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Contrast-enhanced ultrasound for sentinel lymph node mapping in the routine staging of canine mast cell tumours: A feasibility study

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Abstract
Canine mast cell tumours (MCTs) typically spread to lymph nodes (LNs) before reaching distant sites, and LN assessment is an important part of MCT staging. Sentinel LN (SLN) mapping techniques to identify draining LNs are being developed and could improve the accuracy of MCT staging. The primary objective of this feasibility study was to determine the safety and effectiveness of contrast-enhanced ultrasound (CEUS) to identify SLNs. Secondary objectives were to determine if the SLNs identified by CEUS coincided with the regional LN predicted by the anatomical lymphosomes, if previous MCT excision altered CEUS SLN findings, and if CEUS could identify MCT nodal metastases. Between June 2017 and March 2019, 59 dogs with 62 MCTs were enrolled. No adverse events related to CEUS were reported. CEUS detected at least 1 SLN in 59/62 MCTs (95.2%, 95% CI: 86.5-99.0%). In only 32/59 (54.2%) MCTs, clinicians would have correctly predicted the SLN(s) identified by CEUS. Among the 35 MCTs that had histological examination of the SLN(s), the prevalence of metastasis was 60% (95% CI: 42.1-76.1%). Additional staging procedures did not reveal any metastases in dogs with histologically non-metastatic SLNs. Integration of CEUS SLN mapping into the routine staging of MCTs is promising, but future studies are required to refine this procedure and to investigate if it would translate into a clinical benefit.

Key words
lymphosonography, mastocytosis, microbubble, sentinel lymph node

1 INTRODUCTION
Regional lymph node (LN) assessment is an important part of staging in canine mast cell tumours (MCTs), as they are generally considered to be the first site of metastasis with this cancer.1,2 Step-wise staging algorithms taking into account LN assessment have been...
proposed, but there is no consensus defining when and how LNs should be assessed. Currently, the choice of the LN(s) to be sampled relies mainly on our knowledge of anatomical lymphosomes in healthy dogs, but several factors may complicate this initial approach: inter-individual variation in the localisation and number of LNs, and alterations in the lymphatic drainage pattern associated with neoplasia and surgery. Another difficulty may be the occurrence of multiple nodal metastases, and some authors have recommended the systematic extirpation of 4 to 6 LNs for the staging of head and neck canine tumours. With a reported 75% sensitivity of fine-needle aspiration cytology (FNAC) to detect MCT nodal metastasis, LN extirpation has also been recommended to complete MCT staging. Lymphadenectomy is an invasive procedure that often necessitates additional surgical preparation and time, extending time under anaesthesia for dogs and costs for owners. It is therefore sometimes difficult to justify when the LN draining status is uncertain and/or when the LN is particularly difficult to remove.

Sentinel lymph node (SLN) mapping techniques have been developed in human oncology to identify the first LN(s) receiving the lymph from tumours that predominantly spread through the lymphatic system. Regional lymphoscintigraphy combined with intra-operative lymphoscintigraphy and blue dye, the gold standard SLN mapping technique in human oncology, has been successfully incorporated into canine MCT staging in previous studies. Lymphoscintigraphy is however, only available in very few veterinary centres. The use of computed-tomography (CT) SLN mapping is mainly restricted to Japan in human oncology, but is becoming a very popular technique among veterinarians. The main advantages being the wide availability of CT, the ability to visualize an entire area at the same time, and to perform 3D-reconstructions. Disadvantages are the necessity for deep sedation or general anaesthesia, cost, risk of adverse reactions to the iodinated contrast and its possible accumulation within the injected tissues and associated LNs for a prolonged period of time, difficulty in determining the ideal time to scan, and in complying with the As Low As Reasonably Achievable radiation safety principle.

Other SLN mapping techniques have been developed in human oncology, including contrast-enhanced ultrasound (CEUS) or lymphophonography, a non-ionizing and non-invasive technique. The potential of this technique was validated in canine models prior to application in human patients diagnosed with breast cancer and cutaneous melanoma. The main advantages are the wide availability of CEUS, low cost, safety and rapid clearance of the contrast, no requirement for general anaesthetic, quick contrast diffusion to the LNs in real-time, the ability to view the fine details of the lymphatics and LNs, and minimal spillover of the contrast to secondary nodes. The main disadvantages are the need to select which lymphatic basins to scan, and inter-operator variability. This technique seems however particularly advantageous for dogs with MCTs as abdominal ultrasound (US) and liver/spleen US-guided FNAC are already often performed for their staging.

Our hypothesis was that CEUS SLN mapping could be integrated into the routine staging of canine MCTs, from both practical and technical aspects. The primary objective was to determine the safety and effectiveness of CEUS to identify SLNs. Secondary objectives were to determine if the SLNs identified by CEUS coincided with the regional LN predicted by the anatomical lymphosomes, if previous excision of the MCT would alter CEUS SLN findings, and if CEUS uptake patterns could identify MCT nodal metastasis.

## 2 | MATERIALS AND METHODS

### 2.1 | Case selection

All dogs included in this feasibility study were presented to the Specialist Oncology service of the University of Edinburgh between June 2017 and March 2019. Dogs were eligible if they were undergoing staging of a cytologically or histologically diagnosed MCT. Dogs with scars of already excised MCTs were also eligible, providing no regional LN was extirpated. Owners signed an informed consent form before enrolment of their dog. The study design, procedure protocol, and informed owner consent form were approved by the Institutional Veterinary Ethical Review Committee.

### 2.2 | Study design

Clinical information was collected, including: location of the primary MCT recorded on a body map, presence of negative prognostic factors (location, recurrence, clinical behaviour, histological grade, proliferation marker, multinucleation and infiltrative pattern for subcutaneous MCTs), size of the mass and/or scar, subjective palpation of the LNs, closest LN to the MCT and the regional LN predicted by the anatomical lymphosomes. A standard list of lymphatic basins to be scanned per body area was suggested and adapted to each dog (Supplementary form 1). Dogs underwent routine procedures for staging of their MCT including serum biochemistry and haematology, thoracic radiographs, abdominal US, liver/spleen FNAC, and regional LNs' FNAC (including the regional LN predicted by the anatomical lymphosomes, the SLN(s) identified by CEUS, and any other LNs of interest identified by the clinician). Dogs received an intramuscular injection of chlorpheniramine (4 mg for dogs <15 kg, 8 mg for dogs ≥15 kg) before lymphosonography, which was performed at the end of the staging workup. Fine-needle aspiration of the identified SLN(s) or any other LN(s) of interest identified by the clinician were aspirated after lymphosonography. Dogs were checked for any potential adverse events by a clinician immediately after lymphosonography and at least 1 hour later and by owners thereafter. Any identified SLNs were advised to be extirpated, but this was not necessary for study inclusion. When it was performed, SLN(s) extirpation was planned after lymphosonography, together with MCT resection or scar re-excision if indicated. Information on the staging procedures and their results, and histological characteristics of the MCTs were collected. Cytological samples and histological sections from identified SLNs were reviewed and classified according to previously published standardized criteria by a board-certified clinical pathologist (P.C.) and by a board-certified anatomical pathologist (A.M.), respectively. Prior to review, histological sections were stained with toluidine blue.
2.3 | Lymphosonography

The contrast agent SonoVue (Bracco Imaging, Milan, Italy), based on stabilized sulphur hexafluoride microbubbles surrounded by a phospholipid shell with a mean size of 2.5 μm, was used for this study. A commercially available US machine (MyLab Twice Esaote, Genova, Italy) was used with an electric microconvex probe. In all examinations, the transmission frequency ranged from 3 to 10 MHz for B-mode imaging, and the manufacturer’s preset for contrast imaging was used with a low mechanical index of 0.1, a frequency of 3 MHz, gain 50%, and a single focus on the lymph node. Images were registered as still images and video clips.

Dogs were positioned in lateral or dorsal recumbency. The relevant lymphatic basins designated by an oncologist were clipped and examined by B-mode US before injection of the contrast. Using a 25-gauge needle, 1 to 2 mL of contrast was injected peri-tumourally with a linear threading technique from the deepest part of the tumour to the superficial dermis, divided in four quadrants within 5 mm of the edge of the mass or scar. Following injection, an antegrade massage was performed for about 30 seconds, with fingers applying light stroking movements over the skin from the injected site towards the LNs. Contrast injections and antegrade massages were administered by one oncologist (Q.F.), or by another clinician under his direction. A timer was started after contrast injection. The lymphatic basins were scanned according to the ordered list provided by the clinician, and the sites of contrast injection were scanned in an attempt to follow afferent lymphatic tracts. A second contrast injection was authorized if no SLN was initially identified.

Only two radiologists (F.T., M.L.) performed all ultrasonographic examinations, and all recorded images from the SLNs were retrospectively blindly reviewed by a board-certified radiologist (F.T.). Collected B-mode and post-contrast sonographic features included: long and short axis diameters, shape, architecture, nodal border characteristics, perinodal steatitis, intranodal calcification or cystic areas, SLN detection time, afferent and efferent lymphatic tracts, enhancement and filling patterns, and acoustic shadowing artefacts.

2.4 | Data analysis

The safety of lymphosonography was assessed based on the documentation of adverse events reported by the clinicians and owners. The effectiveness of lymphosonography was assessed by determining: the SLN detection rate, the prevalence of nodal metastasis detected, and any discrepancy recorded during staging (ie, distant or regional MCT metastasis in the absence of SLN metastasis). The SLN(s) identified by CEUS were compared with the regional LN predicted by the anatomical lymphosomes and with the closest LN to the MCT, and were used to build a reconstructed body map of the MCTs and their lymphatic drainage. The impact of previous MCT excision on lymphosonography was assessed by determining any potential difference in SLN detection rate, number of identified SLNs, prevalence of nodal metastasis detected, and SLN sonographic characteristics between the MCTs that were already excised and the ones that were not. The ability of lymphosonography to identify MCT nodal metastasis was assessed by determining any potential association between recorded SLN sonographic characteristics and SLN histology. Enhancement patterns recorded included: Pattern I, SLN completely homogeneously enhanced; Pattern II, SLN partially enhanced with a disorganized architecture; Pattern III, SLN not enhanced with only afferent tract(s) identified; Pattern IV, SLN with a thin and smooth peripheral enhancing rim and hypo-enhancing centre with a preserved architecture.

Pearson’s chi-squared test and Fisher’s exact test were used to compare categorical data as appropriate. Continuous data were evaluated for normality with the Shapiro-Wilk test. The Mann-Whitney and Kruskal-Wallis tests were used to compare ordinal and continuous non-normally distributed data between two or more groups, respectively. Student’s t test and One-Way Analysis of Variance were used to compare continuous normally distributed data between two or more groups, respectively. Statistical analyses were performed by the free statistics software JASP (Version 0.12, JASP Team 2020). A $P$-value <.05 was considered statistically significant for all analyses.

| TABLE 1 | Characteristics of 59 dogs and 62 mast cell tumours |
| Parameter | Characteristics of 59 dogs and 62 mast cell tumours |
| Age (years) | Median (range) 8.8 (0.3-13.7) |
| Sex | Male 27 (45.8%) |
| | Female 32 (54.2%) |
| Weight (kg) | Median (range) 30.45 (2.5-50.3) |
| Breed | Labrador 18 (30.5%) |
| | Staffordshire terrier 4 (6.8%) |
| | Boxer 4 (6.8%) |
| | Golden retriever 3 (5.1%) |
| | Weimaraner 3 (5.1%) |
| | Other breeds (±2) 16 (27.1%) |
| | Cross breed 11 (18.6%) |
| Mast cell tumour | Subcutaneous 20 (32.3%) |
| | Cutaneous 42 (67.7%) |
| | Patnaik Grade 1 3 (7.1%) |
| | Grade 2 29 (69.1%) |
| | Grade 3 5 (11.9%) |
| | Unknown 5 (11.9%) |
| | Kiupel Low grade 29 (69.0%) |
| | High grade 9 (21.4%) |
| | Unknown 4 (9.6%) |
3 | RESULTS

Fifty-nine dogs with 62 MCTs were enrolled in this study (Table 1) and had CEUS SLN mapping integrated into their routine staging procedures (Figure 1). Two dogs had two concurrent MCTs in different body areas, microbubble destruction naturally occurred between both examinations by switching back to B-mode and waiting 30 minutes before the second CEUS SLN mapping; one dog developed a second MCT in another body area about a year following his first enrolment. Twenty-four out of 62 (38.7%) MCTs were already resected. Median time between previous MCT excision and CEUS SLN mapping was 27 days (range, 13-98). No adverse reaction to CEUS SLN mapping was reported.

3.1 | SLN detection and localisation

A median of five lymphatic basins (range, 2-9) were examined per dog. At least one SLN was identified in 59/62 MCTs (Figure 1). CEUS had a SLN detection rate of 95.2% (95% CI: 86.5-99.0%). Seventy-four SLNs belonging to 1 or 2 lymphatic basins were identified in 48/62 (77.4%) and 11/62 (17.8%) of MCTs, respectively (Figure 1). In four dogs, two nodes were identified as SLNs within the same basin. No contralateral SLN was identified. Previously excised MCTs had a significantly lower number of SLN(s) identified ($P = .034$).

Palpation revealed enlargement of 7/74 (9.5%) SLNs; 18/74 (24.3%) were considered within normal limits and 49/74 (66.2%) were non-palpable. The closest LN to the MCT was the only SLN in 31/59 (52.5%) cases, one of several SLNs in 7/59 (11.9%) cases, and not the SLN in 35.6% (21/59) cases. By following the anatomical lymphosomes of the MCTs, clinicians would have sampled all SLNs in 32/59 (54.2%) cases, one but not all SLNs in 6/59 (10.2%) cases, and no SLN in 21/59 (35.6%) cases. The location of SLNs differed even for MCTs in similar anatomic locations (Table 2). "Zones of ambiguity" and aberrant skin lymphatic drainage were also noted (Figure 2).

3.2 | SLN pathology and clinical staging

A diagnostic cytological sample and histological sample were obtained in 42/74 (56.7%) and 41/74 (55.4%) SLNs, respectively. Overall, a diagnostic sample (cytology or histology) was obtained in 59/74 (79.7%) SLNs (Figure 1). No adverse event associated with FNAC was
reported, whilst mild self-resolving (within 14 days) postoperative seromas were noted on three occasions following extirpation of two mandibular LNs, one mandibular and medial retropharyngeal LNs, and one superficial inguinal LN (ipsilateral popliteal LN also extirpated). When the original histological diagnosis was based on haematoxylin & eosin stained sections, the agreement with the reviewed diagnosis was only moderate (n = 29), whereas it was perfect when sections were already stained with toluidine blue (n = 12; Supplementary form 2). Using the previously proposed cytological and histological classification schemes,\textsuperscript{41,42} cytologically probable and certain metastases accurately identified early and overt histological metastases (n = 6), respectively; but the other cytological classes were unable to differentiate pre-metastasis from early metastasis in this small sample (n = 15; Supplementary form 3).

Diagnostic cytological and/or histological samples were obtained in 18 non-sentinel LNs, and revealed metastasis in nine of them: five medial iliac and one axillary associated with metastatic superficial inguinal SLNs; one superficial inguinal associated with

| TABLE 2 Distribution of identified lymphatic basin(s) for 59 mast cell tumours |
|-----------------|-----------------|-----------------|
| MCT localisation | Lymphatic basin(s) | Number of MCTs |
|-----------------|-----------------|-----------------|
| Head/Neck (n = 10) | Muzzle | Mandibular | 3 |
|                   | Forehead | Mandibular | 1 |
|                   |         | Parotid | 1 |
|                   | Ear base | Medial retropharyngeal | 1 |
|                   | Pinna | Superficial cervical | 2 |
|                   | Neck (ventral) | Medial retropharyngeal | 1 |
|                   |         | Superficial cervical | 1 |
| Forelimb (n = 12) | Shoulder | Superficial cervical | 4 |
|                   |         | Superficial cervical; Axillary | 1 |
|                   | Elbow | Superficial cervical | 1 |
|                   |         | Axillary | 1 |
|                   |         | Superficial cervical; Axillary | 2 |
|                   | Antebrachium | Superficial cervical | 1 |
|                   |         | Axillary | 1 |
|                   | Forepaw | Superficial cervical | 1 |
| Trunk (n = 21) | Thoracic wall | Axillary | 9 |
|                   |         | Axillary; Accessory axillary | 1 |
|                   |         | Superficial inguinal | 1 |
|                   |         | Medial iliac | 1 |
|                   |         | Medial iliac; Colic | 1 |
|                   | Back | Medial iliac; Femoral | 1 |
|                   | Abdominal wall | Superficial inguinal | 1 |
|                   |         | Medial iliac | 1 |
|                   | Groin | Superficial inguinal | 2 |
|                   | Scrotum | Superficial inguinal | 1 |
|                   | Vulva | Superficial inguinal | 1 |
| Hindlimb (n = 16) | Thigh | Superficial inguinal | 4 |
|                   | Stifle | Superficial inguinal | 1 |
|                   |         | Superficial inguinal; Medial iliac | 1 |
|                   | Hock | Popliteal | 3 |
|                   |         | Superficial inguinal | 2 |
|                   |         | Popliteal; Superficial inguinal | 1 |
|                   |         | Popliteal; Medial iliac | 2 |
|                   | Hindpaw | Popliteal | 2 |

Abbreviation: MCT, mast cell tumour.
metastatic medial iliac SLN; one medial retrophyaryngeal associated with metastatic ipsilateral mandibular SLN; one accessory axillary associated with metastatic ipsilateral axillary SLN. Hepatic and/or splenic metastasis was confirmed in three dogs with SLN metastasis, all of which had advanced multinodal metastases including ipsi- and contralateral superficial inguinal and medial iliac LNs. None of the MCTs with histologically non-metastatic SLN(s) had metastasis diagnosed at the end of staging.

In all four MCTs that had two SLNs extirpated, histological classes of the paired SLNs were identical (early metastasis in two MCTs, premetastasis in the other two MCTs). There was no significant difference in SLN histological classes whether the MCTs had been excised before the lymphosonography was performed or not (P = .204). The prevalence of nodal metastasis among the 35 MCTs that had all their SLNs extirpated was 60% (95% CI: 42.1-76.1%). The only clinical/pathological parameter assessed that was associated with histological nodal metastasis was the size of the primary MCT, and all five MCTs >26 mm were metastatic (Table 3).

3.3 | SLN sonographic characteristics

The median total amount of contrast administered was 2 mL (range, 0.8-3). A contrast injection was repeated a second time in 3/5 cases in which no SLN was initially detected, and SLN(s) were eventually identified in two of these cases. Afferent lymphatic tracts were easily identified from the injection sites but were difficult to follow all the way to the SLN(s). The median time for SLN detection was 1 minute 40 seconds (range, 15 seconds - 6 minutes 25 seconds). Four SLNs could not be seen on B-mode US but became clearly visible after taking up contrast (2 superficial inguinal, 1 accessory axillary, 1 colic). Gas artefact causing distal acoustic shadowing was noted in 17/74 (23.0%) SLNs after injection. Efferent lymphatic tract(s) were noted in 12/74 (16.2%) SLNs.

Four main enhancement patterns were identified (Figure 3): Pattern I, 37/74 (50%); Pattern II, 33/74 (44.6%); Pattern III, 2/74 (2.7%); Pattern IV, 2/74 (2.7%).

Pattern II included SLNs with a broad-range of contrast uptake, and a 3-tier filling score (1 to 3) was established to further characterize it: 3, strong and diffuse filling of the SLN with only minor filling defect(s); 18/33 (54.6%); 2, moderate and obviously heterogeneous filling of the SLN, 7/33 (21.2%); 1, minimal and mainly peripheral filling of the SLN, 8/33 (24.2%). The enhancement pattern was the only sonographic parameter significantly associated with histological nodal metastasis (P = .009), but splitting the enhancement pattern II with a filling score did not help discriminate metastatic SLNs (Table 4). No difference was noted in any of the SLN sonographic characteristics from MCT that had or not already been excised, including enhancement patterns (P = .684).

Considering Patterns II and III as metastatic, CEUS only had a moderate agreement with histology (Table 5). Performing FNAC could increase the sensitivity of CEUS alone from 78.3% (95% CI: 56.3-92.5%) to 94.7% (95% CI: 74-99.9%) with the combination but would not have affected the specificity. All the metastatic LNs with a Pattern I only had early metastasis; with the exception of one LN with overt metastasis, which had large sheets of mast cells mostly within the sinuses and only minor disruption of the nodal architecture.

4 | DISCUSSION

Integration of CEUS SLN mapping to MCT staging is technically feasible in routine practice. Most SLNs were clearly visible, and CEUS even allowed the detection of small SLNs that were not visible on B-mode US. The high SLN detection rate and high SLN positivity rate associated with this technique support its effectiveness in identifying SLNs. Sentinel LN(s) enhancement patterns were associated with the metastatic status of the nodes, but a direct clinical application is nonetheless limited as a result of the low sensitivity and specificity. No adverse event was reported following lymphosonography in client-owned dogs with MCTs, which confirms the safety of this technique.27,29,31,32,44

The 95.2% SLN detection rate obtained in this study compares favourably with the 60% to 96.6% and the 91.1% to 100% SLN detection rates reported with CT lymphangiography.18,19,21,45 and
lymphoscintigraphy/intraoperative methylene blue techniques, respectively. In humans, lymphosonography also identifies at least one SLN in 89% to 97% of patients, which is not different from the traditional techniques. An average of 1.2 (74/62) SLNs per MCT was identified using CEUS, whereas an average of 1.6 (88/54) SLNs per MCT was identified with lymphoscintigraphy/intraoperative methylene blue, when compiling the cases from two studies. It is difficult to compare these figures, but it is possible that the dual-labelled technique truly identifies a higher number of LNs. Indeed, radiopharmaceuticals and blue dyes are associated with significant spill-over resulting in the identification of non-sentinel LNs. Significant spill-over has also been confirmed with iodinated contrast in one study investigating CT lymphangiography in dogs, since second and third order LNs were commonly identified. It is assumed that the large particle size of the contrast agent used with lymphosonography prevents its uptake by second order LNs. In addition, it has also been noted that the nodal macrophages can trap (phagocytose) the contrast agent. Nonetheless, efferent lymphatic tracts were detected in a recent study and in this one, making the detection of second order LNs also possible with CEUS.

Based on the results from this CEUS study, performing SLN mapping is necessary to correctly identify draining LNs, since clinicians would have correctly identified all SLNs in only 54.2% MCTs. This may be partly explained by so-called "zones of ambiguity". Table 3 shows the characteristics of 35 mast cell tumours with sentinel lymph node histology.

| MCT parameter | Histological classification | Histological nodal status | P-value |
|---------------|-----------------------------|---------------------------|---------|
|               | Non-metastatic HN0 (n = 4)  | Non-metastatic HN0/HN1 (n = 14) |         |
|               | Pre-metastatic HN1 (n = 10) | Metastatic HN2/HN3 (n = 21) |         |
|               | Early metastasis HN2 (n = 15) | |         |
|               | Overt metastasis HN3 (n = 6) | |         |
| Skin location | 1.0 | | |
| Cutaneous (n = 20) | 2 | 6 | 8 | 4 | 8/20 (40%) | 12/20 (60%) |
| Subcutaneous (n = 15) | 2 | 4 | 7 | 2 | 6/15 (40%) | 9/15 (60%) |
| Patnaik Grade | 1.0 | | |
| Grade 1 (n = 2) | 0 | 1 | 1 | 0 | 1/2 (50%) | 1/2 (50%) |
| Grade 2 (n = 16) | 2 | 4 | 7 | 3 | 6/16 (37.5%) | 10/16 (62.5%) |
| Grade 3 (n = 2) | 0 | 1 | 0 | 1 | 1/2 (50%) | 1/2 (50%) |
| Kiupel Grade | 1.0 | | |
| Low grade (n = 16) | 2 | 4 | 7 | 3 | 6/16 (37.5%) | 10/16 (62.5%) |
| High grade (n = 4) | 0 | 2 | 1 | 1 | 2/4 (50%) | 2/4 (50%) |
| Mitotic count<sup>a</sup> | .132 | | |
| Median (range) | 1.5 (1-2) | 1 (0-8) | 1 (0-35) | 1 (1-10) | 1 (0-8) | 1 (0-35) |
| Tumour size (mm) | .039 | | |
| Median (range) | 10 (8-26) | 11.5 (2-25) | 17 (3-42) | 23(18-50) | 10 (2-26) | 20 (3-50) |
| Negative prognostic factor<sup>b</sup> | .204 | | |
| Median (range) | 0 (0-1) | 0.5 (0-3) | 1 (0-2) | 1 (0-3) | 0 (0-3) | 1 (0-3) |

<sup>a</sup>Number of mitotic figures per 10 high-power fields.

<sup>b</sup>Location (eg, scrotum, muzzle), recurrence, clinical behaviour (eg, ulceration, rapid growth), histological grade (high grade Patnaik and/or Kiupel), proliferation marker (eg, mitotic count >5 for cutaneous and >4 for subcutaneous MCTs, Ki67 index >1.80% laboratory cut-off), multinucleation and infiltrative pattern for subcutaneous MCTs.

Abbreviation: MCT, mast cell tumour.
lymphatic drainage was also noted with the medial retropharyngeal and colic LNs not expected to drain the skin, and the medial iliac and femoral LNs not expected to drain the skin of the scrotum and the rump, respectively. Aberrant lymphatic drainage and the identification of unexpected SLNs is also a well-known phenomenon in human cancers. The most striking example was a MCT on the caudodorsal thoracic wall, which had colic and medial iliac SLNs identified. Remarkably, after completion of this feasibility study, another of our canine patients with a large MCT on the thigh also had a colic SLN identified. We also report an accessory axillary SLN as an interval node to the axillary SLN. Both axillary and accessory axillary nodes had histological early metastasis, which highlights the importance of identifying interval nodes even if they probably occur less frequently in dogs than in human.

The prevalence of histological nodal metastasis in dogs with MCTs was 60%, which is similar to what was previously reported using regional lymphoscintigraphy combined with intra-operative lymphoscintigraphy and blue dye. It is however higher than the 42% previously reported in our institution when SLN mapping was not part of the routine staging, and the 45.9% reported in another study. This supports the effectiveness of CEUS SLN mapping; but unlike in these two retrospective studies, in this current study all histological sections were stained with toluidine blue, which may also have affected these results.

Remarkably, the prevalence of nodal metastasis for subcutaneous MCTs was 60%, whilst it was previously reported to be only 4%. The prevalence of nodal metastasis for cutaneous MCTs was also higher in our study compared with another large retrospective study, in particular for Patnaik grade 2 MCTs (16.2% in previous publication and 62.5% in this study). It is difficult to draw any conclusion on the impact of SLN mapping from this, since the numbers in this feasibility study were small and the quality of LN assessment was variable in these retrospective studies. Nonetheless, this highlights the marked difference in the reported prevalence of nodal metastasis depending on the regional LN(s) staging procedures performed. Following the use of CEUS SLN mapping and standard histological assessment, none of the grading systems nor any other prognostic factors assessed were significantly associated with nodal metastasis. Only the size of the primary tumour was associated with LN metastasis, but tumours as small as 3 mm could still be metastatic. In two previous studies using SLN mapping and standard histological assessment, the presence of recognized prognostic factors did not correlate with nodal metastasis either. Although this feasibility study is not adequately powered to analyse the association between prognostic factors and nodal metastasis, these results suggest extirpation of SLN(s) may be required in all MCTs to achieve an accurate clinical staging. Future studies are needed to confirm these findings and determine if this procedure would also be accompanied with an improved outcome.

In this study, there was no significant difference in SLN histological and sonocharic characteristics whether the MCTs had been excised before the lymphoscintigraphy was performed or not. Sentinel LN biopsy after previous wide local excision of cutaneous melanomas in humans also seems to accurately identify nodal metastases. MCTs that had already been excised had however significantly fewer SLNs identified, which could be explained by the surgical disruption of the natural lymphatic drainage. If this finding is true, then performing wide local excision of the primary tumour before SLN mapping may carry the risk of leaving an undetected metastatic SLN, which could in turn alter the outcome of the dog, since extirpation of metastatic LNs likely has a therapeutic benefit. This should be further investigated as it may have resulted from a selection bias or from a type I error.

**FIGURE 3** Sentinel lymph node enhancement patterns observed with contrast-enhancement ultrasound. Four main enhancement patterns were identified: Pattern I, SLN completely and homogeneously enhanced, A; Pattern II, SLN partially enhanced with a disorganized architecture (D, E, F); Pattern III, SLN not enhanced with only afferent tract(s) identified, B; Pattern IV, SLN with a thin and smooth peripheral enhancing rim and hypoenhancing centre with a preserved architecture, C. Pattern II was further stratified into a 3-tier filling score: 3, strong and diffuse filling of the SLN with only minor filling defect(s) (D); 2, moderate and obviously heterogeneous filling of the SLN (E); 1, minimal and mainly peripheral filling of the SLN, F. For each subfigure, the B-mode image is on the left side, with the corresponding CEUS image on the right side. CEUS, contrast-enhanced ultrasound; SLN, sentinel lymph node.
| US parameter                  | Histological classification | Histological nodal status | P-value |
|------------------------------|----------------------------|---------------------------|---------|
|                              | Non-metastatic HN0 (n = 5) | Pre-metastatic HN1 (n = 13) | Early metastasis HN2 (n = 17) | Overt metastasis HN3 (n = 6) | Non-metastatic HN0/HN1 (n = 18) | Metastatic HN2/HN3 (n = 23) |
| B-mode US                    |                           |                           |                           |                           |                           |                          |
| SLN short axis (mm) Median (range) | 5.3 (3-7.2)               | 4.7 (2.8-6.8)             | 4.8 (1.9-12.4)            | 7.7 (6-19.6)              | 4.4 (2.8-7.2)              | 6 (1.9-19.6)              |
| SLN long axis (mm) Median (range) | 15.7 (11-21)              | 17.75 (10.9-30)           | 15.6 (7.9-22.4)           | 19.9 (10-31.7)            | 17.1 (10.9-30)             | 15.8 (7.9-31.7)           |
| SLN long/short axis ratio Median (range) | 3.15 (2-5.1)              | 3.65 (2.2-5.4)            | 2.33 (1.8-4.9)            | 2.05 (1.7-4)              | 3.45 (2-5.4)               | 2.3 (1-7.4)               |
| Echotexture                  | Homogeneous                | 4                         | 13                        | 15                        | 2                         | 17/34 (50%)               |
|                              | Heterogeneous              | 1                         | 0                         | 2                         | 4                         | 1/7 (14.3%)               |
| Echogenicity                 | Normal                     | 3                         | 11                        | 11                        | 2                         | 14/27 (51.9%)             |
|                              | Hypoechoic                 | 2                         | 2                         | 6                         | 3                         | 4/13 (30.8%)              |
|                              | Hyperechoic                | 0                         | 0                         | 0                         | 1                         | 0/1 (0%)                  |
| Shape                        | Oval                       | 2                         | 11                        | 8                         | 1                         | 13/22 (59.1%)             |
|                              | Oval plump                 | 3                         | 2                         | 7                         | 4                         | 5/16 (31.3%)              |
|                              | Round                      | 0                         | 0                         | 2                         | 1                         | 0/3 (0%)                  |
| Perinodal steatitis          | Present                    | 0                         | 2                         | 2                         | 2                         | 2/6 (33.3%)               |
|                              |Absent                      | 5                         | 11                        | 15                        | 4                         | 16/35 (45.7%)             |
| CEUS                         | Enhancement pattern        |                           |                           |                           |                           |                           |
| Pattern I                    | 5                          | 6                          | 4                          | 4                          | 1                          | 11/16 (68.8%)             |
| Pattern II                   | 0                          | 6                          | 13                         | 13                        | 4                          | 6/23 (26.1%)              |
| Pattern III                  | 0                          | 0                          | 0                          | 0                          | 0                          | 0/1 (0%)                  |
| Pattern IV                   | 0                          | 1                          | 0                          | 0                          | 1                          | 1/1 (100%)                |
| Filling score (Pattern II)   | 3 (strong filling)         | 0                          | 3                          | 6                          | 2                          | 3/11 (27.3%)              |
|                              | 2 (moderate filling)       | 0                          | 2                          | 2                          | 1                          | 2/5 (40%)                 |
|                              | 1 (mild filling)           | 0                          | 1                          | 5                          | 1                          | 1/7 (14.3%)               |

Abbreviations: CEUS, contrast-enhanced ultrasound; SLN, sentinel lymph node; US, ultrasound.
Three of the four enhancement patterns described in this study (Patterns I, II and III) were previously proposed in breast cancer CEUS SLN mapping. We also reported an additional pattern with a thin and smooth peripheral enhancing rim (Pattern IV) which was noted in two SLNs. This was evocative of a particular type of lymph node structure, when the lymphatic vessel runs over the surface of the LN without discharging its content, which is one of the explanations proposed for false-negative SLNs and skip metastasis. This remains speculative, however, and this pattern would need to be reported in other studies before its relevance can be confirmed.

This feasibility study had several limitations. First, SLNs identified by CEUS were generally not marked, and it is therefore difficult to confirm the LNs extirpated were the ones identified by CEUS. This is the disadvantage of preoperative techniques, which could be overcome by deployment of a guidewire for instance. It could also be possible to inject SLNs preoperatively with blue dye, or to combine CEUS with intraoperative blue dye SLN mapping. The systematic combination of CEUS with a blue dye technique could also allow a better visualization of the SLNs to extirpate, as 10 SLNs could not be found in surgery in this study. Second, although we made every effort to be consistent in the selection of the lymphatic basins assessed by CEUS, this procedure remains difficult to standardize, and this may have affected the results of this study. Third, CEUS was not directly compared with other SLN mapping techniques such as lymphoscintigraphy or blue dye injection. Fourth, SLN enhancement Pattern III may be subtle and might have been missed in a few markedly enlarged LNs identified as non-sentinel. This should be more carefully examined in future studies. Fifth, it is difficult to rule out that some medial iliac SLNs may have been second order LNs to popliteal and superficial inguinal SLNs. Sixth, not all SLNs were histologically assessed.

**CONCLUSION**

CEUS SLN mapping is safe and associated with a high SLN detection rate. Given the absence of discrepancy with the rest of clinical staging and the high positive LN rate, this technique seems to accurately predict the metastatic status of dogs with MCTs. Draining LNs are difficult to correctly identify, and SLN mapping should be recommended before sampling LNs in dogs with MCTs. Excising MCTs before CEUS SLN may alter the results. Enhancement patterns only had a moderate agreement with SLN histological metastatic status. Integration of CEUS SLN mapping into the routine staging of MCTs is promising, but future studies are required to refine this procedure and to investigate if it would translate into a clinical benefit.

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