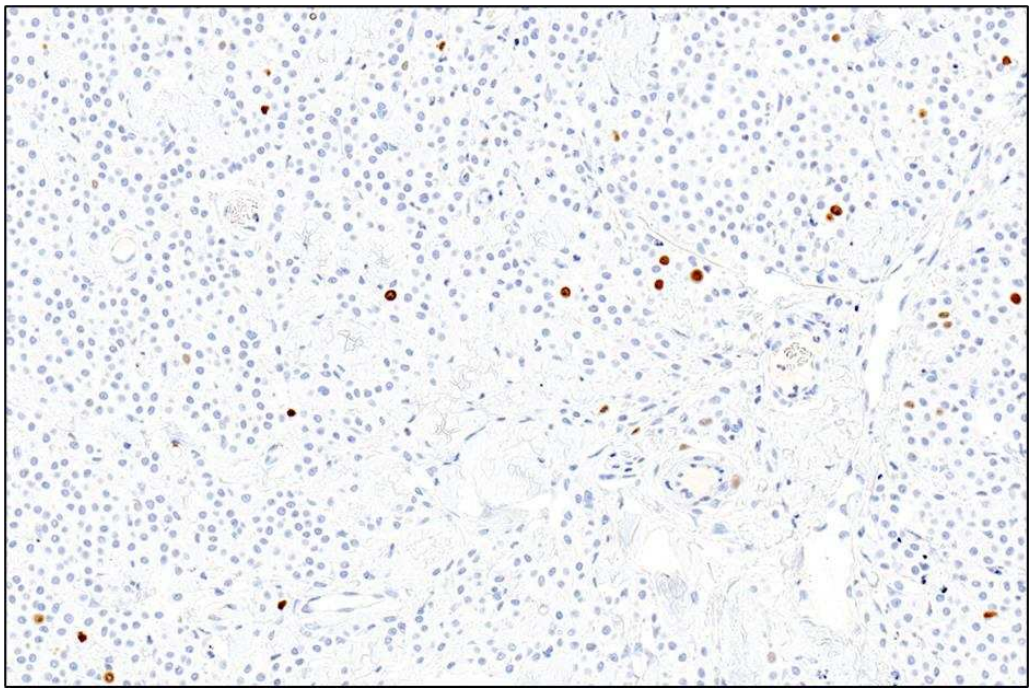


Figure 2 Photomicrograph showing the canine MCT. Ki67 positive nuclei are marked in brown.



The Ki67 index was calculated according to the method proposed by Vascellari et al., reviewing photographs of five randomly selected HPFs (400×) on which the positive cells on a total of 500 cells using ImageJ Image software version 1.53 were counted (Figure 2). For each sample, the Ki67 index was expressed as the average number of Ki67 immunostained cells per 100 cells [111]. Mitotic count was calculated as the absolute number of mitoses in 10 consecutive, nonoverlapping HPF of 0.237 mm² at 40× magnification [109]. Areas of highest mitotic activity were selected, while areas characterized by extensive necrosis and apoptotic or pyknotic nuclei were excluded.

Statistical analysis

Normality of distribution for the numerical variables was assessed with the Shapiro-Wilk test. Continuous variables were expressed as median and range in case of non-normal distribution, and as mean ± standard deviation (SD) in case of normal distribution.

Frequencies are reported for categorical variables. Spearman's correlation coefficient was used to assess potential correlation between Ki67 index and MC.

The date of surgery was used as an entry point for the calculation of the time to local recurrence (TLR), the time to nodal relapse (TNR), the time to distant relapse (TDR) and the survival time (ST), disease-free interval (DFI).

Only dogs deceased of cMCT-related causes were considered as events, dogs without recurrence or disease progression at the date of the last visit, end of the study or death were censored.

Statistical analysis was performed using a commercially available statistical software package (MedCalc Statistical Software version 19.5.1; Ostend, Belgium).

Results

A total of 48 cases were identified. One case was excluded because it was a recurrence, 7 cases were removed from the study due to the histological re-classification of subcutaneous instead of cutaneous cMCT at the histological review; H&E-stained slides and archival tissue blocks were not available for the revision in one case and it was excluded. Finally, 39 cases fulfilled the inclusion criteria.

The study population consisted of 5 (12.8%) castrated males, 14 (35.9%) sexually intact males, 16 (41%) spayed females and 4 (10.3%) sexually intact females, with a mean age of 7.6±2.4 years at the time of diagnosis. The mean weight was 29.5±11.2 Kg. The most represented breeds were Boxer (n=6, 15.4%), Labrador retriever (n=6, 15.4%), mixed breed (n=5, 12.8%), English setter (n=4, 10.3%) and Golden retriever (n=3, 7.7%). Twelve (30.8%) other breeds were represented with 2 dogs each (Beagle, Shar-pei and Weimaraner) and 1 dog each (French bulldog, Appenzeller Mountain dog, Dogo Argentino, Siberian husky, Alaskan malamute, Maltese, Pinscher, Pitbull, Tosa-Inu).

The mean size of the cMCTs was 20.8 mm (SD=11.1); 4 (10.3%) cMCTs were ulcerated. Fourteen (36%) cMCTs were located on the limbs, 11 (28.2%) on the trunk, 7 (18%) on the inguinal region, 4 (10.3%) on the head and neck and 3 (7.7%) on the digital region.

The RLN was clinically enlarged in 15.4% (n=6) of dogs and was normal-sized in 84.6% (n=33) of dogs.

Preoperative SLN mapping and excision were performed in 33 (84.6%) cases. In the remaining 6 (15.4%) cases, a regional lymphadenectomy without preoperative mapping was performed. Sentinel lymph node mapping and extirpation were guided by radiopharmaceutical and methylene blue in 20 (60.6%) cases, in 1 (3%) case the radiopharmaceutical alone was used.

Twelve cases (36.4%) underwent peritumoral injection of aqueous contrast medium and indirect computed tomography lymphangiography to detect SLNs. In 4 of these, peritumoral methylene blue dye was injected to guide lymphadenectomy. Information regarding surgical margins was available for all dogs. In 32 cases (82.1%) cMCTs were completely excised, in 1 (2.6%) case one of the margins was narrow and in 6 cases (15.4%) cMCTs were not completely excised. None of the dogs with narrow or infiltrated margins received further treatment.

The overall median follow-up was 750 days (104-2241 days). During this time, none of the 39 dogs developed local or nodal relapse or metastatic distant disease; therefore, time to local relapse (TLR), time to nodal relapse (TNR) and time to distant relapse (TDR) could not be calculated and mean ST was used as goal for the statistical analysis.

Seven dogs developed one de novo MCT at other locations at 1550, 1307, 857, 804, 473, 428 and 152 days from the primary surgery, respectively; of these, 3 dogs were surgically treated after a new staging and were still alive at the end of the study after 1867, 1781 and 663 days; two dogs died due to splenic hemangiosarcoma and metastatic soft tissue sarcoma 174 and 1003 days after the second surgery, respectively. In two cases, the owners elected against further staging and treatment and these dogs were still alive at the of the study, after 1007 and 1249 days from the first surgery.

At the end of the study, 82% (n=32) of dogs were alive and 18% (n=7) of dogs were dead from unrelated MCT causes. These dogs underwent euthanasia at 1807, 1674, 789, 524, 484, 120 and 104, days for metastatic soft-tissue sarcoma (n=1), metastatic osteosarcoma (n=1), splenic hemangiosarcoma (n=3), right atrial hemangiosarcoma (n=1) and pulmonic stenosis (n=1).

Four dogs (10.2%) were lost to follow-up at 2241, 596, 200 and 180 days. All four dogs were alive, and none had local or nodal relapse or metastatic distant disease at the time of the last contact. The median ST of the entire population was not reached, the mean ST was 893 days (range 104-2241 days, SD±527.5), the mean ST excluding dogs lost to follow-up (n=4) and dogs that died of causes unrelated to the MCT (n=7) was 903 days.

Histopathology

Median MC was 1 (0-2). Median Ki67 index was 3.5 (0.7-14.3). Ki67 index and MC were not significantly correlated (r= -0.05; p=0.76). Due to the lack of events, statistical investigations relating to Ki67 index and outcome were precluded.

Results are summarized in Table 5.

Table 5 Patients' demographics and histological details. Abbreviation: F, female intact; FS, female spayed; M, male intact; MN, male neutered; y, years; MCT, mast cell tumour; MC, mitotic count; Y, yes; N, no; Lfu, lost to follow-up; OSA, Osteosarcoma. All dead dogs died from causes not related to the cMCT.

Case	Signalment	MCT location	Ki67%	MC	Margins	Status	Cause of death	ST (days)
1	Labrador retriever, FS, 8 y	limb	2.5	0	clean	dead	Soft tissue sarcoma	1807
2	Golden retriever, FS, 7 y	limb	1.0	1	clean	alive	-	1931
3	Appenzeller Mountain dog, F, 4 y	limb	1.8	0	clean	alive	-	1867
4	Labrador retriever, MN, 9 y	trunk	1.7	1	clean	dead	OSA	789
5	Boxer, M, 8 y	limb	1.5	1	clean	dead	Splenic hemangiosarcoma	1674
6	Weimaraner, MN, 7 y	limb	3.5	1	clean	alive	-	1781
7	Labrador retriever, FS, 6 y	trunk	0.7	1	clean	alive	-	1756
8	Pitbull, M, 3 y	digit	4.6	1	clean	alive	-	1306
9	English setter, M, 8 y	trunk	1.3	0	clean	alive	-	1249
10	English setter, FS, 11 y	trunk	2.0	0	clean	alive	-	1188
11	Maltese, F, 7 y	head and neck	2.3	0	dirty	alive	-	885
12	Tosa Inu, M, 9 y	inguinal	4.1	0	clean	alive	-	801
13	Dogo Argentino	limb	6.7	1	clean	alive	-	760
14	Mixed- breed, FS, 8 y	limb	5.5	1	clean	alive	-	720
15	Golden retriever, FS, 9 y	limb	3.4	0	clean	dead	splenic hemangiosarcoma	484
16	Mixed-breed, FS, 9 y	trunk	5.9	1	clean	alive	-	690
17	Husky, MN, 9 y	inguinal	6.1	0	clean	alive	-	678
18	Boxer, MN, 8 y	limb	8.7	0	dirty	alive	-	663
19	Labrador retriever, FS, 4.5 y	inguinal	8.8	0	clean	alive	-	613
20	Boxer, M, 8 y	head and neck	11.5	0	clean	dead	Right Atrial hemangiosarcoma	104
21	English setter, FS, 5 y	trunk	14.3	0	clean	alive	-	557
22	Shar-pei, FS, 6 y	limb	9.1	1	dirty	Lfu	-	2241
23	French bulldog, MN, 11 y	digit	5.2	0	dirty	dead	Heart failure (by pulmonic stenosis)	120
24	Shar-pei, F, 10 y	head and neck	1.9	1	dirty	Lfu	-	596
25	Mixed-breed, FS, 11 y	limb	8.4	1	clean	Lfu	-	200
26	Beagle, FS, 13 y	inguinal	3.0	1	dirty	Lfu	-	180
27	Pinscher, M, 5 y	inguinal	2	1	clean	alive	-	935
28	Beagle, M, 10 y	inguinal	3.9	2	clean	alive	-	882

29	Boxer, F, 7 y	limb	2.8	0	clean	alive	-	588
30	Golden retriever, M, 7 y	trunk	1.9	0	clean	alive	-	578
31	Labrador retriever, M, 11 y	head and neck	2.9	0	clean	alive	-	1007
32	Mixed-breed, FS, 9 y	limb	7.4	1	clean	dead	Splenic hemangiosarcoma	524
33	Boxer, FS, 5 y	trunk	8.1	2	clean	alive	-	760
34	Alaskan malamute, M, 5 y	trunk	6.2	1	clean	alive	-	792
35	Labrador retriever, M, 3.5 y	trunk	7	0	clean	alive	-	750
36	English setter, M, 10.5 y	inguinal	3.2	0	clean	alive	-	728
37	Mixed-breed, M, 8 y	digit	4.3	2	clean but close	alive	-	539
38	Boxer, M, 6 y	limb	5.6	1	clean	alive	-	544
39	Weimaraner, FS, 7 y	trunk	3.5	0	clean	alive	-	553

Discussion

MCT is a multifaceted disease which may sometimes behave in an unexpectedly aggressive way, despite the absence of negative clinical factors. Therefore, full staging should be advised in many cases, and the relative costs can be relevant to the owner. A better understanding of the prognosticators may help the clinician in deciding when to ask for a more complete staging. To answer this question, the primary aim of this study was to assess the prognostic significance of Ki67 index in a homogeneous cohort of dogs affected by MCTs and to describe the clinical outcome of this population. To do so, the survival time in a group of dogs with G2/LG cMCTs with HN2 LNs treated with surgery alone, was retrospectively analysed.

To the best of the authors' knowledge, this is the first study in which Ki67 index is investigated in cMCTs with the same histologic grade and the same pattern of LN involvement. The impetus for this study was based on the lack of a unique Ki67 cut-off index useful to help the clinician in defining the prognosis for dogs with this subcategory of cMCTs, despite the large variety of Ki67 cut-offs proposed. This group of cMCTs represents a "grey zone", where the risk of over- or undertreating may be real. A suggestion of the possible benefit of chemotherapy treatments in dogs with HN2 LNs was based on Weishaar's study [7]. Nevertheless, in that study, no difference in disease-free interval and survival time was observed between HN categories. Differences became statistically significant when HN2 cases were merged with HN3. Furthermore, only 6 HN2 nodes were included, only Patnaik grade was considered, and Ki67 index was not reported [7]. In addition, information about the presence of lymph node enlargement, and the exclusion of visceral metastasis, based on spleen and liver cytology was not available [7]. Moreover, even if no survival benefit has been reported in dogs treated with adjuvant systemic chemotherapy after surgical excision of G1-2/LG with HN2 LNs [5], a subset of these cases may have features of high-risk malignancy, such as anatomic location, higher MC and/or Ki67 index, and incomplete excision. In these cases, it could be important to have further standardized parameters to determine which dogs require close monitoring or adjuvant chemotherapy treatment. In addition, the recent Consensus of the Oncology-Pathology Working Group suggested using not only histologic grade but also other clinical and histological markers, such as prognostic factors, since a subset of Kiupel's LG cMCTs could behave aggressively [1].

In the authors' experience, dogs with G2/LG with HN2 LNs, which were not receiving adjuvant chemotherapy for various reasons, had a favourable prognosis. This led to the proposal of this retrospective study in which only dogs affected by low-grade MCTs and early metastatic LNs were included.

Different cut-offs of Ki67 index have been proposed by previous studies [44–52,108,111], but they were assessed considering cMCTs of any histological grade in heterogeneous canine populations; moreover, LNs metastatic status has never been considered. Consequently, the usefulness of this parameter is still not completely clear, at least for low-grade cMCTs.

The results of the present study report that all included dogs treated with surgery of the primary tumour and regional or sentinel lymphadenectomy without neoadjuvant or adjuvant chemotherapy had a good prognosis, regardless of the completeness of the excision, highlighting the low aggressive behaviour of this subcategory, as already reported in the literature [24]. In fact, no dogs developed local and/or nodal relapse, or distant metastasis, and no one died from MCT-related disease. This result may be due to the strict inclusion criteria adopted to exclude possible biases, which may flaw a correct interpretation of the outcome, but it also confirms the behaviour of this subgroup of MCTs. Moreover, the results suggest that Ki67 evaluation in this subset of cMCTs may not be worthwhile, contrasting the suggestion of the Consensus of the Oncology-Pathology Working Group [1].

In this study, the counting method described by Vascellari et al. was applied [111]. According to that study, Ki67 counts ≥ 10.6 were significantly associated with an increased incidence of cMCT-related mortality. The results of the present study mostly support this finding; in fact, the median Ki67 index was relatively low (3.5%), although two of the evaluated tumours expressed a Ki67 index greater than 10.6; anyway, no dogs died due to the cMCT.

In the study of Maglennon et al. (2008), the Ki67 cut-off value of 1.8% proposed by Scase et al. (2006) was confirmed to be associated with a worse prognosis for dogs with G2 cMCTs [48,50]. Also, Berlato et al. (2015) showed that dogs with G2 MCTs, MC> 5 and Ki67> 1.8% had a significantly higher risk of dying from causes related to the cMCTs [45]. In that study, conducted on 49 dogs, Ki67 index was calculated with the same methodology described by Maglennon et al. (2008) and Scase et al. (2006) [45,48,50]. Both MC and Ki67 index were identically useful for distinguishing high-risk from low-risk MCTs, although the probability of dying of MCT was higher for dogs with

increased MC (HR: 15.4 [4.2–56.9]) compared to dogs with increased Ki67 index (HR: 9.8 [2.7–35.7]) [45]. The authors suggested that these markers could be practical to understand which G2 cMCT could benefit from more aggressive treatment, but they did not recommend evaluating proliferation indexes in case of LNs metastases, since these cases have already a worse outcome. Anyway, the Weishaar classification was not yet published and the prognostic role of LNs status was not clarified [45]. In 2018, the same group of authors published a retrospective study on 90 G2 cMCTs, applying the Kiupel classification system, including 82 LG and 8 HG. They reported that 7 of 8 (87%) dogs with HG/MC > 5 died from causes related to the MCT, but also 11 of the 82 (13%) dogs with LG/MC ≤ 5 died. Thus, to improve the sensitivity in finding aggressive disease in this subset of dogs, they split all LG tumours with MC≤5 into two groups, based on the Ki67 index, and they found a higher risk of dying from MCT in dogs with Ki67>1.8% [44]. Anyway, the LNs status was not known, consequently any comparison with our study cannot be made. The data on Ki67 in the population observed in the present study are in contrast with what was reported above [44,45,48,50] since the median Ki67 index was 3.5%, but no negative events were observed.

In the study by Necova et al. (2021), the authors hypothesized that dogs with G2, MI ≤5 and Ki67 index >1.8%, due to the reported high risk of MCT-related death, could benefit from adjuvant lomustine treatment and they found higher survival time compared to previously cited articles. Nevertheless, due to the lack of a control group to assess the benefit of lomustine administration, the regional LNs status assessed only by palpation and ultrasound, and the lack of Kiupel grading system, the authors could not confirm the benefit of chemotherapy [108].

In the present study, a statistical association between MC and Ki67 index was not found. Although it is known that these two parameters may have a nonlinear association, low MI and high Ki67 could be explained by the fact that the Ki67 protein indicates the percentage of cells in the cell cycle and denotes the cells with the capacity to divide. These cells might also stop reproducing or enter apoptosis without going through mitosis [114,115]. Another possible hypothesis of the lack of correlation between the percentage of cells expressing Ki67 and the number of mitoses per field, could be linked to the difference in the choice of the microscopic fields: MC is calculated in the area of the highest mitotic activity (10 consecutive HPF chosen in this area), whereas for Ki67, the HPF fields (5) are selected at random, and not in the areas of

greatest mitotic activity, even though this is indeed the technique described by Vascellari et al., 2013 [111].

The absence of relapse (local, nodal or distant) and the good outcome observed in the study population could be due to the selection of a subset of dogs with low-risk cMCTs (G2/LG). Anyway, since the studies mentioned above [44,45,48,49] did not take into consideration the LNs status, it is not possible to exclude that some of those dogs with G2 tumours actually had metastatic LNs, and their shorter survival could have been due to the undetected nodal metastatic disease, rather than to the high Ki67 index. Moreover, it is reasonable to think that in these studies the Ki67 index was significantly correlated with prognosis because both LG and HG tumours were included in the statistical analysis. This means that, probably, higher Ki67 indexes were found in G2/HG, and lower Ki67 indexes were related to G2/LG [44,45,48,49]. In addition, the present study included dogs in which the regional or sentinel LNs were always removed, and this surgical approach has been recently reported to have a therapeutic impact [5,24]. A study comparing the outcome of dogs with G2/LG MCT in which the regional/sentinel LNs are extirpated or not could help define this impact.

In this study population, 6 cases had infiltrated excision margins, but no adjuvant treatments (re-excision, chemotherapy, radiotherapy) were undertaken; anyway, local recurrence was not detected during the follow-up time. These results are similar to those previously presented by Smith et al. (2017), that reported a low probability of recurrence in G2 MCTs with low Ki67 index (≤23 Ki67-positive cells/grid), even if the threshold adopted was different from the median value (3.5%) observed in the current study [51].

There are some limitations to this study. The lack of events in the study population may have created a bias in the prognostic information, precluding the possibility of adequately evaluating the oncologic outcome of the HN2 LNs category, as well as the role of Ki67 index. Nonetheless, the absence of relapse (local, nodal, or distant) is not a controllable feature, but these results confirm previous studies in which lymphadenectomy showed a therapeutic impact in LG cMCTs with early metastasis [24], also in the absence of adjuvant chemotherapy [5].

Owing to its retrospective nature, survival time was the only measure of outcome in these cases; since none of the included dogs had a nodal or distant relapse, and none of them died from causes related to the cMCT, a median disease-free interval was not reached. On the other hand, despite the retrospective nature, the concordance in the

preoperative staging and postoperative treatment among the 3 institutions, the long median follow-up time, and the homogeneity of enrolled cases may provide for better reliability and significance of these results and may represent a point of strength that should be supported by a larger prospective study.

In conclusion, notwithstanding that the prognosis for cMCTs should be determined by the clinician based on several clinical and histological features, Ki67 index does not have prognostic impact in G2/LG cMCTs with HN2 LNs. Considering the strict inclusion criteria, which represent the strength of this study, dogs affected by low-risk cMCTs (G2/LG) and early metastatic (HN2) lymph nodes treated with curative intent surgery alone may have a good oncologic outcome, and the clinical oncologist may not need to ask for further immunohistochemical evaluation (i.e., proliferation parameters) for prognostication. Anyway, further prospective studies with a longer follow-up and a larger cohort of dogs are required to confirm these findings.

3 The role of lymph node in the staging of mast cell tumours

3.1 Background: sentinel lymph node concept and history of sentinel lymph node mapping in human medicine

In the 19th century, Virchow recognized the role of LNs as filter of lymph and as a barrier to cancer cells. At the same time, Halstead showed that radical mastectomy with *en bloc* axillary lymphadenectomy improved the outcome in 33% of women with breast cancer [13,14,17,19,116–118]. Halsted introduced the concept of "radical surgery" and nonselective lymphadenectomy, which became the standard of care for different epithelial tumours for several years. The inevitable consequence of the resection of all the lymph nodes surrounding the tumour was the increase in morbidity that impacted the quality of life [13,17,116,117].

The term 'sentinel node' (SLN) was coined by Gould in 1960 [14,17,117,119]. He suggested that determining the SLN status for metastatic disease could help in establishing whether a patient needed a radical lymphadenectomy or not [119]. In 1977, Cabanas described for the first time the "SLN biopsy" in patients with penile carcinoma [14,16,17,120]. He published a 100-patient lymphangiogram study resecting only the first lymph node that drained a tumour to determine its metastatic status. Only in case of a positive node, radical lymphadenectomy was recommended. In that study, SLN biopsy was performed without any intraoperative guidance but only using the anatomic landmark [120]. Notwithstanding, the SLN concept proposed by Gould and Cabanas did not consider the variation of lymphatic drainage between patients [119,120].

Despite these revolutionary results, it took more than 40 years for the SLN biopsy to become the standard of care in human oncology.

The intraoperative guided lymphadenectomy was first described by Morton and coworkers in 1992 [14,17,117,118]. In 1993, for the first time, two studies described the use of radioisotope (technetium-99m sulfur colloid) and a hand-held gamma probe to guide the SLN dissection [14,16,117,121,122].

In 1997 Veronesi and colleagues described the preoperative and intraoperative use of lymphoscintigraphy in 163 women with breast cancers, introducing the concept of the "predictive value" of the SLN. They found that in 97.5% of cases, the SLN status

predicted axillary node involvement, thus they concluded that radical axillary dissection was unnecessary for patients with negative sentinel nodes, avoiding the high rate of complications following radical lymphadenectomy in women [18].

3.2 Why is mapping important in veterinary patients?

The lymphatic system plays a pivotal role in the spread of MCTs. MCT has a predilection for the lymphatic route of dissemination, and the LN represents the first station of metastases. Distant metastasis without prior dissemination via the lymphatic vessels has never been reported [20]. Because of this characteristic, MCT represents an ideal model for the development of the sentinel lymph node concept in veterinary medicine.

Even if the metastatic status of regional LNs has been mentioned as part of the clinical stage since 1980, its prognostic importance has consolidated only in the last ten years [87].

The European consensus document published in 2012 suggested to assess the "draining" lymph node status by palpation and cytological sampling [57]. Nevertheless, which LNs must be assessed was not clearly defined, since they were alternatively named as "regional", "draining", or "local". Moreover, this consensus highlighted the difficulty in diagnosing metastatic LNs both cytologically and histologically, due to the lack of standardized criteria [57]. Two studies proposed diagnostic criteria for both cytologic and histologic assessment, respectively [3,7]. Weishaar's and Kirck's classifications are two important steps toward the standardization of nodal assessment in MCT, but the limits between classes are narrow thus further studies are needed to better clarify the proposed criteria.

With the observation that the SLN were not the expected nearest LNs in 42% of MCT, Worley pioneered the "mapping era" in veterinary medicine [9]. The author aimed to adopt the same technique to determine the SLN, and to reach the same clinical benefits from the SLN mapping and elective lymphadenectomy in veterinary medicine as used in human medicine. In fact, in human medicine, complications associated with lymphadenectomy have been extensively evaluated and described [123–127]. These complications could have a severe impact on the quality of life of human patients. The sentinel lymphadenectomy avoids morbidity, especially in patients without nodal metastases.

Since the publication of the milestone study of Worley, a part of the veterinary oncologists has started to systematically incorporate LN mapping in the staging of MCT [9]. On the other hand, the utility of mapping and lymphadenectomy has been questioned. The debate about SLN and lymphadenectomy is still ongoing. The main concerns are about the real advantages of lymphadenectomy for survival, especially in case of cytologically negative LNs or in case of clinically benign tumours (i.e. low- grade MCT or grade I soft tissue sarcoma). Another concern is that lymphadenectomy of small LNs, especially in some locations as the thoracic and abdominal cavities or the axillary region, could be challenging, adding surgical time, costs, and morbidity (lymphedema, seroma, haemorrhage) [30]. In worst cases, surgery is terminated without finding the SLN. Another reason of scepticism is the variability of the current literature. Indeed, the definition of RLN is different between articles, some authors called RLN “the nearest” lymph node to the tumour, while others referred to the Suami description of lymphosomes, increasing the variability of non-correspondence rate [15,26,27,30]. According to recent studies on MCT with different mapping techniques, a discrepancy of 27–63% between RLN and SLN using different mapping techniques has been reported [9,15,26,30,128].

On the other hand, because of the paramount importance of accurate nodal staging, the choice of which LN should be removed is required to avoid under-staging of the disease [15]. In 2020, Ferrari et al. reported that SLNs differed from RLNs in 19/30 tumours, and 32/57 LNs were histologically classified as HN2 and HN3, highlighting the low accuracy of palpation to identify metastatic LNs [22]. Individual and anatomical differences among animals of different breeds have not been studied yet, and only healthy dogs have been used in studies on the lymphatic system. Moreover, tumour-induced lymphangiogenesis creating new connections with unexpected lymphatic basins has been described. Cancerous and inflammatory cells of the tumour microenvironment release factors, such as vascular endothelial growth factor (VEGF) C and D, which promote lymphatics remodelling and lymphangiogenesis [129,130]. In 2003, Pereira et al. proved this theory comparing the lymphatic drainage of healthy and neoplastic mammary glands in 46 female dogs. A larger number of lymphatic anastomoses was found in neoplastic mammary glands than healthy glands, concluding that the regional lymphatic drainage could change during tumorigenesis [131].

An important update on the knowledge of the lymphatic system was provided by Suami et al., that described the lymphatic territories distribution in dogs and cats [110,132,132–

134]. The authors identified 10 lymphatic basins in the canine body: submandibular, parotid, dorsal superficial cervical, axillary, medial iliac, lateral sacral, hypogastric, popliteal, superficial inguinal, and ventral superficial cervical. Interestingly, the authors also described the presence of perforating lymph vessels in the lumbar and gluteal regions that could partially explain why some tumours drained directly into the abdominal cavity. These vessels originated from the skin, penetrated the abdominal wall, and drained into paraaortic LNs instead of the axillary and inguinal ones [132]. Clinicians should take into consideration these perforating vessels when assessing for SLN identification.

The authors also found some similarities with the human lymphatic system: the presence of both a deep and superficial system, the number and diameter of lymphatic vessels, the presence of valves and the absence of vessels crossing the midline [110]. The main difference between human being and dogs is the lymphatic system of the forelimb. In fact, in human beings the axillary lymphocenter is the dominant pathway, while the canine lymphatic system included 3 pathways: the dominant pathway to the ventral cervical node, the residual superficial pathway to the axillary node, and the deep pathway to the same axillary node [135].

This lymphatic topography, although helping in identifying the most probable draining way, also highlighted how the edges of these lymphatic regions are not so clearly distinguishable in the body surface. Thus, in some cases, especially when tumours are located in the “transition” zone, the identification of the RLN could be challenging.

Based on the paper of Suami et al., the lymphatic vessels of the obstructed area connected to the LNs in an adjacent region within 3 weeks following lymphadenectomy [134]. These collaterals probably act as bypasses to prevent the lymphoedema, but they could also operate as new metastatic pathways in case of incomplete tumour resection [22].

Sentinel lymph node mapping is considered useful to avoid unnecessary and extensive lymphadenectomy, minimizing surgical time, tissue trauma and dissection, complications and post operative morbidity. Moreover, some studies provided evidence that LNs extirpation may improve outcome in MCTs [75,136–138]. The results of the meta-analysis conducted by Bae et al., on low-grade cMCTs with either RLN or distant metastasis (so called histologically low-grade, yet biologically high-grade) demonstrated that lymphadenectomy confers a survival advantage (median 637 vs 247 days) [65]. Nowadays, information about the long-term impact of SLN resection on the outcome of

patients affected by MCT are lacking, thus future studies evaluating the impact of SLN lymphadenectomy on the outcome are necessary.

3.3 Sentinel lymph node mapping techniques

Different techniques of SLN mapping have been described in veterinary medicine [9,12,22,30,36,43,139–141]. Imaging techniques vary widely, from cheaper and more accessible methods (i.e. indirect radiographic lymphography [IRL], indirect computed tomographic lymphography [ICTL], contrast-enhanced ultrasound [CEUS], injection of blue dyes), to more expensive and sophisticated methods (pre- and intraoperative lymphoscintigraphy [LS], and the fluorescent dyes for near-infrared [NIRF]).

The rationale behind all the techniques is the peritoumorally injection of a tracer which is drained by lymphatic vessels to the first lymphatic station, making it readily visualized. The type of tracer differs from each technique.

The choice of the technique is based on the institutional availability and owner financial concerns. As in human medicine, lymphoscintigraphy is considered the gold standard. However, due to cost and legal restrictions (i.e. special authorization for the management of technetium is required), lymphoscintigraphy is only available in few highly specialised veterinary centres. Different alternatives are currently being evaluated, of which IRL and ICTL are the most employed. Based on the published results, it is recommended to combine a preoperative to an intraoperative mapping technique to optimize the accuracy for SLN detection and the ease in the surgical extirpation [12,28,141].

The first preliminary study on SLN mapping dates to 2002, when Balogh and coworkers described the preoperative use of human serum albumin colloid labelled with ^{99m}Tc and intraoperative use of both patent blue solution and gamma probe on 24 client-owned dogs with different tumours [12]. The authors focused more on the description of the technique rather than on the agreement between RLN and SLN detection. They reported a total of 35 SLNs containing metastases at histological examination, 77% (n=27) of which were blue-stained and 97% (34) were detected by gamma probe, although only 89% (n=31) were also identified by preoperative scintigraphy.

Only after the unexpected data published by Deanne Worley in 2014, the “mapping era” has officially begun [9]. However, due to its explorative nature, Worley's study has

several limitations: the absence of reported clinical status (if enlarged or not) of the RLN, the absence of Patnaik grade-1 tumours, the histological slides were not review and graded according to the Kiupel system, and Weishaar classification for nodal metastases was not available yet [7,11]. This could have led to a less objective identification of nodal metastasis and to the probability of an overtreatment of some of the enrolled dogs, as the author reported.

3.3.1 Pre- and intraoperative lymphoscintigraphy

This method is considered as the gold standard in human and veterinary medicine due to the high detection rate (90-100%) [9,22]. This technique consists in the peritumoral injection of a variable dose of a radionuclide (technetium-99 labelled nano-sized human serum albumin) in four sites under general anaesthesia. The use of preoperative planar imaging and/or intraoperative portable gamma camera guides the surgeon to the radiolabelled “hot” SLN and in the dissection of soft tissues around the SLN. Generally, the gamma probe is used in combination with a vital dye as direct visual guidance. Even if this technique has shown the highest agreement rate, it seems unreasonable to recommend only scintigraphy to identify SLN, because of the limited number of veterinary centres able to offer this service. In fact, one of the aims of veterinary medicine is to provide less expensive but accurate and safe care to all patients [14]. In addition, lymphoscintigraphy does not offer a high-resolution imaging of the body, thus making the identification of SLN and adjacent nodes difficult.

In 2020, Ferrari et al. applied the same technique described by Worley in 30 MCTs with non-palpable/normal-sized RLN. The authors find a 100% correlation between “hot” and “blue” SLNs [22]. The agreement between RLN and SLN was total in 11/30 tumours, partial in 6/30 and absent in 13/30. The RLN was classified as the closest to the MCT location, as previously done by Worley [9]. Another interesting information provided by this study was that with low-grade MCT the SLNs were overtly metastatic (HN3) in 6 cases. This result proved that also low grade MCTs could metastasize without evident lymph node enlargement.

3.3.2 Indirect Lymphangiography

The term indirect is used to define the deposit of a contrast medium in the periphery of lymphatic vessels. After injection, the contrast is absorbed and drained by the lymphatic vessels and reaches the SLN [28]. Shooting serial radiographs or TC scans after the contrast injection allows the tracking of the contrast medium along the lymphatic draining system to the corresponding LN (SLN).

3.3.3 Radiographic lymphangiography

This technique is non-invasive and easily accessible, since digital radiographs are widely diffused also in small veterinary practices. Also, it requires a short learning curve. The main limits of this technique are that it does not provide intraoperative guidance (as well as ICTL) and, being bidimensional and less accurate than CT, small LNs may not be detected [27,28,142].

In 2017 Brissot and Edery described a protocol to detect SLN with peritumourally or intratumorally injection of iodized oil (IO) as contrast medium for radiographic (R-IL) or computer tomography (CT-IL) lymphography [28]. This study included 30 solid tumours in 29 dogs. Of these, 15 were MCTs and 4 were scars of previously incompletely excised MCTs. A total of 2 to 4 mL of IO were injected over 1-5 minutes and R-IL or CT-IL was performed 24 hours later. They also compared the corresponding detection rate between preoperatively lymphangiography and methylene blue (MB) in the same population. The detection rate was 96.6% (29/30 tumours) for preoperative lymphangiography and 96% (25/26) for intraoperative MB, while the agreement between IL and MB was 84.6% (23/26) [28]. Recently, Annoni and colleagues described the use of preoperative radiographic lymphangiography with iomeprole (a water soluble iodinate contrast agent) in 138 cMCTs [27]. This contrast agent allowed for a more rapid identification of SLN, avoiding the 24 hours delay reported by Brissot and Edery. Thus, in case of failure, the mapping procedure could be repeated. In fact, the SLN was identified at 1 minute post injection in 90% of the cases and at 3 minutes in the remaining 10%. This study reported an overall detection rate of 95%, and the SLN differed from the RLN in 57% of cases [27]. Even if a high success rate has been reported in the aforementioned work, a study conducted on eight intact healthy male Beagles, compared the detection rate of lymphoscintigraphy with radiographic lymphangiography, highlighting differences in SLN detection [142]. Dogs underwent to lymphoscintigraphy first, and to lymphography four days later. The contrast agents were

injected around a cutaneous area of 2cm x 1.5cm on the thoracic limb. Lymphography was performed using 4 ml of 350 mgI/mL of Iohexol. The agreement between the two techniques was complete in 3 dogs (37.5%), partial in 4 dogs (50%) and absent in 1 dog (12.5%) [142]. Based on these findings, SLN identification differs among mapping techniques and clinicians should be considered it before applying this technique.

3.3.4 Computed tomography lymphography

Indirect computed tomography lymphography (ICTL) provided better spatial resolution, high resolution images and efficient identification of lymphatic structures than radiographic lymphography and is now widely available in many veterinary clinics.

Both oil-based and water-soluble contrast agents can be used for CT lymphangiography, but water-soluble agents allow for SLN identification within minutes, compared to hours or days with oil-based agents. The main limits of the technique are the lack of intraoperative guidance, the radiological exposure of the operator and the higher costs compared to the radiographic lymphangiography [26,35,140]. Several studies provided different protocols, with different timing for the scans and dilutions of the contrast agent reaching similar detection results. Thus, the choice of the protocol depends on the preference of the clinician [26,32,33,35,140].

In 2017, Grimes and colleagues described the use of ICTL in 18 dogs with different head and neck tumours, comparing two different doses of injected iohexol. One ml of contrast was injected in 9 dogs, and 2 ml in other 9 dogs. Scans were performed every minute after contrast injection until the SLN was identified. The detection rate was 89% and median time to identification was 0 minutes. SLN was identified in all dogs receiving 2 ml, and in 7 dogs that received 1 ml. Despite this result, the authors recommend the use of 1 ml of contrast because larger volume could be difficult to inject in difficult areas, such as the gingiva or palate. Interestingly, all but one neoplasia drained to the expected mandibular RLN. Moreover, in one case the SLN was the contralateral, while in one dog SLNs were both ipsilateral and contralateral [33].

In a recent work published by Randall and coworkers the intratumoral injection of 0.75 mL of iohexol (350 mgL/mL) was compared with the peritumoral injection of 0.25 mL of the contrast in 20 dogs with head and neck tumours. The results showed that the peritumoral injection provided a better success rate than the intratumoral injection [140]. Moreover, ICTL was compared to lymphoscintigraphy and blue dye injection to determine which technique more reliably identified the SLN. This study provided the

evidence that lymphoscintigraphy is the gold standard technique, since it identified SLNs in 100% of cases, compared with the 94.4% obtained by the blue dye injection [140].

3.3.5 Methylene blue dye (MB)

This intraoperative technique does not require specialized equipment and helps the surgeon to better visualize the blue-stained lymphatic vessels and/or the lymph nodes. MB could not be technically defined a mapping tracer like indocyanine green, since it does not allow the visualization of lymph nodes and lymphatic vessels through the intact skin, except in very thin dogs (Figure 3). Another disadvantage is that MB could stain the surgical gloves, the instruments, the skin and the peritumoral tissues, making difficult the correct identification of the margins around the tumour (Figure 4) [38].
Several studies showed that the detection rate improved to 100% when MB is combined with preoperative lymphoscintigraphy and/or indirect lymphangiography [22,27,31,140,141]. Thus MB can be considered a good complimentary technique to preoperative LN mapping, but it has a minor efficacy when used alone compared, with combined techniques [31,141].

Figure 3 Picture showing blue-stained lymphatic vessels from MCT in the right flank fold to the right axillary lymph node.

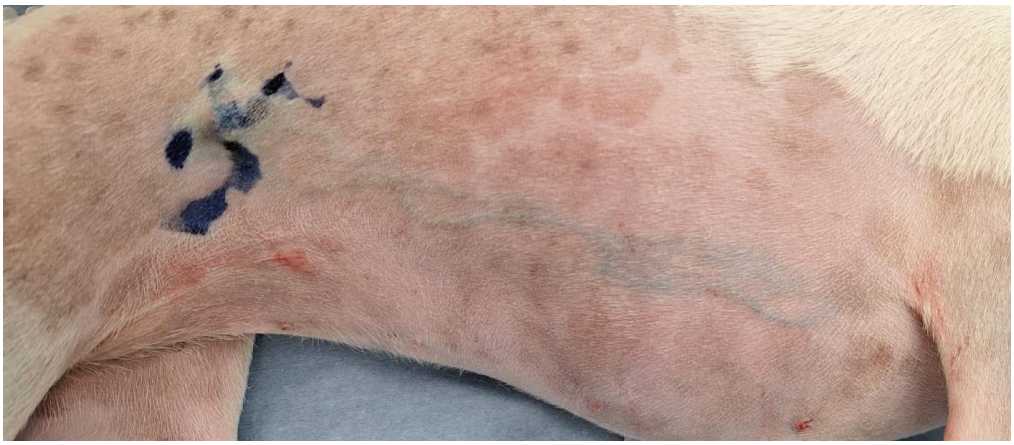
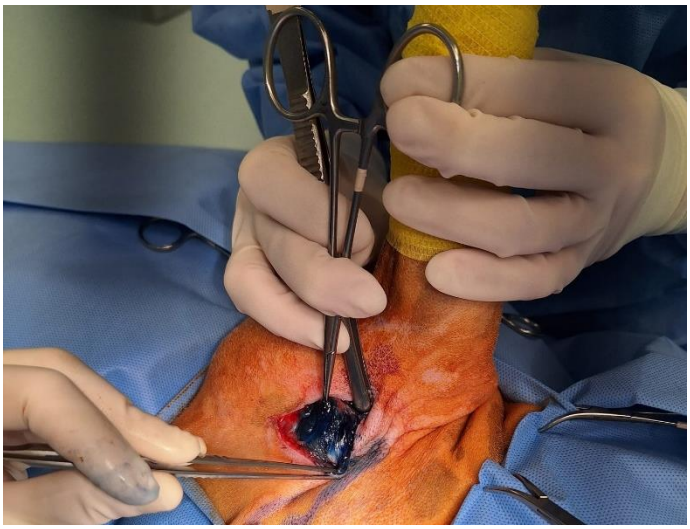


Figure 4 Picture showing Methylene Blue staining the surgical field.



Project 2: Use of indirect computed tomography lymphangiography and methylene blue dye to identify sentinel lymph nodes in 61 canine mast cell tumours.

The primary aim of this study was to determine the rate of agreement between sentinel lymph node (SLN) and regional lymph node (RLN) detected preoperatively with indirect computer tomography lymphangiography (ICTL) and intraoperatively with methylene blue dye (MB) in dogs with MCTs.

According to the previous published studies, the non-concordance rate is largely variable, ranging from 22.3% to 63% [21,26,27,37,143]. Based on our clinical experience, we hypothesized that the rate of agreement between RLN and SLN is higher than 50% with both techniques when RLN is identified according to Suami *et al.* (2013). Moreover, we speculated that the disagreement between RLN and SLN depends on the anatomical location of the tumour. We expected a higher rate of disagreement in the head and neck region where the lymphatic drainage is more complex than the thoracic region.

The second aim was to describe the rate of agreement between the technique of preoperative mapping and intraoperative colorimetric mapping, hypothesizing a level of agreement higher than 80%. The main emphasis of published articles has been on the disagreement between RLN and SLN, but few of them have investigated the factors of failure of the mapping [144].

The third aim was to describe all the possible causes of SLN detection failure during preoperative indirect lymphangiography with aqueous contrast medium injection. We theorized that the presence of greatly enlarged lymph nodes (megalic), high grade of the MCT, and MCT greater than 3 cm may be associated with the failure of the SLN detection with ICTL.

Material and Methods

Study Population

Client-owned dogs with cytological or histological diagnosis of MCT, presented to the Veterinary Teaching Hospital of the University of Parma from November 2020 to December 2023 were prospectively enrolled in the study. All owners signed an informed consent form to all the preoperative and intraoperative procedures and to use data and excised tissues for scientific purposes. All the procedures were performed following Italian guidelines for animal welfare.

Dogs with cutaneous (cMCT), subcutaneous (scMCT) and mucosal MCT (mMCT), both untreated and previously excised that experienced recurrence, dogs treated with neoadjuvant chemotherapy after diagnosis of MCT and preoperative identification of SLN(s) were included. Patients with multiple MCTs were excluded.

The RLN was predicted based on the lymphosomes' concept developed by Suami *et al.* (2013) and recorded [110]. The sentinel node was defined as the first lymph node in the lymphatic pathway to uptake contrast/blue stain.

In case of biologically aggressive tumours, ultrasound-guided fine-needle aspirates of the spleen and liver were performed during the same anaesthetic episode of preoperative ICTL. Sternal lymphadenopathy was evaluated by thoracic TC scan.

For each enrolled dog the following data were collected: breed, sex, age, body weight, type of MCT (cutaneous, mucosal, subcutaneous), tumour size (maximum gross diameter in cm), method of diagnosis (biopsy/cytology), presence of ulceration, speed of growth (fast, slow), anatomic location and laterality of MCT, if the RLN were non-palpable, palpable, or megalic based on physical examination and diagnostic imaging findings. The RLN was defined as palpable in case of asymmetry in comparison with the contralateral, while LN were defined as megalic in case of marked increase in the physiologic diameter. The results of spleen and liver cytology, histopathologic grade of MCTs (including Patnaik and Kiupel grading for cMCTs), mitotic count (MC), histopathologic evaluation of surgical margins (complete, clean but close [tumour cells extending within 2 mm of at least one margin], incomplete) and of the SLN (according to Weishaar); neo- and adjuvant medical treatments, when performed, death for any cause and MCT related death were also recorded [11,99,145].

Adjuvant chemotherapy was always proposed in case of:

- HN3 SLN, independently of histological grade of the primary tumour

-mucosal MCT, independently of the histological status of SLN
-high grade cMCT (both G3/HG and G2/HG), independently of histological status of SLN as recommended [5,10,74,80,137].
Follow-up information by abdominal ultrasound and clinical examination was collected every 3 months for the first year, then every 6 months for the second year. Dogs that were treated with adjuvant chemotherapy were re-checked 2 weeks after the end of the protocol and every 3 months for the first-year post-surgery.

Preoperative assessment of SLN by ICTL

All ICTLs were performed under general anaesthesia. Dogs were premedicated using different drugs at the discretion of the anaesthesiologist and induced with propofol (Proposure 10 mg/mL, Boehringer Ingelheim Animal Health Italia S.p.A., Milan, Italy) at 4 mg/Kg IV.
Chlorphenamine maleate (Trimeton 10 mg/1 mL, Bayer S.p.a., Milan, Italy) was intramuscularly injected in all dogs at the dose of 4 mg/animal, before premedication, to prevent the onset of side effects due to histamine release during the MCT manipulation.
All dogs were firstly positioned in sternal recumbency and scanned to evaluate the lungs and sternal lymphadenopathy, then positioning was changed based on the anatomic location of the MCT, to minimize the compression on the lymphatic pathways around the MTC. CT images were acquired using a 20-slices detector (Siemens, SOMATOM sensation open, Munich, Germany) with 5 mm slice thickness, collimation 24x1.2mm, pitch 0.7, kVp 140, mAs 150 and tube rotation of 0.5 seconds. Images were reconstructed at 1 mm with standard and sharp reconstruction algorithm. The peritumoral area was shaved to better visualize the sites of injection and the boundaries of the MCT. A total of 1 mL of undiluted and warmed at 38°C Iomeprole, a water-soluble iodinate contrast agent (IOMERON® 300, Bracco, Italy, iodine concentration 300 mg/ml) was injected peritoumorally (0.25 ml on each quadrant), 5 mm from the macroscopic boundaries of the tumour. Iomeprole was injected into the dermis for cutaneous MCT, into the subcutis for scMCT and submucosally for mMCT with a 25-gauge needle. TC scan length of the area of interest was set based on the anatomic location of the tumour and the expected RLN/SLN(s). A timer was started after injection and TC scans were obtained at 0-, 2-, 5- and 10-minutes post-injection in all cases. If no lymphatic uptake was visualized at 5 minutes post-injection, the peritumoral area was gently massaged for 5 minutes, taking care not to touch the tumour, to prevent

degranulation of mast cells. Then a scan 5- and 10-minutes post-massage was repeated. All the injections and massages were administered by the same operator.
If the MCT was in the ventral part of the body, dogs were placed in dorsal recumbency, while sternal recumbency was selected for MCT located in the oral cavity and in the dorsal part of the body. For oral tumours, a support phantom was placed under the chin. If the MCT was in the right or left part of the body, dogs were positioned in the contra-lateral recumbency. Dogs with MCT in the medial part of limbs or when inguinal/axillary where the expected SLNs, were placed in lateral recumbency with a positioning device elevating the medial face of the limb to minimize compression of tumour and lymphatic pathways (Figure 5).

Figure 5 Picture showing the support phantom elevating the right hindlimb to avoid compression of the tumour located in the medial face of the left limb.

Image analysis

All the CT scans were visualized with the Horos Imaging Software v 1.1.7, Open-Source License and evaluated by at least one radiologist and one surgeon. The SLN identified by ICTL was called “white”.

Data collected included date of ICTL, change of recumbency (if performed), massage (if performed), any adverse events associated with peritumoral injection, time to contrast enhancement of the lymphatic duct and SLN, *exitus* of ICTL (contrast uptake to the LN, duct, or failure), “White” SLN(s) detected and the rate of agreement between RLN and SLN. If a clear pathway draining to a LN was visualized without ever reaching the corresponding node, the mapping technique was not considered unsuccessful since a precise suggestion of which LN was the SNL was provided. When no SLN/sentinel duct was visualized, regional lymphadenectomy was offered.

Intraoperative identification of SLN by Methylene Blue Dye

Surgery was scheduled on a different day from ICTL to reduce the duration of anaesthesia.

Peritumoral injection of 0.8 mL (0.2 mL on each quadrant) of sterile methylene blue 1% (SALF S.p.A.; Cenate Sotto, Bergamo, Italy) was performed just before scrubbing the skin, approximately 15 minutes before skin incision. Sentinel lymphadenectomy was performed before tumour excision to prevent contamination of the surgical field by tumour cells and MB. Moreover, instruments and gloves were changed after the excision of the MCT, before wound reconstruction. The surgical dose (proportional or wide) was decided according to the cytological grading, clinical characteristics size, and location of the tumour. Histopathological analyses were performed for all excised primary tumours and SLN/RLNs.

Data collected were: “Blue” SLN (those which showed the uptake of the blue dye), correspondence between “Blue” SLN and RLN and between “White” SLN and “Blue” SLN. Dogs were hospitalized for at least 24 hours after surgery and were re-checked 3-7 and 10 days after discharge.

Statistical Analysis

For both ICTL and MB, the agreement between RLN (according to Suami et al. 2013) and SLN was considered complete when the SLN and RLN coincided [110]. Partial agreement occurred when:

- MCT was in an anatomic region belonging to different lymphosomes and draining only to one of the lymphocenters,
- MCT was located on the median plane and drained only to one of the two RLNs (left or right).

Absence of agreement occurred when the SLN differed from the expected RLN detected by either technique, or when one of the mapping techniques (ICTL and MB) failed to detect the SLN; in the latter case the concordance could not be defined.

Agreement between ICTL and MB was complete if the same SLN was identified by both techniques, partial if one of the two techniques identified an adjunctive SLN, and absent if different or no SLN was detected by one of the techniques.

The axillary and pre-axillary (or accessory axillary) are two anatomically different lymphocenters but, according to the color-coded diagram published by Suami et al., they drain the same territory, thus, we considered the two lymphocenters as one and agreement was considered total even if only one of the two lymphocenters was the sentinel [110].

Statistical analysis was performed using “R-4.0.5 for Windows” available under General Public license (R Core Team; 2013). Significance was set at a level of $p\ value < 0.05$.

Total counts and percentages were used to describe categorical data, the RLN-SLN agreement for both ICTL and MB, the agreement between ICTL and MB and the detection rates of both ICTL and MB.

Numerical variables were firstly tested for normality with the Shapiro Wilk normality test. Normally distributed variables were expressed as mean and standard deviation (± SD). Non-normally distributed variables were expressed as median and range. Either Chi-square test or Fisher's exact test was used for testing the significance of the association between categorical variables (count data). Fisher exact test was specifically used when the expected frequencies in a contingency table are small, making the chi-square test potentially unreliable. Logistic regression was used to assess if the diameter of the tumour significantly influenced the outcome of the mapping techniques.

Results

Patient population

Sixty-one dogs were assessed for enrolment. Mean age was 8.65 years (SD ±2.54) and mean weight was 28 kg (SD ± 10.9).

Twenty-six dogs (42.6%) were spayed females, 13 (21%) were intact females, 5 (8%) were neutered males and 17 (28%) were intact males. There were 21 (34%) mixed-breed dogs and among the remaining 40 dogs, the most represented breeds were Labrador retriever (n=10), Jack Russell terrier (n=6), Golden retriever (n=4), Boxer (n=4), Miniature Pischer (n=2) and Siberian Husky (n=2).

Eighty-seven percent (53/61) of MCTs were cutaneous, 10% (6/61) were scMCTs and 3% (2/61) were mucosal.

The median of maximum diameter was 2 cm (range 0.2-15 cm).

Twenty-three percent (14/61) of MCTs were ulcerated at the first presentation. In 84% (51/61) of cases, MCT grew slowly, while in 16% (10/61) the growth was fast.

Ninety-five percent (n= 58) of MCT were diagnosed by fine-needle aspiration, 5% (n=3) by incisional biopsy.

The most common primary tumour location was the cutaneous thoracic region (n=8), followed by femoral (n=5), scapular (n=5), lateral neck (n=3), popliteal (n=3) and preputial (n=3) region. (Table 6).

Table 6 Primary tumour location.

ANATOMICAL region	Left	Median	Right	Total
ABDOMINAL MAMMARY REGION			1	1
ANTEBRACHIAL REGION	1			1
ANTERIOR DIGITAL	1			1
AXILLARY REGION	1			1
BRACHIAL REGION			1	1
CAUDAL MAMMARY REGION	1			1
THORACIC REGION	3		5	8
CRANIAL ANTEBRACHIUM			1	1
CRANIAL STIFLE			1	1
FEMORAL	1		4	5
GLUTEAL			1	1
INTERDIGITAL			1	1
IPOCRONDRIAL			1	1
LATERAL ABDOMINAL			2	2
LATERAL ANTEBRACHIAL	1			1
LATERAL ELBOW	1			1
LATERAL flank FOLD	2			2
LATERAL NECK	3			3
LATERAL STIFLE			1	1
LATERAL TARSAL			1	1
MEDIAL METATARSAL	1			1
MIDDLE ABDOMINAL	1			1
PECTORAL	2			2
PERINEAL	1			1
POPLITEAL	2		1	3
PREPUTIAL	1	1		2
RIGHT MANDIBULAR	1			1
SCAPULAR	3		2	5
SCROTAL		1	1	2
STIFLE	1			1
UPPER LABIAL	1		1	2
TARSAL/METATARSAL	1			1
TEMPORAL			1	1
VULVAR	2	1		3

A total of 88 RLNs were identified during the physical examination. Fifty-one (58%) of the 88 RLNs were considered non-palpable, 11 (12,5%) were megalic and 26 (29,5%) were palpable. Based on Fisher's Exact Test for Count Data, there was no significant association (p-value=0.24 and p-value=0.49) between megalic LN and the failure of both mapping techniques.

SLN detection and localization by Indirect computed tomographic lymphography

Indirect computed tomographic lymphangiography was performed for all 61 enrolled cases. No adverse events were noted after peritumoral injection of Iomeprole.

ICTL had a SLN detection rate of 80% (49/61). At least one “white” SLN was identified in 65% (n=40) of cases, while in 15% (n=9) of cases contrast draining from the tumour site in a pathway sufficient to indicate a SLN without ever reaching that node was visualized. In these cases, adjunctive scans did not identify any lymphatic flow progression toward the LNs. Based on Chi-square test for independence, there was a higher probability of mapping success than failure using ICTL (p-value = 0.0054).

In 20% (n=12) of cases neither the SLN nor the lymphatic duct could be identified. In 50% (n=6) of these 12 cases, recumbency was changed 10 minutes after injection but no SLN was identified.

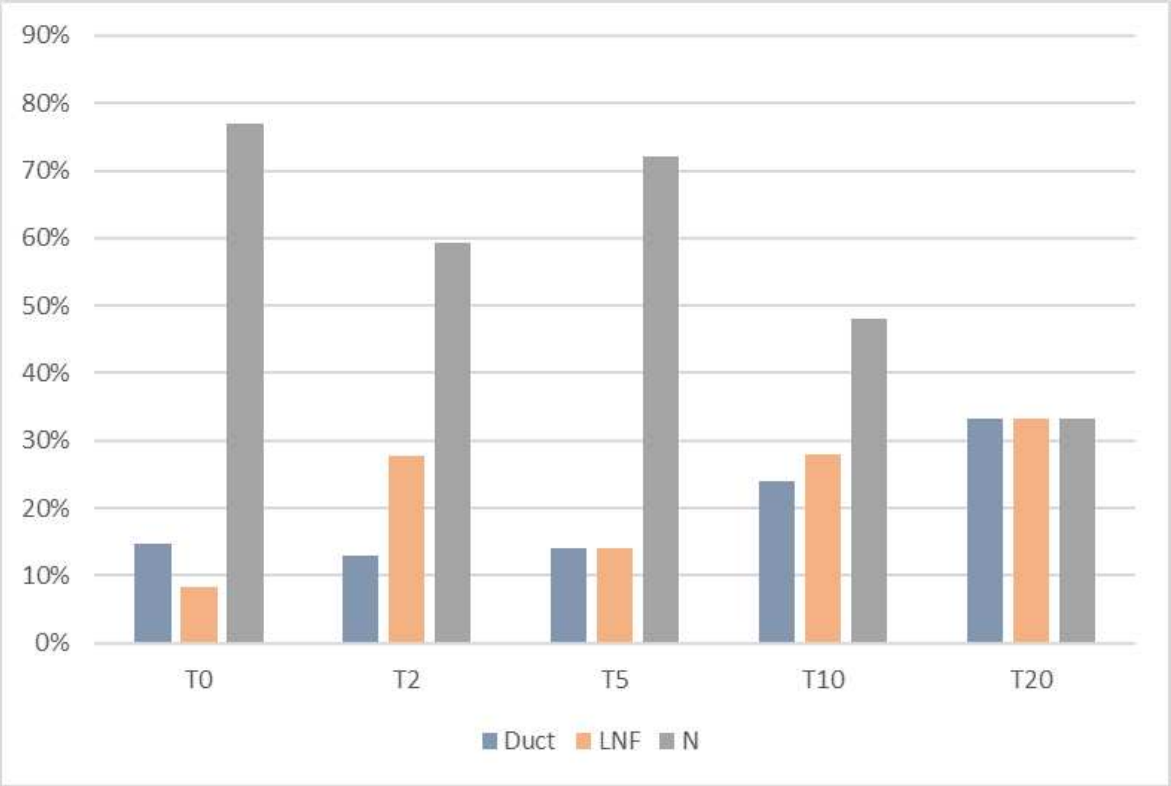
In 50% (n=6) of these 12 cases, MCTs were surrounded by an area of fat stranding, skin thickening, and subcutaneous oedema that was called “*peritumoral halo*” (Image) which could have interfered with the contrast uptake, as discussed further on. A total of 11.7% (n=7) cases presented this tomographic sign. Only in one case, despite the presence of the “peritumoral halo”, there was a contrast uptake of the LNs. The dog was referred to our hospital after the third recurrence of an incompletely excised high grade MCT located in the left scapular region and the SLN mapping and lymphadenectomy have never been performed before. Interestingly, the MCT was encircled by small satellite nodules and bilateral superficial cervical LNs were both giant and both considered sentinel at T10 scan. In this case we speculated that, as previously described [146], the lymphatic drainage was disrupted by the previous surgical episodes and took a different way of drainage. The increase of the lymphatic pathways could explain the unexpected enhancement of both SLNs. This dog did not undergo a new surgery, and he died due to progressive disease 3 weeks later, therefore, no MB

information is available. In four of the six dos with “peritumoral halo” also the RLN was megalic. None of these dogs underwent surgery.

The SLN-RLN agreement was complete in 64% (n=39) and partial in 10% (n=6) cases. Based on Chi-square test for independence, there is a significant concordance between RLN and SLN (p-value= 0.007597). In 26% (n=16) of cases the concordance was absent.

As showed in Graphic 1, SLN enhancement occurred more frequently at T20 (33%), T10 (28%) and T2 (28%) (Figure 6).

Figure 6 Percentages of identification of Duct, Sentinel lymph node (LNF) and not found (N) for each scan time.



Sentinel lymphadenectomy and histological assessment

Of the 61 dogs that undergo to the ICTL, 11 did not receive surgery because of either rapidly progressing disease (8 cases) or owner refusal (3 cases).

Forty-seven of the 50 dogs which were operated has SLN mapped by intraoperative MB, because in 3 cases MB was not injected. In one of these three cases, MCT was in the right popliteal region and RLN was the right popliteal, but the “white” SLN was the right inguinal. Both inguinal and popliteal LN were excised, and histological examination

reported inguinal LN as HN0 and the popliteal LN as HN2. In the second case, the primary MCT was on the left scapular region and the “white” superficial cervical was not found in surgery, thus no histological examination was performed; in the third case, the MCT was located in the left lateral fold region but the “white” left inguinal SLN was removed and was diagnosed as HN2.

Overall, 68 SLNs were removed from 50 dogs. In seventy-five percent (n=35) of the 47 cases the SLN-RLN concordance was total, in 19% (n=9) was partial and in 6% (n=3) absent.

Six of the 11 dogs that did not undergo surgery showed the “peritumoral halo”, ICTL failed to detect the SLN, and they were treated with a mean of 4 neoadjuvant doses of vinblastine (2 mg/m^2 IV once per week) to reduce or control the MCT before surgery. All these dogs died of progressive disease before surgery could be performed. In one of these cases bilateral superficial cervical SLNs were detected by ICTL, but the dog died of severe progressive disease unresponsive to any medical treatment after 3 weeks. In one case, while the dog was shaved prior to surgery, small satellites nodules were noted along the lymphatic vessels, and the owners elected not to pursue surgery.

One of the 6 dogs with the “peritumoral halo” underwent surgery and the RLN was excised. The “peritumoral halo” was histologically diagnosed as small-vessel vasculitis, cellulitis, lymphedema and collagenolysis with eosinophilic infiltration associated with high grade/grade III MCT with HN LN0.

Based on the Chi-square test for independence, there was a significant concordance between SLN-RLN identified by MB (p-value= 0.0054).

In 32 of 47 cases (68%) the agreement between ICTL and MB was total, since all the white SLN showed blue colouration, while the agreement was partial in 3 cases (6.4%). In the remaining 12 cases (25.5%) there was a complete disagreement.

A total of 50 MCT were excised. Forty-one were cMCT, 36 of which Grade 2/Low grade, 3 were Grade3/High grade and 2 Grade 2/High grade. Six MCT were subcutaneous, and three mucosal.

Median MC was 1 (SD±1.28). In 45 cases margins were classified as clean and in 5 cases one margin was defined as “clean but close”. None of the dogs with clean margins experienced local recurrence. Two of five dogs with “clean but close” margins died due to the MCT. Both cases were Grade III/High grade cMCTs. In one of these cases, the excised SLNs were classified as HN2 and HN3, and the dog experienced both local recurrence and metastatic disease during the fifth administration of adjuvant

vinblastine and died 96 days after surgery. In the second case, the SLN was HN0, and dog died due to local recurrence 148 days after surgery. This dog was treated with toceranib and vinblastine.

All dogs with G2/LG cMCTs, scMCTs and mucosal were alive at the end of this dissertation after a median of 698 days (486-1016 days), except for one with a HN3 SLN. In this case, the owner refused the adjuvant treatment proposed and the dogs died of progressive disease after 151 days.

A total of 68 SLNs were removed and histologically analysed (Table 7). Eight of these were classified as HN3 (11.7%). Interestingly, 6/8 SLNs corresponded to the RLN, in one case the correspondence was partial and in one case was absent.

Table 7 Number and Histopathological status of each excised SLN.

Histopathological status	*Total number*	*White &Blue*	*Only white*	*Only Blue*
HN0	21	15	2	4
HN1	17	14	2	1
HN2	22	13	3	3
HN3	8	3	2	3

A total of 11 dogs (16%) received adjuvant Vinblastine (2 mg/m^2) and prednisolone (1 mg/Kg orally). In six of these11 dogs the SLN was HN3, in one dog with scMCT the SLN was not found in surgery but the Ki67% was 25; two cases were mucosal MCT, and two cases were high grade cMCTs. Also, 10 dogs (16%) received neoadjuvant Vinblastine 2 mg/m^2. Of these, 6 were not operated.

To verify the hypothesis that the disagreement between RLN and SLN is anatomical region-dependent, we grouped the previously listed anatomical regions (according to Suami et al. 2013) into the following “macro anatomical regions” to obtain a greater sample for each class:

Abdominal (n=7), hindlimb (n=16), forelimb (n=11), perineal (n=1), scrotal (n=2), vulvar (n=3), thoracic (n=12), inguinal (n=3) and head& neck (n=7) regions.

In the case of head and neck and thoracic location, in all but one case there was complete agreement between SLN-RLN (86% and 92% respectively). In contrast, the

concordance rate was 57% in the abdominal region, 36% in the forelimb and 56% in the hindlimb regions, highlighting that SLN-RLN concordance is highly dependent on the anatomical region of interest (Table 8).

Table 8 Concordance between sentinel lymph node and regional lymph node for each anatomic region using ICTL.

Concordance SLN-RLN with ICTL				
Anatomic region	**ABSENT**	**PARTIAL**	**TOTAL**	**Grand Total**
Abdomen	3		4	7
Forelimb	3	4	4	11
Head and neck	1		6	7
Inguinal		1	2	3
Hindlimb	7		9	16
Perineal		1		1
Scrotal			1	1
Thorax	1		11	12
Vulvar	1		2	3
Grand Total	**16**	**6**	**39**	**61**

Similar patterns were found when considering the concordance between SLN-RLN using MB as shown below. We did not further discuss anatomic region with less than three observations (Table 9).

Table 9 Concordance between sentinel lymph node and regional lymph node for each anatomic region using MB.

Concordance SLN-RLN with MB				
Anatomic region	**ABSENT**	**PARTIAL**	**TOTAL**	**Grand Total**
Abdomen	1		4	5
Forelimb	1	3	3	7
Head and neck		1	5	6
Inguinal		2	1	3
Hindlimb		2	9	11
Perineal		1		1
Scrotal			1	1
Thorax	1		10	11
Vulvar			2	2
Grand Total	**4**	**9**	**35**	**47**

We hypothesized that the presence MCT greater than 3 cm was associated with the failure of the SLN detection by ICTL. Interestingly, on the Logistic regression, the

diameter of the tumour significantly influenced the results of the outcome of the mapping (p-value= 0.00152), and the regression coefficient was equal to -0.4854, which means that the increase in the size of the tumour determines a decrease in the success of the mapping. When the inverse logit transformation was used, a cut-off value for mapping failure was set at a diameter of 6 cm, while no significance was found using the cut-off value of 3 cm.

As previously reported [15], MCT greater than 3 cm are more aggressive. Based on Fisher's Exact Test for Count Data, there was a tendency, although the significance was not reached (p-value=0.11), for the association between tumour diameter greater than 3 cm and aggressiveness based on the combination between Kiupel and Patnaik classifications (Figure 7). Based on Pearson's Chi-squared test with Yates' continuity correction, a lack of significance was found when using only Kiupel grading system (p-value=0.15) (Figure 8).

A correlation between increase of the primary tumour diameter, increase of the aggressiveness and decrease of positive ICTL yields was speculated and discussed further in the discussion chapter.

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Figure 7 Association between tumour diameter and aggressiveness based on the combination of Kiupel and Patnaik classifications.

Figure 8 Association between tumor diameter and aggressiveness based on Kiupel classification.

Discussion

Sentinel lymph node identification and its therapeutic impact represent one of the most important discoveries in human oncology and have recently gained increased attention in veterinary surgical oncology, representing a revolution in the management of MCT in dogs [16]. Even acknowledging that lymphoscintigraphy is the gold standard in human medicine, its limited availability due to costs and the use of radioactive tracers has led to find alternative methods in veterinary medicine, to ensure health care access to all patients. Indirect computed tomography lymphangiography is one of the most widespread alternatives and allows for an accurate localization of SLN(s) and for a concomitant surgical planning [26,33,141,143]. Methylene blued dye is a cheap, easily available blue stain that allows for the direct visualization of lymphatic pathways and SLN after skin incision.

The primary aim of this article was to demonstrate that the strict adherence to the lymphosomes scheme proposed by Suami et al. (2013), could lead to the agreement between the regional and sentinel lymph nodes detection in more than 50% of the cases, using either ICTL or MB [110]. Moreover, we speculated that this agreement could be higher in some anatomical region such as the thorax, where the axillary lymphocenter is the main draining one. In contrast, we expected a higher rate of non-concordance in the head and neck region, as previously described, since three main lymphocenters drain this region [21,147]. The results of this prospective study showed that the SLN-RLN agreement was complete in 64%, partial in 10% and absent in 26% of 61 MCTs evaluated by preoperative ICTL, thus confirming our hypothesis. These results are in accordance with those published by Lapsley and colleagues (2021) and Alvarez-Sanchez and colleagues (2023), that reported a disagreement rate of 27% and 25% respectively using ICTL [26,128]. A possible explanation for this similar results is that, even if they used a different contrast agent (iopamidol), differently diluted [26], (50:50 with sterile saline) and a different total injection volume (2 ml), the authors identified the RLN according Suami and colleagues [110]. Another study reported a higher rate of non-concordance, but they identified the RLN either as the nearest to the tumour or referring to the Suami's scheme [105]. However, the authors did not specify how they elected one method over the other when the RLN did not match. For example, they identified the parotid RLN as SLN for a MCT located in the pinna. Using the system of "proximity" the RLN is the parotid LN, but according to Suami's scheme the RLN is the superficial cervical LN. The ICTL identified the superficial cervical as SLN but they

classified this agreement as absent [105]. This information proved our speculation that the lack of standardization of the definition of RLNs increased the non-concordance rate. Some studies considered the RLN as the nearest [9,22,105], while others used the classification of Suami et al. [26,27,128,139], some other the one proposed by Rogers et al. [42,148] and the subjective assumption of “unpredictable”, when referring to MCTs located on the midline of the body, for which the left or right LN could unpredictably become the SLN [21,105]. In the present study we considered those cases as “partial concordance” since both sides could be elected as sentinel.

The second aim was to describe the rate of agreement between the technique of preoperative mapping and intraoperative guidance, hypothesizing a level of agreement higher than 80%. We did not confirm our second hypothesis. In fact, our study reported a lower agreement between the “white” and “blue” SLN than that previously reported (84.6%-89% vs 68%) [28,149].

We did not expect that the SLN-RLN agreement was higher using MB than ICTL. In fact, in seventy-five percent (n=35) of the 47 cases the SLN-RLN concordance was total and in 19% (n=9) was partial using MB. This unexpected result could be related to the lower number of dogs (n=50) that underwent surgery compared to the total of enrolled cases (n=61). In fact, of 11 cases that did not undergo to surgery, six presented the “peritumoral halo” that impeded to detect the SLN with ICTL. We also could not exclude that this result could be related to errors occurred during the performance of the ICTL.

The third aim was to describe all the possible causes of SLN detection failure during preoperative indirect lymphangiography with aqueous contrast medium injection. We theorized that the presence of megalic LN, the high grade of the MCT, and MCT greater than 3 cm may be associated with the failure of the SLN detection by ICTL.

The ICTL detection rate was 80%; in 20% cases neither SLN nor lymphatic pathways were identified. Previous studies conducted on different tumours histotypes reported a detection rate range from 60 to 90% using ICTL [26,33,143]. This variable range is reasonably the consequence of inclusion of different tumours with a different biology. Nevertheless, the detection rate reported here is lower than that reported in ICTL studies conducted only on MCT (90-100%) [26,105,128,149].

We observed that ICTL failed to identify SLN when the MCT had a diameter greater than 6 cm. Moreover, even if not statistically significant due to the limited number of cases, we reported a tendency to failure in case of the presence of the tomographic sign of “peritumoral halo”. We are not aware of any publications describing this

“peritumoral halo” sign as cause of failure in the detection of SLN in dogs with MCT undergoing ICTL, but this could be an interesting point of discussion with clinical implications. Moreover, this is the first study describing the “peritumoral halo” sign. One recent study conducted on healthy dogs stated that the viscosity of contrast agent did not determine efficacy and speed of identification of SLN [144]. Other authors explained the lack of success in theorizing the obstruction of lymphatic flow due to the positioning or due to the abundant adipose tissue, but in many cases, the reason remains unclear and no evidence was provided [33,105]. We considered these finding to be important for the planning of SLN mapping, since clinicians should predict the low probability of the identification of SLN by ICTL when dogs presented with MCT greater than 6 cm or when the “peritumoral halo” sign is present. Only one dog with a MCT with the “peritumoral halo” sign was surgically treated in this study, since the others died before surgery. In this case, the halo was histologically described as an area of small-vessel vasculitis, cellulitis, lymphedema and collagenolysis with eosinophilic infiltrations associated with grade 3/high grade MCT. Since the other dogs did not undergo either surgery or necroscopy, we cannot technically confirm that this sign is always associated with high-grade MCTs, even though the clinical outcome of these cases may support our hypothesis. We are aware that the small sample size of the population impacts the lack of significance, thus we encourage further studies with a larger population and other mapping techniques to confirm our data. We cannot exclude that other mapping techniques such as NIRF and lymphoscintigraphy could invert our results.

Despite both the peritumoral massage and the change of recumbence performed 10 minutes after the contrast injection, in 6 cases any contrast uptake was visualized. Thus, we hypothesized that a flow obstruction could have occurred. To reduce lymphatic flow compression, we used a supporting tool that was placed under the hindlimb/forelimb when dogs were in lateral recumbency, and axillary and inguinal LN could be expected as SLN. In that way we reached a success rate of 50% (5 of 10 dogs) with MCT located in the limb region. The other 50% of dogs presented both palpable/megalic LNF or “peritumoral halo”. Therefore, even if we are aware of the crucial role of patient’s positioning to avoid flow compression due to the body weight on the tissues, we speculated that the LN size and the tumour diameter could have played a more important role in the failure of the mapping technique. In human medicine the phenomenon of tumour blockage and rerouting of lymphatic drainage was demonstrated for LNs with extensive metastatic involvement causing a false-negative

result [150]. Our study did not confirm this evidence, even if the small sample size could have biased the result. Although not all the failures could be due to the metastatic engorgement of the LN in our cases, the evidence of lack of SLN identification in case of “peritumoral halo” sign was not reported in previous studies, and we speculated that the higher rate of failure of our study could be related to the high number of enrolled cases with this characteristic peritumoral pattern, which can be associated with a more malignant behaviour and LN metastasis. In one dog which showed the “peritumoral halo” pattern, the ICTL identified bilateral SLN at the same time (T10). This dog had been previously surgically treated 3 times with a marginal resection of both the primary MCT and the two recurrences. A recent study conducted on healthy dogs demonstrated that SLN identification occurs faster if performed postoperatively and also identified adjunctive LNs other than that preoperatively identified [146]. Thus, we theorized that surgeries may have altered the lymphatic drainage of the area and that the lymphangiogenetic process determined the success of ICTL. We partially demonstrated our hypothesis that SLN-RLN agreement is dependent on the regional area of the body. Three studies conducted on dogs with tumours located in the head and neck region reported a contralateral SLN in 22%–62% of dogs with lateralized tumours and a rate of SLN-RLN disagreement of 52% [21,147,151]. Thus, we expected a high rate of disagreement with contralateral SLN in the head and neck region. Our results showed a complete agreement rate between SLN and RLN in the head and neck and thoracic regions of 86% and 92% respectively, using ICTL and 83% and 90% using MB. Our results are in contrast with that reported in the cited studies, but we speculated that this could be due to the low number of enrolled cases. In fact, we enrolled only 7 dogs with MCTs located in the head and neck region, that drained to the ipsilateral expected RLN. In contrast, the concordance rate was equal to 36% and 43% in the forelimb region using ICTL and MB respectively. This results supported the recent evidence provided by Hlusko and colleagues [142] that the drainage pattern from the forelimb can be variable also in healthy dogs. This result seems to be related to the presence of MCT in an area of “overlapping” lymphatic territories, the so-called “zone of ambiguity” of lymphatic drainage described on the forelimb between superficial cervical and axillary LNs, and in the hindlimb between superficial inguinal, medial iliac and popliteal LNs. In these cases, performing preoperative mapping is useful to correctly identify the draining LNs and to avoid excision of both lymphocenters, increasing the risk of lymphedema. One area of unclear description of the lymphatic territory is the pre-axillary (or accessory)

lymphocenter that Fournier and colleagues (2021) defined as “interval node” [30]. The axillary and pre-axillary are two anatomically different lymphocenters, but according to the color-coded diagram published by Suami et al. (2013), they drain the same territory [110]. Thus, we considered the two lymphocenters as one, and the agreement was considered total even if only one of the two was the sentinel. In other studies concordance was considered total when the SLN was the axillary, partial when both axillary and pre-axillary were the SLN, and absent if the SLN was the pre-axillary [22,128]. This discrepancy inevitably influenced the rate of agreement between SLN and RLN. The optimal time for detecting SLN during ICTL in this study was 20 minutes after the injection of the contrast agent; this was longer than the timeframe (0-10 minutes) reported in other studies [26,33,128]. A possible explanation is that we did not massage the MCT after peritumoral injection but 5 minutes later if no contrast migration in the lymphatic vessels was observed. Grimes reported that massaging immediately after contrast injection increased the lymphatic flow. Considering the strong evidence of the usefulness of the post-contrast massage and of the results reported here, we suggest massaging the injection immediately after contrast injection, in order to reduce the radiation exposure and anaesthesia time.

This study had several limitations. The main is the low number of enrolled cases that precluded the significancy of some of the statistical analyses. We did not perform a power analysis, but we enrolled all dogs throughout the 37-month period. Secondly, the heterogenicity of anatomic location of MCTs impeded to identify if there were some body locations were SLN mapping is not necessary. Thirdly, to avoid the prolongation of anaesthesia time we did not change recumbency in all failed ICTL, which may have improved our detection rate.

In conclusion, we described for the first time that in case of MCT bigger than 6 cm and/or with a peritumoral halo the ICTL has a high failure rate, questioning its usefulness in these cases. Since SLN has many clinical implications, its correct detection is paramount, and we demonstrated that strictly referring to the Suami scheme, the rate of agreement between SLN and RLN detection by ICTL and MB is equal to 64%.

Project 3: Superficial sentinel lymphadenectomy in 163 dogs: complications and comparison between intraoperative guiding techniques

At present, a limited number of studies have specifically analysed the complications associated with SLN extirpation in dogs [21,40,42]. In one study, the overall incidence of complications following 245 sentinel lymphadenectomies guided by intraoperative γ-probing and methylene blue dye in 113 tumour-bearing dogs was 21.2%. Interestingly, 91.7% of reported complications were classified as minor [43].

Another study reported no difference in the incidence of complications by comparing unassisted lymphadenectomy with intraoperative guidance with anchor wire or methylene-blue dye [40].

The principal aim of this study was to describe the incidence rate and the severity of the complications following superficial sentinel lymphadenectomy in dogs, comparing three different techniques: intraoperative γ-probing and methylene blue dye, methylene blue dye guidance only, and unguided lymphadenectomies. The authors hypothesize that any intraoperative guidance techniques reduced the rate of postoperative complications. The second aim was to investigate if the incidence of complication was influenced by the number, the size, and the location of SLN, theorizing that a lower number, being palpable, and certain anatomic locations were associated with a reduced risk of complications.

Materials and methods

Medical databases of three Veterinary Teaching Hospitals (University of Parma, University of Milan, University of Turin) and two private Veterinary Clinics (Clinica veterinaria Nervianese and Centro Veterinario Pisani-Carli-Chiodo) were retrospectively searched for client-owned dogs that underwent both pre-operative SLN mapping and lymphadenectomy between December 1, 2020, and April 30, 2023.

Dogs were eligible for inclusion if peripheral SLN excision was performed both with or without intraoperative guidance, and if a minimum follow-up of 30 days, and complete information about post-operative treatment and complications were available.

Intracavitary LNs and any situations where more than one lymphocenter draining the same anatomic district (e.g. *en bloc* extirpation of mandibular and retropharyngeal LNs) were excised were excluded.

All owners signed a written informed consent, agreeing to submit their dogs to the procedures described for medical reasons, and for their data to be used for scientific purposes. All the procedures were conducted as part of standard treatment protocols for oncologic diseases, in accordance with the Institutional guidelines for animal welfare, under the control of the National Ministry of Public Health. No extra animal discomfort was caused for the purpose of the study.

All lymphadenectomies were performed by a group of qualified surgeons with variable surgical skills.

Data retrieved for each patient included: breed, sex, age, body weight, body condition score (BCS, range 1–9), anatomic location and laterality of SLNs, if they were clinically palpable or not during physical examination. Lymph nodes were defined as palpable if detected by the surgeon by manual palpation of the anatomic area in which it was located. Type of pre-operative mapping technique (lymphoscintigraphy [LS] or peritumoral injection of aqueous contrast and indirect computed tomographic lymphography [ICTL]), whether intraoperative guidance for SLNs excision was performed, and the type of guidance (colorimetric mapping with methylene blue dye [MBD], or a combination of MBD and hand-held γ-probing), number of excised SLNs and their maximum diameter (measured ex vivo with a calliper after surgical resection), postoperative treatment(s), number and type of intra- and post-operative complications observed and any further surgical intervention or medical treatment.

The complications evaluated were infection, dehiscence, seroma, hematoma, lymphoedema, lameness, haemorrhage, and brachial plexus injuries (BPI). Oedema was defined as fluid accumulation at the surgical site, lymphoedema was defined as spillage of lymph fluid from the tissues of the region drained by the excised lymphocenter [152].

Post-operative complications were defined and graded into five grades of severity: mild (grade 1), moderate (grade 2), severe (grade 3), life-threatening (grade 4) and death (grade 5), according to LeBlanc et al. (2021) [152].

Based on the occurrence of any complication, the population was split into either “Complicated group”, when at least one complication was reported, or “Uncomplicated group”, if no complication was reported.

For each complication, the onset from the day of surgery, duration (days) until complete resolution and type of treatment required were reported. In case of infection, positive bacterial cultures and antibiotic susceptibility tests were collected.

In case of axillary LNs excision, due to the possible brachial plexus injury, thoracic limb reflexes were evaluated before and after surgery and any alteration recorded. Pre-existing lameness was recorded and re-assessed after surgery. As a standard procedure, perioperative antimicrobial prophylaxis and NAIDS (for 2-5 days after surgery) were administered in all the institutions. Post operative antimicrobial administration was continued depending on the concurrent surgery performed.

For statistical purposes, each dissected lymphocenter was considered as a single case, when each lymphadenectomy required different skin incisions on the same dog.

Cases without recorded time for lymphadenectomy (LT) were excluded. LT was recorded from the skin incision over the lymphocenter till the end of the skin suture.

Statistical Analysis

Statistical analysis was performed using “R-4.0.5 for Windows” available under General Public license (R Core Team; 2013).24 The correlation between the presence or absence of complication and all the variables were investigated using a logistic regression model, p was set at a level < 0,05. As a second step, a decision tree algorithm was applied, identifying a threshold in the continuous or discrete variables below and above which the probability of showing a complication became significantly lower or higher compared with the probability of non-developing any complication.

Results

A total of 201 peripheral lymphadenectomies from 163 dogs were included.
Median age of enrolled dogs was 8 years (range 2-16 years), and mean body weight was 24.8 Kg (range 3-55 Kg, SD 12.02). BCS data were available for 133 (81.5%) dogs and were grouped into three categories (underweight BCS <4; normal weight BCS = 4-5; overweight BCS >5). Data regarding breed, sex, and BCS are listed in Table 10.

Table 10 Signalment data of the entire population (Abbreviations: NA, not available)

Patient signalment	**Frequency (n of dogs)**
Breed	
-Crossbreed	31
-Golden retriever	20
-Labrador Retrievers	22
-Boxer	14
-Jack Russel Terrier	9
-French bulldog	9
-Other (<6 dogs each)	67
Sex	
-Female	38
-Female spayed	45
-Male	66
-Male castrated	14
Body condition score	
-3	2
-4	41
-5	65
-6	22
-7	3
-NA	30

The 201 LN extirpation procedures were grouped as follows: 70 (34.8%) inguinal, 44 (21.9%) axillary, 7 (3.5%) pre-axillary, 38 (18.9%) superficial cervical, 19 (9.5%) popliteal, 17 (8.5%) mandibular, 5 (2.5%) retropharyngeal and 1 (0.5%) parotid.

Bilateral lymphadenectomy was performed in 6 (3%) cases, five of which were inguinal LNs. The side of LN was left in 88 (43.8%) and right in 107 (53.8.1%) cases. At physical examination, 51 (25.4%) LNs were considered clinically palpable, and 150 (74.6%) non-palpable. Of the 51 palpable LNs, 16 were superficial cervical LNs, 9 axillary LNs, 10 popliteal LNs, 7 inguinal LNs, 6 submandibular LNs, 2 retropharyngeal and 1 pre-axillary LN. Of the 150 non-palpable, 63 were inguinal, 35 axillary LNs, 22 superficial cervical LNs, 11 mandibular LNs, 9 popliteal LNs, 6 pre-axillary LNs, 3 retropharyngeal and 1 parotid LN.

The median number of excised SLNs was 1 (range 1-4 LNs). In 16 (7.9%) cases, more than 3 LNs were extirpated from a single lymphocenter. The median maximum diameter of the LNs was 15 mm (range 1 – 49 mm). The mean LT was 18.6 minutes (range 1.5 - 83 minutes, SD 12.2).

No intraoperative complications occurred. Of the 201 surgical procedures performed, 93% (n=187) did not develop any postoperative complications (Uncomplicated group), while 7% (n=14) were allocated in the complicated group; therefore, no statistical evaluation could be performed between the two groups.

The overall incidence of complications was 7.4% (15/201), since a total of 15 complications in 14 surgeries were reported. The most frequent postoperative event was seroma of the surgical wound, that developed in 5/201 (2.5%) cases, followed by 3 lymphoedemas (1.5%), 3 dehiscences (1.5%), 3 lamenesses (1.5%) and 1 hematoma (0.5%).

Time of onset and resolution of complications were available in all 201 cases. The median time to onset of the complication was 3 days (range 1-15 days), and the median duration was 4 days (range 1-10 days). Of the 15 complications, 12 (80%) were mild (grade 1) and self-limiting, and 3 (20%) were moderate (grade 2) and required medical intervention. No severe (grade 3), life-threatening (grade 4) and death (grade 5) complications were recorded.

The median time of onset of the seroma formation was 7 days (3-15 days) and the median duration was 6 days (4-10 days). It was classified as grade 1 in 3 cases and grade 2 in 2 cases, treated with prolonged administration of NAIDS and seroma drainage. Two cases of suture dehiscence occurred both 7 days after surgery and

resolved in 4 days without any treatment (grade 1), one case occurred 4 days after surgery and was treated with a primary closure with skin staplers without the need for revision surgery (grade 2). All three cases of lymphoedema were classified as grade 1 and were self-limiting. Also, hematoma and lameness were self-limiting. All 3 cases that developed transient lameness underwent axillary lymphadenectomies. Neurologic complications (e.g. permanent nerve injury), haemorrhage, and infection were not observed in any patient.

Complications occurred after lymphadenectomies of the inguinal lymphocenter in 4/70 cases, mandibular lymphocenter in 2/17 cases, axillary lymphocenter in 3/44 cases, pre-axillary lymphocenter 0/7, superficial cervical lymphocenter in 3/38 cases, retropharyngeal in 2/5 lymphocenter, popliteal in 0/19 cases and parotid 0/1. Of the lymphadenectomies that resulted in complications, 1 was bilateral (1/6), 7 left-sided (7/89) and 6 right-sided (6/106). Complications occurred in 10/150 lymphadenectomies of non-palpable (6.6%) LNs and in 4/51 of palpable LNs (7.8%); in 12/185 of cases when 2 or less LNs were removed (6.4%), and in 2/16 when 3 or more LNs were removed (12.5%). Three dogs with BCS=4, 5 with BCS=5, 4 with BCS=6, and 1 with BCS=7 developed complications

In the logistic regression model, when considering the presence or absence of surgical complications as the dependent variable, BCS, body weight, gender, location, laterality, size of LNs and number of LNs removed, guiding techniques and time of lymphadenectomy were identified as non-significant factors, and did not significantly influence the occurrence of complications ($p > 0.05$).

Preoperative peritumoral injection of aqueous contrast and ICTL was used in 121 (60%) cases, of which 72 (59%) lymphadenectomies were performed without intraoperative guidance and 49 (41%) with intraoperative peritumoral injection of MBD. In 80 (40%) lymphadenectomies, preoperative LS and intraoperative MBD and γ-probe were used.

Patient characteristics, physical examination findings, lymph node characteristics, duration of lymphadenectomy, and postoperative complications were classified based on the intraoperative mapping techniques (unguided, MBD and LS). Interestingly, all SLN excised with LS were classified by the surgeons as non-palpable. No significant difference in all the considered variables were found among the three techniques (Table 11).

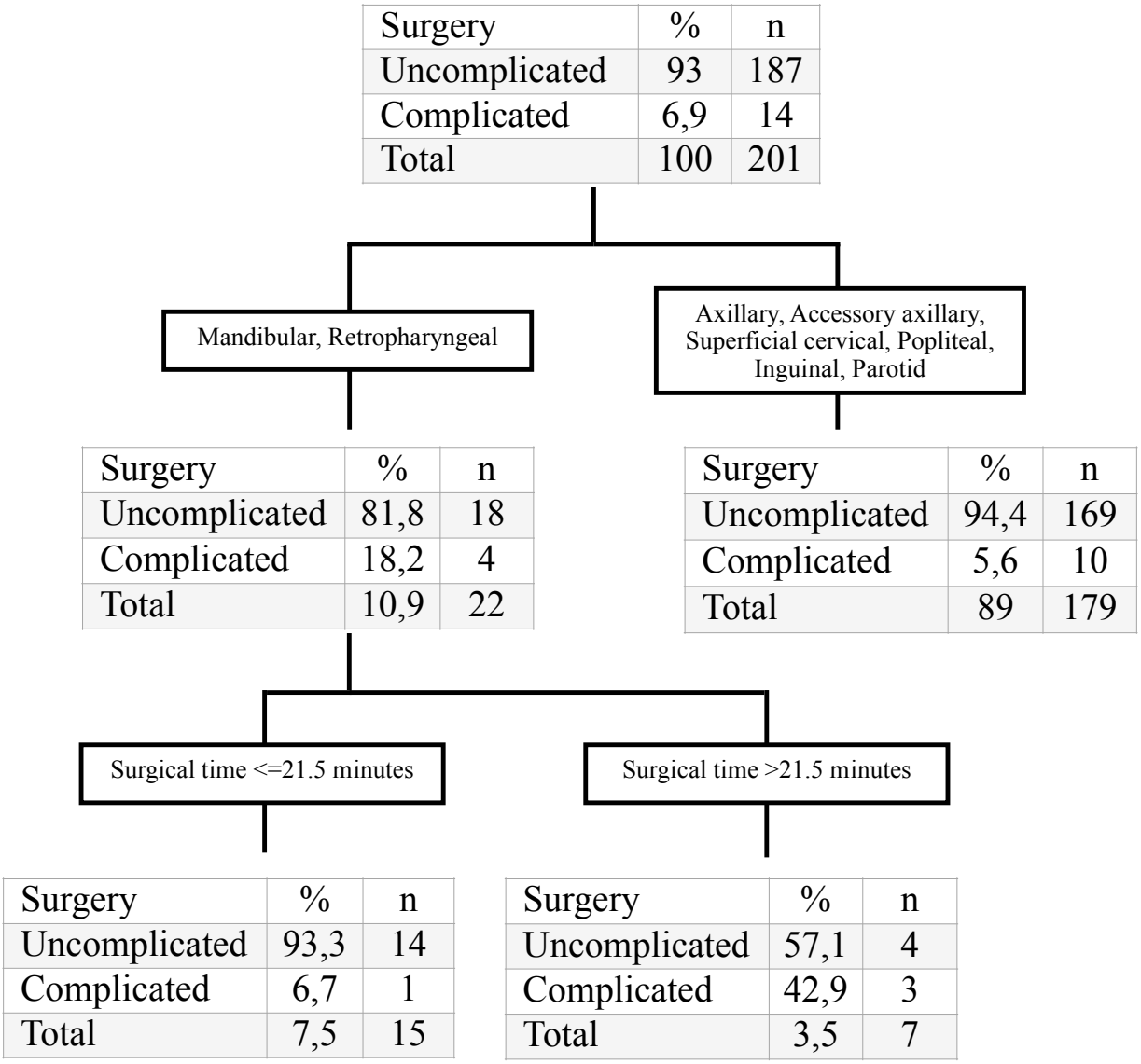
Table 11 Patient characteristics, physical examination findings, lymph node characteristics, duration of lymphadenectomy, and postoperative complications classified by lymph node localization techniques.

Abbreviations: MBD, methylene blue dye

Variable	Unguided	MBD	LS
Female	13	20	10
Spayed females	19	13	21
Males	36	14	38
Neutered males	4	2	11
Age (years)	8	9	8
Median Body weight (kg)	29.2	28	27
Body condition score (1/9)	4	5	5
Palpable (n)	29	21	0
Non-palpable (n)	43	28	80
Lymph node diameter (mm)	14	11.5	16
Lymph node excision No.			
≤2	69	42	74
>2	3	7	6
Lymph nodes excised No. (median)	1	1	1
Duration of lymphadenectomy (min)	13.5	13	15
Accessory Axillary	2	0	5
Axillary	15	15	13
Inguinal	29	17	24
Mandibular	4	2	11
Parotid	1	0	0
Popliteal	8	4	7
Retropharyngeal	2	1	2
Superficial Cervical	11	9	18
Complications, No.	2	6	6

Using the decision tree model, two nodes were found to present a significantly higher incidence of complications in comparison to the others. The two nodes were the LN localization (mandibular and retropharyngeal) and the LT for the latter localization (Figure 9). The highest reported incidence of complications was for mandibular and retropharyngeal (4/22), while only 10/179 of pre- and axillary, superficial cervical, popliteal, inguinal and parotid lymphadenectomies were complicated. Moreover, a surgical time greater than 21.5 minutes increased the risk of complications after mandibular and retropharyngeal lymphadenectomy.

Figure 9 Decision tree model



Discussion

The present study aims to investigate the rate of post-operative complications following superficial lymphadenectomy by comparing three different intraoperative detection techniques.

Few studies have specifically investigated the possible risk factors and the incidence of intra- and postoperative complications after sentinel lymphadenectomy, even if there are many studies about the mapping methods of SLNs in veterinary medicine.

The overall incidence of complications reported in this study was 7.5%, and no one of these was severe or fatal. This is a lower incidence compared to previous studies in which the overall complication rate ranged from 16% to 27% [9,22,40,42,43]. This finding corroborates the assumption that superficial LNs excision is well tolerated in dogs and shows a complication rate similar to other clean surgical procedures.

Several intraoperative guidance techniques have been described in the last ten years. These techniques may improve the successful rate of the lymphadenectomy, expedite lymph node localization and limit the time and the manipulations of the surrounding tissues required for the LNs excision [9,40,42,43,153,154]. To the authors' knowledge, this is the first study that compares unguided lymphadenectomies with guided lymphadenectomies using a combination of peritumoral injection of MBD and gamma probe and MBD alone in a large cohort of dogs. It may be argued that MBD is not a real guiding technique for this purpose, since it is not visible thorough the intact skin; anyway, once the lymph centre is located by preoperative techniques, it allows to follow the blue lymphatic vessels to the LN, through the dissected skin over the lymph centre, thus speeding up the process and limiting the amount of dissection, especially in case of small LNs in difficult locations.

Preoperative LS was used in 80 (40%) lymphadenectomies, and intraoperative MBD and γ-probe were subsequently used to guide dissection. Preoperative peritumoral injection of aqueous contrast and ICTL was used in 121 (60%) cases, of which 72 (59%) were performed without intraoperative guidance and 49 (41%) with intraoperative MBD guidance. Interestingly, the use of intraoperative guidance did not significantly reduce the rate of complications, although the low overall number may have skewed the results. These results are in accordance with those reported by Rossanese and colleagues, even if the intraoperative guidance techniques slightly varied [154]. In the aforementioned study, the authors compared peripheral unguided lymphadenectomies

with those guided by MBD or anchor wire, and intra- and post-operative complications did not significantly differ between groups. Anyway, the success of the LN excision was significantly higher when MBD (87%) and anchor-wire (94%) were used compared to the unassisted excisions (72%) [154].

The failure to detect and remove one or more SLNs could result in downstaging of the patient, therefore, intraoperative detection techniques are commonly employed to improve the success rate of lymphadenectomies [14,21,22,31,40]. Gariboldi et al (2023) [31] reported a success rate of 95% for SLN excision when guided by a γ-probe in combination with MBD; this figure was higher than that reported for MBD alone or other guiding techniques [22,40,42,154]. Similar results were described in the study of Beer et al (2022), in which the authors compared near-infrared fluorescent image-guided LN dissection with locoregional lymphadenectomies without intraoperative guiding techniques, and no difference in complication rate between the two techniques were reported [42]. In the current study the overall success rate of lymphadenectomies was not investigated, since only cases where lymphadenectomy was completed were included.

Overall, 15 complications were reported; they were classified as grade I according to LeBlanc et al (2021) [152] in 85.7% (12/15) of cases; all were self-limiting and resolved after a median time of 4 days. The remaining 3 complications were graded as grade II: 2 cases of seroma that required a drainage and a prolongation of the NSAIDs therapy, and 1 dehiscence that was treated with application of skin staplers without the need for a revision surgery under general anaesthesia.

Considering the presence or absence of surgical complications as the dependent variable, all the examined variables were identified as non-significant factors ($p>0.05$). To the authors' opinion, this result could be related to the overall low number of complications observed and to the low number of LNs removed from each lymphocenter (only in 7.9% of the cases more than 2 LN were excised).

The rate of complicated surgeries was lower in case of palpable lymph node extirpation, even if there was not a statistically significant difference. The excised SLNs were non-palpable in 10/14 (71.4%) and palpable in 4/14 (28.5%) of the complicated surgeries. This result is not unexpected since, according to the authors' experience, the excision of palpable lymph nodes is easily performed regardless of the technique, while the extirpation of non-palpable LNs may be more challenging, with higher surgical time and morbidities [40]. Interestingly, considering only the non-palpable lymphadenectomies,

no difference between guided and unguided techniques was found, although these results could be biased by the low number of complications.

Methylene blue dye and unguided techniques were used for the extirpation of non-palpable LNs in 57% and 59% of the cases, respectively, while γ-probe plus MBD was used for the excision of only non-palpable lymph nodes. Thus, a difference in complications rate between palpable and non-palpable lymphadenctomies could not be assessed.

Considering the rarity of the complications in our study and the low number of the surgeries that experienced at least one complication (14/201), we used a decision tree method to analyse our data. The probability of developing any complications was significantly dependent on the site of the lymphadenectomy; the excision of mandibular or retropharyngeal LNs had a 22.2% probability of developing at least one complication compared to 5.2% for the other superficial LNs. Furthermore, a cut-off value of 21.5 minutes was found to be the most predictive value of developing complications among mandibular and retropharyngeal lymphadenectomies. These results are probably related to the anatomy of the neck, which is characterized by a great tissue laxity, and it requires wider surgical exposure and tissue manipulation to reach these lymphocenters. Furthermore, the complication rate associated with the excision of mandibular or retropharyngeal LNs reported in our study (22.2%) was similar to that of other surgeries of the neck [155–157].

Seroma was the most common complication in this study, although not frequently encountered. In human medical literature, seroma and infection are the most common complications [125,158,159]. In a recent study on the complication rate associated to the number of LNs excised through two different skin incisions in inguinal-femoral lymphadenectomies for vulvar cancer in humans, the surgical technique that allowed the extirpation of the largest number of LNs was the one with the higher rate of major (grade 3) complications [160]. In the present study, no correlation between the number of harvested LNs and the likelihood of experiencing at least one complication was demonstrated, as reported in previous veterinary studies [40,43,161]. This is probably due to the low number of lymph nodes excised from a single lymphocenter in dogs compared to human beings [126,127].

Lymphoedema was reported in 3 lymphadenectomies in this study, two of which included the bilateral extirpation of inguinal lymph nodes in a dog. These findings support the fact that lymphedema is probably related to the extirpation of all the

lymphocenters draining a specific area. Anyway, lymphoedema was always temporary and reversible, in accordance with the results reported in previous veterinary reports [40,43]; the two dogs that experienced this complication healed spontaneously within 7 days, as opposed to what is reported in human patients [162,163]. The main difference between lymphedema reported in this study and human lymphedema may be the greater extent of lymphadenectomies and number of LNs excised in human beings [162], but also the differences in the lymphatic drainage between dog and man. Human researchers strongly work on finding animal models for the study and the treatment of lymphoedema [164]. The dog was the first animal used to investigate the secondary lymphoedema in human beings and was validated as a model by recent studies [110,164]. However, it seems clear that causing a stable lymphoedema in the canine limb requires the extirpation of all lymphocenters and the disruption of both the superficial and deep lymphatics of the limb [110,164].

Nerve injuries were never reported in case of axillary surgeries, although axillary paraesthesia is a very common complication in human patients [123,124]. The diagnosis of axillary paraesthesia in people relies heavily on self-reporting, something the veterinary patients are not able to do [79]. In dogs, assessment of neuropathic pain is difficult to perform, therefore, only the alterations of the main reflexes of the thoracic limb were assessed in case of axillary lymphadenectomy. In human medicine, brachial plexus injury is a serious but rare complication [124]. The rarity of this major nerve damage and the difficulty in the diagnosis of axillary paraesthesia in the dog is probably the reason why neurologic dysfunctions have not been reported in the study population. A mild lameness (grade I and II) was reported in three dogs that underwent axillary lymphadenectomies. This may be related to the wound in the axilla or to a stretching of nerves or muscles in this district. However, lameness resolved spontaneously within 5 days in all cases.

There was no statistically significant difference between institutions where the lymphadenectomies were performed in this study. This is a relevant observation, since it may reflect the uniformity of the surgeons' experience among the centres and, consequently, provide for better reliability and significance of these results. Conversely, this may also imply that lymphadenectomy is a procedure which requires a short learning curve, and it may be easily performed, once acquired [165,166]. Moreover, the procedures performed by 6 different surgeons may mirror the "real life" scenario.

One limitation of this study was its retrospective nature and the subjective assessment of the complications, which may have resulted in a non-uniform judgement of the severity of the same event. Moreover, given the rarity of the complications associated with lymphadenectomy, a larger study population may be necessary to better analyse the difference in complication rate between guided and unguided LNs excision and reach a statistical relevance.

In conclusion, in the study population lymphadenectomy of superficial SLNs was associated with a low rate of self-limiting mild complications that usually resolved in few days. The complication rate among the different guiding techniques was not statistically significant, thus, the first hypothesis of this study was rejected. Mandibular and retropharyngeal lymphadenectomies that lasted more than 21.5 minutes were more prone to develop at least one complication.

Further prospective, randomized clinical trials are required to compare guided and unguided lymphadenectomies of non-palpable SLN, and to better analyse the effect of the site of the lymph nodes removed and the surgical time on the presence or absence of complications.

Overall, lymphadenectomy of the superficial SLNs is a safe procedure, easy to perform in most cases and worth being undertaken for both diagnostic and therapeutic purposes. Gamma probe and MBD and MBD alone are both safe techniques and should be employed when required, although their use does not prevent the risk of rare complications.

Conclusions

The assessment of LN status is a crucial step to establish the extent of cancer disease, to suggest proper treatment, and to accurately predict prognosis in veterinary oncology. The identification and excision of the so called “sentinel lymph nodes” is a well-consolidated concept in human medicine, while is gaining increased attention in veterinary oncology, representing a revolution in the management of many tumours, but mainly in canine MCT. The number of studies focused on the research of sentinel lymph node has enormously increased in the last years in the veterinary practice, but a limited number of them have specifically analysed the possible causes of failures of SLN identification associated with SLN extirpation in dogs.

In this scenario, the results of this PhD project provided important information on SLN identification. Our result proved that the strict adherence to the lymphosomes scheme proposed by Suami et al. (2013) could lead to the higher agreement between the regional and sentinel lymph nodes detection than that previously reported, using either ICTL or MB. This information proved our speculation that the lack of standardization of the definition of RLNs increased the non-concordance rate. Moreover, we described for the first time that in case of MCT larger than 6 cm and/or with a “peritumoral halo” the ICTL has a high failure rate, questioning its usefulness in these cases. We are not aware of any publications describing this “peritumoral halo” sign as cause of failure in the detection of SLN in dogs with MCT undergoing ICTL, but this could be an interesting point of discussion with clinical implications.

Patnaik grade 2/Kiupel low-grade (G2/LG) represent the majority of diagnosed cMCTs, with a reported incidence of 53.6–57.6%. It has been reported that a variable percentage of dogs (3–17%) with G2/LG dies from tumour-related causes. Ki67 and mitotic count are the two most investigated proliferation markers in cMCTs. However, different Ki67 cut-offs have been proposed by previous studies, and have always been assessed in cMCTs of different histological grade in heterogeneous canine populations; moreover, the LNs metastatic status has never been included. One of the objectives of this PhD project was to answer to the following clinical question: Is Ki67 useful to identify a subset of more aggressive G2/LG with HN2 lymph node? Moreover, we speculated that the reported aggressiveness could have been due to the undetected nodal metastatic disease, rather than to the high Ki67 index since that studies were conducted when the role of lymph nodes in the management of MCT was marginal. Our

results showed that all dogs with G2/LG with HN2 LN treated with surgery alone had a good prognosis; no local, nodal or distant metastasis occurred in the timespan considered, and the Ki67 index did not have prognostic impact. These results remarked the importance of the histological status of both primary tumour and draining lymph node to better define the prognosis while Ki67 index does not provided additional information.

Lastly, while most of the literature on SLN mapping is focused on describing the rate of disagreement between RLN and SLN, we investigated the incidence rate and the severity of the complications following peripheral lymphadenectomy in dogs. We hypothesized that the rate of complications could increase when any intraoperative guidance was used during lymphadenectomy. Interestingly, we reported a low overall incidence of complications (7.5%) and, as expected, the majority was classified as mild (85.7%). Moreover, the use of intraoperative guidance did not reduce the rate of complications, although the probability of developing any complications was significantly dependent on the site of the lymphadenectomy.

In conclusion, sentinel lymph node mapping and excision plays an important role in the diagnosis and therapy of canine MCTs, the technique used for the detection is crucial and should be chosen based on clinical presentation of the tumour to avoid failures. Anyway, the lymphadenectomy *per se* is usually a rather safe procedure, rarely associated with severe complications.

Scientific projects unrelated to the PhD project

During the research training, the PhD student has been involved in other parallel projects in the surgical oncology field. Hence, these studies will be presented in this section.

Prognostic Value of Ki67 and Other Clinical and Histopathological Factors in Canine Apocrine Gland Anal Sac Adenocarcinoma.
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Animals (Basel). 2021 Jun 2;11(6):1649. doi: 10.3390/ani11061649. PMID: 34199347; PMCID: PMC8228493.

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CANCRO
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