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Weishaar's classification system for nodal metastasis in sentinel lymph nodes: Clinical outcome in 94 dogs with mast cell tumor

Damiano Stefanello[1] | Elisa M. Gariboldi[1] | Patrizia Boracchi[2] | Roberta Ferrari[1] | Alessandra Ubiali[1] | Donatella De Zani[1] | Davide D. Zani[1] | Valeria Grieco[1] | Chiara Giudice[1] | Camilla Recordati[1] | Mario Caniatti[1] | Luigi Auletta[1] | Lavinia E. Chiti[1,3]

[1]Dipartimento di Medicina Veterinaria e Scienze Animali, Università degli Studi di Milano, Lodi, Italy

[2]Dipartimento di Scienze Biomediche e Cliniche, Università degli Studi di Milano, Milano, Italy

[3]Clinic for Small Animals Surgery—Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

Correspondence
Damiano Stefanello, Dipartimento di Medicina Veterinaria e Scienze Animali—Università degli Studi di Milano, via dell'Università 1, 26900 Lodi, Italy.
Email: damiano.stefanello@unimi.it

Abstract

Background: The therapeutic role and prognostic relevance of lymphadenectomy in mast cell tumor (MCT) has historically been evaluated on regional rather than sentinel lymph nodes.

Hypothesis/Objectives: To update information about the association of histological nodal (HN) classes with clinical outcome in dogs with MCT after tumor excision and extirpation of normal-sized sentinel nodes (SLN) guided by radiopharmaceutical.

Animals: Ninety-four dogs with histologically-confirmed treatment-*naïve* MCT (71 cutaneous, 22 subcutaneous and 1 conjunctival MCT) were included if without: distant metastases, lymphadenomegaly, concurrent mixed cutaneous, and subcutaneous MCT.

Methods: This was a monoistitutional cohort study. Tumors characteristics were retrieved and SLNs were classified according to Weishaar's system. Incidence of MCT-related events (local, nodal, distant relapse), de novo MCT or other tumors and death (MCT-related and non-MCT-related), were recorded. Incidence curves were compared among the HN classes.

Results: Twenty-seven dogs had HN0, 19 HN1, 37 HN2, and 11 HN3 SLN. Thirteen (2 HN0, 4 HN2, and 7 HN3) received adjuvant chemotherapies. Kiupel high grade, increasing number of SLN and lymphocentrums were associated with higher HN classes. Five dogs died for MCT-related causes: 1 low-grade (HN0) and 1 subcutaneous (HN3) had a local relapse, 2 high-grade had distant relapse (HN3-HN0) and 1 dog developed disease progression from a de novo subcutaneous MCT. No nodal relapse was registered. Fourteen dogs developed de novo MCTs.

Conclusion/Discussion: Low grade/low-risk MCT with nonpalpable and normal sized SLN have a favorable outcome independently from the HN. Result should be

Abbreviations: HN, histological node; MCT, mast cell tumor; RLN, regional lymph node; SLN, sentinel lymph node; SLC, sentinel lymphocentrum; WHO, World Health Organization.

Damiano Stefanello and Elisa M. Gariboldi contributed equally as first authors.

considered strictly related to the successful SLN detection guided pre- and intraoperative by radiopharmaceutical markers.

KEYWORDS
canine, mapping, mast cell, prognosis, surgery

1 | INTRODUCTION

The presence of lymph node metastasis is a well-accepted negative prognostic factor for mast cell tumors (MCT) in dogs.[1-4] The histological system for the classification of nodal metastasis proposed by Weishaar et al has lately become pivotal for staging, adjuvant treatments recommendations and prognostication.[3,5-8] According to the current body of literature, occult early/overt (HN2/HN3) metastasis in nonpalpable, clinically normal lymph nodes (regional or sentinel) can occur in 33%-68% of MCT bearing dogs.[9-14] Hence, lymph node extirpation has become routinary in the surgical treatment of MCT.[9,11-15] In studies on MCT in dogs, sentinel lymph node (SLN) can be found at different locations compared to the anatomically expected regional lymph node (RLN) in 28%-63% of cases.[9,11-13,15] Furthermore, non-guided intraoperative identification of the lymph nodes could be hampered by several factors, such as anatomical localization, small size and presence of overlaying adipose tissue.[11,16,17] These considerations promoted scientific interest on pre- and intraoperative mapping techniques for SLN identification and extirpation.[18,19]

In the last 5 years, the metastatic status of RLN and SLN have been separately evaluated in cutaneous and subcutaneous MCT. Regarding cutaneous MCT, lymphadenectomy in stage I and II has a therapeutic effect improving the prognosis.[5-8,20] In Weishaar's study MCT with early (HN2) or overt nodal metastasis (HN3) seemed to bear a worse prognosis, despite recently they seem to show a more favorable outcome.[3,7] For instance, Kiupel low grade MCTs with overt nodal metastases (HN3) treated with MCT excision plus lymphadenectomy and adjuvant chemotherapy displayed improved outcome (in term of disease free interval) compared to Kiupel high grade MCT.[21] Kiupel low grade MCT and early nodal metastasis (HN2) have a favorable outcome, even without adjuvant chemotherapy.[7] For subcutaneous MCT information on the prognostic relevance of Weishaar's classification for nodal metastasis[12,14,22] and outcome[22-24] are limited to a few recent studies.

Studies have explored the favorable therapeutic role of lymphadenectomy mainly by including RLN instead of SLN, whereas the effect on clinical outcome of nodal metastases as classified by Weishaar's study[3] has not been specifically evaluated on SLN yet. In the current scenario where surgical management of cutaneous or subcutaneous MCT in dogs includes more and more often removal of nonpalpable and normal-sized SLN guided by various mapping techniques, an update of the clinical outcome of each histologic metastatic class according to Weishaar's classification is due.

This monoistitutional cohort study aimed to increase knowledge about the role of histological nodal metastasis (HN) classes on clinical outcome in dogs with cutaneous and subcutaneous MCT undergoing tumor excision and radiopharmaceutical-guided extirpation of nonpalpable and normal sized SLN.

2 | MATERIALS AND METHODS

Client-owned dogs with single or multiple concurrent MCT at first presentation that underwent curative intent local surgical treatment and extirpation of nonpalpable and normal sized SLN, from May 2017 to October 2022, were enrolled. The SLN was defined as the first lymph node/s receiving drainage from the primary tumor and was thus expected to be first site of metastasis[9] conversely the regional lymph node was defined as the anatomically closest lymph node according to Suami's study.[25] To be eligible for inclusion, histopathological report of the excised MCT and SLN had to be available for review.

Dogs were excluded if at admission they presented: distant metastasis, visceral MCT, local relapse, regional lymphadenomegaly with or without cytological diagnosis of nodal metastasis. They also were excluded if underwent previous regional or sentinel lymphadenectomy, neoadjuvant chemotherapy or neoadjuvant locoregional radiotherapy. Lastly, dogs were excluded if they presented a de novo MCT after a previously excised MCT; had concurrent histologically confirmed cutaneous and subcutaneous MCT; had cutaneous MCT for which combined histological grading classification of Patnaik[26] and Kiupel[27] was not available[26,27]; had subcutaneous MCT with no information available regarding the histological pattern.[28]

All owners signed a written informed consent for SLN mapping, surgery and data collection. All the procedures were performed in accordance with the Italian National legislation for animal welfare (DL 14th March, 2014 n.26) and best standard of veterinary practice (Good Veterinary Practice from the Federation Veterinary Europe—Italian National Federation of the Veterinary' Orders [FNOVI] 29th January, 2005).

The following information were retrieved: dogs signalment (breed, sex, age, bodyweight) and MCT clinical characteristics (single or multiple presentation, anatomical location, size, ulceration). Anatomical location was categorized in high-risk (head, neck, digital, inguinal/perineal region) and low-risk (trunk, abdominal wall, and limbs excluding digital region).[29,30]

Preoperative work-up included complete blood cell count with differential, serum biochemistry, and oncological staging including 3-views thoracic radiographs, abdominal ultrasound and fine-needle aspiration of liver and spleen regardless of their sonographic appearance.[31]

Included dogs underwent SLN mapping and removal, concurrent with the excision of the primary MCT[9,11,32] or of the scar from an excised tumor.[33] For SLN mapping and extirpation, preoperative planar lymphoscintigraphy[32] was associated with intraoperative detection using a hand-held gamma probe (Crystal probe SG04, Crystal Photonic GmbH, Berlin, Deutschland) and direct visualization with methylene blue (SALF S.p.A; Cenate Sotto, Bergamo, Italy) in all the dogs.[11] The preoperative technique was used for identification of sentinel lymphocentrum (SLC), while the gamma probe and methylene blue guided its surgical exploration and SLN removal. All lymph nodes that were visually identified during the surgical dissection of the SLC were removed, even if they were not blue, nonradioactive or both. Lymphadenectomy was considered complete when the radioactive count of the SLC was less than 10% of the hottest SLN extirped and no other lymph nodes were visible or palpable. Lymph nodes related variables were recorded: number of SLCs and correspondence with the regional ones,[25] total number and size of extirpated SLNs.

Surgical treatment of MCT was categorized as follows: radical (excision of an entire compartment or anatomical structure excision); wide excision (2-3 cm or proportional lateral margins laterally and at least 1 uninvolved fascial layer for deep margin)[34,35]; planned narrow excision, (if wide resection was not possible, the widest margins were applied based on tumor dimension and localization). In dogs with World Health Organization (WHO)[36] stage 0 MCT, a wide re-excision of the scar was performed, if feasible, concurrently with SLN extirpation.

All MCTs, scars and SLNs removed were submitted to the veterinary pathology unit of our institution for histopathological examination. All specimens were fixed in neutral-buffered 10% formalin, routinely processed for histology and embedded in paraffin. For primary tumors or scars, a complete longitudinal section was examined while the status of surgical margins was assessed using combined 3 dimensional and radial-tangential techniques.[37,38] Histological margins were defined as noninfiltrated if cancerous cells were ≥1 mm away from the margin and infiltrated if they were <1 mm or there was evidence of disease at the periphery of the sample.[39,40]

Grading was determined based on Patnaik and Kiupel grading systems for cutaneous MCT.[26,27] Subcutaneous MCT were categorized according to Thompson et al[28] and conjunctival MCT based on Fife et al.[41] Available data related to mitotic count, assessed at 400× in 2.37 mm^2 according to Meuten et al,[42] presence of multinucleate cells, and Ki67 index[43] were also collected.

Each lymph node was trimmed following the same procedure: the lymph-node was divided in two halves with a longitudinal cut through the hilus. When the lymph-node was thicker than 3 mm (minor axis), additional parallel cuts were performed obtaining multiple slices (1.5 mm-thick each) from each half. The whole sample was processed and for each slice, 2 serial microtomic sections were cut and stained with hematoxylin-eosin and Giemsa stain, respectively. The HN status was assigned to all SLNs according to criteria outlined in Weishaar et al.[3] Lymph nodes and MCT grades were examined independently and blinded by 3 experienced pathologists, 2 of them diplomate, and revised then collegially for the purpose of the study.

Adjuvant chemotherapy treatment was offered in Kiupel high grade/Patnaik grade III cutaneous MCT, and in all cases with HN3 SLN. In low grade cutaneous MCT and in subcutaneous MCT with SLN classified as HN <3 and infiltrated margins a surgical revision was proposed if feasible, otherwise radiation therapy was the first treatment suggested and if refused by the owner chemotherapy was offered. In Kiupel low grade/Patnaik grade I-II cutaneous MCT and low risk subcutaneous MCT completely excised and with SLN classified as HN2, adjuvant treatments were proposed until 2020, thereafter no adjuvant therapy was proposed based on Marconato et al.[7]

After surgical treatment, each dog was reevaluated at 7 and 14 days (or more frequently if required) to assess the healing status of the surgical wounds and any occurrence of complication. Dogs were followed-up by means of clinical reevaluation every 3 months for the first year, and every 6 months thereafter until 2 years from surgery, regardless of adjuvant treatment. Reevaluation consisted with physical examination and staging in order to check local, nodal and distant relapse, and the presence of a de novo MCT. Local relapse was defined as cytological evidence of recurrence within 2 cm of the primary tumor surgical scar. Nodal relapse was defined as the presence of newly diagnosed metastatic lymph nodes confirmed by cytology in previously surgically explored sentinel lymphocentrum. Distant relapse was defined as the cytologically confirmed occurrence of visceral metastasis. Abdominal ultrasound was repeated every 6 months in the first 2 years or earlier in the case of symptoms suggestive of recurrence or progressive disease.

For statistical purpose, in dogs with concurrent multiple MCTs, all the highest negative clinical (dimension, anatomical location and ulceration) and histopathological (histological grade, mitotic count, Ki67 index, presence of multinucleated cell, infiltrated margins, infiltrative pattern) prognostic factors were selected in each dog. Also, because of multiple SLN extirpation for each dog, the HN classes considered was the highest 1.

For the distribution of the variables on a categorical scale, the absolute frequencies and the percentages were reported for each category. For the variables measured on a numerical scale, the distributions were summarized reporting minimum, first quartile (Q1), median, mean, third quartile (Q3) and maximum. For categorical variables the association between variables and HN classes was analyzed by Fisher's exact test. The comparison of the distributions of numerical variables between the HN classes was done by nonparametric analysis of variance (Kruskall-Wallis test).

Time to death was calculated from the date of SLN excision to death. The causes of death were classified in MCT-related or non-MCT-related. The neoplastic events were combined for the following composite endpoints: time to MCT relapse was calculated from the date of surgery to the first occurrence of the target events (local relapse, nodal relapse or distant metastasis), and time to MCT disease was calculated as time to MCT relapse, including time to de novo MCT among the target events. Occurrence of a new tumor different from MCT was considered an event.

For each end-point, the cumulative incidences for the whole case series and for the HN classes were graphically represented. The estimates at 12, 24, 36, 48 months are reported with the 95% confidence interval.

Incidences of death were estimated by Kaplan-Meier method (1 minus the estimated survival probability). The incidence curves for single events and for the composite end-points, were estimated by a method that takes into account the presence of competing risks. In fact, the occurrence of death in the case of a subject who had no other neoplastic events before death prevented the observation of the time to occurrence of the endpoint of interest (competition with the event of interest). Comparison of incidence curves in the categories of HN and chemotherapy was done with Gray's test.[44,45]

The significance level was set at 5%. All analyses were performed with analysis software (R-software); library survival and cmprsk were used to estimate and to compare incidence curves (www.r-project.org).[46,47]

3 | RESULTS

Ninety-four out of 127 dogs with histological diagnosis of MCT met the inclusion criteria. Thirty-three dogs were excluded: 3 dogs presented distant metastasis; in 1 dog the owner declined staging procedures; 5 dogs presented recurrent MCTs; 2 dogs had enlarged and cytologically metastatic regional lymph node at admission; 3 dogs underwent neoadjuvant chemotherapy; 3 dogs did not receive SLN extirpation (2 because of the pitfall of the mapping technique and 1 because the owner did not accept the axillary lymphadenectomy), 10 dogs were bearing a de novo nonsynchronous MCT; 6 dogs had multiple mixed concurrent cutaneous and subcutaneous MCT.

Mixed breed was the most represented breed in the study sample (24/94; 26%), followed by Labrador retriever (12/94; 13%), Golden retriever (7/94; 8%), Boxer (7/94; 8%), English Setter (5/94; 5%), Pug (4/94; 4%), Jack Russel Terrier (3/94; 3%); American Staffordshire Terrier (3/94; 3%), Maltese (2/94; 2%), French Bouledogue (2/94; 2%) Pitbull (2/94; 2%), and other breeds 1 for each (23/94; 24%). Sex was: intact male (43/94; 46%); neutered male (11/94; 12%); intact female (6/94; 6%) and spayed female (34/94; 36%); Mean and median age were 7.34 and 7 years (range, 0.7-14 years), and mean and median bodyweight were 24.92 and 26.2 kg (range, 3-62 kg). Eighty-six dogs (91.49%) had a single MCT, while 8 dogs (8.51%) had multiple concurrent MCTs (6 dogs had 2 MCTs; 2 dogs had 3 MCTs). Of the dogs with multiple MCT, 7 had multiple cutaneous MCT and 1 had multiple subcutaneous MCT. In 62 dogs (66%) MCTs were located in a low-risk anatomical site while 32 dogs (34%) presented at least 1 MCT in a high-risk location. Mean and median tumor maximum diameter were 21.5 and 20 mm (range, 2-150 mm). Tumors were nonulcerated in 78 dogs (83%), while there was at least 1 ulcerated MCT in 16 dogs (17%). At inclusion, considering the exclusion criteria adopted for this study, 83 dogs had a measurable MCT while 11 dogs had a scar of MCT previously excised in other facilities. Between the latter, 6 dogs had scars with histopathological infiltrated margins (WHO stage 0), while the remaining 5 did not present neither macroscopic nor microscopic disease (based on histopathological reports). Of the 83 dogs with macroscopic tumor 10 received a radical surgery, 56 a wide excision, and 17 a planned narrow excision. In the 6 cases with WHO stage 0 MCT a wide margins re-excision of the scar was performed.

TABLE 1 Details of distribution of histological characteristics in the study sample.

Histological pattern		Number of patients and % of different histological grade/pattern
Cutaneous mast cell tumor (n = 71)	Patnaik	Grade I: 12 (16.9%)
		Grade II: 56 (78.87%)
		Grade III: 3 (4.23%)
	Kiupel	Low-grade: 68 (95.77%)
		High-grade: 3 (4.23%)
	Patnaik/Kiupel combined	Patnaik grade I/Kiupel low-grade: 12 (16.9%)
		Patnaik grade II/Kiupel low-grade: 56 (78.87%)
		Patnaik grade III/Kiupel high-grade: 3 (4.23%)
Subcutaneous (n = 22)		Infiltrative pattern: 13 (59.09%)
		Expansive pattern: 2 (9.09%)
		Combined pattern: 7 (31.81%)
Conjunctival (n = 1)		(1.06%)

A total of 126 SLCs were detected and surgically explored: 64 dogs (68%) had a single SLC and 30 dogs (32%) had multiple SLCs (28 dogs had 2 SLCs, and 2 dogs had 3 SLCs). In 48 dogs (51%) at least 1 SLC did not correspond with the regional 1. A total of 186 SLNs were excised, and the mean and median maximum diameter of the nodes were 15.28 and 13 mm (range, 2-60 mm).

At histopathological analysis 71 dogs (76%) had a cutaneous MCT, 22 dogs (23%) had subcutaneous MCT, and 1 dog (1%) had a conjunctival MCT (Table 1). In 16 dogs (17%) MCT had multinucleate cells (14 cutaneous and 2 subcutaneous); 88 dogs had a mitotic count ≤1 (66 cutaneous, 21 subcutaneous, 1 conjunctival), 4 dogs had a mitotic count >1 (all cutaneous) and in 2 dogs mitotic count was not available. The immunohistochemical information of Ki67 index was available in 50% of MCTs: 34 cutaneous (7 of them showed a value >93/1000), 12 subcutaneous (6 of them showed a value >23/1000) and 1 conjunctival (1/1000). In 16 dogs (17%) surgical margins resulted infiltrated: 11 dogs (7 cutaneous and 4 subcutaneous MCTs) received a planned narrow excision (4 in high-risk anatomical location and 7 in low-risk anatomical location), 4 dogs (all cutaneous MCTs) a radical excision and 1 dog (subcutaneous MCT) a wide excision.

Between the 186 excised SLNs, 72/186 (38.7%) were HN0, 35/186 (18.8%) were HN1, 60/186 (32.3%) were HN2, and 19/186 (10.2%) were HN3. Considering the higher HN in each dog, 27/94

TABLE 2 Association between histological nodal classes and clinical, pathological variables: categorical variables.

Categorical variables	HN0	HN1	HN2	HN3	Fisher exact test
Anatomical location low-risk	18 (66.7%)	13 (68.4%)	25 (67.6%)	6 (54.5%)	$P = .87$
Anatomical location high-risk	9 (33.3%)	6 (31.6%)	12 (32.4%)	5 (45.5%)	
Not ulcerated	23 (85.2%)	18 (94.7%)	28 (75.7%)	9 (81.8%)	$P = .36$
Ulcerated	4 (14.8%)	1 (5.3%)	9 (24.3)	2 (18.2%)	
Single presentation	27 (100%)	18 (94.7%)	32 (86.5%)	9 (81.8%)	$P = .09$
Multiple presentation	0 (0%)	1 (5.3%)	5 (13.5%)	2 (18.2%)	
Cutaneous MCT	22 (81.5%)	15 (78.9%)	29 (78.4%)	5 (45.5%)	$P = .13$
Subcutaneous MCT	5 (18.5%)	4 (21.1%)	8 (21.6%)	5 (45.5%)	
Conjunctival MCT	0 (0%)	0 (0%)	0 (0%)	1 (9.1%)	
Patnaik grade I	7 (31.8%)	2 (13.3%)	3 (10.3%)	0 (0%)	$P = .05$
Patnaik grade II	13 (59.1%)	13 (86.7%)	26 (89.7%)	4 (80%)	
Patnaik grade III	2 (9.1%)	0 (0%)	0 (0%)	1 (20%)	
Kiupel low-grade	20 (90.9%)	15 (100%)	29 (100%)	4 (80%)	$P = \mathbf{.049}$*
Kiupel high-grade	2 (9.1%)	0 (0%)	0 (0%)	1 (20%)	
Patnaik grade I/Kiupel low-grade	7 (31.8%)	2 (13.3%)	3 (10.3%)	0 (0%)	$P = .05$
Patnaik grade II/Kiupel low-grade	13 (59.1%)	13 (86.7%)	26 (89.7%)	4 (80%)	
Patnaik grade III/Kiupel high-grade	2 (9.1%)	0 (0%)	0 (0%)	1 (20%)	
SC-MCT Infiltrative pattern	4 (80%)	2 (50%)	6 (75%)	1 (20%)	$P = .24$
SC-MCT Expansive pattern	0 (0%)	0 (0%)	0 (0%)	2 (40%)	
SC-MCT Combined pattern	1 (20%)	2 (50%)	2 (25%)	2 (40%)	
Absence of multinucleate cells	21 (77.8%)	16 (84.2%)	32 (86.5%)	9 (81.8%)	$P = .82$
Presence of multinucleate cells	6 (22.2%)	3 (15.8%)	5 (13.5%)	2 (18.2%)	
Not infiltrated surgical margins	23 (85.2%)	17 (89.5%)	30 (81.1%)	8 (72.7%)	$P = .57$
Infiltrated surgical margins	4 (14.8%)	2 (10.5%)	7 (18.9%)	3 (27.3%)	
Mitotic count ≤1	26 (96.2%)	18 (94.7%)	34 (97.1%)	10 (90.9%)	$P = .81$
Mitotic count >1	1 (3.8%)	1 (5.3%)	1 (2.9%)	1 (9.1%)	
Single SLC	23 (85.2%)	14 (73.7%)	23 (62.2%)	4 (36.4%)	$P = \mathbf{.02}$*
Multiple SLC	4 (14.8%)	5 (26.3%)	14 (37.8%)	7 (63.6%)	
Single SLN	16 (59.3%)	10 (52.6%)	11 (29.7%)	3 (27.3%)	$P = .06$
Multiple SLN	11 (40.7%)	9 (47.4%)	26 (70.3%)	8 (72.7%)	

Abbreviations: MCT, mast cell tumor; SC-MCT, subcutaneous mast cell tumor; SLC, sentinel lymphocentrum; SLN, sentinel lymph node.
*p-values referes to all statistically significant values.

dogs (29%) were HN0, 19/94 dogs (20%) were HN1, 37/94 dogs (39%) were HN2, 11/94 dogs (12%) were HN3 (Table 2).

Of the 25 dogs eligible for adjuvant therapy based on the aforementioned criteria, none of the owners accepted adjuvant local radiation therapy. Thirteen dogs received various adjuvant chemotherapy protocols: 11 vinblastine plus prednisone (2.0 mg/m^2 weekly for 4 treatments then biweekly for 4 treatments), 1 lomustine alone (90 mg/m^2 PO every 3 weeks) and 1 chlorambucil (5 mg/m^2 PO every other day) plus prednisone. Reasons for administering chemotherapy were: infiltrated margins in 5 dogs (4 HN2; 1 HN0), HN3 SLNs in 4 dogs, both HN3 and infiltrated margins in 2 dogs, Patnaik grade III/Kiupel high grade MCT and HN3 1 dog, and lastly 1 dog (HN0) had Patnaik grade III/Kiupel high grade and infiltrated margins. Four dogs with HN3 SLN, and 1 dog with high grade/III Patnaik (HN0) did not receive adjuvant chemotherapy because the owner declined it. Among the 8 dogs with infiltrated margins not receiving chemotherapy, 3 received re-excision (2 HN1; 1 HN2), while 5 dogs (2 HN2, 2 HN0; 1 HN3) did not receive any treatment because of the owner's refusal. Overall, 93% of dogs with HN0 (25/27), 100% of dogs with HN1 (19/19), 89% of dogs with HN2 (33/37), and 36% of dogs with HN3 (4/11) did not receive adjuvant chemotherapy treatment.

A statistically significant association was found between the presence of more than 1 draining SLCs and increase of HN classes ($P = .02$). A significance was also found when considering increasing number of extirped SLN and HN classes ($P = .03$), and Kiupel grade and HN classes ($P = .049$). No other variables were significantly associated with HN classes (Tables 2 and 3).

Continuous variables	HN0	HN1	HN2	HN3	Kruskal-Wallis test
Age					$P = .21$
Min	1.0	4.0	3.0	0.7	
Q1	6.2	5.0	6.0	6.1	
Median	8.3	6.0	7.6	7.0	
Mean	8.1	6.7	7.2	7.2	
Q3	10.0	7.5	9.0	9.0	
Max	13.0	13.0	12.0	14.0	
Body weight					$P = .87$
Min	3.2	5.0	3.0	3.5	
Q1	9.2	16.2	15.0	17.5	
Median	26.0	27.0	29.6	26.4	
Mean	23.3	25.9	26.1	23.4	
Q3	32.7	34.5	34.0	30.0	
Max	45.0	55.0	62.0	35.0	
Tumor dimension					$P = .10$
Min	2.0	10.0	4.0	3.0	
Q1	10.0	10.5	10.0	17.5	
Median	10.0	20.0	20.0	30.0	
Mean	15.5	18.7	24.5	31.1	
Q3	20.0	24.0	30.0	43.5	
Max	35.0	40.0	150.0	76.0	
Number of SLN					$P = .03$*
Min	1.0	1.0	1.0	1.0	
Q1	1.0	1.0	1.0	1.5	
Median	1.0	1.0	2.0	2.0	
Mean	1.6	1.7	2.2	2.5	
Q3	2.0	2.5	3.0	3.5	
Max	4.0	3.0	5.0	4.0	

*Statistically significant.

TABLE 3 Association between histological nodal classes and clinical, pathological variables: continuous variables.

TABLE 4 Cumulative incidence of occurrence of events (relapse, de novo MCT, and other tumors different from MCT) at 12, 24, 36, and 48 months.

Events	Months	Cumulative incidence	Lower 95% CI	Upper 95% CI
Relapse occurred (local and distant) (n = 4 events)	12	2.20%	0.42	7.01
De novo MCT (n = 14 events)	12	5.70%	2.10	11.98
	24	14.63%	7.26	24.46
	36	18.87%	10.01	29.86
	48	26.58%	14.02	40.91
Other tumors different from MCT (n = 13 events)	12	3.55%	0.94	9.19
	24	11.71%	5.32	20.85
	36	13.93%	6.60	23.95
	48	26.09%	12.52	41.95

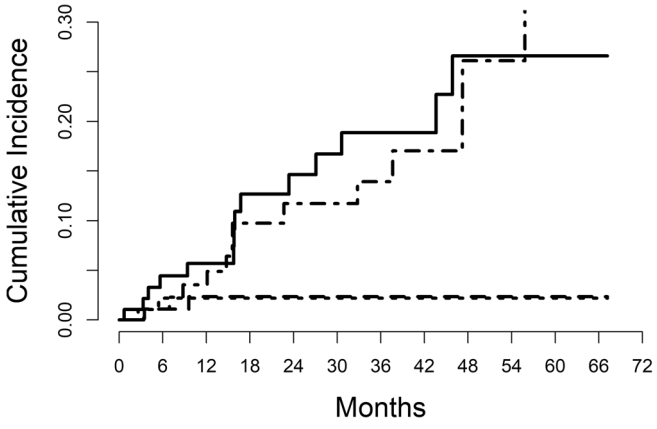

FIGURE 1 Cumulative incidence of different neoplastic events (relapse, de novo MCT, and other tumors different from MCT). Dashed line: local relapse (LR); dotted line: distant metastasis (DM); continue line: de novo MCT; line-dot line: other tumors different from MCT.

The events registered were 31 out of 94 dogs. Four dogs had progressive disease of MCT, all within the first 12 months after surgery: 2 dogs had local relapse (1 HN3 and 1 HN0), 2 dogs had distant relapse (1 HN3 and 1 HN0) (occurrence range, 80-292 days), while neither of dogs presented nodal relapse. Furthermore, 14 dogs presented de novo MCT (range of 21-1399 days) and 13 dogs developed a new tumor different from MCT (range of 103-1703 days).

At the end of the study period, 73 dogs were still alive in good clinical condition without local, nodal and distant relapse and non-MCT-related diseases. Twenty-one dogs died, of which 5 for MCT-related causes. Four dogs died after progressive disease: 2 of them developed a local relapse, and 2 had distant metastasis. One dog died after de novo MCT progressive disease. The remaining dogs died for non-MCT-related causes: 9 for new tumor different from MCT (2 soft tissue sarcoma, 2 hemangiosarcoma, 1 each of multicentric lymphoma, cardiac tumor, plasmacytoma, perivascular wall tumor, adrenal neoplasia), and 7 dogs died for other causes (4 cardiac failure, 1 each of severe hepatopathy, idiopathic epilepsy, thrombotic disease).

The cumulative incidence of different neoplastic events (local and distant relapse, de novo MCT and others tumor different from MCT) in the study sample is reported in Table 4 and Figure 1. Because of

TABLE 5 Cumulative incidence of events for time to MCT relapse, time to MCT disease.

	Months	Cumulative incidence of events	Lower 95% CI	Upper 95% CI
Time to MCT relapse (n = 4 events)	12	4.65%	1.49	10.64
Time to MCT disease (n = 18 events)	12	10.28%	5.01	17.75
	24	19.26%	10.82	29.52
	36	23.51%	13.75	34.79
	48	31.27%	17.91	45.57

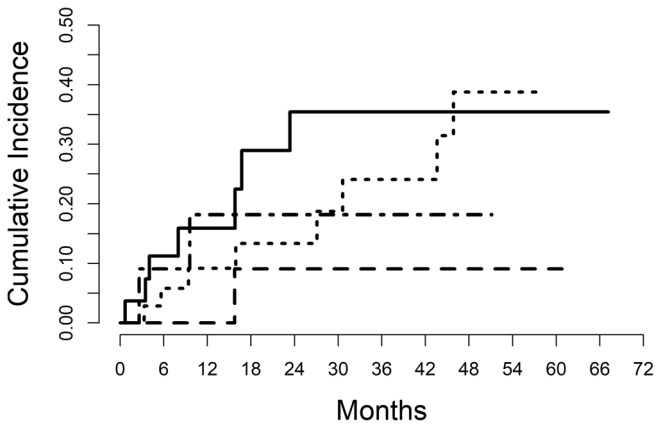

FIGURE 2 Cumulative incidence of MCT disease (composite end-point with target events: local relapse, nodal relapse, distant metastases, and de novo MCTs) in each HN classes. Continue line: HN0; dashed line: HN1; dotted line: HN2; line-dot line: HN3.

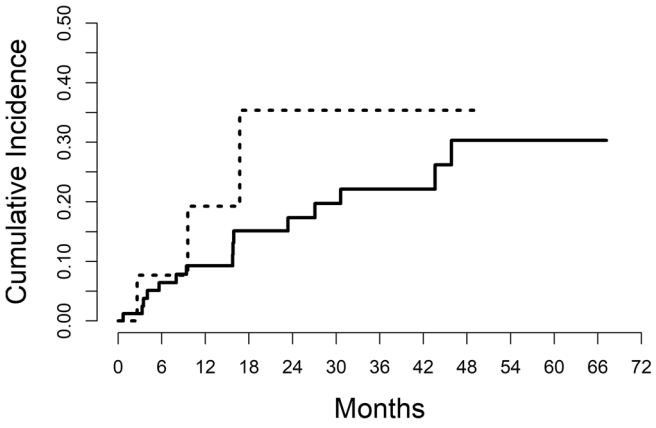

FIGURE 3 Cumulative incidence of MCT disease (composite end-point with target events: local relapse, nodal relapse, distant metastases, and de novo MCTs) in dogs with and without adjuvant chemotherapy treatment. Continue line: without chemotherapy; dashed line: with chemotherapy.

TABLE 6 Cumulative incidence of MCT disease (composite end-point with target events: local relapse, nodal relapse, distant metastases, and de novo MCTs) in each HN classes.

HN classes	N° of events occurred	N° of dogs in each HN classes	Months	Cumulative incidence	Lower 95% CI	Upper 95% CI
HN0	7	27	12	15.94%	4.76	33.02
			24	35.45%	14.13	57.72
HN1	1	19	12	0%	–	–
			24	9.09%	0.43	34.38
HN2	8	37	12	9.19%	2.27	22.20
			24	13.37%	3.98	28.49
			36	24.07%	8.89	43.26
			48	38.79%	15.86	61.44
HN3	2	11	12	18.18%	2.49	45.54

Note: The estimates are reported only for months intervals within new events occurred (see Figure 2).

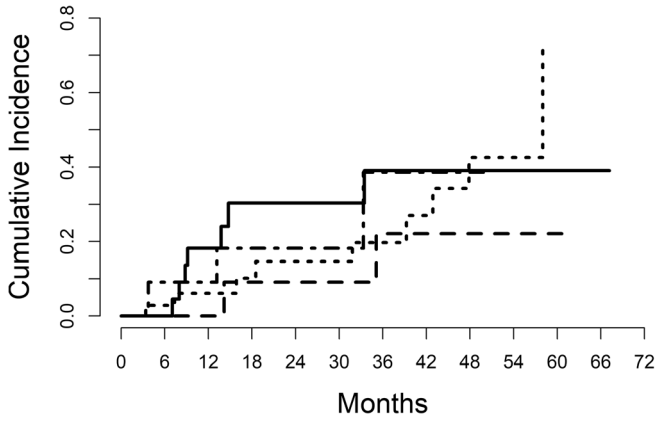

FIGURE 4 The incidence of death from all causes (MCT-related and non-MCT-related), for each HN classes. Continue line: HN0; dashed line: HN1; dotted line: HN2; line-dot line: HN3.

the small number of events related to MCT relapse (n = 4 events), the analysis of the incidence of the events on the different HN classes was not performed. The cumulative incidence of events in all HN classes during the endpoints (time to MCT relapse; time to MCT disease), is reported in the Table 5.

Cumulative incidence of MCT disease (composite end-point with target events: local relapse, nodal relapse, distant metastases and de novo MCTs) in each HN classes in Figure 2 and Table 6 (for each HN category). No significant differences were found ($P = .40$). Cumulative incidence of MCT disease (composite end-point with target events: local relapse, nodal relapse, distant metastases and de novo MCTs) in dogs with and without adjuvant chemotherapy treatment is showed in Figure 3 and there was no significant difference in the incidence of events between treated and untreated dogs ($P = .43$).

Three events (1 local relapse, 1 distant metastasis and 1 de novo MCT) were recorded in dogs with chemotherapy, all within the firsts 24 months. Events registered in dogs without adjuvant chemotherapy were: 1 local relapse, 1 distant metastasis, 10 de novo MCT, 10 new tumors, while 3 dogs developed both a de novo MCT and new non-MCT tumor. There was no significant difference in the incidence of events between treated and untreated dogs ($P = .97$).

Because of the small number of MCT-related death (n = 5), it was not possible to perform an analysis of the incidence of death on the different HN classes. It was possible, instead, to calculate the incidence of death for both MCT-related and non-MCT-related causes, categorized in HN classes (Figure 4). There were no statistically significant differences between the HN classes and incidence of death ($P = .65$).

4 | DISCUSSION

Histological analysis and classification of nodal metastasis according to Weishaar et al is considered a standard approach for staging and prognostication of dogs with MCT.[3] While a better outcome is expected in dogs with HN0-HN1 lymph nodes, a worse prognosis is associated with HN2-HN3 lymph nodes.[3] However, Weishaar's paper included both regional and sentinel lymph nodes without distinguishing between clinically normal and abnormal lymph nodes.[3] Lately, mapping and excision of clinically nonpalpable and normal-sized SLN instead of RLN has become a part of surgical treatment of MCT in dogs because of the high discrepancy between sentinel and regional nodes and to allow for early diagnosis of nodal metastases.[9,11-13,15] Hence, the correlation between the histological nodal status and outcomes needs to be updated. Accordingly, the present study focusses on a specific cohort of dogs with MCT at first presentation with no signs of nodal and distant metastasis, amenable to curative-intent surgery and SLN extirpation.

In contrast to Weishaar's results, in this study low grade/low risk MCT with HN classes 2 showed a clinical outcome as good as HN0 and HN1, even if for most of them adjuvant chemotherapy was not administered. Dogs with low grade/low risk MCT and HN3 SLN receiving adjuvant chemotherapy had a good clinical outcome as well. Only 5 dogs died for causes related to MCT, and this event was not significantly correlated to the HN classes (2 HN3, 2 HN0 and 1 HN2). It should be mentioned that all these tumors displayed 1 or more negative prognostic factor (such as infiltrated margins, Patnaik grade III/Kiupel high grade, high Ki67 or large dimension), which may have biased the evaluation of the pure effect of HN status of SLN on outcome. For the remaining 89 MCT (of which 36 HN2 and 9 HN3) the combined curative intent surgery and SLN removal seemed to favor a good clinical outcome regardless of the metastatic status of extirped nodes. In fact, 14 dogs developed new MCTs, 13 developed a new tumor and 16 dogs died for MCT-unrelated causes during follow-up independently to HN classes.

To date, Weishaar's study is the only 1 that investigated the role of different HN on the clinical outcome of dogs with MCT. However, from that study it cannot be extrapolated the proportion of SLN or RLN removed. Furthermore, the authors reported that 15 out of 41 dogs died for MCT-related causes, 8 of which were Patnaik grade III (half of which with HN3 lymph nodes), 6 were Patnaik grade II (half of which with HN3 lymph nodes), and 1 was Patnaik grade 1 with an HN3 lymph node. Although the high number of Patnaik grade III and HN3 could explain the difference in the oncological outcome between Weishaar's study and the present study, it looks less obvious how to discuss the worse prognosis in Patnaik grade II with early or overtly nodal metastasis. A hypothesis is that no data on Kiupel grading are available in Weishaar's study, leading to a possible underestimation of a Patnaik grade II tumors could be reclassified as Kiupel high-grade,[48] as well as an underestimation of HN classes because of the extirpation of RLN leaving possible positive SLN in the body. Lastly, the higher number of deaths related to MCT in Weishaar's paper could be ascribed to the inclusion of local recurrences or dogs with established distant metastases, which were instead excluded from our study sample.[30,31,49]

Although Weishaar classification system has been applied in several previous studies, the comparison between the outcome results that we report and previous data may also be difficult because of different inclusion criteria among studies. In fact, previous literature has mostly focused either on the regional lymphadenectomy in stage I and

II MCT or on the prognostic role of specific HN classes[7,20,21] in cutaneous or subcutaneous MCT.[22,24] Certainly, our results confirm the good prognosis previously reported in dogs with stage I[20] and stage II cutaneous[6,7] and subcutaneous MCT that underwent RLN removal.[22,24] The only paper focusing on SLN reports a similar good clinical outcome for low grade MCT even in presence of HN3 lymph nodes, but it also showed 18% of nodal relapse.[21] Conversely, no nodal relapses were observed in any of the 94 dogs included in the present study, independently from HN classes and histological grade. A possible explanation is that in Guerra et al the nodal relapse may have developed from an occult metastatic not-removed second tier lymph node.[21] That could be related to the high number of Kiupel high-grade MCTs and the only inclusion of HN3 SLN, rather than actual false negative rate. Another hypothesis for such dissimilarity is linked to the different SLN mapping technique applied. In fact, in Guerra et al[21] the majority of cases were mapped by only intraoperative direct visualization of lymph nodes with methylene blue. In contrast, in the present paper, the combined use of planar lymphoscintigraphy for preoperative identification of the SLC and the intraoperative gamma probe and blue dye for guiding the removal of SLN led to the successful removal of all SLN within each identified SLC.[11,19,50] Indeed, the number of SLC identified and of SLN removed is correlated with an increase in the HN class, suggesting that a single MCT could be engaged with 1 or more SLC, similarly to what is reported in humans bearing melanoma.[51] In fact, while preoperative mapping might identify 1 or more SLC, the employment of intraoperative gamma probe in association with blue dye ensures an accurate exploration of the SLC and removal of multiple SLN hidden within the adipose tissue or unstained, with a detection rate that ranged among 91%-98%.[11,50] In addition, intraoperative gamma probe use allowed the measurement of residual radioactivity that led to straight-forward decision-making during the dissection of the SLCs, since its absence indicates that there are no more SLN to be removed within the SLC. On the other hand, the inability to directly visualize a node, either blue or nonblue, does not exclude the presence of other "hot" SLNs within the same SLC,[11,50] and the use of intraoperative gamma probe allows avoiding missing any lymph node[52] that could belong to a different HN class.[53] For these reasons, the surgeons might be enticed in exploring more SLCs and remove more SLNs when using a high-performance tracer that allows for an intraoperative guidance such as Technetium-99 or indocyanine green in near-infrared fluorescence.[19,54,55]

Adjuvant treatments were not standardized, because of the evolution in chemotherapy recommendations over the study period. Considering the very low rate of MCT-related events recorded, no conclusion can be drawn on the potential improvement on clinical outcome given by chemotherapy. Medical treatment was suggested in all cases with HN3, or in dogs considered at high metastatic risk because of the histologic high-grade of the tumor. On the other hand, most of HN2 Kiupel low-grade and Patnaik grade I/II MCT did not receive adjuvant chemotherapy since 2020, according to published evidence.[7] When considering the management of infiltrated margins, local recurrence was recorded in both dogs that did or did not receive adjuvant chemotherapy. All of them developed distant metastasis and died for causes related to MCT. When chemotherapy was chosen to treat infiltrated margins, it was only because primary re-excision was judged not possible and radiation therapy was declined by the owner. The role of primary re-excision or radiation therapy has been proven to reduce local recurrence,[56] whereas the usefulness of adjuvant chemotherapy in the treatment of at-risk margins has been questioned.[57] Proliferation index such as Ki67 was available in half of cases while the AgNOR x Ki67 (Ag67) values was not investigated, therefore its influence on clinical outcome could not be extrapolated in the present study. The low rate of local recurrence achieved could be explained by a high number of low-risk MCTs (either cutaneous or subcutaneous) and to an adequate surgical dose applied.[58,59] Nevertheless, the real underlying explanation for this finding warrants further investigation.

Several clinical and pathological variables were recorded in this study, but because of the low rate of events related to MCT the evaluation of their prognostic influence was limited to a few of them. Statistical significance was found for the following: Kiupel high-grade, number of SLC, and number of SLN. Contrary to previous studies on RLN[11] and SLN[12,14] that identified a correlation between tumor dimension and early/overtly lymph nodes metastasis, such correlation was lacking in the present study. Taken in consideration that lymphadenectomy has a staging and therapeutical positive effect on MCT, further studies should explore the predictive value of clinical and pathological variables on the detection of early or overtly SLN metastasis in the preoperative setting, in order to identify the dogs that could benefit from SLN mapping and extirpation and those that could be excluded.[14] It should be noted that in the present sample only 3 cutaneous MCT belonged to the Kiupel high-grade/Patnaik Grade III category. The absence of a relevant number of cases in this category might have limited our ability in identifying their effective behavior in terms of metastatic potential, SLN HN status, recurrence rate and death associated to MCT disease.

In conclusion, dogs with low grade/low risk MCT with clinically non-palpable and normal-sized regional lymph nodes and without distant metastasis have a favorable outcome independently from the HN classes of their SLNs. At the moment, adjuvant chemotherapy should be suggested in dogs with HN3 SLN, otherwise seems not necessary in HN2, SLN. However, considering different performances among the mapping techniques, it is worthy to underline that these results should be considered strictly related to the use of preoperative planar lymphoscintigraphy and intraoperative gamma probe plus blue dye for SLN detection.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

Damiano Stefanello https://orcid.org/0000-0003-2726-0366
Elisa M. Gariboldi https://orcid.org/0000-0002-6705-6653
Alessandra Ubiali https://orcid.org/0000-0002-5704-6590
Luigi Auletta https://orcid.org/0000-0002-1624-4240

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