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The 2-tier grading system identifies canine cutaneous and/or subcutaneous mast cell tumors with aggressive biological behavior regardless of growth model

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1 **Title page**

2

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

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19 **Abstract**

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

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

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

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

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

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

44

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

47

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

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

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

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

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

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

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

95

96 **Materials and Methods**

97 *Study design and inclusion criteria*

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

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

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

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

110 MCTs were excised according to recent recommendations, with lateral surgical margins
111 proportional to the widest tumor diameter, and deep margins including at least one fascial
112 plane.^{7,19}

113 The obtained surgical samples were subjected to histologic evaluation. In the presence of
114 high-grade tumors⁹ and/or overt nodal metastasis,²⁵ adjuvant vinblastine treatment was
115 recommended, in accordance with prior literature.¹² The remaining dogs were monitored.

116 Dogs were withdrawn from the study if they were lost to follow-up within 120 days of
117 surgery.

118

119 *Histopathologic examination*

120 Samples were fixed in 10% neutral buffered formalin, processed, and embedded in
121 paraffin using a standardized protocol. Four- μ m-thick histologic sections of the primary
122 tumor, stained with hematoxylin and eosin, were microscopically examined for the
123 assessment of tumor GM, mitotic count (MC), histologic grade according to the two-tier
124 system⁹ and surgical margins.

125 The histologic GM was assessed as detailed in Table 1. Six categories were defined:
126 solely dermal MCT (C-SC₀), dermal MCT with superficial or deep subcutaneous
127 involvement (C-SC₁ and C-SC₂, respectively), solely subcutaneous MCT (SC-C₀) and
128 subcutaneous MCT with deep or superficial infiltration of the overlying dermis (SC-C₁ and
129 SC-C₂, respectively). MC was expressed as the total number of mitotic figures in a 2.37
130 mm² area and assessed in the areas of highest mitotic activity.¹⁴ The two-tier histologic
131 grading system was applied on all tumors, regardless of the GM. Surgical margins were
132 inked by the pathologist, assessed histologically with combined radial and tangential
133 sections and defined as complete, clean but close (tumor cells at 1-3 mm from the surgical
134 margins) or incomplete.

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

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

138

139 *Statistical analysis*

140 Descriptive statistics were used in the analysis of dogs and tumor characteristics. When
141 appropriate, data sets were tested for normality by use of the D'Agostino and Pearson
142 omnibus test. None of the numeric variables had a normal distribution and, therefore, the
143 median and range are used as summary statistics.

144 Information recorded for all dogs included signalment, tumor anatomic location, largest
145 diameter, clinical site (cutaneous or subcutaneous), ulceration, substage, GM, MC, two-tier
146 grade, surgical margins, extent of nodal involvement, date of tumor progression (if any),
147 date of death and cause of death (if any).
148 Time to progression (TTP) was calculated from the date of surgery to the first occurrence
149 of one or more of the following: local recurrence, nodal metastasis and distant spread.
150 Dogs with no recurrence or disease progression at the date of the last visit or death were
151 censored.
152 Tumor-specific survival (TSS) was calculated from the date of surgery to the date of death
153 or to the date of the last visit if death did not occur. Only dogs deceased due to MCT-
154 related causes were considered as events.
155 Survival curves for each GM-defined group were obtained with the Kaplan-Meier method
156 and compared with the log-rank test. One-year survival rates were also calculated for each
157 group.
158 Data were analyzed by use of commercial software programs (SPSS Statistics v. 26, IBM,
159 Somers, NY). P values ≤ 0.05 were considered statistically significant.

160

161 **Results**

162 *Dogs' and tumors' characteristics*

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

169 The tumors were located on trunk and tail (n = 30; 33.0%), limbs (n = 28; 30.8%), head
170 and neck (n = 14; 15.4%), inguinal/perineal area (n = 10; 10.9%), mammary region (n = 7;
171 7.7%), and digital region (n = 2; 2.2%). From a clinical perspective, 53 (58.2%) tumors
172 were cutaneous and 38 (41.8%) were subcutaneous. The median tumor diameter was 1.5
173 cm (range, 0.2-16.0 cm). Four (5.3%) dogs were symptomatic (substage b).

174

175 *Treatment*

176 Seven (9.2%) dogs received neoadjuvant chemotherapy, consisting of vinblastine
177 administered intravenously (IV) every two weeks at the dose of 3 mg/m² for dogs weighing
178 ≥ 20 kg and 2.5 mg/m² for those weighing < 20 kg. Dogs also received daily oral
179 prednisolone (1 mg/kg), oral cetirizine (1 mg/kg) twice daily and oral famotidine (2 mg/kg)
180 twice daily.

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

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

187

188 *Histologic analysis*

189 Eighty-one (89.0%) tumors were low grade and 10 (11.0%) were high grade. The median
190 mitotic count was 0 (range, 0-14). Surgical margins were histologically complete in 76
191 (83.5%) MCTs, clean but close in 12 (13.2%) cases and incomplete in 3 (3.3%) cases.

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

194 According to the histologic GM, 11 tumors (12.1%) were classified as C-SC₀, 12 (13.2%)
195 as C-SC₁, 15 (16.5%) as C-SC₂, 21 (23.0%) as SC-C₀, 15 (16.5%) as SC-C₁, and 17
196 (18.7%) as SC-C₂ (Figure 1).

197 The main characteristics for each GM-defined group are listed in Table 2. Tumors treated
198 with neoadjuvant chemotherapy were distributed as follows: SC-C₂: n = 3; SC-C₀: n = 2; C-
199 SC₂: n = 2. High grade MCTs included 5 C-SC₂, 2 SC-C₀, 2 SC-C₂ and 1 SC-C₁. Tumors
200 with at least one HN3 lymph node included 6 SC-C₀, 5 C-SC₂, 3 SC-C₂, 2 C-SC₁ and 1
201 SC-C₁.

202 When specifically considering tumors with a massive involvement of both cutis and
203 subcutis, C-SC₂ MCTs had a median diameter of 1.4 cm (range, 0.5-7.0) and had been
204 described clinically as a cutaneous nodule in 14 (93.3%) cases and as a subcutaneous
205 nodule in 1 (6.7%) case. Two (13.3%) tumors were ulcerated. SC-C₂ MCTs had a median
206 diameter of 2.0 cm (range, 0.5-6.0) and had been described clinically as a cutaneous
207 nodule in 13 (76.5%) cases and as a subcutaneous nodule in 4 (23.5%) cases. Two
208 (11.8%) were ulcerated (Table 2).

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

211

212 *Outcome and prognostic factors*

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

214 Seven (9.2%) dogs experienced disease progression after a median of 230 days (range,
215 49-666), consisting of visceral metastasis (n = 3), nodal metastasis (n = 3) and local
216 recurrence with nodal metastasis (n =1). All progressions were confirmed by means of

217 cytologic evaluation. The median TTP could not be assessed since the estimated survival
218 curve did not fall below 0.5. The GM of tumors associated with disease progression was
219 as follows: C-SC₂ (n = 3), SC-C₀ (n = 2), SC-C₁ (n = 1), and SC-C₂ (n = 1).

220 At the end of the study, 63 (82.0%) dogs were alive and 7 (9.2%) died because of MCT-
221 unrelated causes, including one each of splenic hemangiosarcoma, heart failure,
222 brachycephalic airway obstructive syndrome, pancreatitis, pancreatic insulinoma, brain
223 neoplasia and degenerative myelopathy. Six (7.9%) dogs died because of MCT-related
224 causes after 139, 191, 219, 321, 349 and 422 days, respectively, due to visceral
225 metastasis. The median TSS could not be estimated. The GM of MCTs in the dogs dead
226 of tumor-related causes was as follows: C-SC₂ (n = 2), SC-C₀ (n = 2), SC-C₁ (n = 1), and
227 SC-C₂ (n = 2).

228 The one-year survival rate was 100% for C-SC₀ and C-SC₁ MCTs, 95.2% for SC-C₀
229 MCTs, 93.8% for SC-C₂ MCTs, 93.3% for SC-C₁ MCTs and 85.7% for C-SC₂ MCTs (Table
230 2).

231

232 **Discussion**

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

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

241 The category of primarily cutaneous MCTs with deep subcutaneous infiltration (C-SC₂)
242 displayed the highest proportion of high-grade tumors (33%) and overt metastasis to
243 lymph nodes (33%); moreover, dogs within this group had a higher frequency of tumor
244 progression (20%) and the lowest one-year survival rate (86%). The C-SC₂ GM has been
245 previously acknowledged as a negative prognostic factor, as the replacement of
246 subcutaneous and deep tissues is considered a feature of grade III tumors in the three-tier
247 grading system.¹⁸

248 In contrast, the category of scMCTs with superficial dermal involvement (SC-C₂) had lower
249 proportions of high-grade tumors, HN3 lymph nodes, tumor progression and MCT-related
250 deaths. Based on these results, it may be worthwhile to differentiate between the C-SC₂
251 and SC-C₂ GMs, rather than generically describing concurrent cutaneous and
252 subcutaneous infiltration. Clinically, most of the tumors with these two GMs were identified
253 as cutaneous, appearing macroscopically indistinguishable. Therefore, histology frequently
254 plays a crucial role in discerning between them.

255 scMCTs may infiltrate the adjacent tissues, including a variable degree of dermal invasion.
256 As a result, all MCTs in which the main bulk of the tumor is in the subcutis are likely to
257 originate subcutaneously and SC-C₀₋₂ tumors clearly represent a continuum. By excluding
258 cases with secondary dermal invasion from studies investigating the biological behavior of
259 scMCTs, similar to what other authors have done in the past, we could potentially be
260 omitting cases of advanced disease and/or locally aggressive forms, leading to an
261 underestimation of the true biological behavior of these tumors.

262 Notably, even considering the category of solely scMCTs (SC-C₀), 6 (29%) cases had
263 overt metastasis to lymph nodes and 2 (10%) tumor-related deaths were recorded. These
264 tumors have been historically associated with a good prognosis, with low metastatic and
265 recurrence rates despite incomplete surgical removal.¹⁷ More recently, several authors
266 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-C₀ 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

293 Finally, significant differences were observed between cutaneous and subcutaneous
294 MCTs regarding the histologic completeness of surgical excision. The removal of tumors
295 belonging to the subcutaneous categories more frequently resulted in clean but close or
296 incomplete margins (SC-C₀, 24%; SC-C₁, 27%; SC-C₂, 18%). This observation is in line
297 with previous studies, which have highlighted greater difficulties and less reproducibility in
298 surgical margin planning for scMCTs.^{15,20} Furthermore, in anatomic sites where
299 maintaining sufficient margins becomes challenging due to limited soft tissue available for
300 deep excision, the subcutaneous localization further reduces the chances of obtaining
301 adequate deep margins. These results could also be explained by the larger size of
302 scMCTs compared to cMCTs in this study. However, regardless of surgical margins, tumor
303 progression was only detected in high-grade MCTs. This is consistent with previous
304 studies and provides increasing evidence of the importance of histologic grade in canine
305 MCTs.^{1,8}

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

311 A further limitation may be the inclusion of dogs receiving neoadjuvant chemotherapy.
312 Although no alterations of the histologic parameters included in the formulation of tumor
313 grade have been reported after neoadjuvant treatments,^{10,11} these therapies could alter the
314 actual GM of the tumor, potentially affecting the results of this study. However, the
315 exclusion of these cases might have resulted in the preferential selection of mostly low-
316 malignancy tumors, limiting representation of the entire spectrum of MCTs in the study.
317 Third, tumors with primary muscular location were not included in the analysis, nor were
318 mucosal MCTs or MCTs located in mucocutaneous junctions. Consequently, it remains to

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

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

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

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

334

335 **Authors' contributions**

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

340

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412

413 **Figure legends**

414 **Figure 1.** Representative examples of canine mast cell tumor growth models: C-SC₀ (A),
415 C-SC₁ (B), C-SC₂ (C), SC-C₀ (D), SC-C₁ (E), SC-C₂ (F).