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3 **Subcutaneous mast cell tumors: a prospective SIONCOV multi-institutional clinico-**
4 **pathologic and prognostic study on 43 dogs**

5

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30

31 **Abstract**

32 **Background.** Canine subcutaneous mast cell tumors (ScMCTs) have a good prognosis.

33 **Methods.** A multi-center prospective study was conducted to identify new prognostic markers.

34 Dogs with a firstly-occurring ScMCT were enrolled upon primary tumor removal and regional
35 lymphadenectomy. In the absence of metastasis dogs were monitored; dogs with overtly metastatic
36 lymph nodes (HN3) received adjuvant vinblastine.

37 **Results.** Forty-three dogs were enrolled: 15 (34.9%) had at least one HN3 LN and received
38 vinblastine, 28 (65.1%) were monitored. Three tumors harbored exon 8 and 9 c-kit mutations. Eight
39 (18.6%) dogs experienced tumor progression, 5 (11.6%) died of MCT-related causes. One- and
40 two-year survival rates were 90% and 77%, respectively. Variables significantly associated with an
41 increased risk of progression included high cytograde, mitotic count (MC) >4/10hpf and Ki67-
42 index >23; MC >4/10hpf was associated with an increased risk of tumor-related death.

43 **Limitations.** Regional rather than sentinel lymphadenectomy was performed. Dogs were enrolled
44 in oncology referral centers, constituting a different population compared to previous studies.

45 **Conclusions.** ScMCTS have a good prognosis. However, metastatic rate at admission was higher
46 than previously reported and a subset of tumors were associated with a fatal outcome despite
47 multimodal treatment. Proliferative activity and cytograding may anticipate a more aggressive
48 biologic behavior.

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51 **Keywords**

52 Canine, subcutaneous, mastocytoma, cytograding, lymph node metastasis, prognosis

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Introduction

In dogs, subcutaneous mast cell tumors (ScMCTs), unlike cutaneous MCTs, develop in the subcutaneous fat and have been historically associated with a good prognosis.¹⁻³ Indeed, across the veterinary literature, studies have documented that ScMCTs seem to have a low metastatic potential and a favorable outcome if treated with surgery only, regardless of histologic margins. Survival figures following surgery alone ranged from 61% (at 3.3 years) and more than 66% at 4 years.^{1,4} In a recent retrospective study, a very good prognosis was confirmed for 43 dogs with ScMCTs undergoing surgery only, as median disease-free interval and survival time were not reached at >5.4 years.³ According to the literature, the recurrence rate is low, regardless of surgical margins. Notably, only 12-21% of dogs with incompletely resected ScMCTs experienced local relapse.^{1,3} Likewise, a 4-6% metastatic rate was reported after surgery.^{1,4} Although no specific histologic grading scheme was developed for ScMCTs, a mitotic count (MC) >4/10 high-power fields (hpf), presence of multinucleation, and an infiltrative growth were reported as negative prognostic factors, yet the population of dogs showing these features was small (6%).¹ Works on proliferation markers gave conflicting results: in an earlier study⁴ they were not prognostically relevant, while in subsequent studies, high Ki67, AgNOR, and PCNA indices negatively impacted outcome.^{3,5} KIT diffuse cytoplasmic immunohistochemical expression was also associated with local recurrence and metastasis. Exon 8 and 11 ITD mutations have been occasionally reported.^{5,22} Unfortunately, all the aforementioned studies suffer from bias related to their retrospective nature.¹⁻⁵ Cases were mainly selected from the archive of pathology laboratories, and information regarding

80 tumor features and follow-up was obtained from veterinary clinics in the form of a questionnaire or
81 telephone interview. Therefore, staging information may have not been gathered from all dogs, and
82 follow-up data may have been subject to inaccuracies and incomplete reporting.
83 Thus, a prospective study was conducted on dogs with newly-diagnosed ScMCT, undergoing tumor
84 removal and regional lymphadenectomy, aimed at providing more insight into their biologic
85 behavior, clinico-pathologic features and clinical outcome, possibly identifying new prognostic
86 factors.

87
88

89 **Materials and methods**

90 The study did not fall within the application areas of the Italian law which governs the protection of
91 animals used for scientific purposes; therefore, ethical approval was waived for this study. All dogs
92 enrolled received the current standard of care at the participating institutions. Owners gave their
93 written informed consent to participate with their dogs in the study.

94

95 *Study design and inclusion criteria*

96 Members of SIONCOV and affiliated with different oncology centers were asked to participate to
97 this prospective, multi-institutional study.

98 Client-owned dogs presented between January 2017 and December 2020 were eligible for
99 recruitment if they had a previously untreated, cytologically confirmed, single ScMCT.

100 Dogs were excluded from enrollment for any of the following reasons: multiple or recurrent
101 ScMCT; concurrent cutaneous MCTs; comorbidities limiting life expectancy; neoadjuvant
102 chemotherapy or radiation therapy.

103 Initial staging included history and physical examination, complete blood cell count with
104 differential, serum biochemistry, coagulation profile, cytological evaluation of the subcutaneous
105 nodule, cytological evaluation of the regional lymph node (LN) according to Krick,⁸ thoracic

106 radiographs (3 views), abdominal ultrasound and fine-needle aspiration of liver and spleen
107 regardless of their sonographic appearance.

108 The regional LN was defined as the first LN in the expected lymphatic drainage,⁹ and was identified
109 either by palpation or ultrasound.²⁸ Any abnormality in LN size or consistency was subjectively
110 categorized into mild, moderate or severe.

111 All dogs underwent surgical removal of the primary tumor and regional lymphadenectomy, as later
112 detailed, and samples were subjected to histological examination for the confirmation of the
113 subcutaneous location of the tumor according to Thompson et al.,¹ and for the assessment of (1)
114 surgical margins, (2) histologic prognostic factors, (3) nodal status according to Weishaar et al.,⁷ and
115 (4) c-kit mutational analysis.

116 In the case of infiltrated margins, dogs had to undergo scar re-excision and were ultimately enrolled
117 only upon the histologic confirmation of adequately locally controlled ScMCT.

118 In the presence of overt (histological node 3, HN3)⁷ nodal metastasis, dogs received adjuvant
119 vinblastine, as later detailed, in accordance with previous literature focusing on cutaneous
120 MCTs.^{24,29} The remaining dogs were monitored.

121 Dogs were withdrawn from the study if owners refused any of the proposed therapeutic procedures,
122 or if they were lost to follow-up before the end of the study period.

123

124 *Treatment and follow-up*

125 Removal of the primary tumor and regional lymphadenectomy were performed within 10-14 days
126 from the staging diagnostic, regardless of the cytological LN diagnosis. Whenever possible, the
127 surgical dose consisted of 20-30 mm lateral margins and one fascial plane deep.

128 In dogs with HN3 LNs, adjuvant vinblastine was administered within 10-14 days from surgery
129 every two weeks, for 8 total cycles at the dose of 3 mg/m² IV for dogs weighing >15 kg or 2.5
130 mg/m² for those ≤15 kg. Dogs also received concomitant medications with H1-/H2-antagonists and
131 corticosteroids at the dose of 1 mg/kg orally once daily for 2 weeks and then gradually tapered to

132 the final dose of 0.5 mg/kg every other day. These drugs were discontinued at the end of the
133 protocol. The protocol was selected based on preference of the clinicians participating to the study.
134 The assessment schedule is summarized in Tables 1-2. Physical examinations were performed at all
135 visits. The owner was asked to record episodes of perceived nausea, vomiting, and diarrhea in the
136 intervals between all visits if medical treatment was administered.

137 Further concomitant medications were recorded at all visits. Concomitant medications that were not
138 permitted as first-line treatment during the study period included: homeopathic or alternative
139 therapies, chemotherapy other than vinblastine, investigational medications and tigilanol tiglate.

140 End-staging was carried out at the end of medical treatment and consisted of physical examination,
141 blood analysis and abdominal ultrasound.

142 All toxicities were graded according to the Veterinary Comparative Oncology Group.¹⁰

143 Dose reductions or delays of vinblastine were permitted. In particular, dose reductions of 10% and
144 20% were calculated following the occurrence of any grade 2 and hematologic grade 3 toxicity,
145 respectively. Permanent discontinuation of medical treatment was undertaken in the case of dose-
146 limiting toxicity defined as grade 3 gastrointestinal or grade 4 hematologic toxicity.

147 Dogs that showed progression of any type and at any time during the trial were allowed to receive
148 any additional treatment, either local or systemic, that was felt appropriate.

149

150 Table 1. Schedule for Monitoring Group (no adjuvant medical treatment).

Event	Day	PE	FNA MCT	FNA LN	CBC	Serum biochemistry	Coag profile	Thoracic radiograph s	Abd US	FNA liver	FNA spleen	BM	Surgery
Screening	-21 to -16	+	+	+	+	+	+	+	+	+	+	+	
Surgery	-14 to -10	+											+
Visit 0	0	+			+								
Visit 1	28 (± 2)	+			+								
Visit 2	56 (± 2)	+			+	+			+				
Visit 3	84 (± 2)	+			+	+							

Visit 4	98 (± 2)	+			+								
Visit 5	126 (± 4)	+			+	+			+				
Visit 6	154 (± 4)	+			+	+							
Visit 7	182 (± 4)	+			+	+			+				
Visit 8	210 (± 4)	+			+	+							
Visit 9	308 (± 7)	+			+	+			+				
Visit 10	406 (± 7)	+			+	+			+				
Visit 11	504 (± 7)	+							+				

Visit 12	602 (± 7)	+							+				
Visit 13	700 (± 7)	+											
Visit 14	728 (± 7)	+							+				

151 PE: physical examination; FNA: fine-needle aspirate; CBC: complete blood cell count; Coag: coagulation; Abd US: abdominal ultrasound; BM:

152 bone marrow

153

154 Table 2. Schedule for Vinblastine Group.

Event	Day	PE	FNA MCT	FNA LN	CBC	Serum biochemistry	Coag profile	Thoracic radiograph s	Abd US	FNA liver	FNA spleen	BM	Surgery	VBL*	AE report
Screening	-21 to -16	+	+	+	+	+	+	+	+	+	+	+			
Surgery	-14 to -10	+											+		

Visit 0	0	+			+									+	+
Visit 1	14 (± 2)	+			+									+	+
Visit 2	28 (± 2)	+			+									+	+
Visit 3	42 (± 2)	+			+									+	+
Visit 4	56 (± 2)	+			+	+			+					+	+
Visit 5	70 (± 2)	+			+									+	+
Visit 6	84 (± 2)	+			+	+								+	+
Visit 7	98 (± 2)	+			+									+	+
Visit 8	126 (± 4)	+			+	+			+						
Visit 9	154 (± 4)	+			+	+									
Visit 10	182 (± 4)	+			+	+			+						
Visit 11	210 (± 4)	+			+	+									
Visit 12	308 (± 7)	+			+	+			+						
Visit 13	406 (± 7)	+			+	+			+						
Visit 14	504 (± 7)	+							+						
Visit 15	602 (± 7)	+							+						
Visit 16	700 (± 7)	+													

Visit 17	728 (± 7)	+							+						
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155 PE: physical examination; FNA: fine-needle aspirate; CBC: complete blood cell count; Coag: coagulation; Abd US: abdominal ultrasound; BM:

156 bone marrow; VBL: vinblastine; AE: adverse event

157

158 *Histopathologic examination*

159 Samples were fixed in 10% buffered formalin, processed, and embedded in paraffin by using a
160 standardized protocol. Four- μ m-thick histologic sections, stained with hematoxylin and eosin, were
161 microscopically examined. The diagnosis of ScMCT was confirmed if the bulk of the tumor was in
162 the subcutaneous tissue, as described by Thompson et al.¹

163 The histologic growth pattern was defined as infiltrative, circumscribed or combined according to
164 Thompson.¹ Surgical margins were examined by tangential and radial sections, and defined as
165 infiltrated or not infiltrated.¹¹ MC was expressed as the total number of mitotic figures in a 2.37
166 mm² area and assessed in the areas of highest mitotic activity.¹²

167 Serial sections of the tumors underwent immunohistochemical analysis using primary antibodies
168 anti-MIB1 and CD117 for the assessment of Ki67-index and KIT-pattern, respectively.

169 Ki67-index was obtained by counting the number of immunopositive cells present in a 10 \times 10 mm
170 grid area using a 1-cm² 10 \times 10 grid reticle at 400x magnification. The number of immunopositive
171 cells per grid area was evaluated over 5 hpf and subsequently averaged.² KIT immunohistochemical
172 staining pattern was assessed as previously described.^{2,13}

173 The extent of LN metastasis according to Weishaar was evaluated on toluidine blue or Giemsa-
174 stained slides.⁷

175 Histologic evaluations were performed by one board-certified and one experienced veterinary
176 pathologist (SS, LA), and final determinations were by consensus.

177

178 *c-kit mutational analysis*

179 Mutation analyses were performed at the Department of Comparative Biomedicine and Food
180 Science, University of Padua (Italy).

181 One tissue core (2-mm diameter) of fresh tissue was obtained from each ScMCT sample.

182 Specimens were submersed in a stabilization and storage solution and refrigerated at -20°C until
183 use. Exons 8, 9 and 11 were screened for mutations by PCR and direct sequencing.¹⁴

184

185 *Data recording*

186 All data were collected through a cloud-based electronic data capture platform (Castor EDC).

187 Records were periodically updated at each follow-up visit. During the conduct of the study, a
188 monitor (FSB) checked the adequacy and quality of collected data.

189 Recorded information included signalment, tumor description (anatomic location, largest diameter),
190 date of initial observation, cytograde,⁶ date of staging, clinical stage and substage, site of metastasis,
191 date of surgery, histologic growth pattern (infiltrative vs circumscribed vs combined), histologic
192 margins (infiltrated vs non infiltrated), MC,¹² Ki67-index,² Kit pattern,² c-kit mutational status,
193 histologic classification of the regional LN,⁷ adjuvant treatment (none vs vinblastine), local relapse
194 (defined as the cytological evidence of recurrence within 2 cm from the primary tumor surgical
195 scar), nodal progression (defined as the presence of further metastatic LNs), distant relapse (defined
196 as the occurrence of visceral metastasis), treatment-related toxicity, date of death or last follow-up
197 examination, and cause of death.

198 Data collection was closed in September 2022, 20 months after enrollment closure.

199

200 *Statistical analysis*

201 Descriptive statistics were used in the analysis of dogs and tumor characteristics. When appropriate,
202 data sets were tested for normality by use of the D'Agostino and Pearson omnibus normality test.

203 No data had normal distribution and were therefore expressed as median (range).

204 Differences in the distribution of deleterious c-kit mutations according to KIT protein expression
205 pattern and LN status at admission were assessed with Fisher's exact test.

206 Time to local recurrence (TLR) was calculated from the date of surgery to the date of cytologically
207 or histologically confirmed local recurrence. Time to nodal relapse (TNR) was calculated from the
208 date of surgery to the date of further cytologically or histologically confirmed nodal involvement.

209 Time to distant relapse (TDR) was calculated from the date of surgery to the date of cytologically

confirmed visceral metastases. Time to progression (TTP) was calculated from the date of surgery to the first occurrence of one or more of local recurrence, nodal and distant relapse. 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 for ScMCT-related causes were considered as events. The influence of potential prognostic variables on tumor progression and tumor-related death was investigated with univariable and multivariable Cox proportional hazards regression analysis. Only covariates that were significant at univariable analysis were included in the multivariable (adjusted) regression model. The considered variables included sex, neutering status, age, weight, anatomic location of ScMCT (biologically aggressive locations, i.e. head and neck, inguinal/perineal area, mammary region and digits), macroscopic tumor largest diameter, high cytological grade,⁶ substage b, presence of HN3 LN at admission,⁷ presence of distant metastases at admission, infiltrative growth pattern, MC >4/10 hpf, Ki67-index >23, KIT expression patterns, c-kit ITD or deleterious missense mutations. For age and weight, the median was used as cut-off value. For tumor diameter, a cut-off value of 3 cm was selected based on previous studies.¹⁵⁻¹⁷ Data were analyzed by use of commercial software programs (SPSS Statistics v. 26, IBM, Somers, NY). P values ≤ 0.05 were considered significant.

228
229

230 **Results**

231

232 *Demographics and staging*

233 A total of 59 dogs were screened: 6 dogs were excluded because of lack of owners' compliance
234 after screening, and 10 dogs were excluded because the MCT also involved the dermis.

235 The remaining 43 dogs were eligible for study enrollment and were included. The most represented
 236 breeds were Labrador retriever (n=6; 14.0%), English setter (n=4; 9.3%), boxer (n=4; 9.3%), and
 237 golden retriever (n=3; 7.0%). Of the remaining, 10 (23.3%) were mixed-breed dogs, and 14 breeds
 238 were represented once or twice.

239 Median age was 8 years (range, 4 to 13) and median weight was 27.4 kg (range, 5.8 to 48.1). There
 240 were 19 females (16 spayed) and 24 males (3 neutered).

241 ScMCTs had been noticed by the owner for a median of 55 days (range, 3 to 540). At the time of
 242 admission, 3 (7.0%) dogs showed systemic clinical signs related to tumor degranulation (substage
 243 b).

244 ScMCTs were located on limbs (n=23; 53.5%), head and neck (n=7; 16.3%), trunk (n=7; 16.3%),
 245 mammary region (n=3; 7.0%), axilla (n=1; 2.3%), inguinal region (n=1; 2.3%) and digit (n=1;
 246 2.3%). Median maximum tumor diameter was 3.0 cm (range, 0.3 to 20.0). According to Camus,⁶ 4
 247 (9.3%) ScMCTs were cytologically high grade.

248 Regional LNs were clinically abnormal in 17 (39.5%) dogs, of which 7, 7 and 3 showed mild,
 249 moderate and severe lymphadenomegaly, respectively.

250 The cytological evaluation of the regional LN yielded a diagnosis of non-metastatic LN in 20
 251 (46.5%) dogs, possible metastasis in 11 (25.6%), probable in 4 (9.3%) and certain in 8 (18.6%).⁸ No
 252 dog had metastatic involvement of spleen and/or liver.

253

254 *Surgical treatment, histopathologic and molecular features*

255 All dogs underwent surgical removal of the primary tumor and lymphadenectomy. Thirty-three
 256 (76.7%) ScMCTs were completely removed, whereas 10 (23.3%) were removed with infiltrated
 257 margins. The latter underwent scar re-excision before enrollment.

258 Twenty-nine (67.4%) dogs had one regional LN removed, whereas 14 (32.6%) underwent multiple
 259 lymphadenectomies (n=12 had 2 LNs removed; n=1 had 3, n=1 had 4). Among dogs having more

260 than 1 LN removed, different lymphocenters were removed in all cases. The following 60 LNs were
261 removed: 16 inguinal, 14 popliteal, 12 prescapular, 10 axillary, 5 submandibular, and 3 medial iliac.
262 According to Weishaar,⁷ there were 20 (33.3%) non-metastatic (HN0) LNs, 9 (15.0%) pre-
263 metastatic (HN1), 14 (23.3%) early metastatic (HN2) and 17 (28.3%) overtly metastatic (HN3)
264 LNs. Among dogs with metastatic LNs, 10 (23.3%) had only HN2 LN and 15 (34.9%) dogs had at
265 least one HN3 LN. Interestingly, out of these 15 dogs, 3 (20.0%) had severe lymphadenomegaly, 5
266 (33.3%) had moderate lymphadenomegaly, 3 (20.0%) had mild lymphadenomegaly and 4 (26.7%)
267 had no LN enlargement.

268 Histologic growth pattern was infiltrative in 23 (53.5%) cases, circumscribed in 9 (20.9%) and
269 combined in 11 (25.6%). Median MC was 1 (range, 0 to 27); 4 (9.3%) dogs had MC >4/10 hpf.
270 The results of immunohistochemical analysis were available for 39 (90.7%) cases; the remaining 4
271 (9.3%) tumors were not immunoreactive. Median Ki67-index was 1 (range, 0 to 50); 3 (7.0%) dogs
272 had Ki67-index >23. Fifteen (34.9%) dogs had KIT pattern 2/3.

273 c-kit mutations were detected in 3 (7.0%) ScMCTs: specifically, 1 ITD in exon 8 and 2 deleterious
274 missense mutations in exon 9. Silent, clinically irrelevant mutations were identified in exon 8 in 12
275 dogs, on exon 9 in 6 dogs, and on exon 11 in 12 dogs. Twenty-three (53.5%) ScMCTs were wild-
276 type.

277 There was no association between c-kit mutational status and KIT protein expression pattern. No
278 relationship was identified between the presence of deleterious c-kit mutations and LN status at
279 admission.

280

281 *Adjuvant treatment*

282 Twenty-eight (65.1%) dogs received no medical treatment and were only monitored, while 15
283 (34.9%) received vinblastine because of the presence of one or more HN3 LNs.

284 Ten (66.7%) of the 15 dogs receiving vinblastine completed the 8 scheduled administrations;
285 among these, one dog needed a 7-days delay and a 20% dose reduction following grade 3

286 neutropenia. One dog experienced grade 1 neutropenia and another had grade 1 gastrointestinal
287 toxicity. Two dogs experienced tumor progression after the completion of vinblastine and received
288 toceranib as a rescue treatment.

289 Among the 5 (33.3%) dogs not completing the vinblastine protocol, 2 stopped treatment after 3 and
290 6 doses, respectively, because of grade 4 neutropenia, and 3 because of tumor progression after a
291 median of one dose (range, 1-3). Among the latter, 2 received toceranib as a rescue treatment.

292

293 *Outcome*

294 Overall, 8 (18.6%) dogs had tumor progression after a median of 237 days (range, 15 to 857).

295 Median TTP was not reached.

296 Specifically, seven (16.3%) tumors recurred locally recurred after a median time of 182 days
297 (range, 15 to 857). Three tumors recurred after a single complete surgery and 4 after revision; 6 had
298 an infiltrative histologic growth pattern. Five (11.6%) dogs experienced further nodal involvement
299 after a median of 82 days (range, 25 to 351); in 4 dogs, a single HN3 LN was removed along with
300 the primary ScMCT, whereas one dog already had 3 HN3 LNs removed. Two (4.7%) dogs
301 developed distant metastasis to the liver and spleen after 84 and 182 days. Both of them had been
302 previously treated with vinblastine.

303 At data analysis closure, 29 (67.4%) dogs were still alive after a median follow-up of 697 days
304 (range, 608 to 2063). Fourteen (32.6%) dogs had died after a median time from enrollment of 382
305 days. Cause of death was tumor-unrelated in 9 of them, whereas 5 (11.6%) dogs died of tumor-
306 related causes after a median of 91 days (range, 82 to 422). Median TSS was not reached.

307 One- and two-year survival rates were 90% and 77%, respectively.

308

309 *Analysis of prognostic factors*

310 Variables significantly associated with an increased risk of local recurrence on univariable analysis
311 were high cytograde (HR, 17.2; P=0.001), substage b (HR, 12.1; P=0.005), MC >4/10 hpf (HR,

312 22.0; P <0.001) and Ki67-index >23 (HR, 21.0; P=0.003; Table 3). On multivariable analysis, high
313 cytgrade (HR, 24.3; P=0.002) and MC >4/10 hpf (HR, 13.5; P=0.042) retained prognostic
314 significance (Table 4).

315 Variables significantly associated with an increased risk of nodal relapse on univariable analysis
316 were high cytgrade (HR, 22.5; P=0.001), substage b (HR, 13.8; P=0.005), MC >4/10 hpf (HR,
317 30.7; P <0.001) and Ki67-index >23 (HR, 58.5; P=0.001; Table 3). On multivariable analysis, high
318 cytgrade (HR, 25.1; P=0.010) and MC >4/10 hpf (HR, 32.1; P=0.013) retained prognostic
319 significance (Table 5).

320 The only variable significantly associated with an increased risk of distant metastasis was MC
321 >4/10 hpf (HR, 21.9; P=0.035; Table 3).

322 Overall, variables significantly associated with an increased risk of tumor progression on
323 univariable analysis were high cytgrade (HR, 11.6; P=0.001), substage b (HR, 12.3; P=0.004), one
324 or more HN3 regional LNs (HR, 13.5; P=0.015), MC >4/10 hpf (HR, 22.4; P<0.001) and Ki67-
325 index >23 (HR, 36.0; P <0.001; Table 3). On multivariable analysis, MC >4/10 hpf (HR, 30.0;
326 P=0.032) and Ki67-index >23 (HR, 26.1; P=0.033) retained prognostic significance (Table 6).

327 Variables significantly associated with an increased risk of tumor-related death on univariable
328 analysis were high cytgrade (HR, 19.0; P=0.001), substage b (HR, 14.8; P=0.004), MC >4/10 hpf
329 (HR, 30.0; P<0.001) and Ki67-index >23 (HR, 20.6; P=0.003; Table 3). On multivariable analysis,
330 only MC >4/10 hpf (HR, 13.5; P=0.045) retained prognostic significance (Table 7).

331

332 **Table 3.** Univariable Cox regression analysis of variables potentially associated with increased risk of local recurrence (LR), nodal relapse (NR),
333 distant metastasis (DM), tumor progression (TP) and tumor-related death (TRD) in 43 dogs with subcutaneous mast cell tumors.

Variable	LR		NR		DM		TP		TRD	
	HR (95% CI)	<i>P</i>	HR (95% CI)	<i>P</i>	HR (95% CI)	<i>P</i>	HR (95% CI)	<i>P</i>	HR (95% CI)	<i>P</i>
Female sex	1.9 (0.4-8.4)	0.407	5.6 (0.6-50.5)	0.122	92.5 (0.1-910.1)	0.445	2.4 (0.6-10.1)	0.228	5.6 (0.6-50.3)	0.124
Neutered	1.6 (0.4-7.3)	0.532	1.9 (0.3-11.6)	0.469	1.3 (0.1-20.6)	0.859	2.0 (0.5-8.6)	0.329	1.9 (0.3-11.4)	0.479
Age > 8 years	7.7 (0.9-64.5)	0.060	75.0 (0.1-970.7)	0.241	72.7 (0.1-993.1)	0.461	88.1 (0.3-793.1)	0.129	74.7 (0.6-980.7)	0.242
Weight > 27.4 kg	0.4 (0.1-2.0)	0.251	0.6 (0.1-3.7)	0.601	0.1 (0.0-992.3)	0.466	0.5 (0.1-2.3)	0.395	0.6 (0.1-3.7)	0.597
Aggressive tumor location	1.0 (0.2-5.1)	0.992	0.7 (0.1-6.0)	0.721	65.8 (0.1-994.1)	0.450	0.8 (0.2-4.1)	0.825	1.7 (0.3-9.9)	0.583
Largest diameter > 3 cm	3.3 (0.6-17.4)	0.151	79.6 (0.6-765.4)	0.236	1.2 (0.8-19.6)	0.886	3.9 (0.8-19.5)	0.097	5.0 (0.6-45.2)	0.149

High cytgrade ⁶	17.2 (3.4-87.9)	0.001*	22.5 (3.7-136.5)	0.001*	13.1 (0.8-218.1)	0.073	11.6 (2.6-52.1)	0.001*	19.0 (3.1-115.2)	0.001*
Substage b	12.1 (2.2-68.2)	0.005*	13.8 (2.2-86.3)	0.005*	0.5 (0.0-969.2)	0.854	12.3 (2.2-69.2)	0.004*	14.8 (2.4-91.6)	0.004*
Overt nodal metastases at admission ⁷	81.8 (0.2-874.5)	0.143	202.3 (0.1-934.1)	0.221	183.7 (0.1-911.0)	0.437	13.5 (1.7-111.0)	0.015*	191.8 (0.4-862.4)	0.219
Infiltrative growth pattern	7.0 (0.8-58.4)	0.074	4.1 (0.5-36.8)	0.208	65.5 (0.1-918.9)	0.471	3.8 (0.7-18.9)	0.109	4.1 (0.5-36.6)	0.209
MC >4/10 hpf	22.0 (4.2-115.3)	<0.001*	30.7 (4.8-194.9)	<0.001*	21.9 (1.2-389.7)	0.035*	22.4 (4.3-117.3)	<0.001*	30.0 (4.7-190.4)	<0.001*
Ki67-index >23	21.0 (2.9-154.5)	0.003*	58.5 (5.8-587.1)	0.001*	0.1 (0.0-922.9)	0.854	36.0 (5.8-222.9)	<0.001*	20.6 (2.8-151.8)	0.003*
KIT cytoplasmic expression patterns	3.0 (0.7-13.3)	0.155	8.3 (0.9-74.3)	0.058	2.1 (0.1-33.4)	0.604	3.7 (0.8-15.6)	0.073	3.1 (0.5-18.6)	0.216
c-kit deleterious mutations	2.0 (0.2-16.5)	0.527	0.4 (0.0-965.8)	0.676	0.4 (0.1-930.4)	0.792	1.8 (0.2-14.4)	0.595	0.4 (0.1-997.7)	0.672

334 Abbreviations: HR, hazard ratio; CI, confidence interval. *statistically significant.

335

336 **Table 4.** Multivariable Cox regression analysis for the risk of local recurrence in 43 dogs with
 337 subcutaneous mast cell tumors. Significant variables at univariable analysis were included in the
 338 model.

Variable	Local recurrence 339	
	HR (95% CI)	P 340
High cytograde ⁶	24.3 (3.1-190.0)	0.002*341
Substage b	2.4 (0.1-290.5)	0.722 342
MC >4/10 hpf	13.5 (1.1-166.2)	0.042*343
Ki67-index >23	6.4 (0.1-863.4)	0.458 344

345

346 Abbreviations: HR, hazard ratio; CI, confidence interval. *statistically significant.

347

348 **Table 5.** Multivariable Cox regression analysis for the risk of nodal relapse in 43 dogs with
 349 subcutaneous mast cell tumors. Significant variables at univariable analysis were included in the
 350 model.

Variable	Nodal relapse 351	
	HR (95% CI)	P 352
High cytograde ⁶	25.1 (2.1-294.9)	0.010*353
Substage b	2.1 (0.1-34.7)	0.605 354
MC >4/10 hpf	32.1 (2.1-499.6)	0.013*355
Ki67-index >23	5.4 (0.2-915.4)	0.872 356

357

358 Abbreviations: HR, hazard ratio; CI, confidence interval. *statistically significant.

359

360 **Table 6.** Multivariable Cox regression analysis for the risk of tumor progression in 43 dogs with
 361 subcutaneous mast cell tumors. Significant variables at univariable analysis were included in the
 362 model.

Variable	Tumor progression 363	
	HR (95% CI)	P 364
High cytograde ⁶	8.3 (0.9-74.7)	0.058 365
Substage b	0.3 (0.1-11.3)	0.477 366
Overt nodal metastases at admission ⁷	5.1 (0.3-76.7)	0.243 367
MC >4/10 hpf	30.0 (1.3-672.5)	0.032*368
Ki67-index >23	26.1 (1.3-521.1)	0.033*369

370

371 Abbreviations: HR, hazard ratio; CI, confidence interval. *statistically significant.

372

373 **Table 7.** Multivariable Cox regression analysis for the risk of tumor-related death in 43 dogs with
 374 subcutaneous mast cell tumors. Significant variables at univariable analysis were included in the
 375 model.

Variable	Tumor-related death 376	
	HR (95% CI)	P 377
High cytograde ⁶	7.6 (0.7-80.0)	0.091 378
Substage b	1.1 (0.1-845.7)	0.970 379
MC >4/10 hpf	13.5 (1.1-170.5)	0.045*380
Ki67-index >23	2.0 (0.1-1502.5)	0.843 381

382

383 Abbreviations: HR, hazard ratio; CI, confidence interval. *statistically significant.

384

385

386 **Discussion**

387

388 Over the last years, methods for screening, diagnosing and managing dogs with MCTs have
389 improved. This is due to our growing understanding of the biology of MCTs. However, while
390 plenty of literature exists focusing on cutaneous MCTs, the subcutaneous counterpart has been less
391 investigated.

392 Several studies based on retrospective series defined ScMCTs as having a low metastatic potential
393 and a favorable outcome if treated with surgery only, regardless of histologic margins and clinical
394 stage.¹⁻⁴ However, staging has continued to evolve as dog cohorts have expanded and additional
395 prognostic features have been described, particularly concerning the histologic nodal status.^{7,18,19}
396 Here, we prospectively enrolled 43 dogs, for which all possible information regarding clinical stage,
397 histologic variables, treatment, and long-term outcome were available. While we confirm that
398 prognosis is overall favorable, our study reveals some differences with previously published data,
399 which are clinically relevant.

400

401 The first striking finding is the metastatic rate at admission: overall, 58% of dogs were diagnosed
402 with a metastatic LN.

403 Approximately 23% of dogs had early metastatic LNs. It is likely that the same finding would have
404 been shown in previous studies if the LN had been removed. However, therapeutic strategies
405 targeting early aspects of the metastatic process may not be relevant to the outcome, resulting in
406 long survival times regardless.²⁹

407 Thirty-five percent of dogs had at least one overtly metastatic regional LN. This remarkable
408 metastatic potential is in disagreement with previous data. A possible explanation again lies in the
409 lack of lymphadenectomy in previous studies.¹⁻⁵ The metastatic rate of 4 to 6% reported in 2 of
410 these studies refers to the post-surgical follow-up period in all but one dog.^{1,4} In the most recent

411 study, nodal metastasis was diagnosed by means of cytology in one out of the 14 dogs undergoing
412 LN sampling.³

413 All these studies suffer from bias and cannot reflect the real metastatic potential of ScMCTs. In
414 addition, 27% of HN3 LNs in the current study were not clinically enlarged, highlighting the poor
415 sensitivity of palpation in predicting the nodal metastatic load and confirming its poor diagnostic
416 accuracy.

417 The current finding has the potential to change therapeutic and prognostic discussions with the
418 owners or encourage oncologists to be more circumspect, as overt nodal metastasis dictates the need
419 for a multimodal approach, consisting of lymphadenectomy and medical treatment in addition to the
420 excision of the primary tumor.²⁰

421 The second relevant finding concerns the first documentation of c-kit exon 9 missense mutations in
422 ScMCT, that were identified in 2 dogs. Neither of them experienced tumor-related events.

423 Similarly, the only dog with an exon 8 ITD did not have a poor outcome, in line with the published
424 literature.^{21,22}

425 We confirmed the prognostic significance of previously reported factors and uncovered new ones.
426 In line with previous reports, a MC >4/10 hpf significantly correlated with local, nodal and distant
427 relapse and tumor-related death.^{2,3} ScMCTs with higher MC were also frequently associated with
428 substage b disease and HN3 LN at admission in the current series (50% and 75%, respectively). The
429 practical implication of having accurate and reliable information about the MC of ScMCTs is that a
430 better estimate of prognosis can be given and more rational treatment planning is possible. An
431 important consideration for wide adoption of this prognostic factor is the reproducibility among
432 pathologists of MC assessment, which may be hampered by variations in the total counting area and
433 in the selection of the counting fields.^{12,23}

434 Similarly, in agreement with previous studies, a Ki67-index >23 was significantly associated with
435 tumor progression.^{3,5} Conversely, tumor growth and KIT pattern, which have been previously
436 reported as a relevant prognostic factor in ScMCT,^{1,2} did not impact on outcome in the current

437 series. This may be due to the high subjectivity level that is inherent in these assessments or to a
438 type II error due to the low population number.

439 Notably, in the present study, all ScMCTs that were cytologically high grade had an overtly
440 metastatic LN. Additionally, cytograding was significantly associated with local recurrence and
441 nodal relapse. Although the Camus cytograding system has been validated for cutaneous rather than
442 subcutaneous MCTs,⁶ here it was shown that it may be useful to identify biologically aggressive
443 ScMCTs as well. This finding is particularly relevant, as the clinical distinction between cutaneous
444 and subcutaneous MCTs is not always straightforward, and cytograding is an additional tool to sort
445 out the more complex cases. Thus, cytograde should be incorporated in the fine-needle aspiration
446 report and this can be of great value in guiding treatment choice.

447 Overall, the outcome was good, with a 1- and 2-year survival rate of 90% and 77%, respectively,
448 confirming that most ScMCTs behave in a benign fashion. However, all dogs underwent wide
449 surgical excision of the primary tumor and had their regional LNs removed, and 35% of the dogs
450 also received medical treatment, based on the presence of overt nodal metastatic disease. It is not
451 known whether medical therapy made a difference and was really indispensable; however,
452 compared to what has been published on cutaneous MCTs,^{20,24,25} we believe that dogs with HN3
453 LNs require a multimodal treatment. This is also supported by the fact that all the 5 dogs dead of
454 MCT-related causes had at least one HN3 LN at admission, highlighting that, albeit rarely, even the
455 subcutaneous localization can have a fatal outcome.

456

457 There are some limitations. First, regional lymphadenectomy was performed in all dogs. The study
458 was designed in 2016 and started in 2017, before emphasis was given and results were available
459 regarding the critical role of the sentinel LN. Indeed, during the past few years, we have seen the
460 pendulum swing from the “regional” to the “sentinel” nodal approach, which may not match in up
461 to 63% of cases.²⁶ So, it may be possible that metastatic LNs were left behind, possibly increasing
462 the rate of nodal progression.

463 Also, some dogs underwent multiple lymphadenectomies. The procedure was not standardized, but
464 was dependent on the surgeons' experience or individual anatomic variation. It remains unclear
465 how many LNs must be removed to accurately predict the nodal status in cutaneous and
466 subcutaneous MCTs. Studies are under way to address this question.²⁷ It must also be stressed that
467 dogs received different treatments (surgery vs surgery and adjuvant chemotherapy), based on
468 clinical stage. Given that the aim of the study was not to report the best possible treatment for
469 ScMCTs, the results document that there are aggressive cases that could benefit from multimodal
470 treatment.

471 Additionally, dogs in this study were enrolled in oncology referral centers, thereby constituting a
472 different population compared to the previous retrospective studies, recruiting animals from
473 primary practice. Cases from referral centers might be subjected to bias (e.g., selection of the most
474 aggressive cases, higher owner compliance) that can influence results and be partially responsible
475 for the observed differences from previously published studies.

476
477 In conclusion, the overall prognosis for dogs with adequately locally controlled ScMCTs is good.
478 However, surgery may not be enough, as approximately 35% of dogs have overt nodal metastatic
479 disease at admission, requiring adjuvant medical treatment. Proliferation markers confirm as a
480 valuable tool in the formulation of prognosis and in the selection of patients requiring additional
481 treatments. Cytograding also provides relevant information regarding the aggressiveness of ScMCT
482 and LN metastasis. Thus, it is suggested that a conscious effort should be made to include the
483 cytological grade in all cytology reports of ScMCTs.

484

485 **Data availability statement**

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

488

489 **Conflict of interest statement**

490 The authors have no conflicts of interest to declare.

491

492 **Authors contribution statement**

493 Study design: LM; Acquisition of data (provided animals, acquired and managed patients, provided
494 facilities): LM, DS, MA; Laboratory investigation: SS, LA, MD, MG, GB, UB, WB; Analysis and
495 interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): SS, EF, LM;
496 Writing, review, and/or revision of the manuscript: LM, SS, EF; Study supervision: LM, FSB

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