**Utility of flow cytometry in canine primary cutaneous and matched nodal mast cell tumor**

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**Original Article**

# Utility of flow cytometry in canine primary cutaneous and matched nodal mast cell tumor

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## Abstract

Mast cell tumors (MCT) are among the most frequent tumors in dogs, but studies regarding mast cell immunophenotype are still lacking. The aim of this study was to assess the feasibility of flow cytometric analysis on MCTs, to describe canine MCTs immunophenotype and to evaluate the ability of flow cytometry to detect mast cells in lymph nodes. Thirty-four primary canine MCTs and 12 draining lymph nodes were evaluated. The expression of CD117, IgE, CD11b, CD18, CD44, CD34, CD25 and CD45 was evaluated. Distinct populations attributable to mast cells and eosinophils were recognized based on scatters and CD117 positivity. Common antigens (CD18, CD45, CD44) and CD117 were detected in all cases; positivity for IgE and CD11b was found in 28 (82%) and 23 (68%) cases respectively, while CD34 and CD25 were occasionally exposed. A single multicolor tube (IgE/CD117/CD11b/CD21&CD5) allowed the identification of mast cells in lymph nodes, showing a high correlation with cytology in quantifying mast cells infiltration. In conclusion, flow cytometric analysis can be applied to characterize canine MCTs and can be used to detect the presence of mast cells in lymph nodes. The immunophenotype abnormalities observed may be useful to confirm the neoplastic nature of such mast cells but the diagnostic usefulness of atypical antigen expression remains to be clarified.



*Keywords: Dog, Mast cell tumor, Lymph node, Flow cytometry*


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## Introduction

Mast cell tumors (MCT) are among the most frequent tumors in dogs, with an overall prevalence of 7-25% of all cutaneous neoplastic disorders (Brodey, 1970; Priester, 1973; Finnie and Bostock, 1979; Rothwell, et al., 1987; Misdrop, 2004).

When a MCT is suspected, the first diagnostic step usually includes the cytological evaluation of a fine needle aspiration biopsy (FNAB) of the cutaneous nodule, providing a diagnosis in 92-96% of cases (Blackwood et al., 2012). Clinical evaluation and staging of the MCT play a prominent role in determining prognosis and dictating therapy. Moreover, histological grading (Patnaik et al., 1984; Stefanelllo et al., 2015) and immunohistochemistry (IHC), including CD117 and Ki67 evaluation, provide important prognostic information (Kiupel et al., 2004; Preziosi et al., 2004; Scase et al., 2006).

In humans, neoplastic disorders of mast cells (MCs) are mostly represented by systemic mastocytosis, and the hallmark of neoplastic MCs is the aberrant expression of CD25 and often CD2. Thus, flow cytometry (FC) plays a major role in the diagnosis, enabling a reliable detection of MCs immunophenotype (Perbellini et al., 2011; Teodosio et al., 2014; Jabbar et al., 2014; Pozdnyakova et al., 2015).

While FC is emerging as a routine technique for the diagnosis of hematopoietic neoplasms in dogs and humans, only few studies have adopted FC to characterize canine MCs. Notably, all the aforementioned studies are based on cultured cells obtained from normal bone marrow CD34 (+) positive cells (Lin et al., 2006), effusions associated with systemic

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mastocytosis (Lin et al., 2009) or normal cutaneous MCs (Kawarai et al., 2010). To the best of the authors' knowledge, studies focusing on FC analysis of non-cultured cells deriving from spontaneous MCTs are lacking. Since MCTs are composed by round and isolated cells, it was hypothesized that canine MCT is suitable for flow cytometric analysis. Furthermore, FC may be particularly appealing due to its affordable costs in relation to the number of detectable markers, the multiparametric analysis, the ability to analyze a wider number of cells and the shorter time to provide results compared with histology.

The aims of the present prospective study were: 1) to evaluate the feasibility of FC analysis on FNABs obtained from canine MCTs 2) to describe the immunophenotype of neoplastic MCs and 3) to identify and quantify MCs in lymph nodes (LNs).

## Materials and methods

*Cases and sample collection.*

Dogs with a previous cytological diagnosis of MCTs and a planned surgical removal of the mass were considered eligible for the study. At the time of surgery, both FNAB and a neoplastic tissue fragment were placed in two separate tubes containing 1ml of RPMI medium enriched with fetal calf serum and sodium azide, while the remaining part of the mass was placed in formalin for the histopathological investigations. Whenever possible, a FNAB from the draining LN was obtained and placed in a third tube and another FNAB was used to produce a smear for cytological evaluation. The samples were sent to the laboratory of the Department of Veterinary Sciences of the University of Turin and processed within 24h from collection. Cases were included in the study if the cytological diagnosis of MCT was confirmed by histology. No exclusions were made based on the anatomic site of origin of the MCTs (cutaneous or

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subcutaneous); however, MCTs had to have the longest diameter of at least 1 cm. All dogs were client-owned and subjected to surgery for diagnostic and therapeutic purposes with the informed consent of the owners. The study did not involve any additional invasive samplings. Thus, a formal approval of the Institution Committee for Animal Care of the University of Turin was not necessary.

*Flow cytometric analysis*

Cell count was performed in all MCTs suspensions using a BD Accury C6 flow cytometer (Becton Dickinson, San Josè, CA). Samples were previously diluted 1:3 with RBC lysing buffer and a morphological gate was applied to exclude debris/background. A minimum of 30 cells/µl was required to consider each sample suitable for the analysis. In case of a cell count between 30/µl and 100/µl, scraping of the piece of the tumor mass with a surgical blade was performed in order to obtain a higher cell concentration. Samples were placed in 3 different tubes and investigated for antigen surface exposition: tube 1 (IgE/CD117/CD11b/CD18); tube 2 (CD44/CD34/CD45) and tube 3 (CD25) (Tab.1). The standard protocol for direct labeling of surface antigens (Riondato et al., 2016) was modified in order to reduce the loss of cells during the washing steps. A previously titred quantity of each antibody was added to fifty microliters of the cell suspension. Titration of the antibodies was carried out on pilot MCT samples (not included in the study) used to optimize procedures and protocols. After incubation for 30 minutes in the dark at 4°C, a 5 minutes RBC lysing step (ammonium chloride lysis buffer) was performed. The sample was then centrifuged, the supernatant discarded, the pellet washed once and suspended in 150µL of phosphate buffered saline. A minimum of 3.000 events in the intact cell gate were acquired with a BD Accury C6 Cytometer (Becton Dickinson, San Josè, CA). The

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intact cell gate was designed on PI-negative events and scatter properties excluding debris and events smaller than a lymphocyte. Analyses were performed with CFlow Plus software (Becton Dickinson, San Josè, CA) activating the described gate.

Lymphocytes were identified as small CD117 (-) negative, CD11b (-) negative, CD18 (+) positive events; MCs and eosinophils were discriminated based on CD117 expression and scatter properties according to the evidences of the sorting experiment reported in the specific paragraph in the Results section. These features were used to design population-specific gates within the previously described intact cell gate. (Fig.1)

Normal eosinophils and lymphocytes were used as internal negative control for CD117 and CD11b respectively. Isotype controls were used for CD34, CD25, CD18, CD44 and CD45. MCs and eosinophils were recorded as positive for each antigen if more than 20% of the events in the respective gate were positive. An example of CD11b analysis is reported in Fig.1.

Labeling for CD18, CD44, CD25 and CD45 was missing in some cases (Tab.2).

The immunophenotype was described in both MCs and eosinophils within each sample. Moreover, the percentage of both populations within the intact cell gate was calculated for each case.

The following multicolor tube was designed for the identification and quantification of MCs percentage in LNs: IgE/CD117/CD11b/CD21&CD5 (fluorochromes are reported in Tab.1). MCs were recognized as CD117 (+) positive, CD21 (-) negative, CD5 (-) negative events showing the same IgE and CD11b pattern observed in the tumor mass. The gating strategy was confirmed artificially by adding mast cells into a reactive lymph node sample (Fig.2).

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*Cytological evaluation*

Lymph node smears were stained with May Grunwald Giemsa (MGG). One cytologist, who was blinded regarding FC data, evaluated all slides. Lymph node infiltration was calculated as the percentage of MCs out of 1000 total intact cells and classified according to the criteria by Krick et al. (2009) into the following categories: reactive lymphoid hyperplasia, probable metastasis, possible metastasis, certain metastasis.

*Histopathology*

The primary cutaneous MCTs were graded according to the Patnaik (Patnaik et al., 1984) and Kiupel systems (Kiupel et al., 2011), whereas the subcutaneous MCTs were classified according to Thompson (Thompson et al., 2011). The draining LNs, if surgically removed, were graded according to Weishaar criteria (Weishaar et al., 2014) as follows: HN0 (non-metastatic), HN1 (pre-metastatic), HN2 (early metastasis), and HN3 (overt metastasis). Histopathological evaluation of MCTs and lymph nodes was performed by two different pathologists.

*Statistical analysis*

Correlation between cytology and FC in quantifying MCs infiltration in LNs was determined using the Pearson correlation coefficient. Comparison of the eosinophils percentage between IgE (+) positive and IgE (-) negative MCTs was performed using Wilcoxon-Mann-Whitney test. The association between unusual immunophenotypes and Kiupel/Patnaik grade was evaluated through Chi-square test. Significance level was set at p=0.05 for all tests.

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## Results

Thirty-eight MCTs were collected. Four samples were considered unsuitable for FC analysis because of a cellularity < 30/µl and a substantial absence of intact PI-negative events. Scraping of the tumor mass was needed only in one case. In total, 34 MCTs were available for the analysis: 29 cutaneous and 5 subcutaneous.

A minimum of 5000 total cells with a minimum of 1500 MCs were analyzed in 30 cases; 4 cases had a minimum of 3000 total cells and/or 1000 MCs.

According to Patnaik, 22 out of 29 cutaneous MCTs were classified as grade II, and 7 out of 29 were classified as grade III. According to Kiupel, 22 MCTs were low-grade, and 7 were high-grade. All Patnaik grade II MCTs were Kiupel low-grade, and all Patnaik grade III MCTs were Kiupel high-grade.

According to Thompson, 3 subcutaneous MCTs were classified as infiltrative and 2 as circumscribed; all were cytologically well differentiated.

*Mast cells and eosinophils identification*

We preliminary evaluated a set of MCTs to optimize cell collection and to titre monoclonal antibodies. During this pilot study two main populations characterized by different scatter properties and CD117 exposure were observed. We hypothesized CD117 (+) positive events to be MCs and CD117 (-) negative events to be eosinophils. The two populations were sorted with a BD FACSAria III cell sorter (Becton Dickinson), and MGG-stained cytospins of each purified population was evaluated confirming our hypothesis. (Fig.3)

These results lead to the definition of the two specific gates of analysis for MCs and eosinophils. (Fig.1)

*Immunophenotype of MCT populations and eosinophilic infiltration*

Mast cells were always positive for CD117, CD18, CD45 and CD44, while positivity for IgE and CD11b was observed in 28 (82.35%) and 23 (67.64%) cases, respectively. A significant association (p=0.018) between CD11b expression and Kiupel/Patnaik grade was detected: low-grade MCTs represented 90% of CD11b (+) positive cases and only 50% of CD11b (-) negative cases. Four out of 29 MCTs (13.9%) were CD34 (+) positive and one out of 17 (5.8%) was CD25 (+) positive. Eosinophils were always positive for CD11b, CD18, CD45, CD44 and negative for CD117, CD34, CD25 and IgE (Tab. 2). More than 90% of both MCs and eosinophils within the respective gates were antigen-positive or antigen-negative in most of the cases; cases showing positivity between 20% and 90% are reported in Tab.2.

Median percentage of eosinophils was 27% (range 0-65%), with a significant difference (p=0.03) between IgE (-) negative (median = 8%; range 0-51%) and IgE (+) positive (median= 30%; range 2-65%) MCTs. Morphological evaluation of IgE (-) negative MCTs revealed the presence of larger and fewer granules compared with IgE (+) positive cases (Fig.4). In particular, these features were presented by the majority of mast cells in 5 out of 6 IgE (-) negative cases. In the remaining case, MCs with fewer and larger granules were estimated to be 40%. Conversely, they were always < 20% in IgE (+) positive MCTs.

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*Lymph nodes results*

A lymph node cell suspension was available for 16 cases. They were all suitable for FC analysis, but 4 cases were excluded because the smear was considered inappropriate for cytological evaluation. Cytologically, mast cells were detected in all cases. According to Krick et al. (2009) there were 3 reactive lymphoid hyperplasia, 2 possible metastasis, 3 probable metastasis and 4 certain metastasis.

Six dogs underwent lymphadenectomy; according to Weishaar, there were 1 pre-metastatic and 5 metastatic lymph nodes. A high correlation was observed between FC and cytology in quantifying the percentage of MCs in the LNs ($r=0.989$, $p < 0.01$). Percentages of single cases and Weishaar classification are reported in Table 3 and examples of high and low infiltration are illustrated in Fig. 5.

**Discussion**

Despite the high prevalence and the isolated cell nature of canine MCTs, studies focusing on flow cytometric analysis are lacking.

The experience gained with this study demonstrates that FC can be performed on canine MCTs. Fine needle aspiration biopsy provided samples of good quality in most cases, leading to the consideration that the analysis could be run together with cytology in a diagnostic setting.
The most common immunophenotype of MCs in this case series was CD117, IgE, CD11b, CD44, CD45, CD18 (+) positive and CD34, CD25 (-) negative.

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CD117 is considered as a unique marker for MCs in human medicine (Orfao et al., 1996; Escribano et al., 1998; Escribano et al., 2004) and has been previously described in cultured canine MCs derived from both normal and neoplastic cells (Lin et al., 2006; Lin et al., 2009; Kawarai et al., 2010). Our results confirm that CD117 is a reliable marker for canine mast cells *in vivo*.

As expected, IgE appeared as a second marker characteristic for MCs. It is well known that IgE and its specific receptor (FcεRI) on MCs have a great reciprocal affinity (Tizard, 2004). FcεRI and IgE are usually bound, even in the absence of the antigen (Coico and Sunshine, 2009), and the presence of the FcεRI on the MCs surface ensures that MCs are constantly coated with IgE (Tizard, 2004).

Surprisingly, six IgE-negative cases were recorded. The absence or low concentration of IgE has previously been described in canine cultured MCs (Brazis et al., 2002). One of the hypotheses for the absence of IgE is the immaturity of these cells. Indeed, FcεRI is not usually presented on MCs in the early phases of development (Dahlin and Hallgren, 2015). The low number of granules and their bigger size presented by MCs in IgE (-) negative cases in this series corroborates this hypothesis, even though CD34 was expressed only by one out of five of these cases. In fact, the atypical morphological features detected have been linked to immature cells in humans (Georgin-Lavialle et al., 2013).

A second hypothesis is the loss of FcεRI due to genetic mutations (Sundstorm et al., 2003) or its downregulation in response to low IgE levels. In fact, both *in vivo* and *in vitro* levels

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of FcεRI surface expression on MCs positively correlates with the concentration of IgE in serum and supernatant, respectively (Metz et al., 2013). Moreover, one study hypothesized that culturing MCs without IgE-rich serum would lead to the loss of IgE receptors (Brazis et al., 2002). The determination of IgE serum concentration in future cases will help in checking this hypothesis.

In addition, the IgE-negative cases showed a significantly lower eosinophilic infiltration compared with the IgE-positive cases. This is not unexpected because eosinophils are recruited by the degranulation of MCs (Day, 2014) and one of the main mechanisms of MCs degranulation is the crosslinking of IgE. Further analyses are needed to evaluate if the lack of IgE is due to the absence or downregulation of the specific receptor FcεRI.

Fourteen percent of MCTs was CD34 (+) positive in this study. CD34 is a known marker of progenitor cells and it is not expressed on mature cells in peripheral tissues with the exception of endothelial cells. Considering the above, CD34 is used in the diagnosis of acute leukemia both in dogs and humans (Vernau and Moore, 1999; Be'ne et al., 2011). Similarly, its expression on mature MCs in peripheral tissues represents aberrancy and might therefore be used as a marker of malignancy. A comparative analysis of normal mature MCs in canine peripheral tissues is mandatory to confirm this hypothesis.

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CD25 expression was detected only in one MCT. This result disagrees with the findings in human mastocytosis and with a study in veterinary medicine in which most of the MCTs were CD25-positive on immunohistochemistry (Meyer et al., 2012). This discrepancy may be due to

the different technique and CD25 clone utilized. Furthermore, only 50% of cases were tested and only superficial expression of CD25 was investigated in this study, and an intracytoplasmic expression cannot be excluded.

The expression of CD11b was significantly associated with Kiupel low-grade and Patnaik grade II classification, but the sensibility was not high. No other associations were observed between aberrant immunophenotypic features with neither Kiupel/Patnaik classification for MCTs or Weishaar classification for LNs. The limited number of cases and the fact that the histologic classification was performed by two different pathologists represent a limitation of the study potentially affecting these results. Thus, further investigations are needed to confirm these findings.

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The multicolor tube adopted for the analysis of LNs allowed an easy identification of MCs and the FC results showed a good correlation with the cytological quantification. Therefore, flow cytometry is able to identify and quantify MCs in LNs. Nevertheless, there was no evident association between the degree of infiltration of LNs and the Weishaar classification. Most likely this is attributable to the low number of cases undergoing lymphadenectomy and subsequent histopathological node classification. On the contrary, higher flow cytometric percentages of MCs were associated to a higher probability of metastasis according to Krick (2009). Further studies are needed to confirm this finding and to evaluate the possible role of FC in detecting nodal metastases.

Similarly to cytology and histopathology, the major limitation of the flow cytometric approach is the inability to assess the neoplastic nature of the detected MCs. Anyway, the

immunophenotypic abnormalities showed by MCs in the primary mass could be used as a surrogate marker of neoplastic infiltration if detected in the corresponding draining LN. Immunophenotypic description of non-neoplastic non cultured canine MCs is needed to validate this assumption.

## Conclusions

To the best of the authors' knowledge this is the first study where FC is used to describe neoplastic non cultured MCs. The results demonstrate that flow cytometric analysis of canine MCTs is feasible in a routine diagnostic setting. The description of the antigenic pattern of MCs in the mass could be a valuable tool to detect the infiltration of tissues. Identification and quantification of MCs in LNs can be performed by FC, and a further prospective study is currently ongoing to assess a possible correlation between cytology, histopathology and FC results. Furthermore, immunophenotypic abnormalities may be useful to confirm the neoplastic nature of MCs detected in tissues such as liver and spleen.

Further studies are warranted to better define the association between immunophenotype and histopathology, metastatic potential or propensity to recur and their possible role in the prognostic assessment.

**Conflicts of interest:** None

## Acknowledgements

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**Table 1**

List of the antibodies used for the study

| Antibody | Source | Clone | Quantity (ul/tube) | Conjugated |
|---|---|---|---|---|
| **IgE** | Bio-Rad | Polyclonal | 1 | FITC |
| **CD117** | BD pharmingen | ACK45 | 0.5 | PE |
| **CD11b** | Abcam | M1/70 | 0.5 | PE/Cy5 |
| **CD18** | Bio-Rad | CA1.4E9 | 1 | AlexaFluor647 |
| **CD44** | Thermo-Fisher | IM7 | 1 | FITC |
| **CD45** | ABD-Serotec | YKIX716.13 | 1 | AlexaFluor647 |
| **CD34** | BD Pharmingen | 1H6 | 1 | PE |
| **CD25** | E-Bioscience | P4A10 | 5 | PE |
| **CD21** | Bio-Rad | CA2.1D6 | 0.5 | AlexaFluor647 |
| **CD5** | Bio-Rad | YKIX322.3 | 1 | AlexaFluor647 |

**Comment [UdMO37]:** Tab.1 of YTVJL-D-17-00795
Deleted – 1st column (Tube)

**Comment [UdMO38]:** Tab.1 of YTVJL-D-17-00795
Deleted – AlexaFluor488

**Table 2**

Neoplastic mast cells immunophenotype, eosinophilic infiltration and Kiupel/Patnaik grade.

| | | Mast Cell Imunophenotype | | | | | | | | Kiupel Grade | Patnaik Grade |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cases** | **Eos %** | **CD117** | **IgE** | **CD11b** | **CD18** | **CD45** | **CD44** | **CD25** | **CD34** | | |
| **MCT01** | 23 | + | + | +* | + | + | + | - | +* | SC | SC |
| **MCT02** | 30 | + | + | +* | +* | + | + | NA | - | Low | II |
| **MCT03** | 40 | + | + | + | + | + | + | - | - | Low | II |
| **MCT04** | 10 | + | - | +* | NA | + | + | - | - | High | III |
| **MCT05** | 42 | + | + | + | + | + | + | NA | - | Low | II |
| **MCT06** | 27 | + | + | + | + | + | + | - | - | SC | SC |
| **MCT07** | 65 | + | + | +* | + | + | + | - | - | Low | II |
| **MCT08** | 50 | + | + | +* | + | +* | + | - | - | Low | II |
| **MCT09** | 50 | + | + | +* | + | + | + | NA | NA | SC | SC |
| **MCT10** | 7 | + | - | - | + | + | + | - | + | Low | II |
| **MCT11** | 51 | + | - | +* | + | + | + | - | - | Low | II |
| **MCT12** | 45 | + | + | +* | + | NA | NA | NA | NA | Low | II |
| **MCT13** | 25 | + | + | - | NA | NA | NA | NA | NA | High | III |
| **MCT14** | 4 | + | - | - | + | + | + | + | - | Low | II |
| **MCT15** | 10 | + | + | + | + | + | + | - | - | Low | II |
| **MCT16** | 45 | + | + | - | +* | + | + | NA | - | High | III |
| **MCT17** | 17 | + | - | - | + | +* | + | NA | NA | High | III |
| **MCT18** | 25 | + | + | + | + | + | + | NA | NA | Low | II |
| **MCT19** | 60 | + | + | +* | +* | + | + | NA | - | Low | II |
| **MCT20** | 23 | + | + | + | + | + | + | NA | - | Low | II |
| **MCT21** | 7 | + | +* | +* | + | + | + | NA | - | Low | II |
| **MCT22** | 26 | + | + | - | +* | + | + | NA | - | Low | II |
| **MCT23** | 45 | + | + | +* | +* | + | + | NA | - | Low | II |
| **MCT24** | 60 | + | + | + | + | + | + | NA | - | High | III |
| **MCT25** | 7 | + | + | - | NA | + | NA | NA | - | High | III |
| **MCT26** | 30 | + | + | +* | NA | + | +* | NA | - | Low | II |
| **MCT27** | 2 | + | + | - | NA | + | + | NA | + | Low | II |
| **MCT28** | 30 | + | + | - | + | +* | + | - | + | SC | SC |
| **MCT29** | 0 | + | - | - | + | + | + | - | - | Low | II |
| **MCT30** | 21 | + | + | +* | + | + | + | - | - | SC | SC |
| **MCT31** | 15 | + | + | + | + | + | + | - | - | Low | II |
| **MCT32** | 16 | + | + | - | + | + | + | - | - | High | III |
| **MCT33** | 35 | + | + | +* | + | + | + | - | - | Low | II |
| **MCT34** | 59 | + | + | + | + | + | + | - | - | Low | II |

(+) positive; (-) negative; SC - Sub Cutaneous; NA – Not analyzed

* positive events within the mast cells gate of analysis <90%.

**Table 3**

Percentage of mast cells in lymph nodes detected by means of cytology and flow cytometry. Nodal classification according to Weishaar et al. (2014) and Krick et al. (2009) is reported.

Comment [UdMO39]: Line 432-433 of YTVJL-D-17-00795
Deleted – and Weishaar classification

| Sample | Cytology (%) | Flow cytometry (%) | Weishaar Classification | Krick Classification |
|---|---|---|---|---|
| 1 | 0 | 0.2 | HN3 | Reactive Lymphoid Hyperplasia |
| 2 | 0 | 5 | HN2 | Reactive Lymphoid Hyperplasia |
| 3 | 0.2 | 0.1 | HN2 | Reactive Lymphoid Hyperplasia |
| 4 | 1.6 | 0.6 | | Probable Metastasis |
| 5 | 2 | 0.4 | | Possible Metastasis |
| 6 | 2 | 1.8 | HN1 | Probable Metastasis |
| 7 | 5.5 | 8.9 | HN2 | Certain Metastasis |
| 8 | 6 | 3.6 | | Possible Metastasis |
| 9 | 7.7 | 2.5 | HN2 | Probable Metastasis |
| 10 | 35.2 | 42.4 | | Certain Metastasis |
| 11 | 51.5 | 68.1 | | Certain Metastasis |
| 12 | 88.8 | 83.1 | | Certain Metastasis |

**Figure legends**

Fig.1. Gating strategy. A: an "intact cell gate" was created on FSC vs SSC plot (gate P1); B: within gate P1, specific gates for mast cells (R1), eosinophils (P2) and lymphocytes (P3) were depicted on FSC vs CD117-PE; C: morphological gates for mast cells (P5) and eosinophils (P4) were created around the colored events corresponding to populations in gates R1 and P2. Antigen expression analysis on mast cells and eosinophils was carried out activating gates R1 and P2, respectively, in tube 1 and gates P4 and P5, respectively, in both tubes 2 and 3. An example of CD11b analysis is reported in plots D-F: the marker for discriminating negative and positive events was set on lymphocytes within gate P3 (D); percentage of positive mast cells (E) and eosinophils (F) was calculated activating gates R1 and P2, respectively.

Fig.2. Artificial nodal mast cells infiltration. Scatter characteristics (left) and immunophenotypic features (right) of the population of a reactive lymph node before (A) and after (B) the addition of mast cells suspension from a MCT. Q2-UR quadrant was set based on the presentation of mast cells in the MCT aspirate. IgE (+) positive CD117 (-) negative cells in Q2-LL quadrant are B lymphocytes.

Fig.3. Mast cells and eosinophils identification. Scatters properties and IgE vs CD117 exposure of the two main populations in one MCT sample (A and B, respectively). The two populations were sorted with a BD FACSAriaIII cell sorter. Cytospins of unsorted and sorted populations were prepared and stained with MGG. C: cytospin of the population prior to sorting; both MCs and eosinophils are present. D: cytospin of sorted CD117 (+) positive events (B: gate R1); only mast cells are recognizable. E: cytospin of sorted CD117 (-) negative events (B: gate R2); only eosinophils are detected. Original magnification 600x. May-Grumwald Giemsa stain.

Fig.4. Morphological features of mast cells. A: IgE (+) positive MCT showing cells with a high number of small sized granules. B: IgE (-) negative MCT showing a low number of larger granules. Original magnification 1000x. May-Grumwald Giemsa stain.

**Comment [UdMO40]:** Line 438 of YTVJL-D-17-00795 Deleted – 1

**Comment [UdMO41]:** Line 438 of YTVJL-D-17-00795 Deleted – MCTs

**Comment [UdMO42]:** Line 439 of YTVJL-D-17-00795 Deleted – MCTs

Fig.5. Evaluation of mast cells infiltration in lymph nodes. A: lymph node showing low infiltration both at flow cytometric (left, 3.6%) and cytological evaluation (right, 6%). B: lymph node showing high infiltration both at flow cytometric (left, 83.1%) and cytological evaluation (right, 89%). Cytology: original magnification 400x; May-Grumwald Giemsa stain. Flow cytometry: mast cells are detected as events expressing the same phenotype as in the tumor mass (A: CD117 (+) positive, IgE (+) positive; B: CD117 (+) positive, IgE (-) negative). The populations in the lower-left quadrant are eosinophils (1), B-lymphocytes (2) and T-lymphocytes (3).

**Comment [UdMO43]:** Line 441 of YTVJL-D-17-00795
Deleted – 2

**Comment [UdMO44]:** Line 441-444 of YTVJL-D-17-00795
Deleted – Flow cytometric analysis (left) and cytological presentation (right; original magnification 400x) of two lymph nodes showing low (A: 6%) and high (B: 89%) infiltration at the cytological evaluation. Mast cells are detected as CD117+IgE+ (A: Q8-UR quadrant) and CD117+ IgE- (B: Q8-UL quadrant) events.

**Figure 1**

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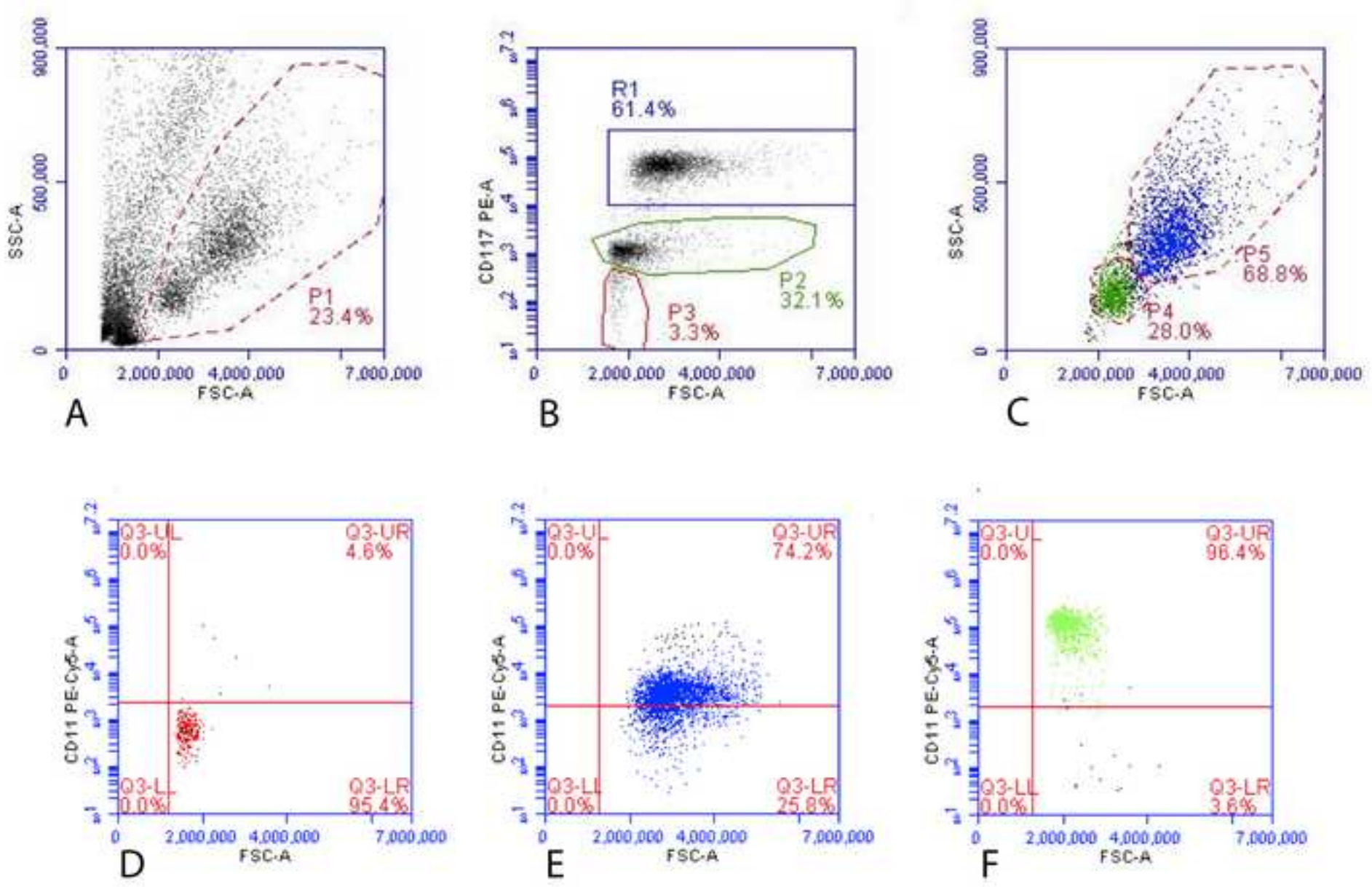

**Figure 2**

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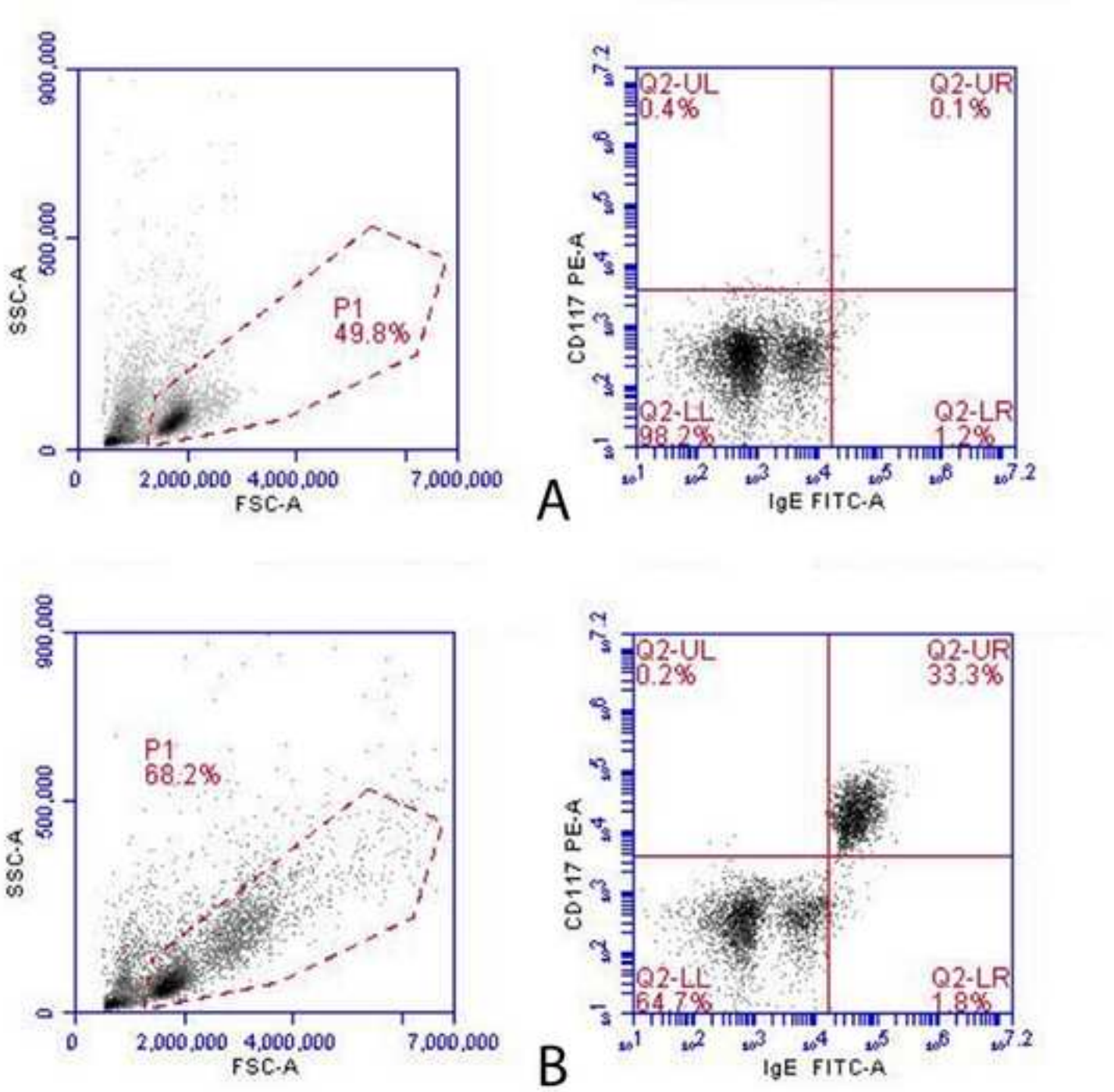

**Figure 3**

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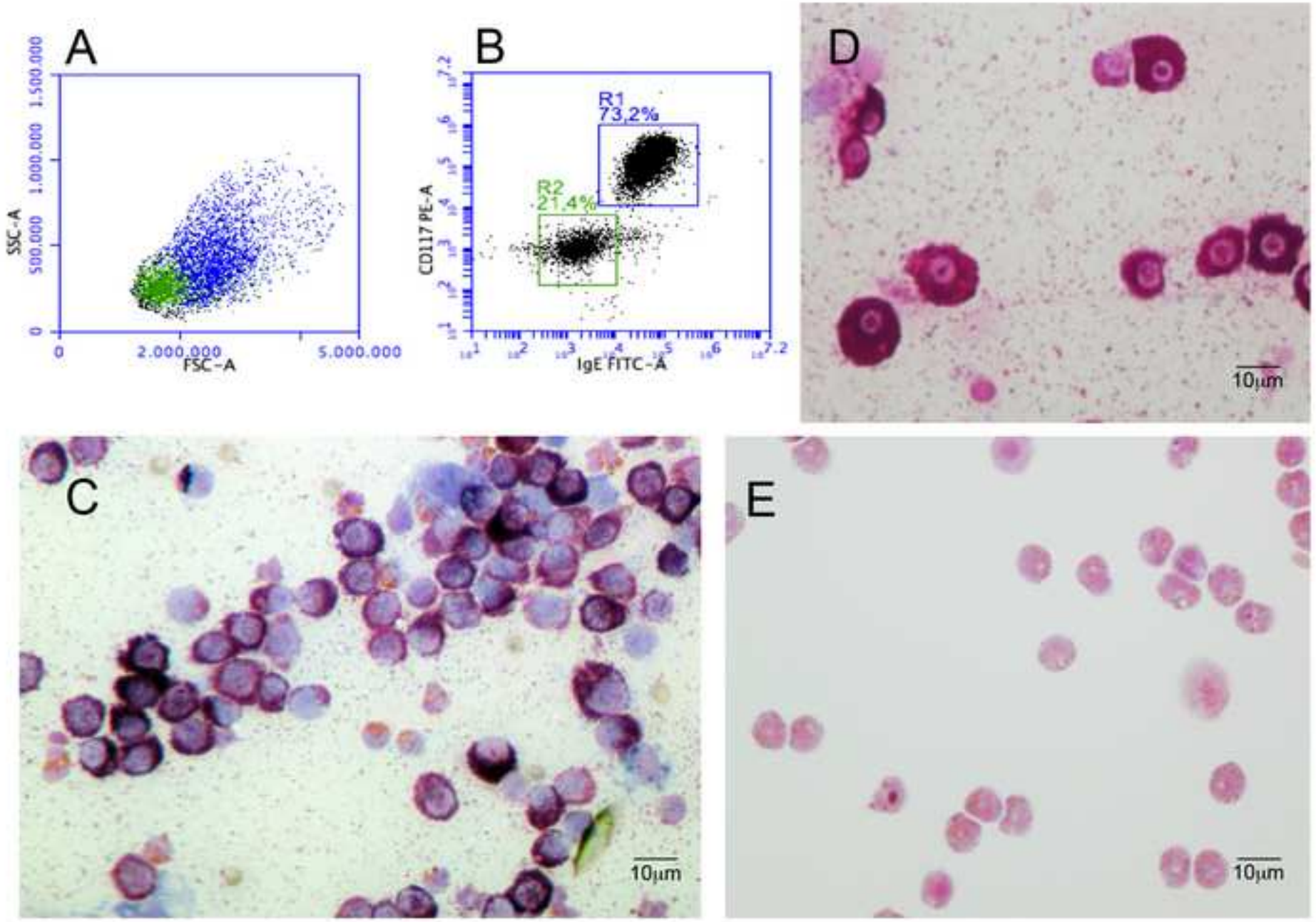

**Figure 4**

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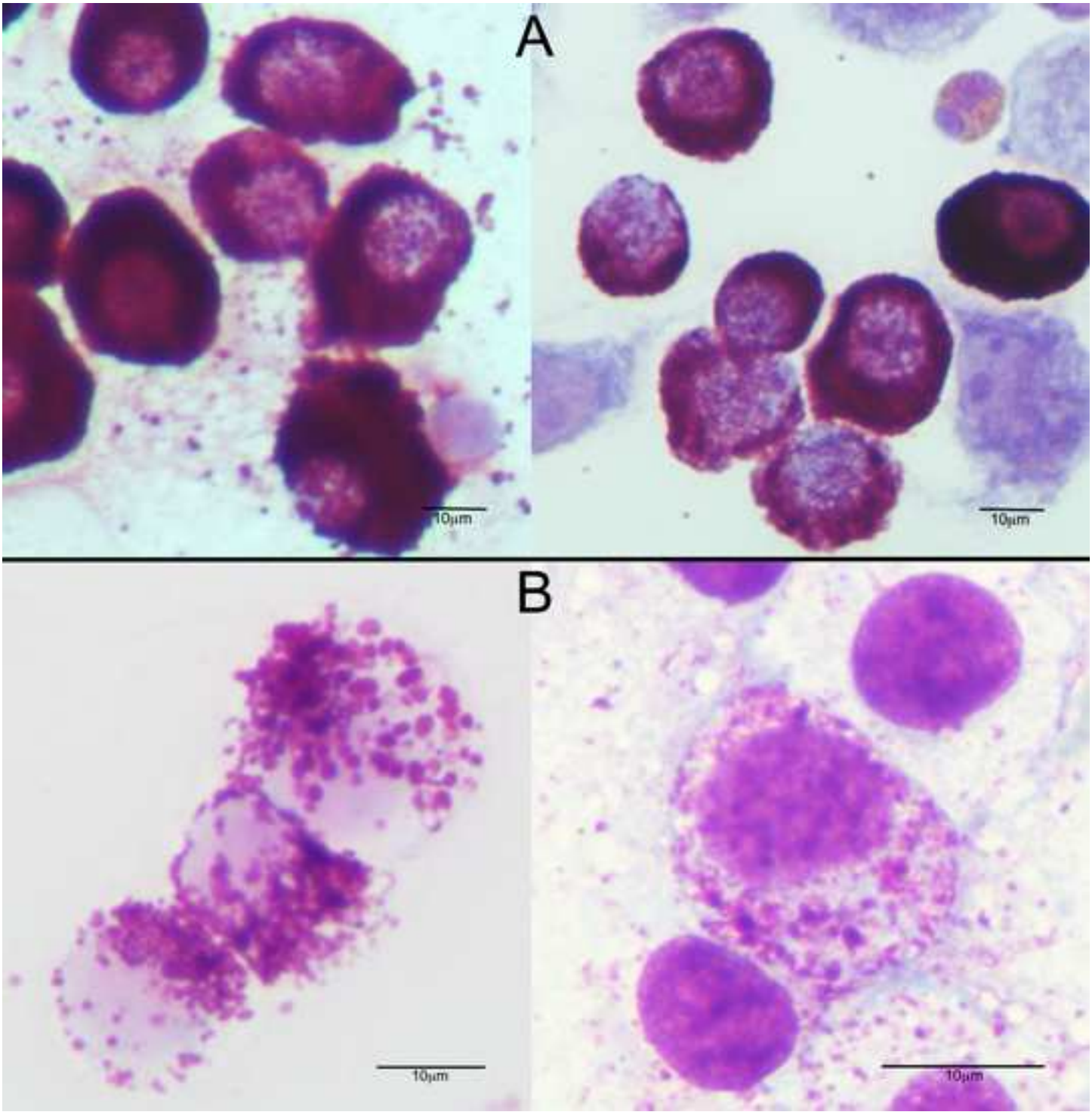

**Figure 5**

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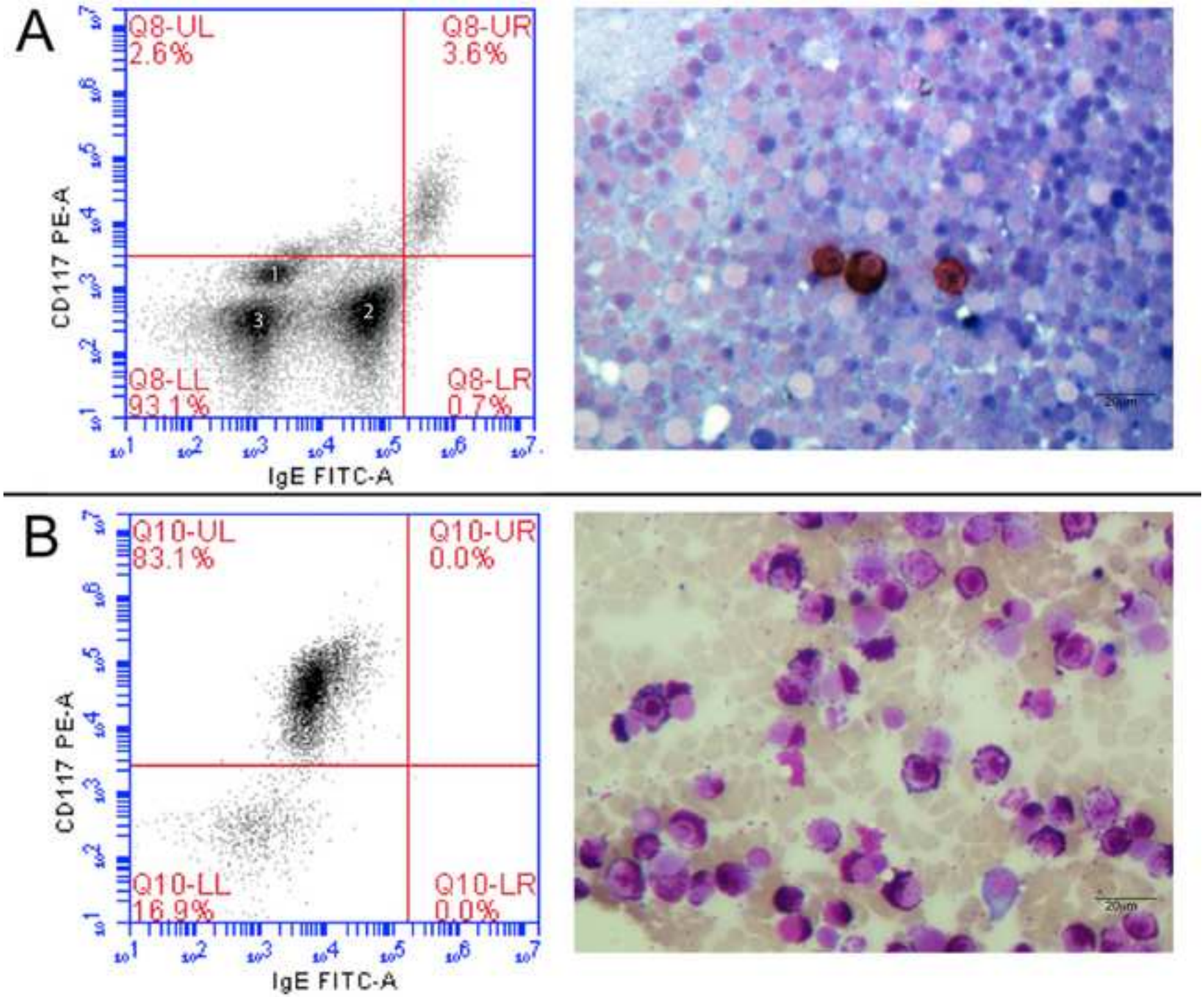


***Highlights (for review)**

Flow Cytometric analysis of canine mast cell tumors can be run in routine diagnostic settings

Mast cells can be detected in lymph nodes by flow cytometry

Immunophenotypic features are useful to detect mast cells in tissues

*Revision Note

Dear Editor,
each comment has been identified with a letter or a number. Answers to each specific comment or question have been provided using red type. Line numbers in the answers refer to the current lines in the revised manuscript
best regards,
Fulvio Riondato, on behalf of the authors

**Reviewer #1:**

General:
The manuscript presented for review is an original article of identifying and immunophenotyping mast cells, which were aspirated from cutaneous and subcutaneous mast cell tumors from dogs. An additional goal of the article is to use the flow cytometry to identify the presence of mast cells in lymph nodes that are draining the primary mass.

The goal of this article is well described and the scientific idea is significantly important, mainly when considering the possible clinical diagnostic utility. In addition a lot of work has put into this study with logical thinking. However, my main concern is that the data as provided is lacking to prove that the cells characterized and identified by the flow cytometry are specifically the mast cells. In addition, I do not quite agree that the data presented is convincing enough to conclude that "flow cytometry can be used to detect the presence of mast cells in lymph nodes", and in my opinion more data needs to be provided before such an unambiguous statement can be presented.
AUTHORS: The manuscript has been integrated with additional data. Our conclusions are now more robust. Please, see answers to specific comments

The main section that needs to be better described and include more detail with major corrections is the flow cytometric analysis in the material and methods
Done following indications of specific comments

Note: the highlights and abstract should also be revised according to the corrections suggested in the body of the manuscript

Specific comments:

<u>Introduction:</u>

A. Line 34: Please change "mast cell tumor is"… to mast cell tumors are, with adjusting the following sentence accordingly as mast cell is not one specific tumor, but a group of tumors.

Done (line 33). We changed the abstract accordingly (line 15). Thank you for the suggested clarification.

B. Line 40: the paper Dodd et al is cited but not found in the references.

It was a refusal and has been deleted. Thank you for catching this up

C. Line 43-44: Can the author provide a reference for the statement: "immunohistochemistry… provide important prognostic information"

References have been added

D. Line 47: Please change from "aberrant expression of CD25 and CD2" to "aberrant expression of CD25 and often CD2", as not all neoplastic mast cells in humane express CD2.

Done. Thank you for the suggested clarification.

E. Line 59-61: Could the author rephrase the advantages of using a flow cytometry as a tool for characterizing and diagnosis of mast cell tumors. I do believe flow has unique abilities, however, I less agree that this is a low cost test nor less operator dependency. In my opinion a significant challenge we deal with in flow cytometry is gaining the knowledge to choose the correct panel and interpreting the data reliably, both which are dependent on the operator.

We agree with the reviewer. Thank you for your comment. We rephrased as follows: "Furthermore, FC may be particularly appealing due to its affordable costs in relation to the number of detectable markers, the multiparametric analysis, the ability to analyze a wider number of cells and the shorter time to provide results compared with histology" (Line 58-61)

<u>Material and Methods:</u>

Flow cytometry analysis:

I find this section very confusing and requires attention, as previously mentioned. The core of the study is based on the results and interpretation of the flow cytometry data. Thus, in my opinion, the data as provided is lacking to prove the hypothesizes of this study.

A. Line 89-90: "The standard protocol for direct… briefly…" is not adequate for describing the method. Could the author please describe all the steps taken, for running the sample through the flow cytometry analyzer? This section was well described in the cited paper however the methods used in this study should be independently described.

The paragraph has been modified adding the requested information and according to the following comments n.1, 2 and 3

The following comments/questions are a guide of more specific information that needs to be provided in this section:

1. I am not familiar with the step of counting cells/ ul. Though I can understand the logic, did you differentiate counting nucleated cells from other cells? How did you exclude the possibility that the counted cells are not RBC?
We used the same approach that we routinely apply for lymphomas. We run a tube with an aliquot of cell suspension diluted 1:3 with lysis buffer in order to get a first indication on cellularity, quality of the sample and scatter characteristics. The ratio is sufficient, because usually blood contamination is absent or very limited. Our cytometer gives direct absolute count of total or gated events. The counted cells cannot be RBC or debris/background because of the lysing step and the application of a morphological gate. Based on our experience with lymph nodes, we arbitrarily set the two thresholds reported in the text (30 – 100 cells/uL) in order to maximize the number of reliable samples. The text has been integrated with this information (Line 86-87)

2. How was the titer quantity of the antibody (as described in table 1) chosen, with mast cells samples?
We titred the antibodies using the pilot MCT cases. We routinely use most of the described antibodies in the panels for the characterization of lymphomas and leukemias. Thus, they were already titred to be used in lymph node aspirates, peripheral blood, bone marrow, spleen and liver FNABs, effusions and (sub)cutaneous masses. We preliminarily titred the antibodies on MCTs that were not included in the current study starting from the concentrations used for lymph nodes and peripheral blood. A reference to these pilot MCT samples has been added (Line 95-96).

3. Why was the RBC lysis step taken after incubating the cells with the antibodies and not prior to that?
MCT aspirates often have a low cellularity and we need to save some cells to carry out different analyses that were not included in the present study (ongoing research). Thus, we decided to run the lysis step after incubating the cells with the antibodies in order to reduce the loss of cells during the washing steps (centrifugation). MCT samples are always free of erythrocytes or have a very low contamination. Hence, we tested the possibility to use the lysis buffer in place of PBS to wash the unbound antibody and simultaneously obtaining the lysis of possibly present RBCs.

4. Was the suspended cells washed only once, and if so how did you evaluate that it is sufficient?
We performed only 1 washing step in order to save cells (see answer to comment n.3), since we compared 1 vs 2 washings when standardizing the method and we found that no changes were observed.

5. Could the author provide more information regarding how the cells were identified by the flow cytometry? Presenting the SSC vs FSC dot plot and SSC vs CD117 can be very helpful to better understand this process. In addition it was not mentioned if gating excluded cell debris and smaller cells.
We added a specific paragraph (providing images) in the Results ("Mast cells and eosinophils identification"). Here we report the sorting experiment we did in order to identify the two main

populations (mast cells and eosinophils) and leading to the depiction of the specific gates of analysis.
We refer to this new paragraph in the Material & Methods (lines 10106-107). Furthermore, we added a figure explaining the gating strategy (Fig.1).

6. Line 34: "A minimum of 3,000 events"... it seems a bit low mainly as it doesn't clarify how many of these events were CD117 positive (considered to be mast cells)
We actually agree that a minimum of 3,000 events is a bit low, but we set this threshold in order to save samples with low cellularity. According to our experience, in the analysis of other types of samples it is sufficient to get reliable results. Anyway, the total analyzed cells were > 5000 in all MCTs but 2 cases and MCs were > 1500 in all cases but 4 (> 1000 cells). We reported these numbers in the Results (lines 152-153)

7. Line 96-98:" normal eosinophils and lymphocytes were used..." could the author clarify how the normal eosinophils and lymphocytes were identified?
Done: (Line 104-108)

8. Line 98: "MCs and eosinophils were recorded positive if antigen was presented in >20%.."
Could the author clarify this statement and present it by showing the dot plot graph of these gated populations.

The statement has been rephrased (line 111-112). In our opinion the inclusion of pictures representative of positive vs negative results for each antibody would be a bit burdening for the study. Fig.1 includes one analysis of CD11b and can serve as reference. Anyway, more than 90% of cells were positive or negative in most of cases. The principal exception was CD11b that was expressed at low intensity by MCs in a high number of cases. In the CD25 (+) positive case, 100% of mast cells were positive. CD34 (+) positive MCs were 100%, 100%, 91.9%, 80.8% in the 4 positive cases, respectively. We added a statement in the text (lines 180-182) and an indication of cases with <90% in Tab.2 in order to give the reader a clearer idea of mast cells positivity.

9. May the author explain why isotype controls were not used for all antibodies?
CD117 and CD11b are two antibodies that we have been using for a long time in our daily practice in other tissues verifying that the two populations used are adequate as negative controls. In particular, we considered very important to have a CD117 negative granulocyte control population since we observed that the fluorescence of granulocytes changes compared to the isotype control. However, the concept presented here has been more deeply developed in the subsequent comments.

10. Line 103-106: May the author expand about the gate strategy used in this paper as in my opinion it is essential for the interpretation of the results and conclusions.
Done (Line 100-108 and Fig.1). Please, refer to the answer to previous comment n.5

11. Line 106: could the author describe how the population percentage of eosinophils and mast cells calculated?

Percentages were calculated within the intact cell gate (now specified in line 100-102). The method to identify the two populations has been added and a specification of the gate used for the analyses has been introduced. Please, refer to the answer to previous comment n.5

12. Line 108-109: could the author describe the multicolor tube designed for identifying MCs in the lymph node, what fluorochromes were used and were positive controls used?

The multicolor approach used for the LN has been described in lines 119-120 and conjugated fluorochromes are reported in Tab.1

To validate our approach we added mast cells in an aspirate from a reactive lymph node (Line 122-123). A figure has been added (Fig.2).

In lymph nodes samples, lymphocytes served as internal control for IgE, CD5 and CD21, and eosinophils for CD11b, while it was impossible to have an internal positive control for CD117; anyway we looked at the eosinophils as negative threshold for mast cells.

Furthermore, MCTs and LNs were always labeled together using the same protocol. Thus, the MCT tube can be considered a positive control for the procedure.

Results:

Immunophenotyping of MCT population and eosinophilic infiltration:

A. Line 150-151: Were all the cells identified as IgE and CD11b positive co-express CD 117- confirming these are mast cells?

Yes, they were all CD117-positive cells. The gating strategy includes CD117 positivity for mast cells.

B. Lines 150-161: Please be consistent with presenting the immunophenotyping data (some places positive presented as + and some as "-positive"). I would maybe combine (+) positive, (-) negative. The author can choose how present it as long as it is consist

Thank you. We appreciate your suggestion. We adopted (+) positive, (-) negative. The consistency has been verified throughout all the manuscript.

C. Line 160: "morphological evaluation of IgE-negative…presence of larger and fewer.." Was this a consistent finding with all cases of mast cell tumors that were IgE negative? In the discussion you mention it was seen in the majority of the cases, what percentage of cases?

Yes, the feature was a consistent finding in all IgE-negative cases, but in one of them the cells with fewer and larger granules didn't represent the majority of the population. The text has been integrated accordingly (lines 187-190 and line 231)

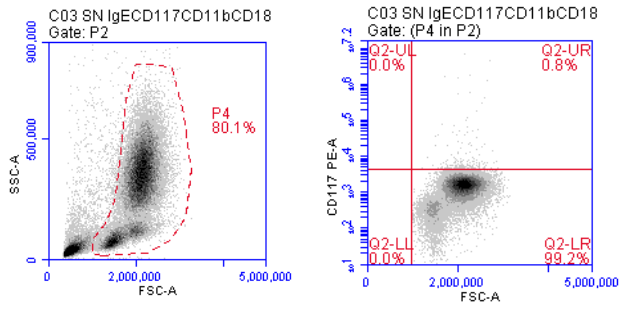
Lymph node results:

D. Line 166-167: "mast cells aggregates.." could the author expand how many cells together were considered to be an aggregate and how many minimum aggregates needed to be seen?

The cytological evaluation of lymph nodes has been performed according to Krick et al. (2009). The manuscript has been updated accordingly (M&M: lines 128-130; Results: lines 196-198 and Tab.3; Discussion: lines 276-277)

E. Line 170-174: The conclusion of "high correlation was observed between FC and cytology…" should be re-evaluated when considering the dot plot presented in Fig 2 (A). The data is presented as 6% of mast cells in the LN, however from the dot plot it seems there is a separated population that is positive to CD117 (though it is presented under the detected line it seems separated from a deferent population and most consistent with CD117 positive), and an additional population that is CD117 (-) negative and IgE + positive. Could the author give his opinion on what these cells are and why they were not considered to be mast cells?

The different populations are now described in the figure legend (Fig.5). The second population (CD117 (-) negative and IgE (+) positive) is composed by B-Lymphocytes. They are CD21 (+) positive and CD5 (-) negative and show scatter properties consistent with lymphocytes. The first population is represented by eosinophils. As explained in comment number 9, we decided to use a granulocytic population as internal negative control. In our experience granulocytes show a higher CD117-fluorescence compared to lymphocytes and isotypic control (as you can see in the pictures from a peripheral blood of a healthy dog below) but they should be considered negative based on the evidence that mature myeloid cells do not express CD117 because they lose it during maturation (Wojciech et al. 2011. Immunophenotypic Pattern of Myeloid Populations by Flow Cytometry Analysis. Methods in cell biology 10, 221-266).
Furthermore, we never identified CD117-positive eosinophils at IHC done on MCT slides (we are running a parallel study on the level of expression of CD117 and other antigens by mast cells).
Considering all evidences we stick to our decision to consider eosinophils as CD117 (-) negative. Anyway, the interpretation of CD117 signal by eosinophils was not the goal of the present study. No matter how they are considered, the strategy to identify mast cells in lymph nodes would not be affected because it is dependent on the pattern described in the tumor mass and mast cells always show the highest CD117-fluorescence




Discussion :

A. Line 189: "our results confirm that CD117 is a reliable…". From the data presented I think we should be very cautious to commit that CD117 is a reliable marker for canine MCT unless a

second method was used to confirm that the CD117 positive cells were mast cells, and that all mast cells will show positive expression of CD117 on flow cytometry. Usage of cell sorter can be a good method to confirm this process. Usage of cell sorter was mentioned in this paper but not detailed.

We identified the mast cell population in the mass during a pilot test on a MCT not included in the current study, and we only cited this test in line 104-106 of the original manuscript. We have now added a specific section in the Results reporting evidences of this test ("Mast cells and eosinophils identification" – Line 163-171 and Fig.3). Briefly, we sorted the two main populations that were present in the MCT aspirates by gating on CD117 and scatter properties and confirmed to be MCs and eosinophils through cytospin morphologic evaluation.
Moreover we added mast cells in a reactive lymph node to confirm that we were able to identify MC in the lymph nodes. These results have been reported in the manuscript (lines 122-123; Fig.2)

B. Line 192: " IgE appears as a second distinctive MC marker". From the dot plot presented in Figure 2 it seems there is a population that is IgE (+) positive and CD117 (-) negative. I would encourage to look into it and give the opinion regarding that population, and be more cautious with this statement.

The questioned population is made of CD21 (+) positive B lymphocytes (please, refer also to the answer to comment E in the Results section). That is why we used a multicolor tube comprising lymphoid marker. We wanted to be sure to correctly detect all populations present in the lymph node. Our approach allows to discriminate lymphocytes (CD5+ or CD21+ and negative to all the others with B lymphocytes showing variable expression of IgE), neutrophils and macrophages (CD11b+ and negative to all the others and with CD117 higher than lymph), eosinophils (CD11b+hi and CD117 higher than lymph), and mast cells (CD117+, negative for CD5 and CD21 and with IgE and CD11b positive or negative according to the expression detected in the mass). The different populations are now described in the figure legend (Fig.5)
Anyway, we agree with the clarification by the Reviewer: IgE is not distinctive of MCs because we can find it on lymphocytes. Thus, we change the text in "IgE appeared as a second marker characteristic for MCs" (line 221)

C. Line 199-200: "absence of IgE is the immaturity of the cells". Could the author comment if these cases with IgE (-) negative were also the cases with the cells that expressed CD34 (+) positive?

The question is appropriate, because CD34 would have confirmed the hypothesis of immature cells. Unfortunately, only 1 case was IgE (-) negative and CD34 (+) positive. The data are reported in Tab.2. We added a statement to specify it (lines 232-233). Anyway, the absence of CD34 does not exclude a more immature cell.

D. Line 202: "Majority of IgE-negative" What percentage of these cases had large granules

Please, see answer to Comment C of Results

E. Line 220-226: What percentage of mast cells expressed CD 34 (+) positive?

Please, see answer to Comment n.8 of Materials and Methods

F. Line 228-232: Did a CD25 immunohistochemistry stain done on the histology sections of the mast cell tumors, to evaluate the presented hypothesis?

No, we didn't plan IHC investigation for CD25 because our first aim was to validate the flow approach and we did not have the possibility to run it now. Anyway, on the basis of the present results we will evaluate CD25 expression by IHC in a prospective study

G. Line 239-244: As previously mentioned, I think we should be cautious to state that flow can allow an easy identification of MCs mainly when considering both the presented dot plot of case A (fig 2) and the fact that this was not supported by the histological sections as mentioned by the author in line 241-242.

As reported in the answer to Comment A in the Discussion section, we are confident in the identification of MCs in lymph node. We believe that the results of both the sorting procedure and the addition of MCs in lymph node are sufficient to support it. Concerning fig.2A (now fig.5), please refer to answers to Comment E in the Results section and to Comment B in the Discussion section.

The aim of the study was to identify mast cells in lymph nodes and we totally agree that our data do not demonstrate the possibility of detecting nodal metastases by flow cytometry at the moment. The statement at lines 277-279 is intended to underline this point. A specific study to evaluate the usefulness of flow cytometry in diagnosing metastatic lymph node is ongoing in our institution.

Figure legends:

Fig 1: Please add stain used. A. Consistency with IgE (+) as previously mentioned in the text. Please correct MCTs to MCT as only one tumor is presented here. B. Consistency with IgE (-) negative. Correct MCTs to MCT as above

Done (now Fig.4)

Fig 2. Please mention stain used. In the flow of sample A, didn't mention including Q8- UL, in addition to UR. In the image the scale bar is not easily seen.

The figure legend has been completely rephrased (now Fig.5). Scale bars have been modified

**Reviewer #2:**

The topic of the submitted article fits very well into the general scope of „The Veterinary Journal". The information is very valuable, interesting and helpful for the scientific community and opens new diagnostic and prognostic perspectives in canine mast cell tumor. However some methodological aspects have to be mentioned in more detail. Also one additional Figure describing the MCT and LN results would be valuable.

Some detailed questions and comments to the Manuscript:

Materials and Methods:

1. Page 4 line 69-76: please clarify, was the solid material, after using the part for FCM used for histology as mentioned in line 76), or was this a different part of the tumor?
   Was the solid part only used in case the FNA showed no sufficient cell material?

Two different parts of the mass were sampled for flow cytometry and histopathology, respectively. We better specified it in the text (Line 70-73).
Yes, the solid part was only used in case the FNA showed a cell count <100/uL (Line 88-90). However, the FNA was sufficient in most of the cases and scraping of the solid part was only necessary in one case (Line 150)

2. Page 5 line 85-86: Please clarify the minimum cell count needed - in line 85 the cell count is more than 30/µl but in the next line it says that <100/µl were not enough and an enrichment from the solid mass was added.

samples with a count <30/µl were no further processed and were excluded; samples with >100/ul were processed without scraping of the solid part; samples with a cell count between 30/ul and 100/ul were subjected to scraping of the solid part in order to increase the cellularity of the suspension. The statement has been modified so as to be clearer (Line 88-90).

3. Page 5 line 91: What was the cell count required for one FC tube?

The minimum cell count for one FC tube was 5000 cells (50ul of cell suspension with 100 cells/µl). that is: the total number of cells to be labeled in each tube was 5000. After labeling, the minimum of cells to be acquired was 3000.

4. Page 5 line 92: Was lysing done on all samples, also if they were not bloody?

Yes, lysis was performed on all samples as it served also as a washing step as well as lysing step. (please, see also answer to comment n.3 of Material and Methods section by Reviewer 1)

5. Page 5 line 99: The positivity for eosinophils and MC is reported with a cut off of 20% - does this refer only to the lymph node infiltration or also to the tumor? Wouldn't it be more logical to put the cut off for the tumors at 80 or 90% for the MCT?

Percentage of positive events within the mast cell gate was used to define MCs as positive or negative in the primary mass. The goal for the lymph node was not to describe MC

immunophenotype; it was to detect events with the same antigen expression described in the mass.
We agree that using a higher cut off (80%) would have guaranteed a more specific result. We choose a low cutoff because we were interested in recording dim expression in particular of antigens such as CD25. Anyway, most of the antigens were positive in >90% of MCs in the majority of cases (please, refer also to answer to comment n.8 of Material and Methods section by Reviewer 1)

6. Page 6 line 102: Please add a FSC/FSC picture to the figures to explain the gating in more detail. This is difficult to reconstruct. This should be done for the MCTs and the lymph node infiltration presenting one representative example.

The gating strategy is now better described in M&M (lines 100-108) and a figure has been added (Fig.1).
We added a specific paragraph (providing images) in the results section ("Mast cells and eosinophils identification" – lines 163-171). Here we report the sorting experiment performed in order to identify the two main populations (mast cells and eosinophils) and leading to the depiction of the specific gates of analysis. We refer to this new paragraph in the Material & Methods (lines 106-108)

7. Page 6 line 105-106: please describe in a little more detail how the negative controls were sorted.

A specific section describing the sorting experiments has been added (lines 163-171 anf Fig.3)

8. Page 6 line 118: Which part was used for Histopathology - the remaining from the sold material or a separate one? Was this part then from the same tumor? Was there also a requirement for a minimal size of the MCT sampled?

A little piece of the solid tumor was sampled for flow cytometry; all the remaining part (same tumor) was used for histopathology. It is now specified in M&M (lines 71-73). MCTs had to have a longest diameter of at least 1 cm. This has also been added (line 78-79).

Results:

1. Page 8 line 164: Please describe in more detail: Was the cytological evaluation done from a cytospin of the FNA or was it an impression smear of the solid mass?

Lymph node smears used to evaluate the correlation of mast cells infiltration between cytology and flow cytometry were obtained by direct smear of fine needle aspiration. Cytospins were not used in this study. It is now clarified in M&M (line 74-75).

Discussion:

1. Page 9 line 184: Was the positivity cut off for the MCTs also 20 percent here?

Yes, neoplastic cells were considered positive to each antigen if >20% of the mast cells in the mass were positive. It has been better described in M&M (lines 110-112) and in Results (lines 180-182 and Tab.2)

Figures:

Figure 2

1. Please explain the percent mentioned in the figure legend. They are different from the Figure (A: 3.6%; B: 83.1%)

The percentages mentioned in the figure legend refer to the cytological evaluation, while the numbers on the figure refer to the flow cytometric analysis. The whole figure legend has been rephrased in order to be clearer.

2. Please also clarify and describe the 3 different populations in Fig 2 A in quadrant Q8-LL. Is one of them maybe CD117+ and IgE-?

Please, refer to the answers to Comment E in the Results section and to Comment B in the Discussion section by Reviewer 1. The different populations are now described in the figure legend (Fig.5)

I recommend the manuscript YTVJL-D-17-00795 to be published in "The Veterinary Journal" after MAJOR REVISION.

Editorial comments:

1. Line 96/97, line 103, line 154, line 158: Regarding the CD11b+CD45+CD18+ cells defined as 'eosinophils': how were neutrophils excluded from this population? And why was an anti-human CD11b antibody (M1/70) used when there is an anti-canine (CA16.3E10) available?

Actually neutrophils are very rare to be found in both tumor mass and lymph nodes. Moreover they have particular scatters which allow their identification in combination with the CD11b, CD45 and CD18 expression levels. This has been confirmed also by cytological evaluation of the smears.

We used the cited CD11b antibody because it is part of our panels since a long time. We choose this particular antibody many years ago because it was the only one we found in the market already conjugated and detected in FL3. We tested the product and it works very similarly to anti-canine antibody.

2. Line 118: Was histopathological grading done by a single operator or by multiple operators? If multiple operators performed the grading, this needs to be mentioned as a limitation of the study in the discussion (interobserver variation).

The histopathological grading was done by two different pathologists. We agree with the Reviewer: this limitation must be mentioned. A specific sentence has been added in the M&M section (Lines 137-138) and in the Discussion (lines 266-268)

3. Line 130: 'Aberrant' phenotype can only be assessed when the phenotype of non-neoplastic canine mast cells is known.

We based our definition on the existing reports describing exposure of some of the markers we used by normal human and canine mast cells and on the consistent results on the majority of the analyzed samples. However, we agree with the Reviewer and that's why we concluded the discussion stating that immunophenotyping of non-neoplastic non-cultured canine mast cells is mandatory to definitely use the described abnormalities as surrogate marker of neoplasia. Thus, we changed the word 'aberrant' to 'unusual' (Line 144). Thank you for the clarification.

4. Line 163 (lymph node results): Were all lymph nodes (cytology and/or histopathology) diagnosed as metastatic mast cell tumor? This is not clear from the text. Also, please briefly explain the meaning of HN1, HN2, and HN3 lymph nodes for the readers (line 170/171).

We added a brief description of Weishaar grading in the M&M (Lines 136-138) and made the sentence more descriptive in the results (Lines 200-201)

5. Line 228: CD25 expression was assessed in only 50% of cases - this needs to be discussed here as well.

Done (Line 260).

6. Line 220/221: As a note - endothelial cells in peripheral tissue are CD34+.

Thank you for your clarification. The text has been rephrased accordingly (line 250-251).

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