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

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1

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

4

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

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

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

16 **Results:** Forty-nine dogs were included: 18 were assigned to group A and 31 to group B. Median
17 time to progression was significantly shorter in group A (150 days, 95%CI: 129-170) than in group
18 B (229 days, 95%CI: 191-266), as well as median survival time (250 days, 95%CI: 191-308 versus
19 371 days, 95%CI: 311-430, respectively).

20 On multivariable analysis, lack of lymphadenectomy was associated with higher risk of overall
21 tumour progression (hazard ratio [HR]: 2.05, 95%CI: 1.02-4.13), nodal progression (HR: 3.4, 95%CI:
22 1.65-7.02) and tumour-related death (HR 3.63, 95%CI: 1.72-7.66), whereas tumour size was
23 associated with higher risk of local recurrence (HR: 3.61, 95%CI: 1.06-13).

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

26 **Introduction**

27 Treatment recommendations and prognosis for canine cutaneous mast cell tumours (cMCTs) are
28 based on the combination of clinical staging and histologic grade (Patnaik *et al.* 1984; Kiupel *et al.*
29 2011; Blackwood *et al.* 2012; Weishaar *et al.* 2014; Lejeune *et al.* 2015; Miller *et al.* 2016; Horta *et*
30 *al.* 2018; Marconato *et al.* 2018; Pizzoni *et al.* 2018; Marconato *et al.* 2020).

31 High-grade (Kiupel high-grade [K-HG] and Patnaik grade 3 [P-G3]) cMCTs have a poorer prognosis
32 than low grade (Kiupel low-grade [K-LG] and Patnaik grade 1 [P-G1]) cMCTs, due to the higher rate
33 of recurrence and metastasis, with regional lymph nodes (RLNs) being the most commonly reported
34 site for metastasis, occurring in 30-60% of dogs (Krick *et al.* 2009; Hume *et al.* 2011; Kiupel *et al.*
35 2011; Donnelly *et al.* 2015; Stefanello *et al.* 2015; Horta *et al.* 2018).

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

44 It has been recently shown that the removal of metastatic RLNs is associated with a better outcome
45 in canine cMCTs (Hume *et al.* 2011; Baginski *et al.* 2014; Marconato *et al.* 2018; Mendez *et al.*
46 2020;). Hume *et al.* (2011) showed that adequate treatment of metastatic RLN (either with surgery or
47 RT) significantly improved survival in dogs with stage II, P-G3 MCTs, with a median survival time
48 of 240 days.

49 A previous study by Marconato et al. reported that surgical extirpation of a metastatic lymph node
50 ([LN] early -HN2- or overt -HN3- LN metastasis according to Weishaar et al. [2014]) alongside the
51 resection of the primary cMCT significantly improved outcome. In the aforementioned study, dogs
52 with both high-grade and low-grade cMCTs treated with adjuvant chemotherapy, had a mean time to
53 progression (TTP) of 1461 days and a median tumour-specific survival (TSS) of 2213 days
54 (Marconato *et al.* 2018). However, most dogs that underwent lymphadenectomy had K-LG cMCTs,
55 and stratification according to histologic grade was not performed in the survival analysis; therefore,
56 no further information could be specifically provided for dogs with K-HG cMCTs (Marconato *et al.*
57 2018).

58 In a more recent study, RLN removal with or without RLN bed irradiation resulted in a significant
59 prolongation of progression-free survival (PFS) and overall survival (OS) in dogs with stage II high-
60 grade cMCTs, with a median PFS and OS of 125 and 330 days, respectively (Mendez *et al.* 2020).

61 Collectively, the above data support the beneficial effect of lymphadenectomy on the outcome of
62 dogs with stage II high-grade cMCTs; however, none of these studies have specifically focused on
63 dogs with K-HG cMCTs and HN3 LNs.

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

67

68 **Material and methods**

69

70 **Study design**

71 A multi-institutional retrospective cohort study

72

73 **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 *et 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.

100

101 **Staging and treatment**

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

105 Adjuvant medical treatment consisted of vinblastine ([Velbe; EuroGenerici] 2-3 mg/m² IV every 2
106 weeks for a total of eight doses) and prednisolone ([Prednicortone; Dechra] 1 mg/kg orally once daily
107 for the duration of the protocol), toceranib phosphate ([Palladia; Zoetis] 2.4-2.8 mg/kg orally on
108 Monday, Wednesday, Friday schedule for 6 months) or both (vinblastine [1.6 mg/m² IV every 2 weeks
109 for a total of eight doses] and toceranib phosphate [2.4-2.8 mg/kg orally on Monday, Wednesday,
110 Friday for the duration of the course]). Dogs also received additional medications during their
111 treatment for prophylactic management of paraneoplastic conditions associated with cMCTs,
112 consisting of chlorpheniramine ([Chlorphenamine; Crescent] 0.2-0.5 mg/kg orally twice daily) and
113 ranitidine ([Zantadine; CEVA] 2-4 mg/kg orally twice daily).

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

119

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

122

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

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

130

131 **Data extracted**

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

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

149

150 **Statistical analysis**

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

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

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

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

176 Survival plots were generated according to the Kaplan-Meier product-limit method. Survival
177 estimates were presented as medians with the corresponding 95% confidence intervals (95% CIs).
178 TTP and ST of both groups obtained with the Kaplan-Meier method were compared by use of log-
179 rank test.
180 Statistical analysis was performed with SPSS Statistics v.25 (IBM, Armonk, NY, United States).
181 Significance was set at $P < .05$.

182

183 **Results**

184 - **Patient data and tumour characteristics**

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

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

193

194 1. Group A – dogs that did not undergo lymphadenectomy

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

201 All dogs were asymptomatic at presentation (substage a).

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

205 Data on tumour diameter was available for 17 dogs. Median tumour diameter was 2 cm (range, 1-
206 4.5). At presentation, 6 (33.3%) tumours were ulcerated.

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

208 Metastatic RLNs included inguinal (n=6; 33.3%), popliteal (n=4; 22.2%), superficial cervical (n=3;
209 16.7%), axillary (n=3; 16.7%), and mandibular (n=2; 11.1%) LN.

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

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

214 KIT staining pattern was available for 6 (33.3%) cases: 2 cMCTs had pattern III, 2 had pattern II, and
215 2 had pattern I. Mutational analysis was available for 12 (66.7%) cMCTs: 2 had an ITD on exon 11,
216 1 had ITD on exon 8, and 9 were wild type.

217

218 2. Group B – dogs that underwent lymphadenectomy

219 Among the 31 dogs undergoing lymphadenectomy, there were 17 males (of which 9 castrated) and
220 14 females (of which 11 spayed). At the time of diagnosis, the median age was 10 years (range, 5-
221 15), and the median weight was 23 kg (range, 4.9-55). Represented breeds included: mixed breed
222 (n=10; 32.2%), miniature Pinscher (n=4; 12.9%), cane corso (n=3; 9.7%), golden retriever (n=2;
223 6.45%), and one (3.2%) each of Labrador retriever, Shar pei, American Staffordshire terrier, Bichon,
224 Bernese Mountain dog, Doberman pinscher, German shepherd, Jack Russell terrier, Weimaraner,
225 Maltese terrier, and Griffon.

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

228 (n=8; 25.8%), inguinal/perineal region (n=6; 19.3%), head and neck (n=3; 9.7%), and digits (n=3;
229 9.7%). Median tumour diameter was 3 cm (range, 2-4.2). At presentation, 12 (38.7%) tumours were
230 ulcerated.

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

232 A total of 52 RLNs were removed, including inguinal (n=16; 30.8%), superficial cervical (n=14;
233 26.9%), axillary (n=10; 19.2%), popliteal (n=6; 11.5%), mandibular (n=3; 5.9%), retropharyngeal
234 (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%)
235 dogs 2 LNs were removed, and in 2 (7.9%) dogs 4 LNs were removed.

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

238 Based on histopathology reports, there were 23 (74.2%) K-HG/P-G3 cMCTs, and 8 (25.8%) K-HG/P-
239 G2 cMCTs. Surgical margins were complete in 22 (71%) cMCTs, clean but close margins in 5
240 (16.1%) cases, and incomplete in 4 (12.9%) cases.

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

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

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

245

246 - **Treatment and outcome**

247 1. Group A – dogs that did not undergo lymphadenectomy

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

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

254 surgical margins. All (100%) dogs experienced nodal progression after a median of 148 days (range,
255 30-511), and 7 (39%) dogs developed distant metastasis after a median of 180 days (range, 72-205).
256 Median TTP was 150 days (95% CI, 129-170 days; Figure 1). Four (22%) dogs received an additional
257 medical treatment at the time of disease progression: 1 dog received lomustine ([Lomustine;
258 medac]70 mg/m² orally every 4 weeks) and prednisolone, and 3 dogs received toceranib.
259 At the end of the study, all dogs had died because of cancer-related (n=17; 92%) or unrelated (n=1;
260 8%) causes. The latter dog died due to gastric dilation volvulus after 140 days.
261 Median ST was 250 days (95% CI, 311-430 days; Figure 2).

262

263 2. Group B – dogs that underwent lymphadenectomy

264 Lymphadenectomy was well tolerated in all cases and no major complications were reported. Thirty
265 (97%) dogs were treated with systemic chemotherapy consisting of vinblastine and prednisolone,
266 while one dog (3%) was treated with vinblastine and toceranib. The latter dog had an ITD mutation
267 on exon 11. Overall, 17 (54.8%) dogs developed progressive disease. Of those, 8 (25.8%) dogs
268 experienced local recurrence after a median of 218 days (range, 160-536); two of these 8 dogs had
269 their cMCT removed with incomplete surgical margins. Thirteen (41.9%) dogs experienced nodal
270 relapse after a median of 228 days (range, 97-287), and 12 (38.7%) dogs developed distant metastasis
271 after a median of 267 days (range, 120-371). Median TTP was 229 days (95% CI, 191-266 days;
272 Figure 1).

273 Six (35%) dogs received an additional medical treatment at the time of disease progression: 2 dogs
274 received lomustine (60 mg/m² orally every 4 weeks and 70 mg/m² orally every 4 weeks, respectively)
275 and prednisolone, and 4 dogs received toceranib.

276 At data analysis closure, 13 (41.9%) dogs were alive, with a median follow-up of 180 days (range,
277 123-594), while 18 (58.1%) dogs had died because of cancer-related (n=15; 48.4%) or unrelated (n=3;
278 9.7%) causes. Two dogs died due to acute pancreatitis, and one dog due to heart failure.

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

280

281 **Analysis of outcome and prognostic variables**

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

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

288

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

292 When recurrence/progression characteristics were evaluated separately, tumour diameter >3 cm (HR:
293 5.53, 95%CI: 1.73-17.72; $P=0.004$) and incomplete surgical margins (HR: 3.99, 95% CI: 1.38-11.57;
294 $P=0.011$) were associated with a higher risk of local recurrence on univariable analysis (Table 2).

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

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

300 Lack of lymphadenectomy was the only variable associated with a higher risk of tumour-related death
301 both in univariable (HR: 3.57, 95% CI: 1.70-7.48; $P=0.001$) and multivariable (HR: 3.63, 95% CI:
302 1.72-7.66; $P=0.001$) analyses (Tables 5 and 6).

303

304 **Discussion**

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

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

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

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

331 2020). It has been shown that tangential sectioning detected more incomplete surgical margins than
332 radial sectioning, because the former evaluates a considerably greater percentage of the total margin
333 surface area (Dores *et al.* 2017). Moreover, it could be hypothesized that K-HG cMCTs > 3 cm are
334 associated with more infiltrative growth patterns. In these case, radial sections might be expected to
335 have even poorer precision in detecting incomplete surgical margins (Dores *et al.* 2017). Thus, it is
336 possible that the number of surgical margins determined to be complete in the current study as well
337 as in the Hume study was overestimated, thereby skewing the results. Further studies are required to
338 establish the impact of the trimming technique, tumour size and of histologically free-surgical
339 margins on local recurrence in dogs with K-HG MCTs.

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

354

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

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

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

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

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

374

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

382

383 **Figure legends**

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

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

393

394 **Table legends:**

395 Table 1: Demographic information and distribution of variables potentially associated with
396 prognosis in 49 dogs with high grade mast cell tumours and metastatic regional lymph nodes.

397 Differences in data distribution were assessed with Chi-square test/Fisher's exact test (categorical
398 variables) or Mann-Whitney U test (continuous variables). † cutaneous mast cell tumour; ‡ tyrosine
399 kinase inhibitors, *significant.

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

404 Table 3: Multivariable Cox regression analysis for risk of tumour progression. Variables with a
405 significance level of $P \leq 0.1$ at univariable analysis were included in the model. Abbreviations: CI,
406 confidence interval. *significant.

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

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

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

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