Subcutaneous mast cell tumours: A prospective multi-institutional clinicopathological and prognostic study of 43 dogs

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

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Abstract

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

Methods. A multi-center prospective study was conducted to identify new prognostic markers. Dogs with a firstly-occurring ScMCT were enrolled upon primary tumor removal and regional lymphadenectomy. In the absence of metastasis dogs were monitored; dogs with overtly metastatic lymph nodes (HN3) received adjuvant vinblastine.

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

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

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

Keywords

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

Word count: 4245

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 study[4] 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

tumor features and follow-up was obtained from veterinary clinics in the form of a questionnaire or telephone interview. Therefore, staging information may have not been gathered from all dogs, and follow-up data may have been subject to inaccuracies and incomplete reporting.

Thus, a prospective study was conducted on dogs with newly-diagnosed ScMCT, undergoing tumor removal and regional lymphadenectomy, aimed at providing more insight into their biologic behavior, clinico-pathologic features and clinical outcome, possibly identifying new prognostic factors.

Materials and methods

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

Study design and inclusion criteria

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

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

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

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

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

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

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

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

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

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

Treatment and follow-up

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

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

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

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

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

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

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

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

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

Event	Day	PE	FNA MCT	FNA LN	CBC	Serum biochemistry	Coag profile	Thoracic radiographs	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)	+							+				

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

Table 2. Schedule for Vinblastine Group.

Event	Day	PE	FNA MCT	FNA LN	CBC	Serum biochemistry	Coag profile	Thoracic radiographs	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)	+							+						

PE: physical examination; FNA: fine-needle aspirate; CBC: complete blood cell count; Coag: coagulation; Abd US: abdominal ultrasound; BM: bone marrow; VBL: vinblastine; AE: adverse event

Histopathologic examination

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

The histologic growth pattern was defined as infiltrative, circumscribed or combined according to Thompson.[1] Surgical margins were examined by tangential and radial sections, and defined as infiltrated or not infiltrated.[11] 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.[12]

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

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

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

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

c-kit mutational analysis

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

One tissue core (2-mm diameter) of fresh tissue was obtained from each ScMCT sample. Specimens were submersed in a stabilization and storage solution and refrigerated at -20°C until use. Exons 8, 9 and 11 were screened for mutations by PCR and direct sequencing.[14]

Data recording

All data were collected through a cloud-based electronic data capture platform (Castor EDC). Records were periodically updated at each follow-up visit. During the conduct of the study, a monitor (FSB) checked the adequacy and quality of collected data.

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

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

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).

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

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

Results

Demographics and staging

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

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

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

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

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

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

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

Surgical treatment, histopathologic and molecular features

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

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

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

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

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

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

Adjuvant treatment

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

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

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

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

Outcome

Overall, 8 (18.6%) dogs had tumor progression after a median of 237 days (range, 15 to 857). Median TTP was not reached.

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

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

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

Analysis of prognostic factors

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

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

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

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

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

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

Table 3. Univariable Cox regression analysis of variables potentially associated with increased risk of local recurrence (LR), nodal relapse (NR), 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)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
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 cytograde[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.

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

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

The second relevant finding concerns the first documentation of c-kit exon 9 missense mutations in ScMCT, that were identified in 2 dogs. Neither of them experienced tumor-related events. Similarly, the only dog with an exon 8 ITD did not have a poor outcome, in line with the published literature.[21,22]

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

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

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

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

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

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

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

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

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

Data availability statement

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

Conflict of interest statement

The authors have no conflicts of interest to declare.

Authors contribution statement

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

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