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The impact of extirpation of non-palpable/normal-sized regional lymph nodes on staging of canine cutaneous mast cell tumours: A multicentric retrospective study

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THE IMPACT ON STAGING OF EXTIRPATION OF NON-PALPABLE/NORMAL SIZED REGIONAL LYMPH NODE IN CANINE CUTANEOUS MAST CELL TUMORS: A MULTICENTRIC RETROSPECTIVE STUDY

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3 1 **THE IMPACT ON STAGING OF EXTIRPATION OF NON-PALPABLE/NORMAL-**
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5 2 **SIZED REGIONAL LYMPH NODE IN CANINE CUTANEOUS MAST CELL**
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7 3 **TUMOURS: A MULTICENTRIC RETROSPECTIVE STUDY**
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11 5 ABSTRACT

12 6 Metastasis to regional lymph nodes (RLN) in cutaneous mast cell tumour (cMCT) in
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14 7 dogs have been correlated with shorted of survival time and higher risk of spread to
15
16 8 distant sites. In the present study, extirpation of no-palpable or normal-sized regional
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18 9 RLNs was included in the surgical management of cMCT in dogs. Correlations
19
20 10 between histological nodal status (HN0-3) and tumour variables were analyzed.
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22

23 11 Ninety-three dogs with single cMCT without distant metastasis that underwent wide
24
25 12 surgical excision of the primary tumour and extirpation of no-palpable or normal-sized
26
27 13 RLN were included. The association between HN (HN0 vs HN>0; HN0-1 vs HN2-3)
28
29 14 and tumour variables (site, dimension, ulceration, 3-tier and 2-tier histological grades)
30
31 15 was analysed by a generalized linear model with multinomial error.
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33

34 16 Thirty-three (35.5%) RLN were HN0, 14 (15%) were HN1, 26 (28%) were HN2 and
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36 17 20 (21,5%) were HN3. The presence of positive (HN>0) RLN was significantly
37
38 18 associated with cMCT larger than 3 cm. No other association was statistically
39
40 19 significant. Mean and median follow-up time were 695 and 504 days, respectively
41
42 20 (range, 10-2429). Seven dogs developed metastatic spread to other lymph nodes
43
44 21 and/or other organs.
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47 22 Non-palpable/normal-sized RLN in dogs with cMCT can harbor histologically
48
49 23 detectable metastatic disease in nearly half of the cases. Further studies should
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51 24 evaluate the possible therapeutical effect of the tumour burden reduction obtained by
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53 25 extirpation of a positive RLN.
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27 Keywords: Dogs, Lymph node excision, Neoplasm staging, Lymphatic metastasis,

28 Mastocytoma

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30 Introduction

31 Lymph node (LN) metastasis is a well-known negative prognostic indicator in canine
32 cutaneous mast cell tumours (cMCTs).¹⁻⁹ The presence of LN metastasis implies a
33 higher risk of distant spread and the need for adjuvant chemotherapy, regardless of
34 the characteristics of the primary tumour, such as histological grade and proliferation
35 indexes.⁹ Needless to say, an early detection of nodal metastasis is crucial for prompt
36 and adequate therapeutic proposal, as well as for a correct staging and
37 prognostication. It is accepted that palpation has a limited value in predicting lymph
38 node metastasis in cMCT¹⁰⁻¹²; cytology as well has been associated with a high
39 proportion of both false positive and negative results.¹³ Furthermore, not all regional
40 lymph nodes (RLN) are feasible for immediate fine-needle aspiration due to their
41 anatomical location or size.¹⁴⁻¹⁶ Histopathology remains the gold standard for the
42 diagnosis of RLN metastasis¹⁰ but the role of lymphadenectomy of non-palpable or
43 normal-sized lymph nodes in increasing diagnostic accuracy and delineating
44 prognosis in canine cMCT has not been reported yet. Notably, some authors have
45 recently explored the utility of some diagnostic and surgical procedures in an attempt
46 to remove regional or sentinel LNs that were not clinically suspected for metastasis in
47 cMCTs and other canine malignancies in order to obtain an early detection.^{15,17-20}
48 Due to inconsistency in LN sampling inside the population enrolled, selection of
49 different inclusion criteria for the study population (e.g. high-risk cMCT or Patnaik
50 grade II cMCT only) and different sampling methods (cytology vs. histology) within
51 and among studies, it is difficult to extrapolate from the literature the exact rate of
52 metastatic nodal involvement in canine cMCT.^{3,11,14-16,21-23} In a recent paper, the
53 reported rate of nodal metastasis for canine cMCT at first presentation confirmed by
54 means of cytology was 18.1%,²³ this rate increased to 61% in the study by Baginsky

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3 55 and colleagues (2014) that included 90 dogs with grade 2 MCTs, of which 55 had an
4
5 56 enlarged RLN.¹¹
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7
8 57 One of the major concerns encountered in the histological diagnosis of nodal
9
10 58 metastasis in cMCTs is the interpretation of individual mast cells or small aggregates
11
12 59 within the LN.^{8,15} Recently, standardized histological criteria have been described to
13
14 60 document nodal involvement, consisting of 4 histological patterns that correlated with
15
16 61 outcome.²⁴ Based on this novel categorization²⁴, the purpose of the current study
17
18 62 was to assess the metastatic rate of non-palpable or normal-sized, surgically
19
20 63 removed, RLNs in canine cMCT. It was hypothesized that non-palpable or normal-
21
22 64 sized RLNs may often harbor histopathologically detectable metastatic disease. The
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24 65 RLN status was then correlated with tumour variables, including both
25
26 66 histopathological grading systems,^{25,26} in an attempt to find a possible predictive
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28 67 association.
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33 34 69 **Materials and Methods**

35 36 37 38 71 Case selection and data collection

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40 72 Medical records of client-owned dogs with a single cMCT referred to the XXX, YYY
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42 73 and ZZZ were reviewed. Dogs with multiple concurrent or subcutaneous MCTs were
43
44 74 excluded. To be eligible for inclusion all dogs had to be staged negative at admission
45
46 75 for distant metastasis, and the primary tumour and the RLN had to be surgically
47
48 76 removed. The excision of the primary tumour included from 2 to 3 cm of normal
49
50 77 tissue around the palpable edge of the mass and at least 1 deep fascial plane. Dogs
51
52 78 were included if the RLN identified as the anatomically closest LN to the primary
53
54 79 cMCT was non-palpable or normal-sized (not clinically enlarged, and equal to the
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3 80 contralateral). To exclude distant metastasis, thoracic radiography (3 views),
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5 81 complete blood cell count and biochemistry evaluation, ultrasound-guided cytology of
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7 82 spleen and liver regardless of their ultrasonographic appearance, with or without
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9 83 bone marrow cytologic evaluation were performed, as previously described.²⁷⁻²⁹ All
10
11 84 histopathological samples had to be available for review in order to apply the 3-tier
12
13 85 and 2-tier histological grading systems on the primary MCT²⁵⁻²⁶, and the Weishaar
14
15 86 histological classification on the RLN.²⁴
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18 87 Additional retrieved information included breed, age, weight, sex, presentation (first
19
20 88 vs recurrence), anatomic location of cMCT, maximum diameter of cMCT, presence of
21
22 89 ulceration, histological margin status (infiltrated vs not infiltrated), RLN location and
23
24 90 adjuvant treatment, if performed.
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27 91 All dogs were re-checked (physical examination, fine-needle aspiration of new
28
29 92 lesions) every 3 months during the first year, and every 6 months thereafter. A re-
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31 93 staging was always performed in the case of new or recurrent cMCT or LN
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33 94 metastasis. For dogs undergoing adjuvant medical therapy, clinical evaluation was
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35 95 repeated at every scheduled administration, or once a month in the case of
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37 96 continuous oral administration.
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40 97 Local recurrence was defined as the occurrence of a cMCT located less than 2 cm
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42 98 from the previous scar. Loco-regional progression was defined as the presence of
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44 99 metastatic disease to LNs other than the RLN, assessed via cytology and/or
45
46 100 histology. Distant progression was defined as the development of distant metastatic
47
48 101 disease to any organ with the exception of LNs, assessed via cytology and/or
49
50 102 histology. Time between RLN extirpation and loco-regional or distant progression
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52 103 was calculated. Overall survival was defined as the time from surgery to death. In
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54 104 case of death, the cause (related or not to cMCT) was retrieved.
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3 105 Statistical analysis
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5 106 The association between histopathological node (HN) category (Weishaar et al,
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7 107 2014) and clinicopathological variables was evaluated by generalized linear models
8
9 108 with binomial error. Two separate analysis were performed: the first for HN0 vs.
10
11 109 HN>0 and the second for HN0-HN1 vs. HN2-HN3. Model response was the HN
12
13 110 category, coded as 0 if HN0 and 1 if HN>0 for the first analysis, and coded as 0 if
14
15 111 HN0-HN1 and 1 if HN2-HN3 for the second analysis. Explanatory variables were
16
17 112 both categorical and continuous. Categorical variables (location, ulceration, Patnaik
18
19 113 grade and Kiupel grade) were considered as dummy variables, thus for a categorical
20
21 114 variable with K categories, K-1 dummy variables were included into the regression
22
23 115 model and one of the categories was considered as reference one. The variable
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25 116 "location" was categorized in 2 groups: sites historically associated with worse
26
27 117 prognosis (head and neck genital [including inguinal, scrotal, perivulvar and perineal]
28
29 118 and digit) vs. sites historically associated with better prognosis (lateral thorax and
30
31 119 abdomen, and limb, excluding digits).³⁰ Maximum tumour diameter was included in
32
33 120 its original measurement scale and also considered as categorical variable, coded as
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35 121 0 if < 3 cm and 1 if > 3 cm.²³
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40 122 Firstly, univariate analysis was performed for each of the above-mentioned variables,
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42 123 and then multivariate analysis was performed to evaluate the joint role of the
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44 124 variables. To obtain reliable results in the multivariate analysis, the maximum number
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46 125 of explicative variables was decided according to the rule suggesting a ratio of at
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48 126 least 10 between the number of subjects with model response coded as 1, and the
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50 127 number of regressors.³¹ To reach this aim the following variables, considered as
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52 128 related to each other, were evaluated in the multivariate analysis: maximum tumour
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54 129 diameter, location and Kiupel grade.
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3 130 Results of the regression model were reported as odds ratio (OR) with corresponding
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5 131 95% confidence intervals. The odds is the ratio between the proportion of subjects
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7 132 with $HN>0$ (or $HN2-HN3$) and the proportion of subjects with $HN=0$ (or $HN0-HN1$).
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9 133 For each categorical variable with K categories $K-1$ odds ratios are reported, each
10
11 134 one representing the ratio between the odds for the category and the odds for the
12
13 135 reference category. If $OR>1$, the estimated proportion of subject with $HN>0$ (or $HN2-$
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15 136 $HN3$) in the category is greater than that in the reference category (and vice-versa).
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17 137 In the absence of association between a variable and HN , OR is expected to be 1.
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19 138 The null hypothesis of $OR=1$ was tested by Wald statistics. As odds ratio is a
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21 139 measure of the association that is not of a direct clinical interpretation, the risk ratio
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23 140 corresponding to the odds ratio was also provided for the comparison discussed into
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25 141 results section.^{32,33}
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29 142 Analysis of outcome was explored. Time to event was calculated as the time elapsed
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31 143 from surgery to the date of distant or loco-regional progression or death (in absence
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33 144 of previous disease progression). For dogs being alive at the end of the study
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35 145 (censored data), time was calculated from the date of surgery to the one of the last
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37 146 clinical examination. Survival and event-free probabilities were estimated by the
38
39 147 Kaplan-Meier method. The correct application of log-rank test was investigated by
40
41 148 examining the relative shape of Kaplan-Meier estimated curves. In the case of
42
43 149 crossing curves, log-rank is not an adequate test. Specific modelling techniques
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45 150 based on the adjustment of log-rank weights are available but they are not
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47 151 suitable in the case of low number of events. Thus, in the presence of crossing
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53 152 hazard, an approach based on Landmarking was considered for explorative aim³⁴.
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3 153 Follow-up time was partitioned in intervals of 25 days and for each interval the
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7 154 hazard ratio was estimated from a Cox model on subjects at risk to the beginning
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10 155 of the interval. The approach allowed to show the time dependent pattern of HN
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13 156 prognostic impact.

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16 157 Median, first and third quartile for follow-up time were estimated by the reverse
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18 158 Kaplan-Meier method.³⁵

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21 159 All analyses were performed with a software package (R-Software; www.r-
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23 160 project.org) and a $p \leq 0.05$ was considered significant.

24 25 161 **Results**

26 27 162 Patient population

28
29 163 Ninety-three dogs fulfilled the inclusion criteria. There were 21 (22.6%) mixed-breed
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31 164 dogs, 25 (26.9%) Retrievers, 11 (11.8%) Boxers, 4 (4.3%) Shar-pei and 32 (34.4%)
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33 165 dogs belonging to other pure breeds (from 1 to 3 dogs for each breed). Thirty-six
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35 166 (38.7%) dogs were males (10 neutered), and 57 (61.3%) were females (41
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37 167 neutered). Mean and median age was 7.5 and 7 years, respectively (range 1-14
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39 168 years). Mean and median weight was 23.8 and 25.6 kg, respectively (range 2.9-47
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41 169 kg).

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45 170 Ninety (96,8%) cMCT represented a first presentation, whereas 3 cMCT (3.2%) were
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47 171 a cMCT recurrence after previous surgery. Eleven (11.8%) cMCT were ulcerated.

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49 172 Twenty-two (23.7%) cMCTs were located on the head, 4 (4.3%) on the neck, 25
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51 173 (26.8%) on the trunk (including above knee and elbow joint, lateral thorax and lateral
52
53 174 abdomen), 20 (21.5%) on the distal limb (distal to elbow and knee joints), 5 (5.4%) on
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55 175 the digit and 17 (18.3%) in the genital region (scrotal, perineal, perivulva, prepuzial,
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3 176 inguinal region). Mean and median dimension were 1.83 and 1.5 cm, respectively
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5 177 (range 0.2 – 5.3 cm).

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7 178 Histologically, there were 7 (7.5%) Patnaik grade I cMCTs, 81 (87.1%) Patnaik grade
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9 179 II and 5 (5.4%) Patnaik grade III cMCTs; using the 2-tier grading system, 83 (89.3%)
10
11 180 cases were low-grade cMCTs, and 10 (10.7%) were high-grade tumours. All Patnaik
12
13 181 grade I cMCTs were Kiupel low-grade, and all Patnaik grade III cMCTs were Kiupel
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15 182 high-grade. Seventy-six of the 81 (93.8%) Patnaik grade II cMCTs were Kiupel low-
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17 183 grade, while 5 (6.2%) Patnaik grade II cMCTs were Kiupel high-grade tumours. In 24
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19 184 (25.8%) cases, the margins were infiltrated (all Patnaik grade II; 23 Kiupel low grade
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21 185 and 1 Kiupel high grade).

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23 186 The extirpated RLN included 24 (25.8%) mandibular nodes, 20 (21.5%) prescapular
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25 187 nodes, 28 (30.1%) popliteal nodes, 18 (19.3%) superficial inguinal nodes, 2 (2.2%)
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27 188 axillary nodes and 1 (1.1%) axillary accessory node. Histologically, 33 (35.5%) LNs
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29 189 were classified as HN0, 14 (15%) as HN1, 26 (28%) as HN2 and 20 (21.5%) as HN3
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31 190 (Table 1).

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38 192 *Association between clinicopathological variables and HN category (HN0 vs HN>0)*

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40 193 Results of univariate analysis are summarized in Table 2. Only dimension of the
41
42 194 primary tumour was associated with RLN status: dogs with cMCT bigger than or
43
44 195 equal to 3 cm had a higher probability to have HN>0 LN if compared to dogs with
45
46 196 smaller tumours (risk ratio=1.42).

47
48 197 Despite no statistically significant Patnaik grade II and III cMCT tended to have a
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50 198 greater probability of HN>0 compared to Patnaik grade I tumours (risk ratio=1.56
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52 199 and risk ratio=1.40, respectively), and the same consideration held true for Patnaik
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3 200 grade II/Kiupel low-grade and Patnaik grade III/Kiupel high-grade cMCT if compared
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5 201 to Patnaik grade I/Kiupel low-grade tumour. (risk ratio= 1.60 and risk ratio=1.40
6
7 202 respectively) Unexpectedly, Kiupel high-grade MCTs had a risk of having a RLN
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9 203 HN>0 about a quarter lower than that of Kiupel low-grade cMCT (risk ratio=0.76).
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11 204 By multivariate analysis, dimension remained a significant prognostic variable for
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13 205 HN>0 (risk ratio=1.43, Table 3).
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18 207 Association between clinicopathological variables and HN category (HN0-1 vs HN2-
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21 208 HN3)

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23 209 Results of univariate analysis are summarized in Table 4. Despite the absence of
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25 210 statistical significance for all variables, cMCT bigger than 3 cm, ulcerated or of
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27 211 Patnaik grade III tended to have a higher risk for RLN categorized as HN2-3 (risk
28
29 212 ratio=1.28, risk ratio=1.34, and risk ratio=1.40, respectively).
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31 213 No significant statistical association was found by multivariate analyses (Table 5). A
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33 214 HN2-HN3 RLN tended to be more likely for cMCTs > 3 cm (risk ratio =1.40).
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38 216 Outcome

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40 217 Forty-nine dogs (52.7%) did not receive any adjuvant therapy. The remaining 44
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42 218 (47,3%) dogs received adjuvant chemotherapy: 29 (31.2%) received vinblastine-
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44 219 prednisone, 6 (6.5%) vinblastine-prednisone in association with tyrosine kinase
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46 220 inhibitors (TKI), 6 (6.5%) TKI only, and 3 (3.2%) received other chemotherapeutic
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48 221 agents (n=2 chlorambucil, and n=1 lomustine). The LN status of these 44 dogs
49
50 222 included 6 HN0, 6 HN1, 15 HN2 and 17 HN3.
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52 223 Seven (7.5%) dogs were lost to follow-up at a mean and median time of 458 and 650
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54 224 days, respectively (one of which with metastatic disease at 302 days, the remaining
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225 had no sign of disease). Overall median follow-up was 596 days, 25% of cases were
226 observed for a period longer than 1188 days, and 75% of cases were observed for a
227 period longer than 266 days.

228 Local recurrence was detected in 2 dogs after 29 and 337 days from surgery,
229 respectively. Seven dogs experienced metastatic disease. Loco-regional progression
230 with a positive LN was detected in 5 cases after a range of 52 to 1071 days from
231 surgery (Table 6). Distant progression to spleen and liver was identified in 5 dogs
232 after a range of 52 to 1071 days (3 out of this 5 dogs had also simultaneous loco-
233 regional relapse) (Table 6). The RLN status of dogs with loco-regional and/or distant
234 relapse included 4 HN3, 1 HN2, 1 HN1, and 1 HN0 (Table 6).

235 Sixty-eight dogs were still alive at the end of the study, 13 dogs were dead for causes
236 unrelated to cMCT, and 5 dogs were dead due to cMCT. The survival probability at
237 730 and at 1460 days was 0.856 (95% confidence interval: 0.776-0.945) and 0.591
238 (95% confidence interval: 0.434-0.803), respectively. Considering the first event
239 (loco-regional or distant metastasis or death in absence of disease progression)
240 analysis, 20 cases with events were observed (13 cases were dead without loco-
241 regional or distant progression, 5 cases were dead after loco-regional or distant
242 progression, and 2 cases were alive despite distant or loco-regional progression).
243 The probability of remaining free from event was 0.833 (95% confidence interval:
244 0.750-0.925) at 730 days, and 0.577 (95% confidence interval: 0.424- 0.785) at 1460
245 days.

246 The prognosis for HN0-HN1 cases was better than the prognosis for HN2-HN3 cases
247 up to 1000 days; however, after this follow-up time a reverse pattern was observed
248 (Figure 1). The probability of remaining free from event at 730 days was 0.902 (95%
249 confidence interval: 0.801-1.000) and 0.766 (95% confidence interval: 0.6422- 0.915)

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3 250 for HN0-HN1 and HN2-HN3, respectively, whereas the probability of remaining free
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5 251 from event at 1460 days was 0.434 (95% CI:0.226-0.834) and 0.719 (95%CI: 0.5781
6
7 252 -0.893) for HN0-HN1 and HN2-HN3, respectively (Figure 1). Because of the wide
8
9 253 confidence intervals of the event free survival curve, this reverse pattern should be
10
11 254 considered with caution. The event-free survival curves of HN0-HN1 vs HN2-HN3
12
13 255 were not statistically significant. The landmarking approach suggested a risk of event
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15 256 for dogs with HN2-HN3 higher than that for dogs with HN0-HN1 in the early period
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17 257 (before 175 days the estimated HRs decreased from 1.2 to 1.1); following which a
18
19 258 reverse pattern was estimated and the risk of event was higher for dogs with HN0-
20
21 259 HN1 (the estimated HRs decreased from 0.987 to 0.170 at 825 days).

25 260 **Discussion**

26
27 261 In the present study, 93 dogs with a single cMCT and non-palpable or normal-sized
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29 262 RLNs underwent LN extirpation. Surprisingly, half of the RLNs were documented as
30
31 263 metastatic, based on histopathology (HN2 and HN3). When including the pre-
32
33 264 metastatic status, this percentage increased to 65%. These data are similar to those
34
35 265 reported by Worley (2012) in a smaller case-series, in which 12 out of 19 cases had
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37 266 a positive sentinel LN, even if the histopatological categorization of nodal metastasis
38
39 267 was not available at that time.¹⁵ Based on the documented prognostic value of HN2
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41 268 and HN3 reported by Weishaar and colleagues (2014),²⁴ our results have a
42
43 269 significant clinical impact, because in the absence of histopathological evaluation of
44
45 270 the RLN, all these cases would have been incorrectly staged, possibly overestimating
46
47 271 prognosis and undertreating dogs. Actually, the histological grading of the primary
48
49 272 cMCT is considered one of the most important prognostic factors guiding
50
51 273 treatment.^{8,36} Surprisingly, only a small proportion of dogs with HN2 and HN3 had a
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53 274 Kiupel high-grade (n=5; 5.3%) or Patnaik grade III cMCT (n=3; 3.2%). Consequently,
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3 275 in a big proportion of dogs with Kiupel low grade (n= 41; 44.1%) and Patnaik grade I
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5 276 (n= 3; 3.2%), or Patnaik grade II cMCT (n=40; 43%) a systemic adjuvant treatment
6
7 277 would have not been offered if the LN was not removed.
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9
10 278 The association between clinicopathological variables of cMCTs and the histological
11
12 279 LN status²⁴ was analysed as an initial step for a possible prediction model for non-
13
14 280 palpable/normal-sized LN metastasis, possibly dictating surgical decisions (lymph
15
16 281 node extirpation versus no lymphadenectomy). Unfortunately, the low number of
17
18 282 dogs included in each category precluded the possibility to analyse each group
19
20 283 separately. The role of the pre-metastatic HN1 LNs is still under debate.^{24,36}
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22
23 284 Therefore, two different analyses were performed by including HN1 cases with HN2-
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25 285 HN3 and with HN0.

26
27 286 The statistical analysis failed to associate the RLN status with other
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29 287 clinicopathological variables, including both histological grading systems. Only
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31 288 tumours bigger than 3 cm were statistically correlated with a higher probability of
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33 289 RLN classified as HN>0. However, this significant correlation was not confirmed
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35 290 when the pre-metastatic status (HN1) was considered combined with HN0.
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37
38 291 Nevertheless, some aspects must be underlined. Although there is no general
39
40 292 agreement for evaluating odds ratio in terms of strength of association, some authors
41
42 293 reported an *odds ratio* greater than 1.6 and lower than 3.0 as moderate association
43
44 294 for epidemiologic studies.³⁷ Considering the number of dogs included in the present
45
46 295 study, such estimates cannot result as “statistically significant”, because a sample of
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48 296 about 354 cases, equally subdivided in the 4 categories of histological node status,
49
50 297 would have been required to obtain a 90% power of the test. Taking into
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52 298 consideration the aforementioned statement, further studies should be designed to
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54 299 better explain the negative prognostic correlation between Patnaik grade II and III
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3 300 cMCT and nodal metastasis, and the low rate of nodal metastasis for Kiupel high
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5 301 grade tumours reported in the present work. Notably, the application of both grading
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7 302 system simultaneously also failed to clarify the prognostic role on non-palpable and
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9 303 normal-sized RLN metastasis detection.^{23,38} These results highlight the complexity
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11 304 relationship and maybe the independency between staging and grading in cMCT in
12
13 305 dogs.

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15 306 Which LN should be removed is currently based on its anatomical proximity to the
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17 307 tumour rather than on the assessment of the lymphatic drainage pathway with
18
19 308 sentinel LN mapping methods. A recent study considering different malignancies on
20
21 309 the head (including 3 cMCTs) found a high frequency of medial retropharyngeal
22
23 310 lymph node metastasis with contralateral dissemination.¹⁸ In the study of Worley
24
25 311 (2012), 8 out of 19 dogs with MCTs had a sentinel LN recognized by
26
27 312 lymphoscintigraphy that differed from the anatomically identified RLN.¹⁵ Nonetheless,
28
29 313 due to the high rate of nodal involvement retrieved in the present study, it may be
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31 314 hypothesized that the detection of draining LNs with mapping techniques matches
32
33 315 quite well with the anatomical selection. Further studies on the application of sentinel
34
35 316 LN mapping techniques should be performed to elucidate the real advantages of this
36
37 317 extra diagnostic procedure and the possible error related to the anatomical detection.
38
39 318 The analysis of outcome was not a primary aim of the study due to its retrospective
40
41 319 nature and the heterogeneity of treatment and follow-up examinations. However,
42
43 320 some results are of interest and should be further explored. First of all, the number of
44
45 321 cases with metastatic progression (n= 7) was low if considering the high number of
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47 322 metastatic LN at admission (n=46; 49.5%); also, it was lower than what reported by
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49 323 Weishaar and colleagues (2014).²⁴ It is possible that the inclusion of non-palpable or
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51 324 normal-sized RLN in the current study may have selected "early" cases, thus carrying
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3 325 a better prognosis than dogs with clinically enlarged RLNs. Baginsky and colleagues
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5 326 (2014) also hypothesized that the reduction of tumour burden by means of extirpation
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7 327 of metastatic LNs in Patnaik grade II cMCT may prolong survival, and this may be
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9 328 emphasized in the case of early micrometastasis.¹¹ Whether chemotherapy should
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11 329 be administered in dogs with metastatic non-palpable or normal-sized lymph node
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13 330 remains to be elucidated. However, based on the current results and in agreement
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15 331 with the Weishaar's study,²⁴ medical antitumour treatment should be offered and
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17 332 undertaken in the case of HN2-HN3 LNs, regardless of their size. Dogs with HN0-
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19 333 HN1 nodes tended to progress at a later stage compared to dogs with HN2-HN3
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21 334 nodes. The progression rate was too low to reach definitive evidence, and further
22
23 335 studies should verify a possible diversification of time to progression between the
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25 336 different categories of histopathological nodal metastasis. At the same time, the
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27 337 relative high rate of progression for dogs with HN3 RLN (4 out of 7) compared to
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29 338 dogs with HN2 RLN (1 out of 7), prompts to verify the role of each histological node
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31 339 category.

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36 340 Even if the collaboration of 3 veterinary referrals permitted to collect almost 100
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38 341 cases, this value was still low precluding the possibility to analyse each HN category
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40 342 as a unique variable. In addition, the relative high number of dogs with HN1 and its
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42 343 unclear prognostic role^{24,36} prevented to achieve a correct results interpretation.
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44 344 Further studies should focus on the prognostic role of RLN status. The different post-
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46 345 surgical treatment approach and the influence of owner's decision did not permit to
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48 346 draw conclusion on the possible therapeutic role of metastatic non-palpable or
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50 347 normal-sized RLN extirpation. Finally, the identification of the RLN by means of
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52 348 anatomical evaluation rather than sentinel LN mapping techniques may have led to
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3 349 selection bias and limited the number of dogs enrolled, as only cases in which the
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5 350 RLN was recognizable and removable were included in the current study.
6

7 351 In conclusion, non-palpable or normal-sized RLN may harbour occult metastatic
8
9 352 disease in dogs with cMCT, regardless of the histological grade of the primary cMCT.
10
11 353 The extirpation of non-palpable or normal-sized RLNs permitted an early detection of
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13 354 nodal metastasis and a more accurate tumour staging. Even if size of the primary
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15 355 tumour tended to correlate with a positive node, no significant correlation with
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17 356 clinicopathological variables was found. Further prospective studies are needed to
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19 357 elucidate the therapeutic role of lymphadenectomy of metastatic non-palpable or
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21 358 normal-sized RLN.
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23 359 **Conflict of Interest Statement**

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27 360 The authors declare no conflicts of interest.
28

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477 **Tables**

478 Table 1. Distribution of histological lymph node status among tumour variables.

479

cMCT variables	HN0	HN1	HN2	HN3
Site				
Not associated with worse prognosis	16	7	16	7
Associated with worse prognosis	17	7	10	13
Dimension				
Median (cm)	1.3	1.9	1.7	1.75
3 cm cut-off				
<3cm	30	10	18	16
>=3cm	3	4	8	4
Ulceration				
Yes	3	1	2	5
No	30	13	24	15
Patnaik				
I	4	0	1	2
II	27	14	23	17
III	2	0	2	1
Kiupel				
Low grade	28	14	23	18
High grade	5	0	3	2
Patnaik-Kiupel				
I-low grade	4	0	1	2
II-low grade	24	14	22	16
II-high grade	3	0	1	1
III-high grade	2	0	2	1

481

482

483 Table 2. Association between cMCT clinicopathological variables and HN category
 484 (HN0 vs. HN>0): Univariate analysis.
 485

cMCT variables	Odds Ratio	95% C.I.	Z	p	Risk Ratio
SITE					
Not associated vs. associated with worse prognosis	1.06	0.45-2.49	0.14	0.89	1.02
DIMENSION					
Increasing of 1 cm > 3 cm vs. ≤ 3cm	1.30 3.64	0.88-1.93 0.97-13.58	1.33 1.92	0.19 0.05	1.42
ULCERATION					
Yes vs. no	1.54	0.38-6.25	0.60	0.55	1.15
PATNAIK					
II vs. I	2.67	0.56-12.78	1.23	0.22	1.56
III vs. I	2.00	0.19-20.62	0.58	0.56	1.40
KIUPEL					
High vs. low grade	0.51	0.14-1.91	-1.00	0.32	0.76
HISTOLOGICAL GRADE					
II-low grade vs. I-low grade	2.89	0.60-13.93	1.32	0.19	1.60
II-high grade vs. I-low grade	0.89	0.09-9.16	-0.10	0.92	0.93
III-high grade vs. I-low grade	2.00	0.19-20.62	0.58	0.56	1.40

486
 487 Legend: Odds Ratio = ratio between Odds HN>0 of each category and Odds HN>0
 488 of reference category; 95% C.I. = 95% confidence interval of Odds Ratio; Z= Wald
 489 Statistics; Risk Ratio = ratio between proportion of HN>0 of each category and
 490 proportion HN>0 of reference category.

491

492 Table 3. Association between cMCT clinicopathological variables and HN category
 493 (HN0 vs. HN>0): Multivariate analysis.

494

cMCT variables	Odds Ratio	95% C.I.	Z	p	Risk Ratio
SITE Not associated vs. associated with worse prognosis	0.69	0.27-1779	-0.76	0.45	0.88
DIMENSION > 3 cm vs. ≤ 3cm	4.28	1.07-17.21	2.05	0.04	1.46
KIUPEL High vs. low grade	0.43	0.11-1.75	-1.18	0.24	0.66

495

496 Legend:Odds Ratio = ratio between Odds HN>0 of each category and Odds HN>0 of
 497 reference category; 95% C.I. = 95% confidence interval of Odds Ratio; Z= Wald
 498 Statistics; Risk Ratio = ratio between proportion of HN>0 of each category and
 499 proportion HN>0 of reference category.

500

501 Table 4 . Association between cMCT clinicopathological variables and HN category
 502 (HN0-1 vs. HN2-3): Univariate analysis.

503

cMCT variables	Odds Ratio	95% C.I.	Z	p	Risk Ratio
SITE Not associated vs. associated with worse prognosis	1.04	0.46-2.35	0.10	0.92	1.02
DIMENSION Increasing of 1 cm > 3 cm vs. ≤ 3cm	1.14 2.02	0.81-1.62 0.72-5.70	0.74 1.32	0.46 0.19	1.38
ULCERATION Yes vs. no	1.93	0.52-7.10	0.99	0.32	1.34
PATNAIK II vs. I III vs. I	1.30 2.00	0.27-6.18 0.19-20.61	0.33 0.58	0.74 0.56	1.15 1.40
KIPEL High vs. low grade	1.02	0.28-3.81	0.04	0.97	1.01
HISTOLOGICAL GRADE II-low grade vs. I-low grade II-high grade vs. I-low grade III-high grade vs. I-low grade	1.33 0.89 2.00	0.28-6.36 0.09-9.16 0.19-20.62	0.36 -0.10 0.58	0.72 0.92 0.56	1.17 0.93 1.40

504

505 Legend:Odds Ratio = ratio between Odds HN>1 of each category and Odds HN>1 of
 506 reference category; 95% C.I. = 95% confidence interval of Odds Ratio; Z= Wald
 507 Statistics; Risk Ratio = ratio between proportion of HN>1 of each category and
 508 proportion HN>1 of reference category.

509

510 Table 5. Association between cMCT clinicopathological variables and HN category
 511 (HN0-1 vs. HN2-3): Multivariate analysis.

512

513

cMCT variables	Odds ratio	95% C.I.	Z	p	Risk Ratio
SITE Not associated vs associated with worse prognosis	0.87	0.36-2.10	-0.31	0.76	0.93
DIMENSION > 3 cm vs ≤ 3cm	2.13	0.71-6.33	1.36	0.18	1.40
KIUPEL High vs low grade	0.98	0.25-3.80	-0.03	0.98	0.99

514

515 Legend: Odds Ratio = ratio between Odds HN>1 of each category and Odds HN>1
 516 of reference category; 95% C.I. = 95% confidence interval of Odds Ratio; Z= Wald
 517 Statistics; Risk Ratio = ratio between proportion of HN>1 of each category and
 518 proportion HN>1 of reference category.

519

520 Table 6. Cases with loco-regional and/or distant metastatic progression.

521

Case	MCT size (cm)	MCT site	RLN	3-tier grading	2-tier grading	HN	Chemotherapy	MCT progression (days)	Survival
1	5	neck	mandibular	II	low	3	Vinblastine + prednisone	LRP (52) DP (52)	Death due to cMCT
2	1,5	inguinal	inguinal	III	high	3	Vinblastine + prednisone + TKI	LRP (415)	Death due to cMCT
3	2,2	head	mandibular	II	low	3	Vinblastine + prednisone + TKI	LRP (126)	Alive
4	3,2	trunk	prescapular	II	low	2	TKI	DP (218)	Death due to cMCT
5	1,5	head	mandibular	II	low	1	no	DP (759)	Death due to cMCT
6	1,2	head	mandibular	II	high	3	Vinblastine + prednisone + TKI	LRP (293) DP (302)	Alive
7	1,5	perineal	inguinal	II	low	0	no	LRP (1071) DP (1071)	Death due to cMCT

522

523 Legend: TKI= Tyrosin-kinase inhibitor; LRP= loco-regional progression; DP= distant

524 progression.

525

1
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3 526 **Figure legends**
4

5 527 Figure 1. Kaplan-Meier curves described the probability to be free of event (loco-
6 regional progression or distant progression or death without progression) for dogs
7 528 regional progression or distant progression or death without progression) for dogs
8 with lymph node status HN0-HN1 (solid line) and for dogs with lymph node status
9 529 with lymph node status HN0-HN1 (solid line) and for dogs with lymph node status
10 HN2-HN3 (dotted line). Vertical lines are censored data (case alive at the end of the
11 530 HN2-HN3 (dotted line). Vertical lines are censored data (case alive at the end of the
12 study or lost to follow-up in absence of disease progression).
13 531 study or lost to follow-up in absence of disease progression).
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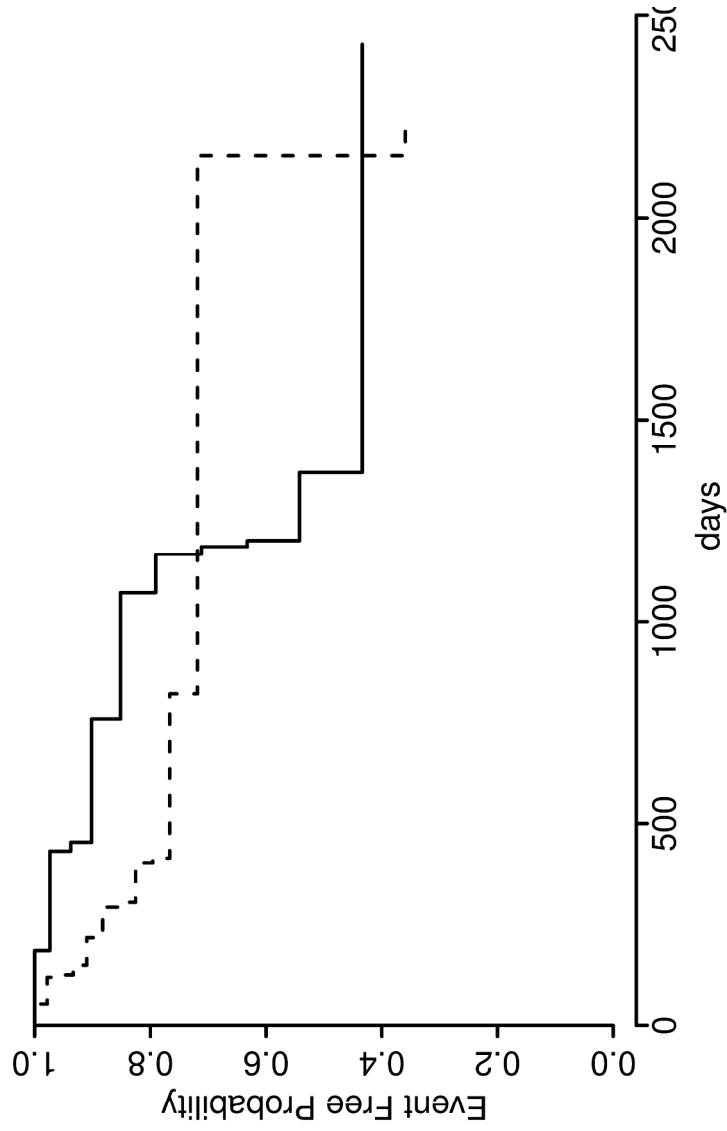


Figure 1. Kaplan-Meier curves described the probability to be free of event (loco-regional progression or distant progression or death without progression) for dogs with lymph node status HN0-HN1 (solid line) and for dogs with lymph node status HN2-HN3 (dotted line). Vertical lines are censored data (case alive at the end of the study or lost to follow-up in absence of disease progression).

279x361mm (300 x 300 DPI)