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Prognostic impact of mast cell infiltration detected by flow cytometry on excised sentinel lymph nodes in dogs with newly diagnosed mast cell tumors

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Abstract

Lymph node (LN) metastasis is a negative prognostic factor in canine mast cell tumors (MCTs). Flow cytometry (FC) can identify and quantify mast cells (MCs) in LNs. This tri-institutional prospective study aimed to evaluate the impact of previously reported MC cut-off values in sentinel LNs (SLNs) detected by FC on clinical outcomes in dogs with cutaneous or subcutaneous MCTs, and to determine a prognostically significant cut-off. Dogs with newly diagnosed, completely staged, single MCT scheduled for primary tumor excision and sentinel lymphadenectomy were enrolled. The SLNs of enrolled dogs were analyzed using cytology and FC after excision. MCs were quantified as a percentage using FC, and SLNs were cytologically and histologically classified according to the Krick and Weishaar systems, respectively. The influence of potential prognostic variables, including MCs cut-offs >1.1% and >4%, on tumor progression and tumor-specific survival (TSS) was evaluated using Cox regression and log-rank analysis. The optimal cut-off for predicting tumor-related death was determined via ROC curves.

A total of 64 dogs were enrolled. Dogs with nodal MC infiltration exceeding 1.1% and 4% were 10 and 40 times more likely, respectively, to experience tumor progression. SLN MC infiltration >4% and HN3 SLN were associated with shorter TSS (median, 327 versus not reached; $P<0.001$). The optimal SLN cut-off for predicting tumor-related death was 16%. These findings suggest that previously established cut-offs may hold prognostic value. Additional studies performing in vivo sampling with a larger number of events are necessary to validate this proposed cut-off.

Key words: canine, flow cytometry, mastocytoma, nodal, prognosis

Introduction

Interest in the prognostic role of lymph node (LN) status in dogs with cancer has increased over the past decade. LN metastasis is a well-known negative prognostic factor in canine mast cell tumors (MCTs),¹⁻⁷ as it indicates aggressive biologic behavior and predicts disease relapse and death.¹⁻⁸ Approximately 20 to 70% of dogs with MCTs have nodal metastasis at initial diagnosis.⁷⁻¹⁵ Therefore, an accurate staging to determine the extent of nodal metastases is of utmost importance to guide treatment recommendations and predict prognosis. Traditionally, the regional LN has been assessed during nodal staging. More recent studies indicate that sentinel LN (SLN) assessment offers greater accuracy in staging MCTs in dogs, as the SLN is the first LN to receive lymphatic drainage from the primary tumor and is therefore the most likely initial site of metastasis.^{11,15-18} Several studies have suggested that SLN and regional LN do not match in 28-63% of cases, highlighting the importance of SLN mapping during the staging work-up.^{11,15-18} The histologic examination of resected SLNs is crucial for verifying nodal metastasis, as it accurately detects metastatic involvement and provides essential prognostic information.¹ Nevertheless, preoperative prediction of SLN status can assist clinicians in treatment decision-making, optimizing patient care by guiding the need for adjuvant chemotherapy and ultimately improving outcome.^{7,19-23} Although fine-needle aspiration (FNA) cytology of LNs may assist in the preoperative prediction of nodal status, it has a 25% false negative rate.^{24,25} Flow cytometry (FC) is a safe and minimally invasive technique, offering the advantage of analyzing a large number of cells and providing timely results.²⁶ Recent studies demonstrated that FC is able to correctly identify and quantify mast cells (MCs) in LNs of dogs with MCTs.^{25,27} Furthermore, when using histopathology as the gold-standard, FC can effectively distinguish between metastatic from non-metastatic SLN.²⁵ The combined use of FC

and cytology enhances diagnostic accuracy in differentiating metastatic from non-metastatic nodes in dogs with MCTs during clinical staging.²⁵ However, the prognostic significance of different levels of SLN infiltration detected by FC remains undetermined.

This pilot prospective study aimed to preliminarily assess whether nodal infiltration cut-off values identified through FC in the preoperative setting were associated with clinical outcomes in dogs with cutaneous or subcutaneous MCT. To achieve this, the SLNs of enrolled dogs were analyzed using cytology and FC after excision. First, the impact of previously reported cut-off values for the percentage of MCs in SLN detected by FC²⁵ was evaluated in relation to time to progression (TTP) and tumor-specific survival (TSS). A secondary aim was to establish a cut-off of prognostic relevance. If specific cut-off values for the percentage of MCs in SLNs are found to be meaningful for predicting prognosis, further validation will be needed by testing these cut-off values on in vivo SLNs.

Methods

Case selection and enrollment

We conducted a tri-institutional prospective study. Client-owned dogs with newly diagnosed, single cutaneous or subcutaneous MCTs that were scheduled for primary tumor excision and sentinel lymphadenectomy were eligible for inclusion.

Dogs were enrolled at (“masked for review”), between (“masked for review”), following informed written consent from the dog owner, in accordance with the ethical committee approval of (“masked for review”) protocol number (“masked for review”), of (“masked for review”) protocol

number (“masked for review”), and of (“masked for review”) protocol number (“masked for review”).

To be eligible for inclusion, dogs had to undergo a complete clinical staging including history and physical examination, complete blood cell count with differential, serum biochemistry profile, thoracic radiographs, abdominal ultrasound, cytologic evaluation of the liver and spleen regardless of their sonographic appearance, SLN mapping via radiographic indirect lymphography (RIL)²⁶ or computed tomography lymphography (lympho-CT)²⁷, cytology and FC of SLN.

The choice of SLN mapping technique varied based on each institution’s preferences. In certain cases, methylene blue was also used intraoperatively to aid in identifying SLN/s that were either too small or located in anatomically challenging areas, regardless of the primary mapping technique employed.

Dogs were excluded if they had concurrent allergic dermatologic disease, an acute flare-up of their chronic allergic disease,³⁰ multiple MCTs, or visceral metastasis. Dogs were also excluded if they had received neoadjuvant chemotherapy.

Sentinel lymph node sampling and processing

At the time of surgery, 2-5 FNAs were performed ex vivo on excised SLNs using a 2.5 ml syringe and a 22G needle. The aspirated material was then transferred in 1 ml of RPMI medium enriched with sodium-azide and fetal calf serum (FCS) to obtain cell suspensions for FC analysis. Samples for FC analysis from the different institutions were shipped to (“masked for review”) and processed within 24 hours from sampling.

Additionally, one to two smears for each SLN were prepared for cytologic evaluation.

Excised SLNs were then placed in 10% neutral-buffered formalin for histopathologic evaluation.

Cytologic and histopathologic classification of SLN

Cytologic evaluation of SLNs was performed by the same cytologist (“masked for review”) using May-Grünwald Giemsa-stained smears. Each SLN was categorized according to the classification system by Krick² into one of the following categories: normal or reactive LN, possible metastasis, probable metastasis, and certain metastasis.

For histologic examination, after formalin-fixation, SLNs were bisected along their longest axis through the hilum, and both halves were included in paraffin. Paraffin blocks were then cut as previously described.³¹ Histologic classification was performed on toluidine blue-stained sections by a board-certified veterinary pathologist (“masked for review”) blinded to cytology and FC results.³¹ Each SLN was categorized according to the classification system by Weishaar¹ into one of the following categories: HN0, HN1, HN2 or HN3.

Flow cytometry analysis

Flow cytometric analysis was conducted using a CytoFLEX flow cytometer (Beckman Coulter) following previously described methods.^{25,29} Briefly, antibodies against CD117, IgE, CD5, and CD21, each conjugated with different fluorochromes, were added to lymph node aspirate in a single tube. Following a 30-minute incubation in the dark, red blood cells were lysed with lysis buffer for 5 minutes, followed by washing step with PBS. Samples were then acquired on the flow cytometer. Isotype controls were used to assess non-specific binding. A compensation matrix was previously established using VersaComp beads (Beckman Coulter), and fluorescence minus one tubes were used to set the gate for analysis. Mast cells were identified as CD117+ IgE+ CD5- CD21- events and recorded as a percentage of the total intact cells. A minimum of 10000 events in the gate of analysis was acquired. All samples were analyzed on CytExpert software (Beckman Coulter) by

the same operator (“masked for review”). The list and the specifications of the antibodies, along with an illustration of the gating strategy, are provided in the supplementary file “Flow Cytometry”

Treatment and follow-up

All dogs underwent wide surgical excision of the primary MCT within 7-14 days from staging, defined as a lateral margin of 2-3 cm and a deep margin extending to one fascial plane, depending on tumor size and location. The primary tumor was graded according to the 2-tier histologic grading system^{32,33} by a single board-certified veterinary pathologist (“masked for review”). For dogs with incompletely excised MCTs, scar re-excision or definitive-intent radiation therapy was recommended to ensure local tumor control. If further local treatments were declined by the owner, Ag67 score was determined to predict the risk for local recurrence in dogs with low-grade MCTs.³⁴ Dogs were eligible for adjuvant medical treatment in case of high-grade MCTs, regardless of their SLN status, or when the SLN was classified as HN3, regardless of the MCT histologic grade. Adjuvant chemotherapy consisted of vinblastine administered every two weeks for 8 total cycles, starting within 10-14 days from surgery. The dosage was 3 mg/m² for dogs weighing >15 kg and 2.5 mg/m² for dogs weighing ≤15 kg. Dogs treated with vinblastine also received concomitant corticosteroids (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), and H1- and H2-antagonists. These drugs were discontinued at the end of the protocol. All toxicities were graded according to the Veterinary Comparative Oncology Group – Common Terminology Criteria for Adverse Events.³⁵

While under medical treatment, dogs were clinically monitored every 2 weeks; all dogs, regardless of whether they received medical treatment, were followed up monthly during the first year and every 2 months thereafter.

Abdominal ultrasound was performed after the fourth vinblastine, and end-staging was carried out at the completion of medical treatment. End-staging included physical examination, blood analysis and abdominal ultrasound. Additionally, abdominal ultrasound was performed every 6 months regardless of previous treatments. FNA and cytology of any suspected lesion, during re-staging and follow-up, was performed as needed.

Dogs that showed progression of any type and at any time during the study were eligible to receive any additional local and/or systemic treatment.

Data collection

Information recorded for each dog included: signalment (i.e., breed, age, sex, weight), clinical substage (i.e., asymptomatic[a] or symptomatic [b]), primary MCT description (i.e., location, maximum diameter, presence of ulceration), SLN location, clinical characteristics (dimension and, for extra-cavitary SLN, consistency and mobility), number of removed SLN, percentage of MCs infiltrating the SLN detected by FC, SLN status according to Krick (for cytology samples) and Weishaar (for histology samples) classifications, histologic grade of MCT, histopathologic evaluation of MCT surgical margins (complete vs incomplete), Ag67 score (if performed), postoperative complications, medical treatment (chemotherapy vs none), additional treatment/s after progression of any type (i.e., surgery, chemotherapy, and/or tyrosine kinase inhibitors).

To assess outcome, the following information were also recorded: local recurrence (defined as MCT relapse at or with 2 cm of surgical scar, confirmed by cytology or histopathology), nodal progression (defined as the presence of new metastatic LN/s, confirmed by cytology or histopathology), distant progression (defined as the occurrence of cytologically confirmed metastasis at distant organs), date and cause of death.

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.

TTP was calculated from the date of surgery to the first occurrence of at least one of the following: local recurrence, nodal progression or distant metastasis. Tumor-specific survival (TSS) was calculated from the date of surgery to the date of death due to tumor-related causes.

Dogs without tumor progression or tumor-related death were censored in the respective statistical analysis.

Survival estimates were presented as medians with the corresponding 95% confidence intervals (CI).

The influence of potential prognostic variables on tumor progression and tumor-related death was investigated with Cox proportional hazards regression analysis.

Assessed variables included aggressive tumor anatomic location (head and neck, digits, inguinal, and perineal regions), tumor ulceration, tumor diameter ≥ 3 cm, histologic high-grade, incomplete surgical margins, and presence of one or more HN3 LNs.

Regarding the percentage of LN infiltration assessed by FC, the previously identified cut-offs of MCs $>1.1\%$ and $>4\%$ were evaluated for prognostic significance.²⁵ For dogs with more than one SLN excised, the one with the highest percentage of MC infiltration was considered for the analysis.

In case no events were registered in one group (complete separation), variables were compared using log-rank test.

Additionally, the optimal cut-off for SLN infiltration detected by FC to predict tumor-related events was identified by receiver operating characteristic (ROC) curves.

Data were analyzed using a commercial software (SPSS Statistics v. 26, IBM, Somers, NY). P-values ≤ 0.05 were considered significant.

Cell line statement

No cell lines were used in this study.

Results

The study group included 64 dogs previously described in a prior study.²⁵ A summary of the dogs and tumor characteristics is provided in Table 1.

SLN mapping and characteristics

All dogs underwent SLN mapping by means of lympho-CT (n=40; 62.5%) or RIL (n=24; 37.5%). Methylene blue was used as a supplementary intraoperative tool to identify SLNs in 33 (61.5%) cases.

A total of 117 SLN were identified, with a median of 1 SLN (range, 1-7) for each dog. SLN included inguinal (n=37; 31.7%), axillary (n=19; 16.2%), superficial cervical (n=17; 14.5%), popliteal (n=14; 11.9%), mandibular (n=13; 11.1%), medial retropharyngeal (n=7; 6%), medial iliac (n=7; 6%), sacral (n=2; 1.7%), and parotid (n=1; 0.9%).

Information on SLN characteristics was available for 59 (92%) of the cases, as 5 (8%) had intracavitary SLN. From a clinical perspective, 48 (75%) dogs had normal-sized SLN/s, whereas 16 (25%) dogs had enlarged SLN/s; among these, 2 (12.5%) dogs had enlarged, firm and fixed SLN/s.

Data of individual cases are described in Table 2.

229

230 *Treatment*

231 All dogs underwent wide surgical excision of their primary MCT along with sentinel
232 lymphadenectomy. Three (4.7%) dogs underwent reconstructive surgery via axial flaps or free
233 grafts due to anatomic location (distal to mid antebrachium) and dimensions of the tumors.

234 Post-operative complications were observed in 8 (12.5%) dogs. Of these, 6 (75%) developed a
235 seroma, and 2 (25%) experienced flap dehiscence. Among dogs with seroma, 4 (66.6%) developed
236 it at the primary tumor removal site, while 2 (33.3%) had seroma at both the primary tumor and
237 SLN sites. All seromas resolved without medical treatment. The 2 dogs with flap dehiscence were
238 treated with antibiotics (amoxicillin and clavulanic acid) and healed by second intention, achieving
239 full resolution after 8 and 10 weeks.

240 Thirteen (20.3%%) dogs received adjuvant vinblastine. Of those, 8 had low-grade MCT and at least
241 one HN3 SLN, 4 had a high-grade MCT and at least one HN3 SLN, and 1 had a high-grade MCT
242 and HN2 SLN.

243 Three (20%) dogs experienced toxicity. Overall, there were 5 episodes of self-limiting grade 1
244 diarrhea.

245

246 *Histopathologic evaluation of MCTs*

247 Among cutaneous MCTs, 42 (89.4%) were low-grade and 5 (10.6%) were high-grade. All 17
248 (100%) subcutaneous MCTs were low-grade.

249 Surgical margins were histologically complete in 58 (90.6%) cases, and incomplete in 6 (9.4%),
250 all of which were low-grade MCTs. Further local therapy was declined by the owners in all cases.

251 Ag67 score was determined in 4 of these 6 cases, with a median value of 10 (range, 8-34.4).

252

Cytology, flow cytometry and histology of SLNs

The cytologic nodal status was available for 102/117 (87.2%) SLNs. Fifteen (12.8%) SLNs were deemed inadequate due to insufficient cellularity. Overall, 65 (63.7%) SLNs were classified as normal or reactive, 8 (7.8%) as possible metastasis, 10 (9.8%) as probable metastasis and 19 (18.6%) as certain metastases.

FC results were available for all 117 SLNs. The median percentage of MCs in the SLNs was 0.075 (range, 0-33.1).

Histologically, there were 39 (33.3%) HN0, 41 (35%) HN1, 18 (15.4%) HN2, and 19 (16.3%) HN3 SLNs. Four (6.2%) dogs had more than 1 HN3 SLN: 3 dogs had 2 HN3 SLNs, and 1 dog had 4 HN3 SLNs.

Outcome

During follow-up, 6 (9.4%) dogs experienced tumor progression (local, n=1; nodal n=2; nodal and distant, n=3). In all cases, disease progression was confirmed through cytology.

For all dogs with local and/or nodal progression, further local therapy (surgery or radiation therapy) was recommended but declined by the owners.

One dog with an incompletely excised low-grade MCT and an HN3 sentinel lymph node (SLN) developed nodal progression and visceral metastases (liver and spleen) 233 days after diagnosis. The dog was subsequently treated with toceranib but was euthanized 352 days after diagnosis due to poor quality of life.

Another dog with a completely resected low-grade MCT and an HN2 sentinel lymph node developed nodal progression 398 days after surgery. The owner declined further treatment, and the dog remained alive at data closure, with a follow-up of 1,050 days.

A dog with a high-grade MCT and HN3 sentinel lymph node developed distant progression (skin) 99 days after surgery. The dog was initially treated with lomustine and, after three cycles, switched to toceranib. However, 235 days after diagnosis, due to deteriorating quality of life marked by dyspnea and lethargy, treatment was discontinued, and the dog was euthanized.

A dog with a completely resected high-grade MCT and HN3 sentinel lymph node developed nodal progression 190 days after surgery. The owner declined further treatment, and the dog was euthanized 210 days after diagnosis due to poor quality of life, worsened by increasing lymph node size and skin ulceration.

A dog with a completely resected high-grade MCT and an HN3 sentinel lymph node developed nodal and distant (splenic) progression 219 days after diagnosis and was treated with lomustine. The dog was euthanized 327 days after initial diagnosis due to poor quality of life, characterized by vomiting, anorexia, and severe lethargy.

Finally, a dog with a completely resected high-grade cutaneous MCT and an HN2 sentinel lymph node developed local progression 211 days after surgery. The owner declined further local treatment, and the dog was managed with toceranib. At the time of data closure, the dog was alive with stable local disease, with a follow-up of 423 days.

Individual event data are summarized in table 3

At data analysis closure, 5 (7.8%) dogs were dead due to tumor-related causes after a median of 325 days (range, 210-352), and 5 (7.8%) due to unrelated causes after a median of 465 days (range, 63-544), including diffuse large B-cell lymphoma, retroperitoneal hemangiosarcoma, laryngeal mass with airway obstruction, vertebral sarcoma, and acute pancreatitis.

The remaining 56 (87.5%) dogs were alive, after a median follow-up time of 453 days (range, 194-1250). Overall median TTP and TSS were not reached.

None of the dogs with subcutaneous MCT developed tumor progression or tumor-related death.

Analysis of prognostic factors and correlation with FC

In univariable analysis, tumor ulceration (HR: 16.2; 95% CI: 2.9-91.5; P=0.002), high-grade MCT (HR: 31.2; 95% CI: 5.5-176.2; P<0.001), HN3 nodal status (HR:12.9; 95% CI:2.2-75.6; P=0.004), SLN MC infiltration >1.1% (HR: 9.9; 95% CI: 1.8-56.0; P=0.009) and >4% (HR: 41.0; 95% CI: 4.0-426.3; P=0.002) were associated with an increased risk of tumor progression. Tumor ulceration (HR: 30.7; 95% CI: 3.4-279.3; P=0.002), tumor diameter ≥ 3 cm (HR: 6.2; 95% CI: 1.0-37.0; P=0.047) and high-grade MCT (HR: 46.5; 95% CI: 5.1-421.5; P=0.001) were associated with an increased risk of tumor-related death.

As no MCT-related deaths were recorded in dogs without HN3 LNs or in those with SLN infiltration percentages below 4% (complete separation), the log-rank test was used to assess the impact of those variables on outcome. Variables significantly associated with shorter TSS included the presence of at least one HN3 LN (median TSS: 327 versus not reached; P<0.001) and SLN MC infiltration >4% on FC (median TSS: 327 versus not reached; P<0.001; Figure 1).

Among the 3 dogs that died from tumor-related causes, the median nodal MC infiltration was 26% (range, 18.4-33.1), while the median for the remaining dogs was 0.1% (range, 0-13.7).

According to ROC curve analysis, the optimal cut-off value of SLN infiltration to predict tumor-related death was 16%, with sensitivity and specificity of 100% (AUC: 1; P<0.001).

Discussion

Nodal metastasis plays a crucial role in the staging, treatment planning and prognosis of dogs with MCTs, with histopathology regarded as the gold standard for accurately identifying metastatic involvement of the LN.^{1-7,19-23}

Previous studies demonstrated that FC is able to correctly identify and quantify MCs in LNs of dogs with MCTs.^{25,29} A recent study identified cut-offs values of 1.1% and 4%, with a sensitivity of 84.2% and 68.4, and a specificity of 98% and 100%, respectively, for detecting overt metastasis (HN3) in dogs with MCTs.²⁵ To verify whether these cut-off values have prognostic significance, in the current study we subjected the SLNs to cytometric evaluation after their excision. We are aware that it is not always easy to preoperatively sample SLNs: mapping techniques often identify the sentinel lymph center, but it is during the intraoperative phase, using vital dyes, that the draining LNs are visualized. However, before undertaking a technically challenging study, we decided to first validate the previously identified cut-off values from a prognostic standpoint, with the intention of applying them in the future to non-excised LNs.

The results of the current study indicate that both proposed cut-offs have prognostic significance in predicting tumor progression. Specifically, dogs with nodal MC infiltration levels exceeding 1.1% and 4% were found to be nearly 10 and 40 times more likely, respectively, to experience tumor progression, which includes local, nodal, or distant **failure**. Using these cut-offs, clinicians can categorize dogs into high-risk (>4%), intermediate-risk (1.1%-4%), and low-risk (<1.1%) groups, allowing them to provide more informed prognostic information and set expectations for disease progression in the pre-operative setting. Dogs in the high and intermediate-risk groups may benefit from more personalized treatment and/or more frequent follow-ups to identify disease progression early, enabling timely interventions that could ultimately lead to improved outcomes. In this study, no MCT-related deaths **occurred** in dogs that either **lacked a detectable** HN3 SLN or exhibited SLN infiltration levels below 4%. This observation indicates a complete separation in

outcomes between these groups. Consequently, a log-rank test was utilized to evaluate the impact of these variables on the overall outcome. The results indicated that both SLN MC infiltration levels exceeding 4% and the presence of at least one HN3 SLN were significantly associated with a shorter TSS.

This finding suggests that FC can serve as a valuable prognostic tool for predicting survival outcomes. Its application could have significant clinical implication, not only in cases involving a single MCT, but also in situations with simultaneous multiple MCTs where several LNs may be involved. Additionally, this tool may prove useful in scenarios where extensive surgical efforts are needed for lymphadenectomy, offering a timely and non-invasive method to assess prognosis.

The presence of at least one HN3 SLN was found to be associated with shorter TSS, which aligns with previous studies.^{21,22,33}

Given the high specificity of FC in identifying HN3 LN when MC infiltration exceeds 4%,²⁵ all dogs in the current study with >4% MC in the SLN were also classified as having HN3 nodes. This correlation may help explain the prognostic impact of both variables reported in this study concerning TSS.

The optimal cut-off value for SLN infiltration to predict tumor-related death was established at 16%, achieving both 100% specificity and sensitivity. This finding suggests that FC can be used as a prognostic tool for predicting tumor-related death.

Although subcutaneous MCTs have historically been considered less aggressive, recent studies have shown that they may display variable biological behavior, including nodal metastasis and tumor-related death.^{36,37} Moreover, the clinical distinction between cutaneous and subcutaneous MCTs does not always correspond to the actual histologic growth pattern, as tumors may involve both dermal and subcutaneous layers to varying degrees. The Kiupel grading system has also been shown to be applicable to both forms.³³ Given these findings and considering the uniform staging

and treatment protocols applied in our study, subcutaneous MCTs were included in the overall analysis. However, no events of tumor progression or tumor-related death occurred within this subgroup precluding a separate outcome analysis.

While FC is a safe and minimally invasive technique that delivers timely results, it has several limitations compared to histopathology. First, fine-needle aspiration can yield random and variable cellularity, potentially leading to low or absent samples, particularly in normal-sized or intra-cavitary LNs. Second, FC analysis must be conducted within 24 hours from sampling which may pose technical challenges in clinical settings. Additionally, the relatively high costs of cytometers and the limited availability of the analysis in laboratories may hinder its widespread adoption.

Hence, while FC is a valuable prognostic tool, lymphadenectomy and histologic examination of the SLN remain essential for accurately evaluating nodal involvement and informing therapeutic and prognostic decisions.

This study has some limitations. First, the small number of events led to limited statistical power, despite the relatively large number of analyzed cases. This could be attributed to several factors: 1) the majority of dogs had low-grade MCTs which are associated with better outcome;^{19,22,33} 2) SLN mapping was performed in all cases, ensuring that no potential metastatic LNs were overlooked; 3) surgical margins were complete in the majority of cases; 4) SLNs were surgically removed in all cases, which has been shown to lead to better outcome even in high-grade MCTs; and lastly, all dogs with high-grade MCT and/or at least one HN3 SLN received adjuvant chemotherapy.^{13,19,21,22} These combined factors may have contributed to the improved outcome observed in this study population. Second, the optimal cut-off value to predict tumor-related death may have been influenced by the small number of dogs that died from tumor-related causes, potentially overestimating the infiltration level required to predict tumor-related death.

Given the limited number of events, the statistical power of survival analyses is inherently constrained. As such, the observed associations, especially those derived from Cox regression and ROC analysis, should be interpreted with caution and considered exploratory, pending validation in larger cohorts. Third, the samples for FC were obtained directly from excised LNs which may yield different results compared to in vivo sampling. In vivo sampling may result in inconclusive FC analysis due to insufficient cellularity, especially in deeply located or small-sized LNs, potentially reducing the sensitivity of this technique. A future study with in vivo LNs sampling is planned to validate these findings. Fourth, two different SLN mapping techniques (RIL and lympho-CT) were used, chosen based on anatomic complexity and financial considerations. While this approach introduces variability, it reflects real-world clinical practice. Importantly, standardized diagnostic protocols were applied after SLN identification, minimizing downstream variability. Finally, the FC markers utilized in this study do not distinguish between non-neoplastic and malignant MCs.²⁵ Thus, to reduce the risk of including dogs with a higher percentage of non-neoplastic MCs in LNs, we excluded dogs with concurrent allergic dermatologic conditions and those experiencing an acute flare-up of their chronic allergies from the analysis.³⁰

Although the flow cytometric approach used in this study correlates well with the histologic reference classification this limitation may impact the test's specificity when applied to staging neoplastic infiltration of lymph nodes.²⁵ Flow cytometry has the potential to identify neoplastic mast cells through the detection of aberrant immunophenotypes. In humans, for example, systemic mastocytosis is characterized by aberrant expression of CD2 and CD25.³⁸ In dogs, markers such as CD34 and CD25 may assist in distinguishing neoplastic from non-neoplastic mast cells; however, these markers are expressed in only a minority of tumors.²⁹ Therefore, further comprehensive studies are needed to better characterize the immunophenotype of canine mast cells, which would improve the specificity and diagnostic utility of flow cytometric analysis in this context.

In conclusion, this study highlights the prognostic relevance of previously established cut-off values for MC infiltration levels in SLNs of dogs with MCTs as assessed by FC. Clinicians could categorize dogs into high-risk (>4%), intermediate-risk (1.1%-4%), and low-risk (<1.1%) groups based on these cut-offs, with those having MC infiltration levels exceeding 4% more likely to experience shorter survival times. By stratifying patients into these risk categories preoperatively, clinicians could offer critical prognostic insights and implement tailored monitoring and treatment strategies aimed at enhancing outcomes. A cut-off value of 16% for MCs in SLNs aspirates accurately predicted tumor-related events. Additional studies performing in vivo sampling with a larger number of events are necessary to validate this proposed cut-off. The authors emphasize the importance of histologic evaluation of SLN, as it offers essential prognostic information and provides therapeutic benefit.

Data availability statement

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

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540 **Tables:**

541 **Table 1.** Breed, sex, age (in years) of the 64 dogs included in the study and histologic information
 542 (localization and 2-tier grade).

Individual information for included cases					
DOG	BREED	SEX	AGE	C/SC	K
1	Mixed Breed	SF	11	C	L
2	Mixed Breed	M	15	C	L
3	Golden Retriever	SF	7	C	L
4	Boxer	F	7	C	L
5	Mixed Breed	SF	16	SC	L
6	Mixed Breed	SF	13	C	L
7	Labrador Retriever	F	4	SC	L
8	English Setter	F	6	SC	L
9	English Setter	SF	12	SC	L
10	Shi-tzu	SF	9	C	L
11	Golden Retriever	F	5	C	L
12	Boxer	SF	5	C	L
13	Golden Retriever	F	4	C	L
14	Mixed Breed	NM	6	SC	L
15	Labrador Retriever	SF	9	SC	L
16	Mixed Breed	SF	9	SC	L
17	American Staffordshire Terrier	SF	7	SC	L
18	French Bouledogue	M	10	C	L
19	Mixed Breed	NM	8	C	L
20	Golden Retriever	F	6	C	L
21	Mixed Breed	SF	7	C	L
22	Golden Retriever	M	6	SC	L
23	Boxer	SF	7	C	L
24	French Bouledogue	NM	11	C	L
25	Mixed Breed	M	10	C	H
26	Weimaraner	F	4	C	L
27	Boxer	M	6	C	L
28	Mixed Breed	SF	11	SC	L
29	Boxer	SF	6	C	L
30	Dachshund	M	13	C	H
31	Weimaraner	F	12	C	L
32	Jack Russel	M	7.5	C	H
33	Golden Retriever	NM	7	C	L
34	Golden Retriever	F	11	C	H

35	Bernese Mountain Dog	M	7.5	C	L
36	Pug	M	9	C	L
37	Golden Retriever	F	2	C	L
38	Maltese Dog	M	13	C	L
39	Pitt Bull	NM	8	C	L
40	Mixed Breed	M	5.5	C	L
41	Weimaraner	SF	9	C	L
42	Mixed Breed	SF	11	C	L
43	Mixed Breed	NM	5	C	L
44	French Bouledogue	F	8	C	H
45	Maltese Dog	M	7	C	L
46	Mixed Breed	F	12	C	L
47	Cocker Spaniel	M	13	C	L
48	Corso	M	6	SC	L
49	American Staffordshire Terrier	M	9	C	L
50	Bernese Mountain Dog	NM	9	SC	L
51	Golden Retriever	M	4.5	C	L
52	Mixed Breed	NM	7	C	L
53	Boxer	NM	7	C	L
54	Golden Retriever	M	7	SC	L
55	Mixed Breed	F	2.5	C	L
56	Labrador Retriever	F	10	SC	L
57	Maltese Dog	F	7	C	L
58	Mixed Breed	NM	4	SC	L
59	Boxer	M	6	SC	L
60	Mixed Breed	SF	12	C	L
61	Dogo Argentino	SF	8	C	L
62	Mixed Breed	SF	8	SC	L
63	Mixed Breed	M	10	C	L
64	American Bulldog	M	10	C	L

Abbreviations: M, intact male; NM, neutered male; F, intact female; SF, spayed female; C, cutaneous mast cell tumor;

SC, subcutaneous mast cell tumor; K, Kiupel's two-tier grading classification (L: low grade; H: high grade).

Table 2. Sentinel lymph node mapping technique, number of sentinel lymph nodes for each dog and individual results of cytologic, histologic and flow cytometric analysis for each lymph node.

Dog	SLN mapping technique	Number of SLN	Cytology	Histology	FC (MC%)
1	RIL	2	reactive/normal	HN0	0.09
	RIL		certain metastasis	HN3	20.5
2	RIL	7	reactive/normal	HN0	0.01

	RIL		reactive/normal	HN0	0.01
	RIL		possible metastasis	HN1	0.2
	RIL		reactive/normal	HN1	0.01
	RIL		reactive/normal	HN1	0.01
	RIL		possible metastasis	HN1	0.01
	RIL		reactive/normal	HN1	0.01
3	RIL	2	reactive/normal	HN1	0.02
	RIL		certain metastasis	HN3	0.8
4	RIL	1	reactive/normal	HN2	0.25
5	RIL	4	reactive/normal	HN1	0.3
	RIL		reactive/normal	HN1	0.24
	RIL		possible metastasis	HN2	0.66
	RIL		probable metastasis	HN2	0.55
6	RIL	4	reactive/normal	HN0	0.12
	RIL		reactive/normal	HN0	0.1
	RIL		reactive/normal	HN0	0.2
	RIL		reactive/normal	HN0	0.03
7	RIL	4	reactive/normal	HN0	0.01
	RIL		reactive/normal	HN1	0.1
	RIL		reactive/normal	HN1	0.3
	RIL		reactive/normal	HN0	0.8
8	lympho-CT	4	reactive/normal	HN0	0.05
	lympho-CT		reactive/normal	HN0	0.05
	lympho-CT		reactive/normal	HN0	0.03
	lympho-CT		reactive/normal	HN0	0.02
9	RIL	4	reactive/normal	HN1	0.06
	RIL		reactive/normal	HN2	0.06
	RIL		certain metastasis	HN3	0.4
10	lympho-CT	1	probable metastasis	HN2	1.2
11	RIL	2	reactive/normal	HN1	0.01
	RIL		probable metastasis	HN2	0.3
12	RIL	2	reactive/normal	HN1	0.01
	RIL		nd	HN1	0.01
13	RIL	3	nd	HN1	0.02
	RIL		reactive/normal	HN2	0.01
	RIL		probable metastasis	HN2	0.31
14	lympho-CT	1	nd	HN1	1
15	RIL	2	reactive/normal	HN1	0.38
	RIL		probable metastasis	HN2	0.82
16	lympho-CT	1	certain metastasis	HN2	0.22
17	lympho-CT	1	reactive/normal	HN0	0.04
18	RIL	2	nd	HN0	0.02
	RIL		possible metastasis	HN1	0.1
19	lympho-CT	1	reactive/normal	HN0	0.02

20	lympho-CT	2	reactive/normal	HN0	0.01
	lympho-CT		reactive/normal	HN0	0.02
21	lympho-CT	1	possible metastasis	HN1	0.02
22	lympho-CT	2	reactive/normal	HN0	0.1
	lympho-CT		reactive/normal	HN2	0.02
23	lympho-CT	1	reactive/normal	HN0	0.03
24	RIL	4	reactive/normal	HN0	0.02
	RIL		reactive/normal	HN0	0.02
	RIL		reactive/normal	HN0	0.02
	RIL		reactive/normal	HN0	0.02
25	lympho-CT	4	certain metastasis	HN3	8
	lympho-CT		certain metastasis	HN3	11.1
	lympho-CT		certain metastasis	HN3	28.4
	lympho-CT		certain metastasis	HN3	33.1
26	lympho-CT	1	reactive/normal	HN0	0.04
27	lympho-CT	1	certain metastasis	HN3	9.6
28	RIL	2	nd	HN0	0.01
	RIL		reactive/normal	HN1	0.3
29	lympho-CT	2	nd	HN2	0.05
	lympho-CT		nd	HN3	2
30	lympho-CT	2	certain metastasis	HN3	2.2
	lympho-CT		certain metastasis	HN3	29.1
31	RIL	3	reactive/normal	HN0	0.01
	RIL		reactive/normal	HN1	0.1
	RIL		possible metastasis	HN1	0.47
32	lympho-CT	1	certain metastasis	HN3	18.35
33	lympho-CT	1	reactive/normal	HN0	0.4
34	RIL	3	nd	HN1	0.17
	RIL		nd	HN1	0.02
	RIL		certain metastasis	HN3	26
35	lympho-CT	1	reactive/normal	HN0	0.01
36	lympho-CT	2	reactive/normal	HN0	0.03
	lympho-CT		possible metastasis	HN1	0.03
37	lympho-CT	1	possible metastasis	HN2	0.39
38	RIL	2	nd	HN1	0.1
	RIL		nd	HN1	0.01
39	lympho-CT	1	certain metastasis	HN2	1.1
40	lympho-CT	1	reactive/normal	HN2	0.03
41	RIL	1	reactive/normal	HN0	0.02
42	lympho-CT	1	reactive/normal	HN0	0.09
43	lympho-CT	1	nd	HN1	0.05
44	lympho-CT	1	reactive/normal	HN1	0.01
45	lympho-CT	1	nd	HN1	0.12
46	lympho-CT	1	reactive/normal	HN1	0.13

47	RIL	1	reactive/normal	HN2	0.2
48	lympho-CT	1	reactive/normal	HN1	3.96
49	lympho-CT	2	certain metastasis	HN3	11.67
	lympho-CT		certain metastasis	HN3	5.2
50	RIL	1	probable metastasis	HN3	0.04
51	lympho-CT	3	probable metastasis	HN1	0
	lympho-CT		reactive/normal	HN1	0.04
	lympho-CT		reactive/normal	HN2	0.02
52	lympho-CT	2	certain metastasis	HN3	11.55
	lympho-CT		certain metastasis	HN2	0.25
53	RIL	1	reactive/normal	HN0	0
54	RIL	1	reactive/normal	HN0	0.02
55	lympho-CT	2	probable metastasis	HN1	0.04
	lympho-CT		probable metastasis	HN1	0.06
56	RIL	2	probable metastasis	HN3	1.17
	RIL		certain metastasis	HN3	13.7
57	lympho-CT	1	reactive/normal	HN1	0.02
58	lympho-CT	1	nd	HN1	0
59	lympho-CT	1	reactive/normal	HN0	0.03
60	lympho-CT	2	reactive/normal	HN0	0.05
	lympho-CT		reactive/normal	HN0	0.2
61	lympho-CT	1	nd	HN1	0.03
62	lympho-CT	1	reactive/normal	HN1	0
63	lympho-CT	1	reactive/normal	HN1	0.06
64	lympho-CT	2	reactive/normal	HN0	0
	lympho-CT		reactive/normal	HN0	0.1

Abbreviations: RIL, radiographic indirect lymphography; lympho-CT, computed tomography lymphography; SLN sentinel lymph node ;FC, flow cytometry; MC, mast cells; nd, unavailable data.

Figure legends

Figure 1. Kaplan-Meier curves of tumor-specific survival in dogs with MCTs, based on flow cytometry-detected nodal mast cell (MC) infiltration. Dogs with MC infiltration >4% (solid line) had a median TSS of 327 days, while those with infiltration ≤4% (dashed line) did not reach median survival ($P < 0.001$). Tick marks indicate censored observations.