

**Integrative Evaluation of Prognostic Factors in Surgically
Resected Canine Pulmonary Adenocarcinoma**

(犬の切除肺腺癌における予後因子の統合的評価に関する研究)

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GENERAL INTRODUCTION

Lung cancer in humans remains a leading cause of cancer-related death worldwide (Bray, F. *et al.*, 2024). Approximately 80–85% of lung cancers are classified as non-small cell lung cancer (NSCLC) (Sher, T. *et al.*, 2008), and lung adenocarcinoma is the most common histologic subtype within NSCLC (Travis, WD. *et al.*, 2011). Adenocarcinoma accounts for about 40% of all lung cancers and therefore constitutes a major component of NSCLC (Zappa, C. and Mousa, SA., 2016, Denisenko *et al.*, 2018). In view of this substantial incidence and mortality burden, optimization of treatment strategies based on accurate stage assessment is essential in NSCLC. Clinical staging according to the tumor–node–metastasis (TNM) classification provides the central framework for prognostic stratification and directly guides therapeutic decision-making (Schneider, BJ. *et al.*, 2008, Detterbeck, FC. *et al.*, 2018, Matilla, JM. *et al.*, 2020). Complete surgical resection is the standard curative-intent treatment and offers the most effective option for long-term disease control, particularly in early-stage NSCLC (Van Schil, PE. *et al.*, 2013, Zer, A. *et al.*, 2025). Nevertheless, disease progression after curative-intent surgery, here defined as recurrence and/or distant metastasis, still occurs in a substantial proportion of patients. Postoperative disease progression has been reported in approximately 30–55% of resected NSCLC cases overall (Uramoto, H. *et al.*, 2014), and the 5-year cumulative incidence of recurrence is approximately 20% even in pathologic stage I–II disease (Potter, AL. *et al.*, 2023). Thus, although staging lies at the core of prognostication and treatment planning, considerable heterogeneity in recurrence risk and survival persists among patients within the same stage. Accordingly, there is a continuing need for additional predictive factors that can identify high-risk subsets and refine treatment selection beyond TNM-based stratification. In recent years, molecular profiling and assessment of the tumor microenvironment (TME) have gained attention as

complementary approaches to address this stage-internal heterogeneity. Molecular profiling has identified genetic alterations that drive tumor growth and metastasis, commonly referred to as driver mutations. In NSCLC, *epidermal growth factor receptor (EGFR)*, *human epidermal growth factor receptor 2 (HER2)*, and *Kirsten rat sarcoma viral oncogene homolog (KRAS)* mutations represent major driver abnormalities (Zhu, QG. *et al.*, 2017). Analyses of resected cohorts, in which the influence of targeted therapies is limited, have shown that the prognostic impact of *EGFR* mutations is inconsistent (Zhang, Z. *et al.*, 2014, Takamochi, K. *et al.*, 2017), whereas *KRAS* mutations are associated with a higher risk of recurrence and poorer survival even within the same stage, as reported in multiple studies including meta-analyses (Pan, W. *et al.*, 2016, Zhang, SM. *et al.*, 2018). Moreover, the clinical efficacy of EGFR tyrosine kinase inhibitors (TKIs) in advanced *EGFR*-mutant NSCLC (Mok, TS *et al.*, 2009, Maemondo, M. *et al.*, 2010, Ramalingam, SS. *et al.*, 2020), together with the survival benefit of adjuvant osimertinib in resectable *EGFR*-mutant disease (Wu, YL. *et al.*, 2020, Tsuboi, M. *et al.*, 2023), demonstrates that treatment individualization based on molecular profiles can improve outcomes. In parallel, components of the TME, such as tertiary lymphoid structures (TLS) and tumor-infiltrating lymphocytes (TILs), have been evaluated as factors that may further explain prognostic differences within a given stage. Tertiary lymphoid structures are organized lymphoid aggregates containing B-cell follicles, T-cell zones, and high endothelial venules; they serve as local sites of anti-tumor immune responses (Hiraoka, N. *et al.*, 2016, Schumacher, TN. *et al.*, 2022, Fridman, WH. *et al.*, 2023). In resected NSCLC, the presence of TLS correlates with improved disease-free and overall survival, and higher TLS maturity has also been associated with favorable outcomes (Dieu-Nosjean, MC. *et al.*, 2008, Sun, X. *et al.*, 2022). Similarly, dense

intratumoral CD8⁺ T-cell infiltration tends to associate with favorable outcomes (Chen, B. *et al.*, 2020), whereas increased Foxp3⁺ regulatory T-cell (Treg) infiltration is often linked to higher recurrence risk and poorer survival (Shimizu, K. *et al.*, 2010). Collectively, integrated evaluation of these immune microenvironmental features may contribute to more precise stratification of outcome variability among patients classified within the same pathologic stage. In human thoracic oncology, clinical stage, tumour genomic profiles, and immune microenvironmental features are increasingly integrated to refine risk stratification and support treatment individualisation.

Canine primary pulmonary carcinomas (cPPCs) are rare compared with human lung cancer but represent clinically relevant malignancies in older dogs (Culp, WTN. and Rebhun, RB., 2020). Canine pulmonary adenocarcinoma (cPAC) is the most common histologic subtype, and surgical lobectomy remains the standard local treatment when imaging indicates resectability (Ogilvie, GK. *et al.*, 1989, Hahn, FF. *et al.*, 1996, McPhetridge, JB. *et al.*, 2021). The median survival time for surgically resected cPPCs has been reported to range from 92 to 399 days (McNiel, EA. *et al.*, 1997, Sabattini, S. *et al.*, 2014, Polton, G. *et al.*, 2018, Lee, BM. *et al.*, 2020, McPhetridge, JB. *et al.*, 2021), and from 131 to 339 days for cPACs (Ogilvie, GK. *et al.*, 1989, Polton, GA. *et al.*, 2008, Able, H. *et al.*, 2021). However, postoperative courses still vary widely, and clinicians lack reliable tools to identify dogs at high risk for early progression. A World Health Organization (WHO) staging system for canine lung tumours was introduced in 1980, although tumour size was not incorporated in that framework (Owen, LN. and World Health Organization., 1980). Later investigations showed that tumour volume correlates with survival in cPPCs (Mehlhaff, CJ. *et al.*, 1984, Rose, RJ. and Worley, DR., 2020), indicating that tumour size likely carries prognostic value. These findings led to the

proposal of the canine lung carcinoma stage classification (CLCSC) in 2020 (Lee, BM. *et al.*, 2020), which was modelled on the human TNM system (Brierley, J. *et al.*, 2017). The CLCSC stratifies tumour size into four tiers (≤ 3 cm, >3 to ≤ 5 cm, >5 to ≤ 7 cm, and >7 cm) (Lee, BM. *et al.*, 2020). Importantly, tumour size, nodal status, and distant metastasis within the CLCSC have each been associated with survival, and the overall staging system has remained prognostically relevant in later large-scale studies (McPhetridge, JB. *et al.*, 2021). However, unlike humans, dog breeds vary widely in body size. In the two reports that evaluated CLCSC, the median body weight of the dogs was >20 kg (Lee, BM. *et al.*, 2020, McPhetridge, JB. *et al.*, 2021). To date, the prognosis of surgically resected PACs in small-breed dogs has not been clarified, and the applicability of the CLCSC tumour size classification to PACs in small-breed dogs has not been examined. This uncertainty motivated the first component of the present thesis, which aimed to determine whether CLCSC tumour-size categories provide prognostic stratification in small-breed dogs (<15 kg) with surgically resected PACs and to identify additional clinicopathological risk factors in this group.

Alongside clinical staging, molecular studies have identified genomic features of cPACs that differ from human NSCLC and may offer comparative and therapeutic insights. Among these, *HER2* mutations are one of the most consistently reported findings in dogs. Previous canine series have reported *HER2* mutations in approximately 8% to 38% of cPACs (Lorch, G. *et al.*, 2019, Brunetti, B. *et al.*, 2024), and most variants involve the recurrent V659E substitution in the transmembrane domain (TMD). This distribution contrasts with human NSCLC, in which *HER2* mutations occur in about 2% to 4% of adenocarcinomas (Shigematsu, H. *et al.*, 2005, Tomizawa, K. *et al.*, 2011, Arcila, ME. *et al.*, 2012) and typically localize to the kinase or extracellular domains, whereas V659E is

exceptionally rare in large human cohorts (Ou, SI. *et al.*, 2017, Jia, Z. *et al.*, 2021). Experimental work using canine lung cancer cell lines further indicates that HER2 V659E promotes ligand-independent signalling with downstream AKT activation and confers sensitivity to HER2-targeted TKIs (Lorch, G. *et al.*, 2019). These observations suggest that *HER2*-mutant cPACs constitute a biologically distinct subgroup and may serve as a spontaneous comparative model for HER2-altered lung cancer. However, despite the relatively high mutation frequency and supporting functional data, the clinical significance of HER2 alterations in dogs remained uncertain. In particular, it was not established whether *HER2* mutation status or HER2 protein expression provides prognostic information beyond established clinicopathological factors, including CLCSC staging, in surgically resected cPACs. This gap motivated the second component of the present thesis, which aimed to define the frequency and spectrum of *HER2* mutations in surgically resected cPACs, to characterize HER2 immunoreactivity, and to assess their prognostic relevance.

In addition to clinicopathological staging and oncogenic driver profiling, increasing attention has recently been directed toward the TME in dogs (Gray, M. *et al.*, 2020, Carvalho, MI. *et al.*, 2021). Across several canine tumour types, higher densities of CD8⁺ TILs are associated with improved clinical outcomes, whereas enrichment of Foxp3⁺ TILs is often linked to immunosuppression and shorter survival (Pinard, CJ. *et al.*, 2022). In cPACs, earlier studies suggested an adverse prognostic role for Foxp3⁺ TILs (Sakai, K. *et al.*, 2018), yet comprehensive evaluation of immune architecture in larger, surgically treated cohorts has remained limited. In particular, TLS are well established in human oncology, whereas in dogs rigorous TLS identification remains challenging. Therefore, comparable organised immune structures have been reported

mainly as lymphoid aggregates (LAs) or TLS-like formations. Only recently have TLS-consistent LAs been described in a limited number of canine tumour types, including mammary carcinomas (Giambrone, G. *et al.*, 2022) and cutaneous or subcutaneous sarcomas (Rugh, KM. *et al.*, 2025), supported by immunohistochemical and gene-expression evidence. Nevertheless, no studies have confirmed whether intratumoural LAs or TLS-like formations are present in cPACs, nor has their clinical significance been evaluated in comparison with established prognostic variables. Accordingly, it remains unknown whether such immune structures provide prognostic information independent of recognised clinicopathological factors and HER2 status. This uncertainty motivated the third component of the present thesis, which aimed to quantify intratumoural CD8⁺ and Foxp3⁺ TIL densities, to identify and define intratumoural LAs as CD20⁺ B cell-rich clusters with admixed T cells, and to determine whether these immune features provide prognostic information independent of established factors and HER2 status in surgically resected cPACs.

In this thesis, I aimed to refine prognostic evaluation in surgically resected cPACs by integrating clinicopathological staging, oncogenic driver profiling, and TME assessment. Chapter 1 examines whether CLCSC, incorporating tumour-size categories, stratifies prognosis in small-breed dogs commonly encountered in Japan. Chapter 2 evaluates the relationships between HER2 alterations and clinicopathological factors, including CLCSC tumour size, and assesses HER2 alterations as a prognostic marker beyond these variables. Chapter 3 quantifies CD8⁺ and Foxp3⁺ TILs and evaluates intratumoural LAs, testing their associations with the factors identified in Chapters 1 and 2 and their independent prognostic value. Collectively, these studies clarify postoperative

outcome heterogeneity in cPACs and provide a basis for improved risk stratification and future comparative research.

Chapter 1

Clinicopathological prognostic factors in surgically resected pulmonary
adenocarcinoma in small-breed dogs

SUMMARY

Tumour size is an important prognostic factor in canine primary lung cancer, and the CLCSC was recently proposed to subdivide tumour size, but its applicability to small-breed dogs has not been clarified. This retrospective, single-centre cohort study evaluated whether CLCSC tumour-size categories are prognostic for progression and survival in small-breed dogs with surgically resected PAC. Between 2005 and 2021, medical records of client-owned dogs weighing <15 kg that underwent lung lobectomy for histologically confirmed PAC were reviewed. Fifty-two dogs met the inclusion criteria. Tumour sizes of ≤ 3 cm, >3 to ≤ 5 cm, >5 to ≤ 7 cm, and >7 cm were recorded in 15, 18, 14, and 5 dogs, respectively. The median PFI and overall survival time (OST) were 754 and 716 days. In univariable analyses, clinical signs, lymph node metastasis, incomplete surgical margins, and higher histologic grade were associated with shorter PFI, whereas older age, clinical signs, incomplete margins, and lymph node metastasis were associated with shorter OST. CLCSC tumour-size category was associated with PFI across all size groups, and tumour size >7 cm was associated with OST. In multivariable analysis, tumour size >5 to ≤ 7 cm and incomplete margins remained independently associated with shorter PFI, whereas older age was independently associated with shorter OST. These findings indicate that CLCSC tumour-size categories are prognostically relevant in small-breed dogs with surgically resected PAC and support the use of this size-based system for risk stratification in this population.

MATERIALS AND METHODS

Case selection

A retrospective cohort study was performed at the Japan Small Animal Cancer Center (JSACC) between April 1, 2005, and March 31, 2021, in dogs weighing <15 kg that had undergone surgical resection of pulmonary masses diagnosed as PACs by histologic examination. Dogs with a prior history of adenocarcinoma and concomitant malignancies other than PACs on the day of initial examination were excluded.

Medical records review

Information from medical records was obtained on signalment, clinical signs at initial examination, clinical pathology results, including hematology and urinalysis, radiography, abdominal ultrasonography, computed tomography (CT), cytology, histology, postoperative chemotherapy, date that tumor recurrence occurred, and presence of distant metastases. Staging, including history, physical examination, blood test results, urinalysis, thoracic and abdominal radiography, and abdominal ultrasound examination, was performed in all cases. The diagnostic methods and date and cause of death (PAC-related, other causes, unknown causes, and last day of confirmed survival if the date of death was unknown) also were collected. When follow-up data were limited, medical records were obtained from the primary veterinarian, or updates were obtained by telephone from the owner. The presence of clinical signs was defined as the initial presentation with any potential clinical sign associated with a lung tumor, including coughing, tachypnea, dyspnea, lethargy, exercise intolerance, anorexia, or hemoptysis, whereas no clinical signs at diagnosis were defined as incidental identification of a lung tumor on imaging with no clinical signs associated the lung tumor (Lee, BM. *et al.*, 2020).

The CLCSC system was used for staging and tumor size classification (Lee, BM. *et al.*, 2020). The anatomic location and longest tumor diameter of PAC and lymph node status were recorded according to the CT data that was reassessed by a single diagnostic radiologist. If the CT data were incomplete, the data were supplemented with the original diagnostic imaging reports. If heterogeneous contrast-enhanced lymph node patterns were seen, the longest lymph node diameter was >12 mm, or a lymph node/vertebral body ratio was >1.05 on CT, lymph node metastasis was suspected (Ballegeer, EA. *et al.*, 2010). Diagnosis, lymph node status, presence, and location of intrapulmonary nodules separate from the primary tumor, tumor invasion into specific organs, and surgical margins were recorded from the original histopathologic reports. Because the histologic pattern and grade of PAC were not evaluated in the original histopathology report, those were reassessed by 2 pathologists. The PAC histologic grade was based on the PPC grading system (McNiel, EA. *et al.*, 1997). Complete resection was defined as a lack of tumor cells at the margins. Perioperative death was defined as death within 14 days after surgery. Adjuvant chemotherapy was defined as cytotoxic chemotherapy administered within 4 weeks after surgery. Surgical complications and adverse events (AEs) of adjuvant chemotherapy were assessed using the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events (VCOG-CTCAE) version 1.1 (Veterinary Co-operative Oncology Group., 2004).

Computed tomography examination

For the CT scanner at the JSACC, I used a 4 multidetector-row (MD) CT scanner (Asteion Super4 Edition TSX-02B/6; Toshiba Medical Systems, Otahara City, Japan) from 2005 to January 2015 and an 80MDCT scanner (Aquilion prime TSX-303A;

Toshiba Medical Systems, Otahara City, Japan) from February 2015 to 2021. Contrast-enhanced CT of the chest was performed under general anesthesia. The CT scanning and reconstruction parameters used at the JSACC for the 4MDCT scanner were 120 kVp, 120 mA, rotation time = 0.75 seconds, slice thickness = 1-2 mm, beam pitch = 1.374, reconstruction interval = 1-2 mm, and a standard algorithm, whereas for the 80MDCT scanner these were 120 kVp, 350 mA, rotation time = 0.5 seconds, slice thickness = 0.5 mm, beam pitch = 1.388, reconstruction interval = 0.5 mm, and a standard algorithm. Iopamidol (Oypalomin; Fuji Pharma) was used as the contrast agent for CT. This contrast agent was administered at a dosage of 2-2.5mL/kg (600-750 mg/kg) for >10 seconds via the cephalic vein, and images were taken 120 seconds after administration.

Statistical analysis

PFI was defined as the time from surgery to progressive disease (PD). PD was defined as recurrence, dissemination, metastasis, or malignant pleural effusion noted postoperatively and was confirmed when the diagnosis was based on imaging, cytologic findings or histologic findings. Dogs were censored from PFI analysis if no detectable PD was noted at the last follow-up or death. OST was defined as the time from surgery to death from any cause. Dogs that were lost to follow-up or remained alive were censored from OST analysis at the date of the last contact.

The Kaplan-Meier method was used to estimate and depict PFI and OST. Interval or survival between groups was compared using the log-rank method. Age, weight, clinical signs at the initial examination, lymph node status (N0 vs N1, 2), completeness of tumor resection (margin), histologic grade (grade 1-3), histologic patterns (papillary vs other), tumor size classification of CLCSC (≤ 3 cm, >3 cm to ≤ 5 cm,

>5 cm to ≤ 7 , or >7 cm), and adjuvant chemotherapy were analyzed as variables to identify potential risk factors associated with PFI and OST. These data were reported as median and interquartile range (IQR). Lymph node metastasis was evaluated by histopathologic examination, and as in previous reports, for dogs in which lymph node resection or biopsy was not performed, lymph node status was recorded as N0 (Lee, BM. *et al.*, 2020, McPhetridge, JB. *et al.*, 2021). Dogs that died perioperatively were excluded from the analysis of PFI and OST of adjuvant chemotherapy. To determine potential risk factors associated with PFI and OST, univariable and multivariable analyses were performed using the Cox proportional hazards regression model. Multivariable analyses included variables for which $P < .05$ was obtained in univariable analyses. These results were presented as hazard ratio (HR) and 95% confidence intervals (CI). The potential risk factors between the dogs receiving or not receiving adjuvant CBDCA were compared using Fisher's exact test. P values $< .05$ were considered significant. Statistical analysis was performed using EZR software (Saitama Medical Centre, Jichi Medical University, Saitama, Japan).

Histology and CLCSC staging

The primary PAC was completely resected in 45 dogs (86.5%). The histologic grades of PAC were grade 1, 2, and 3 in 35 (67.3%), 15 (28.9%), and 2 (4%) dogs, respectively. Vascular invasion was noted in 8 (15.4%) dogs. There were 14, 17, 13, and 8 dogs for the T1, T2, T3, and T4 stages of CLCSC, respectively. In 5 dogs, T stage did not correlate with the tumor size classification of CLSC because of the presence of either intrathoracic organ invasion or a lung nodule. Lymph node metastases were observed in 9 of 37 dogs (24.3%), of which 6 dogs had suspected lymph node metastases on CT.

Thirteen, 28, and 11 dogs were classified as CLCSC stages 1, 2, and 3, respectively. No dogs were classified as stage 4.

Chemotherapy

Adjuvant chemotherapy was administered to 19 dogs, and carboplatin (CBDCA) was used in all dogs. The CBDCA was administered to 3 of the 19 dogs by a referring veterinarian. At the JSACC, CBDCA was administered IV 4-6 times at an interval of every 3 weeks and at a dosage of 200-250 mg/m² for 30-60 minutes. The CBDCA administration had been proposed to the owner at the discretion of each attending or referring veterinarian. A median initial dose of 225 mg/m² (range, 180-250 mg/m²) was administered, and dogs received a median of 4 doses (range, 1-6). Grade 4 neutropenia and thrombocytopenia occurred in 10 (58.8%) and 8 (42.1%) dogs, respectively. Neutropenia with fever occurred in 6 dogs (31.6%), with all dogs recovering. Grade 3 or 4 gastrointestinal toxicity occurred in 2 dogs (10.5%). Differences in baseline prognostic factors were present between dogs that did and did not receive chemotherapy (Table I-1). One dog did not receive adjuvant CBDCA but was given thalidomide postoperatively. The affected dog had multiple lung metastases on Day 193 and died on Day 220.

RESULTS

Patient population

Fifty-two client-owned small-breed dogs met the criteria for inclusion. There were 23 castrated males, 26 spayed females, and 3 intact males. The median age was 12 years (IQR, 10.0-13.0 years). Seventeen dog breeds were included: Toy Poodle (n = 10), Miniature Dachshund (n = 10), Chihuahua (n = 7), Maltese (n = 4), Shih Tzu (n = 4), and 17 dogs of other breeds, each with fewer than 3 dogs per breed (n = 17). The median body weight was 5.6 kg (IQR, 4.4-7.0 kg) and the numbers of dogs ≤ 5 kg, >5 kg to ≤ 10 kg, and >10 kg to ≤ 15 kg were 19, 29, and 4, respectively. Clinical signs at initial presentation were noted in 33 dogs (63.5%): coughing (n = 32), anorexia (n = 9), exercise intolerance (n = 9), lethargy (n = 3), hemoptysis (n = 2), tachypnea (n = 1), and dyspnea (n = 1).

Diagnostic testing and staging

Contrast-enhanced thoracic CT was performed in all cases. Three patients had CTs performed at other institutions. The CT data were partially or totally lost for 2 dogs and could not be reviewed, and thus the description from the original diagnostic imaging report was used. Those reports described the anatomic location and the longest diameter of the mass but did not describe the details of the lymph nodes. The median longest diameter of the lung masses was 41.0 mm (IQR, 30.0-54.0 mm). The lung mass was solitary in 49 dogs, and satellite nodules were found in the same lobes as the lung mass in 3 dogs. The numbers of dogs with tumor size ≤ 3 cm, >3 cm to ≤ 5 cm, >5 cm to ≤ 7 cm, or >7 cm were 15 (28.8%), 18 (34.6%), 14 (26.9%), and 5 (9.6%), respectively. Lung masses occurred in the cranial lobe of the right lung in 8 dogs (15.4%), the middle lobe

of the right lung in 5 dogs (9.6%), the caudal lobe of the right lung in 13 dogs (25.0%), the accessory lobe in 3 dogs (5.8%), the cranial lobe of the left lung in 15 dogs (28.8%), and the caudal lobe of the left lung in 8 dogs (15.4%). Fourteen of 50 dogs (28.0%) were diagnosed with suspected lymph node metastasis. Other CT findings included a small amount of effusion in 4 dogs, 1 of which had lung lobe torsion. Pleural fluid examinations were performed in 2 of 4 dogs; both dogs were found to have transudative pleural effusion and no tumor cells. Distant metastases were not found in any of the cases.

Surgery

Forty-six dogs underwent resection of 1 lung lobe. Because of the anatomical location of the tumor and the pulmonary vessels and trachea, 3 dogs underwent resection of the accessory lobe and right caudal lung lobe, and 3 dogs underwent left lung resections. Lymph nodes were removed in 37 dogs (71.2%); the regional tracheobronchial lymph nodes were removed in 35 dogs. In the other 2 dogs, cranial mediastinal and sternal lymph nodes were removed in 1 dog, and cranial mediastinal lymph nodes were removed in the other dog. One dog had a nodule in the caudal mediastinum that was removed. Lymph nodes were removed in 12 of 14 dogs with suspected metastases on CT. Intraoperative complications occurred in 6 (11.5%) dogs, postoperative complications occurred in 19 (36.5%) dogs, and perioperative death was observed in 4 dogs (7.7%; Table I-2).

Postoperatively, clinical signs improved in 27 of 33 dogs (84.3%). Six dogs showed no improvement in coughing after surgery, and the suspected causes of coughing (including causes affecting >1 dog) were tracheal or bronchial-related disease (n = 3), myxomatous degenerative mitral valve disease (n = 3), carcinomatous pleuritis (n = 1), pneumonia (n = 1), and pneumothorax (n = 1). The remaining 3 dogs, in which improved

coughing could not be confirmed, died during the perioperative period, and therefore it was not possible to assess whether coughing had improved.

Outcomes

Regarding outcomes at the time of analysis, 41 dogs had died (78.8%), 6 were still alive (11.5%), and 5 were lost to follow-up (9.6%).

PD of PAC was reported postoperatively in 24 dogs (46.2%). The sites of PD after surgery were as follows (including sites affecting multiple dogs): lung (n = 13), malignant pleural effusion (n=6), pleura (n=3), humerus (n=2), ribs (n=2), dorsal mediastinum (n = 1), subcutaneous (n = 1) and right eye (n = 1). Progressive disease was diagnosed by diagnostic imaging alone in 8 dogs, and in the remaining 16 dogs, PD was diagnosed by diagnostic imaging and cytology in 14 dogs and diagnostic imaging and histopathology in 2 dogs. The median time from surgery to PD was 208 days (IQR, 52-387.3 days). Twenty-two of the dogs with PD had died by the date of the last confirmation. The causes of death in these dogs were PAC (n = 18), non-neoplastic diseases (n = 2), and other neoplastic diseases (oral malignant melanoma, n = 1), and in 1 case, the cause of death was unknown. Non-neoplastic diseases were congestive heart failure (n = 1) and sepsis (n = 1) associated with pyelonephritis.

Except for dogs that died in the perioperative period, 16 of 25 dogs without PD had died. The causes of death in these dogs were suspected to be non-neoplastic disease (n = 9) and other neoplastic diseases (cutaneous epitheliotropic lymphoma, n = 1), and the cause of death in 6 dogs was unknown. Non-neoplastic diseases included congestive heart failure (n = 2), seizures (n = 2), immune-mediated hemolytic anemia (n = 1),

obstructive hydrocephalus (n = 1), pneumonia (n = 1), chronic kidney disease (n = 1), and protein-losing enteropathy (n = 1).

Median PFI and OST were 754 (95% CI, 354-Not reached days) and 716 days (95% CI, 326-1009 days), respectively. Median PFIs for dogs with tumor sizes ≤ 3 cm, >3 cm to ≤ 5 cm, >5 cm to ≤ 7 cm, or >7 cm were not reached, 754, 365.5, and 95 days, respectively (Figure I-1). Median OSTs for dogs with tumor sizes ≤ 3 cm, >3 cm to ≤ 5 cm, >5 cm to ≤ 7 cm, or >7 cm were 1064, 704, 574, and 110 days, respectively (Figure I-2). The 1-year survival rate was 63.5%, and the 2-year survival rate was 49.0%. Median follow-up time was 544 days (IQR, 229.3-954 days). Median follow-up time for censored dogs was 710 days (IQR, 581.5-865 days).

Results of univariable and multivariable analyses investigating the association of PFI and OST with potential risk factors are shown in Tables I-3 and I-4, respectively. In univariable analysis, potential risk factors associated with PFI were clinical signs, lymph node metastasis, margin, and histologic grade, and those associated with OST were age, lymph node metastasis, and margin. Tumor size classification of CLCSC was associated with PFI in all categories, and only tumor size >7 cm was associated with OST. In multivariable analysis, potential risk factors associated with PFI were tumor size >5 cm to ≤ 7 cm (adjusted HR, 13.68; 95% CI, 1.66-112.5; $P = .01$) and margin (adjusted HR, 5.85; 95% CI, 1.45-23.51; $P = .01$), and the only potential risk factor associated with OST was age (adjusted HR, 1.28; 95% CI, 1.07-1.53; $P = .01$).

Outcome after disease progression

Of the 24 dogs with PD, 12 dogs received rescue treatment: toceranib phosphate (TOC; n = 9), surgery (n = 2), and CBDCA (n = 1). Surgeries on 2 dogs were performed

by referring veterinarians; 1 dog underwent right eye removal, and the other underwent lobectomy of the right caudal lung lobe, both of which were diagnosed as PAC metastases on histopathologic examination. One dog that had received CBDCA as the primary rescue treatment had TOC as a further rescue treatment after recurrence of malignant pleural effusion.

DISCUSSION

In my study, the median PFI and OST for surgically resected PACs in small-breed dogs were 754 and 716 days, respectively. In multivariable analysis, tumor size >5 cm to ≤ 7 cm and margin were associated with PFI, and age was associated with OST. Risk factors associated with progression outcomes and survival time in small-breed dogs with surgically resected PACs have not been reported previously.

In small-breed dogs with surgically resected PACs, tumor size classification of CLCSC was associated with PFI. No significant difference was found between tumor sizes >3 cm to ≤ 5 cm and >5 cm to ≤ 7 cm, and between >5 cm to ≤ 7 cm and >7 cm. However, these results potentially could be attributed to Type II statistical error resulting from inadequate sample size. In addition, because the inclusion criteria in my study were limited to dogs in which surgical resection was performed, non-surgical treatment might have been selected for dogs with larger tumor sizes. Overall survival time was not associated with tumor size classification of CLCSC. In my study, the association between tumor size classification of CLCSC and OST may have been influenced by the fact that 16 of 48 dogs (33.3%), excluding deaths in the perioperative period, died without PD of PACs and half of the PD cases received rescue treatment. The median OST for my study was 716 days, indicating relatively long survival. The median survival time for surgically resected PPCs in dogs has been reported to be 92-399 days (McNiel, EA. *et al.*, 1997, Sabattini, S. *et al.*, 2014, Polton, G. *et al.*, 2018, Lee, BM. *et al.*, 2020, McPhetridge, JB. *et al.*, 2021) and 131-339 days for PACs (Ogilvie, GK. *et al.*, 1989, Polton, GA. *et al.*, 2008, Able, H. *et al.*, 2021). Although it is difficult to identify the specific reasons for long-term survival, population characteristics defined by inclusion criteria (ie, restricted to PACs and small-breed dogs) and the absence of cases with distant metastases were

considered factors for long-term survival in my study. Additionally, age was associated with OST in both univariable and multivariable analyses, with a 1.28-fold increased risk of death for each additional year. In general, the number of dogs with comorbidities increases with age. The median age at initial presentation in my study was 12 years, and the dogs survived for a relatively long time after surgery, which suggests that the risk of death from diseases other than PAC increases with age and that age may have been associated with OST.

Surgical margins were associated with PFI in both univariable and multivariable analyses in my study. Previous studies have reported that surgical margins of PPCs or primary pulmonary neoplasia (PPN) are associated with OST (Lee, BM. *et al.*, 2020, Able, H. *et al.*, 2021, McPhetridge, JB. *et al.*, 2021) and tumor-specific survival (Lee, BM. *et al.*, 2020, Able, H. *et al.*, 2021) in univariable analysis but not in multivariable analysis (Lee, BM. *et al.*, 2020, McPhetridge, JB. *et al.*, 2021). These reports suggested that increased tumor size and invasiveness and location within the lung lobes (hilar and peripheral) may have been associated with surgical margins and influenced the results of multivariable analysis (Lee, BM. *et al.*, 2020, McPhetridge, JB. *et al.*, 2021). I only investigated some of those previously reported factors, making it difficult to determine the confounding factors between surgical margins and the other factors used in my study. All 7 dogs in my study with incomplete margins were found to have PD postoperatively, and the PFI was significantly shortened. Therefore, when surgical margins are incomplete, postoperative progression should be carefully monitored, and adjuvant therapy should be considered.

The lymph node metastatic rate determined by histology in my study was 24.3%, which was comparable to previously reported lymph node metastatic rates ranging

from 22.4% to 32.7% for PPCs (Ogilvie, GK. *et al.*, 1989, Lee, BM. *et al.*, 2020, Rose, RJ. and Worley, DR., 2020). Previous reports found that N stage affects survival in PPCs or PPNs (Ogilvie, GK. *et al.*, 1989, McNiel, EA. *et al.*, 1997, Polton, GA. *et al.*, 2008, Lee, BM. *et al.*, 2020, Rose, RJ. and Worley, DR., 2020). In my study, N stage was associated with PFI and OST in univariable analysis but not in multivariable analysis. However, in my study, 15 dogs in which lymph nodes could not be resected were considered N0. Therefore, histologic lymph node evaluation was not available in all dogs, and the impact of lymph node metastasis may have been underestimated. Interestingly, in 3 of the 9 dogs with histologic lymph node metastasis, no abnormalities were found in the lymph nodes on CT or observed intraoperatively. Similarly, the previous study of PPN reported discrepancies between preoperative CT and histology finding in 28.1% of dogs (McPhetridge, JB. *et al.*, 2021). Therefore, in the future, to accurately determine the prognostic impact of lymph node metastasis of PAC in small-breed dogs, the regional lymph nodes should be removed regardless of preoperative CT and intraoperative macroscopic findings.

In small-breed dogs with surgically resected PACs, I found PD in 46.2% of the dogs. The incidence of PD in dogs with PPC has been reported previously to be 43.3%-50.7% (McNiel, EA. *et al.*, 1997, Lee, BM. *et al.*, 2020), which was comparable to what I observed. Therefore, adjuvant chemotherapy should be considered, especially in dogs with poor prognostic factors. To date, the benefit of adjuvant chemotherapy in dogs with PPC has not been shown (Lee, BM. *et al.*, 2020, Rose, RJ. and Worley, DR., 2020, McPhetridge, JB. *et al.*, 2021). In my study, CBDCA was used in all dogs that received adjuvant chemotherapy, and no significant differences in PFI and OST were observed between the groups treated with surgery and CBDCA vs surgery alone. The retrospective

nature of my study and lack of an analysis to avoid type II statistical error could be reasons for the lack of differences detected in my study. At my institution, I propose using adjuvant CBDCA in dogs with potential risk factors, such as advanced stage, tumor size >5 cm, and highly malignant PAC as determined by histology. In addition, I found significant differences for age and weight in dogs that received adjuvant CBDCA compared to those that did not. These selection biases could have influenced the observed effect of adjuvant CBDCA. Prospective studies should be conducted in larger populations to evaluate the effect of adjuvant CBDCA on PAC in dogs with potential risk factors.

My study had some limitations. First, the study was conducted at a single institution with a relatively small sample size. Second, because of the retrospective study design, some diagnostic methods were incomplete; The CT equipment was modified during the study, and some data was lost; thus, there was incomplete reassessment of CT images. The distance between the surgical margins and the tumor on histologic tissue sections was not evaluated, and therefore the prognostic impact could not be assessed. Third, the PD evaluations were problematic; 9 dogs were diagnosed with PD using only thoracic radiography to identify lung masses. In these dogs, no cytologic or histologic assessments of the lung masses were performed. I also did not perform necropsies on these dogs. It is possible that the lung masses diagnosed as PD were diseases other than PAC. Routine monitoring was recommended, but the follow-up schedule was not standardized. Fourth, because of the long study period, treatment methods were not consistent; surgical techniques for lobectomy were changed (eg, from suture ligation to Endo GIA Stapler) and rescue therapy options after PD were added (eg, TOC).

In conclusion, the tumor size classification of CLCSC can be an important prognostic factor in small-breed dogs with surgically resected PACs. Given that

postoperative PD was found in 46.2% of the dogs in my study, a randomized trial with a larger sample size is warranted to determine the benefit of adjuvant chemotherapy for dogs with potential risk factors.

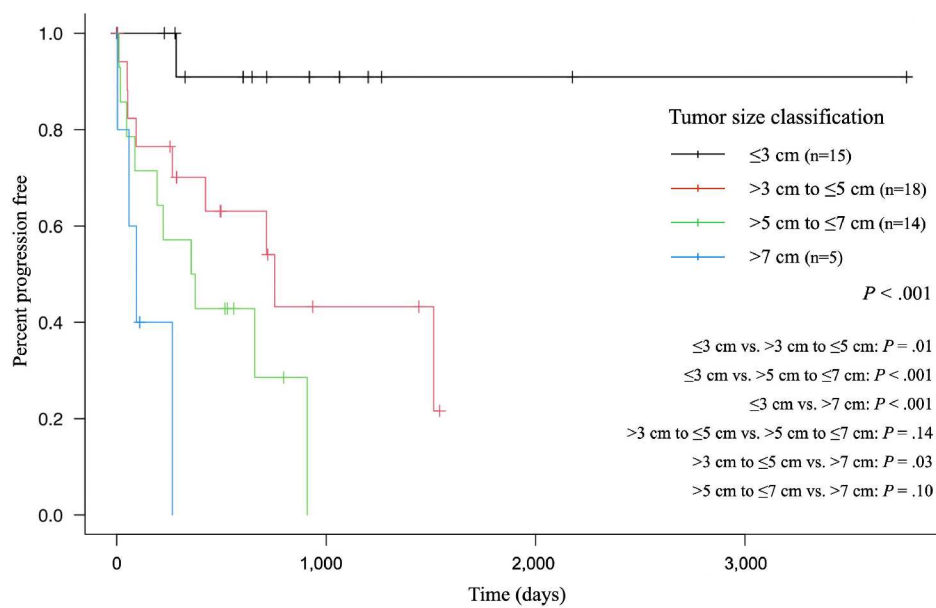


Figure I-1. Kaplan–Meier curve showing the progression-free interval of small-breed dogs with surgically resected pulmonary adenocarcinomas by tumor size classification. Significant differences were found between ≤ 3 cm and > 3 cm to ≤ 5 cm ($P = .01$), ≤ 3 cm and > 5 cm to ≤ 7 cm ($P < .001$), ≤ 3 cm and > 7 cm ($P < .001$), and > 3 cm to ≤ 5 cm and > 7 cm ($P = .03$). Vertical lines indicate censoring.

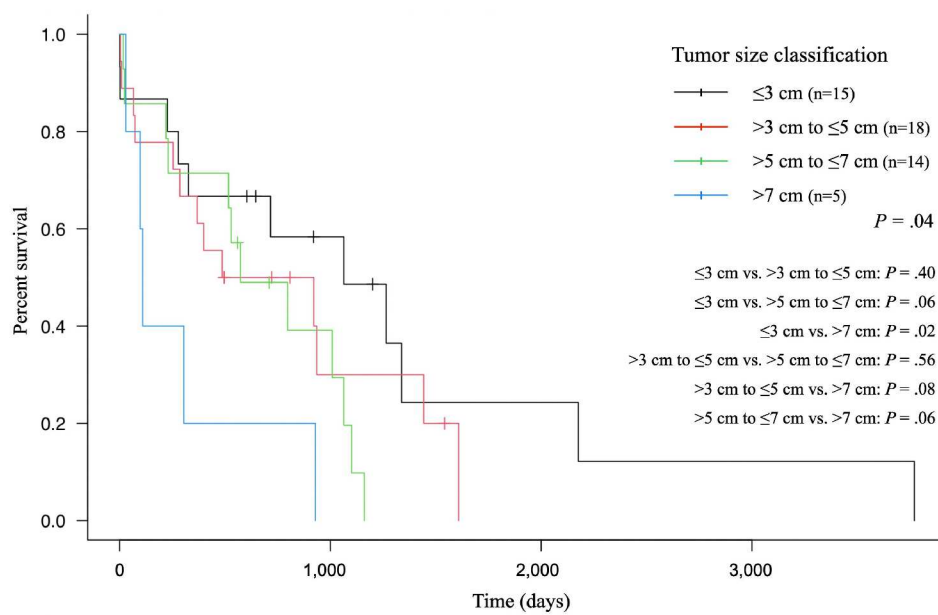


Figure I-2. Kaplan-Meier curve showing the overall survival time of small-breed dogs with surgically resected pulmonary adenocarcinomas by tumor size classification. There was a significant difference only between ≤ 3 cm and > 7 cm ($P=0.02$). Vertical lines indicate censoring.

Table I-1. Comparisons of potential risk factors in small-breed dogs with PACs treated with surgery alone or surgery plus adjuvant chemotherapy.

Variables	Surgery only (n=29)	Surgery + chemotherapy (n=19)	P-value
Median age, years (IQR)	11.5 (11.0-13.0)	10.0 (9.5-11.5)	0.049
Median body weight, kg (IQR)	5.3 (4.1-6.50)	6.3 (5.2-8.95)	0.046
Clinical signs			
No	10 (20.8%)	8 (16.7%)	0.76
YES	19 (39.6%)	11 (22.9%)	
Median tumor size, mm (IQR)	38.0 (25.0-52.0)	51.0 (35.5-55.5)	0.11
Lymph node status			
N0	23 (47.9%)	17 (35.4%)	0.45
N1, 2	6 (12.5%)	2 (4.1%)	
Margin			
Complete	26 (54.2%)	16 (33.3%)	0.67
Incomplete	3 (6.3%)	3 (6.3%)	
CLCSC stage			
Stage 1	10 (20.8%)	1 (2.1%)	0.07
Stage 2	13 (27.1%)	13 (27.1%)	
Stage 3	6 (12.5%)	5 (10.4%)	
Stage 4	0	0	
Histologic grade			
Grade 1	20 (41.7%)	13 (27.1%)	1.0
Grade 2	8 (16.7%)	5 (10.4%)	
Grade 3	1 (2.1%)	1 (2.1%)	
Histologic classification			
Papillary	26 (54.2%)	15 (31.3%)	0.41
Others	3 (6.3%)	4 (8.3%)	

Table I-2. Intra- and postoperative complications in small-breed dogs with surgically resected PACs.

Complications	Grade	No. of dogs
Intraoperative		
Minor hemorrhage	1	4
Air leakage	1	1
Hemorrhage	2	1
Postoperative		
Seroma	1	4
Subcutaneous emphysema	1	5
Reaccumulation of pleural effusion after removal of chest drain	1	2
Hypoxemia requiring oxygen supplementation (>24 h)	3	1
Acute congestive heart failure (pulmonary edema)	3	1
Aspiration pneumonia	3	1
Pleural effusion requiring repeated thoracocentesis	4	1
Hemorrhage requiring revision surgery	4	1
Suspected acute congestive heart failure	5	1
Unknown cause of death	5	1
Acute respiratory distress syndrome and disseminated intravascular coagulation	5	1
Acute respiratory distress syndrome due to noncardiogenic pulmonary edema	5	1

TABLE I-3. Univariable Cox regression analysis of potential risk factors for the Progression-free interval and Overall survival time in 52 small-breed dogs with surgically resected pulmonary adenocarcinomas.

Variable	N	Progression-free interval		Overall survival time	
		Hazard ratio (95% confidence interval)	P-value	Hazard ratio (95% confidence interval)	P-value
Age	52	1.03 (0.88-1.31)	0.48	1.27 (1.07-1.50)	0.006
Body Weight	52	0.97 (0.83-1.13)	0.69	0.92 (0.80-1.06)	0.26
Clinical signs	52				
No	19	Reference		Reference	
YES	33	2.67 (1.05–6.81)	0.03	2.13 (1.05-4.29)	0.03
Tumor size classification	52		<0.001		0.04
≤3 cm	15	Reference		Reference	
>3 cm to ≤5 cm	18	8.87 (1.12-70.09)	0.04	1.62 (0.68-3.81)	0.27
>5 cm to ≤7 cm	14	17.49 (2.19-139.80)	0.007	2.13 (0.87-5.23)	0.10
>7 cm	5	43.12 (4.47-416.20)	0.001	4.76 (1.51-15.06)	0.01
Lymph node status	52				
N0	43	Reference		Reference	
N1, 2	9	3.44 (1.33-8.90)	0.02	2.2 (1.03-4.70)	0.04
Margin	52				
Complete	45	Reference		Reference	
Incomplete	7	12.54 (4.37-36.02)	<0.001	2.92 (1.25-6.82)	0.01
Histologic grade	52		0.002		0.20
Grade 1	35	Reference		Reference	
Grade 2	15	3.09 (1.31-7.27)	0.01	1.78 (0.90-3.52)	0.10
Grade 3	2	7.36 (1.57-34.56)	0.01	2.40 (0.56-10.41)	0.24
Histologic classification	52				
Papillary	45	Reference		Reference	
Others	7	0.56 (0.16-1.92)	0.36	0.44 (0.18-1.11)	0.08
Adjuvant chemotherapy	48				
No	19	Reference		Reference	
Yes	29	1.33 (0.58-3.05)	0.50	0.78 (0.39-1.53)	0.46

TABLE I-4. Multivariable Cox regression analysis of potential risk factors for the Progression-free interval and Overall survival time in 52 small-breed dogs with surgically resected pulmonary adenocarcinomas.

Variable	N	Progression-free interval		Overall survival time	
		Hazard ratio (95% confidence interval)	P-value	Hazard ratio (95% confidence interval)	P-value
Age	52	–	–	1.28 (1.07-1.53)	0.01
Clinical signs	52				
No	19	Reference		Reference	
YES	33	1.42 (0.45-4.51)	0.55	1.82 (0.80-4.14)	0.15
Tumor size classification	52				
≤3 cm	15	Reference		Reference	
>3 cm to ≤5 cm	18	5.74 (0.69-47.52)	0.11	1.67 (0.69-4.04)	0.25
>5 cm to ≤7 cm	14	13.68 (1.66-112.50)	0.01	1.51 (0.57-4.00)	0.41
>7 cm	5	6.55 (0.47-91.08)	0.16	2.00 (0.47-8.42)	0.35
Lymph node status	52				
N0	43	Reference		Reference	
N1, 2	9	2.40 (0.75-7.66)	0.14	2.31 (0.99-5.41)	0.05
Margin	52				
Complete	45	Reference		Reference	
Incomplete	7	5.85 (1.45–23.51)	0.01	1.20 (0.40-3.55)	0.75
Histologic grade	52				
Grade 1	35	Reference		–	
Grade 2	15	2.61 (0.94-7.23)	0.07	–	–
Grade 3	2	1.94 (0.20-18.99)	0.57	–	–

Chapter 2

Prognostic relevance of HER2 alterations in surgically resected
canine pulmonary adenocarcinoma

SUMMARY

HER2 gene mutations have been reported in 8% to 38% of cPACs, most commonly as V659E mutations in exon 20. However, their prognostic and predictive significance remains unclear. This retrospective, single-centre cohort study investigated the frequency of *HER2* mutations in surgically resected cPACs and their association with clinical outcomes. Between 2005 and 2021, lung masses histologically diagnosed as cPACs were collected and subjected to direct sequencing of *HER2* exons 20 and 21. A total of 72 dogs were enrolled, with successful *HER2* gene analysis in 69 cases. *HER2* exon 20 missense mutations were identified in 20 dogs (29.0%), including 18 harbouring the previously reported V659E hotspot mutation within the transmembrane domain. Homozygous mutations were detected in 13 dogs. Univariable analysis revealed associations between PFI and clinical signs, tumour size classification, lymph node metastasis, surgical margin status, and histologic grade. Overall survival time was associated with age, clinical signs, tumour size > 7 cm, histologic subtype, lymph node metastasis, and margin status. In multivariable analysis, tumour size classification and margin status remained significantly associated with PFI, while age, tumour size >7cm, and histologic subtype were independently associated with OST. Notably, the presence of *HER2* mutations was significantly associated with prolonged PFI in both univariable and multivariable analyses, although no significant association with OST was observed. These findings suggest that *HER2* mutation status may serve as a favourable prognostic marker for disease progression in surgically resected cPACs.

MATERIALS AND METHODS

Study Design

This retrospective cohort study was performed at the JSACC between April 2005 and December 2021. Dogs included in the study underwent surgical resection of pulmonary masses subsequently diagnosed as PACs by histological evaluation. Fifty-two cases overlapped with those from a prior study on clinicopathological prognostic factors in cPACs, the JSACC but the present study specifically focused on evaluating the prognostic significance of *HER2* mutation status and HER2 immunoreactivity scores. Medical records were reviewed to collect clinical, histopathological, and survival data. Tumour grades and histological patterns were reassessed by two pathologists at the JSACC using the PPC grading system (McNiel, EA. *et al.*, 1997). Data on the date and cause of death (classified as PAC-related, other, unknown, or last confirmed survival date) were also collected. For incomplete follow-up data, information was obtained from referring veterinarians or obtained through phone interviews with the owner. The presence of clinical signs was defined based on previously published criteria (Lee, BM. *et al.*, 2020). Tumour staging and size classification followed the CLCSC system (Lee, BM. *et al.*, 2020). Tumour size was defined as the maximum diameter of the primary lung lesion measured on preoperative CT images.

HER2 Mutations Analysis

Genomic DNA was extracted from FFPE samples (10µm/slide ×4 slides) using the QIAamp DNA FFPE Tissue Kit (Qiagen), following the manufacturer's instructions. Exons 20 and 21, including the V659E hotspot mutation, were amplified using primers YTM2319 (5'-AAACATCAGGGACCTGCCTA-3') and YTM2352 (5'-

AGCAGCCTCCGCATAGTGTA-3') with MightyAmp DNA polymerase Ver.3 (TAKARA BIO INC.). PCR products were extracted from agarose gels, followed by direct sequencing with primer YTM2319 and comparison to the reference canine genomic sequence (CamFam3.1).

Immunohistochemistry

Paraffin-embedded tissue sections (4µm) were mounted on MAS-coated slides (Matsunami Glass Kogyo, Osaka, Japan), deparaffinised in xylene, and rehydrated in ethanol. Antigen retrieval was performed by autoclaving in pH9.0 Target Retrieval Solution (Dako) for 20 min at 121°C. Endogenous peroxidase activity was blocked using 3% H₂O₂ in methanol for 30 min at room temperature (RT), followed by phosphate-buffered saline (PBS) rinsing. Subsequently, sections were blocked with 5% bovine serum albumin in PBS for 30 min at RT and incubated overnight at 4°C with anti-ErbB2 antibody (clone CB11, Abcam) or mouse IgG1 (ebioscience) as a control. After three 5-min PBS washes, sections were treated with Histofine Simple Stain MAX PO (Nichirei Corporation, Tokyo, Japan) as a secondary antibody for 30 min at RT. After another wash in PBS, diaminobenzidine (Nacalai Tesque, Kyoto, Japan) was used for visualisation, followed by counter-staining with Mayer's haematoxylin.

HER2 immunoreactivity was scored based on previous studies (Yoshimoto, S. *et al.*, 2020). Scoring was as follows: 0 (no reactivity), 1+ (weak, incomplete immunoreactivity in <10% of tumour cells), 2+ (strong, incomplete immunoreactivity in 10% ≤ of tumour cells), and 3+ (strong, complete immunoreactivity in 10% ≤ of tumour cells). Scores of 0 and 1+ were considered negative, whereas scores of 2+ and 3+ were considered positive. Histopathologic evaluation of H&E sections was performed by a

board-certified pathologist. HER2 immunoreactivity was scored by the first author, who did not participate in the histopathologic evaluation. The scorer was not blinded to clinical outcomes. The anti-ErbB2 antibody used in this study (clone CB11, Abcam) is a mouse monoclonal antibody raised against the internal domain of human HER2. Its cross-reactivity with canine HER2 was validated in our laboratory by Western blotting using the cPAC cell line CLAC, with and without transfection of a canine HER2 expression construct. The antibody detected an endogenous HER2 band at the expected molecular weight (~185kDa) in CLAC cells and an additional band corresponding to the overexpressed HER2 protein in transfected cells, confirming its specificity for canine HER2 (data not shown).

Statistical Analysis

PFI was defined as the time from surgery to the onset of PD, including recurrence or metastasis, confirmed by diagnostic imaging, cytology, or histopathology. OST was defined as the time from surgery to death from any cause. Dogs lost to follow-up or still alive were censored at the date of last contact.

PFI and OST were analysed using the Kaplan–Meier method and compared using the log-rank test. Variables assessed included general clinical parameters (age and body weight), *HER2* mutation status, HER2 immunoreactivity score, and clinicopathologic factors previously reported as potential negative prognostic indicators in cPACs (clinical signs, lymph node status, surgical margin, histologic grade and pattern, and tumour size (CLCSC classification)). Cox proportional hazards regression was used to identify variables associated with PFI and OST. Only variables with $p < 0.05$ in univariable analysis were entered in multivariable models. No automated stepwise

selection procedure was applied; all variables meeting the inclusion criterion were entered simultaneously, and the final model included only those variables that remained statistically significant ($p < 0.05$). Fisher's exact test or the Mann–Whitney U test was used to assess associations between *HER2* mutation status and other variables. Results were reported as median or IQR. A p -value <0.05 was considered statistically significant. Statistical analysis was conducted using EZR (Saitama Medical Centre, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R.

RESULTS

Patient Population

Seventy-two client-owned dogs met the inclusion criteria. Of these, 29 were castrated males, 36 spayed females, 5 intact males, and 2 intact females. The median age was 12 years (IQR, 9.8–13.0years). Twenty-five dog breeds were represented, including Toy Poodle (n=11), Miniature Dachshund (n=10), Chihuahua (n = 7), Shih Tzu (n = 5), and Maltese (n = 4). An additional 35 dogs belonged to various other breeds, each represented by fewer than three individuals. The median body weight was 6.1kg (IQR, 5.0–9.3kg). Clinical signs at the time of initial presentation were recorded in 41 dogs (56.9%).

CLCSC Staging and Histology

Lung masses were located in the cranial lobe of the right lung in 12 dogs (16.7%), the middle lobe of the right lung in six dogs (8.3%), the caudal lobe of the right lung in 16 dogs (22.2%), the accessory lobe in three dogs (4.2%), the cranial lobe of the left lung in 20 dogs (27.8%), and the caudal lobe of the left lung in 15 dogs (20.8%). The median maximum diameter of the lung masses was 39.5 mm (IQR, 26.0–54.0 mm). The numbers of dogs with tumours ≤ 3 cm, >3 to ≤ 5 cm, >5 to ≤ 7 cm, or >7 cm were 24 (37.5%), 22 (30.6%), 17 (23.6%), and 9 (12.5%), respectively.

Complete surgical resection of the primary PAC was achieved in 63 dogs (87.5%). Papillary adenocarcinoma was diagnosed in 62 dogs (86.1%). Histological grading of the tumours revealed 48 dogs with grade 1 (66.7%), 22 dogs with grade 2 (30.6%), and two dogs with grade 3 tumours (2.8%). Vascular invasion was noted in 10 dogs (13.9%). Lymph node metastases were present in 12 of the 50 dogs for whom lymph

node status was available (24.0%). According to CLCSC staging, 19, 37, and 16 dogs were categorised as stage 1, 2, and 3, respectively. No dogs were classified as stage 4.

Postoperative Treatment and Outcomes

Adjuvant chemotherapy was administered to 23 dogs (31.9%), with 22 receiving carboplatin alone and one dog receiving a combination of carboplatin and doxorubicin.

Regarding outcomes at the time of analysis, 58 dogs had died (80.6%), 7 were still alive (9.7%), and 7 were lost to follow-up (9.7%). The median follow-up time for censored cases was 783.5 days (IQR, 642.5–1151.5 days). Progressive disease of cPAC was documented postoperatively in 34 dogs (47.2%). Among these 34 dogs, 25 (73.5%) had died from cPAC by the date of last confirmation, 3 had died from other causes, 4 were alive at last follow-up, 1 died in the perioperative period, and 1 died of unknown cause. Among the dogs with PD, nine received toceranib phosphate and one dog was treated with an anti-canine programmed cell death protein 1 antibody as rescue therapy. No HER2-targeted therapies were administered during the study period. Across all deaths (n = 58), non-cPAC-related causes were identified in 18 dogs (31.0%), the cause of death was unknown in 11 dogs (19.0%), and 4 dogs (6.9%) died in the perioperative period. Non-cPAC-related causes comprised congestive heart failure (n = 3), seizure disorder (n = 3), pneumonia (n = 2), and single cases of protein-losing enteropathy, chronic kidney disease, airway obstruction-related respiratory failure, urinary tract infection-associated sepsis, obstructive hydrocephalus, immune-mediated hemolytic anaemia, right-sided heart failure-associated respiratory failure, melanoma, cutaneous epitheliotropic lymphoma, and osteosarcoma. Median PFI and OST were 785 days (95% CI, 375–not

reached) and 797 days (95% CI, 399–936), respectively. The 1-year survival rate was 66.4%, and the 2-year survival rate was 50.4%.

HER2 Mutation Status and HER2 Immunoreactivity Scoring

Sequencing was successful in 69 of the 72 dogs that underwent PCR. In the remaining three cases, PCR amplification failed or sequencing data were of insufficient quality for analysis. *HER2* mutations were identified in 20 dogs (26.0%). A summary of the detected mutations is presented in Figure II-1. Mutations such as A664T and K676E, previously reported in other studies, were not identified in this cohort. The V659E mutation was detected in 18 dogs—12 homozygous and six heterozygous—while the V659G mutation was found in two dogs, one homozygous and one heterozygous. Additionally, two synonymous mutations, p.Q680Q and p.K681K, were detected in 15 and 17 dogs, respectively.

HER2 IHC staining was successfully performed in 71 of the 72 dogs (Figure II-2). HER2 expression analysis showed that 20 samples (28.2%) were scored 3+, 30 samples (42.3%) as 2+, and 21 samples (29.6%) as 1+. No cases were scored as 0.

Analysis of Prognostic Factors

The results of univariable and multivariable analyses assessing the association of potential risk factors with PFI and OST are summarised in Tables II-1 and II-2. In univariable analysis, factors significantly associated with PFI included clinical signs, lymph node metastasis, surgical margin, tumour size classification (CLCSC), histologic grade, and *HER2* mutation status. Factors associated with OST included age, clinical signs, lymph node metastasis, surgical margin, tumour size classification (CLCSC), and

histologic patterns (Table 1). In multivariable analysis, *HER2* mutation status, surgical margin, and tumour size classification were independently associated with PFI. For OST, significant factors included age, tumour size > 7 cm, and histologic patterns (Table 2). Dogs with *HER2* mutations did not reach a median PFI, whereas dogs without the mutation had a median PFI of 632 days (Figure II-3).

The relationship between *HER2* mutation status and various clinicopathological factors is shown in Tables II-3. No statistically significant association was found between *HER2* mutation status and *HER2* expression levels. However, tumours harbouring a *HER2* mutation tended to exhibit lower histological grade compared to the wild-type group. Similarly, analysis of *HER2* expression in relation to other prognostic variables (Tables II-4) revealed no significant associations with clinicopathological features.

DISCUSSION

In this retrospective cohort study of 72 dogs with surgically resected cPACs, I expanded upon previous report on the clinical features of cPACs in JSACC by including a larger sample size and providing a more comprehensive clinical characterisation. More importantly, I analysed *HER2* mutations, focusing on exons 20 and 21, and evaluated *HER2* protein expression via immunohistochemistry (IHC).

First, the clinical characteristics of the cases remained largely consistent with earlier findings, despite the increased cohort size. I also compared our results with those of Treggiari *et al.*, published recently (Treggiari, E. *et al.*, 2025). As in their study, tumour size emerged as a significant prognostic factor. My study further identified significant differences in PFI across all tumour size categories. While the overall PFI appeared slightly longer in my cohort, outcomes for dogs with tumours smaller than 5 cm were similar across both studies. The high proportion of small-breed dogs in my cohort may partly account for this difference.

HER2 mutations were identified in 29.0% of cases, consistent with prior reports of mutation frequency ranging from 8% to 38% in canine populations (Lorch, G. *et al.*, 2019, Brunetti, B. *et al.*, 2024). However, those studies did not evaluate the relationship between *HER2* mutation status, *HER2* expression, or clinical outcomes. In my study, *HER2* mutation was independently associated with prolonged PFI in both univariable and multivariable analyses, indicating a favourable prognostic impact. No significant association was found with OST (Figure S1), which may be attributed to age-related comorbidities or non-cancer-related mortality in this older canine population. Although the prognostic implications of *HER2* mutations in human NSCLC remain controversial, with several studies linking them to poor prognosis (Suzuki, M. *et al.*, 2015, Wei, XW.

et al., 2020) and resistance to targeted therapies (Nützinger, J. *et al.*, 2023), I initially hypothesised a similar outcome in cPACs. Contrary to expectations, *HER2* mutations in my cohort were associated with a favourable prognosis. The underlying reasons remain unclear, but several possible explanations exist. Tumours harbouring *HER2* mutation tended to exhibit lower histologic grades, which may have contributed to the improved PFI, although other clinicopathologic factors did not differ significantly. Notably, *HER2* mutation remained significantly associated with prolonged PFI even when histologic grade was included in the multivariable model, suggesting that the prognostic effect of *HER2* mutation may not be fully explained by grade alone.

HER2 immunoreactivity did not correlate with *HER2* mutation status, suggesting that protein overexpression and gene mutation may occur through distinct mechanisms in cPACs. This finding aligns with observations in human NSCLC, where *HER2* amplification (Arcila, ME. *et al.*, 2012, Li, BT. *et al.*, 2016), overexpression (Li, BT. *et al.*, 2016), and mutation do not necessarily co-occur. In my study, *HER2* immunoreactivity was present in all tumours, with IHC scores ranging from 1+ to 3+, consistent with prior studies of *HER2* overexpression in 43% to 73% of cases (Yoshimoto, S. *et al.*, 2020, Brunetti, B. *et al.*, 2024). Although *HER2* overexpression is typically attributed to gene amplification or transcriptional upregulation, I did not assess *HER2* copy number in this study. Consequently, the underlying mechanism of protein overexpression remains undetermined. Moreover, functional activation of *HER2* signalling by mutation may not result in increased total protein levels; rather, phosphorylation status may better reflect biological activity. Due to the lack of validated antibodies for phosphorylated *HER2* in canine tissue, this analysis could not be performed. While *HER2* mutations in cPACs have been linked to PI3K/ AKT pathway activation

(Lorch, G. *et al.*, 2019), the downstream signalling consequences of HER2 overexpression remain poorly defined. In this study, *HER2* mutations—not HER2 overexpression—were associated with improved prognosis, suggesting that mutation-driven HER2 activation may exert distinct biological effects compared to protein overexpression. This distinction may relate to differences in signalling intensity, duration, or cellular context, even if both mechanisms converge on pathways such as PI3K/AKT and MAPK/ERK.

Although HER2-targeted therapies were not used in this cohort, the high frequency and conserved location of the *HER2* V659E mutation in the TMD suggest that cPACs may serve as a spontaneous model for evaluating HER2-targeted treatments. In human NSCLC, TMD mutations such as V659E and G660D function as oncogenic drivers and demonstrate sensitivity to afatinib in both preclinical and clinical settings (Ou, SI. *et al.*, 2017). Additionally, cPAC cell lines harbouring the *HER2* V659E mutation exhibit constitutive AKT signalling and increased sensitivity to HER2 inhibitors, including lapatinib and neratinib (Lorch, G. *et al.*, 2019), supporting their utility as translational models for therapeutic development.

This study has several limitations. First, its retrospective nature may have introduced selection bias, limiting our ability to determine whether the *HER2* mutation directly influenced prognosis. Second, improvements in surgical techniques and the introduction of molecularly targeted agents such as TOC over the long study period may have impacted clinical outcomes. Third, the absence of a standardised follow-up protocol may have affected the consistency of progression and survival evaluations. Fourth, variable-quality archived FFPE samples, including potential DNA degradation, likely contributed to failed amplifications in some cases, and prolonged storage may have

affected IHC staining quality, potentially influencing HER2 expression assessment. Fifth, no correction for multiple comparisons (e.g., false discovery rate adjustment) was applied in the univariable analysis, which may have increased the risk of type I error. Finally, I did not assess phospho-HER2 or downstream signalling markers, which could have provided further insight into the functional consequences of *HER2* mutations.

In conclusion, *HER2* mutation was a common and recurrent finding in cPACs and was associated with longer PFI following surgical resection. These results suggest that *HER2* mutation status may serve as a useful prognostic marker in cPACs and may have immunologic and therapeutic relevance.

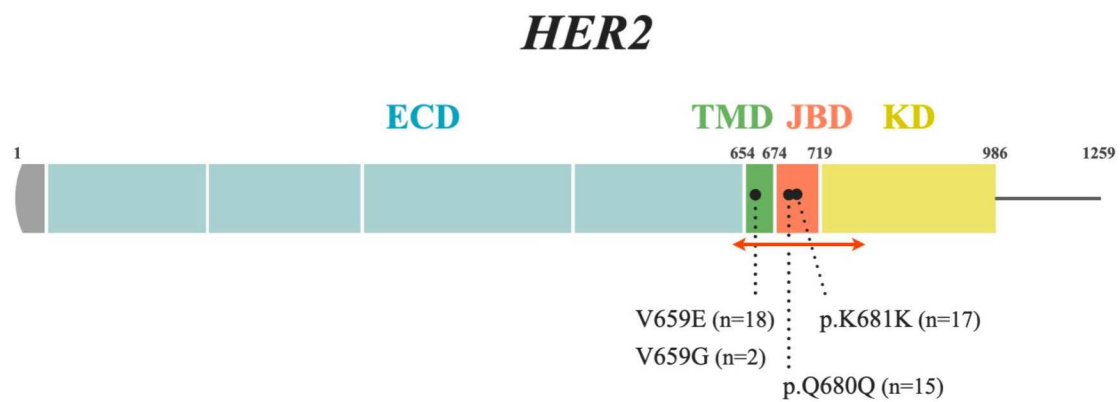


Figure II-1. Schematic representation of *HER2* mutations identified in canine pulmonary adenocarcinomas, showing their distribution within the *HER2* protein. Numbers showed above the boxes indicated the amino acid positions of the *HER2* protein. The red arrow denotes the region of amplification (amino acids 650–736). ECD, extracellular domain; JMD, juxtamembrane domain; KD, kinase domain; TMD, transmembrane domain.

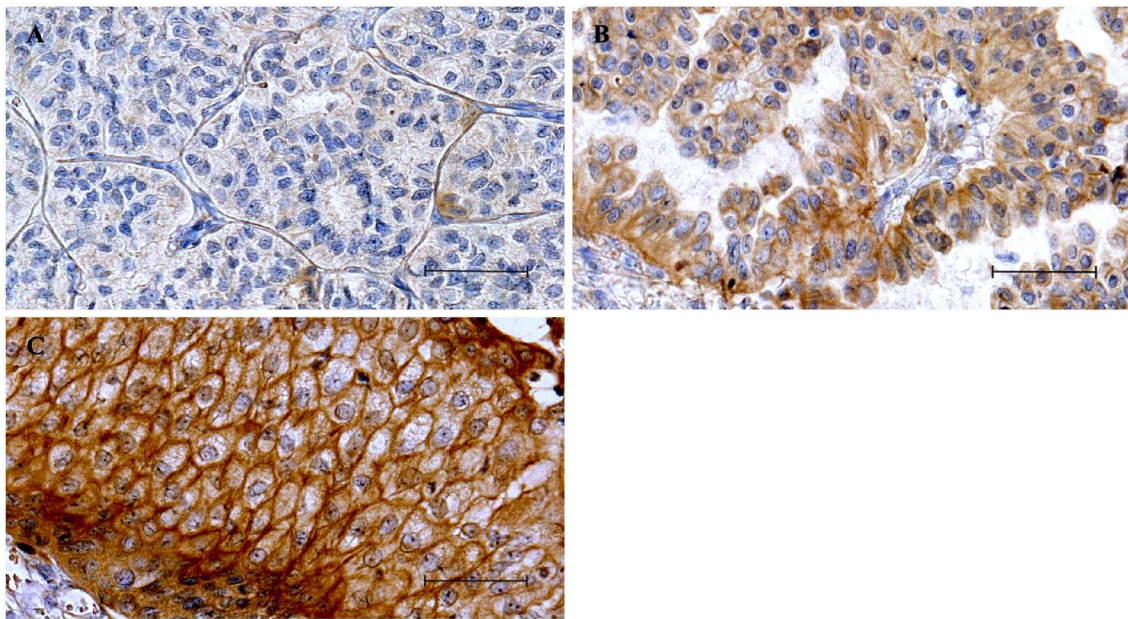


Figure II-2. Immunohistochemical staining of HER2 protein in canine pulmonary adenocarcinomas. Representative images are shown with HER2 expression scored as 1 (A, negative), 2 (B, positive), and 3 (C, positive). Diaminobenzidine (DAB) was used as the chromogen, and sections were counterstained with Mayer's haematoxylin. Scale bar = 50 μ m

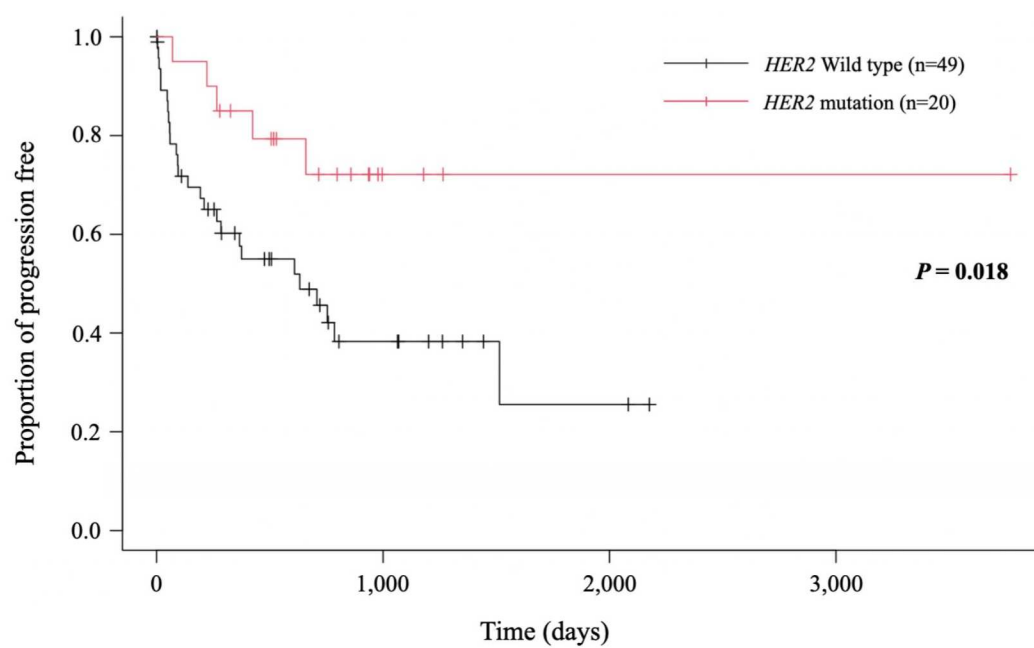


Figure II-3. Kaplan–Meier curves of progression-free interval (PFI) in dogs with surgically resected pulmonary adenocarcinomas based on *HER2* mutation status. Dogs harbouring the *HER2* mutation (red line) had significantly longer PFI than those without the mutation (black line). Median PFI was not reached in the mutant group, whereas it was 632 days in the wild-type group.

TABLE II-1. Univariable Cox regression analysis of potential risk factors for the progression-free interval and overall survival time in dogs with surgically resected pulmonary adenocarcinomas.

Variable	N	Progression-free interval		Overall survival time	
		Hazard ratio (95% confidence interval)	<i>P</i> -value	Hazard ratio (95% confidence interval)	<i>P</i> -value
Age	72	1.03 (0.88-1.31)	0.69	1.27 (1.07-1.50)	0.006
Body Weight	72	1.00 (0.96–1.05)	0.86	0.92 (0.80-1.06)	0.26
Clinical signs	72				
No	31	Reference		Reference	
YES	41	2.50 (1.21–5.15)	0.01	2.13 (1.05-4.29)	0.03
Tumor size classification	72		<0.001		0.04
≤3 cm	24	Reference		Reference	
>3 cm to ≤5 cm	22	7.56 (1.69–33.78)	0.008	1.62 (0.68-3.81)	0.27
>5 cm to ≤7 cm	17	14.94 (3.34–66.81)	<0.001	2.13 (0.87-5.23)	0.10
>7 cm	9	21.82 (4.46–106.80)	<0.001	4.76 (1.51-15.06)	0.01
Lymph node status	72				
N0	60	Reference		Reference	
N1, 2	12	4.77 (2.16–10.57)	<0.001	2.2 (1.03-4.70)	0.04
Margin	72				
Complete	63	Reference		Reference	
Incomplete	9	12.27 (4.91–30.69)	<0.001	2.92 (1.25-6.82)	0.01
Histologic grade	72		0.004		0.20
Grade 1	48	Reference		Reference	
Grade 2	22	2.18 (1.07–4.45)	0.03	1.78 (0.90-3.52)	0.10
Grade 3	2	6.47 (1.46–28.75)	0.01	2.40 (0.56-10.41)	0.24
Histologic classification	72				
Papillary	62	Reference		Reference	
Others	10	0.61 (0.21–1.75)	0.36	0.44 (0.18-1.11)	0.08
HER2 mutation	69				
Negative	49	Reference		Reference	
Positive	20	0.33 (0.13-0.87)	0.03	0.86 (0.47-1.54)	0.61
HER2 expression	71				
Negative	21	Reference		Reference	
Positive	50	1.44 (0.64-3.21)	0.37	1.13 (0.62-2.05)	0.68
Adjuvant chemotherapy	68				
No	45	Reference		Reference	
Yes	23	1.33 (0.68-2.75)	0.38	0.78 (0.39-1.53)	0.46

TABLE II-2. Multivariable Cox regression analysis of potential risk factors for the Progression-free interval and Overall survival time in 52 small-breed dogs with surgically resected pulmonary adenocarcinomas.

Variable	N	Progression-free interval		Overall survival time	
		Hazard ratio (95% confidence interval)	P-value	Hazard ratio (95% confidence interval)	P-value
Age	72	–	–	1.18 (1.04–1.34)	0.01
Clinical signs	72				
No	31	Reference		Reference	
YES	41	1.17 (0.46–2.96)	0.75	1.60 (0.88–2.91)	0.12
Tumor size classification	72				
≤3 cm	24	Reference		Reference	
>3 cm to ≤5 cm	22	5.79 (1.27–26.41)	0.02	–	–
>5 cm to ≤7 cm	17	17.84 (3.56–89.44)	<0.001	–	–
>7 cm	9	8.64 (1.43–50.23)	0.02	1.96 (0.83–4.61)	0.13
Lymph node status	72				
N0	60	Reference		Reference	
N1, 2	12	2.47 (0.85–7.14)	0.10	2.40 (1.13–5.08)	0.01
Margin	72				
Complete	63	Reference		Reference	
Incomplete	9	4.23 (1.22–14.67)	0.02	3.24 (1.50–7.00)	0.02
Histologic grade	72				
Grade 1	48	Reference		–	
Grade 2	22	1.37 (0.55–3.34)	0.50	–	–
Grade 3	2	1.07 (0.14–8.08)	0.95	–	–
HER2 mutation	69				
Negative	49	Reference		Reference	
Positive	20	0.31 (0.13–0.87)	0.04	–	–

TABLE II-3. Comparisons of potential risk factors in dogs with surgically resected pulmonary adenocarcinomas based on *HER2* V659E mutation status.

Variables	<i>HER2</i> wild type (n=49)	<i>HER2</i> mutation (n = 20)	<i>P</i> -value
Median age, years (IQR)	12.0 (10.0–13.0)	11.0 (9.0–12.25)	0.72
Median body weight, kg (IQR)	5.7 (5.0–8.2)	7.5 (4.0–12.7)	0.52
Clinical signs			
No	21 (30.4%)	10 (14.5%)	0.61
YES	28 (40.6%)	10 (14.5%)	
Median tumor size, mm (IQR)	26.0 (9.0–40.0)	27.9 (15.0–31.2)	0.60
Tumor size classification			
≤3 cm	16 (23.2%)	8 (11.6%)	0.08
>3 cm to ≤5 cm	16 (23.2%)	5 (7.2%)	
>5 cm to ≤7 cm	8 (11.6%)	7 (10.1%)	
>7 cm	9 (13.0%)	0	
Lymph node status			
N0	39 (56.5%)	19 (27.5%)	0.16
N1, 2	10 (14.5%)	1 (1.4%)	
Margin			
Complete	41 (59.4%)	20 (29.0%)	0.09
Incomplete	8 (11.6%)	0	
Histologic grade			
Grade 1	28 (40.6%)	18 (26.1%)	0.02
Grade 2	19 (27.5%)	2 (2.9%)	
Grade 3	2 (2.9%)	0	
Histologic classification			
Papillary	41 (59.4%)	19 (27.5%)	0.27
Others	8 (11.6%)	1 (1.4%)	
HER2 expression			
Negative	13 (18.8%)	7 (10.1%)	0.56
Positive	36 (52.2%)	13 (18.8%)	
Adjuvant chemotherapy			
No	31 (47.7%)	12 (18.5%)	0.57
Yes	14 (21.5%)	8 (12.3%)	

TABLE II-4. Comparisons of potential risk factors in dogs with surgically resected pulmonary adenocarcinomas based on HER2 expression status.

Variables	HER2 expression Negative (n=21)	HER2 expression Positive (n=50)	<i>P</i> -value
Median age, years (IQR)	12.0 (10.0–13.0)	11.0 (9.0–12.8)	0.35
Median body weight, kg (IQR)	5.9 (5.2–12.2)	6.2 (4.93–8.58)	0.56
Clinical signs			
No	11 (15.5%)	20 (28.2%)	0.43
YES	10 (14.1%)	30 (42.3%)	
Median tumor size, mm (IQR)	30.0 (23.0–51.0)	41.0 (25.0–54.0)	0.26
Tumor size classification			
≤3 cm	11 (15.5%)	13 (18.3%)	0.16
>3 cm to ≤5 cm	4 (5.6%)	18 (25.4%)	
>5 cm to ≤7 cm	3 (4.2%)	13 (18.3%)	
>7 cm	3 (4.2%)	6 (8.5%)	
Lymph node status			
N0	16 (22.5%)	43 (60.6%)	0.32
N1, 2	5 (7.0%)	7 (9.9%)	
Margin			
Complete	18 (25.4%)	45 (63.4%)	0.69
Incomplete	3 (4.2%)	5 (7.0%)	
Histologic grade			
Grade 1	14 (19.7%)	33 (46.5%)	0.79
Grade 2	6 (8.5%)	16 (22.5%)	
Grade 3	1 (1.4%)	1 (1.4%)	
Histologic classification			
Papillary	15 (21.1%)	46 (64.8%)	0.055
Others	6 (8.5%)	4 (1.4%)	
HER2 mutation			
Wild type	13 (18.3%)	36 (50.7%)	0.56
Positive	7 (9.9%)	13 (18.3%)	
Adjuvant chemotherapy			
No	14 (20.9%)	30 (44.8%)	0.78
Yes	6 (9.0%)	17 (25.4%)	

Chapter 3

Prognostic significance of the tumour microenvironment in resected
canine pulmonary adenocarcinoma

SUMMARY

The prognostic relevance of the TME in surgically resected cPACs remains uncertain. This retrospective single-centre cohort study evaluated associations between intratumoural immune features and postoperative outcomes. Dogs treated during 2005–2021 with histologically confirmed cPACs after surgical excision and with an evaluable tumour microenvironment were included. IHC for CD8, Foxp3, CD3, and CD20 was performed. Tumour-infiltrating lymphocyte densities for CD8+ and Foxp3+ cells were quantified in five intratumoural hotspots of 0.237 mm² each. LAs were defined as intratumoural aggregates of at least 50 CD20+ B cells with intercellular spacing ≤ 10 μ m and with admixed CD3+ T cells. Primary outcomes were progression-free interval and overall survival time. A total of 71 dogs were enrolled. LAs were identified in 49 dogs (69.0%). Median CD8+ density was 130.0 cells/mm² and median Foxp3+ density was 79.3 cells/mm². Tumours with LAs showed higher Foxp3+ density than tumours without LAs. Median PFI and OST were 754 days (95% CI, 375–not reached) and 716 days (95% CI, 399–936), respectively. In multivariable analysis, tumour size classification, lymph node metastasis, surgical margin, and the presence of LAs were independently associated with shorter PFI. Age and lymph node metastasis were independently associated with OST. The present findings indicate that IHC features provide additional prognostic information for postoperative risk stratification in cPACs, supplementing established clinicopathological factors.

MATERIALS AND METHODS

Study Design

This retrospective cohort study was performed at JSACC between April 2005 and December 2021. Dogs were enrolled if they underwent surgical excision of a pulmonary mass, were histologically diagnosed with PAC, and had TME data available for evaluation. Almost all cases overlapped with those from a prior study in cPACs, but the present study specifically focused on evaluating the prognostic significance of the TME. The TME was assessed for TIL density and for the presence of LAs. Medical records were reviewed to collect clinical, histopathological, *HER2* mutation, *HER2* immunoreactivity scores and survival data. The *HER2* immunoreactivity scores were determined as previously described (Yoshimoto, S. *et al.*, 2020). Tumour grades and histological patterns were reassessed by two pathologists using the PPC grading system (McNiel, EA. *et al.*, 1997). Data on the date and cause of death (classified as PAC-related, other, unknown, or last confirmed survival date) were also collected. For incomplete follow-up data, information was obtained from referring veterinarians or obtained through phone interviews with the owner. The presence of clinical signs was defined based on previously published criteria (Lee, BM. *et al.*, 2020). Perioperative death was defined as death within 14 days after surgery. Adjuvant chemotherapy was defined as cytotoxic chemotherapy administered within 4 weeks after surgery. Tumour staging and size classification followed the CLCSC system (Lee, BM. *et al.*, 2020).

Immunohistochemistry processing

IHC staining was performed to evaluate tumour-infiltrating lymphocyte subsets, including CD3+, CD8+, CD20+, and Foxp3+ cells, as previously described with minor

modifications (Nururrozi, A. *et al.*, 2024). Briefly, formalin-fixed, paraffin-embedded tumour samples were sectioned at 4 μm thickness and mounted onto glass slides. Following deparaffinization in xylene and rehydration through graded ethanol series to distilled water, antigen retrieval was carried out by heating the sections in 0.01 M citrate buffer (pH 6.0) at 121 $^{\circ}\text{C}$ using an autoclave. Endogenous peroxidase activity was quenched by incubating sections in 3% hydrogen peroxide in methanol for 30 min at room temperature. Non-specific protein binding was blocked with 5% skim milk and/or bovine serum albumin diluted in PBS for 30 min. The slides were then incubated with primary antibodies specific for canine CD3 (clone CD3-12, Abcam, Tokyo, Japan), CD8 (rat anti-canine CD8 monoclonal antibody [clone F3- B2] (Sakai, O. *et al.*, 2020), CD20 (rabbit anti-CD20 polyclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA), or Foxp3 (mouse/rat anti-FOXP3 monoclonal antibody (clone FJK-16s, Invitrogen, Carlsbad, CA, USA)) at appropriate dilutions overnight at 4 $^{\circ}\text{C}$. After washing, sections were treated with a species-appropriate secondary antibody reagent (Histofine[®], Nichirei Bioscience Inc., Tokyo, Japan) for 30 min at room temperature. Visualization was achieved using a diaminobenzidine (DAB) chromogenic substrate (Nacalai Tesque Inc., Kyoto, Japan), and the slides were counterstained with Mayer's hematoxylin (FUJIFILM Wako, Osaka, Japan). For each staining run, isotype-matched antibodies were used as negative controls to confirm specificity.

Immunohistochemistry quantification and analysis

I prepared IHC- stained sections of PAC, scanned the tumour slides at $\times 200$ using a whole-slide scanner (Hamamatsu NanoZoomer; NDP.scan), and digitized and

analyzed the images with NanoZoomer Digital Pathology software (NDP.view2; Hamamatsu, Japan).

LAs were defined, with reference to reports in NSCLC (Peyraud, F. *et al.*, 2025) and canine soft tissue sarcoma (Rugh, KM. *et al.*, 2025), as intratumoural aggregates composed of at least 50 CD20+ B cells with intercellular spacing $\leq 10 \mu\text{m}$, admixed with variable proportions of CD3+ T cells.

For TIL density, I selected, for each case, five hotspots within tumour nests and intratumoural stroma where CD8+ T cells and Foxp3+ cells were most abundant. Following prior reports, I excluded regions with necrosis, crush artifact, fibrosis, peribronchial areas, and regions with very high lymphocyte density from TIL assessment (Obeid, JM. *et al.*, 2017, Kim, A. *et al.*, 2019). Each hotspot was defined as a circular region of 0.237 mm^2 (radius $\approx 274 \mu\text{m}$). I performed two manual counts at high power and converted the counts to cell density (cells/ mm^2) for analysis. LAs and TIL densities in cPACs were assessed by the first author, who did not take part in histopathological evaluation and was not blinded to clinical outcomes.

Statistical Analysis

PFI was defined as the time from surgery to the onset of PD, including recurrence or metastasis, confirmed by diagnostic imaging, cytology, or histopathology. Overall survival time was defined as the time from surgery to death from any cause. Dogs lost to follow-up or still alive were censored at the date of last contact.

PFI and OST were analyzed using the Kaplan–Meier method and compared using the log-rank test. Variables assessed included age, body weight, clinical signs, *HER2* mutation status, *HER2* immunoreactivity score, TIL density, the presence of LAs,

tumour size classification (CLCSC), lymph-node status (N0 vs. N1, N2), surgical margin status, histologic grade and pattern, and adjuvant chemotherapy. HER2 immunoreactivity was scored on a scale from 0 to 3, with scores of 0 and 1 classified as negative, and scores of 2 and 3 classified as positive (Yoshimoto, S. *et al.*, 2020). Lymph node metastasis was evaluated by histopathologic examination, and as in previous reports, for dogs in which lymph node resection or biopsy was not performed, lymph-node status was recorded as N0 (Lee, BM. *et al.*, 2020). Dogs that died perioperatively were excluded from survival analyses of adjuvant chemotherapy. Correlation between intratumoural CD8+ T cells and Foxp3+ cells was examined using Spearman's correlation test. Cox proportional hazards regression was used to identify variables significantly associated with PFI and OST. Variables with $p < 0.05$ in univariable analysis were included in multivariable models. Fisher's exact test or the Mann-Whitney U test was used to assess associations between the presence of LAs and other variables. Results were reported as median or IQR. A p -value < 0.05 was considered statistically significant. Statistical analysis was conducted using EZR (Saitama Medical Centre, Jichi Medical University, Saitama, Japan).

RESULTS

Patient Population

Seventy-one client-owned dogs met the inclusion criteria. Detailed clinicopathologic variables, including age, breed, tumour size classification, lymph-node status, and histologic grade, are summarized in Table III-1. At the time of analysis, 57 dogs (80.3%) had died, seven dogs (9.9%) were alive, and seven dogs (9.9%) were lost to follow-up. Postoperative PD was documented in 33 dogs (46.5%), and no dogs received HER2-targeted therapy. Median PFI and OST were 754 days (95% CI, 375–not reached) and 716 days (95% CI, 399–936), respectively.

Tumour microenvironment profiles

The median densities of intratumoural CD8⁺ and Foxp3⁺ TILs were 130.0 cells/mm² (IQR, 82.3-189.5 cells/mm²) and 79.3 cells/mm² (IQR, 41.8-129.5 cells/mm²), respectively. A weak but significant positive correlation was observed between CD8⁺ T-cell and Foxp3⁺ cell counts ($r = 0.306$, $p = 0.009$, Figure III-1). Representative CD3 and CD20 immunostaining of intratumoural LAs are shown in Figure III-2. LAs were identified in 49 dogs (69.0%), and typically consisted of tightly clustered CD20⁺ B cells with admixed CD3⁺ T cells. Tumours with LAs showed significantly higher Foxp3⁺ cell density than those without LAs ($p < 0.05$), whereas CD8⁺ density did not differ between groups (Table III-2).

Prognostic analysis

The results of univariable and multivariable analyses are summarized in Supplementary Table S2, Tables 2 and 3. Univariable findings are presented separately

for clinicopathological variables (Table III-3) and for *HER2* mutation and IHC variables (Table III-4). In the clinicopathological set, PFI was associated with clinical signs, tumour size classification (CLCSC), lymph node metastasis, surgical margin status, and histological grade, whereas OST was associated with age, clinical signs, tumour size >7 cm, and surgical margin status. These determinants were largely consistent with those identified in Chapter 2, confirming reproducibility of baseline prognostic factors within this cohort. Among variables relating to *HER2* status and IHC, PFI was associated with *HER2* mutation status and the presence of LAs, whereas no association with OST was observed.

In multivariable models (Table III-5), tumour size classification (CLCSC), lymph node metastasis, surgical margin status, and the presence of LAs were independently associated with shorter PFI; for OST, age and lymph node metastasis were independent prognostic factors, also in agreement with previous findings. Dogs with LAs had a median PFI of 632 days, whereas the median PFI was not reached in dogs without LAs (Figure III-3). Taken together, these findings indicate that intratumoural LAs represent an immunological feature linked to early disease progression after surgical resection, providing prognostic information complementary to established clinicopathological and molecular variables.

DISCUSSION

I found that intratumoural LAs are common in cPACs and that their presence independently associates with shorter PFI after surgical resection. This association persisted after adjustment for established clinicopathological determinants, including CLCSC tumour size category, lymph-node status, and surgical margin status. These findings indicate that immune architecture within the tumour compartment carries prognostic information that is not captured by conventional factors.

In my cohort, the presence of LAs was associated with a shorter PFI, whereas many human NSCLC studies link TLS with better outcomes (Dieu-Nosjean, MC. *et al.*, 2008, Sun, X. *et al.*, 2022, Ma, L. *et al.*, 2024). A likely explanation is that the structures we labelled as lymphoid aggregates may not have been mature TLS. I defined aggregates by visual review on CD3 and CD20 sections and we did not apply NanoString-based gene-expression or other transcriptomic assays, and our scoring was visual rather than software-assisted (Rugh, KM. *et al.*, 2025). In dogs, reliable IHC for follicular dendritic cell markers and for high endothelial venules is difficult due to lack of an antibody specific to canine molecules, so I could not confirm these components or assign a maturation stage. These methodological and technical constraints make it likely that my aggregates included immature or functionally different structures within tumour nests. This scenario aligns with human immuno-oncology reviews that describe immature TLS as unlikely to induce effective antitumour responses and as linked to T-cell exhaustion and an immunosuppressed TME (Chen, Y. *et al.*, 2024).

cPACs with LAs showed a higher intratumoural Foxp3⁺ cell density. Enrichment of regulatory T cells can suppress local cytotoxic activity and antigen presentation within tumour nests and can promote features of T-cell dysfunction,

including an exhaustion-like state. Such suppression may permit microscopic residual disease to expand after resection and bring forward the time to progression, consistent with studies in resected NSCLC showing that higher intratumoural Foxp3+ T-cell density is associated with increased recurrence risk and shorter recurrence-free survival (Petersen, RP. *et al.*, 2006, Shimizu, K. *et al.*, 2010). This pattern is also consistent with veterinary data that associate higher intratumoural Foxp3+ counts with adverse outcomes in malignant tumours, including PAC (Sakai, K. *et al.*, 2018). I did not measure immune-checkpoint expression, ligand co-localization, or the functional state of T cells, so I could not confirm exhaustion directly. Prospective studies that pair aggregate status with maturation markers, checkpoint and ligand profiling, and functional T-cell assays are needed to test this mechanism.

This study has limitations. First, the retrospective design introduces potential selection bias. Second, the long enrolment period spans advances in surgical technique and the introduction of targeted drugs such as toceranib, which may have influenced outcomes. Third, the absence of a standardized follow-up protocol may have affected consistency in progression and survival assessment. Fourth, in 22 dogs for whom lymph nodes were not resected, lymph-node status was recorded as N0. Consequently, histological assessment was not available for all dogs and the impact of nodal metastasis may have been underestimated. Fifth, long-term storage of formalin-fixed paraffin-embedded material may have affected IHC staining quality. Sixth, univariable analyses were not adjusted for multiple comparisons, which increases the risk of type I error.

In surgically resected cPACs, LA assessment provides the TME information that complements clinicopathological variables in postoperative risk stratification. When combined with CD8 and Foxp3 counts, LA status may inform studies that assess adjuvant

or immunomodulatory therapies. Future work should include multicentre validation with standardized definitions, evaluation of markers for high endothelial venules and follicular dendritic cells, and blinded digital and spatial analyses with inter-observer agreement testing.

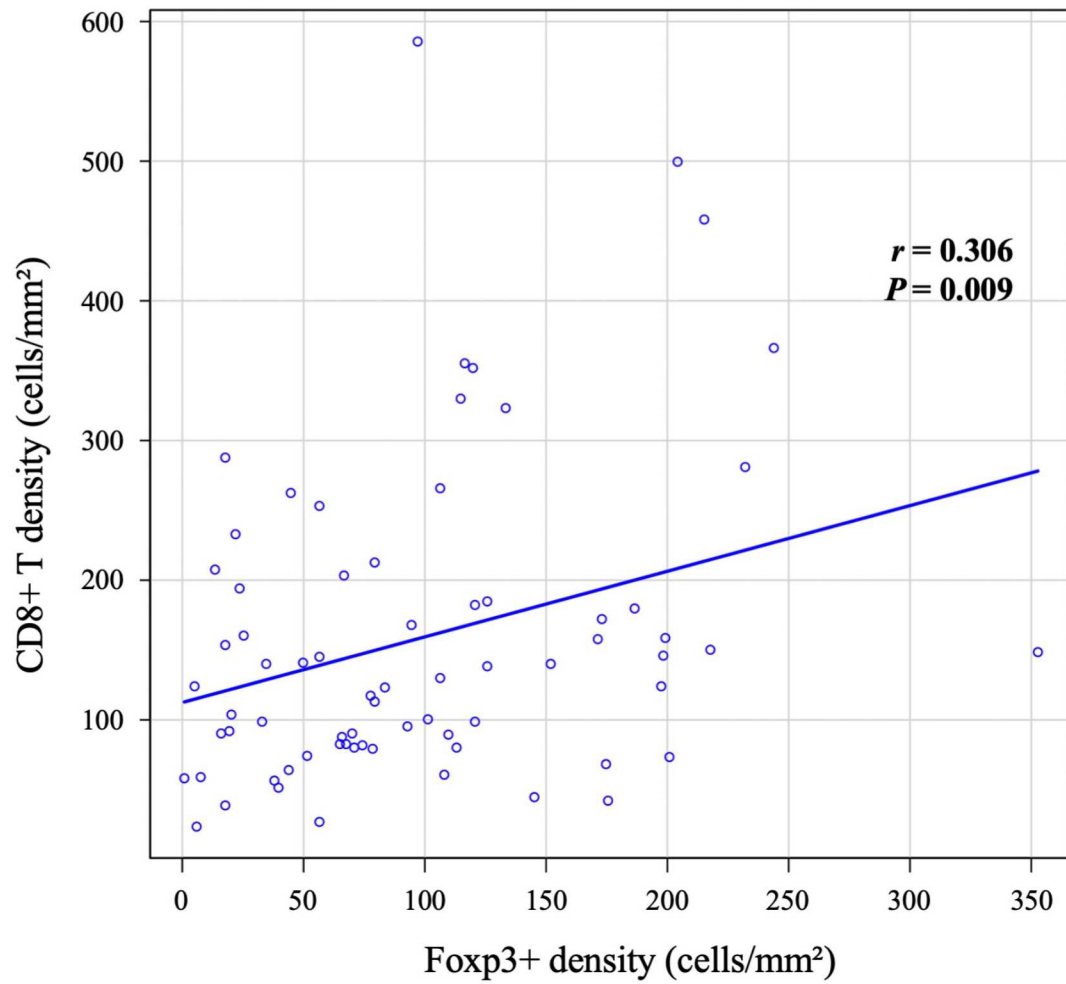


Figure III-1. Correlation between CD8+ T cells and Foxp3+ cells in dogs with surgically resected pulmonary adenocarcinoma.

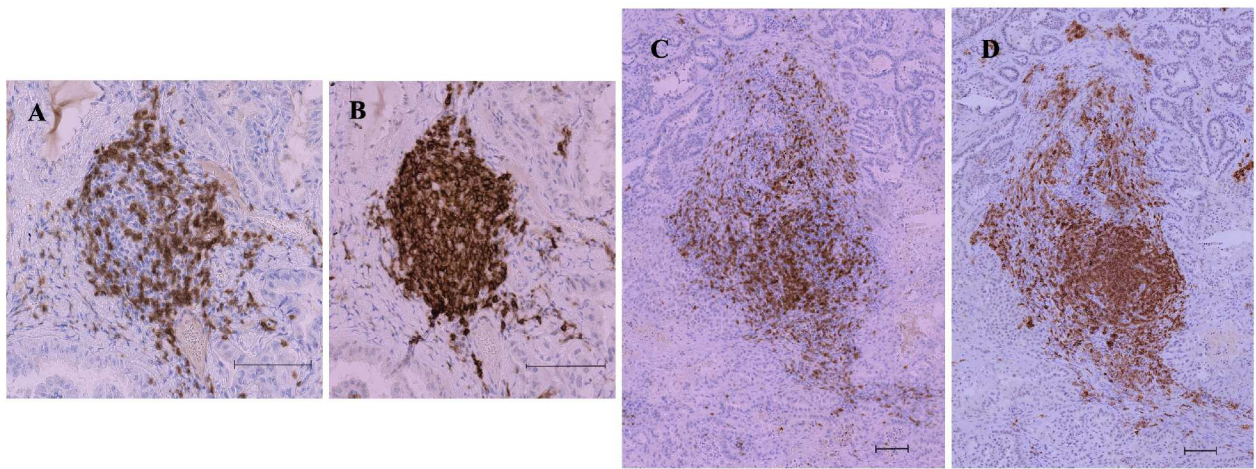


Figure III-2. Serial sections from two dogs with surgically resected canine pulmonary adenocarcinomas demonstrating intratumoural lymphoid aggregates (LAs). (A,B) Typical LA from one dog. (A) Anti-CD3 immunohistochemistry demonstrating the distribution of T cells. (B) Anti-CD20 immunohistochemistry demonstrating the distribution of B cells. (C,D) Large LA from a different dog. (C) Anti-CD3 demonstrating the distribution of T cells. (D) Anti-CD20 demonstrating the distribution of B cells. Diaminobenzidine was used as the chromogen, and sections were counterstained with Mayer's haematoxylin. Scale bars = 100 μ m.

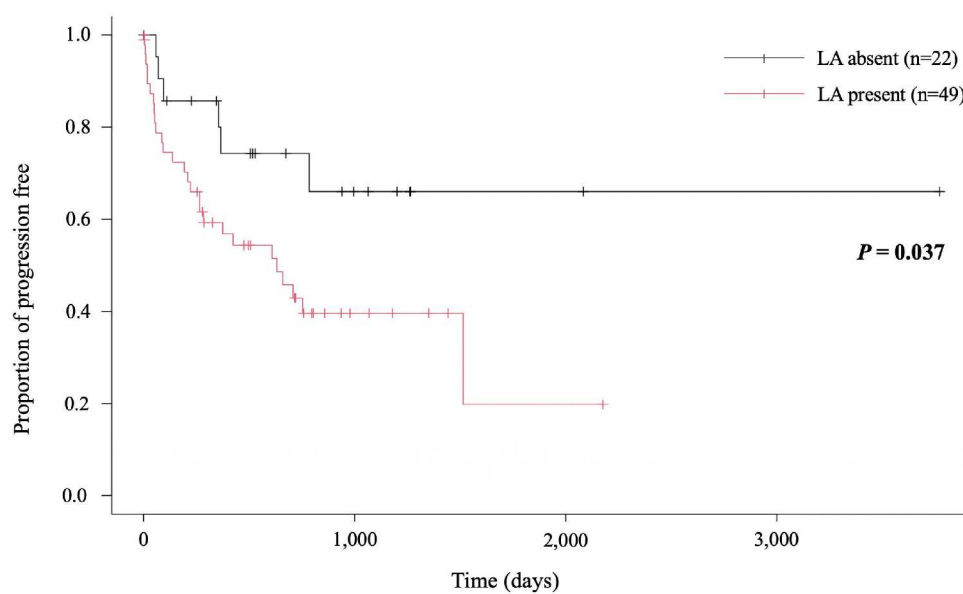


Figure III-3. Kaplan–Meier curves of progression-free interval (PFI) in dogs with surgically resected canine pulmonary adenocarcinomas stratified by the presence of intratumoural lymphoid aggregates (LAs). Dogs with LAs (red line) had significantly shorter PFI than those without LAs (black line). Median PFI was 632 days in the LA-present group, whereas it was not reached in the LA-absent group.

Table III-1. Clinicopathological characteristics of all dogs with surgically resected pulmonary adenocarcinomas.

Variables	Dogs (n=71)
Median age, years (IQR)	12.0 (9.5-13.0)
Median body weight, kg (IQR)	6.2 (5.0-9.3)
Clinical signs	
No	31 (43.7%)
YES	40 (56.3%)
Median tumor size, mm (IQR)	39.0 (26.0-54.0)
Tumor size classification	
≤ 3cm	24 (33.8%)
>3 cm to ≤5 cm	22 (31.0%)
>5 cm to ≤7 cm	16 (22.5%)
>7 cm	9 (12.7%)
Primary tumour location	
Left cranial lung lobe	19 (26.8%)
Left caudal lung lobe	15 (21.1%)
Right cranial lung lobe	12 (16.9%)
Right middle lung lobe	6 (8.5%)
Right caudal lung lobe	16 (22.5%)
Accessory lung lobe	3 (4.2%)
Lymph node status	
N0	12 (16.9%)
N1, 2	59 (83.1%)
Margin	
Complete	62 (87.3%)
Incomplete	9 (12.7%)
CLCSC Staging	
Stage 1	19 (26.8%)
Stage 2	36 (50.7%)
Stage 3	16 (22.5%)
Stage 4	0
Histologic grade	
Grade 1	47 (66.2%)
Grade 2	22 (31.0%)
Grade 3	2 (2.8%)
Histologic classification	
Papillary	61 (85.9%)
Others	10 (14.1%)
Vascular invasion	
No	61 (85.9%)
Yes	10 (14.1%)
Adjuvant chemotherapy	
No	44 (62.0%)
Yes	23 (32.6%)

Table III-2. Comparison of clinical, histological, and molecular characteristics according to the presence of intratumoural lymphoid aggregates in dogs with surgically resected pulmonary adenocarcinoma.

Variables	LA absent (n=22)	LA present (n=49)	P-value
Median age, years (IQR)	11.5 (9.0-12.75)	12.0 (10.0-13.0)	0.546
Median body weight, kg (IQR)	5.61 (4.13-9.35)	6.3 (5.1-13.0)	0.723
Clinical signs			1.0
No	10 (14.1%)	21 (29.6%)	
YES	12 (16.9%)	28 (39.4%)	
Median tumor size, mm (IQR)	50.0 (22.25-63.5)	38.0 (28.9-51.0)	0.452
Lymph node status			0.089
N0	21 (29.6%)	38 (53.5%)	
N1, 2	1 (1.4%)	11 (15.5%)	
Margin			0.711
Complete	20 (28.2%)	42 (59.2%)	
Incomplete	2 (2.8%)	7 (9.9%)	
Adjuvant chemotherapy			0.587
No	15 (22.4%)	29 (43.3%)	
Yes	6 (9.0%)	17 (25.4%)	
Histologic grade			0.184
Grade 1	18 (25.4%)	29 (40.8%)	
Grade 2	4 (5.6%)	18 (25.4%)	
Grade 3	0	2 (2.8%)	
Histologic classification			1.0
Papillary	19 (26.8%)	42 (59.2%)	
Others	3 (4.2%)	7 (9.9%)	
HER2 expression			0.849
1+	7 (9.9%)	14 (19.7%)	
2+	10 (14.1%)	20 (28.2%)	
3+	5 (7.0%)	15 (21.1%)	
HER2 mutation			0.387
Negative	13 (18.8%)	36 (52.2%)	
Positive	8 (11.6%)	12 (17.4%)	
Median CD8+ cells, /mm² (IQR)	120.68 (80.59-168.78)	140.08 (87.76-203.38)	0.526
Median Foxp3+ cells, /mm² (IQR)	69.20 (19.20-107.59)	94.51(56.54-151.90)	0.04

Table III-3. Univariable Cox regression analysis of clinicopathological risk factors for the progression-free interval and overall survival time in dogs with surgically resected pulmonary adenocarcinomas.

Variable	N	Progression-free interval		Overall survival time	
		Hazard ratio (95% confidence interval)	P-value	Hazard ratio (95% confidence interval)	P-value
Age	71	1.03 (0.87-1.21)	0.74	1.19 (1.0-1.36)	0.01
Body Weight	71	1.00 (0.96-1.05)	0.85	1.00 (0.97-1.03)	0.94
Clinical signs	71				
No	31	Reference		Reference	
YES	40	2.54 (1.21-5.27)	0.01	2.00 (1.15-3.49)	0.02
Tumor size classification	71		<0.001		0.02
≤3 cm	24	Reference		Reference	
>3 cm to ≤5 cm	22	7.55 (1.69-33.75)	0.01	1.10 (0.55-2.21)	0.79
>5 cm to ≤7 cm	16	15.59 (3.45-70.51)	<0.001	1.91 (0.92-3.95)	0.08
>7 cm	9	21.89 (4.47-107.30)	<0.001	2.89 (1.27-6.60)	0.01
Lymph node status	71				
N0	59	Reference		Reference	
N1, 2	12	4.85 (2.18-10.79)	0.001	2.40 (1.22-4.70)	0.01
Margin	71				
Complete	62	Reference		Reference	
Incomplete	9	12.93 (5.20-32.19)	<0.001	3.44 (1.64-7.20)	0.001
Histologic grade	71		0.003		0.05
Grade 1	47	Reference		Reference	
Grade 2	22	2.24 (1.09-4.59)	0.03	1.62 (0.92-2.84)	0.10
Grade 3	2	6.45 (1.45-28.74)	0.01	2.33 (0.55-9.83)	0.25
Histologic classification	71				
Papillary	61	Reference		Reference	
Others	10	0.63 (0.22-1.81)	0.39	0.46 (0.21-1.02)	0.05
Adjuvant chemotherapy	67				
No	44	Reference		Reference	
Yes	23	1.36 (0.67-2.75)	0.39	0.89 (0.50-1.57)	0.69

Table III-4. Univariable Cox regression for progression-free interval and overall survival time by human epidermal growth factor receptor 2 mutation status and immunohistochemical variables in dogs with surgically resected pulmonary adenocarcinoma.

Variable	N	Progression-free interval		Overall survival time	
		Hazard ratio (95% confidence interval)	P-value	Hazard ratio (95% confidence interval)	P-value
CD8+ cells	71	1.00 (1.00-1.01)	0.11	1.00 (0.997-1.003)	0.99
Foxp3+ cells	71	1.00 (0.997-1.01)	0.57	1.00 (0.996-1.004)	0.95
HER2 mutation	69				
Negative	49	Reference		Reference	
Positive	20	0.33 (0.13-0.87)	0.03	0.86 (0.47-1.54)	0.61
HER2 expression	71				
Negative	21	Reference		Reference	
Positive	50	0.95 (0.61-1.46)	0.80	0.92 (0.65-1.28)	0.61
Presence of LA	71				
No	22	Reference		Reference	
Yes	49	2.49 (1.03-6.05)	0.04	1.33 (0.75-2.36)	0.33

Table III-5. Multivariable Cox regression analysis of risk factors for the progression-free interval and overall survival time in dogs with surgically resected pulmonary adenocarcinomas.

Variable	N	Progression-free interval		Overall survival time	
		Hazard ratio (95% confidence interval)	P-value	Hazard ratio (95% confidence interval)	P-value
Age	71	–	–	1.18 (1.04-1.35)	0.01
Clinical signs	71				
No	31	Reference		Reference	
YES	40	1.02(0.40–2.60)	0.96	1.63 (0.89-2.97)	0.11
Tumor size classification	71				
≤3 cm	24	Reference		Reference	
>3 cm to ≤5 cm	22	5.71(1.26-25.98)	0.02	–	–
>5 cm to ≤7 cm	16	18.14 (3.58-91.78)	<0.001	–	–
>7 cm	9	22.80 (2.91-167.60)	0.003	1.75 (0.74-4.16)	0.20
Lymph node status	71				
N0	59	Reference		Reference	
N1, 2	12	1.73 (0.56-5.36)	0.34	2.20 (1.03-4.69)	0.04
Margin	71				
Complete	62	Reference		Reference	
Incomplete	9	5.93 (1.60-21.98)	0.008	1.42 (0.56-3.57)	0.46
Histologic grade	71				
Grade 1	47	Reference			
Grade 2	22	1.44 (0.58-3.57)	0.43	–	–
Grade 3	2	0.29 (0.03-2.92)	0.29	–	–
HER2 mutation	69				
Negative	49	Reference		–	
Positive	20	0.38 (0.13-1.15)	0.09	–	–
Presence of LA	71				
No	22	Reference		–	
Yes	49	4.15 (1.15-15.01)	0.03	–	–

GENERAL DISCUSSION

Surgical resection is the main curative approach for cPACs, yet postoperative outcomes remain heterogeneous even among dogs treated with comparable intent. This thesis sought to clarify the sources of that heterogeneity by evaluating three prognostic dimensions in an integrated manner: clinicopathological staging, oncogenic driver alterations, and features of the TME. Across Chapters 1 to 3, tumour burden and local control, as assessed by CLCSC tumour size and surgical margin status, consistently stratified risk. Against this clinicopathological background, *HER2* mutations, predominantly V659E, identified a subgroup with delayed postoperative progression. In parallel, immune architecture within the TME—particularly the presence of intratumoural LAs accompanied by regulatory T-cell enrichment—further explained early progression independently of tumour size, nodal status, and *HER2* status. Taken together, these findings support a layered prognostic model for resected cPACs that combines stage-based assessment with molecular and immune context.

Chapter 1 establishes clinicopathological staging as the foundation for postoperative risk assessment in cPACs, even within the small-breed population that predominates in Japan. By restricting the cohort to dogs weighing less than 15 kg, this study directly tested whether the CLCSC tumour-size scheme retains prognostic value in a population that differs markedly from the original CLCSC cohort (Lee, BM. *et al.*, 2020). The initial CLCSC study was conducted in the United States and, among eligible cases, included a relatively high proportion of larger dogs, resulting in a median body weight exceeding 20 kg. In the present work, tumour-size categories stratified PFI in a stepwise manner, and size greater than 5 cm remained an independent adverse factor after accounting for other variables. Notably, whereas the initial CLCSC report showed limited separation between tumours ≤ 3 cm and those >3 to ≤ 5 cm, the present series consistently

demonstrated a tendency for dogs with tumours ≤ 3 cm to experience longer PFI than those in the next size category, and a similar pattern was observed across Chapters 1 to 3. Although both the present work and the original CLCSC studies are retrospective cohorts and cannot be compared directly, the consistent separation at the ≤ 3 cm boundary suggests that body-size background may influence the prognostic meaning of absolute tumour dimensions. Canine lung volume scales approximately in direct proportion to body weight, implying that identical absolute tumour diameters may represent different relative burdens across body sizes (Gehr, P. *et al.*, 1981). Thus, the same absolute increase in diameter may correspond to a greater proportional tumour burden in small-breed dogs, potentially contributing to the clearer outcome differences seen between the smallest and the next size categories. Consistently, the expanded cohorts analysed in Chapters 2 and 3 showed a stepwise adverse effect of tumour size on PFI across T1 through T4 categories. Accordingly, tumour size appears to function as a practical prognostic indicator across CLCSC stages, with larger tumours corresponding to progressively shorter PFI. In small-breed dogs in particular, remaining within the ≤ 3 cm category may provide an additional, clinically meaningful benchmark for preoperative risk assessment and postoperative monitoring.

Chapter 2 showed that molecular profiling refines prognosis beyond clinicopathological staging. *HER2* mutations were detected in a substantial subset of tumours, most commonly the recurrent V659E substitution in the TMD. Dogs with *HER2*-mutant tumours experienced significantly longer PFI, even after adjustment for CLCSC variables, histological grade, and margin status. By contrast, *HER2* protein expression by IHC did not mirror the prognostic effect of mutation status. These findings indicate that *HER2* genotyping captures tumour behaviour that is not reflected by routine

clinicopathological variables or by protein expression levels alone. Preclinical work in lung cancer research has shown that cell line models engineered to express *HER2* V659E or related TMD/JMD mutations exhibit oncogenic activation and respond to *HER2*-targeted TKIs, including lapatinib, afatinib, and neratinib (Pahuja, KB. *et al.*, 2018). Subsequent experimental studies in canine cPAC cell lines demonstrated that naturally occurring *HER2* V659E likewise drives ligand-independent signalling and confers sensitivity to *HER2*-targeted TKIs such as lapatinib and neratinib (Lorch, G. *et al.*, 2019). In addition, a real-world study of Chinese patients with *HER2* TMD-mutant lung adenocarcinoma, predominantly V659E, reported that *HER2*-targeted TKIs with high binding affinity, particularly afatinib and pyrotinib, achieved meaningful clinical benefit in this setting (Jia, Z. *et al.*, 2021). Taken together, the relatively high frequency of V659E in cPAC, its favourable association with outcome in this thesis, and its concordant drug sensitivity across canine cPAC cells and *HER2* TMD-mutant experimental models suggest that *HER2*-mutant cPAC may serve as a useful spontaneous counterpart to these systems and as a potential comparative model of *HER2* TMD-mutant human PAC. Although *HER2*-mutant cPACs as a group showed more favourable postoperative courses, 5 of 20 dogs with *HER2*-mutant tumours still developed PD after surgery, most often characterised by distant metastasis, indicating that *HER2*-mutant disease is not uniformly indolent. These findings suggest that *HER2*-targeted therapy could benefit dogs that develop postoperative metastatic disease. In addition, for dogs with unresectable or metastatic cPAC in which *HER2* mutations are identified, *HER2*-targeted TKIs might represent a rational systemic option alongside or beyond conventional cytotoxic chemotherapy. From a clinical standpoint, incorporating *HER2* mutation testing into postoperative evaluation and into the work-up of advanced cases may improve

counselling and help identify candidates for future HER2-targeted trials in dogs and for comparative therapeutic studies.

Chapter 3 extended this work to the TME and showed that organised immune structures within the tumour carry additional prognostic information. Intratumoural LAs were frequently identified in cPACs, and their presence was associated with shorter PFI and OST, even after accounting for CLCSC stage, margin status, and HER2 alterations. Thus, LA assessment provided prognostic information that was not captured by conventional clinicopathological or molecular factors. When intratumoural TILs were quantified within tumour parenchyma, LA-positive tumours contained higher numbers of Foxp3⁺ cells than LA-negative tumours, indicating that the presence of LAs was linked to increased regulatory T-cell infiltration at the whole-tumour level. These findings suggest that, in cPACs, intratumoural LAs are not simply markers of productive anti-tumour immunity but may be associated with a more immunosuppressive TME. This pattern differs from many reports in human NSCLC, in which canonical, B-cell follicle-rich TLS are often associated with more favourable survival (Dieu-Nosjean, MC. *et al.*, 2008, Sun, X. *et al.*, 2022), but is compatible with emerging data that some TLS can be functionally exhausted or immunosuppressive depending on their cellular composition and context (Xin, S. *et al.*, 2024). Within the broader framework of this thesis, Chapter 3 indicates that simple, reproducible readouts of the TME—such as the presence of LAs and intratumoural Foxp3⁺ TIL burden—can refine risk stratification in surgically resected cPACs beyond clinicopathological staging and HER2 status, and may help to identify dogs with an immunosuppressive TME who might derive the greatest benefit from future immunomodulatory strategies.

Overall, this thesis shows that prognosis after surgical resection of cPACs is influenced by tumour size and local control, *HER2* mutation status, and the TME. CLCSC-based tumour-size categories and margin status provide a practical foundation for baseline risk assessment, *HER2* mutations define a subgroup with more favourable postoperative courses, and the presence of intratumoural LAs identifies dogs at higher risk of early progression. Considering these clinicopathological, molecular, and immune factors in combination allows more refined stratification than any single parameter alone and may inform decisions on monitoring and the development of future adjuvant or systemic therapies in dogs with cPACs.

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REFERENCES

- Able H, Wolf-Ringwall A, Rendahl A, *et al.* Computed tomography radiomic features hold prognostic utility for canine lung tumors: An analytical study. *PLoS One*. 2021;16(8):e0256139.
- Arcila ME, Chaft JE, Nafa K, *et al.* Prevalence, clinicopathologic associations, and molecular spectrum of ERBB2 (HER2) tyrosine kinase mutations in lung adenocarcinomas. *Clin Cancer Res*. 2012;18(18):4910-4918.
- Ballegeer EA, Adams WM, Dubielzig RR, Paoloni MC, Klauer JM, Keuler NS. Computed tomography characteristics of canine tracheobronchial lymph node metastasis. *Vet Radiol Ultrasound*. 2010;51(4):397-403.
- Bray F, Laversanne M, Sung H, *et al.* Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2024;74(3):229-263.
- Brierley J, Gospodarowicz MK, Wittekind C. *TNM Classification of Malignant Tumours*. 8th ed. Chichester, West Sussex, UK; Hoboken, NJ: John Wiley & Sons, Inc.; 2017.
- Brunetti B, de Biase D, Millanta F, *et al.* Protein Expression, Amplification, and Mutation of *HER2* Gene in Canine Primary Pulmonary Adenocarcinomas: Preliminary Results. *Animals (Basel)*. 2024;14(18):2625.
- Carvalho MI, Raposo TP, Silva-Carvalho R, *et al.* The Dog as a Model to Study the Tumor Microenvironment. *Adv Exp Med Biol*. 2021;1329:123-152.
- Chen B, Li H, Liu C, *et al.* Prognostic value of the common tumour-infiltrating lymphocyte subtypes for patients with non-small cell lung cancer: A meta-analysis. *PLoS One*. 2020;15(11):e0242173.
- Chen Y, Wu Y, Yan G, Zhang G. Tertiary lymphoid structures in cancer: maturation and induction. *Front Immunol*. 2024;15:1369626.
- Culp WTN, Rebhun RB. Pulmonary neoplasia. In: Vail DM, Thamm DH, Liptak JM, eds. *Withrow & MacEwen's Small Animal Clinical Oncology*. 6th ed. Missouri, MO: Elsevier; 2020:507-523.
- Denisenko TV, Budkevich IN, Zhivotovsky B. Cell death-based treatment of lung adenocarcinoma. *Cell Death Dis*. 2018;9(2):117.
- Detterbeck FC. The eighth edition TNM stage classification for lung cancer: What does it mean on main street?. *J Thorac Cardiovasc Surg*. 2018;155(1):356-359.

Dieu-Nosjean MC, Antoine M, Danel C, *et al.* Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures. *J Clin Oncol.* 2008;26(27):4410-4417.

Fridman WH, Meylan M, Pupier G, Calvez A, Hernandez I, Sautès-Fridman C. Tertiary lymphoid structures and B cells: An intratumoral immunity cycle. *Immunity.* 2023;56(10):2254-2269.

Gehr P, Siegwart B, Weibel ER. Allometric analysis of the morphometric pulmonary diffusing capacity in dogs. *J Morphol.* 1981;168(1):5-15.

Gray M, Meehan J, Turnbull AK, *et al.* The Importance of the Tumor Microenvironment and Hypoxia in Delivering a Precision Medicine Approach to Veterinary Oncology. *Front Vet Sci.* 2020;7:598338.

Giambrone G, Di Giorgio S, Vullo C, *et al.* Does TLS Exist in Canine Mammary Gland Tumours? Preliminary Results in Simple Carcinomas. *Vet Sci.* 2022;9(11):628.

Hahn FF, Muggenburg BA, Griffith WC. Primary lung neoplasia in a beagle colony. *Vet Pathol.* 1996;33(6):633-638.

Hiraoka N, Ino Y, Yamazaki-Itoh R. Tertiary Lymphoid Organs in Cancer Tissues. *Front Immunol.* 2016;7:244.

Kim A, Lee SJ, Ahn J, *et al.* The prognostic significance of tumor-infiltrating lymphocytes assessment with hematoxylin and eosin sections in resected primary lung adenocarcinoma. *PLoS One.* 2019;14(11):e0224430.

Jia Z, Xing J, Li J, *et al.* HER2 transmembrane domain mutation: comprehensive characteristics and real-world evidence of treatment response in Chinese lung adenocarcinoma. *Transl Lung Cancer Res.* 2021;10(3):1383-1396.

Lee BM, Clarke D, Watson M, Laver T. Retrospective evaluation of a modified human lung cancer stage classification in dogs with surgically excised primary pulmonary carcinomas. *Vet Comp Oncol.* 2020;18(4):590-598.

Li BT, Ross DS, Aisner DL, *et al.* HER2 Amplification and HER2 Mutation Are Distinct Molecular Targets in Lung Cancers. *J Thorac Oncol.* 2016;11(3):414-419.

Lorch G, Sivaprakasam K, Zismann V, *et al.* Identification of Recurrent Activating HER2 Mutations in Primary Canine Pulmonary Adenocarcinoma. *Clin Cancer Res.* 2019;25(19):5866-5877.

McPhetridge JB, Scharf VF, Regier PJ, *et al.* Distribution of histopathologic types of primary pulmonary neoplasia in dogs and outcome of affected dogs: 340 cases (2010-2019). *J Am Vet Med Assoc.* 2021;260(2):234-243.

- Maemondo M, Inoue A, Kobayashi K, *et al.* Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med.* 2010;362(25):2380-2388.
- Ma L, Li R, Liu X, *et al.* Prognostic and clinicopathological significance of tertiary lymphoid structure in non-small cell lung cancer: a systematic review and meta-analysis. *BMC Cancer.* 2024;24(1):815.
- Matilla JM, Zabaleta M, Martínez-Téllez E, Abal J, Rodríguez-Fuster A, Hernández-Hernández J. New TNM staging in lung cancer (8th edition) and future perspectives. *J Clin Transl Res.* 2020;6(4):145-154.
- McNiel EA, Ogilvie GK, Powers BE, Hutchison JM, Salman MD, Withrow SJ. Evaluation of prognostic factors for dogs with primary lung tumors: 67 cases (1985-1992). *J Am Vet Med Assoc.* 1997;211(11):1422-1427.
- Mehlhaff CJ, Leifer CE, Patnaik AK, *et al.* Surgical treatment of primary pulmonary neoplasia in 15 dogs. *J Am Anim Hosp Assoc.* 1984; 20:799-803.
- Mok TS, Wu YL, Thongprasert S, *et al.* Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med.* 2009;361(10):947-957.
- Nururrozi A, Miyanishi K, Igase M, *et al.* The Density of CD8⁺ Tumor-infiltrating Lymphocytes Correlated With Akt Activation and Ki-67 Index in Canine Soft Tissue Sarcoma. *In Vivo.* 2024;38(4):1698-1711.
- Nützinger J, Bum Lee J, Li Low J, *et al.* Management of HER2 alterations in non-small cell lung cancer - The past, present, and future. *Lung Cancer.* 2023;186:107385.
- Obeid JM, Wages NA, Hu Y, Deacon DH, Slingluff CL Jr. Heterogeneity of CD8⁺ tumor-infiltrating lymphocytes in non-small-cell lung cancer: impact on patient prognostic assessments and comparison of quantification by different sampling strategies. *Cancer Immunol Immunother.* 2017;66(1):33-43.
- Ogilvie GK, Haschek WM, Withrow SJ, *et al.* Classification of primary lung tumors in dogs: 210 cases (1975-1985). *J Am Vet Med Assoc.* 1989;195(1):106-108.
- Ou SI, Schrock AB, Bocharov EV, *et al.* HER2 Transmembrane Domain (TMD) Mutations (V659/G660) That Stabilize Homo- and Heterodimerization Are Rare Oncogenic Drivers in Lung Adenocarcinoma That Respond to Afatinib. *J Thorac Oncol.* 2017;12(3):446-457.
- Owen LN, and World Health Organization. *TNM Classification of Tumours in Domestic Animals.* 1st. ed., World Health Organization, 1980.
- Pahuja KB, Nguyen TT, Jaiswal BS, *et al.* Actionable Activating Oncogenic ERBB2/HER2 Transmembrane and Juxtamembrane Domain Mutations. *Cancer Cell.* 2018;34(5):792-806.e5.

- Pan W, Yang Y, Zhu H, Zhang Y, Zhou R, Sun X. KRAS mutation is a weak, but valid predictor for poor prognosis and treatment outcomes in NSCLC: A meta-analysis of 41 studies. *Oncotarget*. 2016;7(7):8373-8388.
- Petersen RP, Campa MJ, Sperlazza J, *et al*. Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer*. 2006;107(12):2866-2872.
- Peyraud F, Guégan JP, Rey C, *et al*. Spatially resolved transcriptomics reveal the determinants of primary resistance to immunotherapy in NSCLC with mature tertiary lymphoid structures. *Cell Rep Med*. 2025;6(2):101934.
- Pinard CJ; International Immuno-Oncology Biomarker Working Group, Lagree A, *et al*. Comparative Evaluation of Tumor-Infiltrating Lymphocytes in Companion Animals: Immuno-Oncology as a Relevant Translational Model for Cancer Therapy. *Cancers (Basel)*. 2022;14(20):5008.
- Polton G, Finotello R, Sabattini S, *et al*. Survival analysis of dogs with advanced primary lung carcinoma treated by metronomic cyclophosphamide, piroxicam and thalidomide. *Vet Comp Oncol*. 2018;16(3):399-408.
- Polton GA, Brearley MJ, Powell SM, Burton CA. Impact of primary tumour stage on survival in dogs with solitary lung tumours. *J Small Anim Pract*. 2008;49(2):66-71.
- Potter AL, Costantino CL, Suliman RA, *et al*. Recurrence After Complete Resection for Non-Small Cell Lung Cancer in the National Lung Screening Trial. *Ann Thorac Surg*. 2023;116(4):684-692.
- Ramalingam SS, Vansteenkiste J, Planchard D, *et al*. Overall Survival with Osimertinib in Untreated, *EGFR*-Mutated Advanced NSCLC. *N Engl J Med*. 2020;382(1):41-50.
- Rose RJ, Worley DR. A Contemporary Retrospective Study of Survival in Dogs With Primary Lung Tumors: 40 Cases (2005-2017). *Front Vet Sci*. 2020;7:519703.
- Rugh KM, Ashton LV, Schaffer PA, Olver CS. Lymphoid Aggregates in Canine Cutaneous and Subcutaneous Sarcomas: Immunohistochemical and Gene Expression Evidence for Tertiary Lymphoid Structures. *Vet Comp Oncol*. 2025;23(1):10-19.
- Sakai K, Maeda S, Yamada Y, *et al*. Association of tumour-infiltrating regulatory T cells with adverse outcomes in dogs with malignant tumours. *Vet Comp Oncol*. 2018;16(3):330-336.
- Sakai O, Ii T, Uchida K, Igase M, Mizuno T. Establishment and Characterization of Monoclonal Antibody Against Canine CD8 Alpha. *Monoclon Antib Immunodiagn Immunother*. 2020;39(4):129-134.

Sabattini S, Mancini FR, Marconato L, *et al.* EGFR overexpression in canine primary lung cancer: pathogenetic implications and impact on survival. *Vet Comp Oncol.* 2014;12(3):237-248.

Schumacher TN, Thommen DS. Tertiary lymphoid structures in cancer. *Science.* 2022;375(6576):eabf9419.

Schneider BJ. Non-small cell lung cancer staging: proposed revisions to the TNM system. *Cancer Imaging.* 2008;8(1):181-185.

Sher T, Dy GK, Adjei AA. Small cell lung cancer. *Mayo Clin Proc.* 2008;83(3):355-367.

Shimizu K, Nakata M, Hiramami Y, Yukawa T, Maeda A, Tanemoto K. Tumor-infiltrating Foxp3+ regulatory T cells are correlated with cyclooxygenase-2 expression and are associated with recurrence in resected non-small cell lung cancer. *J Thorac Oncol.* 2010;5(5):585-590.

Shigematsu H, Takahashi T, Nomura M, *et al.* Somatic mutations of the HER2 kinase domain in lung adenocarcinomas. *Cancer Res.* 2005;65(5):1642-1646.

Sun X, Liu W, Sun L, *et al.* Maturation and abundance of tertiary lymphoid structures are associated with the efficacy of neoadjuvant chemioimmunotherapy in resectable non-small cell lung cancer. *J Immunother Cancer.* 2022;10(11):e005531.

Suzuki M, Shiraishi K, Yoshida A, *et al.* HER2 gene mutations in non-small cell lung carcinomas: concurrence with Her2 gene amplification and Her2 protein expression and phosphorylation. *Lung Cancer.* 2015;87(1):14-22.

Takamochi K, Oh S, Matsunaga T, Suzuki K. Prognostic impacts of EGFR mutation status and subtype in patients with surgically resected lung adenocarcinoma. *J Thorac Cardiovasc Surg.* 2017;154(5):1768-1774.e1.

Travis WD, Brambilla E, Noguchi M, *et al.* International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. *J Thorac Oncol.* 2011;6(2):244-285.

Treggiari E, Romanelli G, Valenti P, Montinaro V, Rossanese M. Evaluation of lung lobectomy and adjuvant treatment for primary pulmonary carcinoma in dogs: 89 cases (2005-2022). *J Small Anim Pract.* 2025;66(9):654-664.

Tsuboi M, Herbst RS, John T, *et al.* Overall Survival with Osimertinib in Resected *EGFR*-Mutated NSCLC. *N Engl J Med.* 2023;389(2):137-147.

Tomizawa K, Suda K, Onozato R, *et al.* Prognostic and predictive implications of HER2/ERBB2/neu gene mutations in lung cancers. *Lung Cancer.* 2011;74(1):139-144.

- Uramoto H, Tanaka F. Recurrence after surgery in patients with NSCLC. *Transl Lung Cancer Res.* 2014;3(4):242-249.
- Van Schil PE, Balduyck B, De Waele M, Hendriks JM, Hertoghs M, Lauwers P. Surgical treatment of early-stage non-small-cell lung cancer. *EJC Suppl.* 2013;11(2):110-122.
- Veterinary Co-operative Oncology Group (VCOG). Veterinary Co-operative Oncology Group - Common Terminology Criteria for Adverse Events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.0. *Vet Comp Oncol.* 2004;2(4):195-213.
- Wei XW, Gao X, Zhang XC, *et al.* Mutational landscape and characteristics of ERBB2 in non-small cell lung cancer. *Thorac Cancer.* 2020;11(6):1512-1521.
- Wu YL, Tsuboi M, He J, *et al.* Osimertinib in Resected *EGFR*-Mutated Non-Small-Cell Lung Cancer. *N Engl J Med.* 2020;383(18):1711-1723.
- Xin S, Wen S, He P, Zhao Y, Zhao H. Density of tertiary lymphoid structures and their correlation with prognosis in non-small cell lung cancer. *Front Immunol.* 2024;15:1423775.
- Yoshimoto S, Kato D, Kamoto S, *et al.* Overexpression of human epidermal growth factor receptor 2 in canine primary lung cancer. *J Vet Med Sci.* 2020;82(6):804-808.
- Zappa C, Mousa SA. Non-small cell lung cancer: current treatment and future advances. *Transl Lung Cancer Res.* 2016;5(3):288-300.
- Zer A, Ahn MJ, Barlesi F, *et al.* Early and locally advanced non-small-cell lung cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol.* 2025;36(11):1245-1262.
- Zhang SM, Zhu QG, Ding XX, *et al.* Prognostic value of *EGFR* and *KRAS* in resected non-small cell lung cancer: a systematic review and meta-analysis. *Cancer Manag Res.* 2018;10:3393-3404.
- Zhang Z, Wang T, Zhang J, *et al.* Prognostic value of epidermal growth factor receptor mutations in resected non-small cell lung cancer: a systematic review with meta-analysis. *PLoS One.* 2014;9(8):e106053.
- Zhu QG, Zhang SM, Ding XX, He B, Zhang HQ. Driver genes in non-small cell lung cancer: Characteristics, detection methods, and targeted therapies. *Oncotarget.* 2017;8(34):57680-57692.