# Canine anal sac gland carcinoma with regional lymph node metastases treated with sacculectomy and lymphadenectomy: prognostic factors and outcome.

Running title: Prognosis of anal sac gland carcinoma with nodal metastases

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**Conflicts of Interests:**

The authors of this manuscript declare that they have no competing interests, financial or non-financial that might have influenced their interpretation or presentation of the data presented in this manuscript.

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**Abstract**

The staging system commonly used in canine anal sac gland carcinoma (ASGC) is a revised Tumour-Node-Metastasis (TNM) system published in 2007. This staging system consists in four stages and, for dogs with nodal metastases, the size of the metastatic lymph node (mLN) defines the N stage. However, we hypothesise that (1) the mLN size has no prognostic significance when the mLN can be excised, (2) a high number of mLNs is associated with poorer prognosis and (3) the measurement of the mLN on imaging is not reproducible.

To investigate these hypotheses, medical records and diagnostic images of dogs with ASGC and mLN, treated with sacculectomy and lymphadenectomy, with or without chemotherapy, were reviewed. Interobserver variability for mLN measurement was assessed. Prognostic factors including mLN size and number were investigated. Time to documented progression (TDP) and disease-specific survival (DSS) were evaluated. Progression-free interval (PFI) was analysed with interval-censored data analysis.

Fifty-seven dogs were included. The median PFI, TDP and DSS were 110 (95%CI 61.5-185.5), 196 (95%CI 162-283) and 340 days (95%CI 321-471), respectively. For measurement of the largest mLN, interobserver agreement was excellent but limits of agreement reached 39.7%. Neither the size of the largest mLN nor the use of adjuvant chemotherapy were associated with outcome. The number of mLNs was associated with outcome and having more than four mLNs was associated with shorter PFI ($p<0.001$), TDP ($p=0.004$) and DSS ($p<0.001$).

While mLN size measurement was not consistently reproducible and did not influence outcome in our cohort, number of mLNs did. Further studies are required for development of a revised staging system.

**Keywords:** anal sac gland carcinoma, lymph node excision, neoplasm, staging

## 1. Introduction

Anal sac gland carcinoma (ASGC) is a common perianal tumour in dogs, accounting for 17% of perianal malignancies[1]. At presentation, metastases to the regional lymph nodes (LNs) are common with rates that range from 26 to 96%[2-8]: medial iliac, internal iliac and sacral LNs are most commonly affected[2-8]. Distant metastases to abdominal organs (spleen, liver), lungs or bone are less common (2-18%) and occur later in the course of the disease[3,4,5,8]. Although there is no gold standard treatment in canine ASGC, treatments such as surgery[2,9], radiation therapy[10-13], and chemotherapy[6,7,14,15] have been described and the treatment of choice depends on the tumour burden, the extent of the disease, the expected prognosis and the owners' intent.

In 2007, Polton and Brearley[3] proposed a TNM (Tumour Node Metastasis) clinical staging system based on the size of the primary tumour, the size of the largest metastatic lymph nodes (mLNs) and the presence of distant metastatic disease (Table 1). This staging system is widely used[2,10,11,14] to inform treatment decisions and provide prognostic information[3]. The N stage is defined according to mLN size assessed via imaging: a mLN cut-off size of 4.5 cm differentiates stage 3a from stage 3b. For stage 3b ASGC, palliative medical treatment is recommended due to the poor prognosis associated with mLN size > 4.5 cm. However, with the advances of imaging modalities and surgical techniques over the last years, the definition of the N stage in ASGC may need to be reconsidered.

The number of mLN has been reported as a negative prognostic factor in canine mammary carcinoma[16] and is a well established risk factor, included in the TNM stage, for many human carcinomas of diverse histotypes[17-30]. Similarly, we have observed that dogs with ASGC and multiple mLNs appeared to have a worse prognosis than those with one single mLN. This clinical observation raised the question whether a N stage based on the number, rather than size, of mLNs could be a more reliable prognostic factor in canine ASGC.

Additonally, the inclusion of a N stage based on mLN size assessed via imaging may not be adequate. Several veterinary studies have shown that the size of both normal and pathological LNs is influenced by animal weight and size[31-36] suggesting that relative LN size may be more relevant than absolute LN size. Moreover, a staging system needs to be reproducible to be useful, and the interobserver variability in the assessment of mLN size can lack reproducibility in humans[37,38] and the same may be true for dogs, although this has not been evaluated so far.

To re-evaluate the definition of the N stage in ASGC proposed by Polton and Brearley[3], we decided to describe the outcome of a cohort of dogs with stage 3a and 3b ASGC treated with anal sacculectomy and lymphadenectomy and to analyse prognostic factors including size and number of mLNs. We also analysed the intersoberver variability for the measurement of the largest mLN. Our hypotheses are (1) the mLN size has no prognostic significance when the mLN can be excised, (2) a high number of mLNs is associated with poorer prognosis and (3) the measurement of the largest mLN on imaging is not reproducible

## 2. Material and methods

### 2.1. Case selection

Medical records of client owned dogs with ASGC admitted to XXXXX and XXXXX between 2010 and 2018 were reviewed. The inclusion criteria were: (1) histologically confirmed ASGC with histologically confirmed LN metastasis (stage 3a and 3b), (2) staging work-up including thoracic imaging (radiography or computed tomography) and abdominal imaging (abdominal ultrasound or computed tomography), (3) surgical excision of the primary tumour and mLN/mLNs, (4) adequate follow-up and (5) more than 1 week of post-operative survival. *Adequate follow-up* (4) criteria were defined as: if no event (death, disase progression) was recorded within the first year of diagnosis a minimum of one-year follow-up was required; if an event (death, disease progression) was recorded within the first year of diagnosis, no minimum follow-up time was required for inclusion. Adjuvant treatment was allowed. Date of disease progression, death and last follow-up were recorded.

Dogs were excluded if they had recurrent disease and/or distant metastasis at initial presentation, if they received radiation therapy as initial treatment or if the number of excised LNs and/or mLNs could not be retrieved from the clinical records. The study was approved by the XXXXXXX Veterinary Ethics Committee (VREC738).

### 2.2. Imaging measurements

Computer tomography (CT) scans (pre- and post-contrast), thoracic radiographs and abdominal ultrasound images were reviewed by a board-certified veterinary radiologist (XXX) and a veterinary radiology resident (XXX) on DICOM (Digital Imaging and Communication in Medicine) viewing software (Osirix, version 7.0.1, Pixmeo, Switzerland). Both imagers were blinded to the initial imaging report and unaware of the medical record for each dog. Imaging equipment and image acquisition characteristics are detailed in Supporting Information 1.

For abdominal ultrasound (US), all the DICOM images containing the scanned abdominal LNs were reviewed. All CT scans of the thorax and abdomen required soft tissue reconstruction algorithms and included series obtained before and after intravenous contrast administration. Multiplanar reconstructions (dorsal, sagittal, and transverse) were used in evaluation of all studies. For both modalities and for each abnormal LN, the length, defined as maximal craniocaudal dimension and the height were measured. On CT scan, the height was defined as the maximal dorsoventral dimension; on US, the height was defined as the maximal dimension perpendicular to the length. For each dog, the maximum measurement (maximum diameter, length and height) was defined as the largest measurement obtained among all the abnormal LNs. For each measurement, the mean of the measurements obtained from both radiologists was calculated. The mean maximum diameter (length or height), the maximum length and the maximum height were obtained for each case.

The number of abnormal LNs were determined based on size, shape and echogenicity (for US) or contrast enhancement (for CT scan). For US, the number of abnormal LNs was determined based on the LN images available for review. The maximum number of abnormal LNs determined by the two observers was considered for statistical analysis.

For each dog, the ventrodorsal diameter of the aorta (Ao) measured at the level of T6-T7 intervertebral disc space and the ventrodorsal diameter of the vertebral body of the 5$^{th}$ vertebra (T5) were measured from either thoracic radiographs (lateral projection) or thoracic CT scan (sagittal reconstruction of post-contrast images). Normalized ratios of LN measurement to the weight, Ao or T5 were obtained.

### 2.3. Histopathology

Histopathology reports issued by private veterinary laboratories and the Veterinary Pathology Diagnostic Service of XXXX were reviewed. These were all reported by board-certified pathologists. The mitotic count (MC) was defined as the number of mitoses counted in 10 high-power fields (x400). Peripheral infiltration and lymphovascular invasion (LVSI) were considered present only if mentioned in the report. Excision was considered complete in cases with histological tumour free margins > 0 mm. The methods of margin assessment were not systematically described. Nuclear pleomorphism, predominant growth pattern and the presence of necrosis were not included in data collection. The histopathology slides were not reviewed.

### 2.4. Treatment and follow-up

Surgical records for both anal sacculectomy and lymphadenectomy procedures were reviewed. Anal sacculectomy and lymphadenectomy were performed either concomitantly or staggered. The decision to stagger surgical procedures and the choice of the first surgery (anal sacculectomy or lymphadenectomy) was left to the surgeon's judgement. All abnormal LNs detected by imaging or on gross examination were excised. If possible, additional grossly normal LNs were also excised but this was not standardised. Adjuvant chemotherapy was performed according to clinicians' judgment and owners' preferences.

Following surgery, repeat staging work-up was recommended every 3 months for a year and subsequently every 6 months for up to 2 years. Information regarding follow-up visits to the referral centre and results of repeat staging undertaken was collected. When necessary, the refering veterinary surgeons were contacted by phone to define date of progressive disease (PD), type of progression, date of death and reason of death if available. Confirmed PD was defined based on clinical examination, imaging findings and/or cytology/histopathology results if available. A strong suspicion of mLN based on imaging was considered sufificent to confirm metastatic nodal progression without the need for cytological/histopathological confirmation. For the purpose of the study,PD was defined as either the appearance of one or more new lesions or at least 20% increase of the sum of diameters of the target lesions[39]. Locoregional failure was defined as local recurrence and/or development of mLNs. Suspected PD was defined as when euthanasia was performed for clinical signs suggestive of PD or when the reason for euthanasia was not specified as another cause.

## 2.5. Data collection

Clinical data were extracted from the medical records and included: signalment (age, weight, sex, breed), clinical signs, tumour location and size, tumour stage[3], hypercalcemia, imaging modality, number of LN(s) excised, histopathology data, adjuvant treatment, follow-up, nature of PD, date of PD and death if available. Measurement of ionized calcium was required to confirm hypercalcemia. Tumour stage (3a or 3b) was defined according to the largest diameter of the largest mLN determined on imaging review[3]. Tumour size was defined as the largest diameter of the neoplastic anal sac mass. The measurements of the primary tumour were obtained with a calliper at the time of the initial clinical examination. For bilateral ASGC, tumour size was defined as the largest diameter of the largest ASGC.

Surgical complications were retrieved from the clinical records. Surgical complications were defined as adverse events temporally associated with, and attributable to, surgical intervention[40]. Intraoperative complications were defined as those that occurred in the time period from skin incision to closure. Postoperative complications were defined as complications that occurred after skin closure: the ones that occurred up to 14 days after skin closure were considered short term, and complications that occurred after 14 days after skin closure were considered long term. Complications were retrospectively graded by a single investigator (XXX) using complication grading systems used in human surgery; the CLASSIC (classification of intraoperative complications)[41] scheme for intraoperative complications (Supporting information, Table 1) and the Accordion Severity Classification of Postoperative Complications (contracted classification)[42] (Supporting information, Table 2). For the purpose of the study, the Accordion Classification was amended with the inclusion of probiotics in the list of drugs allowed to define “mild post-operative complication”.

## 2.6. Statistics

### 2.6.1. Descriptive statistics

Statistical analysis was performed using R version 3.4.2. Continuous variables were tested for normality using the Shapiro Wilk test. As data were non-normally distributed, median and range were reported. Frequencies and percentages were used to describe categorical variables. Survival data were expressed as median times and 95% confidence intervals (CI).

### 2.6.2. Interobserver variability

Interobserver variability between the two observers was assessed using the intraclass correlation coefficient (ICC) with 95% CI in a two-way random model and using Bland-Altman limits of agreement analysis. ICC was interpreted as follows: an ICC of 0-0.20 indicated poor agreement; 0.40-0.75 indicated fair to good agreement; and >0.75 indicated excellent agreement. Independent Student’s t-tests were performed to compare the mean difference of agreement between measurements obtained with CT scan and with ultrasound.

### 2.6.3. Survival analyses

Survival represents an inaccurate endpoint due to the the variability of treatment pursued when tumour progression is identified[2,43]. Progression-free interval (PFI) is more reliable to assess the benefit of initial intervention[43]. However, the adherence to follow-up staging recommendations in ASGC is limited, resulting in an inaccurate estimation of the date of tumour progression[44]. In fact, the exact date of progression falls between the date of the last restaging and the date of documented PD[45]. For the purpose of this study, the use of "time to documented progression" (TDP) was considered more appropriate as this term does not suppose the exact time of PD is known.

Analysing the prognostic factors associated with TDP may lead to an overestimation of the PFI. To limit this, we also assessed PFI with interval-censored data analyses[46] as this method may provide a better way to estimate the date of progression for a population with heterogeneous follow-up[47]. With this method, although the exact date of PD remains unknown, PD is known to have occurred in a defined time interval. The left side of this interval is defined by the date of the last staging or the date of treatment start (TS) and the right side is defined as the date of the documented PD (confirmed or suspected). Treatment start was defined as the day at which all gross disease had been excised: either the date of surgery if sacculectomy and lymphadenectomy were combined or the day of the second surgery if the two surgeries were staggered. As an example: if a dog died from an ASGC-related causes after 10 months without a follow-up appointment, this dog's TDP will be 10 months; if a dog comes for repeat staging at 3 months and PD is documented, the TDP will be 3 months even if death only occurs after 10 months. Conversely, the PFI, analysed with interval-censored data analysis, will be between 0 and 10 months for the first dog and between 0 and 3 months for the second dog. The date of PD will be estimated based on a model derived from the whole population. This method is therefore more robust and less likely to create PFI overestimation[46,47].

The endpoints were defined as follows: PFI was the time from TS to confirmed/suspected PD analysed with interval-censored data, TDP was the time from TS to confirmed/suspected PD, and disease-specific survival (DSS) was the time from TS to death due to or suspected to be due to ASGC.

For PFI, for both univariable and multivariable analyses, a semi-parametric regression model for interval-censored data assuming proportional hazards was used. For TDP and DSS, the log-rank test and Cox proportional hazard ratio were used for univariable analysis and. Cox's proportional hazards models were used for multivariable analyses. For PFI, TDP and DSS, dogs were right censored if they were alive at last contact without evidence of PD or died from a cause clearly unrelated to ASGC. When cause of death was unknown, it was assumed to be due to ASGC-related causes.

For dichotomisation of mLN sizes, a ROC (Receiving Operator Curve) analysis was performed. The area under the ROC curve (AUC) was used to calculate the accuracy of dichotomous results for predicting TDP over the median. Calculation of the cut-off was based on the highest Youden's index.

Each predictor variable showing marginal association with PFI, TDP and DSS ($p<0.1$) in univariable analysis were included in the multivariable model and a backward stepwise fashion selection approach was performed to determine the most appropriate model. For significant variables, p-value, hazard ratio (HR), odds ratio (OR) and 95% CI were reported.

For statistical tests, a p-value < 0.05 was considered significant. The icenReg, survivalROC, OptimalCutPoints, ggplot2 and survminer packages were used[48].

## 3. Cell Line Validation Statement

No cell lines were used in the current study.

## 4. Results

### 4.1. Case selection

Eighty-eight dogs with confirmed stage 3a and 3b ASGC that underwent anal sacculectomy and lymphadenectomy with or without chemotherapy were identified. From these, 23 dogs were excluded due to inadequate follow-up. Three dogs were excluded as the ASGC was a recurrence at the time of presentation. Two dogs were excluded because the number of mLNs could not be retrieved. Three dogs were excluded due to peri-operative deaths.

### 4.2. Study population

Fifty-seven dogs met the inclusion criteria and were included in the study. Characteristics of the population are presented in Table 2. Twenty-four cases (42.1%) were recruited from the XXXX and 33 cases (57.9%) were recruited from XXXX. Clinical signs at presentation involved the presence of an anal sac mass in all dogs, polyuria and polydipsia (PUPD) (n=13, 22.8%), perianal pruritus (n=9, 15.8%), tenesmus (n=8, 14%), lethargy (n=7, 12.3%), pelvic limb weakness (n=5, 8.8%) and abnormal faecal shape (n=2, 3.5%). Thirty-five dogs (61.4%) had at least one clinical sign prior to diagnosis of ASGC. The duration of the clinical signs was known for 34 dogs and the median duration was 22 days (range 3 - 185). Seventeen dogs (29.8%) were hypercalcemic and among them, 11 (64.7%) had PUPD. Two dogs with PUPD did not have plasma ionized calcium measured.

### 4.3. Imaging data

Twenty-nine dogs (50.9%) underwent thoracic radiography and abdominal ultrasonography, 24 dogs (42.1%) underwent thoracic and abdominal CT and 4 dogs (7%) underwent thoracic CT and abdominal ultrasonography. In 9 dogs, only the imaging report was available. Images were reviewed in 48 cases: 45 cases had both thoracic and abdominal images available for review; 3 cases had only abdominal images available for review. Twenty-nine dogs were stage 3a (60.4%) and 19 were stage 3b (39.6%).

Among the 48 dogs with images reviewed, the median number of abnormal LNs was 2 (range 1-6). The largest LN was the medial iliac in 21 cases (43.8%), the internal iliac in 14 cases

(29.2%), the sacral in 7 cases (14.6%) and the para-aortic in 1 case (2.1%). In 5 cases (10.3%), the largest LN could not be assessed as multiple LNs formed one single sublumbar mass. The side of the largest LN was known in 23 cases: it was ipsilateral to the primary ASGC in 16 cases (69.6%) and contralateral in 7 cases (30.4%).

Inter-observer agreement ICC (Table 3) and limits of agreement (Supporting Information, Figure 1) were assessed for measurement of the maximum diameter (D), length (L) and height (H) as well as T5 and Ao. There was an excellent agreement between observers for all the variables measured regardless of imaging modality. For D, L and H, the mean difference of agreement (bias) was 3.2%, 2.4% and 5.2%, respectively. There was a trend for a lower bias for CT than for US: this was only significant for L ($p=0.03$) and close to significance for D ($p=0.058$). The other biases were not significantly different between CT and US for H ($p=0.7$) or between CT and radiographs for T5 ($p=0.19$) and Ao ($p=0.45$). For D, the 95% limit of agreement ranged from -21.3 to 27.7% and this represented an absolute difference of -7.1 to 9.6 mm. The largest 95% limit of agreement was for H (-29.2 to 39.7%).

### 4.4. Treatment performed

All dogs underwent the first surgery within 2 weeks (median 7 days, range 2-14) from imaging. Anal sacculectomy was performed at the referral centre in 49 dogs (86%). All lymphadenectomies were performed at the referral centre. Both anal sacculectomy and lymphadenectomy were performed concomitantly in 41 dogs (71.9%) and were staggered in 16 dogs (28.1%). If staggered, the median time between sacculectomy and lymphadenectomy was 23 days (range 10-50). Sacculectomy was performed first in 11 dogs (19.3%) and lymphadenectomy was performed first in 5 dogs (8.8%). Five dogs had bilateral sacculectomy performed: the 4 dogs with bilateral ASGC and 1 dog according to surgeon's preference. In total, 121 LNs were excised and, among them, 21 (17.4%) were normal in size. The median number of LN excised per dog was 2 (range 1-6). Excision of all the abnormal LNs documented on pre-operative imaging was attempted. Excision of normal-size LNs was performed at the surgeon's discretion. Repeat post-operative imaging was not performed to make sure all abnormal LNs had been excised. Pubic osteotomy was not required for any dog in the sudy.

Thirty-six dogs (63.2%) received adjuvant chemotherapy: 11 dogs (30.6%) received carboplatin alone, 3 (8.3%) received mitoxantrone alone, 17 (47.2%) received alternated carboplatin-mitoxantrone and 5 (13.9%) received toceranib phosphate. One dog received toceranib phosphate as maintenance treatment following a carboplatin-mitoxantrone protocol. Adjuvant chemotherapy started after a median of 18 days (range 13-271). The dog that started chemotherapy after 271 days had a severe post-operative complication following lymphadenectomy. Chemotherapy schedules and doses are summarised in Supporting Information Table 3.

### 4.5. Surgical complications

The reporting of surgical complications was not standardised; only complications mentioned in the medical history were analysed (Table 4). Two dogs (3.5%) experienced grade II (rectal perforation) intraoperative complications during sacculectomy, managed by primary closure. Post-operative complications associated with anal sacculectomy were reported in 22 dogs

(38.6%) and were mild to moderate and short-term in all cases. Faecal incontinence was described as mild in the medical history and no dogs were reported to have experienced long-term faecal incontinence.

Intraoperative complications associated with lymphadenectomy included three (5.2%) grade II haemorrhages, one (1.8%) LN fragmentation (grade II) and included one grade III haemorrhage (1.8%). Post-operative complications included two mild complications (3.5%) and one severe complication (omental herniation). The omental herniation occurred through the laparotomy site and required additional surgery.

Among the 88 dogs with stage 3a/b ASGC initially retrieved, three dogs (3.4%) died post-operatively following lymphadenectomy. Two died from uncontrollable haemoabdomen 24 hours post-operatively and one died three days post-operatively after an initial good recovery. The cause of death was not identified but this dog also had adrenalectomy performed at the time of lymphadenectomy and no haemoabdomen was identified.

### 4.6. Histopathology

The MC was mentioned in 48 histopathology reports. The median mitotic count was 23.5 (range 2 to 120). According to Pradel *et al.*[8], a MC above 8 was associated with a shorter disease-free interval and this cut-off was analysed in this study (Table 6): 10 cases (20.8%) had a MC<8 and 38 cases (79.2%) had MC≥8. Peripheral infiltration and LVSI were described in 26 (46.4%) and 33 cases (58.9%), respectively. Complete histological excision was reported in 19 cases (34%).

Among the 121 LNs excised, 109 (90%) were mLNs on histopathology. Among the 21 normal-sized LNs excised, 19 (90%) were mLNs on histopathology. The median number of mLNs per dog was 2 (range 1-6). When taking into account both enlarged and normal-sized LNs, twenty-six cases (45.6%) had 1 mLN, 18 (31.6%) had 2 mLNs, 8 (14%) had 3 mLNs, 3 (5.2%) had 4 mLNs and one each (1.8%) had 5 and 6 mLNs. In 49 dogs, all the LNs excised were metastatic. In 8 dogs (14%), at least one of the LNs excised was negative.

### 4.7. Follow-up and outcome

Following surgery, 47 dogs (82.4%) had at least one follow-up post-operative appointment and 35 (61.4%) had at least one repeat staging work-up. The first restaging included abdominal ultrasound alone in 14 dogs (40%), thoracic radiographs and abdominal ultrasound in 14 dogs (40%) and thoracic and abdominal CT scan in 7 dogs (20%). The median time from surgery to first restaging was 124 days (range 45 – 335).

Progressive disease was confirmed or suspected in 52 dogs (91.2%). The median PFI and TDP was 110 days (95% CI 61.5-185.5 days) and 196 days (95% CI 162-283 days), respectively (Figure 1). Three dogs were lost to follow-up at the time of last restaging without evidence of PD and two dogs died to causes unrelated to ASGC (1 from metastatic splenic haemangiosarcoma and the other one from metastatic high-grade soft tissue sarcoma). These three cases were censored. The median follow-up was 326 days (range 159-883 days).

Progressive disease was confirmed in 36 cases (63.1%). The first PD was documented at the time of first restaging in 22 cases (73.3%), at the second restaging in 2 cases (6.7%), at the third in 2 cases (6.7%) and at the fourth in 4 cases (13.3%). The median time between the last restaging (or surgery) and confirmed PD was 140 days (range 20-364) and, despite follow-up recommendation, only 9 dogs (25%) had repeat staging 3 months or less before documented PD. In total, fifteen dogs experienced local recurrence, 32 dogs experienced nodal progression and seven experienced distant progression (4 lungs, 1 spleen, 1 liver, 1 sternal LN). All dogs with confirmed PD experienced locoregional failure. Among the dogs with nodal progression, 16 (50%) had nodal progression alone, 12 (37.5%) also had local recurrence, 3 (9.4%) also had distant progression and one (3.1%) had both local recurrence and distant progression. One dog with local recurrence also had distant metastasis without nodal progression. One dog had local recurrence alone. For dogs with confirmed PD, median PFI and TDP was 110 days (95% CI 61.5-216.5) and 178 days (95% CI 153-234), respectively.

Progressive disease was suspected in 16 cases (28.1%) due to euthanasia secondary to clinical signs suggestive of PD including decreased mobility (2), cough (1), dyspnoea and faecal incontinence (1), spinal pain (1), spinal osteolytic lesion with hypercalcemia (1), seizure (1), severe dyschezia (1), because of "progressive disease" mentioned in the history from the referring veterinarian (3) or because of the cause of death was not specified (5). The median time between the last restaging (or surgery) and suspected PD was 219 days (range 58-358) and only one dog had repeat staging 3 months or less before documented PD. For dogs with suspected PD, median PFI and TDP was 93 days (95% CI 29-NR) and 199 days (95% CI 157-539), respectively.

Treatment was performed at the time of first PD in 23 cases (63.4% of the dogs with confirmed PD) and included radiation therapy alone in one case (4.3%), surgery alone in 5 cases (21.7%,), chemotherapy alone in 10 cases (43.5%) and surgery and chemotherapy in 7 cases (30.4%). Subsequently, 19 dogs had further PD documented with a median time between first PD to second PD of 144 days (range 69-305 days). Eight dogs had a third relapse documented and two dogs had a fourth relapse documented. The details of the treatment at PD are summarised in Supporting Information Table 4.

For all dogs, the median DSS was 340 days (95% CI 321-471) (Figure 2). Fifty-two dogs died from ASGC-related or suspected ASGC-related causes. Among the five dogs who were censored: one was still alive at the time of data analysis (550 days after treatment start) and four were euthanised for unrelated causes: one for acute kidney injury following long-term chronic kidney disease (746 days), one for widespread haemangiosarcoma (159 days), one for widespread high-grade sarcoma (883 days) and one for a large ventral neck mass (333 days). The dog that died from acute kidney injury was treated with toceranib but neither disease progresssion nor hypercalcemia were documented at the time of death.

### 4.8. Prognostic factors

*Lymph node size*

None of the LN diameters and their respective ratios (T5, Ao, weight) were associated with PFI, TDP and DSS (Supporting Information, Table 5). The lowest p-value was for the association between the maximum LN diameter:aorta dimension ratio (D:Ao) and PFI (p=0.09) and TDP (p=0.17). ROC analysis indicated that a best cut-off value for D:Ao was 2.53, yielding a sensitivity of 56.5% and a specificity of 59% in predicting TDP above the median (AUC = 0.536, 95% CI 0.362-0.709). Based on this cut-off, the dogs were divided in 2 groups with 23 dogs with small-sized LN (D:Ao < 2.53) and 22 dogs with large-sized LN (D:Ao $\geq$ 2.53).

To reassess the relevance of the staging system proposed by Polton and Brearley[3], ROC analysis was performed for the maximum diameter. ROC analysis indicated that a cut-off value of maximum diameter of 51.6 mm yielded a sensitivity of 46.1% and a specificity of 77.2% in predicting TDP above the median (AUC = 0.533, 95% CI 0.365-0.702). Based on this cut-off, the dogs were divided in 2 groups with 31 dogs with small-sized LN (< 51.6 mm) and 17 dogs with large-sized LN ($\geq$ 51.6 mm).

*Number of metastatic lymph nodes*

In univariable analysis, the number of mLNs (continuous variable) was significantly associated with PFI (p=0.02, OR 2.0, 95% CI 1.03-3.9) and TDP (p=0.02, HR 1.3 95% CI 1.039-1.8). Several cut-offs of mLNs were attempted to dichotomize the population. Dogs with mLNs $\geq$ 4 had a significantly shorter PFI (p < 0.001), TDP (p=0.004) and DSS (p<0.001) than dogs with mLNs < 4 (Table 5). The median PFI for dogs with mLNs $\geq$ 4 was 29 days and it was 110 days if mLNs < 4. Only 5 dogs had 4 or more mLNs, preventing inclusion in multivariable analyses. Therefore, to force the inclusion of the number of mLNs in multivariable analyses, we used a cut-off of 3 or more.

Eight dogs had one negative LN among the LNs excised. The presence of one negative LN was not associated with PFI (p=0.8), TPD (p=0.2) or DSS (p=0.1).

*Other prognostic factors*

The association between possible prognostic variables and outcome is summarised in Table 6.

On univariable analysis, LVSI was significantly associated with PFI (p=0.02). The median PFI for dogs with LVSI was 87 days (95% CI 47.5-154) and the median PFI for dogs without LVSI was 188 days (95% CI 112-652). On multivariable analysis, none of the variables investigated were associated with PFI.

On univariable analysis, abdominal imaging (p=0.02), D:Ao (p=0.04) and LVSI (p=0.01) were associated with TDP. On multivariable analysis, only the side (p = 0.016, HR 2.27 85% CI 1.16-4.44) was significantly associated with TDP. The TDP for left-sided ASGC was longer (231 days, 95% CI 181-367 days) than right-sided ASGC (186 days, 95% CI 160-324 days) (Figure 3a). The TDP of bilateral ASGC was 227 days (124-NA). Bilateral tumours were not analysed in multivariable analyses due to small numbers.

On both univariable and multivariable analyses, only the variable "treatment at PD" was associated with DSS (p<0.001, HR 0.19 95% CI 0.07-0.48). Dogs with treatment at relapse had a median DSS of 490 days (95% CI 469-999) and dogs without treatment at relapse had a DSS of 212 days (95% CI 186-335) (Figure 3b). Among the dogs (n=23) that were treated at relapse, the treatment type (surgery +/- chemotherapy vs chemotherapy alone) was not significantly associated with survival (p=0.24).

## 5. Discussion

In TNM classifications used in humans, the N stage is defined differently for different tumour types; whereas the LN size is used in some cancers, the number of mLNs is more relevant for other[49,50]. An adequate definition of the N stage is essential to predict prognosis accurately and guide treatment decisions. Our study aimed to question the N stage of the TNM staging described by Polton and Brearley in 2007[3]. To do so, we retrospectively analysed outcome and prognostic factors of a cohort of dogs with stage 3a and 3b ASGC treated with surgery and lymphadenectomy with or without chemotherapy. We also assessed the reproducibility of the mLN size measurement via imaging. Our study revealed that the size of the largest mLN may not be the most important factor that influences outcome in canine ASGC: firstly, the measurement of the mLN size between two observers was poorly reproducible and secondly, the size of the largest mLN was not associated with outcome. Conversely, in our cohort, the number of mLNs was significantly associated with PFI and TDP and dogs with mLNs $\geq$4 had significantly shorter PFI, TDP and DSS compared to dogs with mLNs < 4. Further studies are required to confirm this finding.

Previous studies[2-8,11,15,51] have described the clinical outcome of dogs with stage 3 ASGC treated with saccuelectomy and lymphadenectomy with or without chemotherapy but the number of cases has been relatively low with no more than 26 dogs described[2]. Our study included 57 dogs with stage 3 ASGC treated with surgery thus possibly strengthening the reliability of our findings compared to previous studies. In addition, we have used interval-censored data analyses[46] and we believe that this recently developped statistical method is appropriate for ASGC. Indeed, multiple studies have reported progression-free survival or interval[8,11,15], time to progression[7] or disease-free interval[6] in canine ASGC but, as recently described by Chambers *et al.*[44], adherence to follow-up recommendations is suboptimal making the assessment of the exact time of PD largely inaccurate. In Chambers *et al.*'s study[44], 66% of dogs had at least one restaging performed, similar to our population (61.4%). Additionally, although our recommendation was to repeat staging every 3 months for a year, the median time between the last restaging/surgery and confirmed or suspected PD was 140 days and 219 days, respectively. More importantly, the range between last restaging/surgery and PD was wide ranging from 20 to 364 days for confirmed PD and from 58 to 358 days for suspected PD. The exact date of PD therefore falls within these intervals, and this confirms the value of the interval-censored data analysis in our population. As suggested for humans[42,44], we believe this statistical method may be more appropriate and could be considered for future studies investigating PFI, disease-free interval and progression free survival when follow-up appointment intervals are long and heterogeneous. Further studies are required to compare the relevance of predictive/prognostic factors identified with interval-censored data analysis and classical Kaplan-Meier methodology.

To question the definition of the N stage in the staging system of ASGC[3], we reassessed the prognostic value of the mLN size in dogs with stage 3 ASGC treated with surgery with or without chemotherapy. First, we investigated the interobserver variability of the LN measurement in stage 3 ASGC. We have shown that, although the overall interobserver agreement was excellent according to calculation of ICC, the mean difference between observers (95% limit of agreement) for a single measurement ranged from -29.2 to 39.7% for the measurement of the height. This interobserver difference is similar, albeit mildly superior, to that observed in CT measurement of human cervical LNs in head and neck squamous cell carcinoma[52] or in various LNs[37,38] associated with several cancers. This lack of reproducibility between observers may be, at least partly, explained by the difference of experience between the two imagers who reviewed the images but can also be due to the subjectivity (difficulty to define the edges of the LN, adjacent tissue of similar density/echogenicity, angle of the measurement)[38] of mLN measurement on imaging. Therefore, an ASGC staging system in which the N stage is based mLN size may not be reproducible. More importantly, we have shown that, when lymphadenectomy is possible, the size of the largest mLN is not associated with prognosis. As LN size may be associated with body size[31-36] and weight[32] we also assessed the prognostic value of the ratio of the LN size to the aorta[32,36] and vertebral body[31,35] as previously described. The choice of the vertebrae measured and the location of the aorta measurement was dependent on the images available: as most dogs of our study had thoracic radiographs or CT scan available, T5 was elected and Ao was measured at the level of T6-T7 intervertebral disc space. There was no association between outcome and normalised mLN size ratio. These findings suggest that the definition of the N stage in ASGC staging may need to be reviewed.

Our study proposes that a N stage based on the number of mLNs may be more appropriate. This aligns with our clinical observations and multiple studies in human carcinomas[17-30]. For example, in breast carcinoma, the N stage is based on number of mLNs, with N1 (1-3 positive nodes), N2 (4-9 positive nodes) and N3 (more than 9 positive nodes) stages being associated with prognosis[53]. In our study, the total number of mLNs was significantly associated with PFI and TDP. However, in our study, the prognostic value of the number of mLNs was only true for dogs with mLNs $\geq$4 and this group was small (5 cases). Therefore, further investigations with larger sample sizes are required to assess the prognostic value of the number of mLNs in canine ASGC. Should this initial result be confirmed, a revised staging system with N stage defined by number of mLNs could be implemented.

Our study also suggests that surgery with or without chemotherapy may not be sufficient to optimise prognosis in stage 3 ASGC. Indeed, in our cohort, more than 90% of dogs experienced confirmed or suspected PD following surgery with all dogs experiencing locoregional progresssion. This prevalence of locoregional failure was similar to previous studies with dogs with stage 3 ASGC treated with surgery[11] or palliative-intent radiation therapy on gross disease[11,12]. Conversely, dogs with nodal metastases treated by definitive-intent radiation therapy may have a lower risk locoregional failure with only 2/12 (16.7%) experiencing locoregional progression in the study by Turek *et al*.[54] and only 3/11 (27.3%) in the study by Körner *et al*.[13].

In our study, the median PFI was 110 days, the median TDP was 196 days and the median DSS was 340 days. Although it is difficult to compare outcome between retrospective studies, the

data here are in alignment with the literature on dogs with stage 3 ASGC treated with anal sacculectomy and lymphadenectomy with or without chemotherapy[2,6,7,11,51,55]. Conversely, the outcome reported in studies including radiation therapy as part of the treatment of stage 3 ASGC seems to be longer. Indeed, in the study by Turek *et al*.[54], when taking only dogs with stage 3 ASGC treated with anal sacculectomy, radiation therapy and mitoxantrone with or without lymphadenectomy, median PFI was 400 days and median survival time was 677 days and this compares favourably to our study. Similarly, median survival time for dogs with stage 3b ASGC treated with palliative-intent radiation therapy[11] (447 days) or ASGC (9/11 were stage 3) treated with definitive-intent radiation therapy[13] (908 days) were notably longer than outcome reported in our study. Furthermore, the study by Meier *et al.*[11] compared outcomes of dogs with stage 3b ASGC treated with palliative-intent radiation therapy or surgery and found that dogs treated with radiation therapy had a significantly longer outcome.

For dogs with stage 3 ASGC treated with surgery with or without chemotherapy, the high rate of locoregional failure together with the short PFI, TDP and DSS reported in our study and other studies[2,6,7,11,51,55] and the possible improved prognosis reported with radiation therapy[11,13,54] suggest that post-operative radiation therapy could improve prognosis of dogs with stage 3 ASGC treated with surgery. This improvement provided by radiation therapy could stem from the treatment of microscopic disease on the primary tumour site. Indeed, in our study, 66% of dogs had incomplete excision of the primary tumour and the risk of local recurrence has recently been associated with incomplete margins in ASGC[56] . As suggested in many humans carcinomas with positive histological margins[57,58,59], post-operative radiation therapy can be beneficial to decrease the risk of local recurrence and this is likely to be true in ASGC. This is suggested in the study by Turek *et al.*[54], in which only 2/15 (13%) of ASGC of dogs experienced local recurrence after surgery and post-operative radiation therapy whereas recurrence was documented in 15 out of 36 dogs (41.7%) with confirmed PD in our study. Additionally, the benefit provided by radiation therapy could stem from the treatment of microscopic disease in the non-resected normal-sized mLNs. Indeed, as suggested in our study and other canine cancers[60-62], metastasis can be present in normal-sized LNs. If these mLNs are not excised, adjuvant radiation therapy could limit the risk of progression as suggested in various human cancers involving pelvic LNs[63,64]. Alternatively, the high rate of locoregional failure documented in our study could also suggest that the surgical technique could be improved, including a more aggressive excision of the primary tumour and a more thorough dissection of the sublumbar and pelvic LNs as is commonly performed in humans with urologic[65], gynaecologic[66] and, to a lesser degree, rectal cancers[67]. Improving the relevance of the staging system may also help to define the best treatment strategy in dogs with ASGC.

The reporting of surgical complications was not standardised and complication rate was likely underestimated in our study. That said, it is probable that severe/high-grade complications were more likely reported than mild/low-grade complications and, in our study, grade III intraoperative and severe postoperative complications were only reported for one dog each (1.8%). This is similar to Barnes *et al.*[2] who did not describe any severe/high-grade complications associated with lymphadenectomy. Although the severe/high-grade complication rate was low, three dogs among the 88 dogs initially retrieved died following lymphadenectomy and these dogs were not included in the study. Among these dogs, haemoabdomen was confirmed for two dogs (2/88, 2.2%) suggesting a direct complication

from lymphadenectomy. For the third dog, adrenalectomy was performed concomitantly and no haemoabdomen was identified suggesting that adrenalectomy was the most likely cause of death. This suggests that, although generally safe, lymphadenectomy can be associated with severe post-operative complications. Further studies are required to assess the pre-operative variables associated with high-grade intraoperative and post-operative complications associated with sublumbar LN excision.

Our study also corroborates findings previously reported in other studies. First, as previously suggested[6,7], the use adjuvant chemotherapy was not associated with improved outcome. However, this study was not designed to assess the role of adjuvant chemotherapy and the lack of protocol standardisation may have created biases in demonstrating of the benefit of adjuvant chemotherapy in the treatment of metastatic ASGC. Based on the response rate on gross disease described for cisplatin[5], carboplatin[5] and toceranib[15] and the clinical benefit of carboplatin described by Polton *et al.*[3], it is possible that adjuvant chemotherapy is beneficial for a subset of dogs. Further studies investigating the role of chemotherapy in ASGC with nodal metastases and adequate locoregional control are therefore required. Secondly, as previously suggested by Barnes *et al.*[2] , our study has shown that treatment at the time of PD positively influences outcome. This reinforces the use of PFI as a more appropriate endpoint to investigate the role of initial treatment in canine ASGC[43].

Our study also showed that, in multivariable analysis, the side of the ASGC was significantly associated with TDP with right-sided ASGC performing worse than left-sided ASGC. This finding was surprising, and a type I error should be considered a possibility due to the small sample size and the lack of significance obtained for PFI and on univariable analysis. The side could also be a confounder or proxy for an unmeasured variable. However, similar laterality has also been suggested in various human cancers[68] and is most studied in human colorectal cancer (CRC)[69] for which patients with right-sided CRC perform worse than left-sided CRC. This difference is believed to be secondary to different embryologic origins, molecular pathways of oncogenesis, microbiota and mucosal immunology and surgical accessibility. The embryologic hypothesis described in human CRC seems unlikely in canine ASGC given the common ectodermal origin of both anal glands[70]. One study[71] suggested possible differences in the anal sac bacterial and cellular content between left and right anal sac secretions suggesting a possible role of the microbiota in the different behaviours observed between right and left canine ASGC. In humans, it is also reported that the number of pelvic LNs may be higher in the right side compared to the left side[72]. If this difference was verified in dogs, this could also explain the differences observed in our population as more metastatic right-sided LNs may have not been retrieved, thereby reducing the time without progression. Further studies are required to assess the role of the side in the prognosis of canine ASGC.

Our study has several limitations. First, although sample size is higher than previously reported, the population remains small and heterogeneous making type I and type II errors plausible. Second, the retrospective design of the study led to important limitations since the choice of the imaging modalities (US vs CT scan), the number of LNs excised and the time between staging and surgery were not standardized. The number of abnormal LNs assessed with US relied on recorded fixed images. In one study[73] none of the normal sacral LNs were visible on US compared to CT scan and, in another study[36], only 36.4% of abnormal internal iliac LNs and 44.4% of abnormal sacral LNs were visible on US compared to CT. Therefore, it

is probable that the number of abnormal LNs in dogs that had abdominal US was underestimated. Additionally, inguinal LNs can be sentinel LN in normal canine anal sac[74] and these were not consistently assessed with US. Also, the decision on how many LNs had to be excised was at the discretion of the surgeon. Although most surgeons typically remove all the LNs that are grossly abnormal, metastases can also be present in grossly normal LNs as previously discussed. In humans, 10-30 LNs are commonly excised for most cancers involving pelvic LNs[75] which is considerably more than the number of LNs excised in our study. Therefore, it seems likely that many metastatic normal-sized LNs were not excised in our cohort, hindering the evaluation of the prognostic role of the number of mLNs in canine ASGC. All dogs had the first surgery performed within 2 weeks of initial staging and eleven dogs (19.3%) had lymphadenectomy performed after sacculectomy, with a median of 23 days between the first and second surgery. Therefore, although it seems unlikely to change the conclusion of this study, it is possible that the number and size of mLNs at the time of LN excision was higher than at the time of initial imaging. Third, histopathology slides were not reviewed and the association (or lack of thereof) of histological features with outcome has to be interpreted with caution. Besides, only haematoxylin and eosin (H&E) staining was used to detect mLN and, as previously described in canine carcinoma[76], some LNs diagnosed non-metastatic on H&E may be positive when immunohistochemistry is used. Moreover, none of the dogs included had excision of the superficial inguinal LNs, which could be sentinel LN in canine ASGC[74]. Therefore, better standardisation of LN dissection and possible use of sentinel LN mapping could be beneficial to assess the prognostic role of the number of mLNs in canine ASGC. Finally, the survival data described in this study need to be interpreted bearing in mind the strict inclusion criteria that were adopted. Indeed, we focused only on dogs for which lymphadenectomy was successful in order to investigate pre-operative factors influencing PFI, TDP and DSS. Therefore, dogs for which lymphadenectomy was not performed due to an anticipated high-risk of surgical complication or dogs with peri-operative mortality were not included in the study and this is likely to be influenced by the surgeon's skills and experience. Furthermore, we decided to include only dogs with staging/surgery performed within a year of the first event. This selection criteria potentially excluded long-term survivors for which no clinical PD was documented for more than one year post-operatively. However, this population is likely to be minimal and therefore unlikely to dramatically change the conclusions of the study.

In conclusion, our study showed that the definition of the N stage in the current staging system[3] of ASGC may need to be reviewed. This is due to the lack of reproducibility of mLN size measurement and the lack of prognostic value of mLN size. Our study does suggest that a N stage based on the number of mLNs may have a better prognostic relevance although further studies are needed to confirm this. The side of the ASGC could also provide prognostic information but this remains unclear. Furthermore, the use of interval-censored data analysis may be appropriate for studies in which patients have long and heterogeneous intervals between follow-up appointments.

## 6. References

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**7- Data availability statement**

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

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## FIGURES

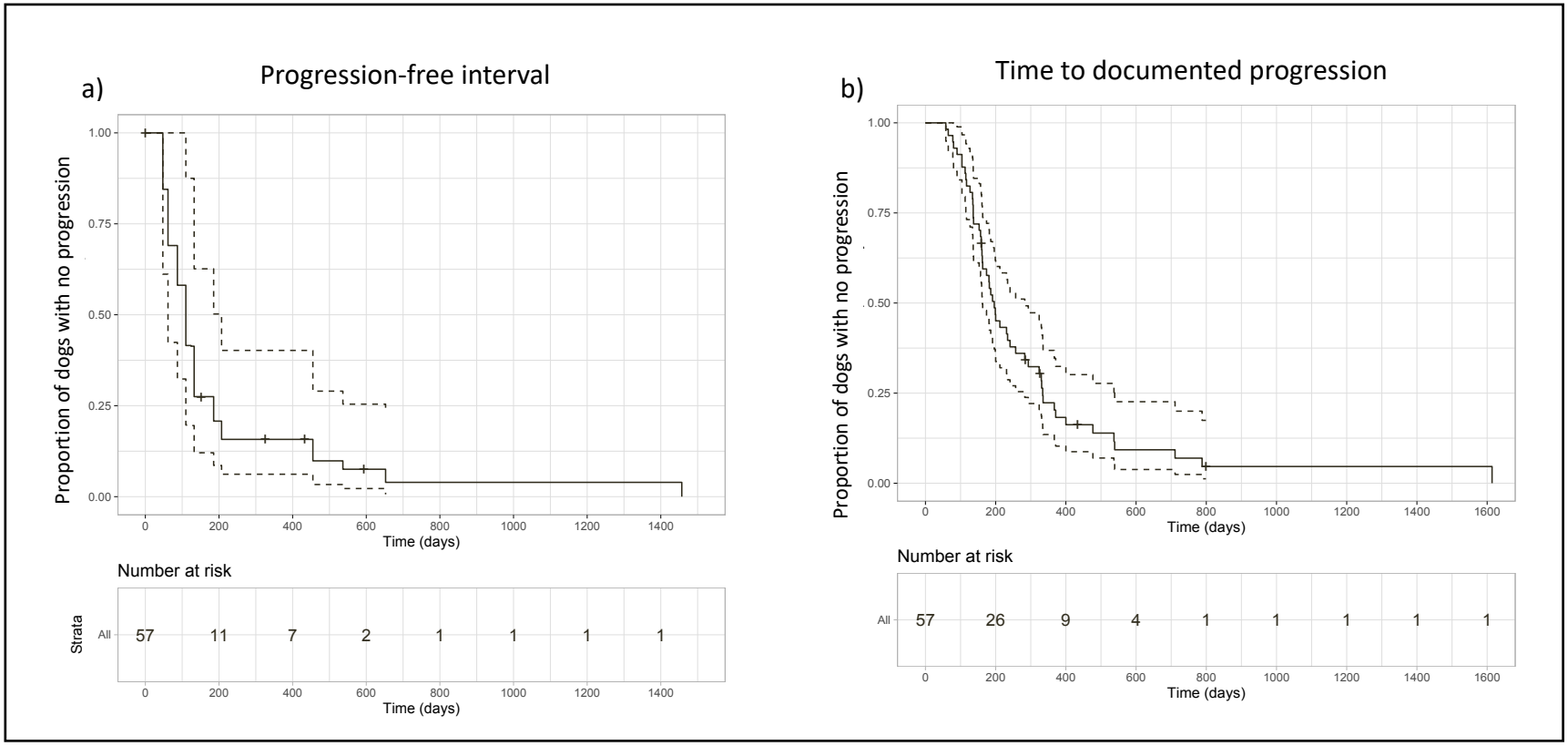



**Figure 1:** Survival curves for (a) progression free survival estimated using interval censored data analysis and (b) time to documented progression estimated using Cox proportional hazard regression model. The dashed lines represent the 95% confidence interval range. The crosses represent censored cases; dogs were censored if they were euthanised for a reason unrelated to ASAC or if they were lost to follow-up without documentation of disease progression.

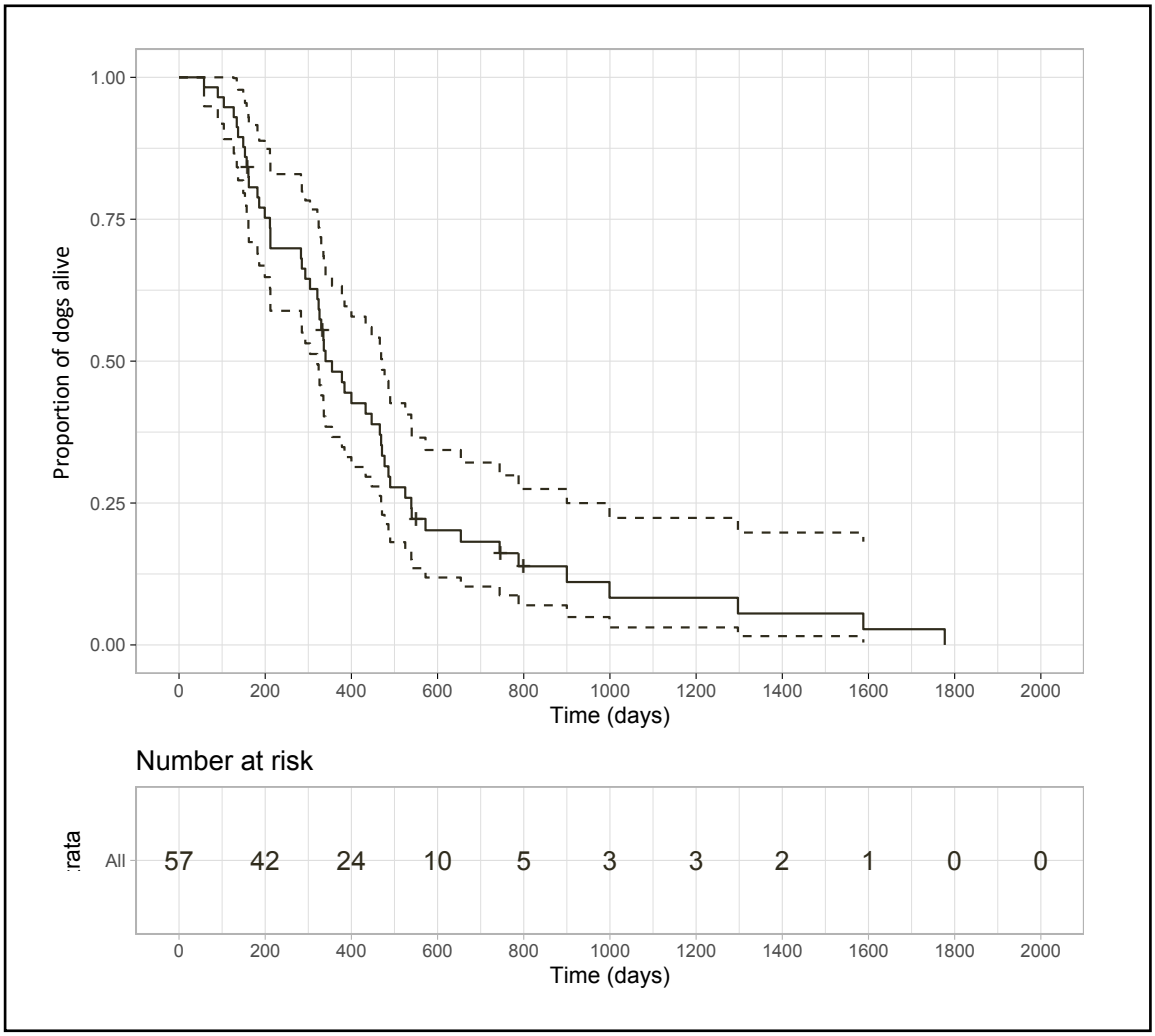



**Figure 2**: Survival curve for disease-specific survival estimated using Cox proportional hazard regression model. The dashed lines represent the 95% confidence interval range. The crosses represent censored cases; dogs were censored if they were alive at time of last follow-up or if they were euthanised for a reason unrelated to ASAC.

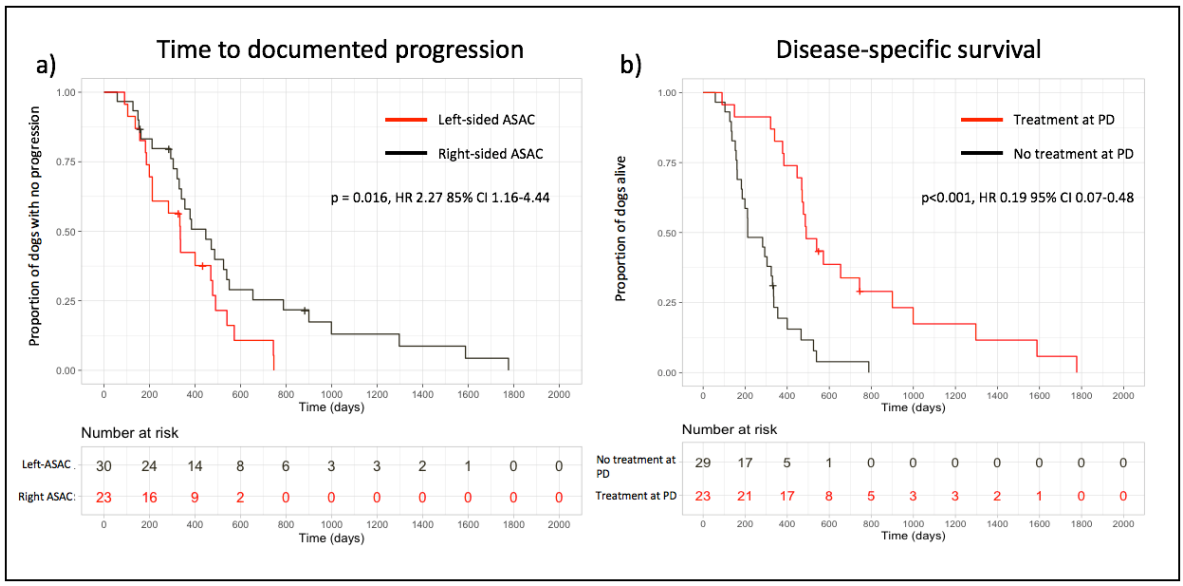



**Figure 3:** Survival curves for the variables significantly associated with (a) time to documented progression and (b) disease-specific survival on multivariable analysis, estimated with Cox proportional hazard regression model. The p-value mentioned were obtained from multivariable Cox proportional hazard and backward selection.

Abbreviation: HR = Hazard Ratio, CI = Confidence Interval, PD = Progressive Disease

# Tables

| Stage | Size primary tumour (maximum diameter) | Size lymph node metastases (maximum diameter) | Distant metastases |
|---|---|---|---|
| 1 | < 2.5 cm | None | None |
| 2 | > 2.5 cm | None | None |
| 3a | Any | < 4.5 cm | None |
| 3b | Any | > 4.5 cm | None |
| 4 | Any | Any | Present |

**Table 1**: Clinical staging scheme for ASGC in dogs proposed by Polton and Brearley[4]

| Variables | Number (percentage) |
|---|---|
| **Age** (y, median with range) | 8.7 (5.9 - 13) |
| **Weight** (kg, median with range) | 22.2 (8.3-60) |
| **Sex** | |
| FE | 3 (5.3) |
| FN | 17 (29.8) |
| ME | 3 (5.3) |
| MN | 34 (59.6) |
| **Breed** | |
| Cocker spaniel | 18 (32) |
| Labrador Retriever | 14 (24) |
| Cross breed | 9 (16) |
| Border Collie, Boxer, CKCS | 2 each (3.5) |
| Other | 10 (17.5) |
| **Primary tumour side** | |
| Left | 30 (52.6) |
| Right | 23 (40.4) |
| Bilateral | 4 (7) |
| **Hypercalcemia** | |
| Yes | 17 (29.8) |
| No | 29 (50.9) |
| Unknown | 11 (19.3) |
| **Primary tumour size** | |
| Median (range, mm) | 30 (5-80) |
| < 25 mm | 22 (38.6) |
| > 25 mm | 27 (47.4) |
| Unknown | 8 (14) |
| **Stage** | |
| 3a | 29 (50.9) |
| 3b | 19 (33.3) |
| Unknown | 9 (15.8) |

**Table 2**: Signalment and clinical characteristics of the 57 dogs with confirmed stage 3a/b ASGC treated with anal sacculectomy and lymphadenectomy

| | Number | Median diameter (range) | ICC (95 % CI) | | | Percentage of limit of agreement (95% percentage limit of agreement) | | |
|---|---|---|---|---|---|---|---|---|
| ***Lymph node measurement*** | | | All | US | CT | All | US | CT |
| **Largest diameter (D)** | 48 | 37.9 (14.4-129.4) | 0.99 (0.98-0.99) | 0.99 (0.97-0.99) | 0.99 (0.98-0.99) | 3.2 (-21.3-27.7) | 5.9‡ (-19.2-31.0) | -0.9‡ (-22.7-20.9) |
| **Largest length (L)** | 48 | 37.0 (15.6-125.5) | 0.99 (0.98-0.99) | 0.99 (0.97-0.99) | 0.99 (0.97-0.99) | 2.4 (-23.8-28.5) | 5.4* (-22.6-33.5) | -2.3* (-22.6-18.0) |
| **Largest height (H)** | 48 | 23.5 (5.0-63.6) | 0.97 (0.94-0.98) | 0.96 (0.92-0.98) | 0.97 (0.94-0.98) | 5.2 (-29.2-39.7) | 4.2† (-32.4-40.7) | 6.8† (-25.1-38.7) |
| ***Other*** | | | All | XR | CT | All | XR | CT |
| **T5** | 45 | 12.2 (7.7 – 22.3) | 0.92 (0.67-0.97) | 0.92 (0.67-0.97) | 0.86 (0.58-0.94) | -8.13 (-25.1-8.8) | -9.7‡ (-27.5-8.2) | -6.2‡ (-21.6-9.2) |
| **Ao** | 45 | 15.5 (7.5-25.2) | 0.91 (0.85-0.94) | 0.84 (0.7-0.92) | 0.95 (0.87-0.98) | -0.99 (-20.1-18.1) | 4.5‡ (-16.5-26.6) | 3.4‡ (-8.4-15.4) |

**Table 3**: Intraclass correlation coefficient and Bland-Altman limit of agreement for multiple measurements of the largest lymph nodes, the vertebral body of T5 and the ventrodorsal diameter of the aorta.

Abbreviation: ICC = Intraclass correlation coefficient, CI = confidence interval, US= ultrasound; XR = radiograph; CT = computer tomography, T5 = ventrodorsal diameter of the vertebral body of T5, Ao = ventrodorsal diameter of the aorta measured at the level of T6-T7.

Footnotes: * the difference of bias between US (or XR) and CT scan is significant ($p \leq 0.05$), † the difference of bias between US (or XR) and CT scan is borderline significant ($0.05 < p\ value \leq 0.1$), ‡ difference of bias between US (or XR) and CT scan is not significant ($p > 0.1$).

| Sacculectomy | | Lymphadenectomy | |
|---|---|---|---|
| Number of patients | 57 | Number of patients | 57 |
| Complications | Number (%) | Complications | Number (%) |
| **Intraoperative** | | **Intraoperative** | |
| *Grade II* | 2 (3.5) | *Grade II* | 4 (7) |
| Rectal perforation | 2 (3.5) | Haemorrhage [§] | 3 (5.2) |
| | | LN fragmentation | 1 (1.8) |
| | | Grade III[¶] | 1 (1.8) |
| | | Haemorrhage | 1 (1.8) |
| **Post-operative** | | **Post-operative** | |
| *Mild*[†] | 18 (31.6) | *Mild*[††] | 2 (3.5) |
| Faecal incontinence | 9 (15.8) | Seroma | 1 (1.8) |
| Diarrhoea | 4 (7) | Urinary incontinence | 1 (1.8) |
| Serosanguinous discharge | 4 (7) | | |
| Perianal hematoma | 1 (1.8) | | |
| *Moderate*[‡] | 6 (10.5) | *Moderate* | 0 (0) |
| Wound dehiscence | 4 (7) | | |
| Perianal dermatitis | 2 (3.5) | | |
| *Severe* | 0 (0) | *Severe* | 1 (1.8) |
| | | Omental herniation[‡‡] | 1 (1.8) |

**Table 4**: Surgical complications associated with sacculectomy and lymphadenectomy.

Footnotes:

[†]All cases of faecal incontinence were short-term, and no specific treatments were used. Diarrhoea was treated with probiotics. No specific treatment was used for both serosanguinous discharge. Analgesia was used for the dog with perianal hematoma

[‡]Wound dehiscence was treated with oral antibiotics and perianal dermatitis was treated with analgesia and topical treatment.

[§] The grade II haemorrhages were managed by pressure, ligation and hemoclip respectively.

[¶] For the grade III haemorrhage, the bleeding vein was packed with swabs and laparotomy was performed the following day to remove the swabs.

[††]Analgesia was used to manage the seroma and no treatment was used for the dog with urinary incontinence as it resolved by itself within a week of surgery.

[‡‡] [‡‡] A second surgery was needed to treat the omental herniation.

Abbreviation: LN = Lymph Node

| | Number | PFI (days, 95% CI) | TDP (days, 95% CI) | DSS (days, 95% CI) |
|---|---|---|---|---|
| Number of metastatic lymph nodes (p-value, HR/OR, 95% CI) | 57 | P-value = 0.03[a]<br>OR 2.0 95% CI 1.03-3.9 | P-value = 0.02[a]<br>HR 1.3 95% CI 1.039-1.8 | P-value = 0.055[b]<br>HR 1.33 95% CI 0.99-1.8 |
| **Cut-off 2** | | P = 0.29 | P = 0.4 | P = 0.6 |
| 1 | 26 | 110 (days 94-433) | 378 (304-539) | 378 (304-539) |
| 2 or more | 31 | 74 (47.5-285) | 330 (304-539) | 330 (285-539) |
| **Cut-off 3** | | P = 0.74 | P = 0.2 | P = 0.3 |
| 1 or 2 | 44 | 110 (94-285) | 200 (174-330) | 355 (324-486) |
| 3 or more | 13 | 74 (29-NR) | 157 (90-NR) | 335 (157-NR) |
| **Cut-off 4** | | P < 0.001[a] | P = 0.004[a] | P < 0.001[a] |
| 1 to 3 | 52 | 110 (87-285) | 200 (174-324) | 384 (330-486) |
| 4 or more | 5 | 29 (NA-NA) | 134 (78-NA) | 157 (134-NA) |

**Table 5:** Association between number of metastatic lymph nodes and outcome.

Cox's proportional hazards models was used to assess the association between number of metastatic lymph nodes (continuous variable)) and TDP/DSS. Log-rank test was performed to assess the association between the different groups of number of metastatic lymph nodes (categorial variables) and TDP/DSS. Semiparametric regression of interval censored data was used to assess the association between number of metastatic lymph nodes (continuous and categorical) and PFI. The p-value of association between the outcome and number of metastatic lymph nodes are mentioned in the table

Abbreviation: PFI = Progression Free Interval; TDP = Time to Documented Progression; DSS = Disease Specific Survival; OR = Odd Ratio; HR = Hazard ratio; CI = confidence interval

Footnotes:

[a] There is a statistically significant association ($p<0.05$) between the number of metastatic lymph nodes and the outcome

[b] There is a borderline significant association ($0.05<p\ value\leq 0.1$) between the number of metastatic lymph nodes and the outcome

| | n | PFI days (95% CI) | PFI p-value | TDP days (95% CI) | TDP p-value | DSS days (95% CI) | DSS p -value |
|---|---|---|---|---|---|---|---|
| Institution | | | 0.15 | | 0.2 | | 0.4 |
| Institution A | 24 | 110 (51.5-652) | | 234 (135-400) | | 400 (340-540) | |
| Institution B | 33 | 80 (80-NR) | | 186 (161-330) | | 326 (211-525) | |
| Breed | | | 0.29 | | 0.7 | | 0.3 |
| Cocker spaniel | 18 | 124 (75-NR) | | 212 (136-NR) | | 326 (199-744) | |
| Other | 39 | 94 (51-536) | | 186 (162-324) | | 378 (324-490) | |
| Age Continuous | 57 | | 0.7 | | 0.9 | | 0.6 |
| ASAC Side[§] | | | 0.17 | | 0.08[†] | | 0.07[†] |
| Left | 30 | 124 (52-652) | | 231 (181-367) | | 384 (330-654) | |
| Right | 23 | 87 (61.5-NA) | | 162 (135-283) | | 335 (212-490) | |
| ASAC Size | | | | | | | |
| < 25 mm | 22 | 128 (69-NR) | 0.28 | 216 (163-788) | 0.1[†] | 336 (304-788) | 0.5 |
| ≥ 25 mm | 27 | 87 (47.5-NR) | | 186 (160-324) | | 378 (293-490) | |
| Abdominal imaging | | | 0.11 | | **0.02*** | | 0.9 |
| Ultrasound | 33 | 87 (79.5-NR) | | 174 (153-212) | | 340 (285-540) | |
| CT scan | 24 | 124 (124-NA) | | 288 (191-400) | | 345 (304-486) | |
| Stage | | | 0.4 | | 0.5 | | 0.4 |
| 3a | 29 | 132 (61.5-652) | | 212 (162-331) | | 400 (321-654) | |
| 3b | 19 | 94 (74-NR) | | 200 (159-335) | | 340 (330-539) | |
| Lymph node: largest diameter | | | | | | | |
| < 51.6 mm | 31 | 132 (112-652) | 0.29 | 212 (174-324) | 0.3 | 469 (335-654) | 0.3 |
| ≥ 51.6 mm | 17 | 94 (39-NR) | | 196 (137-477) | | 340 (293-539) | |
| Lymph node: largest ratio D:Ao | | | 0.08[†] | | **0.04*** | | 0.5 |
| < 2.53 | 23 | 112 (47.5-NR) | | 212 (186-711) | | 378 (321-788) | |
| ≥ 2.53 | 22 | 109 (74-NR) | | 172 (137-283) | | 348 (330-540) | |
| Hypercalcemia | | | 0.4 | | 0.8 | | 0.1[†] |
| No | 31 | 74 (74-353) | | 174 (153-283) | | 304 (199-486) | |
| Yes | 17 | 121 (94-468) | | 200 (162-330) | | 384 (340-NR) | |
| Abnormal lymph nodes | | | 0.4 | | 0.1[†] | | 0.2 |
| < 2 | 19 | 128 (112 -NR) | | 241 (191-711) | | 486 (321-NR) | |
| ≥ 2 | 29 | 109 (47.5-285) | | 181 (137-324) | | 384 (321-NR) | |
| Combined surgeries | | | 0.69 | | 0.7 | | 0.2 |
| No | 16 | 110 (47.5-NR) | | 200 (135-NR) | | 469 (378-1588) | |
| Yes | 41 | 87 (74-484) | | 194 (163-330) | | 333 (293-539) | |
| Metastatic lymph nodes | | | 0.74 | | 0.2 | | 0.2 |
| < 3 | 44 | 110 (94-285) | | 200 (174-330) | | 355 (324-486) | |
| ≥3 | 13 | 74 (29-NR) | | 157 (90-NR) | | 335 (157-NR) | |
| Mitotic count | | | 0.83 | | 0.8 | | 0.4 |
| < 8 | 10 | 188 (29-285) | | 288 (113-NR) | | 340 (283-NR) | |
| ≥ 8 | 38 | 111 (87-154) | | 174 (159-234) | | 378 (321-490) | |
| Infiltration adjacent tissue | | | 0.47 | | 0.9 | | 0.08[†] |
| No | 31 | 124 (94-652) | | 199 (153-293) | | 486 (340-744) | |
| Yes | 26 | 111 (54.5-NR) | | 188 (163-335) | | 335 (304-471) | |
| Lymphovascular invasion | | | **0.02*** | | **0.01*** | | 0.1[†] |
| No | 24 | 188 (112-652) | | 241 (143-711) | | 477 (321-NR) | |
| Yes | 33 | 87 (47.5-154) | | 182 (160-257) | | 340 (324-490) | |
| Margins of excision | | | 0.6 | | 0.08[†] | | 0.2 |
| Complete | 19 | 29 (29-NA) | | 181 (160-330) | | 324 (182-654) | |
| Incomplete | 36 | 132 (94-652) | | 234 (162-367) | | 400 (336-540) | |
| Adjuvant chemotherapy | | | 0.72 | | 0.5 | | 0.9 |
| No | 21 | 128 (111-NR) | | 191 (160-334) | | 447 (321-900) | |
| Yes | 36 | 110 (61.5-455) | | 198 (159-293) | | 378 (304-490) | |
| Treatment at PD | | | NA | | NA | | **<0.001***** |
| No | 29 | | | | | 212 (186-335) | |
| Yes | 23 | | | | | 490 (469-999) | |

1249

**Table 6**: Univariable association between potential prognostic factors and PFI, TDP and DSS.
The log-rank test was used to assess the associations between the various categorical variables and DSS/TDP. Semiparametric regression of interval censored data was used to assess the association between categorical variables and PFI/
Abbreviation: PFI = Progression Free Interval; TDP = Time to Documented Progression; DSS = Disease Specific Survival; CI = confidence interval; NR = not reached; NA = not assessed; CT = Computed Tomography; D:AO = ratio of the largest diameter and the ventrodorsal diameter of the aorta measured at the level of T6-T7, PD = Progressive Disease;
Footnote: * significant association between categorical variable and outcome with p-value ≤ 0.05;

*** significant association between categorical variable and outcome with p-value < 0.001;
† the association between categorical variable and outcome is closed to significance (0.05<p-value≤0.1) and included in multivariable analysis.
§ the survival outcomes of bilateral ASAC were not included in statistical analysis due to their small number (n=4)

# Supporting information

For the XXXXX, computed tomography scans were performed using an 80-slice multidetector CT machine (Aquilion Prime, Toshiba Medical Systems, Tokyo, Japan). All dogs were either deeply sedated or anaesthetised. Reconstructions were generated using a standard (soft tissue) kernel with a slice thickness of 1 to 2mm depending on the patient size. Images were acquired pre-and post-intravenous iodinated contrast medium (Xenetix, iobitridol, 300mg I/ml, Guebert, Roissy CdG, France or Ultravist 300 mg I/ml iopromide, Bayer PLC, Berkshire, UK) administration, at the dose of 600mg I/kg. Depending on the patient, intravenous injection of contrast was performed manually or using a pressure injector (Medrad Stellant CT injection system, Newbury, UK). In all the dogs, venous-phase post-contrast images were acquired approximately 60 to 70 seconds following the contrast medium administration. Ultrasound examinations were performed with a Logiq 7 (General Electric Medical Systems, Milwaukee, USA) or Logiq S7 Expert (General Electric Medical Systems, Milwaukee, USA).

For XXXXX computed tomography scans were performed using Aquilion Lightning, 16-slice multidetector helical scanner (Canon Medial Systems), GE HiSpeed dual slice scanner (General Electric Healthcare, Amersham, United Kingdom). All dogs were either deeply sedated or anaesthetised. Reconstructions were generated using a standard (soft tissue) kernel with a slice thickness of 1 to 2mm depending on the patient size. Images were acquired pre-and post-intravenous iodinated contrast medium (Omnipaque 300 mg I/ml, GE Healthcare, Oslo, Norway). Depending on the patient, intravenous injection of contrast was performed manually or using a pressure injector (Nemoto A-60,Nemoto Kyorindo CO.,LTD, Tokyo, Japan). In all the dogs, the post-contrast images were acquired approximately 60 seconds following the contrast medium administration. Ultrasound examinations were performed with an Aloka IPC-1530 (U1) (Aloka, 6-22-1 Mure, Mitaka-shi, Tokyo, Japan) or EPIQ 5 (Philips Ultrasound, Bothell, WA, USA).

**Supporting information 1**: imaging equipment and image acquisition

| Grade | Definition |
|---|---|
| 0 | No deviation from the ideal intraoperative course |
| I | Any deviation from the ideal intraoperative course<br>• Without the need for any additional treatment or intervention |
| II | Any deviation from the ideal intraoperative course<br>• With the need for any additional treatment or intervention<br>• Not life-threatening and not leading to permanent disability |
| III | Any deviation from the ideal intraoperative course<br>• With the need for any additional treatment or intervention<br>• Life-threatening and/or leading to permanent disability |
| IV | Any deviation from the ideal intraoperative course<br>• With death of the patient |

**Supplmentary table 1:** Classification of Intraoperative Complications (CLASSIC)

Ref 41: Rosenthal R, Hoffmann H, Clavien PA, et al. Definition and Classification of Intraoperative Complications (CLASSIC): Delphi Study and Pilot Evaluation. World J Surg 2015;39:1663-1671.

1. Mild complication

Requires only minor invasive procedures that can be done at the bedside such as insertion of intravenous lines, urinary catheters, and nasogastric tubes, and drainage of wound infections. Physiotherapy and the following drugs are allowed-antiemetics, antipyretics, analgesics, diuretics, electrolytes, and physiotherapy**.**

Amendment: the use of probiotics was included in the list of the possible medications allowed for the definition of mild complications.

2. Moderate complication

Requires pharmacologic treatment with drugs other than such allowed for minor complications, for instance antibiotics. Blood transfusions and total parenteral nutrition are also included.

3. Severe complication

All complications requiring endoscopic or interventional radiologic procedures or re-operation as well as complications resulting in failure of one or more organ systems.

4. Death

Postoperative death.

**Supplementary table 2:** Amended Accordion Severity Classification of Postoperative Complications: Contracted Classification

Ref 42: Strasberg SM, Linehan DC, Hawkins WG. The accordion severity grading system of surgical complications. Ann Surg 2009;250:177-186.

| Number of patients | 36 |
|---|---|
| **Carboplatin – mitoxantrone** | 17 (47.2%) |
| Dose carboplatin (median in mg/m$^2$, range) | 288 (223 – 357) |
| Dose mitoxantrone (median in mg/m$^2$, range) | 5.2 (4.7-6.0) |
| 3 carboplatin – 3 mitoxantrone | 9 |
| 3 carboplatin – 2 mitoxantrone | 3 |
| 2 carboplatin – 1 mitoxantrone | 2 |
| 1 carboplatin – 2 mitoxantrone | 2 |
| 1 carboplatin – 1 mitoxantrone | 1 |
| **Carboplatin alone** | 11 (30.6%) |
| Dose carboplatin (median in mg/m$^2$, range) | 300 - (285 – 310) |
| 4 carboplatin | 10 |
| 6 carboplatin | 1 |
| **Mitoxantrone alone** | 3 (8.3%) |
| Dose mitoxantrone (median in mg/m$^2$, range) | 5.4 (4.9-5.5) |
| 3 mitoxantrone | 2 |
| 1 mitoxantrone | 1 |
| **Toceranib** | 5 (13.9%) |
| Dose (median in mg/kg, range) | 2.5 (2-3.3) |

**Supplementary table 3**: Chemotherapy schedules and doses

| **Progressive disease** | Number (%) |
|---|---|
| **Progressive disease 1** | 36 |
| Treatment at PD1 | 23 (63.4) |
| Sacculectomy alone | 1 |
| Lymphadenectomy alone | 4 |
| Lymphadenectomy and chemotherapy† | 6 |
| Sacculectomy and lymphadenectomy and chemotherapy† | 1 |
| Chemotherapy† alone | 10 |
| Radiation therapy alone* | 1 |
| **Progressive disease 2** | 19 |
| Treatment at PD2 | 8 (42.1) |
| Sacculectomy alone | 1 |
| Lymphadenectomy and chemotherapy | 2 |
| Chemotherapy† alone | 4 |
| Sacculectomy and radiation therapy* and chemotherapy† | 1 |
| **Progressive disease 3** | 8 |
| Treatment at PD3 | 4 (50) |
| Chemotherapy† alone | 3 |
| Lymphadenectomy and chemotherapy† | 1 |
| **Progressive disease 4** | 2 |
| Treatment at PD4 | 1 (50) |
| Lymphadenectomy alone | 1 |

**Supplementary table 4:** Treatment at the time of documented progressive disease

Footnotes:
†Chemotherapy included toceranib, melphalan, carboplatin, metronomic cyclophosphamide alone or together with thalidomide.

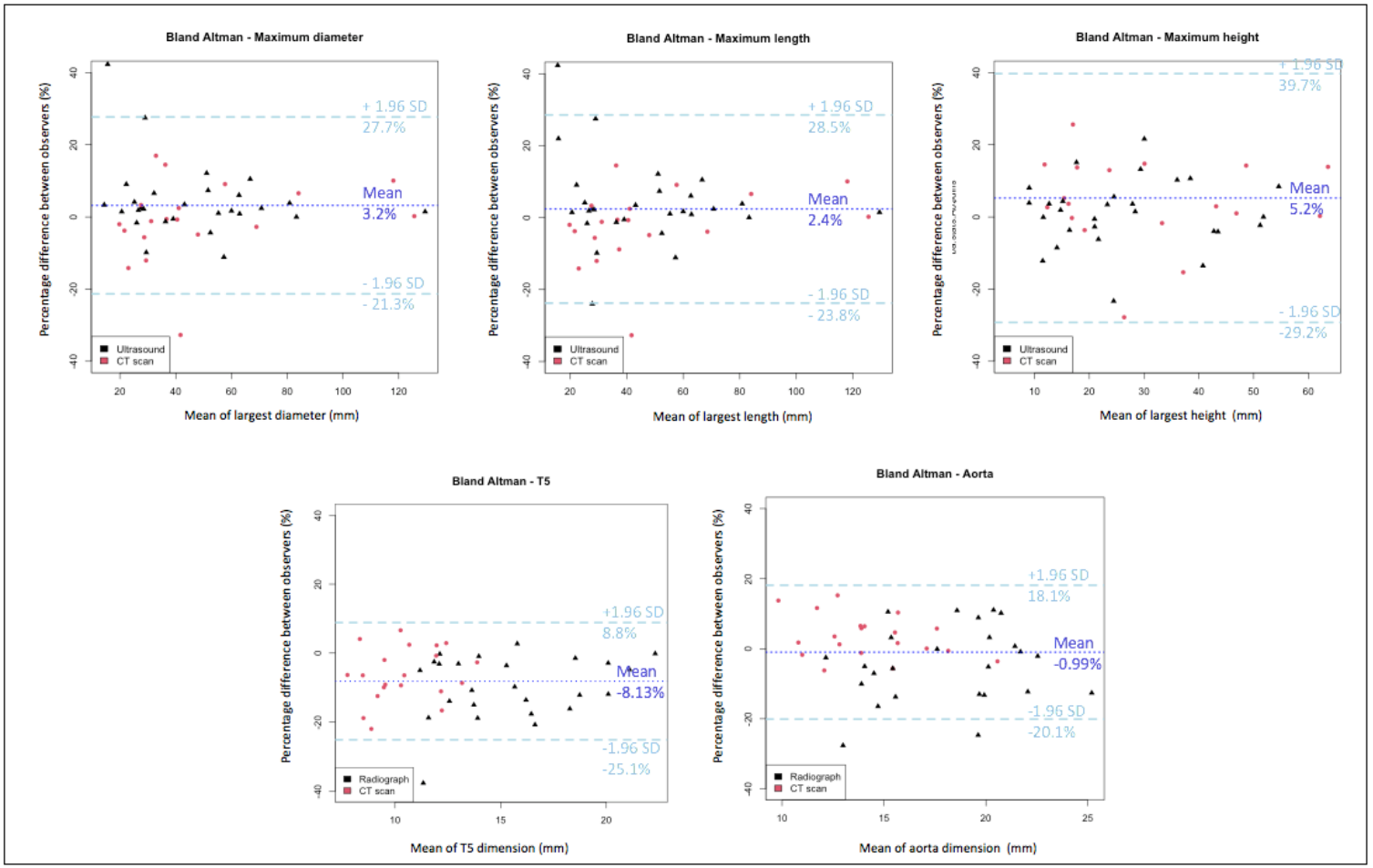
Abbreviation: PD = Progressive Disease

| | PFI (semi-parametric regression - interval censored data) | TDP (log-rank test) | DSS (log-rank test) |
|---|---|---|---|
| Average maximum diameter | 0.21 | 0.6 | 0.68 |
| Ratio Max : T5 | 0.17 | 0.22 | 0.95 |
| Ratio Max : Ao | **0.09**[†] | 0.17 | 0.72 |
| Ratio Max : weight | 0.59 | 0.5 | 0.72 |
| Average maximum length | 0.2 | 0.63 | 0.72 |
| Ratio Length : T5 | 0.19 | 0.3 | 0.98 |
| Ratio Length : Ao | 0.16 | 0.2 | 0.74 |
| Ratio Length : weight | 0.67 | 0.6 | 0.72 |
| Average maximum height | 0.32 | 0.79 | 0.62 |
| Ratio Height : T5 | 0.13 | 0.26 | 0.88 |
| Ratio Height : Ao | 0.17 | 0.18 | 0.76 |
| Ratio Height : weight | 0.36 | 0.62 | 0.87 |

**Supplementary table 5:** P-values of the association between lymph node measurements and their ratios with survival outcomes.
Log-rank test was performed for both TDP and DSS and non-parametric regression model for interval censor data, assuming proportional hazard was performed for PFI.

Abbreviation: PFI = Progression Free Interval; TDP = Time to Documented Progression; DSS = Disease Specific Survival; T5 = ventrodorsal diameter of the T5 vertebral body; Ao = ventrodorsal diameter of the aorta at the level of T6-T7.

Footnote: [†] the p-value association between the lymph node variable and the outcome is $\leq 0.1$.
1




**Supplementary figure 1**: Bland-Altman plots of interobserver agreement for (a) greatest diameter, (b) greatest length, (c) greatest height, (d) ventrodorsal diameter of T5 and (e) ventrodosrsal diameter of the aorta measured at the level of T6-T7. The x-axes show the mean value of the two observers' measurement and the y-axes show differences between the diameters on each set as a percentage of their mean. Solid line indicates absolute differences; dashed line indicates 95% limits of agreement.