Lymphadenectomy improves outcome in dogs with resected Kiupel high-grade cutaneous mast cell tumours and overtly metastatic regional lymph nodes

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Lymphadenectomy may improve outcome in dogs with resected Kiupel high-grade cutaneous mast cell tumours and overtly metastatic regional lymph nodes

Introduction: Historically, the prognosis for dogs with stage II Kiupel high-grade cutaneous mast cell tumours has been considered poor.

Objectives: The aim of this study was to explore the impact of lymphadenectomy on outcome in dogs with Kiupel high-grade cutaneous mast cell tumours and overt regional lymph node metastasis.

Material and methods: Dogs with completely staged Kiupel high-grade cutaneous mast cell tumours with overt and/or certain regional lymph node metastasis undergoing excision of the primary tumours and adjuvant medical treatment were retrospectively enrolled. Dogs were divided into two groups: dogs that had no lymphadenectomy but underwent fine-needle aspiration of the regional lymph node with a cytological diagnosis of certain metastasis in group A, whereas dogs that underwent lymphadenectomy and had a histological diagnosis of overt lymph node metastasis in group B.

Results: Forty-nine dogs were included: 18 were assigned to group A and 31 to group B. Median time to progression was significantly shorter in group A (150 days, 95%CI: 129-170) than in group B (229 days, 95%CI: 191-266), as well as median survival time (250 days, 95%CI: 191-308 versus 371 days, 95%CI: 311-430, respectively).

On multivariable analysis, lack of lymphadenectomy was associated with higher risk of overall tumour progression (hazard ratio [HR]: 2.05, 95%CI: 1.02-4.13), nodal progression (HR: 3.4, 95%CI: 1.65-7.02) and tumour-related death (HR 3.63, 95%CI: 1.72-7.66), whereas tumour size was associated with higher risk of local recurrence (HR: 3.61, 95%CI: 1.06-13).

Clinical significance: Regional lymphadenectomy may improve outcome in dogs with biologically aggressive cutaneous mast cell tumours.

Introduction

Treatment recommendations and prognosis for canine cutaneous mast cell tumours (cMCTs) are based on the combination of clinical staging and histologic grade (Patnaik *et al.* 1984; Kiupel *et al.* 2011; Blackwood *et al.* 2012; Weishaar *et al.* 2014; Lejeune *et al.* 2015; Miller *et al.* 2016; Horta *et al.* 2018; Marconato *et al.* 2018; Pizzoni *et al.* 2018; Marconato *et al.* 2020).

High-grade (Kiupel high-grade [K-HG] and Patnaik grade 3 [P-G3]) cMCTs have a poorer prognosis than low grade (Kiupel low-grade [K-LG] and Patnaik grade 1 [P-G1]) cMCTs, due to the higher rate of recurrence and metastasis, with regional lymph nodes (RLNs) being the most commonly reported site for metastasis, occurring in 30-60% of dogs (Krick *et al*. 2009; Hume *et al.* 2011; Kiupel *et al.* 2011; Donnelly *et al.* 2015; Stefanello *et al.* 2015; Horta *et al.* 2018).

Current treatment recommendations for dogs with high-grade cMCTs, with or without RLN metastasis, include surgical excision of the primary tumour, with or without radiation therapy (RT), followed by systemic chemotherapy (Hayes *et al.* 2007; Hume *et al.* 2011; Blackwood *et al.* 2012; Mendez *et al.* 2019). According to the World Health Organization (WHO) clinical staging system, stage II MCT is defined as a primary single tumour confined to the dermis with nodal metastasis (Owen 1980). The prognosis for dogs with stage II, P-G3 cMCTs treated with surgical excision of the primary tumour and adjuvant systemic chemotherapy is relatively poor, with reported median survival time (mST) ranging from 142 to 194 days (Hayes *et al.* 2007; Hume *et al.* 2011;).

It has been recently shown that the removal of metastatic RLNs is associated with a better outcome in canine cMCTs (Hume *et al.* 2011; Baginski *et al.* 2014; Marconato *et al.* 2018; Mendez *et al.* 2020;). Hume et al. (2011) showed that adequate treatment of metastatic RLN (either with surgery or RT) significantly improved survival in dogs with stage II, P-G3 MCTs, with a median survival time of 240 days.

A previous study by Marconato et al. reported that surgical extirpation of a metastatic lymph node ([LN] early -HN2- or overt -HN3- LN metastasis according to Weishaar et al. [2014]) alongside the resection of the primary cMCT significantly improved outcome. In the aforementioned study, dogs with both high-grade and low-grade cMCTs treated with adjuvant chemotherapy, had a mean time to progression (TTP) of 1461 days and a median tumour-specific survival (TSS) of 2213 days (Marconato *et al.* 2018). However, most dogs that underwent lymphadenectomy had K-LG cMCTs, and stratification according to histologic grade was not performed in the survival analysis; therefore, no further information could be specifically provided for dogs with K-HG cMCTs (Marconato *et al.* 2018).

In a more recent study, RLN removal with or without RLN bed irradiation resulted in a significant prolongation of progression-free survival (PFS) and overall survival (OS) in dogs with stage II high-grade cMCTs, with a median PFS and OS of 125 and 330 days, respectively (Mendez *et al.* 2020). Collectively, the above data support the beneficial effect of lymphadenectomy on the outcome of dogs with stage II high-grade cMCTs; however, none of these studies have specifically focused on dogs with K-HG cMCTs and HN3 LNs.

The aim of this retrospective study was to explore the impact of lymphadenectomy as part of the primary tumour surgery on TTP and ST in dogs with K-HG cMCT and overt (HN3)/certain RLN metastasis while also receiving adjuvant medical treatment as part of their treatment.

Material and methods

Study design

A multi-institutional retrospective cohort study

Study population

74 The electronic medical records of four European institutions (masked for review) were searched
75 retrospectively to identify dogs with firstly occurring, treatment-naïve, histologically confirmed K-
76 HG cMCT with certain and/or overt RLN metastasis confirmed either by cytology (Krick *et al.* 2009)
77 or histology (Weishaar *et al.* 2014), between July 1, 2014 and July 21, 2021. Medical records have
78 been searched by four operators independently doing the same investigation. Searched terms used
79 included "dog", "cutaneous MCTs", "Kiupel high-grade", "nodal or LN metastasis", "certain nodal
80 or LN metastasis", "lymphadenectomy, and "overt/HN3 nodal or LN metastasis".
81 The RLN was defined as the LN draining the anatomical region surrounding the cMCT, and was
82 identified by palpation, ultrasound or surgical exploration.
83
84 **Inclusion criteria**
85 For the purpose of this study, dogs were only included if histopathology or cytology confirmed overt
86 (HN3;) or certain RLN metastasis, respectively, of at least one RLN (Krick *et al.* 2009; Weishaar *et*
87 *al.* 2014). Overt nodal metastasis (HN3) was histologically defined as the disruption or effacement of
88 normal nodal architecture by discrete foci, nodules, sheets of overt masses of mast cells (Weishaar et
89 al. 2014); whereas cytologically certain metastasis was defined as the effacement of lymphoid tissue
90 by mast cells, and/or the presence of aggregated, poorly differentiated mast cells with pleomorphism,
91 anisocytosis, anisokaryosis, and/or decreased or variable granulation, and/or greater than five
92 aggregated for more than three mast cells (Krick el al. 2009).
93 Primary cMCTs and LNs were histologically evaluated by multiple board-certified pathologists and
94 slides were not reviewed.
95 In addition, dogs were eligible for inclusion if they underwent complete clinical staging, surgical
96 excision of the primary cMCT and adjuvant medical treatment. Furthermore, a follow-up of at least
97 4 months from surgery had to be available. Dogs that had disease progression or were dead due to
98 tumour-related causes within 4 months from surgery were included in the analysis. Follow-up
99 information was collected from the clinical records of each institution.

Staging and treatment

Clinical staging included haematological and biochemical analysis, cytological evaluation of the primary cMCT and RLN; thoracic radiographs (3 views), abdominal ultrasound and fine-needle aspiration (FNA) of liver and spleen regardless of their sonographic appearance.

Adjuvant medical treatment consisted of vinblastine ([Velbe; EuroGenerici] 2-3 mg/m^2 IV every 2 weeks for a total of eight doses) and prednisolone ([Prednicortone; Dechra] 1 mg/kg orally once daily for the duration of the protocol), toceranib phosphate ([Palladia; Zoetis] 2.4-2.8 mg/kg orally on Monday, Wednesday, Friday schedule for 6 months) or both (vinblastine [1.6 mg/m^2 IV every 2 weeks for a total of eight doses] and toceranib phosphate [2.4-2.8 mg/kg orally on Monday, Wednesday, Friday for the duration of the course]). Dogs also received additional medications during their treatment for prophylactic management of paraneoplastic conditions associated with cMCTs, consisting of chlorpheniramine ([Chlorphenamine; Crescent] 0.2-0.5 mg/kg orally twice daily) and ranitidine ([Zantadine; CEVA] 2-4 mg/kg orally twice daily).

For MCTs located on either the trunk, proximal part of the limb, inguinal/perineal region, head and neck, and mammary region, excision of the primary tumour included at least 2 cm of macroscopically normal tissue around the tumour and at least one deep fascial plan; for MCTs located on the distal region of the limb, a reconstructive surgery was performed. Finally, for digit MCTs, digit amputation was performed.

Dogs with subcutaneous or multiple MCT/s, and/or with stage IV disease at the time of diagnosis, were excluded from the study. Dogs treated with radiotherapy were also excluded.

In order to evaluate the impact of lymphadenectomy on outcome, dogs were divided into two groups: dogs that had no lymphadenectomy but underwent FNA of the RLN/s with a cytological diagnosis of "certain metastasis" were included in group A, whereas dogs that underwent lymphadenectomy and

had a histological diagnosis of HN3 LN were included in group B. The decision on whether to perform lymphadenectomy of the metastatic RLN was made at the personal discretion of each clinician, as well as the number of LNs sampled or excised when more than one LN was assessed by cytology or histology, respectively.

Data extracted

For each case the following data were recorded: breed, sex, age and weight at presentation, clinical substage (a or b); cMCT anatomic site, size and presence of ulceration; size (recorded as either normal or enlarged, based on physical examination or diagnostic imaging findings), site and number of evaluated RLNs; histologic or cytological results of all excised or sampled LNs, respectively; date of surgery; intra- and postoperative severe complications (severe complications were defined as those that required additional medical treatment and/or surgical revision to resolve; only for dogs in group B), histopathologic evaluation of surgical margins (complete, clean but close [tumour cells extending within 1 mm of any cut margins], incomplete); Ki-67 index/ KIT pattern/ c-kit mutational status (if performed); adjuvant medical treatment (cytotoxic chemotherapy; tyrosine kinase inhibitors [TKIs] or both).

In order to evaluate the impact of lymphadenectomy on TTP and ST, the following information were also retrieved: local recurrence (defined as cMCT relapse at or within 2 cm of the surgical scar, confirmed by cytology), nodal progression (defined as nodal progressive disease according to RECIST criteria for dogs in which lymphadenectomy was not performed [Nguyen *et al*. 2013] or the presence of new metastatic LNs for dogs that undergo lymphadenectomy); distant progression (defined as the occurrence of cytologically confirmed metastasis at distant organs); date of death or last follow-up examination, and cause of death.

Statistical analysis

Descriptive statistic was used in the analysis of dogs and tumour characteristics. Data were tested for normality by use of Shapiro-Wilk normality test. All tested values were not normally distributed and therefore were expressed as median (range).

The $\chi 2$ test/Fisher exact probability test (categorical variables), and the Mann-Whitney U test (continuous variables) were applied to evaluate differences in demographic features and possible prognostic factors between group A and group B. The considered variables included breed (predisposition to biologically aggressive MCTs [i.e., Labrador retriever, golden retriever, Shar pei] vs others [Dobson & Scase 2007]), sex (male vs female), age, body weight, anatomic location of the primary cMCT (sites associated with a worse prognosis [i.e., head and neck, inguinal/perineal region, scrotal, digital, mammary] vs sites associated with a better prognosis [i.e., trunk, limbs excluding digital] [Blackwood *et al.* 2012; Pizzoni *et al.* 2018]), macroscopic tumour longest diameter ($>$ 3 cm vs $\leq$ 3 cm [Mendez *et al.* 2020]), ulceration (yes vs no), substage (a vs b), Patnaik grading (P-G2 vs P-G3). For age and weight, the median was used as cut-off value.

The influence of potential prognostic variables on TTP and ST was investigated with univariable and multivariable Cox's regression analyses. All variables associated with outcome with a P-value $\leq$0.1 at univariable analysis were selected for multivariable analysis.

Outcome was reported as time to local recurrence (TLR), calculated from the date of surgery to the date of local recurrence; time to nodal progression (TNP), calculated form the date of surgery to the date of nodal progression; time to distant progression (TDP), calculated from the date of surgery to the date of diagnosis of distant metastasis; TTP, calculated from the date of surgery to the first occurrence of at least one of the following: local recurrence, nodal progression or distant metastasis; ST, calculated from the date of surgery to the date of death or to the date of the last visit if death did not occur. Only dogs deceased for cMCT-related causes were considered as events. Dogs with no disease progression, still alive or dead for MCT-unrelated causes at the time of data closure were censored from the respective statistical analysis.

Survival plots were generated according to the Kaplan-Meier product-limit method. Survival estimates were presented as medians with the corresponding 95% confidence intervals (95% CIs). TTP and ST of both groups obtained with the Kaplan-Meier method were compared by use of log-rank test.

Statistical analysis was performed with SPSS Statistics v.25 (IBM, Armonk, NY, United States). Significance was set at $P < .05$.

Results

- **Patient data and tumour characteristics**

The electronic medical records search identified 60 dogs potentially suitable for the study. Six dogs were excluded as they had multiple MCTs at presentation and 5 were excluded due to lack of follow-up information.

A total of 49 dogs were eventually included in the study: 18 dogs did not undergo lymphadenectomy (group A) and 31 underwent lymphadenectomy (group B). No significant difference was found among the two groups with respect to demographic features and possible outcome variables, apart from medical treatment (Table 1), as dogs that did not undergo lymphadenectomy were treated more often with TKIs with or without systemic cytotoxic chemotherapy.

1. Group A – dogs that did not undergo lymphadenectomy

Among the 18 dogs that did not undergo lymphadenectomy, there were 9 (50%) females (of which 6 spayed), and 9 (50%) males (of which 7 castrated). At the time of diagnosis, the median age was 10 years (range, 0.5-13), and the median weight was 22.8 kg (range, 2.5-39). Represented breeds included: mixed breed (n=7; 38.9%), Labrador retriever (n=4; 22.2%), golden retriever (n=2; 11.2%), boxer (n=2; 11.2%), and one (5.5%) each of Doberman pinscher, American Staffordshire terrier, and West Highland White terrier.

All dogs were asymptomatic at presentation (substage a).

The most common primary tumour location was trunk (n=6; 33.3%), followed by limbs (n=4; 22.2%), inguinal/perineal region (n=3; 16.7%), head and neck (n=2; 11.1%), digital (n=2; 11.1%), and mammary region (n=1; 5.6%).

Data on tumour diameter was available for 17 dogs. Median tumour diameter was 2 cm (range, 1-4.5). At presentation, 6 (33.3%) tumours were ulcerated.

Four (22%) dogs had normal-sized RLNs, whereas 14 (78%) dogs had an enlarged RLN.

Metastatic RLNs included inguinal (n=6; 33.3%), popliteal (n=4; 22.2 %), superficial cervical (n=3; 16.7%), axillary (n=3; 16.7%), and mandibular (n=2; 11.1%) LN.

Based on histopathology reports, there were 11 (61.1%) K-HG/P-G3 cMCTs, and 7 (38.9%) K-HG/P-G2 cMCTs. Surgical margins were complete in 11 (61.1%) cMCTs, clean but close in 3 (16.7%) cases, and incomplete in 4 (22.2%) cases.

Ki67 immunohistochemistry was available for 5 (27.8%) cases. Ki67 score ranged from 2% to 23%.

KIT staining pattern was available for 6 (33.3%) cases: 2 cMCTs had pattern III, 2 had pattern II, and 2 had pattern I. Mutational analysis was available for 12 (66.7%) cMCTs: 2 had an ITD on exon 11, 1 had ITD on exon 8, and 9 were wild type.

2. Group B – dogs that underwent lymphadenectomy

Among the 31 dogs undergoing lymphadenectomy, there were 17 males (of which 9 castrated) and 14 females (of which 11 spayed). At the time of diagnosis, the median age was 10 years (range, 5-15), and the median weight was 23 kg (range, 4.9-55). Represented breeds included: mixed breed (n=10; 32.2%), miniature Pinscher (n=4; 12.9%), cane corso (n=3; 9.7%), golden retriever (n=2; 6.45%), and one (3.2%) each of Labrador retriever, Shar pei, American Staffordshire terrier, Bichon, Bernese Mountain dog, Doberman pinscher, German shepherd, Jack Russell terrier, Weimaraner, Maltese terrier, and Griffon.

Four (12.9%) dogs showed clinical signs (n=2 pruritus, n=1 vomiting, n=1 diarrhoea; substage b) at presentation. The most common primary tumour location was limb (n=11; 35.5%), followed by trunk

(n=8; 25.8%), inguinal/perineal region (n=6; 19.3%), head and neck (n=3; 9.7%), and digits (n=3; 9.7%). Median tumour diameter was 3 cm (range, 2-4.2). At presentation, 12 (38.7%) tumours were ulcerated.

Six (19.4%) dogs had normal-sized RLNs, whereas 25 (80.6%) dogs had an enlarged RLN.

A total of 52 RLNs were removed, including inguinal (n=16; 30.8%), superficial cervical (n=14; 26.9%), axillary (n=10; 19.2%), popliteal (n=6; 11.5%), mandibular (n=3; 5.9%), retropharyngeal (n=2; 3.8%), and medial iliac (n=1; 1.9%) LN. In 14 (57.9%) dogs 1 LN was removed, in 15 (31.6%) dogs 2 LNs were removed, and in 2 (7.9%) dogs 4 LNs were removed.

Concerning the HN3 LNs, 30 dogs had one RLN classified as HN3, while 1 dog had 2 RLNs classified as HN3. Among the remaining 20 extirpated LNs, there were 8 HN2, 7 HN1 and 5 HN0.

Based on histopathology reports, there were 23 (74.2%) K-HG/P-G3 cMCTs, and 8 (25.8%) K-HG/P-G2 cMCTs. Surgical margins were complete in 22 (71%) cMCTs, clean but close margins in 5 (16.1%) cases, and incomplete in 4 (12.9%) cases.

Ki67 immunohistochemistry was available for 4 (12.9%) cases. Ki67 score ranged from 9% to 29%.

KIT staining pattern was available for 4 (12.9%) cases: 3 had pattern II, and 1 case had pattern I.

Mutational analysis was available for 13 (41.9%) cMCTs: 6 had an ITD on exon 11, 1 had ITD on exon 8, and 6 were wild type.

- **Treatment and outcome**

1. Group A – dogs that did not undergo lymphadenectomy

All dogs received adjuvant medical treatment. Among them, 13 (72.2%) were treated with systemic chemotherapy consisting of vinblastine and prednisone, 3 (16.7%) with toceranib alone, and 2 (15.4) with both. Among the 5 dogs treated with toceranib alone or in combination with vinblastine, 2 had an ITD mutation on exon 11, and 1 dog had an ITD mutation on exon 8.

All dogs developed disease progression. Of those, 8 (44.5%) experienced local recurrence after a median of 170 days (range, 60-511); three of these 8 dogs had their cMCT removed with incomplete

surgical margins. All (100%) dogs experienced nodal progression after a median of 148 days (range, 30-511), and 7 (39%) dogs developed distant metastasis after a median of 180 days (range, 72-205). Median TTP was 150 days (95% CI, 129-170 days; Figure 1). Four (22%) dogs received an additional medical treatment at the time of disease progression: 1 dog received lomustine ([Lomustine; medac]70 mg/m^2 orally every 4 weeks) and prednisolone, and 3 dogs received toceranib.

At the end of the study, all dogs had died because of cancer-related (n=17; 92%) or unrelated (n=1; 8%) causes. The latter dog died due to gastric dilation volvulus after 140 days.

Median ST was 250 days (95% CI, 311-430 days; Figure 2).

2. Group B – dogs that underwent lymphadenectomy

Lymphadenectomy was well tolerated in all cases and no major complications were reported. Thirty (97%) dogs were treated with systemic chemotherapy consisting of vinblastine and prednisolone, while one dog (3%) was treated with vinblastine and toceranib. The latter dog had an ITD mutation on exon 11. Overall, 17 (54.8%) dogs developed progressive disease. Of those, 8 (25.8%) dogs experienced local recurrence after a median of 218 days (range, 160-536); two of these 8 dogs had their cMCT removed with incomplete surgical margins. Thirteen (41.9%) dogs experienced nodal relapse after a median of 228 days (range, 97-287), and 12 (38.7%) dogs developed distant metastasis after a median of 267 days (range, 120-371). Median TTP was 229 days (95% CI, 191-266 days; Figure 1).

Six (35%) dogs received an additional medical treatment at the time of disease progression: 2 dogs received lomustine (60 mg/m^2 orally every 4 weeks and 70 mg/m^2 orally every 4 weeks, respectively) and prednisolone, and 4 dogs received toceranib.

At data analysis closure, 13 (41.9%) dogs were alive, with a median follow-up of 180 days (range, 123-594), while 18 (58.1%) dogs had died because of cancer-related (n=15; 48.4%) or unrelated (n=3; 9.7%) causes. Two dogs died due to acute pancreatitis, and one dog due to heart failure.

Median ST was 371 days (95% CI, 311-430 days; Figure 2).

Analysis of outcome and prognostic variables

Median TTP for dogs that underwent lymphadenectomy (229 days, 95% CI 191-266 days) was significantly longer than median TTP for dogs in which lymphadenectomy was not performed (150 days, 95% CI 129-170 days, P<0.001; Figure 1).

Median ST for dogs that underwent lymphadenectomy (371 days, 95% CI 311-430 days) was significantly longer than median ST for dogs in which lymphadenectomy was not performed (250 days, 95% CI, 191-308 days, P=0.001, Figure 2).

Lack of lymphadenectomy was the only variable associated with a higher risk of overall tumour progression both in univariable (hazard ratio [HR]: 2.19, 95% CI: 1.11-4.33; P=0.024) and multivariable (HR: 2.05, 95% CI: 1.02-4.13; P=0.043) analyses (Tables 2 and 3).

When recurrence/progression characteristics were evaluated separately, tumour diameter >3 cm (HR: 5.53, 95%CI: 1.73-17.72; P=0.004) and incomplete surgical margins (HR: 3.99, 95% CI: 1.38-11.57; P=0.011) were associated with a higher risk of local recurrence on univariable analysis (Table 2).

Lack of lymphadenectomy was the only variable significantly associated with a higher risk of nodal progression (HR: 3.40, 95% CI: 1.65-7.02; P<0.001), while none of the evaluated prognostic variables was associated with an increased risk of distant progression (Table 2).

On multivariable analysis, only tumour diameter >3 cm remained significant for local recurrence (HR: 3.61, 95% CI: 1.06-13; P=0.041; Table 4).

Lack of lymphadenectomy was the only variable associated with a higher risk of tumour-related death both in univariable (HR: 3.57, 95% CI: 1.70-7.48; P=0.001) and multivariable (HR: 3.63, 95% CI: 1.72-7.66; P=0.001) analyses (Tables 5 and 6).

Discussion

In the current study it was documented that dogs with K-HG cMCTs undergoing lymphadenectomy of HN3 LN as part of their primary surgery and adjuvant medical treatment had a significant improvement in TTP and ST compared to those in which the metastatic LN was not excised. These findings further support the therapeutic benefit of lymphadenectomy, also in the face of biologically aggressive cMCTs.

It is widely accepted that canine cMCTs metastasise in a stepwise manner from the primary tumour to the draining LN/s and then systemically to distant sites (Warland *et al.* 2014). Accordingly, the LN involvement is of prognostic importance not only because it indicates a more aggressive tumour behaviour, but also because persistent neoplastic cells in LN/s can be the source of subsequent metastases as proposed by the "Halstedian" theory (Halsted 1907). Considering the above, a reasonable explanation for the beneficial effects of metastatic LN dissection includes the reduction of tumour burden and the elimination of a potential source of neoplastic cells which could result in further spread and fatal outcome (Halsted 1907; Kawada & Taketo 2011).

In order to better define the impact of lymphadenectomy on TTP, we also evaluated separately recurrence/progression characteristics between the two groups. Lack of lymphadenectomy was the only variable significantly associated with a higher risk of nodal progression. These results were not surprising since dogs in which lymphadenectomy was not performed (group A) had persistent metastatic nodal disease, most likely representing the source of the subsequent nodal progression.

On the other hand, tumour diameter was the only variable significantly associated with an increased risk of local recurrence on multivariable analysis. Dogs with tumour diameter >3 cm had an increased risk of local recurrence regardless of histologic margins. This result is in agreement with a previous study in which dogs with P-G3 MCTs greater than 3 cm were at higher risk of local recurrence, despite complete surgical margins (Hume *et al.* 2011). It is important to note that in the aforementioned study, as well as in the current study, the exact technique of surgical trimming as well as the number of sections of surgical margins evaluated in each case were not reported. The impact of specimen trimming technique on margin evaluation has been previously reported (Dores *et al.* 2017; Liptac

2020). It has been shown that tangential sectioning detected more incomplete surgical margins than radial sectioning, because the former evaluates a considerably greater percentage of the total margin surface area (Dores *et al.* 2017). Moreover, it could be hypothesized that K-HG cMCTs > 3 cm are associated with more infiltrative growth patterns. In these case, radial sections might be expected to have even poorer precision in detecting incomplete surgical margins (Dores *et al.* 2017). Thus, it is possible that the number of surgical margins determined to be complete in the current study as well as in the Hume study was overestimated, thereby skewing the results. Further studies are required to establish the impact of the trimming technique, tumour size and of histologically free-surgical margins on local recurrence in dogs with K-HG MCTs.

None of the other evaluated variables, including lack of lymphadenectomy, was significantly associated with an increased risk of developing distant metastasis. There are some potential explanations for this result: first, since all dogs included in this study had biologically aggressive cMCTs, it is possible that, at least in some cases, the metastatic cascade had already initiated, but was not detectable at the time of staging. If this was the case, lymphadenectomy may have not disrupted the metastatic cascade, but it may have contributed to slowing down the metastatic progression. Second, although all dogs in group B underwent lymphadenectomy of at least one overtly metastatic LN, none of them underwent sentinel LN (SLN) mapping. Thus, it is possible that not all SLNs were removed, potentially leaving a source of neoplastic cells which could then spread to distant sites (Wong & Hynes 2006; Kawada & Taketo 2011). Moreover, due to the retrospective nature of this study, the number of LNs excised was not standardized. Indeed, most dogs that underwent lymphadenectomy had only one HN3 LN removed. Since lymphocentra may contain more than one LN, it is possible that some metastatic LNs were left behind and spread to distant organs (Wong & Hynes 2006; Kawada & Taketo 2011; Suam *et al.* 2013).

The current work has several limitations. First, despite performing a multi-institutional study, inclusion criteria were strict, resulting in a total population of 49 dogs only. Second, the retrospective

nature of this study did not allow for obtaining information regarding Ki67 index, KIT-pattern, and c-kit mutational status in all cases, which might have provided further relevant prognostic information.

Third, although all dogs received adjuvant medical treatment, protocols were not standardized, rather the choice of the protocol and dosage were left to the primary clinician, making comparison of the effect of medical treatment on outcome more challenging. Furthermore, decisions regarding whether to perform lymphadenectomy were made according to each clinician's description or owner preferences, rather than random allocation.

Fourth, primary cMCTs and LNs were histologically evaluated by multiple pathologists and slides were not reviewed, potentially affecting study results. Nevertheless, both Kiupel and Weishaar schemes are well described and widely used by pathologist worldwide, as they both rely on reproducible criteria. Additionally, Kiupel's grading system has been proven to have a high interobserver agreement (Kiupel et al. 2011).

Finally, even though lymphadenectomy was well tolerated in all cases, it must be pointed out that most dogs underwent lymphadenectomy of one peripheral LN. It is possible that the dissections of a higher number of LNs or the removal of intracavitary LNs might be associated with an increased incidence of postoperative morbidity.

In conclusion, the present study showed that lymphadenectomy along with the resection of the primary tumour and adjuvant medical treatment improves outcome for dogs with K-HG cMCTs and overt nodal metastasis. The findings of the current study provide additional support for the therapeutic role of lymphadenectomy and further insight into the management of stage II Kiupel high-grade cMCTs. Further prospective studies are warranted to explore the effect of surgical extirpation of metastatic SLN and the number of LNs removed on outcome in dogs with K-HG cMCTs.

Figure legends

Figure 1: Time to progression for dogs with Kiupel high-grade cutaneous mast cell tumours treated by surgical excision of the primary tumour with (group B) or without (group A) lymphadenectomy, and adjuvant medical treatment. Median time to progression for dogs in group B was significantly longer than median time to progression for dogs in group A (229 days versus 150 days, respectively; $P<0.001$)

Figure 2: Survival time for dogs with Kiupel high-grade cutaneous mast cell tumours treated by surgical excision of the primary tumour with (group B) or without (group A) lymphadenectomy, and adjuvant medical treatment. Median survival time for dogs in group B was significantly longer than median survival time for dogs in group A (371 days versus 250 days, respectively; $P=0.001$).

Table legends:

Table 1: Demographic information and distribution of variables potentially associated with prognosis in 49 dogs with high grade mast cell tumours and metastatic regional lymph nodes. Differences in data distribution were assessed with Chi-square test/Fisher's exact test (categorical variables) or Mann-Whitney U test (continuous variables). † cutaneous mast cell tumour; ‡ tyrosine kinase inhibitors, *significant.

Table 2: Univariable Cox regression analysis of variables potentially associated with increased risk of tumour progression, local recurrence, nodal progression and distant progression in 49 dogs with high grade mast cell tumours and metastatic regional lymph nodes. Abbreviations: CI, confidence interval. *significant.

Table 3: Multivariable Cox regression analysis for risk of tumour progression. Variables with a significance level of $P\leq0.1$ at univariable analysis were included in the model. Abbreviations: CI, confidence interval. *significant.

Table 4: Multivariable Cox regression analysis for risk of local recurrence. Variables with a significance level of P≤0.1 at univariable analysis were included in the model. Abbreviations: CI, confidence interval. *significant.

Table 5: Univariable Cox regression analysis of variables potentially associated with increased risk of tumour-related death in 49 dogs with high grade mast cell tumours and metastatic regional lymph nodes. Abbreviations: CI, confidence interval. *significant.

Table 6: Multivariable Cox regression analysis for risk of tumour-related death. Variables with a significance level of P≤0.1 at univariable analysis were included in the model. Abbreviations: CI, confidence interval. *significant.

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