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**Title page**

# The two-tier grading system identifies canine cutaneous and/or subcutaneous mast cell tumors with aggressive biological behavior regardless of growth model

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## Abstract

Histologic grading of canine cutaneous mast cell tumors (cMCTs) has prognostic and therapeutic implications, yet validation for subcutaneous (sc) MCTs is lacking. For scMCTs with or without dermal invasion, determining their biologic behavior remains poorly standardized and sometimes sparks controversy.

This prospective study aims to assess the prognostic utility of the two-tier histologic grading system in MCTs with distinct growth models (GM) and explore the prognostic impact of the GM itself.

Dogs undergoing excision of a cMCT or scMCT and regional/sentinel lymphadenectomy were included. The two-tier grade was applied, and 6 histologic GM categories were defined: solely cMCT (C-SC0), cMCT with superficial (C-SC1) or deep subcutaneous (C-SC2) involvement, solely scMCT (SC-C0) and scMCT with deep (SC-C1) or superficial (SC-C2) infiltration of the dermis. Mitotic count, two-tier grade, nodal involvement, surgical margins, and outcome were stratified according to GM.

Ninety-one MCTs from 76 dogs were examined. GM classification identified 11 (12.1%) C-SC0 tumors, 12 (13.2%) C-SC1, 15 (16.5%) C-SC2, 21 (16.5%) SC-C0, 15 (16.5%) SC-C1, and 17 (18.7%) SC-C2.

The two-tier histologic grading enabled the identification of all MCTs with aggressive biologic behavior, regardless of their cutaneous or subcutaneous location. scMCTs lacking dermal invasion, historically associated with a benign clinical course, had a poor prognosis in 10% of cases.

cMCTs exhibiting deep subcutaneous involvement had the highest occurrence of high-grade tumors (33.3%; P=0.01), overt nodal metastases (33.3%) and the lowest one-year survival rate (85.7%). Histologic grade was confirmed as a relevant prognostic factor, surpassing nodal involvement and histologic margin status.

**Keywords:** dog; grading; histology; lymphadenectomy; mast cell tumor; prognosis; subcutaneous

Mast cell tumors (MCTs) are the most common malignant skin neoplasms in dogs. The prognostication of canine MCTs is strictly dependent on their growth model (GM). For MCTs arising primarily in the dermis (cutaneous MCTs [cMCTs]) two histologic grading systems have been introduced: the Patnaik (three-tier) system, which considers both architectural and morphologic criteria, and the Kiupel (two-tier) system, which exclusively relies on cell morphology.[9,18] According to the three-tier system, cMCTs infiltrating the subcutaneous tissues exhibit a more aggressive behavior and a worse outcome compared with those confined to the dermis.[18]

A subset of canine MCTs is confined entirely within the subcutis, with limited or no involvement of the upper dermal layer (subcutaneous MCTs [scMCTs]). Until recently, these tumors had received limited attention and were either not graded or arbitrarily classified as Patnaik grade II due to their subcutaneous location. Several studies addressing this MCT variant reported extended survival times and low rates of local recurrence and metastasis.[5,17,23] However, recent reports have highlighted a small number of cases displaying aggressive biologic behavior.[3,13,24]

Histologically, several negative prognostic factors have been identified for canine scMCT, including mitotic count, infiltrative growth, and multinucleation.[23] However, no specific grading system has been proposed, and it remains uncertain which combination of negative histologic prognostic factors could be considered equivalent to high grade. In a recent study that applied the two-tier grading system to scMCTs, only one dog was diagnosed with a high-grade tumor, and in no case did the mitotic count reach the cutoff established for high-grade cMCTs.[5] These findings may support the hypothesis that

scMCTs as an entity are generally lower grade. Alternatively, this may suggest that the histologic prognostic factors identified for cMCTs may not be applicable to the subcutaneous variant.

Additionally, in a significant proportion of canine MCTs, the bulk of the tumor resides within the subcutis, but neoplastic cells extend upward to infiltrate the deep or intermediate dermis, or even reach the superficial dermis. From a clinical perspective, most of these tumors cannot be distinguished from primary cMCTs. This particular GM represents a gap that has not been previously addressed in the literature. As a result, the histologic approach to these tumors is quite confusing: many pathologists grade them as if they were cMCTs with deep subcutaneous invasion, while others recognize their subcutaneous origin and apply the prognostic criteria recommended for scMCTs.

If a single grading system could be applied to all canine MCTs, regardless of their primary location and extent of infiltration, this would ensure a reduced level of subjectivity in the histologic interpretation of their biologic behavior, ultimately resulting in more informed clinical management.

In this prospective study, the prognostic impact of MCT GM was assessed in a cohort of dogs undergoing primary tumor removal and regional or sentinel lymphadenectomy. For this purpose, tumors were separated into several histologic GM-defined categories, including solely dermal MCTs, solely scMCTs, primarily dermal MCTs extending into the subcutis, and primarily scMCTs extending into the dermis. The primary aim was to compare the biologic behavior of cMCTs with deep subcutaneous invasion with that of scMCT displaying dermal invasion, to assess whether these GMs merit distinction in histopathologic reports. Additional aims were to investigate if there were differences in the two-tier grade distribution based on tumor GM and if grading correlated with tumor biologic behavior across all categories.

## Materials and Methods

*Study design and inclusion criteria*

Client-owned dogs with cutaneous or subcutaneous MCTs undergoing surgical excision of the primary tumor and regional or sentinel lymphadenectomy at the University Hospital of the Department of Veterinary Medical Sciences (University of Bologna, Italy) between January 2018 and December 2022 were eligible for inclusion.

Dogs with more than two concurrent MCTs, recurrent MCTs, or with mucosal or muscular MCTs were excluded; comorbidities limiting life expectancy to <6 months represented a further exclusion criterion.

Dogs with two concurrent or asynchronous primary MCTs were included if they underwent surgical excision of both tumors and removal of all regional/sentinel lymph nodes.

All dogs had to be staged negative for distant metastases prior to surgery through the completion of a series of diagnostic procedures, including 3-view thoracic radiographs, abdominal ultrasound, and fine-needle aspiration of the liver and spleen.

MCTs were excised according to recent recommendations, with lateral surgical margins proportional to the widest tumor diameter, and deep margins including at least one fascial plane.[7,19]

The obtained surgical samples were subjected to histologic evaluation. In the presence of high-grade tumors[9] and/or overt nodal metastasis,[25] adjuvant vinblastine treatment was recommended, in accordance with prior literature.[12] The remaining dogs were monitored. Dogs were withdrawn from the study if they were lost to follow-up within 120 days of surgery.

*Histopathologic examination*

Samples were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin using a standardized protocol. Four-µm-thick histologic sections of the primary tumor, stained with hematoxylin and eosin, were microscopically examined for the assessment of tumor GM, mitotic count (MC), histologic grade according to the two-tier system[9] and surgical margins.

The histologic GM was assessed as detailed in Table 1. Six categories were defined: solely dermal MCT (C-$SC_0$), dermal MCT with superficial or deep subcutaneous involvement (C-$SC_1$ and C-$SC_2$, respectively), solely subcutaneous MCT (SC-$C_0$) and subcutaneous MCT with deep or superficial infiltration of the overlying dermis (SC-$C_1$ and SC-$C_2$, respectively). MC was expressed as the total number of mitotic figures in a 2.37 $mm^2$ area and assessed in the areas of highest mitotic activity.[14] The two-tier histologic grading system was applied on all tumors, regardless of the GM. Surgical margins were inked by the pathologist, assessed histologically with combined radial and tangential sections and defined as complete, clean but close (tumor cells at 1-3 mm from the surgical margins) or incomplete.

Regional lymph nodes were processed as previously described and stained with toluidine blue for the histologic node status evaluation according to Weishaar *et al.*[21,24]

All histologic evaluations were performed by a board-certified veterinary pathologist (SS).

*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 test. None of the numeric variables had a normal distribution and, therefore, the median and range are used as summary statistics.

Information recorded for all dogs included signalment, tumor anatomic location, largest diameter, clinical site (cutaneous or subcutaneous), ulceration, substage, GM, MC, two-tier grade, surgical margins, extent of nodal involvement, date of tumor progression (if any), date of death and cause of death (if any).

Time to progression (TTP) was calculated from the date of surgery to the first occurrence of one or more of the following: local recurrence, nodal metastasis and distant spread. Dogs with no recurrence or disease progression at the date of the last visit or death were censored.

Tumor-specific survival (TSS) was calculated from the date of surgery to the date of death or to the date of the last visit if death did not occur. Only dogs deceased due to MCT-related causes were considered as events.

Survival curves for each GM-defined group were obtained with the Kaplan-Meier method and compared with the log-rank test. One-year survival rates were also calculated for each group.

Data were analyzed by use of commercial software programs (SPSS Statistics v. 26, IBM, Somers, NY). P values ≤0.05 were considered statistically significant.

## Results

*Dogs' and tumors' characteristics*

Ninety-one MCTs obtained from 76 dogs were included. There were 19 (25.0%) mixed-breed dogs and 57 (75.0%) purebred dogs; among these, the most represented breeds were Labrador retriever (n = 10; 17.5%), French bulldog (n = 8; 14%), boxer (n = 7; 12.3%) and golden retriever (n = 6; 10.5%). There were 39 females (51.3%), of which 28 spayed, and 37 (48.7%) males, of which 8 neutered. The median age at presentation was 8 years (range, 3.5-15.0), and median body weight was 26.5 kg (range, 5.4-58.7).

The tumors were located on trunk and tail (n = 30; 33.0%), limbs (n = 28; 30.8%), head and neck (n = 14; 15.4%), inguinal/perineal area (n = 10; 10.9%), mammary region (n = 7; 7.7%), and digital region (n = 2; 2.2%). From a clinical perspective, 53 (58.2%) tumors were cutaneous and 38 (41.8%) were subcutaneous. The median tumor diameter was 1.5 cm (range, 0.2-16.0 cm). Four (5.3%) dogs were symptomatic (substage b).

*Treatment*

Seven (9.2%) dogs received neoadjuvant chemotherapy, consisting of vinblastine administered intravenously (IV) every two weeks at the dose of 3 mg/m$^2$ for dogs weighing ≥ 20 kg and 2.5 mg/m$^2$ for those weighing < 20 kg. Dogs also received daily oral prednisolone (1 mg/kg), oral cetirizine (1 mg/kg) twice daily and oral famotidine (2 mg/kg) twice daily.

All dogs underwent surgical excision of their MCTs and lymphadenectomy. A total of 169 regional and 19 sentinel lymph nodes were removed, with a median of 2 lymph nodes for each dog (range, 1-6).

Based on the presence of a high-grade tumor and/or HN3 lymph node, 19 (20.9%) dogs received adjuvant chemotherapy, consisting of vinblastine administered as previously described up to a total of 8 doses.

*Histologic analysis*

Eighty-one (89.0%) tumors were low grade and 10 (11.0%) were high grade. The median mitotic count was 0 (range, 0-14). Surgical margins were histologically complete in 76 (83.5%) MCTs, clean but close in 12 (13.2%) cases and incomplete in 3 (3.3%) cases.

Forty-three (47.3%) dogs had at least one HN2 lymph node and 16 (17.6%) had at least one HN3 lymph node.

According to the histologic GM, 11 tumors (12.1%) were classified as C-$SC_0$, 12 (13.2%) as C-$SC_1$, 15 (16.5%) as C-$SC_2$, 21 (23.0%) as SC-$C_0$, 15 (16.5%) as SC-$C_1$, and 17 (18.7%) as SC-$C_2$ (Figure 1).

The main characteristics for each GM-defined group are listed in Table 2. Tumors treated with neoadjuvant chemotherapy were distributed as follows: SC-$C_2$: n = 3; SC-$C_0$: n = 2; C-$SC_2$: n = 2. High grade MCTs included 5 C-$SC_2$, 2 SC-$C_0$, 2 SC-$C_2$ and 1 SC-$C_1$. Tumors with at least one HN3 lymph node included 6 SC-$C_0$, 5 C-$SC_2$, 3 SC-$C_2$, 2 C-$SC_1$ and 1 SC-$C_1$.

When specifically considering tumors with a massive involvement of both cutis and subcutis, C-$SC_2$ MCTs had a median diameter of 1.4 cm (range, 0.5-7.0) and had been described clinically as a cutaneous nodule in 14 (93.3%) cases and as a subcutaneous nodule in 1 (6.7%) case. Two (13.3%) tumors were ulcerated. SC-$C_2$ MCTs had a median diameter of 2.0 cm (range, 0.5-6.0) and had been described clinically as a cutaneous nodule in 13 (76.5%) cases and as a subcutaneous nodule in 4 (23.5%) cases. Two (11.8%) were ulcerated (Table 2).

The highest percentage of high-grade tumors and HN3 lymph nodes (n=5; 33.3) was found in the C-SC2 category.

*Outcome and prognostic factors*

The median follow-up time was 681 days (range, 217-2054).

Seven (9.2%) dogs experienced disease progression after a median of 230 days (range, 49-666), consisting of visceral metastasis (n = 3), nodal metastasis (n = 3) and local recurrence with nodal metastasis (n =1). All progressions were confirmed by means of

cytologic evaluation. The median TTP could not be assessed since the estimated survival curve did not fall below 0.5. The GM of tumors associated with disease progression was as follows: C-$SC_2$ (n = 3), SC-$C_0$ (n = 2), SC-$C_1$ (n = 1), and SC-$C_2$ (n = 1).

At the end of the study, 63 (82.0%) dogs were alive and 7 (9.2%) died because of MCT-unrelated causes, including one each of splenic hemangiosarcoma, heart failure, brachycephalic airway obstructive syndrome, pancreatitis, pancreatic insulinoma, brain neoplasia and degenerative myelopathy. Six (7.9%) dogs died because of MCT-related causes after 139, 191, 219, 321, 349 and 422 days, respectively, due to visceral metastasis. The median TSS could not be estimated. The GM of MCTs in the dogs dead of tumor-related causes was as follows: C-$SC_2$ (n = 2), SC-$C_0$ (n = 2), SC-$C_1$ (n = 1), and SC-$C_2$ (n = 2).

The one-year survival rate was 100% for C-$SC_0$ and C-$SC_1$ MCTs, 95.2% for SC-$C_0$ MCTs, 93.8% for SC-$C_2$ MCTs, 93.3% for SC-$C_1$ MCTs and 85.7% for C-$SC_2$ MCTs (Table 2).

## Discussion

Histologic grading is currently validated exclusively for canine cMCTs, and divergent opinions exist regarding the appropriateness of grading MCTs arising in other sites. In the solely subcutaneous forms, grading might underestimate tumor's biologic behavior, while for scMCTs with secondary dermal invasion, there is a lack of established guidelines, and the decision to apply grading is left to the discretion of the pathologist.

In the present study we have tested the prognostic utility of the two-tier grading system in canine MCTs with different histologic GMs. The prognostic impact of the GM itself was also investigated.

The category of primarily cutaneous MCTs with deep subcutaneous infiltration (C-$SC_2$) displayed the highest proportion of high-grade tumors (33%) and overt metastasis to lymph nodes (33%); moreover, dogs within this group had a higher frequency of tumor progression (20%) and the lowest one-year survival rate (86%). The C-$SC_2$ GM has been previously acknowledged as a negative prognostic factor, as the replacement of subcutaneous and deep tissues is considered a feature of grade III tumors in the three-tier grading system.[18]

In contrast, the category of scMCTs with superficial dermal involvement (SC-$C_2$) had lower proportions of high-grade tumors, HN3 lymph nodes, tumor progression and MCT-related deaths. Based on these results, it may be worthwhile to differentiate between the C-$SC_2$ and SC-$C_2$ GMs, rather than generically describing concurrent cutaneous and subcutaneous infiltration. Clinically, most of the tumors with these two GMs were identified as cutaneous, appearing macroscopically indistinguishable. Therefore, histology frequently plays a crucial role in discerning between them.

scMCTs may infiltrate the adjacent tissues, including a variable degree of dermal invasion. As a result, all MCTs in which the main bulk of the tumor is in the subcutis are likely to originate subcutaneously and SC-$C_{0\text{-}2}$ tumors clearly represent a continuum. By excluding cases with secondary dermal invasion from studies investigating the biological behavior of scMCTs, similar to what other authors have done in the past, we could potentially be omitting cases of advanced disease and/or locally aggressive forms, leading to an underestimation of the true biological behavior of these tumors.

Notably, even considering the category of solely scMCTs (SC-$C_0$), 6 (29%) cases had overt metastasis to lymph nodes and 2 (10%) tumor-related deaths were recorded. These tumors have been historically associated with a good prognosis, with low metastatic and recurrence rates despite incomplete surgical removal.[17] More recently, several authors hypothesized that they may constitute a more aggressive disease than previously

267 reported.[3,13,24] It has been suggested that application of the two-tier grading system might
268 lead to an underestimation of the true biologic behavior of scMCTs, due to lower mitotic
269 rate and different morphologic characteristics.[5] However, no specific grading system is
270 currently available for canine scMCTs, which is a clear limitation in terms of their
271 prognostication and post-surgical treatment decisions. In the present study, both dogs that
272 died due to SC-$C_0$ MCT had high-grade tumors, suggesting that the two-tier system has
273 prognostic value for scMCT prognostication and a validation study should be conducted.
274 Indeed, in the current study, all dogs with disease progression had a high-grade tumor,
275 regardless of their GM. The application of a sole grading system could simplify the work of
276 pathologists, as they would no longer be required to rely on the tumor's location to predict
277 its behavior. This would eliminate a major source of subjectivity in histopathologic
278 reporting, thereby offering clinicians more reproducible information to manage their
279 patients.
280 Furthermore, this may indirectly validate application of the Camus cytologic grading
281 system across all MCTs, as it mostly relies on the same parameters included in the two-
282 tier grading system.[2] This would be of great practical utility, since determining the clinical
283 location of the tumor (cutaneous vs. subcutaneous) might not always be straightforward,
284 as corroborated by the findings of the current study. Together with grading, the presence
285 of one or more lymph nodes with overt metastasis (HN3) lymph nodes has been confirmed
286 as a negative prognostic factor, although not necessarily associated with tumor-related
287 death in low-grade tumors.[6] This finding further supports the previously demonstrated
288 different significance of nodal metastasis in terms of its impact on low-grade and high-
289 grade tumors.[6]
290 Similarly, we confirmed the limited prognostic relevance of early (HN2) nodal metastases,
291 which did not result in disease progression in any of the cases, even without the
292 administration of adjuvant treatments.[12]

Finally, significant differences were observed between cutaneous and subcutaneous MCTs regarding the histologic completeness of surgical excision. The removal of tumors belonging to the subcutaneous categories more frequently resulted in clean but close or incomplete margins (SC-$C_0$, 24%; SC-$C_1$, 27%; SC-$C_2$, 18%). This observation is in line with previous studies, which have highlighted greater difficulties and less reproducibility in surgical margin planning for scMCTs.[15,20] Furthermore, in anatomic sites where maintaining sufficient margins becomes challenging due to limited soft tissue available for deep excision, the subcutaneous localization further reduces the chances of obtaining adequate deep margins. These results could also be explained by the larger size of scMTCs compared to cMCTs in this study. However, regardless of surgical margins, tumor progression was only detected in high-grade MCTs. This is consistent with previous studies and provides increasing evidence of the importance of histologic grade in canine MCTs.[1,8]

This study has several limitations. First, the small number of events (i.e., tumor progression and tumor-related death) resulted in limited statistic power, despite a fairly large number of cases. Among the possible explanations, the radicality of the surgical approach likely contributed to this low progression rate, reaffirming its efficacy as a valid strategy in the treatment of these tumors.

A further limitation may be the inclusion of dogs receiving neoadjuvant chemotherapy. Although no alterations of the histologic parameters included in the formulation of tumor grade have been reported after neoadjuvant treatments,[10,11] these therapies could alter the actual GM of the tumor, potentially affecting the results of this study. However, the exclusion of these cases might have resulted in the preferential selection of mostly low-malignancy tumors, limiting representation of the entire spectrum of MCTs in the study.

Third, tumors with primary muscular location were not included in the analysis, nor were mucosal MCTs or MCTs located in mucocutaneous junctions. Consequently, it remains to

be determined whether the application of the two-tier histologic grading can be extended to tumors in these particular locations.

Fourth, in advanced tumors with massive dermal and subcutaneous involvement, the identification of the GM might not always be feasible. Therefore, the possibility of grading tumors regardless of their GM becomes even more important in such circumstances.

Finally, it is worth considering that further variables related to the growth pattern (e.g. circumscribed/expansile, infiltrative or combined) that have been shown to be prognostic in previous studies could also influence tumor biologic behavior.[23]

In conclusion, regardless of the growth pattern, the two-tier histologic grading appears to accurately identify canine MCTs with aggressive biologic behavior, including scMCTs with or without dermal invasion, for which specific guidelines are currently lacking. Histologic grade was confirmed as the most significant prognostic factor, surpassing nodal involvement and histologic margin status. cMCTs with deep subcutaneous infiltration may exhibit a more aggressive biologic behavior when compared to MCTs with other GMs. Recognizing this distinct pattern could hold prognostic significance.

## Authors' contributions

SS and AB designed the study and performed the experiments; LM and EF enrolled cases and contributed to the experimental design; SS, AB, RZ, and AR performed histologic evaluations; SS performed the statistical analysis; the manuscript was written by SS and AB with contribution from the other authors.

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## Figure legends

**Figure 1.** Representative examples of canine mast cell tumor growth models: C-$SC_0$ (A), C-$SC_1$ (B), C-$SC_2$ (C), SC-$C_0$ (D), SC-$C_1$ (E), SC-$C_2$ (F).