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Incorporation of pre- and intra-operative sentinel lymph node mapping techniques in dogs with apocrine gland anal sac adenocarcinoma

Maureen A. Griffin[1,3*], Brian K. Flesner[1], Deanna R. Worley[2], David E. Holt[1], Nimar Gill[1], Audrey Ghanian[1], Mia Talone[1] and Jennifer Reetz[1]

Abstract

The high incidence of nodal metastasis, variable lymphatic drainage patterns, and prognostic significance of nodal metastasis in dogs with apocrine gland anal sac adenocarcinoma (AGASACA) highlight the need for development of standardized techniques for lymph node staging in canine AGASACA. The aim of this study was to develop and describe the utility of pre- and intra-operative sentinel lymph node (SLN) mapping techniques for subsequent nodal extirpation and histologic assessment in dogs with AGASACA. A prospective clinical trial was performed as a pilot study. Eight client-owned dogs with unilateral AGASACA were enrolled. Preoperative contrast-enhanced ultrasound (CEUS) via transrectal ultrasound (TRUS) and CT-lymphography (CTL) was followed by intraoperative SLN mapping within 7 days utilizing a visible dye (methylene blue [MB]) and a near-infrared (NIR) fluorescent dye (indocyanine green [ICG]) with subsequent nodal extirpation and routine anal sacculectomy. In all dogs, preoperative CTL and intraoperative SLN mapping identified at least one SLN. Pre- and intra-operative findings differed in 4/8 dogs. Preoperative CEUS identified a SLN in 7/8 dogs, but the exact location of the SLN was indeterminate. Extirpated lymph nodes were metastatic in 2/8 dogs. Preoperative CTL and intraoperative MB and ICG were an effective combination of SLN mapping techniques in dogs with AGASACA. These techniques each provided unique advantages. Combined, they identified and guided extirpation of early metastatic lymph nodes. Large-scale, prospective studies utilizing the techniques described are needed to accurately determine the incidence and significance of early/occult nodal metastasis in canine AGASACA.

Keywords Sentinel lymph node, Apocrine gland anal sac adenocarcinoma, Canine, CT-lymphography, Contrast-enhanced ultrasound, Near-infrared, Methylene blue

Introduction

Apocrine gland anal sac adenocarcinoma (AGASACA) is a relatively common tumor affecting the anal sacs of dogs. The incidence of metastasis is high, with 47–96% of dogs reported to have locoregional lymph node metastasis [1–8]. Detection and extirpation of metastatic lymph nodes is important in improving both prognostication and outcomes in dogs with AGASACA. Dogs with metastatic nodes have shorter disease-free intervals and

*Correspondence:
Maureen A. Griffin
mag493@cornell.edu
[1] Department of Clinical Sciences and Advanced Medicine, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA, USA
[2] Department of Clinical Sciences, Flint Animal Cancer Center, Colorado State University, Fort Collins, CO, USA
[3] Current affiliation: Cornell University College of Veterinary Medicine, Ithaca, NY, USA

overall survival times compared to dogs without nodal metastasis, and surgical extirpation of metastatic nodes improves outcomes [7, 9–14].

Determining if and where nodal metastasis has occurred is challenging for several reasons: (1) The locoregional lymph nodes are the iliosacral lymphocentrum, comprised of both ipsilateral and contralateral medial iliac, internal iliac (previously termed hypogastric), and sacral lymph nodes, with considerable individual variation in drainage pattern from the anal sac to these nodes [15–17]. (2) These lymph nodes are generally not palpable due to their deep intraabdominal/intrapelvic locations. (3) Computed tomography (CT) and ultrasonography are relatively inaccurate in detecting early nodal metastasis, and abdominal ultrasonography is unable to visualize sacral lymph nodes within the pelvic canal [18–20]. (4) Iliosacral lymph nodes with early metastatic disease can be difficult to detect intraoperatively owing to the small size of these lymph nodes which are surrounded by retroperitoneal fat and located adjacent to important vascular and urinary structures.

Sentinel lymph node (SLN) mapping techniques that identify the first tumor-draining lymph node(s) in a setting without overt nodal metastasis are utilized for multiple tumor types in veterinary and human oncology, and these techniques have become widely regarded as standard of care for staging in melanoma and breast cancer in people [21, 22]. Preoperative SLN mapping with indirect CT-lymphography (CTL), utilizing locally applied iodinated contrast to detect the SLN by serial CT imaging, is the only SLN mapping technique described for dogs with AGASACA to date [15]. It has a reported success rate of 92% in identifying the SLN [15]. Contrast-enhanced ultrasound (CEUS) is a SLN mapping technique in which a sonographic contrast agent with small microbubbles is injected peritumorally. It has been well described with high SLN detection rates for human breast cancer and is being studied for SLN mapping in dogs with mast cell tumors and other conditions [23–26]. Though routine abdominal ultrasonography cannot evaluate sacral lymph nodes within the pelvic canal, transrectal ultrasound (TRUS) can evaluate intrapelvic disease such as rectal tumors and peri-rectal lymph nodes in people [27]. Its utility has been characterized for canine prostatic tumors and the medial and internal iliac lymph nodes of those dogs [28]. TRUS has not yet been evaluated for sacral lymph node characterization in dogs. In addition, intraoperative SLN mapping techniques involve localized injection of visible dyes (e.g., methylene blue [MB]) and/or near-infrared (NIR) fluorescent dyes (e.g., indocyanine green [ICG]) [21, 26, 29–31]. To date, intraoperative SLN mapping in dogs with AGASACA has not yet been reported in any peer-reviewed manuscript. As pre- and intra-operative SLN techniques have unique strengths and weaknesses, and there is variable agreement between them, a combination of preoperative and intraoperative techniques may provide optimal detection of SLNs. This has been demonstrated for canine oral tumors [29].

Our primary aim was to develop and describe SLN mapping techniques in canine AGASACA via 1) preoperative CEUS with TRUS in conjunction with CTL and 2) intraoperative MB and ICG/NIR. Results of this study will provide insight into the feasibility of SLN mapping in canine AGASACA and allow opportunities to study the long-term outcomes of dogs with early nodal metastasis detected and extirpated via SLN mapping.

Methods

A prospective, pilot clinical trial was performed at the University of Pennsylvania Matthew J. Ryan Veterinary Hospital. Inclusion criteria were as follows: client-owned dogs with unilateral, cytologically confirmed AGASACA amenable to surgical excision, with no prior treatment of the AGASACA via surgery, radiation therapy, or chemotherapy. In addition, as SLN mapping is predominantly useful in a setting without overt nodal metastasis, dogs had to have no definitive evidence of nodal metastasis (characterized by effaced/enlarged lymph nodes $\geq$ 1 cm thickness) on the basis of rectal examination and abdominal ultrasonography performed by a boarded radiologist [32, 33]. Routine preoperative staging including labwork, thoracic radiographs, and complete abdominal ultrasonography were performed in all dogs prior to enrollment, and no overt distant metastatic disease or contraindications to general anesthesia and surgery were needed for enrollment. Written informed client consent was obtained for each dog prior to enrollment. The study protocol was approved by the hospital's Institutional Animal Care and Use Committee Office of Animal Welfare and Privately Owned Animal Protocol (POAP) #638.

Dogs underwent general anesthesia for ultrasonography and CT imaging. A single boarded veterinary radiologist (JR) performed all focal lymph node and transrectal ultrasound scans in conjunction with CEUS and evaluated all CTL studies; machine and transducer information is provided in the supplemental materials. All dogs were fasted for 24 h prior to imaging. Routine abdominal ultrasound (B-mode) was followed by TRUS for characterization of all visible medial iliac, internal iliac, and sacral lymph nodes. Prior to TRUS, dogs were positioned in supported sternal recumbency as previously described for SLN mapping via CTL, and fecal material was digitally evacuated from the rectum [15]. CEUS was performed via peritumoral contrast injection with ultrasonographic imaging of the locoregional lymph nodes [25]. Definity (perflutren lipid microsphere injectable

suspension [Lantheus, Inc.; Billerica, MA, USA]) was used as a sonographic contrast agent for CEUS, and 1 mL was divided equally into four injections and administered percutaneously with a 25 Ga needle into the tissues immediately adjacent to the tumor edge. Injections were performed slowly with negative aspiration prior to injection to avoid injection into vasculature, and a 30 s intrarectal and percutaneous massage of the peritumoral tissues was performed following injection. The iliosacral lymph nodes were then imaged ultrasonographically (via transrectal and potentially also transabdominal imaging) to document afferent lymphatic tracts and SLNs with contrast uptake. Under the same anesthetic event and with the same supported sternal positioning, dogs subsequently underwent routine pre- and post-IV contrast CT of the abdomen and perianal region followed by indirect CTL via peritumoral contrast injection for SLN mapping as previously described [15, 34]. Omnipaque (iohexol 350 mg/mL [GE Healthcare; Chicago, IL, USA]) was used as an iodinated contrast agent for CTL, with 1 mL Omnipaque diluted with 1 mL sterile saline, and a percutaneous peritumoral four quadrant injection was administered with a 25 Ga needle into similar locations and with similar technique as per CEUS. Following a 30 s massage, serial CT images were obtained at 1- and 3-min post-injection, then every 3 min until the SLN was identified, at which time serial imaging was discontinued. AGASACA and lymphatic imaging features were recorded.

Within 7 days of CEUS and CTL, intraoperative SLN mapping with lymph node extirpation was performed followed by standard of care anal sacculectomy for excision of the primary tumor. The individual that administered the peritumoral contrast agent was the same for the CEUS, CTL, and intraoperative SLN mapping studies for each dog to replicate the locations and technique used for each study. Patients were positioned in dorsal recumbency with the rectum near the end of the table, and percutaneous peritumoral four quadrant injection of ICG and MB was performed following (caudal) abdominal exploration; no nodal dissections were initiated prior to injection. A 1 mL solution with 0.5 mg (0.2 mL of 2.5 mg/mL suspension) ICG (Diagnostic Green, LLC; Farmington Hills, MI, USA), 2.0 mg (0.4 mL of 5.0 mg/mL suspension) MB (Cenexi; Fontenay-sous-Bois, France), and 0.4 mL sterile saline was injected with a 25 Ga needle via similar locations and technique as per preoperative SLN mapping, with a subsequent 30 s peritumoral massage. During injection, abdominal organs (including the bladder, colon, and small intestines) were retracted to minimize pressure on the caudal abdominal lymphatics and allow for inspection of the iliosacral lymphatics and lymph nodes. Immediately following injection, the caudal abdomen and pelvic canal was inspected under visible light and NIR imaging. Intraoperative NIR imaging was performed with an IMAGE1 S Rubina and 10 mm NIR-compatible endoscope (Karl Storz; Tuttlingen, Germany). Any lymphatic tracts and lymph nodes identified via NIR fluorescence or visible dye were recorded. Any SLNs detected via intraoperative or preoperative imaging, abnormal lymph nodes on gross or imaging assessment, and other readily identifiable iliosacral lymph nodes were then dissected and extirpated. Semiquantitative scoring of ICG fluorescence (with NIR imaging) and MB staining (with visible light) in each extirpated lymph node was performed both in situ and following extirpation as previously described: 0 = no fluorescence/blue, 1 + = 25% lymph node fluorescent/blue, 2 + = 50% lymph node fluorescent/blue, and 3 + = ≥ 75% lymph node fluorescent/blue [29, 35]. Imaging and scoring of extirpated lymph nodes was performed postoperatively with a VisionSense Iridium NIR imaging system (Medtronic; Swedesboro, NJ, USA). The abdomen was closed routinely, and the dog was repositioned into sternal recumbency with the pelvic limbs hanging and tail positioned cranially, the perianal region was prepared for surgery, and routine closed anal sacculectomy was performed for excision of the primary tumor. The anal sac tumor and all extirpated lymph nodes were submitted for histopathologic analysis. Postoperatively, all dogs were treated and monitored according to standard of care. Any adverse events associated with surgery or SLN mapping techniques were recorded.

Patient demographics and clinical findings were recorded, and descriptive statistics were calculated. Results of each SLN mapping technique were documented, and histopathologic results were recorded.

Results

Eight dogs were included in the study. The following dog breeds were represented: mixed breed (3), German Shepherd (1), Beagle (1), Australian Shepherd (1), Miniature Schnauzer (1), and Yorkshire Terrier (1). Seven dogs were male castrated, and one dog was female spayed. The median age was 10.3 years (range 4.4–12.8). The median body weight was 18.8 kg (range 5.8–44.2).

All dogs had a unilateral anal sac mass with median maximal diameter of 2.8 cm (range 1–6) on physical examination at the time of presentation to the referral hospital. No sacral lymphadenopathy was appreciated, and the rectal wall was noted to be mobile over the anal sac tumor on rectal examination in all cases. No dogs were hypercalcemic on pre-anesthetic bloodwork, and all dogs were deemed to be systemically healthy and amenable to general anesthesia for trial imaging. All dogs underwent three-view thoracic radiographs and

complete abdominal ultrasound performed by a boarded radiologist with no overt evidence of nodal or distant metastatic disease identified in any case.

Pertinent findings of the preoperative imaging studies are reported for each dog in Tables 1 and S1. All lymph node abnormalities on preoperative imaging were deemed to be reflective of benign reactive changes or early metastatic disease, with benign reactive changes generally prioritized. All iliosacral lymph nodes were < 1 cm maximal thickness on preoperative imaging (both ultrasound and CT). B-mode TRUS resulted in visualization of at least one iliosacral lymph node in 3/8 dogs, but the exact lymph node that was being evaluated was not definitively determined in any case. SLN mapping via CEUS with TRUS revealed rapid contrast uptake into lymphatics in all cases and enabled identification of at least one SLN within 3 min of peritumoral injection in 7/8 dogs, although the exact localization of the SLN was not feasible in any case. Figure 1 depicts images from SLN mapping via CEUS with TRUS in one dog. In the smallest two dogs of this study (5.8 kg [Dog 8] and 9.5 kg [Dog 7]), SLN mapping was technically challenging given the transducer relative to rectum size and both transrectal and transabdominal imaging with CEUS were applied, though in both cases a SLN was identified transrectally with the probe manipulated just beyond the anus and oriented to image cranially. On pre-lymphography CT, the median maximal anal sac tumor diameter was 2.8 cm (range 1.5–5.5). SLN mapping via CTL revealed rapid contrast uptake into lymphatics and enabled identification of at least one SLN within 1 min of peritumoral injection in 7/8 dogs and within 3 min of peritumoral injection in the remaining dog. The median number of SLNs identified on CTL was 2 (range 1–5). Identified SLNs on preoperative CTL included sacral lymph nodes (7 dogs), internal iliac lymph nodes (4 dogs), and medial iliac lymph nodes (3 dogs). Lymph nodes identified as sentinel on CTL were only ipsilateral to the tumor in 6 dogs and both ipsilateral and contralateral to the tumor in 2 dogs.

Other imaging/diagnostic findings of note are included in supplemental data. No dogs experienced any adverse events directly related to preoperative SLN mapping, and all dogs recovered uneventfully from general anesthesia. Only one dog (Dog 7) had an iliosacral lymph node that was deemed to be sentinel and/or abnormal in appearance that was amenable to fine needle aspiration (FNA). In this dog, FNA of a right caudal internal iliac lymph node (8 mm maximal thickness with mild heterogeneous contrast enhancement on CT, not identified as a SLN on CTL) was performed, and cytology was consistent with metastatic AGASACA and reactive lymphoid hyperplasia. All other iliosacral lymph nodes were deemed too small or inaccessible to safely obtain an FNA preoperatively.

Surgery with intraoperative SLN mapping and anal sacculectomy was performed 1 day following preoperative SLN mapping in 6/8 dogs, 2 days following preoperative SLN mapping in 1/8 dog, and 6 days following preoperative SLN mapping in 1/8 dog. Intraoperative SLN mapping results are characterized for each dog in Tables 1 and S1. For Dog 2, the initial intraoperative peritumoral injection was not successful in identifying lymphatic tracts or lymph nodes within approximately 5 min. A repeat injection with 1 mL ICG (2.5 mg/mL) was performed in a similar four quadrant peritumoral fashion with a 25 Ga needle, and thereafter intraoperative SLN mapping was effective. SLNs were identified intraoperatively in all dogs. A median of 3 SLNs (range 1–4) were identified intraoperatively, and all intraoperative SLNs were extirpated. Five dogs had additional uptake of ICG and/or MB noted in lymph nodes following SLN identification, and these were deemed secondary (or higher tier) lymph nodes; these secondary lymph nodes were also extirpated, as well as any other iliosacral lymph nodes identified intraoperatively. A median of 4 iliosacral lymph nodes (range 3–6) were extirpated for each dog. Identified intraoperative SLNs included sacral lymph nodes (6 dogs), internal iliac lymph nodes (5 dogs), and medial iliac lymph nodes (5 dogs). Lymph nodes identified as sentinel intraoperatively were only ipsilateral to the tumor in 4 dogs and both ipsilateral and contralateral to the tumor in 4 dogs. Figure 2 depicts images from SLN mapping for one dog.

In 4/8 dogs (Dogs 1, 3, 4, and 8), SLNs identified on preoperative CTL and intraoperative SLN mapping were identical, with 1 SLN identified in two dogs, 3 SLNs identified in one dog, and 4 SLNs identified in one dog. In 3/8 dogs (Dogs 2, 5, and 7), the SLN(s) identified on preoperative CTL (1 in two dogs and 3 in one dog) were also determined to be SLN(s) on intraoperative assessment, but additional SLNs (1 in one dog, 3 in one dog, and 4 in one dog) were also identified intraoperatively. In 1/8 dog (Dog 6), 1 SLN was found to be similar between preoperative CTL and intraoperative SLN mapping, with an additional 4 lymph nodes detected as sentinel on CTL and not identified as sentinel at surgery and an additional 1 lymph node identified as sentinel at surgery and not detected as sentinel on CTL. In this case, only 1 of the 4 SLNs on CTL was identified and extirpated at the time of surgery, and the remaining 3 lymph nodes were not readily identified given their lack of NIR fluorescence or blue coloration (deemed not to be intraoperative SLNs), very

Table 1 Results of preoperative and intraoperative SLN mapping for each dog as well as histology of all extirpated iliosacral lymph nodes. Left and right are denoted as "L" and "R

Dog Number (AGASACA laterality, maximal dimension on CT)	CEUS: SLN	Indirect CTL: SLN	Intraoperative SLN(s): In situ scoring (ICG and MB)	Intraoperative SLN(s): Ex vivo scoring (ICG and MB)	All extirpated iliosacral LNs: Histology results
[a]1 (L, 2.5 cm)	SLN identified and suspected to be a sacral lymph node, but unable to determine laterality	-L sacral -L internal iliac -L medial iliac	-L internal iliac: 3 + MB -L medial iliac: 2 + MB -L sacral: 3 + MB	-L internal iliac: 3 + ICG, 3 + MB -L medial iliac: 2 + ICG, 3 + MB -L sacral: 3 + ICG, 3 + MB	-L internal iliac: metastatic AGASACA -L medial iliac: metastatic AGASACA -L sacral: metastatic AGASACA
2 (L, 3.2 cm)	SLN not identified	-L sacral	-L sacral: 3 + MB, 3 + ICG -L medial iliac: 3 + MB, 3 + ICG -R medial iliac:3 + MB, 3 + ICG	-L sacral: 3 + MB, 3 + ICG -L medial iliac: 3 + MB, 3 + ICG -R medial iliac: 3 + MB, 3 + ICG	-L sacral: drainage reaction -L medial iliac: drainage reaction -R medial iliac: drainage reaction -L cranial medial iliac: drainage reaction
3 (R, 3.2 cm)	SLN identified and suspected to be a sacral lymph node, but unable to determine laterality	-R sacral	-R sacral: 3 + MB, 3 + ICG	-R sacral: 3 + MB, 0 ICG	-R sacral: drainage reaction -L sacral: drainage reaction -R medial iliac: drainage reaction
4 (L, 1.8 cm)	SLN identified and suspected to be a medial or internal iliac lymph node, but unable to determine laterality	-L medial iliac	-L medial iliac: 3 + MB, 3 + ICG	-L medial iliac: 3 + MB, 2 + ICG	-L medial iliac: drainage reaction - L cranial medial iliac: drainage reaction -R medial iliac: drainage reaction
5 (L, 5.5 cm)	SLN identified and suspected to be an internal iliac lymph node, but not definitive and unable to determine laterality	-L sacral	-L sacral: 3 + MB, 3 + ICG -L internal iliac: 3 + MB, 3 + ICG -L medial iliac: 3 + MB, 3 + ICG -R medial iliac: 3 + MB, 3 + ICG	-L sacral: 3 + MB, 3 + ICG -L internal iliac: 3 + MB, 3 + ICG -L medial iliac: 3 + MB, 3 + ICG -R medial iliac: 3 + MB, 3 + ICG	-L sacral: drainage reaction -L internal iliac: drainage reaction -L medial iliac: drainage reaction -R medial iliac: drainage reaction -R internal iliac: drainage reaction
6 (R, 1.5 cm)	SLN identified and suspected to be a sacral lymph node, but unable to determine laterality	-Cranial L sacral -Caudal L sacral -Cranial R sacral -Caudal R sacral -R internal iliac	-L internal iliac: 3 + MB, 1 + ICG -R internal iliac: 3 + MB, 3 + ICG	-L internal iliac: 3 + MB, 1 + ICG -R internal iliac: 3 + MB, 2 + ICG	-L internal iliac: drainage reaction -R internal iliac: drainage reaction -R medial iliac: drainage reaction -R sacral: drainage reaction -L medial iliac: drainage reaction
7 (R, 2.5 cm)	SLN considered to be either/both: sacral lymph node (unknown laterality), cranial R internal iliac lymph node	-R lateral sacral -R medial sacral -R cranial internal iliac	-R cranial internal iliac: 3 + MB, 3 + ICG -R caudal internal iliac: 3 + MB, 3 + ICG -R lateral sacral: 3 + MB, 3 + ICG -R medial sacral: 3 + MB, 3 + ICG	-R cranial internal iliac: 3 + MB, 3 + ICG -R caudal internal iliac: 2 + MB, 3 + ICG -R lateral sacral: 3 + MB, 3 + ICG -R medial sacral: 3 + MB, 3 + ICG	-R cranial internal iliac: metastatic AGASACA -R caudal internal iliac: metastatic AGASACA -R lateral sacral: metastatic AGASACA -R medial sacral: drainage reaction -R medial iliac: drainage reaction -L medial iliac: drainage reaction
8 (R, 3.0 cm)	SLN identified and suspected to be a sacral lymph node, but unable to determine laterality	-R sacral -R internal iliac -R medial iliac -L medial iliac	-R sacral: 3 + MB, 3 + ICG -R internal iliac: 3 + MB, 3 + ICG -R medial iliac: 3 + MB, 3 + ICG -L medial iliac: 3 + MB, 3 + ICG	-R sacral: 3 + MB, 3 + ICG -R internal iliac: 3 + MB, 3 + ICG -R medial iliac: 3 + MB, 3 + ICG -L medial iliac: 3 + MB, 0 ICG	-R sacral: drainage reaction -R internal iliac: drainage reaction -R medial iliac: drainage reaction -L medial iliac: drainage reaction

[a] For Dog 1, the NIR equipment was not functioning appropriately at the time of surgery, so NIR fluorescence of lymph nodes was evaluated postoperatively ex vivo and not in situ

small size (ranging from 1.7 to 4.2 mm maximal thickness), and locations within the pelvic canal.

Extirpated iliosacral lymph nodes were determined to be metastatic AGASACA in 2/8 dogs (Dog 1 and Dog 7). For Dog 1, all lymph nodes (3) deemed to be sentinel on both CTL and intraoperative imaging were found to be metastatic. For Dog 7, 2/3 lymph nodes deemed to be sentinel on both CTL and intraoperative imaging were found to be metastatic, and an additional lymph node deemed to be sentinel on intraoperative imaging alone was found to be metastatic. All other extirpated iliosacral lymph nodes were consistent with nodal drainage reaction on histopathology. Additional abdominal procedures and respective histopathologic findings are summarized in supplemental data.

Following SLN mapping with lymph node extirpations, the abdomen was closed routinely and standard of care anal sacculectomy was performed. MB/ICG stain uptake and slightly edematous peritumoral tissues were noted during dissection (Fig. 3). In all cases, the anal sac and mass were able to be excised en bloc with no residual gross disease, though for one dog (Dog 8), preoperative tumor rupture near the rectal wall and site of peritumoral injection(s) had occurred with leakage of fluid in this site. In this dog, the tumor and anal sac were excised en bloc, and the surgical site was copiously lavaged with sterile saline prior to closure. Samples of the peritumoral tissue were obtained for culture and histopathology, and the dog was maintained on antimicrobials (amoxicillin/clavulanic acid, transitioned to chloramphenicol based upon culture/sensitivity results) for 10 days postoperatively. In addition, one dog (Dog 6) was found to have a discrete nodular structure adjacent to the anal sac mass that was also excised. Histopathology of the anal sac tumor confirmed AGASACA in all cases. The peritumoral tissue near the site of rupture was non-neoplastic in Dog 8, and the adjacent/satellite nodule was confirmed to also be AGASACA in Dog 6. The histologic tumor type was solid in 3/8 dogs and tubular in 5/8 dogs. Lymphovascular invasion was not identified in any primary tumor. The median mitotic count (number of mitotic figures per 10 hpf) was 6 (range 1–20). Histologic margins were complete in 3/8 dogs, and neoplastic cells were identified at the surgical margins (i.e. no intact anal sac capsule/lining peripheral to the mass) in 5/8 dogs. No intraoperative complications occurred.

All dogs recovered uneventfully from anesthesia and surgery. All dogs were discharged with a median of 2 days (range 1–3) hospitalization postoperatively. One dog (Dog 4) developed a superficial surgical site infection of the anal sacculectomy site postoperatively. This was managed successfully with a short course of antimicrobials (amoxicillin/clavulanic acid). No other adverse events associated with anesthesia or surgery occurred in any dog. The dog with the suspected preoperative tumor rupture (Dog 8) had no evidence of surgical site complications postoperatively.

No adjuvant treatments were elected or pursued in any case. Follow-up thoracic and abdominal imaging was performed in 4/8 dogs. Median duration of follow-up was 342 days (range 42–497) postoperatively, and all dogs were alive at last follow-up. All dogs retained fecal continence. At the time of last follow-up, no dogs had documented recurrence of local disease or onset/progression of metastatic disease.

Discussion

This is the first study to describe both preoperative and intraoperative SLN mapping techniques for dogs with unilateral AGASACA with concurrent extirpation of lymph nodes for histologic analysis. In the present trial, we found a combination of preoperative indirect CTL and intraoperative NIR imaging with ICG and visible dye assessment with MB to be an effective combination of SLN mapping techniques. Overall, due to the SLN mapping techniques utilized in this trial, metastatic lymph nodes were identified and removed in 2/8 dogs that did not have overt evidence of nodal metastasis on routine preoperative imaging. This finding should be considered clinically relevant, as nodal metastasis is an important determinant of staging and has been associated with a worse prognosis in dogs with AGASACA [7].

In general, CTL proved valuable in both identifying SLNs preoperatively as well as characterizing the iliosacral lymph node sizes and locations relative to anatomical landmarks and vascular structures (via pre- and post-IV contrast scans) for use as a surgical planning tool. Alternatively, intraoperative mapping techniques proved valuable in both identifying the SLNs as well as guiding dissection for safe and efficient extirpation of these very small lymph nodes deep to retroperitoneal fat and adjacent to vascular structures. Whereas ICG and NIR imaging often quickly identified lymphatic tracts and fluorescence in the region of the SLN(s), once dissection for a given lymph node was initiated, the OR and room lights were turned back on and NIR guidance was no longer feasible with the equipment utilized in this study, such that the findings under visible light with MB identification of lymphatic structures became very useful. When iliosacral lymph nodes were not found to be fluorescent or blue, their identification proved more challenging owing to the very small (not overtly metastatic) nature and anatomical locations. As depicted in Table 1, all intraoperative SLNs identified had both uptake of ICG and MB detected in vivo, though ex vivo, two intraoperative SLNs demonstrated

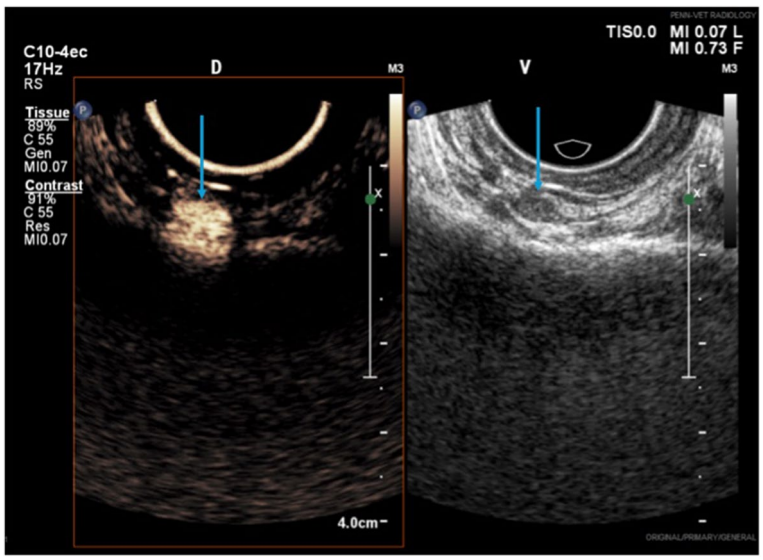

Fig. 1 SLN mapping via CEUS and TRUS in Dog 1. The image on the left is with contrast enhancement, and the image on the right is the concurrent B-mode. The blue arrows in each image point to the lymph node (suspected sacral node) identified to be sentinel on CEUS, with complete and homogeneous enhancement of this node. Images were obtained approximately 2 min post-peritumoral injection

uptake of MB only with suspected washout of ICG from the node occurring in the timeframe for dissections. Ultimately, each of these three techniques (preoperative CTL, intraoperative ICG/NIR, intraoperative MB) in combination provided important information in characterizing the SLNs for each dog and enabling safe nodal extirpations without any observed intraoperative or postoperative adverse events associated with SLN mapping or lymph node extirpation.

This marks the first study to describe the use of TRUS (and CEUS with TRUS) for iliosacral lymph node evaluation in dogs. Challenges were encountered with the ability to identify iliosacral lymph nodes via TRUS, especially anatomic location and laterality. TRUS-relevant results of this study may serve as important pilot data for future studies in which equipment or techniques are modified to enhance the utility and effectiveness of ultrasonographic iliosacral nodal evaluation and SLN mapping in dogs with AGASACA. Findings of this study highlight the need for high resolution, contrast-capable transducers with orientation that allows for visualization of structures immediately dorsal to the rectum as well as small size for use in small breed dogs. In addition, techniques to mark lymph nodes identified as sentinel via CEUS for subsequent localization on cross-sectional imaging or intraoperative dissection may prove valuable. Development of effective TRUS/CEUS protocols that enable accurate identification and localization of SLNs ultrasonographically is important as not all clients will elect preoperative CT, and SLN mapping via TRUS and CEUS may become a useful preoperative imaging strategy for these cases. However, based on the findings of this study, TRUS with CEUS cannot currently be recommended for preoperative SLN mapping in dogs with AGASACA.

Although this study demonstrated general safety and efficacy of SLN mapping as described, further evaluation into the feasibility of minimally invasive intraoperative SLN mapping techniques for iliosacral lymph nodes may be valuable in further reducing the surgical morbidity. However, to date, described techniques for minimally invasive iliosacral lymphadenectomy in dogs do not enable identification or exposure of all iliosacral lymph nodes via a single minimally invasive approach [36, 37]. As the results of this study demonstrate, SLNs associated with AGASACA are highly variable relative to the iliosacral lymphocentrum and intraoperative findings have the potential to vary relative to preoperative CTL findings, such that concurrent evaluation of the entire nodal basin should be performed during intraoperative SLN mapping. Expansion of minimally invasive techniques in dogs to allow for identification of all iliosacral lymph nodes via a single approach would enhance the potential utility of minimally invasive surgery for intraoperative SLN mapping and lymph node extirpation in dogs with AGASACA.

One additional important consideration involves the abnormal appearance in both color and consistency of the peritumoral tissues during routine anal sacculectomy following SLN mapping. However, the gross tumor and anal sac/duct were able to be excised en bloc without

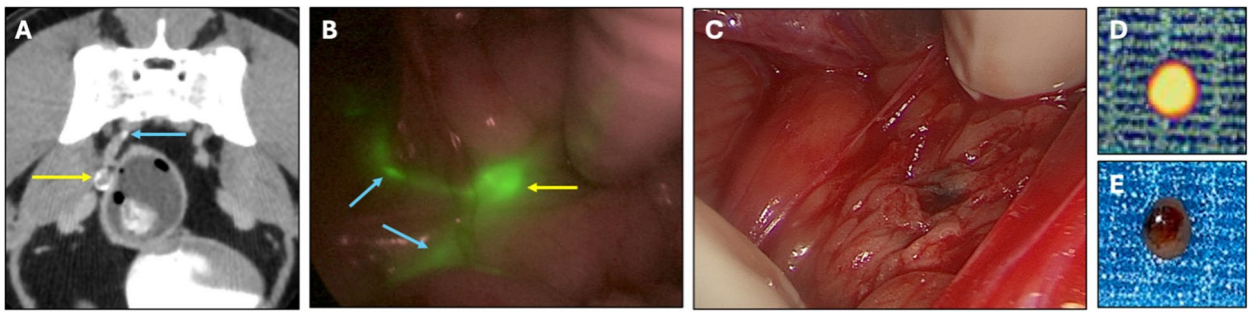

Fig. 2 Sentinel lymph node mapping findings for Dog 7 depicting a right lateral sacral lymph node as sentinel via (**A**) preoperative indirect CTL, (**B**) intraoperative in situ NIR imaging with ICG, (**C**) intraoperative in situ visible dye with MB, (**D**) ex vivo NIR imaging with ICG, and (**E**) ex vivo visible dye with MB. In images A and B, the yellow arrow depicts the sentinel lymph node, and the blue arrow(s) depicts lymphatic tracts

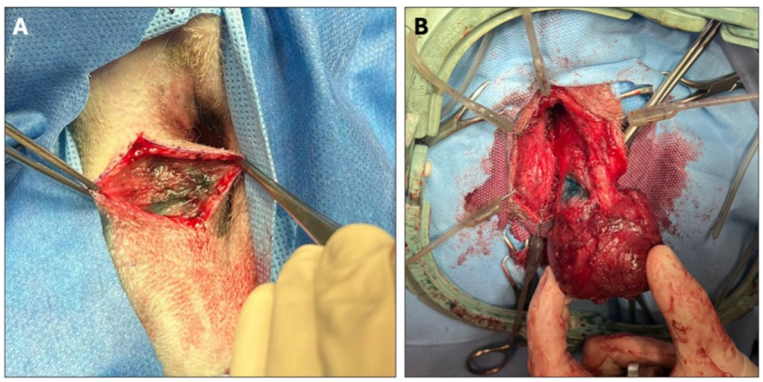

Fig. 3 Intraoperative images during anal sacculectomy of Dog 2 (**A**) and Dog 5 (**B**) demonstrating discoloration of the peritumoral tissues subsequent to peritumoral injections for SLN mapping. In image B, tissue discoloration is noted medially between the anal sac mass and rectal wall as the tumor is retracted cranially

damage to the rectum or neurovascular structures and fecal continence was maintained postoperatively in all cases. This is notable due to the typical indication for a marginal excision in cases of anal sacculectomy for AGASACA, as compared to other tumor types in which SLN mapping is followed by a wide excision. Though no overt adverse events occurred in conjunction with these peritumoral changes, this may be an important consideration in cases with more complex anal sacculectomies, such as dissections that require partial rectal wall resection due to tumor invasion, and any influence on outcomes for such cases remains to be studied.

There were several limitations of this study. First, there was a relatively small sample size. Because of the small sample size, statistical analysis for agreement of techniques was not possible owing to potential for error. In addition, this study was exploratory in nature and has provided important pilot data, but as such, it is possible that protocols or equipment other than those used in our methods, such as for CEUS via TRUS, may yield different results. Also, although the personnel performing the pre- and intra-operative peritumoral injections was the same individual for all studies in any given dog and injections were intended to be performed at similar locations and with similar technique for each dog, it is possible that slight variations in injections relative to the peritumoral tissues may have resulted in differences in findings between SLN mapping techniques. Another consideration relative to divergence in SLN results is the potential for false positive findings and misidentification of secondary or higher tier lymph nodes as sentinel nodes. This cannot be ruled out owing to the generally rapid transit time of ICG and MB, inability to globally evaluate all lymph nodes simultaneously intraoperatively (as compared to via preoperative CTL), and anatomically close interfaces between multiple iliosacral lymph nodes that may not be discernible prior to initiating dissection. However, as divergence between CTL and intraoperative SLN results was typically associated with a greater number of SLNs detected intraoperatively, this suggests that in general, intraoperative SLN mapping via the techniques described may lead to an overestimation (rather than underestimation) of the true number of SLNs. Subsequently, this may result in a greater number of lymph

nodes identified, extirpated, and histologically evaluated, thus potentially resulting in a greater likelihood of correctly identifying (and removing) all metastatic lymph nodes at the time of surgery as compared to if the alternative were true. As an additional limitation, routine histologic assessment of extirpated lymph nodes was performed in this study. Alteration of lymph node assessment techniques, including evaluation of a greater proportion of the extirpated nodes via serial step sectioning and incorporation of immunohistochemistry, may result in identification of early metastatic disease in a greater number of nodes. Finally, although this data is useful in characterizing findings between pre- and intra-operative techniques for SLN mapping and clinical and histologic correlates, the effect of SLN mapping and extirpation of early nodal metastasis on clinical outcomes in dogs with AGASACA is not yet known, and large-scale, properly powered studies are needed to determine this.

In conclusion, the findings of this study support the use of preoperative CTL in conjunction with intraoperative SLN mapping via MB and ICG with NIR imaging for dogs with AGASACA. These techniques may allow for identification and safe extirpation of metastatic lymph nodes in dogs with occult, early metastatic disease. Future, large-scale, multi-institutional studies are needed to determine an accurate incidence of nodal metastasis in dogs with AGASACA without overt metastasis identified on imaging, the effect of SLN mapping with early metastatic lymph node identification and extirpation on oncologic outcomes in dogs with AGASACA, and any potential adjuvant therapies that may play a role in enhancing outcomes in dogs with early metastatic disease.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Conflict of interest
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Authors' contributions
MG: contributed to the study conceptualization/design, data acquisition and analysis, data interpretation, manuscript preparation, and manuscript revision with final manuscript approval. BF: contributed to the study conceptualization/design, data acquisition and analysis, data interpretation, and manuscript revision with final manuscript approval. DW: contributed to the study conceptualization/design and manuscript revision with final manuscript approval. DH: contributed to the study conceptualization/design, data acquisition and analysis, and manuscript revision with final manuscript approval. NG: contributed to data acquisition and analysis and manuscript revision with final manuscript approval. AG: contributed to data acquisition and analysis and manuscript revision with final manuscript approval. MT: contributed to data acquisition and analysis and manuscript revision with final manuscript approval. JR: contributed to the study conceptualization/design, data acquisition and analysis, data interpretation, and manuscript revision with final manuscript approval.

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Availability of data and materials
No additional datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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