Investigation of the utility of lymph node fine needle aspiration cytology for the staging of malignant solid tumors in dogs

Investigation of the utility of lymph node fine needle aspiration cytology for the staging of malignant solid tumors in dogs

Quentin Fournier[1], Paola Cazzini[2], Spela Bavcar[1], Evi Pecceu[1], Clara Ballber[1,3], and Richard Elders[1,4].

Departments of [1]Small Animal Teaching Hospital and [2]Easter Bush Pathology, The Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, Easter Bush, Edinburgh, UK

[3]Dr. Ballber's current address is: Wood Street Veterinary Hospital, London, UK

[4]Dr Elders' current address is: IDEXX Laboratories, Wetherby, UK

Corresponding author:

Quentin Fournier

Small Animal Hospital

Royal (Dick) School of Veterinary Studies

University of Edinburgh

Easter Bush Campus

EH25 9RG

United-Kingdom

qfournie@exseed.ed.ac.uk

19 Keywords: Canine, cytology, lymphatic metastasis, sensitivity

20 Running Head: Lymph node cytology in cancer staging

21 Data presented in part at the joint European Society of Veterinary Oncology and European

22 Society of Veterinary Clinical Pathology Annual Congress, Nantes, France, 2016.

Abstract

Background: Fine needle aspiration cytology (FNAC) of lymph nodes (LNs) is routinely used for staging canine malignant solid tumors, but studies evaluating its efficacy are limited.

Objectives: The primary objectives of this study were to evaluate the sensitivity/specificity of FNAC and the significance of non-diagnostic FNAC when staging canine malignant solid tumors. A secondary objective was to determine the frequency of multiple nodal metastases.

Methods: Histopathological and FNAC assessments of LNs (n = 259) draining malignant solid tumors were included. The sensitivity/specificity of FNAC was determined for 194 LNs with diagnostic FNAC, using histopathology as the gold standard. The proportion of non-diagnostic FNAC and associated histopathological prevalence of metastasis were determined. Among the tumors with multiple LNs assessed (88/189), the prevalence of multiple nodal metastases was determined.

Results: The sensitivity of FNAC was 67% for sarcomas, 100% for carcinomas, 63% for melanomas, 75% for mast cell tumors, and 100% for other round cell tumors. The specificity varied between 83% and 96%. Non-diagnostic FNAC was reported in 25% of LNs sampled, most of which were non-enlarged and/or difficult to access, and 20% of which were metastatic on histopathology. When several LNs were assessed, the prevalence of multiple nodal metastases was 24%.

Conclusions: Histopathologic LN evaluation cannot be robustly substituted with FNAC when staging selected canine solid tumors. When a diagnostic FNAC is elusive, as the prevalence of metastasis remains non-negligible in these cases, histopathological assessment is ideal. Finally, staging should not always be limited to the assessment of one single LN.

Introduction

Assessment of the loco-regional lymph nodes (LNs) is an integral part of the TNM clinical staging system for canine solid tumors, from the original version of the World Health Organization (WHO) staging scheme created in 1980,[1] to the more recently published staging schemes.[2-4] Loco-regional LN metastatic status has been correlated with prognosis in several tumor types, and represents a key element for the clinician to devise an appropriate, bespoke treatment plan for each individual tumor-bearing canine patient.[5, 6] Although histopathologic examination was the method originally recommended in the WHO staging system to assess regional LN status,[1] assessment of the regional LNs in veterinary medicine is often performed using fine-needle aspiration cytology (FNAC). As it is a non-invasive, cost-effective, and rapid technique, FNAC is very appealing. In a large retrospective study, tumor staging was the second most common reason for sampling LNs, leading to the submission of 9.3% of LN FNAC.[7] In a prospective study including 37 dogs diagnosed with a variety of tumors (16 carcinomas, 18 sarcomas, 7 mast cell tumors, 2 melanomas and 1 histiocytic sarcoma), the sensitivity and specificity of FNAC for assessing regional LNs were 100% and 96% respectively, with histopathology used as the gold standard.[8] In another prospective study including 28 dogs with oral or maxillofacial neoplasms (8 squamous cell carcinomas, 5 fibrosarcomas, 5 melanomas and 10 other tumors), the accuracy of FNAC for LN staging was 90.5% when compared with histopathology.[9] Similarly, in a recent large retrospective study, the sensitivity and specificity of FNAC in the detection of LN neoplasia were 66.6% and 91.5%, respectively.[10] However, the latter study was primarily designed to determine the agreement between FNAC and histopathology for diagnosing nodal neoplasia, and was not specifically designed to assess the accuracy of FNAC in the setting of solid tumor staging.[10]

In human oncology practice, LN extirpation followed by histopathologic examination is often performed to achieve an accurate clinical stage. An advantage of histopathologic

examination is the possibility of obtaining multiple tissue sections, and to allow the use of a comprehensive immunohistochemistry panel and/or other further assessments (e.g. PCR-based assessments), which might be necessary to improve the accuracy of staging.[11] However, depending on the location and number of LNs removed, LN extirpation can be associated with complications and can have a negative impact on the quality of life of cancer patients.[12, 13] The value of sentinel LN extirpation for the staging of several tumor types is currently under investigation. Many studies report an increase in the appropriateness of each LN extirpation using this approach, while decreasing the morbidity associated with routine, unguided and extensive LN dissection.[12, 14] Some studies have investigated the possible utility of FNAC in the staging of human breast, head, and neck cancer, but the sensitivity of FNAC to detect metastasis was generally poor.[15-17] However, other studies have suggested a role for ultrasound-guided FNAC of sentinel LNs, in the staging of other tumor types, in particular when used in a step-wise approach.[18-21]

Only two relatively small prospective studies have assessed the accuracy of FNAC for LN staging in dogs, both finding good agreement with histopathology.[8, 9] A more recent, large retrospective study found that FNAC was poorly sensitive in detecting LN neoplasia, however this study was not specifically designed to assess this technique in the setting of routine LN staging.[10] We sought to enhance the evidence base in this area by designing a study to assess the reliability of FNAC for LN staging in dogs presented with a solid tumor. We also sought to elucidate the impact of non-diagnostic FNAC in the routine staging of canine solid tumors, as this limitation of FNAC had not been assessed before. Similarly, as previous studies assessed only one LN per tumor, [8, 10] acknowledging that tumors can metastasize to several LNs, sometimes ”skipping” the anticipated local LN, [22-24] our study included the assessment of several LNs and the impact of such wider LN sampling on staging results. The primary objectives of this study were two-fold: 1) to evaluate the reliability of

FNAC compared with histopathology in the staging of canine malignant solid tumors, and 2) to evaluate the clinical significance of non-diagnostic FNAC in the staging of canine malignant solid tumors. A secondary objective was to determine the impact of multiple LN assessment in the staging of canine malignant solid tumors.

Materials and methods

Data collection

Medical records of dogs presented to the University of Edinburgh Veterinary Teaching Hospital between February 2012 and February 2017 were reviewed to identify dogs with a histopathologic diagnosis of a malignant solid tumor with regional LN sampling. In all cases of histiocytic sarcomas and cutaneous lymphomas included in the study, no sign of distant involvement was noted at initial staging; which included thoracic radiographs, abdominal ultrasound, FNAC of liver and spleen, and bone marrow aspirate. Cases were included in this retrospective study if both FNAC and histopathology of LN(s) were available, and if the interval between FNAC and LN extirpation was <30 days. In some cases, several locoregional LNs were assessed by both methods in staging a single tumor. May-Grünwald-Giemsa-stained FNAC preparations were assessed by board-certified clinical pathologists, while histopathologic sections were routinely stained with H&E and assessed by board-certified anatomic pathologists.

Information collected from the medical records included dog signalment, tumor characteristics (histopathologic diagnosis, anatomic location and lateralization), LN characteristics (anatomic location, size, enlargement status (enlarged/not enlarged), use of ultrasound-guidance for FNAC, date of FNAC, date of extirpation). Tumor types were grouped into: sarcomas, carcinomas, melanomas, mast cell tumors, or other round cell tumors. Lymph node location was classified as: mandibular, prescapular, inguinal, popliteal,

sublumbar, or others. Determination of LN enlargement status was based on clinical examination and was classified as: none, mild, moderate, or marked. Although subjective, enlargement status was preferred to measured size for statistical analysis, as validated information defining the normal size of a normal LN for specific breeds and locations is still lacking, and as such categorization accurately reflects clinical practice. When the cellularity of the sample was too low to allow cytological LN assessment, FNAC was considered non-diagnostic. The LN metastatic status for both cytologic and histopathologic examinations was categorized as positive or negative. When metastasis was only suspected, a positive metastatic status was attributed to the LN. Cytologic and histopathologic criteria for the diagnosis of nodal mast cell tumor metastasis have previously been proposed.[25, 26] These criteria were not systematically used in the original reports, but were retrospectively applied whenever possible.

Reliability of LN FNAC

Only LNs with diagnostic FNAC were selected for this analysis. The sensitivity and specificity of FNAC in the detection of nodal metastasis was determined for all LNs and subsequently for each tumor group, using histopathologic examination as the gold standard. The possible influence of factors such as LN enlargement, time between FNAC and LN extirpation, and LN location, on the failure to obtain agreement between FNAC and histopathology was evaluated. Cases with false-negative and false-positive FNAC reports were reviewed in an attempt to find an explanation for the discrepancy with histopathology.

Significance of non-diagnostic LN FNAC

The proportion of non-diagnostic FNAC was determined overall and for each tumor group. The possible influence of several factors on the failure to obtain a diagnostic FNAC, such as LN enlargement status, ultrasound-guidance, and LN location, was evaluated. The

prevalence of histopathologic metastasis among the LNs with non-diagnostic FNAC was determined and compared to that among the LNs with diagnostic FNAC.

Significance of multiple nodal metastases

Tumors included in the study were reviewed and separated in two groups: tumors which had several LNs aspirated and removed for staging, and tumors which had a single LN assessed. The proportion of each LN location, the prevalence of metastasis, and LN enlargement status were compared between the two groups. Among the tumors with multiple LNs assessed histopathologically, the prevalence of metastasis to several LNs was determined. The patterns of metastasis to several LNs was reviewed.

Statistical analysis

Differences in the prevalence of metastasis between the four subgroups of enlargement status (none, mild, moderate, marked) were assessed using Fisher's exact test on pairwise comparisons and applying the Bonferroni correction. Differences in the prevalence of metastasis between the non-diagnostic and diagnostic FNAC subgroups, the tumors with multiple LNs and tumors with single LN assessed; the difference in overall agreement and agreement within each LN location; the difference in proportion of ultrasound guidance between the non-diagnostic and diagnostic FNAC subgroups; and the difference in proportion of specific LN locations sampled between the tumors with multiple LNs assessed and those with a single LN assessed were analyzed using the chi-square test of homogeneity and Fisher's exact test where appropriate. Mann-Whitney *U* test was used to assess differences in LN enlargement status between the metastatic and non-metastatic LN subgroups; the non-diagnostic and diagnostic FNAC subgroups; the tumors with multiple LNs sampled and those with a single LN sampled; and the correlation of the interval between FNAC and LN extirpation and FNAC-histopathology agreement. The 95 % confidence intervals (CI) were

calculated using the exact binomial method. Statistical analyses were performed using commercially available statistics software (Minitab™ 17 Statistical Software; Minitab Inc., State College, Pennsylvania, PA, USA). A *P*-value of < 0.05 was considered statistically significant for all analyses.

Results

Three hundred and thirty-seven LNs investigated because of neoplasia were initially recruited to the study. Seventy-eight of these cases were excluded, 75 because FNAC had not been attempted prior to LN extirpation, and 3 because the time between FNAC and histopathology was > 30 days. The 259 remaining LNs included in this study were assessed for the staging of 189 tumors in 187 dogs. Primary tumors included a variety of different types, grouped as sarcomas (47 LNs assessed), carcinomas (46 LNs), melanomas (37 LNs), mast cell tumors (110 LNs), and other round cell tumors (19 LNs) (Table 1). One hundred and ninety-four of the FNACs were diagnostic in quality, while the remaining 65 FNACs were non-diagnostic.

The median time between FNAC and LN extirpation was 7 days (1 – 30 days). Ultrasound guidance was used in 16.5% (n=32) of the FNACs. The overall prevalence of metastasis, based on histopathologic examination, was 32.4 % (n=84). The anatomic site of the LNs were mandibular (n = 132), prescapular (n = 51), popliteal (n = 34), inguinal (n = 18), sublumbar (n = 17) and others (n = 7).

Lymph nodes were deemed not enlarged in 58.3% (n=151) of cases, mildly enlarged in 19.7% (n=51) of cases, moderately enlarged in 14.7% (n=38) of cases, and markedly enlarged in 7.3% (n=19) of cases. There was a significant difference in the prevalence of metastasis when the LNs were stratified by enlargement status ($P = 0.006$) (Figure 1). Nodes with metastatic disease were significantly more likely to be deemed enlarged than non-

metastatic LNs in dogs bearing sarcomas ($P = 0.048$), carcinomas ($P < 0.001$), melanomas ($P = 0.035$), and mast cell tumors ($P < 0.001$), although statistics did not yield significant difference in dogs bearing other round cell tumors ($P = 0.351$). All the markedly enlarged LNs were assessed for the staging of dogs bearing either apocrine gland anal sac adenocarcinomas or mast cell tumors. The normal nodal architecture was effaced and replaced by neoplastic cells in all the LNs deemed markedly enlarged, with the exception of only one non-metastatic LN which had moderately disorganized architecture but no neoplastic cells were observed.

Reliability of LN FNAC

Among the 194 LNs with diagnostic FNAC, 35 were from dogs bearing a sarcoma, 34 were from dogs bearing a carcinoma, 30 were from dogs bearing a malignant melanoma, 78 were from dogs bearing a mast cell tumor, and 17 were from dogs bearing another round cell tumor. Using histopathologic examination as the gold standard, the overall sensitivity of FNAC in the detection of LN metastasis was 81% (58/71; CI 70-89%), the overall specificity was 91% (112/123; CI 84 – 95%), and the overall agreement was 88% (170/194; CI 82-92%). The sensitivity and specificity of FNAC were also determined individually for the 5 previously defined tumor groups (Table 2). Grouping FNACs into those which agreed and those which disagreed with histopathology, there was no significant difference in the time between FNAC and LN extirpation ($P = 0.751$), nor in LN enlargement status ($P = 0.587$). When compared to the overall agreement between FNAC and histopathologic examination, there was no significant difference in agreement for each LN anatomic location.

Among the 13 false-negative FNAC results recorded, 2 were from dogs bearing a sarcoma, 3 from dogs bearing a malignant melanoma, and 8 from dogs bearing a mast cell tumor. In one of the sarcoma-bearing dog with a false negative FNAC, the LN was removed

15 days later and histopathologic examination revealed a completely effaced LN by neoplastic tumor cells. The dog was euthanized 3 weeks later due to progressive disease. In the second case, the LN was removed a couple of days after the FNAC, and a 400µm metastatic deposit was noted within the corticomedullary junction. The LN bed was treated with radiation therapy and the dog was still free of disease a year later. All 3 melanoma-bearing dogs with a false negative FNAC were euthanized within a few months of investigation with generalized metastatic disease. In all 3 of these cases, pigmented cells and some large non-pigmented cells were observed on FNAC and interpreted as melanophages or macrophages. In all 8 cases of false-negative FNACs from dogs bearing a mast cell tumor, only small numbers (<1%) of individualized, well-differentiated mast cells were noted on cytology. In most histopathologic examinations of these cases, marked increases in individualized mast cells or mast cell aggregates were noted, sometimes associated with atypical morphology, corresponding to the HN1 and HN2 classes of the histopathologic classification scheme for mast cell tumor LN metastasis previously proposed.[25] In only one such LN did histopathologic examination reveal a focal effacement of the normal nodal architecture by mast cells (consistent with HN3 class).[25] Toluidine blue (TB) staining was performed in 3 LNs from dogs bearing a mast cell tumor, which confirmed pre-metastasis (HN1 class) in one case, early metastasis (HN2 class) in another, and refuted the diagnosis of possible metastasis based on prior H&E in the third case.[25]

Among the 11 false-positive FNACs recorded, 4 were from dogs bearing a mast cell tumor, 2 from dogs bearing a malignant melanoma, 2 from dogs bearing a carcinoma, 2 from dogs bearing non-epitheliotropic cutaneous lymphoma, and 1 from a dog bearing a sarcoma ("high-low" maxillary fibrosarcoma). Interestingly, in the latter case, moderate number of mesenchymal cells with moderate anisocytosis and anisokaryosis were seen on FNAC and were interpreted as neoplastic; however, on histopathologic examination the mesenchymal cells observed, were interpreted as reactive cells related to the presence of multifocal fibrinoid

necrosis of arteriolar walls and associated fibroplasia. At the time of writing, 3 years after the initial diagnosis, this dog continues to be regularly rechecked and remains free of disease, after incomplete excision and definitive-intent radiation therapy of the primary site. In the 2 cases of non-epitheliotropic cutaneous lymphoma with a false-positive LN FNAC, uncertainty regarding the cytologic metastatic diagnosis was mentioned by the clinical pathologist, however metastasis was strongly favored. One case had definitive-intent radiation therapy delivered to a solitary lesion on right carpus based on a lack of dissemination on initial histopathology of the draining LN, but developed disseminated disease 4 months later. The other case had a solitary lesion on the lip which was completely excised, but the dog was lost to follow-up. In the 2 cases of carcinoma with a false-positive FNAC report, cohesive clusters of cells with an appearance compatible with the primary carcinoma (thyroid carcinoma and apocrine gland anal sac adenocarcinoma) were noted on cytology, but similar cells were not observed on histopathology despite requesting additional sections and cytokeratin immunohistochemistry. Both of these dogs were lost to follow-up. In the 2 cases of melanoma with a false-positive FNAC report, uncertainty regarding the cytologic metastatic diagnosis was mentioned by the clinical pathologist. Melanophages and fewer scattered melanocytes were described on both FNAC and histopathology, but in the latter were interpreted as a drainage reaction rather than metastasis as no cellular aggregates or atypia were present. Both of these dogs were lost to follow-up. In all 4 cases of mast cell tumor with a false-positive FNAC, uncertainty regarding the cytologic metastatic diagnosis was expressed by the clinical pathologist; increased number of individualized mast cells (up to 10 per high-power field), aggregates of 2-3 cells or loose groups of up to 8 cells, and moderate anisocytosis were noted on cytology. Unfortunately, the previously proposed cytologic criteria were not applied in the original FNAC reports and could not be retrospectively applied using the detail therein.[26] Although the features noted on FNAC were also described on histopathology, they were

considered not to be consistent with metastasis. Unfortunately, the previously proposed histopathologic criteria were not applied in the original reports and could not be retrospectively applied using the detail therein;[25] but no disruption or effacement of normal nodal architecture was reported. Toluidine Blue staining was performed in one case, and was supportive of the non-metastatic diagnosis made on routine histopathology. Two of these dogs were lost to follow-up after 5 and 7 months, and the other two dogs were free of disease at the time of writing, 1 and 2 years after diagnosis, following complete excision in one case, and incomplete excision with adjuvant definitive-intent radiation therapy on the primary site in the second case.

Significance of non-diagnostic LN FNAC

Twenty-five percent of the FNACs were non-diagnostic. Among the 65 LNs with non-diagnostic quality FNAC, 12 were from dogs bearing a sarcoma, 12 a carcinoma, 7 a melanoma, 32 a mast cell tumor, and 2 another round cell tumor.

Lymph nodes with FNAC of non-diagnostic quality were significantly less likely to be deemed enlarged than were LNs with FNAC of diagnostic quality ($P < 0.001$) (Figure 2). Overall, most of the LNs with FNAC samples of non-diagnostic quality were not (n=50) to mildly enlarged (n=13), with the exception of 2 moderately to markedly enlarged LNs which were sampled using ultrasound guidance. Fine-needle aspiration cytology samples of non-diagnostic quality were significantly more frequently sampled with ultrasound guidance than were FNAC samples of diagnostic quality ($P = 0.002$) (Figure 3). When compared to the overall proportion of FNAC samples of non-diagnostic quality (65/259), the only anatomic site which had a significantly higher proportion of FNAC samples of non-diagnostic quality was the inguinal LN ($P < 0.001$). However, the majority of the inguinal LNs sampled were guided by ultrasound (16/18) and were deemed not enlarged (12/18). The prevalence of histopathologically-proven metastasis was 20.0% among the LNs with non-diagnostic FNAC,

289 and 35.5% among the LNs with diagnostic FNAC, and this was significantly different (P =
290 0.021) (Figure 4).

291 ***Significance of multiple nodal metastases***

292 Among the 189 dogs with a tumor included in the study, 88 cases had at least 2 LNs
293 histopathologically assessed for staging as these LNs were thought to possibly drain the
294 primary mass/be involved in the disease process. The tumors types with multiple LNs
295 assessed included 14 sarcomas, 20 carcinomas, 18 melanomas, 29 mast cell tumors, and 7
296 other round cell tumors. Eighty of these dogs had 2 LNs extirpated, 7 had 3 LNs extirpated,
297 and 1 oral malignant melanoma-bearing dog had 4 LNs extirpated. Lymph nodes removed
298 included those in the mandibular (132, including 64 bilateral and 4 unilateral extirpations),
299 prescapular (13, including 3 bilateral and 7 unilateral extirpations), popliteal (2, unilateral
300 extirpations), inguinal (11, including 5 bilateral and 1 unilateral extirpations), sublumbar (15,
301 including 7 bilateral and 1 unilateral extirpations) and other regions (14). Compared to cases
302 with solitary LNs assessed, among the cases with multiple LNs assessed, there was a lower
303 proportion of popliteal and prescapular LNs, but a higher proportion of mandibular LNs ($P <$
304 0.001). This is in part explained by the frequent bilateral extirpation of mandibular LNs in
305 cases bearing tumors located on the head in the authors' service. The overall prevalence of
306 metastasis was 38.6% (34/88) among the cases with multiple LNs assessed, and 34.7 %
307 (35/101) among the cases with only one LN assessed, which was not significantly different
308 (P = 0.650). There was no significant difference in the enlargement status of the LNs whether
309 cases had single or multiple LNs assessed (P = 0.762). Among the cases with multiple LNs
310 assessed, the prevalence of having ≥2 metastatic LNs was 23.9% (21/88) while 14.8%
311 (13/88) of tumors with multiple LNs assessed had evidence of metastasis in only one LN.
312 Therefore, of those tumors with metastasis which had multiple LNs sampled, 61.8% (21/34)
313 of cases had metastasis to multiple LNs. Tumors types metastasizing to multiple LNs were

carcinomas (n=10), mast cell tumors (n=6), oral malignant melanomas (n=4), and one sarcoma (Table 3).

Discussion

Lymph node enlargement status was significantly associated with tumor metastasis in our study. This was true for all the tumor groups included in our study with the exception of other round cell tumors, possibly because of a type II error. Nevertheless, the prevalence of metastasis among non-enlarged LNs was also substantial (15%). This finding is consistent with one of the two previous studies conducted specifically on malignant melanomas, in which the prevalence of metastasis among non-enlarged LNs was also 15%,[21] but the rate was 40% in the other study.[27]

The overall specificity of FNAC for the detection of tumor metastasis found in our study (91%) was similar to that found in a previous study (91.5%), however, the overall sensitivity was superior in our study (81%) compared to the same previous study (66.6%).[11] This could be explained by differentiating features of this previous study in which cytologic and histopathologic examinations were not always performed on the same LN, the time interval between cytologic and histopathologic examinations was up to 80 days, and cases of multicentric lymphoma were included.[10] Nonetheless, our results concur that although of high value and practicality, LN histopathologic examination cannot always be reliably substituted with FNAC.

The relatively low sensitivity of FNAC for detecting metastatic sarcomas in LNs (67%), also reported in a previous study,[10] could be related, at least in part, to the poorly exfoliative nature of sarcomas limiting the representativeness of FNAC.[28] The relatively poor sensitivity of FNAC for detecting metastatic malignant melanomas in LNs (63%), has also been anecdotally reported before.[27, 29] In one study assessing the efficacy of systemic

adjuvant therapies in dogs with excised oral malignant melanoma, 41 dogs had both cytologic and histopathologic examinations of at least one LN, and the sensitivity and specificity of FNAC were 78.1% and 64.1% , respectively.[29] The specificity found in our study was superior (91%), but these results highlight the difficulty in differentiating melanophages from melanocytes, which is a challenge for both cytologic and histopathologic examinations. The American Joint Committee on Cancer (AJCC) published guidelines for the use of immunohistochemistry in the evaluation of melanoma-draining sentinel LNs in human oncology practice, to facilitate the distinction between melanocytes and histiocytes.[11] The identification of even single-cell metastasis in a sentinel LN is now considered sufficient to categorize patients as having dissemination.[11, 30] With the increasing use of sensitive techniques for the detection of melanoma metastasis (immunohistochemistry, PCR), the challenge to accurately differentiate malignant from benign melanocytes has become even more important. [31, 32] Although these challenges have not been as clearly researched in canine oncology practice, routine histopathologic examination alone is likely to be a suboptimal gold standard for canine melanoma nodal metastasis assessment. This has been highlighted in a recent study, in which the diagnosis of LN melanoma metastasis was changed in 46.9% of dogs upon second opinion histopathology review.[33] This might also explain in part why several studies failed to find a prognostic value of LN metastasis in dogs,[29, 34, 35] while LN metastatic status is of important prognostic value in human melanoma.[36] The robustness of assessment of the diagnostic utility of FNAC for the detection of melanoma nodal metastasis would be enhanced by an optimal gold standard, based on the results of a future comparison of histopathology, immunohistochemistry, PCR, and combinations thereof, incorporating follow-up.

Although one study found a perfect agreement between cytologic and histopathologic examination for the detection of mast cell tumor nodal metastasis,[37] the relatively low

sensitivity (75%) found in our study is more consistent with that reported in another study (68.7%).[10] Cytologically it is often very difficult to differentiate reactive from well differentiated neoplastic mast cells within LN aspirates. Clinical pathologists often rely on the presence of the overall numbers of mast cells, their aggregation, and/or morphologic abnormalities to make such a distinction. It is therefore often difficult to determine the metastatic status of LNs draining canine mast cell tumors either cytologically or histopathologically. Criteria have previously been proposed to standardize the definition of mast cell tumor nodal metastasis for both techniques.[25, 26] These criteria were not systematically applied in our study, which makes it difficult to comment meaningfully on the sensitivity and specificity obtained by using the proposed criteria. A prospective study with the systematic application of these criteria for cytologic and histopathologic examination, together with follow up, would be necessary for a more reliable evaluation of FNAC accuracy. It should also be underlined that in all 4 false-positive FNACs assessing mast cell tumor LN metastases, the clinical pathologist expressed some uncertainty regarding the metastatic status; and for only 1/8 false-negative such FNACs, was the corresponding histopathologic classification HN3 (overt metastasis).[25] Furthermore, TB staining invalidated the diagnosis of LN metastasis made initially on routine histopathology in 1 case, and the systematic use of TB staining might alter the determined sensitivity and specificity of FNAC in the detection of mast cell tumor nodal metastasis.

Non-diagnostic FNAC was reported in 25% of our cases. This is comparable to the results of another study (27.2%) although only 9.3% of the LNs sampled were for tumor staging purposes in that study.[7] In another study, only 5.7% of the cytologic samples were deemed non-diagnostic,[10] but again the study did not exclusively include LNs FNAC sampled for tumor staging as in the current study. The results of our study suggest that FNAC might be technically limited for non-enlarged and/or deep LNs for which a diagnostic-quality

388 sample might not be easily obtainable. Size of the LN is a recognized limiting factor of
389 FNAC in human medicine, and it is often observed that LN < 5 mm are difficult to sample.[16]
390 Histopathologic assessment of local LNs when FNAC is not possible because of a LN's
391 inaccessible location or small size has been recommended by some authors.[22] Our results
392 support this recommendation, as when diagnostic samples could not be obtained by FNAC in
393 such LNs, the prevalence of metastasis remained substantial on histopathology (20%).

394 In previous studies, LN examination for tumor staging was limited to a single LN.[5, 8,
395 10, 26, 37] In our study, 46.5% of the tumors had several LNs assessed for staging. The
396 prevalence of metastasis within several LNs was 23.9%, suggesting that staging should not
397 always be limited to the assessment of a single LN. This is in agreement with the results
398 obtained with routine extirpation of bilateral mandibular and medial retropharyngeal
399 lymphadenectomy for staging of head-based tumours,[38] and with the use of sentinel LNs.[22,
400 23, 39, 40] It remains for further research to investigate whether such cases have a worse
401 prognosis when compared to those cases with a solitary LN metastasis. However, such results
402 are very likely significant from a therapeutic aspect, if the response to an additionally
403 metastatic LN were to be the use of a local therapy modality (i.e. surgical extirpation and/or
404 irradiation) rather than systemic therapy modalities that might already be triggered by even a
405 solitary metastatic LN. In our study, most of the tumors that were investigated for several
406 metastatic LNs were located in the head and involved mandibular LNs, or were anal sac
407 adenocarcinomas involving sublumbar LNs, which is consistent with other studies.[6, 38]
408 Notably, we report the occurrence of bilateral prescapular LN involvement in 3 tumors (2
409 mast cell tumors on the midline neck and 1 thyroid carcinoma), and bilateral inguinal LN
410 involvement in 1 scrotal mast cell tumor. As systematic bilateral assessment of local LNs
411 have been recommended for head-based tumors,[38] bilateral nodal assessment for other
412 locations could be of value, although this requires further investigation.

This study had several limitations, most of them being the consequence of its retrospective design. Cytologic and histopathologic examination was performed by different pathologists all of whom were board certified, although the sections were not systematically reviewed for the purposes of the study, therefore contributing to an inter-observer variation. In particular, previously proposed cytologic and histopathologic criteria for the diagnosis of mast cell tumor metastasis were not systematically applied.[25, 26] Cytological findings in some cases do not allow a certain diagnosis to be reached but they can point to a suspicion that needs to be confirmed through other methods. However, for the purpose of the study, metastatic status was dichotomized into “metastatic” and “non-metastatic”. Because dogs were assessed by different clinicians, the recording of LN enlargement status was subject to inter-observer variation. However, we believe that this effect was minimal as a significant difference in the prevalence of metastasis was noted for each tumor subgroup, and this approach reflects clinical practice. There was a variable interval between cytologic and histopathologic assessments, which could have affected their agreement, although this was intentionally limited. However, there was no significant difference in the interval between FNAC and histopathologic assessments between the LNs with agreement and those with disagreement.

Conclusions

In our study FNAC appeared to be a reliable tool to detect metastatic carcinomas and round cell tumors in LNs. Conversely, the sensitivity of FNAC in the detection of nodal metastasis was relatively low for sarcomas, melanomas and mast cell tumors. Although FNAC remains a non-invasive and affordable test typically obviating general anesthesia, when a negative result is obtained in these tumors, additional histopathologic assessment should be recommended for more robust staging information. Non-diagnostic FNAC reports are frequently encountered (25%) when staging tumor-draining LNs, particularly when the

LNs sampled are non-enlarged and/or have a deep location. Further histopathologic examination should be recommended in these cases, as the risk of metastasis in the non-diagnostic LN aspirates was 20% in our study. Finally, metastasis to multiple LNs seems to be relatively frequent, making investigation of multiple LNs valuable diagnostically and therapeutically.

Acknowledgements

The authors wish to thank Dr. Ian Handel, University of Edinburgh, for his generous assistance with the statistical analysis.

References

1. Owen LN and World Health O. TNM classification of tumours in domestic animals. Geneva, World Health Organization, 1980.
2. MacEwen EG and Withrow SJ. Tumors of the mammary gland. In: *Small Animal Oncology,* 2 edn., EG MacEwen and SJ Withrow, eds., Philadelphia, Saunders Compagny, 1996: 356-72.
3. Liptak JM and Forrest LJ. Soft Tissue Sarcomas. In: *Small Animal Clinical Oncology,* 5 edn., SJ Withrow, DM Vail and RL Page, eds., St. Louis, Missouri, Saunders, 2013: 356-80.
4. Polton GA and Brearley MJ. Clinical Stage, Therapy, and Prognosis in Canine Anal Sac Gland Carcinoma. *Journal of Veterinary Internal Medicine*. 2007; 21(2): 274-80.
5. Baginski H, Davis G and Bastian RP. The prognostic value of lymph node metastasis with grade 2 MCTs in dogs: 55 cases (2001-2010). *J Am Anim Hosp Assoc*. 2014; 50(2): 89.
6. Emms S. Anal sac tumours of the dog and their response to cytoreductive surgery and chemotherapy. *Australian Veterinary Journal*. 2005; 83(6): 340-3.
7. Amores-Fuster I, Cripps P, Graham P, Marrington AM and Blackwood L. The diagnostic utility of lymph node cytology samples in dogs and cats. *J Small Anim Pract*. 2015; 56(2): 125-9.
8. Langenbach A, McManus PM, Hendrick MJ, Shofer FS and Sorenmo KU. Sensitivity and specificity of methods of assessing the regional lymph nodes for evidence of metastasis in dogs and cats with solid tumors. *J Am Vet Med Assoc*. 2001; 218(9): 1424-8.
9. Herring ES, Smith MM and Robertson JL. Lymph node staging of oral and maxillofacial neoplasms in 31 dogs and cats. *J Vet Dent*. 2002; 19(3): 122-6.
10. Ku CK, Kass PH and Christopher MM. Cytologic-histologic concordance in the diagnosis of neoplasia in canine and feline lymph nodes: a retrospective study of 367 cases: Cytologic-histologic concordance in lymph nodes. *Vet Comp Oncol*. 2016.

11. Hutchens KA, Heyna R, 2nd, Mudaliar K and Wojcik E. The new AJCC guidelines in practice: utility of the MITF immunohistochemical stain in the evaluation of single-cell metastasis in melanoma sentinel lymph nodes. *Am J Surg Pathol*. 2013; 37(6): 933-7.

12. Schrenk P, Rieger R, Shamiyeh A and Wayand W. Morbidity following sentinel lymph node biopsy versus axillary lymph node dissection for patients with breast carcinoma. *Cancer*. 2000; 88(3): 608-14.

13. Briganti A, Blute ML, Eastham JH, et al. Pelvic Lymph Node Dissection in Prostate Cancer. *European Urology*. 2009; 55(6): 1251-65.

14. Rubio IT, Korourian S, Cowan C, Krag DN, Colvert M and Klimberg VS. Sentinel lymph node biopsy for staging breast cancer. *The American Journal of Surgery*. 1998; 176(6): 532-7.

15. Shakeel M, Daniel M, Srinivasan S, Koliyan R and Kumar J. Comparative analysis of detecting cervical lymph node metastasis with fine needle aspiration cytology. *Journal of Natural Science, Biology and Medicine*. 2015; 6(3): 7-9.

16. Hoft S, Muhle C, Brenner W, Sprenger E and Maune S. Fine-needle aspiration cytology of the sentinel lymph node in head and neck cancer. *J Nucl Med*. 2002; 43(12): 1585-90.

17. Yu YH, Mo QG, Zhu X, et al. Axillary fine needle aspiration cytology is a sensitive and highly specific technique for the detection of axillary lymph node metastasis: a meta-analysis and systematic review. *Cytopathology*. 2016; 27(1): 59-69.

18. Goel G. Predicting Nodal Burden in Clinically N0 Breast Cancer: a Comparison between Ultrasound Guided Fine Needle Aspiration Cytology and Sentinel Lymph Node Biopsy. edn., 2017: e75-e6.

19. Diaz-Ruiz M, Arnau A, Montesinos J, et al. Diagnostic Accuracy and Impact on Management of Ultrasonography-Guided Fine-Needle Aspiration to Detect Axillary Metastasis in Breast Cancer Patients: A Prospective Study. *Breast Care*. 2016; 11(1): 34-9.

493 20. Kroon BK, Horenblas S, Deurloo EE, Nieweg OE and Teertstra HJ. Ultrasonography-guided
494 fine-needle aspiration cytology before sentinel node biopsy in patients with penile carcinoma. *BJU*
495 *Int*. 2005; 95(4): 517-21.
496 21. Christopher A, Van Akkooi J, Siegel P, et al. Long-term results of ultrasound (US)-guided fine
497 needle aspiration cytology (FNAC) in conjunction with sentinel node biopsy (SNB) to support step-
498 wise approach in melanoma. *Journal Of Clinical Oncology*. 2015; 33(15).
499 22. Worley DR. Incorporation of sentinel lymph node mapping in dogs with mast cell tumours:
500 20 consecutive procedures. *Vet Comp Oncol*. 2014; 12(3): 215-26.
501 23. Beserra HEO, Grandi F, Dufloth RM, et al. Metastasis of Mammary Carcinoma in Bitches:
502 Evaluation of the Sentinel Lymph Node Technique. *Advances in Breast Cancer Research*. 2016;
503 05(02): 58-65.
504 24. Patsikas MN, Karayannopoulou M, Kaldrymidoy E, et al. The Lymph Drainage of the
505 Neoplastic Mammary Glands in the Bitch: A Lymphographic Study. *Anatomia, Histologia,*
506 *Embryologia*. 2006; 35(4): 228-34.
507 25. Weishaar KM, Thamm DH, Worley DR and Kamstock DA. Correlation of Nodal Mast Cells
508 with Clinical Outcome in Dogs with Mast Cell Tumour and a Proposed Classification System for the
509 Evaluation of Node Metastasis. *Journal of Comparative Pathology*. 2014; 151(4): 329-38.
510 26. Krick EL, Billings AP, Shofer FS, Watanabe S and Sorenmo KU. Cytological lymph node
511 evaluation in dogs with mast cell tumours: association with grade and survival*. *Vet Comp Oncol*.
512 2009; 7(2): 130-8.
513 27. Williams LE and Packer RA. Association between lymph node size and metastasis in dogs
514 with oral malignant melanoma: 100 cases (1987-2001). *J Am Vet Med Assoc*. 2003; 222(9): 1234-6.
515 28. Cohen M, Bohling MW, Wright JC, Welles EA and Spano JS. Evaluation of sensitivity and
516 specificity of cytologic examination: 269 cases (1999-2000). *J Am Vet Med Assoc*. 2003; 222(7): 964-
517 7.

518 29. Boston SE, Lu X, Culp WTN, et al. Efficacy of systemic adjuvant therapies administered to
519 dogs after excision of oral malignant melanomas: 151 cases (2001–2012). *JAVMA: Journal of the*
520 *American Veterinary Medical Association*. 2014; 245(4): 401-7.
521 30. Lobo AZC, Luo S, Duncan LM, et al. The distribution of microscopic melanoma metastases in
522 sentinel lymph nodes: Implications for pathology protocols. *American Journal of Surgical Pathology*.
523 2012; 36(12): 1841-8.
524 31. Holt JB, Sangueza OP, Levine EA, et al. Nodal melanocytic nevi in sentinel lymph nodes.
525 Correlation with melanoma-associated cutaneous nevi. *American journal of clinical pathology*. 2004;
526 121(1): 58.
527 32. Taube JM, Begum S, Shi C, Eshleman JR and Westra WH. Benign nodal nevi frequently harbor
528 the activating V600E BRAF mutation. *Am J Surg Pathol*. 2009; 33(4): 568-71.
529 33. Grimes JA, Matz BM, Christopherson PW, et al. Agreement Between Cytology and
530 Histopathology for Regional Lymph Node Metastasis in Dogs With Melanocytic Neoplasms.
531 *Veterinary Pathology*. 2017: 030098581769820.
532 34. Proulx DR, Ruslander DM, Dodge RK, et al. A retrospective analysis of 140 dogs with oral
533 melanoma treated with external beam radiation. *Veterinary Radiology & Ultrasound*. 2003; 44(3):
534 352-9.
535 35. Smedley RC, Spangler WL, Esplin DG, et al. Prognostic markers for canine melanocytic
536 neoplasms: a comparative review of the literature and goals for future investigation. *Veterinary*
537 *Pathology*. 2011; 48(1): 54.
538 36. Morton DL, Thompson JF, Cochran AJ, et al. Sentinel-Node Biopsy or Nodal Observation in
539 Melanoma. *N Engl J Med*. 2006; 355(13): 1307-17.
540 37. Stefanello D, Buracco P, Sabattini S, et al. Comparison of 2- and 3-category histologic grading
541 systems for predicting the presence of metastasis at the time of initial evaluation in dogs with
542 cutaneous mast cell tumors: 386 cases (2009-2014). *J Am Vet Med Assoc*. 2015; 246(7): 765.

543 38. Skinner OT, Boston SE and Souza CHdM. Patterns of lymph node metastasis identified
544 following bilateral mandibular and medial retropharyngeal lymphadenectomy in 31 dogs with
545 malignancies of the head: Patterns of head and neck lymphatic metastasis in dogs. *Vet Comp Oncol*.
546 2016.
547 39. Tuohy JL, Milgram J, Worley DR and Dernell WS. A review of sentinel lymph node evaluation
548 and the need for its incorporation into veterinary oncology. *Vet Comp Oncol*. 2009; 7(2): 81-91.
549 40. Lurie DM, Seguin B, Schneider PD, Verstraete FJ and Wisner ER. Contrast-assisted ultrasound
550 for sentinel lymph node detection in spontaneously arising canine head and neck tumors.
551 *Investigative radiology*. 2006; 41(4): 415.

552 **Tables**

553 **Table 1. Primary tumor types with corresponding number of LNs.**

Tumor type	Number of LNs
Sarcomas	**47**
Osteosarcoma	17
Soft Tissue Sarcoma	10
Sarcoma (not specified)	10
Fibrosarcoma	7
Hemangiosarcoma	2
Chondrosarcoma	1
Carcinomas	**46**
Apocrine gland anal sac adenocarcinoma	16
Oral squamous cell carcinoma	11
Thyroid carcinoma	8
Salivary gland adenocarcinoma	3
Gingival basosquamous cell carcinoma	2
Sebocytic sebaceous carcinoma	2
Mammary carcinoma	1
Pulmonary carcinoma	1
Cutaneous carcinoma (not specified)	1
Hepatocellular carcinoma	1
Malignant melanomas	**37**
Oral	32
Cutaneous	5

Mast cell tumors	**110**
Cutaneous	77
Subcutaneous	20
Mucosal	9
Muco-cutaneous	4
Other round cell tumors	**19**
Oral plasma cell tumor	10
Histiocytic sarcoma	6
Non-epitheliotropic T-cell lymphoma	3

Table 2. Sensitivity, specificity, positive and negative predictive values (and 95% confidence intervals) of LN FNAC in the detection of tumor metastasis.

Tumor types	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Sarcomas	67% (24-94%)	96% (80-99%)	80% (30-99%)	93% (76-99%)
Carcinomas	100% (82-100%)	83% (51-97%)	92% (72-99%)	100% (66-100%)
Malignant melanomas	63% (26-89%)	91% (69-98%)	71% (30-95%)	87% (65-97%)
Mast cell tumors	75% (57-88%)	91% (77-97%)	86% (76-95%)	84% (70-92%)
Other round cell tumors	100% (20-100%)	87% (58-97%)	50 (10-91%)	100% (72-100%)

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval

557 **Table 3. Data regarding the tumors with several metastatic LNs.**

Case Number	Primary tumor			Lymph nodes			
	Tumor type	Location	Lateralization	Location	Lateralization	Enlargement	Metastasis
1	Apocrine gland anal sac adenocarcinoma	Anal sac	R	Medial iliac	R	3	Y
				Medial iliac	L	0	Y
2	Apocrine gland anal sac adenocarcinoma	Anal sac	R	Medial iliac	R	3	Y
				Medial iliac	L	3	Y
3	Apocrine gland anal sac adenocarcinoma	Anal sac	L	Medial iliac	L	3	Y
				Medial iliac	R	2	N
				Sacral	L	2	Y
4	Apocrine gland anal sac adenocarcinoma	Anal sac	R	Medial iliac	R	3	Y
				Medial iliac	L	3	Y
5	Apocrine gland anal sac adenocarcinoma	Anal sac	L	Medial iliac	L	3	Y
				Hypogastric	L	3	Y
6	Apocrine gland anal sac adenocarcinoma	Anal sac	R	Medial iliac	R	3	Y
				Medial iliac	L	3	Y
7	Thyroid carcinoma	Thyroid gland	R	Mandibular	R	2	Y
				Mandibular	L	2	Y
				Prescapular	R	2	Y
8	Thyroid carcinoma	Thyroid gland	L	Medial retropharyngeal	L	1	Y
				Prescapular	L	2	Y
				Prescapular	R	1	Y
9	Squamous cell carcinoma	Tonsil	L	Mandibular	L	2	Y
				Mandibular	R	0	Y
				Medial retropharyngeal	L	3	Y
10	Salivary gland adenocarcinoma	Parotid salivary gland	R	Parotid	R	0	Y
				Medial retropharyngeal	R	1	Y
				Mandibular	R	0	N
11	Malignant melanoma	Maxilla	L	Mandibular	L	2	Y
				Mandibular	R	0	Y
12	Malignant melanoma	Maxilla	R	Mandibular	R	1	Y
				Mandibular	L	1	Y
13	Malignant melanoma	Maxilla	L	Mandibular	L	2	Y
				Mandibular	R	0	Y
14	Malignant melanoma	Mandible	R	Mandibular	R	0	Y
				Mandibular	L	0	Y
15	Mast cell tumor (mucosal)	Upper lip	L	Mandibular	L	2	Y
				Mandibular	R	0	Y
16	Mast cell tumor (cutaneous)	Lower eyelid	L	Mandibular	L	3	Y
				Mandibular	R	3	Y
17	Mast cell tumor (cutaneous)	Scrotum	M	Inguinal	L	0	Y
				Inguinal	R	0	Y
18	Mast cell tumor (cutaneous)	Carpus	L	Prescapular	L	3	Y

				Axillary	L	3	Y
19	Mast cell tumor (cutaneous)	Ventral neck	M	Prescapular	L	1	Y
				Prescapular	R	0	Y
20	Mast cell tumor (cutaneous)	Chest	M	Prescapular	L	2	Y
				Prescapular	R	2	Y
21	Nasal sarcoma	Nasal cavity	R	Mandibular	R	2	Y
				Mandibular	L	2	Y

Lateralization was classified as left (L), right (R) or midline (M). Enlargement was classified as none (0), mild (1), moderate (2), or marked (3).

Figure captions

Figure 1. Prevalence of metastasis stratified by LN enlargement.

The error bars represent 95% confidence intervals.

Figure 2. Lymph node enlargement among non-diagnostic and diagnostic cytology samples.

The error bars represent 95% confidence intervals.

Figure 3. Proportion of FNAC performed *via* ultrasound-guidance among non-diagnostic and diagnostic FNAC samples.

The error bars represent 95% confidence intervals.

Figure 4. Prevalence of metastasis among the LNs with non-diagnostic and diagnostic FNAC samples.

The error bars represent 95% confidence intervals.