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Histologic grade has a higher-weighted value than nodal status as predictor of outcome in dogs with cutaneous mast cell tumors and overtly metastatic sentinel lymph nodes

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Running head: Histological grade beats nodal status

Abstract

In canine cutaneous mast cell tumors (cMCTs), histologic grade and clinical stage are the most important prognostic factors, with high-grade tumors and metastatic lymph nodes (LNs) significantly influencing the evolution of disease. However, it is uncertain whether histologic grade and clinical stage should be given equal weighting value in patient prognostication and management.

Dogs with low- and high-grade cMCTs and at least one overtly metastatic sentinel LN undergoing standardized treatment, consisting of surgical excision of the cMCT, lymphadenectomy and chemotherapy, were retrospectively included. The aim was to determine whether, at the same clinical stage, histologic grade retained prognostic relevance.

Sixty dogs were included: 26 had a high-grade cMCT tumor and 34 had a low-grade cMCT. Median follow-up was 367 days (range, 187-748) in the high-grade group, and 1208 days (range, 180-2576) in the low-grade group. Median time to progression was significantly shorter in the high-grade group than in the low-grade group (214 days versus not reached; P<0.001), as well as tumor-specific survival (545 days versus not reached; P<0.001). On multivariable analysis, a high histologic grade and incomplete margins retained prognostic significance for both tumor progression and tumor-specific death.

In dogs with cMCT and at least one overtly metastatic LN undergoing multimodal treatment, histologic grade significantly correlated with outcome. Overall prognosis was not unfavorable, even in the high-grade group, further supporting that a multimodal therapeutic approach, addressing primary tumor and sentinel LN, should be offered. Whether chemotherapy should be incorporated in the therapeutic planning of low-grade cMCTs remains to be defined.

Keywords

Canine, lymphadenectomy, mastocytoma, Kiupel high-grade, sentinel lymph node metastasis

Word count: 3492

Introduction

Histologic grade and clinical stage are the most important prognostic indicators for outcome in canine cutaneous mast cell tumors (cMCTs).[1-3]

Histologic grade is universally accepted and used by clinicians in treatment decision-making, as several studies have demonstrated its independent prognostic value.[2,4] Since 2011, cMCTs have been classified according to a 2-tier system into low-grade and high-grade tumors.[1]

Clinical stage is also used by clinicians for patient management. WHO stage 2 disease refers to the presence of regional lymph node (LN) metastasis. Recently, standardized histologic criteria have been proposed to more consistently characterize nodal involvement, and 4 histologic patterns have been identified: HN0 (non-metastatic LN), HN1 (pre-metastatic LN), HN2 (early metastasis) and HN3 (overt metastasis).[5]

Presence and extension of LN metastasis influence the evolution of disease as much as other clinical criteria referred to the primary tumor. It could be assumed that the risk of developing metastases increases with histologic grade, because the more undifferentiated the cancer is at diagnosis, the more cells presumably have the capability to metastasize.[1] Regrettably, the relationship between histologic grade and nodal status is not straightforward, as low-grade cMCTs may be overtly metastatic[2,4] and high-grade cMCTs may stage negatively.[7] This can lead to underestimation of the risk in the first case (e.g., assuming that a low-grade cMCT does not spread, leading to undertreatment), and overtreatment in the second, carrying the risk of adverse events in the face of no substantial outcome benefit.

Traditionally, dogs with high-grade cMCTs with or without HN3 LN are not cured by surgery and/or radiation therapy, advocating for the addition of adjuvant chemotherapy.[8,9] On the other hand, based on the current knowledge, it is difficult to anticipate the prognosis for dogs with low-grade cMCTs and HN3 LN. Thus, a frequent clinical dilemma is whether to administer or not chemotherapy to these patients. Furthermore, it has been recently shown that lymphadenectomy is therapeutic for dogs with low-grade cMCT and HN2 LN, and prophylactic for those with HN0/1 LN.[10,11]

In the present study, the prognostic relevance of HN3 LN was analyzed in a series of dogs with low-grade and high-grade cMCTs treated with surgical excision of the primary tumor, sentinel lymphadenectomy and chemotherapy. The aim was to determine whether, at the same clinical stage, histologic grade retains relevance in the decision-making process.

Material and methods

Medical records referred to two Oncology Units (*masked for review*) were reviewed to identify dogs with treatment-naive, firstly occurring, histologically confirmed cMCT of any histologic grade with one or more sentinel HN3 LN according to Weishaar[5] and no distant spread.

To be eligible for inclusion, dogs had to undergo complete staging work-up and wide surgical excision of the primary cMCT and simultaneous lymphadenectomy of the sentinel LN(s), regardless of size and mobility.

Sentinel LNs were identified by means of lympho-CT or scintigraphy and/or nodal methylene blue dye uptake after peritumoral injection.[12,13] All "hot" and/or "blue" LNs were removed and submitted for histopathology; only dogs with at least one HN3 LN were ultimately included.

LNs were histologically evaluated by multiple pathologists that were not aware of sentinel LN mapping results.

Standard recommendations following incomplete resection of high grade MCTs include revision surgery where possible or postoperative definitive radiation therapy. All dog owners were offered re-excision or radiation therapy, but were included following declining pursual of adequate local control.

Information on clinical stage was obtained by means of the following: hematological and biochemical analysis; cytologic evaluation of the cutaneous nodule; thoracic radiographs; abdominal ultrasound, and fine-needle aspirates of liver and spleen regardless of their sonographic appearance. The primary tumor was graded according to Kiupel into low-grade or high-grade.[1]

All dogs received adjuvant medical therapy, consisting of vinblastine (2-3 mg/m^2 IV every 2 weeks, depending on the dog's weight, for 8 cycles) and prednisone (1 mg/kg PO once daily for the entire length of the protocol) or toceranib (2.4 mg/kg on a Monday-Wednesday-Friday

schedule for 6 months), depending on c-kit mutational status, if available, and clinician's and owner's preference.

After completion of treatment, dogs were followed-up by means of clinical rechecks every 3 months for the first year, and every 6 months thereafter. Imaging was repeated whenever indicated. Only dogs with a minimum follow-up time of 180 days from surgery were included in the analysis, unless a documented event (recurrence or death) occurred prior to 180 days.

Dogs with concurrent multiple and/or subcutaneous MCTs, those with distant metastasis or those receiving neoadjuvant or post-operative radiation therapy were excluded from the study.

Background information recorded for each dog included: signalment (i.e, breed, age, sex, weight), clinical substage (i.e, asymptomatic [substage a] or symptomatic [substage b]), primary tumor description (i.e, location, longest diameter, presence of ulceration), presence of clinically altered regional LNs, histologic grade, histopathologic evaluation of surgical margins (complete, or incomplete, if aggregates of mast cells were seen within 1 mm of the surgical margin), Ki-67 index (if performed); c-kit mutational status (exons 8, 9, 10, 11) (if performed), number of removed HN3 LN(s), type of medical treatment.

Regarding outcome, local relapse (LR) was defined as the cytologic evidence of a recurrent cMCT within 2 cm from the previous scar. Nodal relapse (NR) was defined as presence of newly diagnosed metastatic LNs confirmed by cytology. Distant relapse (DR) was defined as the occurrence of cytologically-confirmed visceral metastasis.

Date of death or last follow-up examination, and cause of death were registered.

The care of the dogs included in the study was in accordance with institutional guidelines. All owners provided written informed consent.

Statistical analysis

Descriptive statistics were used in the analysis of dogs and tumor characteristics. When appropriate, data sets were tested for normality by use of the D'Agostino and Pearson omnibus normality test. No data had normal distribution and were therefore expressed as median (range). Dogs were then categorized into low-grade or high-grade groups, and the distribution of demographic features and possible prognostic variables among these groups were assessed with the Mann Whitney U test or the Chi-square test/ Fisher's exact test for continuous and categorical variables, respectively.

Time to progression (TTP) was calculated from the date of surgery to the first occurrence of one or more of LR, NR or DR or to the last visit. Tumor-specific survival (TSS) was calculated from the date of surgery to the date of death for tumor-related causes or to the last visit. If tumor progression or death for tumor-related causes did not occur, dogs were censored for the respective statistical analysis.

Survival plots were generated according to the Kaplan-Meier product-limit method. TTP and TSS of dogs in the low-grade group and those of dogs in the high-grade group were compared by means of the log-rank test.

The influence of potential prognostic variables on tumor progression and tumor-related death was investigated with univariable and multivariable Cox proportional hazards model. Only covariates that were significant at univariable analysis were included in the multivariable (adjusted) regression model. The considered variables included breed (predisposition to biologically aggressive MCTs, i.e. Shar-pei, Labrador retriever and Golden retriever),[36] sex, age, weight, substage, anatomic location of the primary cMCT (biologically aggressive locations, i.e. head and neck, inguinal/perineal area, mammary region and digits), macroscopic tumor longest diameter, ulceration, clinically altered LNs, histologic grade, surgical margins, and number of removed HN3 LNs. For age and weight, the median was used as the cut-off value. For tumor diameter, a cut-off value of 3 cm was selected based on previous studies.[2,14]

Data were analyzed by use of commercial software programs (SPSS Statistics v. 26, IBM, Somers, NY, and Prism v. 5.0, GraphPad, San Diego, CA). P values ≤ 0.05 were considered significant.

Cell Line Validation Statement

No cell lines were used in the current study.

Results

Dogs and tumor characteristics

A total of 60 dogs fulfilled the inclusion criteria: 34 (56.7%) dogs had a low-grade cMCT, and 26 (43.3%) had a high-grade tumor. When comparing demographic features and possible prognostic factors, dogs in the high-grade group were significantly older than those in the low-grade group (P<0.001; Table 1).

Dogs with low-grade cMCTs

In the low-grade group, there were 10 (29.4%) mixed-breed dogs. Among the remaining dogs, the most represented breeds were French Bulldog (n=4; 11.8%), Labrador Retriever (n=3; 8.8%), and Golden Retriever (n=3; 8.8%). Median age was 6.5 years (range, 3-13), and median weight was 18.5 kg (range, 1.9-42.0). Sixteen dogs (47.1%) were males (7 neutered) and 18 (52.9%) were females (16 spayed; Table 1).

Only one (2.9%) dog had signs of systemic effects of cMCT (vomiting, diarrhea, pruritus; substage b), which resolved after surgery. The remaining were asymptomatic (substage a).

The tumors were located on limbs (n=11; 32.3%), head and neck (n=10; 29.4%), inguinal/perineal area (n=7; 20.6%), mammary region (n=4; 11.8%), digital (n =1; 2.9%) and

187 trunk (n=1; 2.9%). Median tumor diameter was 2.4 cm (range, 0.5-9.0); 21 (61.8%) tumors
188 were not ulcerated.
189 Overall, 11 (32.3%) dogs had clinically normal LNs, while 23 (67.7%) dogs had clinical nodal
190 enlargement. Sentinel LN mapping was performed by peritumoral injection of methylene blue
191 in all dogs; 3 (7%) dogs also underwent lympho-CT, and 2 (4.6%) dogs underwent scintigraphy.
192 Twenty-seven (79.4%) dogs had one sentinel HN3 LN removed, 6 (17.6%) dogs had two HN3
193 LNs, whereas one (2.9%) dog had four HN3 LNs. Among dogs having more than 1 HN3 LN
194 removed, a single lymphocenter was involved in 3 cases, and different lymphocenters in 4
195 cases. Overall, removed HN3 LNs included 15 (34.9%) inguinal, 10 (23.3%) popliteal, 7
196 (16.3%) submandibular, 4 (9.3%) prescapular, 3 (7%) retropharyngeal, 2 (4.6%) axillary and 2
197 (4.6%) medial iliac.
198 Histopathologic evaluation revealed complete surgical margins in 25 (73.5%) cMCTs, and
199 incomplete margins in 9 (26.5%) cases. Second surgery or radiation therapy was suggested, but
200 declined by the owners of these 9 dogs. Five (14.7%) dogs had Ki67 evaluated, and one of them
201 had a value >23.[15] Mutational analysis was available for 24 (70.6%) cMCTs: 4 cMCTs were
202 mutated (ITD on exon 11), while the remaining 20 were wild type.
203 Thirty-two (94.1%) dogs received vinblastine and prednisone, and 2 (5.9%) received toceranib.
204
205 *Dogs with high-grade cMCTs*
206 Among dogs with high-grade cMCTs, 9 (34.6%) were mixed-breed. Among the remaining
207 dogs, the most represented breed was the Bernese Mountain dog (n=3; 11.5%). Median age was
208 10 years (range, 3-14), and median weight was 23 kg (range, 4,9-50). Fourteen (53.8 %) dogs
209 were males (6 neutered) and 12 (46.2 %) were spayed females (Table 1).
210 Four (15.4%) dogs were symptomatic. In all these cases, symptoms resolved after surgery.

The cMCTs were located on limbs (n=10; 38.5%), inguinal area (n=7; 26.9%), head and neck (n=5; 19.2%), mammary region (n=2; 7.7%), digits (n=1; 3.9%) and trunk (n=1; 3.9%). Median tumor diameter was 2 cm (range, 1-12); 12 tumors (46.2%) were ulcerated.

Overall, 6 (23.1%) dogs had clinically normal LNs, while 20 (76.9%) dogs had clinical nodal enlargement.

Sentinel LN mapping was performed by peritumoral injection of methylene blue in all dogs; 4 (12.1%) dogs also underwent lympho-CT.

Twenty (76.9%) dogs had one sentinel HN3 LN removed, 5 (19.2%) had two HN3 LNs, and 1 (3.8%) dog had three HN3 LNs. Among dogs having more than 1 HN3 LN removed, a single lymphocenter was involved in 2 cases, and different lymphocenters in 4 cases. Overall, removed HN3 LNs included 15 (45.6%) inguinal, 8 (24.2%) prescapular, 4 (12.1%) submandibular, 3 (9.1%) popliteal, 1 (3%) axillary, 1 (3%) medial iliac, and 1 (3%) retropharyngeal.

Surgical margins were histopathologically complete in 16 (61.5%) dogs and incomplete in 10 (38.5%). Second surgery or radiation therapy was recommended, but declined by the owners of these 10 dogs.

Three (11.5%) dogs had Ki67 evaluated, and none had a value >23. Mutational analysis was available for 12 (46.2%) cMCTs: 7 cMCTs were mutated (ITD on exon 11), while the remaining 4 were wild type.

Twenty dogs (76.9%) received vinblastine and prednisone, and 6 (23.1%) received toceranib.

Outcome

In the low-grade group, the median follow-up time was 1208 days (range, 180-2576). Three (9%) dogs experienced LR after 29, 177, and 216 days; 2 of them had incomplete margins. Seven dogs with incomplete margins did not recur after a median follow-up of 1143 days (range, 180-1650). In six (18%) dogs, NR was registered after a median of 211 days (range, 52-420), while 3 (9%) dogs developed DR after 52, 224, and 241 days. For all dogs experiencing

NR, the lymphocenter closest to the previously removed LN(s) was found involved. Overall, tumor progression was registered in 7 (21%) dogs. One dog developed concurrent LR and NR, and one dog developed concurrent NR and DR. Median TTP was not reached.

Of the 7 dogs with progressive disease, 2 (28.6%) received additional treatment, consisting in lomustine (n=1) and toceranib (n=1). The time to death from progressive disease was 84 and 432 days for these dogs, whereas the median time to death from progressive disease was 45 days (range, 23-170) for the dogs not receiving additional treatment.

At data analysis closure, 22 (65%) dogs were alive, 6 (18%) had died because of tumor-unrelated causes, and 6 (18%) had died because of MCT-related causes. Median TSS was not reached.

In the high-grade group, the median follow-up time was 367 days (range, 187-748). Nine (31%) dogs experienced LR after a median of 162 days (range, 25-663); 6 of them had incomplete margins. Three dogs with incomplete margins did not recur after 292, 848 and 1079 days. In 12 (46%) dogs, NR was registered after a median of 199 days (range, 50-663), while 5 (19%) dogs developed DR after a median of 63 days (range, 34-320). Similarly to dogs in the low-grade group, the lymphocenter closest to the previously removed LN(s) was found involved in those experiencing NR. Overall, tumor progression was registered in 17 (65%) dogs. Three dogs developed concurrent LR and NR, and one dog developed concurrent NR and DR. Median TTP was 214 days (95% CI, 154-274).

Of the 17 dogs with progressive disease, 8 (47.1%) received additional treatment, consisting in surgery (n=1); lomustine (n=1); toceranib (n=3), radiation therapy and lomustine (n=1), radiation therapy and toceranib (n=1), and toceranib and vinblastine (n=1). The median time to death was 160 days (range, 32-602) for these dogs and 26 days (range, 1-144) for the dogs not receiving additional treatment.

At data analysis closure, 8 (31%) dogs were alive, 3 (12%) had died because of tumor-unrelated causes, and 15 (58%) had died because of MCT-related causes. Median TSS was 545 days (95% CI, 187-902).

According to the Kaplan-Meier method with log rank comparisons (Figures 1,2), TTP and TSS were significantly shorter in the high-grade group than in the low-grade group, with a P value lower than 0.001 in both cases.

Univariable analysis using Cox proportional hazards regression model are presented in Table 2. Variables associated with increased risk of tumor progression were age ≥8.5 years (HR: 3.5; P=0.006), female sex (HR: 2.6; P=0.029), presence of clinical signs (HR: 6.5; P=0.001), high-grade (HR: 5.8; P<0.001), and incomplete margins (HR: 2.3; P=0.039). Age ≥ 8.5 years (HR: 3.4; P=0.011), female sex (HR: 2.8; P=0.029), presence of clinical signs (HR: 7.3; P=0.001), high-grade (HR: 6.2; P<0.001), and incomplete margins (HR: 2.9; P=0.014) were significantly associated with an increased risk of tumor-related death.

On multivariable analysis (Tables 3, 4), histologic high-grade and incomplete margins were the only variables retaining prognostic significance for both tumor progression and tumor-specific death.

Discussion

One of the best-established prognostic factors in canine cMCT is histologic grade, which represents the morphological assessment of tumor biologic characteristics and has been shown to be able to generate important information related to the biologic behavior.[1] Although several studies have shown that histologic grade is useful for predicting patient survival,[1,2,4,16] the basic problem remains that the prognostic value of histologic grade has been studied in series of dogs that are heterogeneous in terms of clinical stage and treatment.[17]

Thus, the present study evaluated the prognostic value of histologic grade in dogs with cMCTs and at least one overtly metastatic (HN3) LN concerning tumor progression and TSS. To do so, the study was conducted on a homogeneous population of dogs undergoing complete staging and a multimodal treatment, consisting of resection of the primary tumor, sentinel lymphadenectomy and adjuvant medical treatment, for which long-term follow-up data were available.

All parameters, which are currently applied during routine work-up as staging variables or retrieved after surgery, and are ultimately used to define possible postoperative treatment, were evaluated, including signalment, presence of clinical signs, tumor anatomic location, size, ulceration, and surgical margins.

In this population of dogs, histologic grade and the status of surgical margins significantly correlated with outcome, as at the same clinical stage, dogs with low-grade cMCTs and those with complete surgical margins had a better outcome.

The importance of initial free resection margins has been reported elsewhere.[18.19] Scar re-excision or radiation therapy was offered in the case of incomplete surgical margins; however, reintervention was declined by all owners. Notably, in the low-grade group only 2 out of 9 (22.2%) dogs with incomplete margins recurred, and this local recurrence rate does not differ much from the one obtained after re-excision or radiation therapy.[18,20] Conversely, 6 out of 10 (60%) dogs with high-grade cMCTs and incomplete surgical margins recurred. This is in accordance with the literature, reporting a significantly higher risk of local recurrence in high-grade cMCTs compared with low-grade tumors with equal surgical margins.[21]

In the current series of dogs with overtly metastatic nodal disease, overall prognosis was not unfavorable, not only for dogs with low-grade cMCTs, but also for those with high-grade cMCTs, with a median TSS >500 days in the latter group, despite a higher proportion of ulcerated tumors and systemic signs. This was surprising, as metastatic high-grade cMCTS have been historically considered aggressive and basically incurable.

312 Unfortunately, it is difficult to fully compare our data with historical data, because many
313 published studies have not evaluated a homogeneous patient population, rather have included
314 different grades, different stages and different treatments with no stratification tentative.
315 One of the main differences in the therapeutic approach between the dogs reported in the present
316 study and previously published populations was the focus on obtaining a good regional control,
317 by surgically removing all potentially involved LNs. In the study by Krick *et al*. local/loco-
318 regional control was not a significant prognostic factor for dogs with grade 2 and 3 cMCTs and
319 the median survival time of dogs with stage II tumors was less than one year. However, the
320 treatments received by the dogs in that study were extremely heterogeneous and poorly detailed,
321 and no definition of loco-regional treatment was provided.[22]
322 More recent publications demonstrated that, if treated appropriately, a metastatic LN does not
323 necessarily implicate a worse prognosis;[23,24] and there is increasing evidence that an adequate
324 loco-regional intervention translates into an effective tumor control and improved outcome.[10,18]
325 Thus, this should be a primary aim in the treatment of cMCTs, even more so in high-grade
326 tumors. Given the survival results obtained in the present study, it would seem prudent to
327 recommend surgical removal of the sentinel LNs identified based on mapping procedure, at
328 least of those that are easily accessible. In comparison, radiation therapy may increase costs and
329 prolongs the duration of the overall treatment, in addition to logistic issues regarding the limited
330 availability of radiation therapy facilities. Also, irradiation of regional LNs without LN
331 mapping exposes the patient to an untargeted treatment with possible side effects, may leave
332 disease behind and obviously the histopathologic status (HN1 vs HN2 vs HN3) cannot be
333 determined confidently.
334 Furthermore, according to the literature, outcome of high-grade tumors is improved if a
335 multimodal treatment is carried out. If treated by surgery only at the level of the primary tumor
336 (with no LN removal), high-grade cMCTs have been associated with a median ST of <4
337 months.[1] The addition of chemotherapy to surgery significantly improved outcome. In an early

study, dogs with high-grade cMCTs of different clinical stages (I-II) undergoing surgery and chemotherapy had a median progression-free survival and overall survival of 133 and 257 days, respectively.[8] In a more recent study, 16 dogs with high-grade cMCT and LN metastasis that underwent surgical excision of the primary tumor, irradiation of the metastatic LN and chemotherapy, had a median PFS and OS of 125 and 330 days, respectively.[17]

When evaluating dogs with low-grade cMCTs, median TTP and TSS were not reached, and few events occurred. Those events that did occur, did so late in the disease course. It has been retrospectively shown that dogs with low-grade cMCTs and low-volume metastatic nodal disease (HN2) do not need adjuvant chemotherapy.[10] To avoid treatment-related bias, in this series of dogs, adjuvant medical treatment was always administered, but it is currently unknown whether chemotherapy is really necessary in the case of low-grade cMCTs and HN3 LN, if both the primary tumor and the metastatic node are removed.

This important observation provides further insight into the appropriate management strategies of dogs with cMCTs. High-grade tumors, with their risk of early recurrence and death, require consideration for prompt use of adjuvant chemotherapy, whereas dogs with low-grade cMCTs could be offered a long-term follow-up without chemotherapy. This needs to be confirmed in future prospective trials.

Additionally, based on the current findings, the oncology community should make the effort to revise the current WHO clinical staging system, which has not been updated for decades, by including histologic grade in the staging criteria aimed at allocating patients to risk groups and offering guidance to therapy. We believe that treatment decisions based on the WHO staging system, which measures the anatomic extent of the tumor, can be improved by the addition of histologic grade, which measures the intrinsic biologic features of the tumor and reflects the potential of a cMCT to metastasize or cause death. Integration of histologic grade into the staging system has been accepted for many human cancers, including breast carcinoma and osteosarcoma.[25-27] For cMCTs, the maximum benefit of grade assessment would be in dogs

364 with WHO stage I to III disease. According to a recent prospective study, histologic grade had
365 no prognostic relevance in dogs with stage IV disease. Indeed, in dogs with ascertained visceral
366 metastases, therapy and prognosis did not change according to the histologic grade.[28]
367
368 This study was limited by its retrospective design within this bi-center trial and relatively small
369 size of cases.
370 Further limitations should be noted.
371 First, the identification of sentinel LNs was not standardized. Sentinel LNs are more frequently
372 multiple rather than single. Thus, tumors may be drained by multiple LNs within a single basin
373 or by multiple basins, thereby complicating the recognition and, consequently, the management
374 of the sentinel LN(s).[29] In the current study, peritumoral injection of methylene blue was used
375 to identify sentinel LNs. Even if a careful search was made for blue lymphatic channels leading
376 to blue-stained LNs, a single LN was more often removed. It cannot be excluded that additional
377 sentinel LNs were unrecognized and left behind.
378 While debate exists regarding the optimal technique for sentinel LN mapping, the use of dual
379 methods (dye and advanced imaging) has been suggested to optimize sentinel LN detection.[13,30-
380 31] However, in routine clinical practice, LN mapping is often restricted to methylene blue dye,
381 mainly due to logistic issues related to radioisotopes, technical challenges and financial
382 constraint.
383 In people with various types of cancer, the presence of an overly metastatic LN advocates for
384 further nodal dissection, as it impacts prognosis.[32-34] Although not reported in the veterinary
385 literature, it is likely that the number of HN3 LNs also influences prognosis. For many human
386 cancers, the number of positive LNs is included in the definition of the N categories within the
387 TNM staging system, and the N status shows significant correlation with patient prognosis,
388 dictating the need for further nodal dissection.[35-37] The same may hold true for many canine

solid cancers, including cMCTS. Future studies are warranted to explore the optimal number of LN resection for accurate staging and more survival benefits.

Second, dogs that did not have adequate tumor control were included in the study. While there is plenty of data supporting that adequate local control improves prognosis,[18-20] re-excision of the surgical scar or radiation therapy are not always accepted by owners, thus our population of dogs better reflected daily routine. Also, the two groups were well balanced in terms of completeness of margins.

Third, more than 40% of the dogs with disease progression received additional treatments, which have prolonged survival and may have affected the analysis of prognostic factors.

Fourth, mutational status and Ki67 proliferation activity were not routinely performed. Proliferation markers and the presence of ITD mutations may have added important prognostic information.

Last, we decided to only grade cMCTs according to Kiupel, as it has been shown that the Patnaik grading system is suboptimally reproducible between different pathologists.[38,39] It may be possible that different combinations between the two grading systems may further improve prognostication, as already published.[4]

In conclusion, the results of the current study have unraveled additional characteristics of cMCT biology and have provided further evidence that the biologic features captured by histologic grade are important in determining tumor behavior and in providing predicting tools in clinical practice, even in the presence of other negative prognostic factors.

Data availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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416 **References**

417 1. Kiupel M, Webster JD, Bailey KL, et al. Proposal of a 2-tier histologic grading system
418 for canine cutaneous mast cell tumors to more accurately predict biological behavior.
419 *Vet Pathol*. 2011;48:147-155.
420 2. Stefanello D, Buracco P, Sabattini S, et al. Comparison of 2- and 3-category histologic
421 grading systems for predicting the presence of metastasis at the time of initial evaluation
422 in dogs with cutaneous mast cell tumors: 386 cases (2009-2014*). J Am Vet Med Assoc*.
423 2015;246:765-769.
424 3. Marconato L, Polton G, Stefanello D, et al. Therapeutic impact of regional
425 lymphadenectomy in canine stage II cutaneous mast cell tumours. *Vet Comp Oncol*.
426 2018;16:580-589.
427 4. Sabattini S, Scarpa F, Berlato D, Bettini G. Histologic grading of canine mast cell tumor: is
428 2 better than 3? *Vet Pathol*. 2015;52:70–73.
429 5. Weishaar KM, Thamm DH, Worley DR, Kamstock DA. Correlation of nodal mast cells
430 with clinical outcome in dogs with mast cell tumour and a proposed classification
431 system for the evaluation of node metastasis. *J Comp Pathol*. 2014;151:329-338.
432 6. Ferrari R, Marconato L, Buracco P, et al. The impact of extirpation of non-
433 palpable/normal-sized regional lymph nodes on staging of canine cutaneous mast cell
434 tumours: A multicentric retrospective study. *Vet Comp Oncol*. 2018;16:505-510.
435 7. Moore AS, Frimberger AE, Taylor D, et al. Retrospective outcome evaluation for dogs
436 with surgically excised, solitary Kiupel high-grade, cutaneous mast cell tumours. *Vet*
437 *Comp Oncol*. 2020;18:402-408.
438 8. Hume CT, Kiupel M, Rigatti L, et al. Outcomes of dogs with grade 3 mast cell tumors:
439 43 cases (1997-2007). *J Am Anim Hosp Assoc*. 2011;47:37-44.

9. Hayes A, Adams V, Smith K, Maglennon G, Murphy S. Vinblastine and prednisolone chemotherapy for surgically excised grade III canine cutaneous mast cell tumours. *Vet Comp Oncol.* 2007;5:168-176.
10. Marconato L, Stefanello D, Kiupel M, et al. Adjuvant medical therapy provides no therapeutic benefit in the treatment of dogs with low-grade mast cell tumours and early nodal metastasis undergoing surgery. *Vet Comp Oncol*. 2020;18:409-415.
11. Sabattini S, Kiupel M, Finotello R, et al. A retrospective study on prophylactic regional lymphadenectomy versus nodal observation only in the management of dogs with stage I, completely resected, low-grade cutaneous mast cell tumors. *BMC Vet Res*. 2021;17:331.
12. Manfredi M, De Zani D, Chiti LE, et al. Preoperative planar lymphoscintigraphy allows for sentinel lymph node detection in 51 dogs improving staging accuracy: Feasibility and pitfalls. *Vet. Radiol. Ultrasound*. 2021;62:602–609.
13. Ferrari R, Chiti LE, Manfredi M, et al. Biopsy of sentinel lymph nodes after injection of methylene blue and lymphoscintigraphic guidance in 30 dogs with mast cell tumors. *Vet Surg*. 2020;49:1099-1108.
14. Ferarri R, Boracchi P,Chiti LE, et al. Assessing the Risk of Nodal Metastases in Canine Integumentary Mast Cell Tumors: Is Sentinel Lymph Node Biopsy Always Necessary? *Animals*. 2021;11:2373.
15. Kiupel M, Camus M. Diagnosis and prognosis of canine cutaneous mast cell tumors. *Vet Clin North Am Small Anim Pract.* 2019;49:819–836.
16. Takeuchi Y, Fujino Y, Watanabe M, et al. Validation of the prognostic value of histopathological grading or c-kit mutation in canine cutaneous mast cell tumours: a retrospective cohort study. *Vet J*. 2013;196:492-498.

17. Mendez SE, Drobatz KJ, Duda LE, White P, Kubicek L, Sorenmo KU. Treating the locoregional lymph nodes with radiation and/or surgery significantly improves outcome in dogs with high-grade mast cell tumours. *Vet Comp Oncol*. 2020;18:239-246.
18. Kry KL, Boston SE. Additional local therapy with primary re-excision or radiation therapy improves survival and local control after incomplete or close surgical excision of mast cell tumors in dogs. *Vet Surg*. 2014;43:182-9.
19. Séguin B, Besancon MF, McCallan JL, et al. Recurrence rate, clinical outcome, and cellular proliferation indices as prognostic indicators after incomplete surgical excision of cutaneous grade II mast cell tumors: 28 dogs (1994–2002). *J Vet Intern Med*. 2006;20:933–940.
20. Mason SL, Pittaway C, Gil BP, et al. Outcomes of adjunctive radiation therapy for the treatment of mast cell tumors in dogs and assessment of toxicity: A multicenter observational study of 300 dogs. *J Vet Intern Med*. 2021;35:2853-2864.
21. Donnelly L, Mullin C, Balko J, et al. Evaluation of histological grade and histologically tumour-free margins as predictors of local recurrence in completely excised canine mast cell tumours. *Vet Comp Oncol*. 2015;13:70-76.
22. Krick EL, Billings AP, Shofer FS, et al. Cytological lymph node evaluation in dogs with mast cell tumours: association with grade and survival. *Vet Comp Oncol*. 2009; 7:130-138.
23. Lejeune A, Skorupski K, Frazier S, et al. Aggressive local therapy combined with systemic chemotherapy provides long-term control in grade II stage 2 canine mast cell tumour: 21 cases (1999-2012). *Vet Comp Oncol*. 2015; 13:267-280.
24. Pecceu E, Serra Varela JC, Handel I, et al. Ultrasound is a poor predictor of early or overt liver or spleen metastasis in dogs with high-risk mast cell tumours. *Vet Comp Oncol.* 2020; 18:389-401.

25. Enneking WF, Spanier SS, Goodman MA. A system for the surgical staging of musculoskeletal sarcoma. Clin *Orthop Relat Res.* 1980;153:106-120.
26. Henson DE, Ries L, Freedman LS, et al. Relationship among outcome, stage of disease, and histologic grade for 22,616 cases of breast cancer. The basis for a prognostic index. *Cancer*. 1991;68:2142-2149.
27. Galea MH, Blamey RW, Elston CE, et al. The Nottingham Prognostic Index in primary breast cancer. *Breast Cancer Res Treat*. 1992;22:207-219.
28. Pizzoni S, Sabattini S, Stefanello D, et al. Features and prognostic impact of distant metastases in 45 dogs with de novo stage IV cutaneous mast cell tumours: A prospective study. *Vet Comp Oncol*. 2018;16:28-36.
29. Chagpar AB, Scoggins CR, Martin RC 2nd, et al. Are 3 sentinel nodes sufficient?. *Arch Surg*. 2007;142:456-459.
30. Wan J, Oblak ML, Ram A, Singh A, Nykamp S. Determining agreement between preoperative computed tomography lymphography and indocyanine green near infrared fluorescence intraoperative imaging for sentinel lymph node mapping in dogs with oral tumours. *Vet Comp Oncol*. 2021;19:295-303.
31. Liptak JM, Boston SE. Nonselective Lymph Node Dissection and Sentinel Lymph Node Mapping and Biopsy. *Vet Clin North Am Small Anim Pract*. 2019;49:793-807.
32. Goyal A, Newcombe RG, Mansel RE. Axillary lymphatic mapping against nodal axillary clearance (ALMANAC) trialists group. Clinical relevance of multiple sentinel nodes in patients with breast cancer. *Br J Surg*. 2005;92:438-442.
33. Morton DL, Thompson JF, Cochran AJ et al. Final trial report of sentinel-node biopsy versus nodal observation in melanoma. *N Engl J Med*. 2014;370:599–609.
34. Bonneau C, Bendifallah S, Reyal F, et al. Association of the number of sentinel lymph nodes harvested with survival in breast cancer. *Eur J Surg Oncol*. 2015;41:52-58.

35. Kodera Y, Yamamura Y, Shimizu Y, et al. The number of metastatic lymph nodes: A promising prognostic determinant for gastric carcinoma in the latest edition of the TNM classification. *J Am Coll Surg.* 1998;187:597-603.

36. Nakata M, Saeki H, Kurita A, et al. Prognostic significance of the number of metastatic lymph nodes in surgically resected non-small cell lung cancer. *Haigan*. 1999;39:421-427.

37. Ueda K, Kaneda Y, Sakano H, et al. Independent predictive value of the overall number of metastatic N1 and N2 stations in lung cancer. *Jpn J Thorac Cardiovasc Surg*. 2003;51:297-301.

38. Northrup NC, Harmon BG, Gieger TL, et al. Variation among pathologists in histologic grading of canine cutaneous mast cell tumors. *J Vet Diagn Invest*. 2005;17:245-248.

39. Northrup NC, Howerth EW, Harmon BG, et al. Variation among pathologists in the histologic grading of canine cutaneous mast cell tumors with uniform use of a single grading reference. *J Vet Diagn Invest*. 2005;17:561-564.

40. Dobson JM, Scase TJ. Advances in the diagnosis and management of cutaneous mast cell tumours in dogs. *J Small Anim Pract*. 2007; 48:424-431.

546
547
548 **Table 1.** Demographic information and distribution of variables potentially associated with
549 prognosis of 60 dogs with cutaneous mast cell tumors and overtly metastatic regional lymph
550 node(s). Differences in data distribution were assessed with Chi-square test/Fisher's exact test
551 (categorical variables) or Mann-Whitney U test (continuous variables).
552

Variable	**Low grade cMCTs** (*n* = 34)	**High grade cMCTs** (*n* = 26)	***P***
Breeds predisposed to biologically aggressive cMCTs			
Yes	7 (20.6%)	2 (7.7%)	0.166
No	27 (79.4%)	24 (92.3%)	
Sex			
Male	16 (47.1%)	14 (53.8%)	0.602
Female	18 (52.9%)	12 (46.2%)	
Age (years)			
Median (range)	7 (3-13)	10 (3-14)	<0.001*
Weight (kg)			
Median (range)	18.5 (1.9-42.0)	23.0 (4.9-50.0)	0.416
Substage			
a	33 (97.1%)	22 (84.6%)	0.084
b	1 (2.9%)	4 (15.4%)	
Anatomic location			
Trunk, limbs	12 (35.3%)	11 (42.3%)	0.580
Others	22 (64.7%)	15 (57.7%)	
Diameter (cm)			
Median (range)	2.4 (0.5-9.0)	2.0 (1.0-12.0)	0.614
Ulceration			
Yes	13 (38.2%)	12 (46.2%)	0.538
No	21 (61.8%)	14 (53.8%)	
Number of removed LN(s)			
1	22 (64.7%)	13 (50.0%)	0.252
>1	12 (35.3%)	13 (50.0%)	
Margins			
Complete	25 (73.5%)	16 (61.5%)	0.322

Incomplete	9 (26.5%)	10 (38.5)	
Number of HN3 LN(s)*			
1	5 (38.5%)	7 (53.8%)	0.431
>1	8 (61.5%)	6 (46.2%)	

41. *: percentage calculated on the total number of dogs having >1 LNs removed.

Table 2. Univariable Cox regression analysis of variables potentially associated with increased risk of tumor progression and tumor-specific survival in 60 cutaneous mast cell tumors and overtly metastatic regional lymph node(s).

Variable	**Tumor progression**		**Tumor-specific death**	
	Hazard Ratio (95% CI)	*P*	Hazard Ratio (95% CI)	*P*
Breed predisposed to biologically aggressive cMCTs	0.94 (0.32-2.76)	0.905	0.68 (0.20-2.32)	0.535
Female sex	2.60 (1.10-6.12)	0.029*	2.79 (1.11-6.98)	0.029*
Age >8.5 years	3.51 (1.44-8.58)	0.006*	3.41 (1.32-8.80)	0.011*
Weight >20.0 kg	1.53 (0.68-3.45)	0.306	1.05 (0.45-2.49)	0.906
Substage b	6.55 (2.16-19.83)	0.001*	7.29 (2.34-22.76)	0.001*
Biologically aggressive anatomic location	0.89 (0.40-2.02)	0.788	0.79 (0.33-1.88)	0.590
Tumor diameter ≥3 cm	1.35 (0.60-3.06)	0.467	1.58 (0.66-3.77)	0.304
Ulcerated tumor	1.97 (0.88-4.39)	0.100	1.88 (0.80-4.45)	0.148
Removal of >1 LN	1.54 (0.67-3.50)	0.309	1.26 (0.51-3.13)	0.613
High grade tumor	5.79 (2.31-14.50)	<0.001*	6.18 (2.29-16.70)	<0.001*
Incomplete margins	2.34 (1.04-5.23)	0.039*	2.94 (1.25-6.95)	0.014*
Presence of >1 HN3 LN†	2.04 (0.83-5.01)	0.122	2.52 (0.93-6.84)	0.070

Abbreviations: CI, confidence interval. *significant

† evaluated in dogs having >1 LN removed.

Table 3. Multivariable Cox regression analysis for risk of tumor progression. Significant variables at univariable analysis were included in the model.

Variable	Tumor progression	
	Hazard Ratio (95% CI)	*P*
Female sex	1.72 (0.71-4.19)	0.231
Age >8.5 years	2.18 (0.78-6.05)	0.135
Substage b	2.35 (0.70-7.86)	0.166
High grade tumor	3.30 (1.19-9.15)	0.022*
Incomplete margins	2.40 (1.03-5.63)	0.044*

Abbreviations: CI, confidence interval. *significant

Table 4. Multivariable Cox regression analysis for risk of tumor-specific death. Significant variables at univariable analysis were included in the model.

Variable	Tumor-specific death	
	Hazard Ratio (95% CI)	*P*
Female sex	1.96 (0.78-4.94)	0.154
Age >8.5 years	2.52 (0.82-7.72)	0.106
Substage b	2.51 (0.75-8.40)	0.134
High grade tumor	3.44 (1.19-10.00)	0.023*
Incomplete margins	2.78 (1.11-7.01)	0.030*

Abbreviations: CI, confidence interval. *significant

Figure.

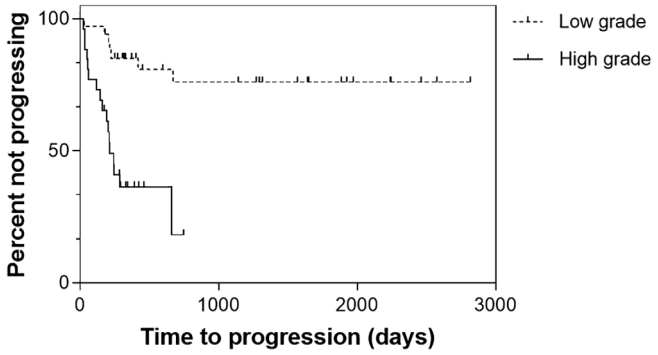

Figure 1. Time to progression (TTP) for 60 dogs with cMCT and HN3 sentinel lymph node undergoing surgical excision of the primary tumor, lymphadenectomy and chemotherapy. TTP is significantly shorter for dogs with high-grade cMCT (solid line) than for dogs with low-grade tumors (dashed line, P<0.001).

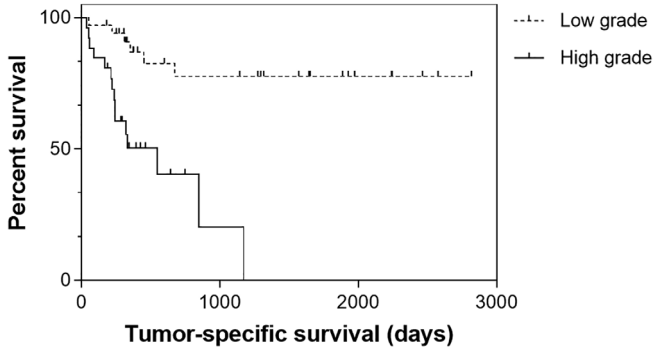

Figure 2. Tumor-specific survival (TSS) for 60 dogs with cMCT and HN3 sentinel lymph node undergoing surgical excision of the primary tumor, lymphadenectomy and chemotherapy. TSS is significantly shorter for dogs with high-grade cMCT (solid line) than for dogs with low-grade tumors (dashed line, P<0.001).