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The distribution of pulmonary metastases in dogs with osteosarcoma

Immunohistochemical characterization of TP3 and PCNA; a novel method for studying the distribution of pulmonary micrometastases.

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Preface

We chose cancer to be the theme of our thesis, due to our interest in oncology and comparative medicine. We have always been interested in small animal medicine and so we felt it was natural to write about our canine companions. Comparative medicine has become in important aspect of cancer research and so, as veterinary students, we wanted to contribute to the growing body of knowledge and to better understand oncogenesis. After several interesting and inspiring conversations with our main supervisor, Professor Lars Moe, we ended up choosing osteosarcoma as the model for our study.

Summary

Title: The distribution of pulmonary metastases in dogs with osteosarcoma.

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Osteosarcoma is a highly devastating disease that occurs in both humans and dogs. Despite aggressive treatment, affected patients often succumb to the disease shortly after it has been diagnosed. Many molecular and genetic factors have been linked to the pathogenesis of metastatic disease and their use in diagnostics have made it easier to better predict patient outcome. Many of these factors have also been investigated for their potential as therapeutic targets. Immunohistochemistry has been shown to be a valuable tool to study protein expression in tissues. In order to shed light on the pathogenesis of early metastatic disease, we investigated the expression of Tumour Protein 3 (TP3) binding antigens and Proliferating Cell Nuclear Antigen (PCNA), by means of immunohistochemistry (IHC), in lung tissue derived from dogs. We wanted to evaluate whether the lungs were colonized with metastatic tumour cells, before macroscopic metastases were present. The dogs included in the current study consisted of one healthy control dog and two dogs with osteosarcoma, one of which had metastatic disease while the other did not, based on routine autopsy and histopathology. PCNA expression was investigated in the dog with osteosarcoma, with visible metastases and in the control dog. TP3 expression was evaluated in all three dogs included in the study. We found that tumour cells in pulmonary metastatic lesions expressed both TP3 binding antigens and PCNA. Furthermore, we found that TP3 and anti-PCNA immunolabelling was useful in

visualising small metastatic lesions and that this method could potentially be more sensitive in detecting such lesions than routine histologic examination using HE-slides. Single cells in the lung parenchyma also expressed TP3 binding antigens in all three dogs. The natures of these cells could not always be determined. However, some of these cells were characterized as leukocytes, demonstrating for the first time that TP3 antibodies bind to epitopes on these cells as well. In order to conclude whether the remainder of these single cells do in fact represent tumours cells or not, further studies using double staining techniques are warranted. This is important for the analysis of whether tumour cells are present as micrometastases in the lungs in the early phase of osteosarcoma or not. We believe that further studies are warranted to shed light on the potential extrapulmonary distribution of tumour cells and their potential to redistribute to the lungs.

Abbreviations

25X: Twenty-five times magnification

100X: Hundred times magnification

200X: Two hundred times magnification

400X: Four hundred times magnification

630X: Six hundred and thirty times magnification

AEC: Aminoethyl carbazole

AgNOR: Argyrophilic Nucleolar Organizer Region

BSA: Bovine serum albumin

DAB: 3, 3'-diaminobenzidine

DNA: Deoxyribonucleic acid

FFPE: Formalin-fixed paraffin embedded

G0: Arrested cells in the cell cycle

G1: Growth phase 1 of the cell cycle

G2: Growth phase 2 of the cell cycle

HE: Haematoxylin & Eosin

HER-2: Human epidermal growth factor 2

HGF: Hepatocyte growth factor

HIER: Heat induced epitope retrieval

HRP: Horseradish peroxidase

IGF-1R: Insulin-like growth factor-1 receptor

IgG: Immunoglobulin G

IHC: Immunohistochemistry

LOH: Loss of heterozygosity

M: Mitotic phase of the cell cycle

mAB: Monoclonal antibody

MET: Met proto-oncogene/receptor tyrosine kinase

MHC: Major Histocompatibility Complex

MST: Median survival time

mTOR: Mammalian target of rapamycin

OS: Osteosarcoma

p53: Protein 53

PBS: Phosphate buffered saline

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase chain reaction

PTEN: Phosphate and tensin homolog

RB1: Retinoblastoma 1 gene

RHOC: Ras homolog family member C gene

RUNX2: Runt related transcription factor 2 gene

S: Synthesis phase of the cell cycle

SLE: Systemic Lupus Erythematous

TBS: Tris buffered saline

TP1: Tumour protein 1

TP3: Tumour protein 3

Trk: Tropomyosin-related kinase

TUSC3: Tumour suppressor candidate 3

α: Alfa

δ: Delta

ε: Epsilon

^{239}Pu: Plutonium 239 isotope

^{226}Ra: Radium 226 isotope

^{90}Sr: Strontium 90 isotope

Introduction

Osteosarcoma

Osteosarcoma is the most common bone tumour in dogs, accounting for 80-85% of all malignant bone tumours. It mainly occurs in large and giant breeds, including Saint Bernard, Great Dane, Irish Setter, Rottweiler, German Shepherd, Boxer, Greyhound, Doberman Pinscher and Golden Retriever (1-4). The disease generally affects middle-aged to older dogs, with a median age of 7 years (range 6-10 years old), although there seems to be a second peak in frequency in younger individuals as well (range 1-2 years old) (1, 2, 4).

The tumour most commonly arises in the appendicular bone (64-75%), but not uncommonly does it arise in the axial skeleton (25-28.5%) and more uncommonly in extra-skeletal sites (up to 7.5% of reported cases) (1, 4-6). The tumours of the appendicular bone usually affect the metaphyseal region of long bones, appearing in the front legs roughly twice as often than in the hind legs, with distal radius and proximal humerus being the most common locations, followed by distal femur, distal tibia and proximal tibia (7-9). Of cases with primary osteosarcoma in the axial skeleton, it has been reported that 27% were located in the mandible, 22% in the maxilla, 15% in the spine, 14 % in the cranium, 10% in the ribs, 9% in the nasal cavity and paranasal sinuses, and 6% in the pelvis (10). Although rare, primary osteosarcoma in extraskeletal sites have been reported in the adrenal gland, eye, gastric ligament, ileum, kidney, liver, spleen, testicle, vagina, mammary tissue and subcutaneous tissue (9, 11, 12).

Risk factors

The etiology of osteosarcoma in dogs is largely unknown, but there are several risk factors that have shown to predispose to its development. Dogs weighting more than 40 kg are more predisposed than smaller dogs and the general risk of developing osteosarcoma increases with age, increasing bodyweight, increasing standard weight and increasing standard height, with height seeming to have a stronger correlation than weight for developing the disease (13, 14). It has been theorized that multiple minor trauma and subsequent injury to sensitive cells in the late-closing physeal regions of major weight-bearing bones might be the reason why heavy dogs are predisposed, but an in vitro study found no difference in the incidence of microdamage between small- and large-breed dogs, when examining the radius post mortem (15). Most studies indicate that males are more often affected then females (1.1-1.5:1 ratio) (3, 8, 16). There are also reports that indicate a correlation between castration and a higher risk of developing osteosarcoma. Male and female dogs than underwent gonadectomy before the age of 1 year were approximately four times more likely to develop osteosarcoma during their lifetime than dogs that were intact (17). It has also been shown that metallic implants for fracture repair, chronic osteomyelitis, fractures without metallic implants and bone allografts all have been associated with the development of osteosarcoma (18-22). Ionizing radiation has also been shown to induce osteosarcoma in dogs (23-28). Dogs either fed or injected with ^{239}Pu, ^{226}Ra or ^{90}Sr showed a dramatic increase in the prevalence of osteosarcoma, with a distribution pattern being very different from that of naturally occurring osteosarcoma, showing an increased frequency in the axial skeleton (24-26). Osteosarcoma has also been shown to occur at sites of therapeutic radiation in dogs, however it has been shown to be a rare complication to radiation therapy (reported in 3.4% of treated dogs), often occurring several years after treatment (range 1.7-5 years) (23). In another report including 57 dogs receiving radiation therapy for oral acanthomatous epulis, similar numbers (2 dogs, 3.5% of

treated dogs) developed a second tumour (sarcoma and osteosarcoma) in the radiation field, 5.2 and 8.7 years after treatment (28). There is evidence that shows some genetic and hereditable factors may predispose for the development of osteosarcoma. One of the most studied genes involved in the formation and progression of osteosarcoma is the tumour suppressor gene *p53* (29-34). Several studies have identified missense mutations involving exons 4 to 8 of the *p53* gene in 24-47% of spontaneous arising osteosarcomas in dogs (29, 33, 34). Studies involving immortalized canine osteosarcoma cell lines have also demonstrated that the functionality of the p53 gene was defective (31). In another study, 59 samples from spontaneously arising canine osteosarcoma were assessed for mutations in the *p53* gene. Mutations in the *p53* gene were identified in 41% of the samples (30). Another study evaluating *p53* expression by immunohistochemistry, showed that 67% of osteosarcoma samples (103 samples) stained positively for the *p53* protein and that samples from appendicular osteosarcomas had a significantly higher *p53* index than samples from axial osteosarcomas (32). Other studies have reported similar differences in the *p53* index depending on tumour location (35). It was also shown that *p53* index was higher in Rottweiler dogs than in other commonly affected breeds, which in turn might support the theory that mutations in the *p53* gene may be associated with some breeds' predisposition for osteosarcoma (17, 32). Another tumour suppressor gene that has shown to have loss of heterozygosity (LOH) in up to 62.9% of human osteosarcoma samples, is the *RB1* gene (36). The *RB1* gene has also been showed to have aberrations in spontaneous arising canine osteosarcoma, showing loss of gene copies in 29% of the analysed samples (37). Abnormalities in the tumour suppressor gene phosphatase and tensin homolog (*PTEN*) has also been shown to be present in 30-42% of spontaneous arising canine osteosarcoma samples, leading to the suspicion that this gene may also participate in the genetic pathogenesis of canine osteosarcoma (37-39). Other genes that have been shown to be

aberrant in canine osteosarcoma and that might be involved in its genetic pathogenesis include the *RHOC* gene, the *RUNX2* gene and the *TUSC3* gene (39).

In addition to genetic factors, several molecular factors have been shown to have some significance in the etiopathogenesis of canine osteosarcoma. The *MET* proto-oncogene encodes a tyrosine kinase receptor, that on ligation with hepatocyte growth factor (HGF) mediates several intracellular pathways. It has been demonstrated that dysregulation and overexpression of this protein promotes tumorigenic phenotypes in cell lines (40, 41). It has also been shown that 71-100% of tumour samples show expression of the *MET* proto-oncogene, demonstrated by northern blot, real-time PCR and western blot analysis (42, 43).
Several other molecular factors have been shown to be aberrantly expressed or overexpressed in canine osteosarcoma. This includes the expression of the insulin-like growth factor-1 receptor (IGF-1R), overexpression of the human epidermal growth factor-2 (HER-2) protein, expression of the mammalian target of rapamycin (mTOR) protein, expression of the tropomyosin-related kinase (Trk) receptor and expression of telomerase (44-48).

Pathophysiology

Osteosarcoma is a locally aggressive tumour, causing bone lysis, production of bone, or both (49). There is often significant soft tissue swelling around the lesion and it is not uncommon for pathological fractures to occur in the affected bone. It is rare for osteosarcoma to cross joint surfaces. Metastases are very common in canine osteosarcoma, even though only 15% of affected dogs will have radiographically detectable metastases (in the lungs or in bone) at presentation. Dogs receiving limb amputation as sole treatment for osteosarcoma will have a median survival time (MST) of 19 weeks, with metastatic disease usually occurring to the lungs (3, 8). Metastasis usually occurs through the haematogenous route, however metastases to regional lymph nodes are sometimes seen and carry a poor prognosis despite treatment (MST of 59 days) in comparison to those without lymph node involvement (MST of 318 days) (50). Even though metastases usually occur in the lungs, there are several reports of metastases occurring to other sites, including bone and other soft tissue sites (51). A difference in metastatic behaviour has been reported, depending on the anatomic location of the primary osteosarcoma (52, 53). It has been shown that mandibular osteosarcomas may have a less aggressive metastatic behaviour than tumours arising in appendicular sites, but other reports have shown conflicting results. There is a rapport of 4 cases of canine osteosarcoma undergoing spontaneous regression, without any antitumor treatment, a phenomenon having also been described in human osteosarcomas (54).

Osteosarcoma is a malignant mesenchymal tumour arising in primitive bone cells and they produce an extracellular matrix consisting of osteoid (49). The presence of osteoid is the basis for the histologic diagnosis, making it possible to differentiate osteosarcomas from other mesenchymal tumours of the bone. It is important to obtain biopsies of appropriate size, due to the variable histological patterns seen in between tumours and within the tumour itself,

in order not to misdiagnose the tumour. It is recommended to do a histologic analysis of the entire tumour after excision, to confirm the diagnosis. The use of alkaline phosphates staining, both on histologic samples and on cytologic specimens, has been shown to be a highly sensitive (100% sensitivity) and specific (89% specificity) way to help differentiate osteosarcoma from other mesenchymal tumours (55-57). Canine osteosarcoma can be further classified histologically, based on the type and amount of extracellular matrix and cellular characteristics. These include osteoblastic, chondroblastic, fibroblastic, poorly differentiated and telangiectatic (49). A difference in biologic behaviour based on histologic subclassification has not been observed, however histologic grading has been shown to be a better predictor of tumour behaviour (58). The tumour can be histologically graded from I to III, based on pleomorphism, mitotic index, the amount of extracellular matrix, tumour cell density and the level of necrosis.

Diagnosis

A presumptive diagnosis of osteosarcoma can be made, based on signalment, medical history, physical examination and radiographic findings (49). The appearance of osteosarcoma can be quite variable, but there are some features that are commonly seen. These include cortical lysis, sometimes being severe enough to cause pathologic fractures, soft tissue swelling and new bone formation, either from the tumour or from reactive bone, organizing in a palisading pattern radiating from the axis of the cortex (often termed "sunburst"). There are often deposits of periosteal new bone on the cortex, at the periphery of the lesion, creating a triangular-appearing deposition (often termed "Codman's triangle). The radiographic appearance of osteosarcoma is similar to that of osteomyelitis, in particular those of fungal etiology and so biopsies may be warranted (59). Other differential diagnosis with similar radiographic appearance (lytic, proliferative or mixed aggressive bone lesions) include other primary bone tumours (chondrosarcoma, fibrosarcoma, hemangiosarcoma), metastatic cancer, multiple myeloma, lymphoma, bacterial osteomyelitis and bone cysts (49).

To further support the diagnosis of osteosarcoma, ultrasound guided fine-needle aspirate cytology of the lesion can be evaluated. This method has been shown to have a sensitivity of 97% and a specificity of 100%, in regard to differentiating sarcomas from other lesions, and has shown to accurately diagnose osteosarcoma in 85% of samples (56, 57, 60). Concurrent alkaline phosphatase staining of cytologic samples appears to be a highly sensitive (sensitivity of 100%) and fairly specific (specificity of 89%) marker for diagnosing osteosarcoma (55-57). However, in most cases, a definitive diagnosis is often made by procurement and analysis of tissue samples for histopathology, either by sending in the entire lesion after surgical excision, or by procuring a bone biopsy, either with a trephine or a bone marrow biopsy needle (Jamshidi bone marrow needle) (49). Biopsies gathered with a trephine has been reported to yield a diagnostic accuracy rate of 93.8%, yet carries a great risk of

creating pathologic fractures (61). Biopsies gather using a bone marrow biopsy needle has been shown to yield a diagnostic accuracy of 91.9% when it comes to detecting tumours and an accuracy of 82.3% when it comes to accurately diagnose specific tumour subtypes (62).

Following diagnosis, patients should be staged and assessed for any concurrent disease. This is usually done by careful clinical examination, followed by a thorough orthopaedic examination, in order to uncover enlarged lymph nodes or pain in the skeleton related to sites of metastasis (49). Staging should include high-detail thoracic radiographs, although pulmonary metastases are uncommonly detected at the time of diagnosis (<10% of dogs) and are not detectable until lesions have reached 6-8 mm in diameter. More advanced imaging (including computer tomography) has shown a higher sensitivity at detecting pulmonary nodules than thoracic radiographs and may play a role in patient staging and monitoring during treatment, but further studies are warranted (63, 64). Bone survey radiographs have been shown to be a valuable tool in detecting metastatic bone lesions, and has been reported to yield a higher incidence of metastatic lesions (6.4%) than thoracic radiographs (4%) at the time of presentation (65).

Treatment and prognosis

There are many treatment options for canine osteosarcoma, including surgery, radiotherapy and chemotherapy, or usually a combination of these (49, 51). Surgery is the first-line procedure, which increases survival, brings pain relief, thereby delaying euthanasia (3, 4, 51). Surgery can be done either by amputation of the effected limb, or by limb-sparing surgery.

Amputation is considered the standard surgical treatment for appendicular osteosarcoma and most dogs, including large- and giant-breed dogs, will function well and have a good quality of life after surgery (66, 67). It completely removes the primary tumour, decreases the risk of postoperative complications, shortens time of anaesthesia and decreases expenses compared to limb-sparing procedures (68). Limb sparing surgery is a procedure in which the bone tumour is resected without limb amputation, by reconstructing the excised bone segment, thus preserving the limb (69-73). Limb function has been shown to be good to excellent in most dogs undergoing limb sparing procedures and removal of the primary tumour with marginal resection has not shown to have an adverse effect on survival time (74). Limb sparing surgery should be considered in patients with pre-existing orthopaedic or neurologic disease, or in patients with owners who will not permit amputation (49). Patients that undergo limb sparing procedures should be in general good health, the tumour should be clinically and radiographically confined to the limb, in which the primary tumour affects less than 50% of the affected bone. There are several limb sparing procedures described, including allograft limb sparing, metal endoprosthesis limb sparing, pasteurized tumoral autograft, longitudinal bone transport osteogenesis and ulna transposition limb sparing, all with their own advantages and limitations, but often associated with complications (wound infections, implant failures and tumour reoccurrence) (75-79). However, dogs treated with surgery alone have a poor prognosis (3). This was shown in a study with 162 dogs treated with amputation alone, where they found a median survival time of 19.2 weeks.

Thus, patients with osteosarcoma are usually treated with adjuvant therapy, including chemotherapy, which have shown to greatly improve survival time. (80-82). The most commonly used cytostatic drugs are carboplatin, cisplatin and doxorubicin. Dogs treated with adjuvant carboplatin after surgery have shown to have a median survival time ranging from 258 to 479 days, depending on number of treatments and dosage (81, 83, 84). Dogs receiving cisplatin as adjuvant treatment after surgery have shown to have a median survival time of 262 to 325 days (70, 85). Treatment with doxorubicin as a single agent adjuvant treatment to surgery has shown to have a median survival time of 240 to 366 days (80, 86). Adjuvant treatment using alternating or concurrent combinations of doxorubicin, with either cisplatin or carboplatin, have been investigated in several studies (81, 83, 84, 87, 88). However, it has shown to have little effect on disease free interval and median survival time, when compared to single agent protocols, nevertheless the frequency of negative side effects was significantly lower for these protocols.

Radiation therapy is considered a palliative method of treatment for canine osteosarcoma, with the intent of providing pain relief and prolonging the patient's life, when surgery is not an option (49, 89, 90). Fractionated tumour irradiation with curative intent seems to be ineffective, however local control dog of the tumour can be achieved by stereotactic radiosurgery or intraoperative extracorporeal irradiation when performing limb sparing procedures (89). However, radiation therapy is not easily accessible and requires general anaesthesia. In a study comparing palliative radiation therapy with and without chemotherapy, there was a significant difference in median survival time. (90) Dogs receiving radiation therapy as the sole treatment for osteosarcoma had a median survival time of 178 days, while those undergoing radiation therapy and chemotherapy had a median survival time of 307 days.

Comparative aspect

Canine osteosarcoma shares many similarities with human osteosarcoma in terms of genetic similarities, clinical presentation, biologic behaviour and metastatic progression, and has been shown to be a valuable comparative model for studies (91-93). Osteosarcoma is more common in dogs than in humans, making it easier to gather patients and samples, it has a more rapid progression in dogs than in humans and so results from treatment trials can be reported earlier than in human trials. Research costs for clinical trials in dogs are significantly less than those in human trials, making it easier to develop new treatments. In contrast to studies done on laboratory animals, dogs with spontaneous occurring osteosarcoma will develop the disease naturally, reducing the need to induce the disease in healthy animals.

Tumour protein (TP)

Tumour protein (TP) is a group of IgG monoclonal antibodies that bind with a high specificity to some human neoplasms of mesenchymal origin, including osteosarcoma (94). The antibodies, consisting of TP1 (IgG-2a) and TP3 (IgG-2b), are produced by immunization of mice with human osteosarcoma cells (94, 95). It has been shown that both TP1 and TP3 bind specifically to two different epitopes on the same antigen in some subtypes of human sarcomas (94). The antigen to which TP1 and TP3 bind is a monomeric polypeptide with a molecular weight of approximately 80kDa and its function is unknown. This antigen has been present in all human osteosarcoma samples examined and it binds strongly with both TP1 and TP3 antibodies, as demonstrated with immunohistochemistry (IHC). TP1 and TP3 have also shown affinity to other sarcomas, including pleomorphic undifferentiated sarcoma, malignant hemangiopericytomas and synovial sarcomas (Table 1) (94, 95). Other sarcomas, non-sarcomas and a wide variety of normal tissues have not been shown to react with TP1 and TP3 in human studies (94). However, TP1 and TP3 show reactivity towards some normal tissues, as shown with IHC, including clusters of cells in the adrenal medulla and in the proximal renal tubules (Table 2). Endothelial cells in proliferating capillaries in placental tissue and in most tumour tissue also stain. Staining is absent in resting osteoblastic cells, but is present in actively proliferating osteoblast (94).

In a study conducted on canine tissues, similarities and dissimilarities to human neoplasms and normal tissues were assessed (96). As in humans, all investigated tissues from canine osteosarcomas show positive staining (94, 96). Osteoblastic subtypes of osteosarcomas have been shown to stain more intensely than chondroblastic and fibroblastic subtypes (96). In addition, TP3 stained a higher number of cells and did so more intensely than TP1. This phenomenon was not observed in human samples (94, 96). This may indicate that there is a

difference in the amino acid sequence of the TP binding antigen between humans and dogs, resulting in a variation in the affinity towards TP1 and TP3 (96). Whether TP binds the same antigen in humans and dogs has not been investigated. Osteoid surrounding the tumour cells did not stain. Chondrosarcomas have also shown reactivity towards both TP1 and TP3 (Table 1). Unlike in human tumours, TP1 and TP3 have shown to stain certain types of canine carcinomas, including lung carcinoma, squamous cell carcinoma, thyroid carcinoma and prostatic carcinoma (94, 96). In addition, tumours in which there is proliferation of myoepithelial cells show reactivity with the antibodies (96). As in humans, little staining was observed in a wide variety of normal canine tissues. Staining was, however, observed in myoepithelial cells in the mammary glands, at the brush border of ciliated respiratory epithelium and endothelium in some normal organs (Table 2) (94, 96). The staining of endothelial cells was, as observed in humans, present in proliferating capillaries in tumours.

Table 1: The table shows the distribution of immunohistochemical staining of human and canine tumours with TP monoclonal antibodies (94, 96).

Tumour type	Human	Canine
Sarcomas	Osteosarcoma Chondrosarcoma Pleomorphic undifferentiated sarcoma Malignant hemangiopericytoma Synovial sarcoma	Osteosarcoma Chondrosarcoma
Carcinoma	No staining	Lung Carcinoma Squamous Cell Carcinoma Thyroid Carcinoma Prostatic Carcinoma

In humans, TP only reacts with some types of sarcomatous neoplasms, while in dogs, they have been shown to bind neoplasms of both epithelial and mesenchymal origin. Most human and canine normal tissues show little reactivity with the antibodies, but there are some similarities and dissimilarities, as mentioned above. The fact that TP reacts strongly with osteosarcoma in both humans and dogs, indicates that there is a antigenic similarity in between these two species (96). These antibodies may play a role in both diagnosis and treatment of osteosarcoma in the future.

Table 2: The table shows the distribution of Immunohistochemical staining of normal human and canine tissues with TP monoclonal antibodies (94, 96).

Tissue	Human	Canine
Adrenal medulla	Clusters of cells	Negative
Proximal renal tubules	Clusters of cells	Negative
Trachea	Negative	Apical surface of ciliated epithelium
Bronchus	Negative	Apical surface of ciliated epithelium
Lung	Negative	Apical surface of ciliated epithelium
Cerebrum	Negative	Endothelial cells
Cerebellum	Negative	Endothelial cells
Mammary gland	Myoepithelial cells	Myoepithelial cells
Bladder	Negative	Endothelial cells
Pancreas	Negative	Endothelial cells
Placenta	Endothelial cells	Negative

Proliferating Cell Nuclear Antigen (PCNA)

Proliferating Cell Nuclear Antigen (PCNA) is an evolutionary well-preserved nuclear polypeptide present in all dividing cells, ranging from high-class plant cells to human cells (97). PCNA was first discovered as an antigen in humans in association with the autoimmune disease Systemic Lupus Erythematous (SLE) (98). Later, an acidic polypeptide that showed an uneven distribution through the cell cycle was discovered using gel electrophoresis and was given the name cyclin (99). The protein cyclin was soon associated with both cell proliferation and neoplastic transformation (99, 100). In 1984, it was found that cyclin and PCNA are in fact the same protein (101). PCNA/cyclin is now usually referred to PCNA, while the name cyclin is given to another class of proteins involved in cell cycle control (102). Later, it has been shown that PCNA has a major role in the cell cycle as a cofactor for DNA polymerase δ (103, 104). It has also been shown that PCNA is organized as a ring that can encircle the DNA and work as a sliding clamp for polymerase δ and ε, as well as a docking station for other proteins involved in DNA replication, DNA repair and cell cycle control (105, 106). PCNA also plays a role in chromatin assembly and remodelling, sister chromatin cohesion, transcription and other functions (97, 102).

The cell cycle is divided into four phases, the G1, S (DNA synthesis), G2 and M (mitosis) phase (Figure 1) (107). G0 cells are non-proliferating and are arrested between the M and S phases. The period between two mitoses is called the interphase and involves the G1, S and G2 phase (107, 108). During the G1 phase the cells contains one diploid copy of the genome and are sensitive to mitogenic signals (107). During the S phase, the genome replicates to become two sister chromatids, which are two identical copies of the original two stranded DNA. After the S phase, the cell enters the G2 phase, which is a shorter phase than G1. After G2, the cell enters the M phase and completes the mitosis.

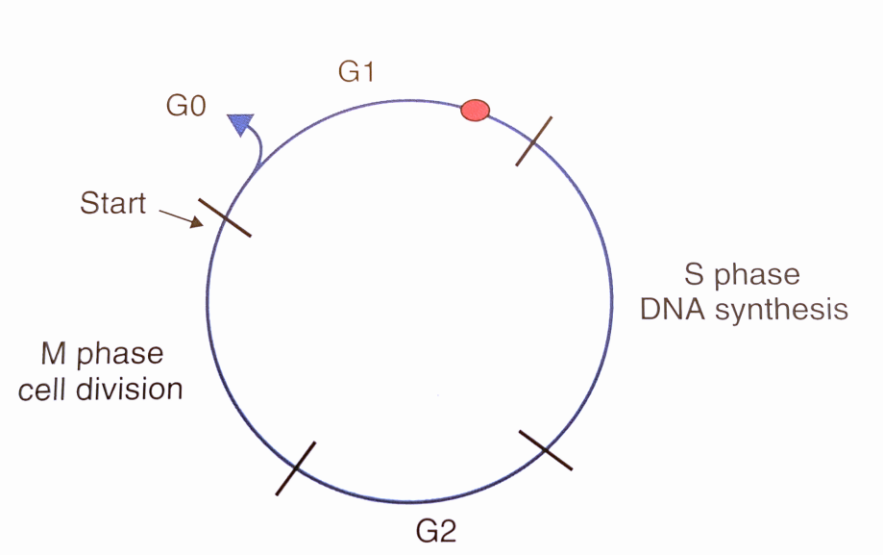

Figure 1: The figure shows a schematic presentation of the cell cycle (107). The cell cycle is divided into four phases, the G1, S, G2 and M phase. The G0 represents arrested cells.

DNA replication is dependent on DNA polymerase α and δ, and since PCNA is a cofactor for polymerase δ, it is a necessary factor in order for cell replication to occur (109, 110). There is an increase in the amount of PCNA in dividing cells during the traversing from cell cycle phase G1 to S (111, 112). It has been shown that human anti-PCNA monoclonal antibodies react with the nucleus in proliferating cells in the S phase in humans, as well as in animals, plants and yeast (97). More recently, PCNA has become a cell cycle marker, both diagnostically and prognostically in human medicine (113). The amounts of PCNA has been shown to be increased in malignant cells and can be used as a prognostic marker in some human gastric and renal cancers (114, 115). PCNA has also been evaluated as a prognostic marker in canine mast cell tumours and has been correlated with histologic grading, mitotic index and AgNOR counts (116).

Indirect method of immunohistochemistry

Immunohistochemistry (IHC) is a suitable method for studying protein expression in tissues, as well as aiding in the classification of tumours in both human and veterinary medicine (117). The method relies on specific interactions between an antibody and its antigen (118-120). The antibodies used can be either polyclonal or monoclonal. IHC can be performed on either frozen tissues or formalin-fixed paraffin embedded (FFPE) tissues (117, 120).

Polyclonal antibodies used on canine tissues are produced by immunizing another species with the antigen of interest (e.g. a rabbit) (118-120). If this species recognizes the antigen as foreign, its lymphocytes will produce antibodies against it (118). Because different lymphocyte clones will recognize different epitopes on the same antigen, they will produce different antibodies, hence the name polyclonal (118, 119). These antibodies can be collected from the animal's plasma (118).

The production of monoclonal antibodies is a more complex procedure. First, the antigen of interest is injected into a mouse, the activated lymphocytes are then extracted a few days later and they are then introduced to a culture with lymphocytic tumour cells (118, 119). These two cell types are then fused and become hybridoma cells (119). The different hybridoma clones can then be isolated and cultured separately to produce one specific type of antibodies, hence the name monoclonal (118).

The name of an antibody against protein X is given according to the species it was produced on, e.g. mouse anti-X monoclonal antibodies. The advantages of using monoclonal instead of polyclonal antibodies are that they bind to the antigen more specifically and in a stronger

fashion (118, 119). This will reduce the amount of non-specific binding to antigens with similar epitopes to the antigen of interest (118).

When performing IHC, a tissue section of interest is incubated along with antibodies (117, 118, 120). These antibodies are called primary antibodies (118). When performing an indirect method of immunohistochemistry, sections are incubated with a secondary antibody that will bind to the primary antibodies bound to the antigen of interest (118-120). Secondary antibodies are produced by immunizing a different species with immunoglobulins from the same species the primary antibodies were produced in (119). This will result in the production of antibodies against the immunoglobulin class to which the primary antibody belongs (118). Secondary antibodies are often made in rabbits to be used with primary antibodies made in mice (119). These antibodies are also labelled with a tag, often a peroxidase enzyme, which can oxidase an added substrate (117-119). Since peroxidase is an enzyme that is present in certain types of normal cells, one must prepare the tissue so that these enzymes do not participate in substrate oxidation during the IHC procedure (118, 119). Two common substrates are 3, 3'-diaminoazobenzidine (DAB) and aminoethyl carbazole (AEC), which results in a brown and red staining in the presence of peroxidase, respectively. The sections are incubated with the substrate to permit precipitation of the dye, before the sections are counterstained with Meyers Haematoxylin (119). This permits the microscopic evaluation of individual cells expressing the antigen of interest, as well as the cells that do not (120).

A common error when performing IHC is non-specific background staining. As mentioned earlier, some normal cells contain endogenous peroxidase, in particular red blood cells, neurons and granulocytes (119). When exposed to substrates such as DAB or ACE, these

cells will react with the substrate and produce staining indistinguishable from specific immunostaining. Most of the endogenous peroxidase is destroyed during formalin fixation, but further inhibition is often required to abolish the peroxidase activity (119). This can be done by incubating the sections in hydrogen peroxide (H_2O_2), diluted in either methanol, azide or distilled water, or by incubating the sections in phenylhydrazine (119). Another source of non-specific background staining can be hydrophobic interactions of proteins. The use of normal serum or protein solutions is a common method for blocking these interactions and reducing non-specific background staining (119).

Seed and soil hypothesis

In 1889, the human doctor Stephen Paget realized that the distribution of metastases was no matter of chance and that specific sites were more prone to develop secondary growths (121). He observed that certain organs were more prone to metastasis than others. Furthermore, he observed that specific tumours seemed to have specific sites to which they metastasized and that the distribution was very different from bacterial embolisms seen in pyaemias. Based on the theories of Langenbeck, along with his own observations, he devised the notion of "the seed and soil". By comparing tumour cells with plant seeds and the organs to which they metastasize with the soil where plants thrive, he proclaimed that certain tumour cells could only grow in certain organs, even though they could spread to every reach of the organism, like seeds that can only grow if they fall on the species' preferred soil. This way of thinking would demand that the tumour cell could fulfil certain requirements, giving it the ability to colonize the target organ, like a seed which has all the necessary components to grow. A lot of research has been performed since Paget formed his ideas. This hypothesis hold even more ground now than it did in his time, given that we know that most primary malignant tumours, both of epithelial and mesenchymal origin, shed cells that can be detected in the blood stream, long before distant metastases appear.

Aim of the study

The aim of our study is to characterize the distribution of pulmonary metastases in early metastatic disease in dogs with osteosarcoma, and by doing so, shed light on the mechanisms involved in the development of metastatic disease. Despite removal of the primary tumour, most canine patients will develop pulmonary metastases within a short time, even though macroscopic metastases are not evident at time of diagnosis, based on routine autopsy and histopathology. This leads to the following questions: Are tumour cells present in the lungs before the development of visible metastases? If not, where are they harboured and what makes them redistribute to the lungs, and when does this occur? To address these questions, we wish to study the distribution of tumour cells by characterizing the expression of TP3 and PCNA in lung tissue from dogs with and without visible metastatic disease, based on routine autopsy and histopathology. By doing so, we wish to evaluate the potential for these markers to be used in the detection of early metastatic disease.

Materials and methods

Study design

The current study was designed as a pilot study and thus few individuals were included. For our results to be validated, further studies with a larger material would be needed.

Animals and collection of tissue specimens

Three (n=3) dogs, aged six to nine years old, were used in the present study. Dogs included as cases in the study (n=2) were middle age to geriatric dogs with osteosarcoma (OS) in the skeleton. One dog (M+) had macroscopic metastases in the lungs, while the other did not (M-), as verified by autopsy and routine histopathological examination. The dog that was included as a control dog (n=1) had been euthanized and autopsied for other reasons than OS, without any concurrent pulmonary disease, as verified by histopathological examination. Dogs with neoplastic disease other than OS, with or without metastatic diseases and/or concurrent pulmonary pathology were excluded from the study.

Relevant data for the dogs included in the present study are shown in Table 3. The dogs were euthanized, after sedation, with intravenous pentobarbital and autopsied within two hours post mortem at the Faculty of Veterinary Medicine, Norwegian University of Life Sciences. The control dog was an eight-year-old Dalmatian dog, euthanized due to urolithiasis (cysteine) and urinary tract obstruction, with no other macroscopic or microscopic pathology, confirmed by histopathologic examination. One of the cases (M+) was a nine-year-old Rottweiler dog, euthanized due to appendicular OS with macroscopic metastatic lesions in the lungs, confirmed by autopsy and histopathologic examination. The other case (M-) was a six-year-old Leonberger dog with appendicular OS without macroscopic lesions in the lung,

confirmed by autopsy and histopathologic examination. No other neoplastic disease was observed in any of the 3 dogs.

Table 3: The table shows relevant data for the dogs used in the present study

	Research number	Breed	Age	Sex	Weight	Euthanized	Time from euthanized to autopsy
Case (M+)	F-2013-00028	Rottweiler	9 years	Male	50 kg	15.05.2013	Within 1.5 hours
Case (M-)	F-2016-00853	Leonberger	6 years	Male	56.5 kg	03.10.2016	Within 2 hours
Control dog	F-2016-00854	Dalmatian	8 years	Male	24 kg	03.10.2016	Within 2 hours

Autopsies were carried out following a routine protocol (Appendix 1) to make sure the dogs had no other concurrent macroscopic neoplastic disease or other pulmonary pathology. Routine tissue samples were collected from every organ system, fixed in 10% neutral buffered formalin, before being embedded in paraffin. Additionally, tissue samples (1x1 cm) were taken from all seven lung lobes, as well as pulmonary and tracheobronchial lymph nodes per a standardised protocol (Appendix 2). Pulmonary samples for formalin-fixed paraffin embedded (FFPE) sections were immediately fixed in a 10% buffered formalin solution, before being embedded in paraffin and stored at room temperature until further preparation. Samples for cryosectioning were immediately snap frozen in isopropanol chilled with liquid nitrogen. Samples were then transferred and stored at -80°C until further preparation.

Antibodies used for labelling

The primary antibodies used in this study are shown in Table 4. The secondary antibodies used for immunohistochemistry (IHC) was Daco EnVision ™ + System-HRP (DAB), Ref K4007 (Daco).

Table 4: The table shows the different primary antibodies used in this study. IHC, immunohistochemistry: mAb, monoclonal antibody.

Antibody	m/p	Isotype	Cellular expression in dogs	Source	Used for	Cross reactivity	References
TP-3	mAb	IgG-2b	Sarcomatous and carsinomatous cells	Oslo University Hospital, Rikshospitalet	IHC	Humans	(94-96)
Anti-PCNA	mAb	IgG-2a	Proliferating cells	Dako Norway	IHC	All species	(113-116)

Histology techniques

Haematoxylin & Eosin (HE)

We placed the formalin-fixed paraffin embedded (FFPE) samples at -20ºC for 1 hour, before they were sliced with a microtome at 2µm thickness. Sections were made from all 14 tissue samples of every lung lobe from all three dogs. The sections were placed on standard microscopy slides. They were then put in a heating chamber at 37ºC overnight and then stored at 4ºC until further preparations. Next, the sections were placed in a heat chamber at 58ºC for 30 minutes, then deparaffinized, stained with Haematoxylin & Eosin, dehydrated and mounted using a standardized protocol (Appendix 3).

Immunohistochemistry (IHC)

Formalin-fixed paraffin embedded (FFPE) sections

We placed the FFPE samples at -20ºC for 1 hour, before they were sliced with a microtome at 2µm thickness. Sections were made from one of the fourteen lung samples, chosen at random, from the M+ case and the control dog. The sections were placed on poly-lysine coated slides (Superfrost^{TM} Plus, Thermo Fisher Scientific) and stored at 4ºC until further

preparation. The sections were placed in a heat chamber at 58ºC for 30 minutes, then deparaffinized, using a standardized protocol (Appendix 4). All washes performed during the IHC procedure were done by immersion of the slides in three changes of phosphate-buffered saline (PBS), for 5 minutes each at room temperature, with the slides placed in a glass container on a rotation table (Heidolph Rotamax 120). All incubations were done at room temperature, in a moisture chamber on a rotation table.

Proliferating Cell Nuclear Antigens (PCNA)

We placed the sections in a Citrate buffer (pH = 6,0) and we autoclaved them for 15 minutes at 121ºC for antigen damasking (heat-induced epitope retrieval - HIER). The sections were then rinsed with distilled water, before being washed. The samples were stored in buffered PBS at 4ºC overnight. The sections were then inhibited using a 3% H_2O_2 solution in methanol for 10 minutes in a fume hood. The sections were then rinsed carefully in distilled water, before being washed. The sections were placed in a moisture chamber for the remainder of the process and the sections were encircled with a water repelling Dako Pen (Dako). The sections were blocked for non-specific binding with 2% normal goat serum in 5% bovine serum albumin (BSA)/tris bufferd saline (TBS) and incubated for 20 minutes. The blocking agent was then gently tapped off, before the primary antibody (Mouse anti-human PCNA) with a concentration of 1:5000 with 1% BSA/TBS was applied on the slides and incubated for 1 hour. This antibody concentration was chosen based on earlier staining of canine tissues at the University's histology laboratory. The sections were then carefully rinsed with PBS, before being washed. 2-3 drops of a room tempered secondary antibody (Daco EnVision ™ + System-HRP (DAB), Ref K4007) were applied on the slides and incubated for 30 minutes. The sections were then carefully rinsed with PBS, before being washed. Finally, immunolabelled cells and tissues were made visible using an aminoethyl carbazole (AEC) Substrate Chromogen (Daco EnVision ™ + System-HRP (AEC), Ref K4009), incubated for

9 minutes. Excess AEC-solution was carefully washed off with distilled water (3 x 3min) to stop further staining. Tissues were then counterstained with Meyer's Haematoxylin dye for 20 seconds before they were washed in distilled water for 5 minutes. The slides were then mounted with cover slips (Menzel-Gläser 24 x 32 mm #1) using a water-soluble mounting medium (Aquatex®) and left to dry in room temperature overnight.

Cryosections

We sliced the frozen samples into 7μm thick cryosections with a cryostat at a temperature set to –25°C. Sections were made from one of the fourteen lung samples, chosen at random, from the M+ case, M- case and the control dog. The cryosections were mounted on poly-lysin-coated slides (Superfrost^{TM} Plus, Thermo Fisher Scientific) and dried in room temperature for one hour before being stored at –80°C until further preparation. All washing procedures during the IHC procedure were done by immersion of the slides in three changes of phosphate-buffered saline (PBS), for 5 minutes each at room temperature, with the slides placed in a glass container on a rotation table (Heidolph Rotamax 120). All incubations were done at room temperature, in a moisture chamber placed on a rotating table.

Tumour Protein 3 (TP-3)

We dried the sections at room temperature for one hour before being fixed in 0.25% glutaraldehyde in cold (4°C) PBS for 5 minutes. The sections were carefully rinsed in PBS, before they were washed. The sections were then placed in a 0.25 % phenylhydrazine/PBS solution for 40 minutes in a heating chamber, to inhibit endogenous peroxidase. This solution was made by adding phenylhydrazine to preheated (37°C) PBS while stirring vigorously with a magnet stirrer. After being washed, the sections were transferred to a moisture chamber and the sections were encircled with a water repelling Dako Pen (Dako). The sections were then blocked for non-specific binding with normal goat serum 1:50 in 5% BSA/TBS and

incubated for 30 minutes. The blocking agent was then gently tapped off, before the primary antibody (TP-3) was applied to the slides and incubated for 1 hour. The TP3 solutions used in this step were made by diluting the original solution, using 0.1% BSA/TBS, to the following concentrations: 10µg/ml, 20µg/ml and 100µg/ml. These concentrations were based on results from earlier reports from staining of canine osteosarcoma samples, where 100µg/ml was successfully used for staining (96). We chose to add additional concentrations (10µg/ml and 20µg/ml) to see if any of these could give similar staining, while producing less background staining. The sections were then carefully rinsed with PBS, before being washed. 2-3 drops of a room tempered secondary antibody (Daco EnVision™ + System-HRP (DAB), Ref K4007) were applied on the slides and incubated for 30 minutes. The sections were then carefully rinsed with PBS, before being washed. Finally, immunolabelled cells and tissues were made visible using an AEC Substrate Chromogen (Daco EnVision™ + System-HRP (AEC), Ref K4009), incubated for 9 minutes. Excess AEC-solution was carefully washed off with PBS (3 x 3min) to stop further staining. Tissues were then counterstained with Meyer's Haematoxylin dye for 20 seconds before being washed in distilled water for 5 minutes. The slides were then mounted with cover slips (Menzel-Gläser 24 x 32 mm #1) using a water-soluble mounting medium (Aquatex®) and left to dry in room temperature overnight.

Microscopic analysis

Microscopic analysis of HE slides

The HE stained sections were examined by light microscopy. All slides were examined by a University pathologist to make sure the dogs had no other concurrent microscopic disease. In addition to routine sections from all organ systems, one central and peripheral sample from each of the 7 lung lobes were examined.

Microscopic analysis of immunolabelled slides

We examined the IHC stained sections using a Leica DM 2500 microscope, equipped with a Leica DFC295 camera, coupled with a Leica Application Suite 4.8 image acquiring software. Images were taken from all sections at different magnifications (25X, 100X, 200X, 400X and 630X).

Results

Haematoxylin & Eosin (HE) sections

HE sections from the M+ case, M- case and control dog were available for assessment

HE coloured sections from the M+ case, M- case and control dog were available for evaluation.

The metastatic lesions had a random distribution throughout the pulmonary parenchyma in the M+ case

The distribution of metastatic lesions showed no preference for any part of the pulmonary parenchyma. Of the 14 lung samples taken from the M+ case, metastases were present in 10. The metastases were of different sizes. Metastases were also present in blood vessels (Figure 2; B and C), however, the majority of metastases were present in the pulmonary parenchyma (Figure 2; A).

The primary tumour in the M+ case was characteristic of fibrosarcomatous osteosarcoma

Sections from the primary tumour showed an infiltrate of pleomorphic cells and contained smaller areas of connective tissue and necrosis. The cells had small nuclei rich in chromatin, whereas some cells had a fibroblast- to fibrocyte-like appearance. There were some areas with minor calcification surrounded with osteoid-like material.

Other findings in the M+ case

Sections taken from the left, middle and right tracheobronchial lymph nodes and the pulmonary lymph node showed cell rich areas in all parts of the node consisting of primarily small lymphocytes. These changes are indicative of lymphoid hyperplasia. There was no evidence of metastases in any of the lymph nodes examined. Sections taken from the left and right kidney showed minor infiltrates of lymphocytic cells in the interstitium of the renal cortex. These findings are indicative of a moderate focal interstitial nephritis and are considered incidental findings.

There were no major findings in the lungs in the M- case

There were no findings in 11 of the 14 lung samples taken from the M- case. In one lung sample, there was a small area with calcification, which is considered an incidental finding. In another lung sample, there was a mononuclear cell infiltration around a smaller vessel. In the last lung sample, a vessel was found to have a thickened muscular layer, with subsequent decreased lumen.

Other findings in the M- case

Sections taken from the left, middle and right tracheobronchial lymph nodes showed cell rich areas in the medullary part of the nodes. There was no evidence of metastases in any of the lymph nodes examined.

There were no major findings in the lungs in the control dog

There were no findings in 11 of the 13 lung samples evaluated from the control dog (Figure 3; A, B and C). One of the 14 lung samples disappeared during labelling and processing. In one of the lung sample, there was a small cell rich area in the parenchyma, which is

considered an incidental finding. In the other lung sample, a vessel was found to have a thickened muscular layer, with subsequent decreased lumen. Anthracosis was evident in several lung samples.

Other findings in the control dog

Sections taken from the bladder wall showed signs of bleeding and hyperaemia. Hypertrophy of the muscular layers was present. There was a moderate mononuclear cell reaction in between the muscular layers in certain areas. Sections taken from the duodenum showed a moderate mononuclear cellular infiltrate in the mucosa. Sections taken from the ileal lymph nodes showed a dilated marginal sinus, filled with macrophages and blood, and a cell rich medulla. These findings are indicative of an acute moderate reactive hyperplasia. Sections taken from the prostate showed broad strands of connective tissue around the glandular tissues with no cellular reaction. These findings are indicative of a chronic prostatitis. Sections taken from the liver showed moderate fibrosis around several central veins. Sections from the heart showed marked fibrosis and thickening of the valves and a few cardiac vessels with mucoelastic thickening.

Immunohistochemistry (IHC) sections

FFPE sections immunolabelled with anti-PCNA antibodies

Anti-PCNA immunolabelled sections were available for the M+ case and control dog

Sections that were available for interpretation were those from the M+ case and control dog. No sections from the M- case were immunolabelled with anti-PCNA.

There was a similar distribution of stained cells in the M+ case and the control dog after immunolabelling with anti-PCNA, however the density was different.

In both the M+ case and in the control dog, there was a similar distribution of staining single cells throughout the pulmonary parenchyma, however the density of stained cells was higher in the control dog than in the M+ case. The distribution seemed homogenous throughout the pulmonary parenchyma. Furthermore, there were multiple metastatic lesions in the M+ case that showed a high density of stained cells. Similar lesions were not present in the control dog. The metastatic lesions appeared evenly distributed in the lung sample studied, thus the distribution was random.

Metastatic lesions in the M+ case stained with anti-PCNA antibodies

Metastatic lesion showed significant staining when labelled with anti-PCNA antibodies (Figure 4; A, C, E and G). Staining was specific for nuclei and variation in staining intensity was seen between tumour cells. A subset of cells in between staining tumour cells showed no staining. Anti-PCNA was useful in visualizing small metastatic lesions. Samples that were prepared without a primary antibody showed no staining (Figure 4; B, D, F and H) demonstrating that staining was a result of anti-PCNA immunoreactivity.

Single cells in the parenchyma in the M+ case stained with anti-PCNA antibodies

Single cells in the pulmonary parenchyma outside metastatic lesions stained when labelled with anti-PCNA antibodies (Figure 5; A, C and E). Staining was specific for nuclei and was less variable in intensity than the staining observed in metastatic lesions. Some of the stained cells were characterized as macrophages, while in others the origin could not be established, however nuclear size and chromatin density was similar to that of alveolar epithelial cells. Samples that were prepared without a primary antibody showed no staining (Figure 5; B, D and F) demonstrating that staining was a result of anti-PCNA immunoreactivity

Single cells in the parenchyma in the control dog stained with anti-PCNA antibodies

Similarly, single cells in the pulmonary parenchyma in the control dog stained when labelled with anti-PCNA antibodies (Figure 6; A, C and E), as they did in the pulmonary parenchyma outside metastatic lesions in the M+ case. Staining was specific for nuclei and was more homogenous than the staining described in metastatic lesions. Some of the stained cells were characterized as macrophages, while in others the origin could not be established, however nuclear size and chromatin density was similar to that of alveolar epithelial cells. Interestingly, the density of stained cells was subjectively higher than that of the density observed in the pulmonary parenchyma outside metastatic lesions in the M+ case. Samples that were prepared without a primary antibody showed no staining (Figure 6; B, D and F), demonstrating that staining was a result of anti-PCNA immunoreactivity.

Columnar ciliated epithelium in the control dog stained with anti-PCNA antibodies

Columnar epithelium in the bronchi in the control dog stained when labelled with anti-PCNA antibodies (Figure 7; A, C, E and G). Staining was specific for nuclei and there was a

variation in staining intensity between cells. A subset of epithelial cells did not stain. Samples that were prepared without a primary antibody showed no staining (Figure 7; B, D, F and H), demonstrating that staining was a result of anti-PCNA immunoreactivity.

All results from the immunolabelling with anti-PCNA antibodies are summarized in Table 5.

Table 5: The table shows the results from the immunolabelling of lung tissue from the M+ case and control dog with anti-PCNA antibodies.

Structures	**M+ case**		**Control dog**	
	With anti-PCNA	Without anti-PCNA	With anti-PCNA	Without anti-PCNA
Metastatic lesions	+	-	None present	None present
Single cells in the parenchyma	+	-	+	-
Columnar ciliated epithelium	None present	None present	+	-

Cryosections immunolabelled with TP3 antibodies

Sections immunolabelled with TP3 were available for the M+ and M- case, as well as for the control dog

Sections that were available for interpretation were those from the M+ case, M- case and control dog. Sections immunolabelled with TP3 at a concentration of 20µg/ml gave the best staining results and were used for interpretation.

There was variation in the distribution of TP3 positive cells when comparing the M+ case, M- case and the control dog

Stained single cells were found throughout the pulmonary parenchyma, with a similar and evenly scattered distribution in all three dogs. Furthermore, there were multiple metastatic lesions in the M+ case that showed a high density of stained cells. Similar lesions were not present in the remaining dogs. The distribution of metastatic lesions seemed random.

Metastatic lesions in the M+ case stained with TP3 antibodies

Metastatic lesions showed strong staining when immunolabeled with TP3 antibodies (Figure 8; A, C, E and G). The stain was mainly cytoplasmic, with a granular pattern, however staining was also observed in nuclei. The stain showed a homogenous intensity throughout the metastatic lesions, however the periphery of the lesions sometimes showed a more intense staining. Smaller metastatic lesions often showed more intense staining than larger ones (Figure 9; A and B). Samples that were prepared without a primary antibody showed no staining of metastatic lesions (Figure 8; B, D, F and H), demonstrating that staining was a result of TP3 immunoreactivity.

Single cells in the parenchyma in the M+ case stained with TP3 antibodies

Single cells in the pulmonary parenchyma outside metastatic lesions stained when immunolabelled with TP3 antibodies (Figure 10; A, B and C). Staining was sometimes observed in the nuclei, but mostly it was present in the cytoplasm. The stain in these cells did not have the same granular appearance as observed in cells in metastatic lesions, instead it was more homogenous and globular in appearance. The stained cells outside metastatic lesions could not always be differentiated, however some of the stained cells were characterized as different subtypes of leukocytes (neutrophils, alveolar macrophages and lymphocytes), while the remainder of the cells had a nuclear size and chromatin density similar to alveolar epithelial cells.

Single cells in the parenchyma in the M+ case stained without TP3 antibodies

Single cells in the pulmonary parenchyma outside metastatic lesions stained even thought they were not immunolabeled with TP3 antibodies (Figure 11; A, B and C). The stain had a cytoplasmic distribution and had a homogenous and globular appearance. The stained cells could not always be differentiated, however most of them were characterized as different subtypes of leukocytes (neutrophils, alveolar macrophages and lymphocytes), while the remainder of the cells had a nuclear size and chromatin density similar to alveolar epithelial cells. The density of stained cells where, however, lower than in samples immunolabelled with TP3 antibodies.

Some normal structures in the M+ case stained with TP3 antibodies

Some normal structures showed significant staining when immunolabelled with TP3 antibodies (Figure 12; A and B). These structures included the walls of vascular structures (endothelial cells) and cells within pulmonary vessels (leukocytes). The vascular walls

showed a mild staining, with a homogenous appearance. Samples that were prepared without TP3 antibodies showed no staining of these structures, demonstrating that staining was a result of TP3 immunoreactivity.

Single cells in the parenchyma in the control dog stained with TP3 antibodies

As with the M+ case, single cells in the pulmonary parenchyma stained when labelled with TP3 antibodies in the control dog (Figure 13; A, C, E and F). Staining was sometimes observed in nuclei, but mostly it was present in the cytoplasm. The stain in these cells did not have the same granular appearance as observed in cells in metastatic lesions, instead it was more homogenous and globular in appearance. The stained cells could not always be differentiated, however some of the stained cells were characterized as different subtypes of leukocytes (neutrophils, alveolar macrophages and lymphocytes), while the remainder of the cells had a nuclear size and chromatin density similar to alveolar epithelial cells. Samples that were prepared without a primary antibody showed no staining (Figure 13; B, D, F and H), demonstrating that staining was a result of TP3 immunoreactivity.

Some normal structures in the control dog stained with TP3 antibodies

Some normal structures showed significant staining when immunolabelled with TP3 antibodies (Figure 14, A, B and C). These structures included brush borders of ciliated columnar epithelium in bronchioles, as well as the walls of vascular structures (endothelial cells). The brush borders showed an intense staining, with a granular to confluent appearance, while the stain observed in the vascular walls was milder and had a more homogenous appearance. Samples that were prepared without TP3 antibodies showed mild staining of the brush border (Figure 14; D). A small agglomeration of stained cells was observed in one of the samples, but the nature of these cells could not be determined for sure (Figure 15; A, B

and C). They did, however, show similarities to the staining brush border of columnar epithelial cells observed in bronchioles (Figure 15; D).

Single cells in the parenchyma in the M- case stained with TP3 antibodies

As observed in the M+ case and the control dog, single cells in the pulmonary parenchyma stained when immunolabelled with TP3 antibodies in the M- case (Figure 16; A, C, E and F). Staining was sometimes observed in the nuclei, but mostly it was present in the cytoplasm. The stain in these cells did not have the same granular appearance as observed in the metastatic lesions in the M+ case; instead it was more homogenous and globular in appearance. The stained cells outside metastatic lesions could not always be differentiated, however some of the stained cells were characterized as different subtypes of leukocytes (neutrophils, alveolar macrophages and lymphocytes), while the remainder of the cells had a nuclear size and chromatin density similar to alveolar epithelial cells. No overt metastatic lesions were visualized after immunolabeling with TP3 antibodies, nor were there observed any cells with similar staining intensity and pattern as seen in metastatic lesions in the M+ case. Samples that were prepared without TP3 antibodies showed no staining of these single cells, demonstrating that staining was a result of TP3 immunoreactivity (Figure 16; B, D, F and H).

All results from the immunolabelling with TP3 antibodies are summarized in Table 6.

Table 6: The table shows the results from the immunolabelling of lung tissue from the M+ case, M- case and the control dog, with TP3 antibodies.

Structures	**M+ case**		**Control dog**		**M- case**	
	With TP3	Without TP3	With TP3	Without TP3	With TP3	Without TP3
Metastatic lesions	+	-	None present	None present	None present	None present
Single cells in the alveolar structures	+	+	+	-	+	-
Brush border of columnar ciliated epithelium	None present	None present	+	+	None present	None present
Endothelium	+	-	+	-	+	-

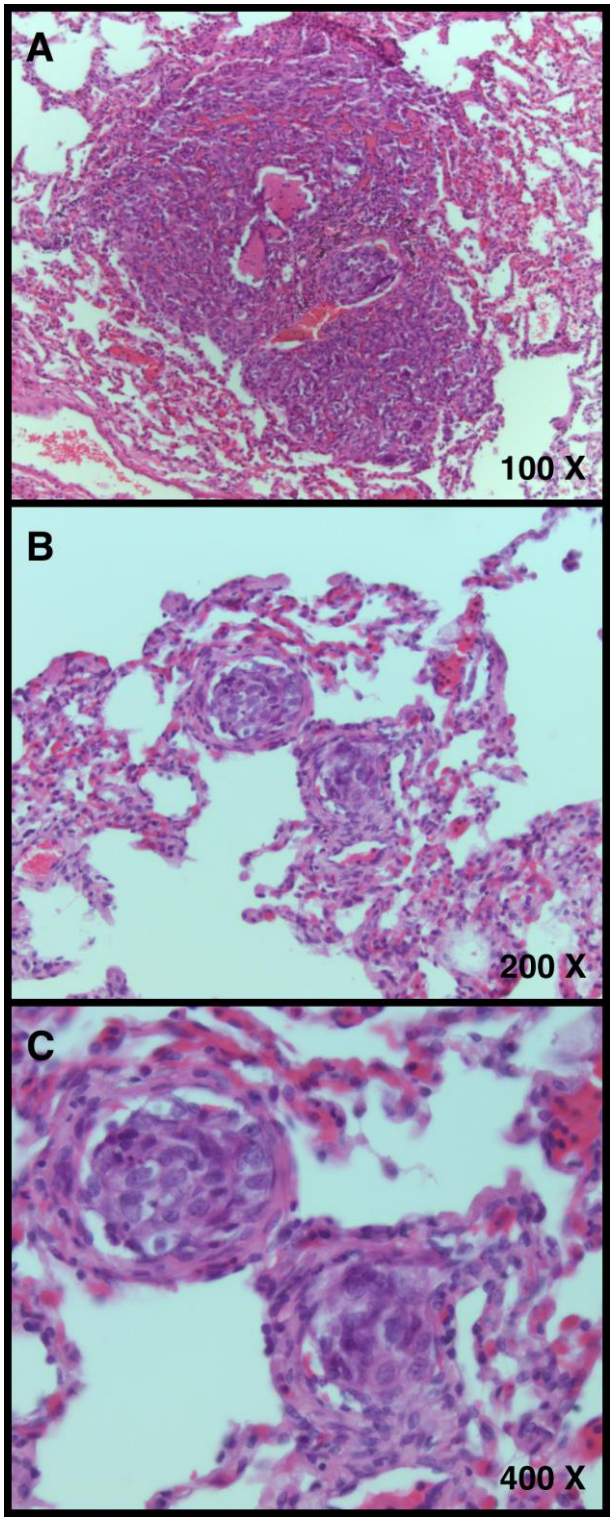

Figure 2: The figure shows the formalin-fixed paraffin embedded sections of lung tissue from the M+ case, stained with haematoxylin & eosin, at different magnifications. Picture A shows a metastasis with central necrosis. Picture B and C show metastatic cells present in vessels at different magnifications.

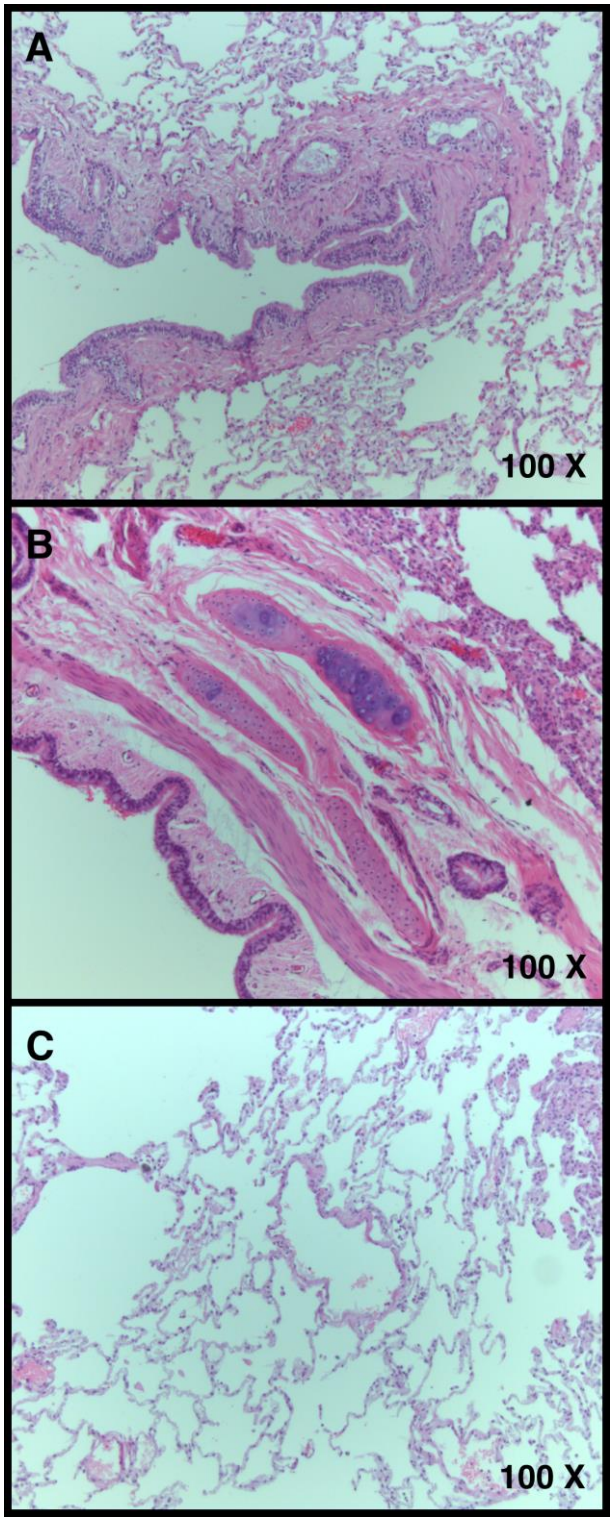

Figure 3: The figure shows the formalin-fixed paraffin embedded sections of lung tissue from the control dog, stained with haematoxylin & eosin, at different magnifications. Picture A shows a normal bronchiolar wall and alveolar structures. Picture B shows a normal bronchi wall. Picture C shows normal alveolar structures.

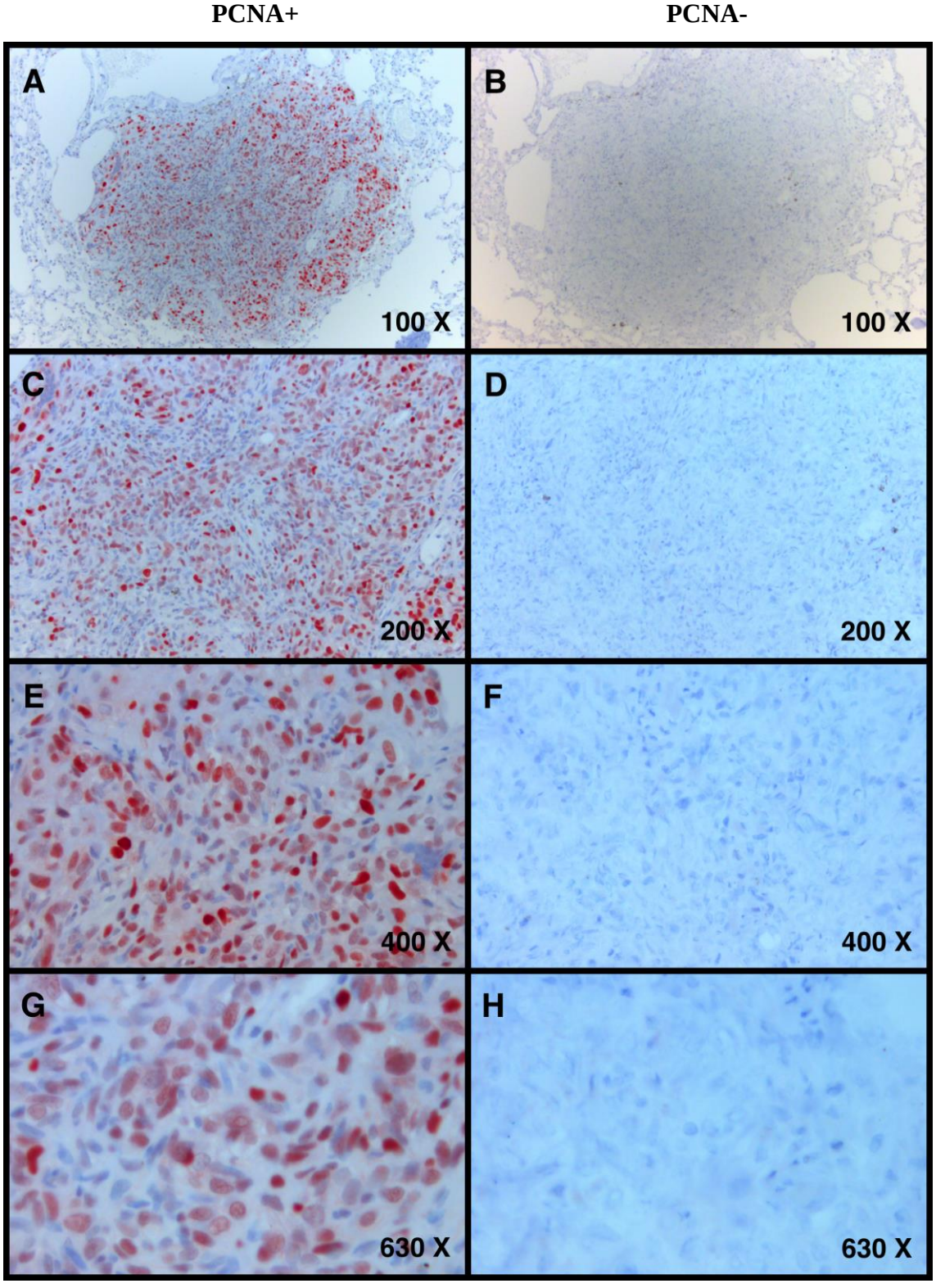

Figure 4: The figure shows the formalin-fixed paraffin embedded lung sections from the M+ case at different magnifications. Picture A, C, E and G show a metastatic lesion with positive staining following immunolabelling with anti-PCNA. Picture B, D, F and H show a metastatic lesion with no staining following preparation without anti-PCNA.

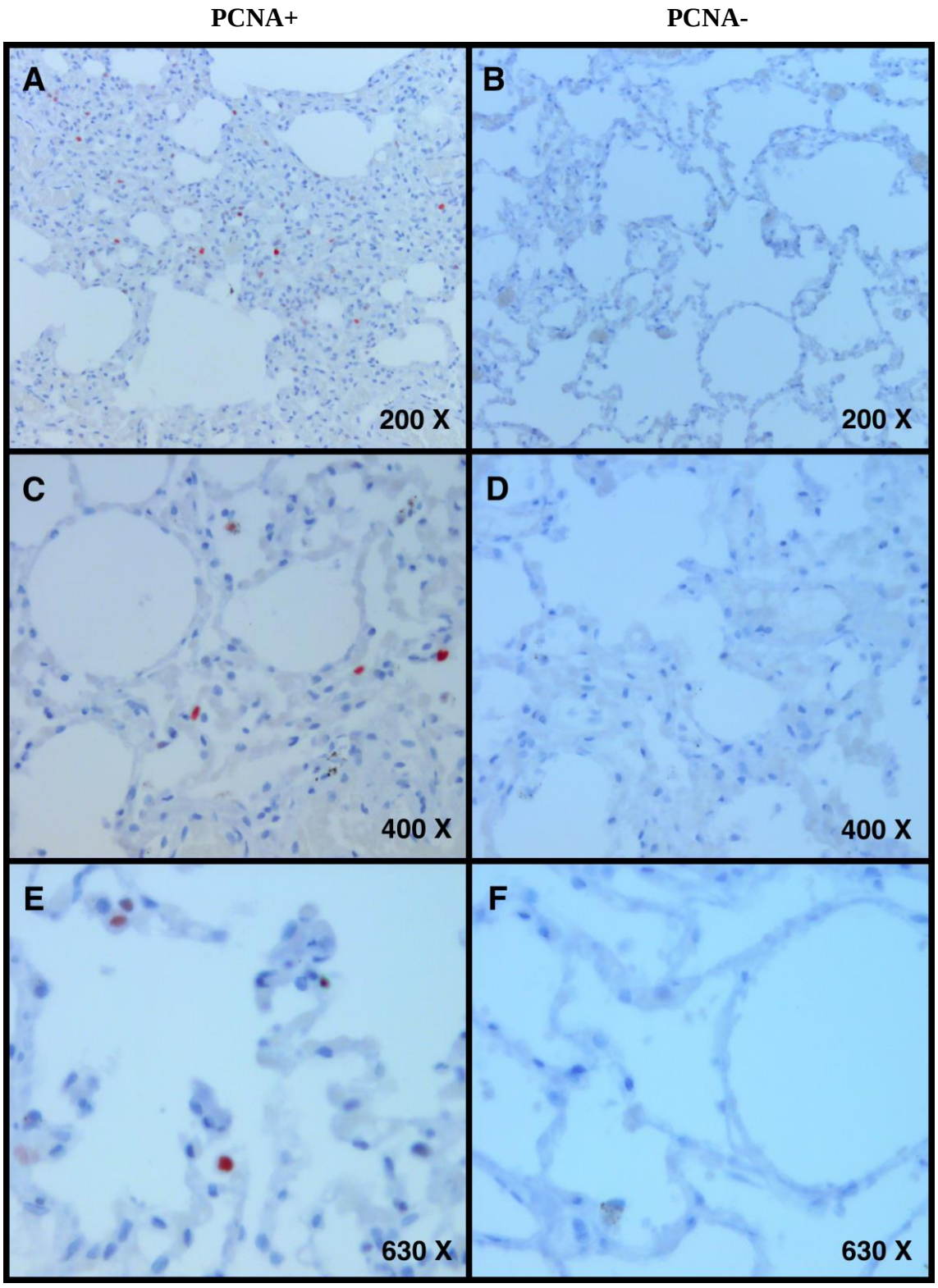

Figure 5: The figure shows the formalin-fixed paraffin embedded lung sections from the M+ case at different magnifications. Picture A, C and E show alveolar structures with positive staining of single cells following immunolabelling with anti-PCNA. Picture B, D and F show alveolar structures with no staining following preparation without anti-PCNA.

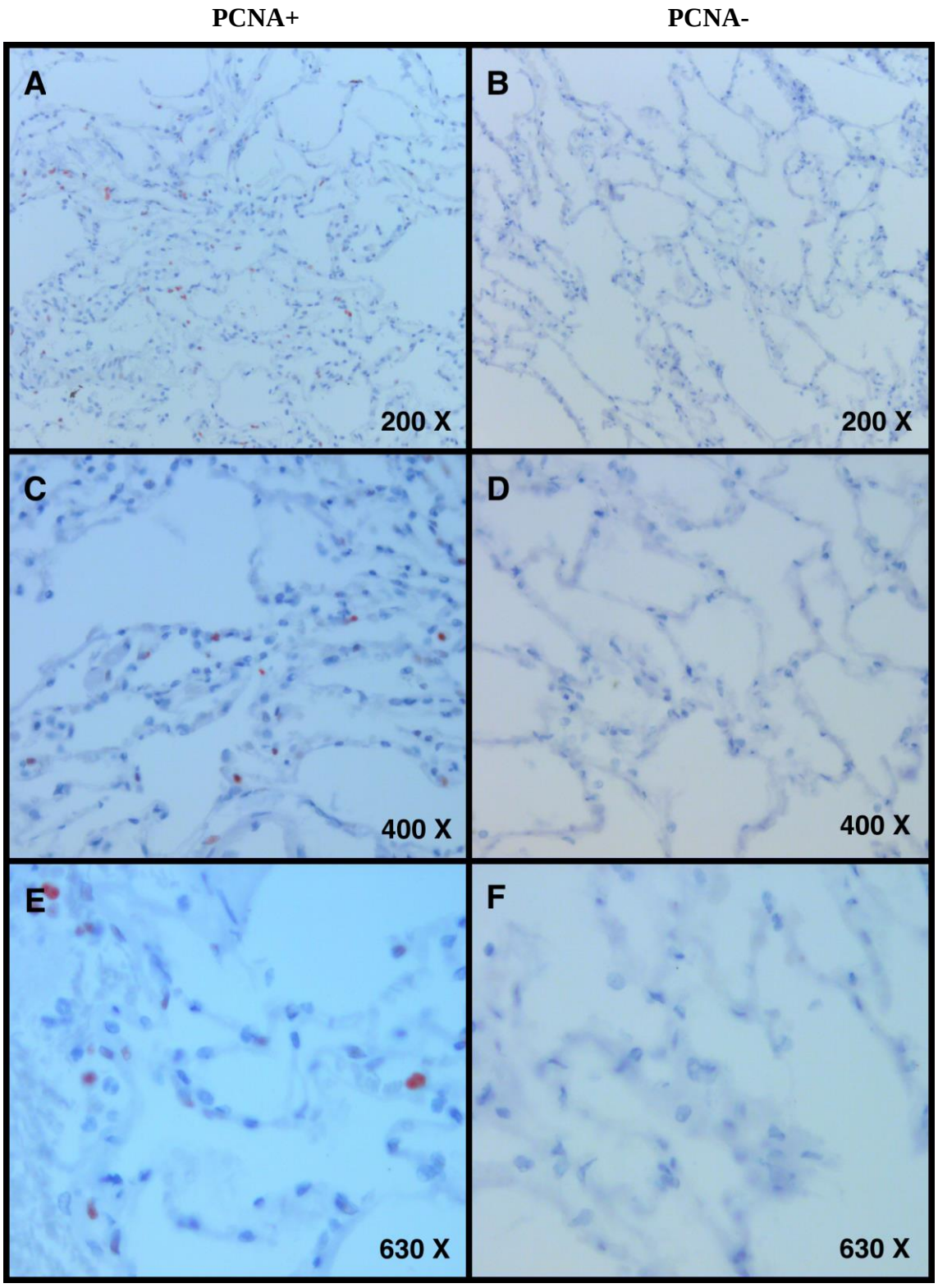

Figure 6: The figure shows the formalin-fixed paraffin embedded lung sections from the control dog at different magnifications. Picture A, C and E show alveolar structures with positive staining of single cells following immunolabelling with anti-PCNA. Picture B, D and F show alveolar structures with no staining following preparation without anti-PCNA.

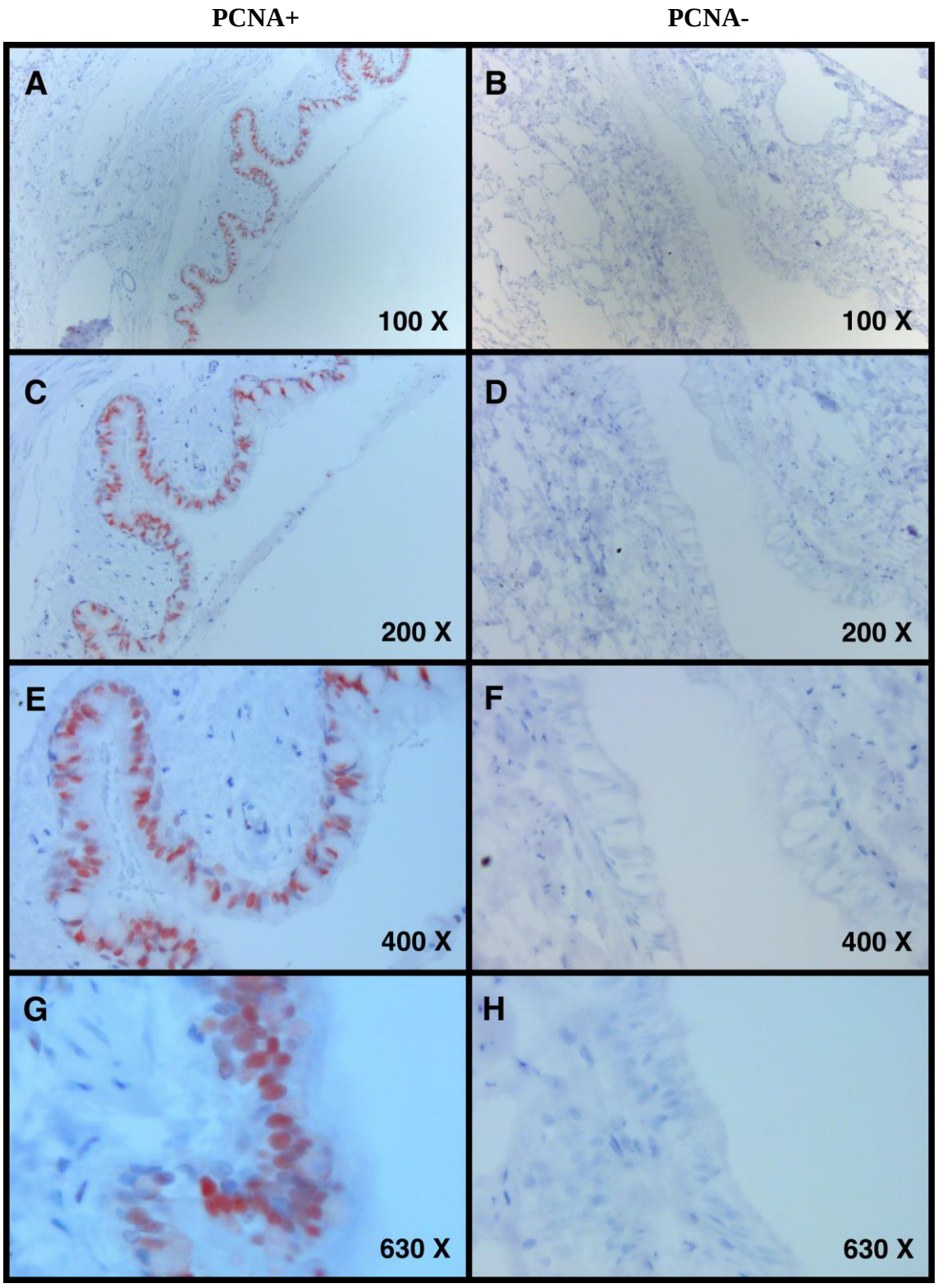

Figure 7: The figure shows the formalin-fixed paraffin embedded lung sections from the control dog at different magnifications. Picture A, C, E and G show columnar ciliated epithelium with positive staining following immunolabelling with anti-PCNA. Picture B, D, F and H show columnar ciliated epithelium with no staining following preparation without anti-PCNA.

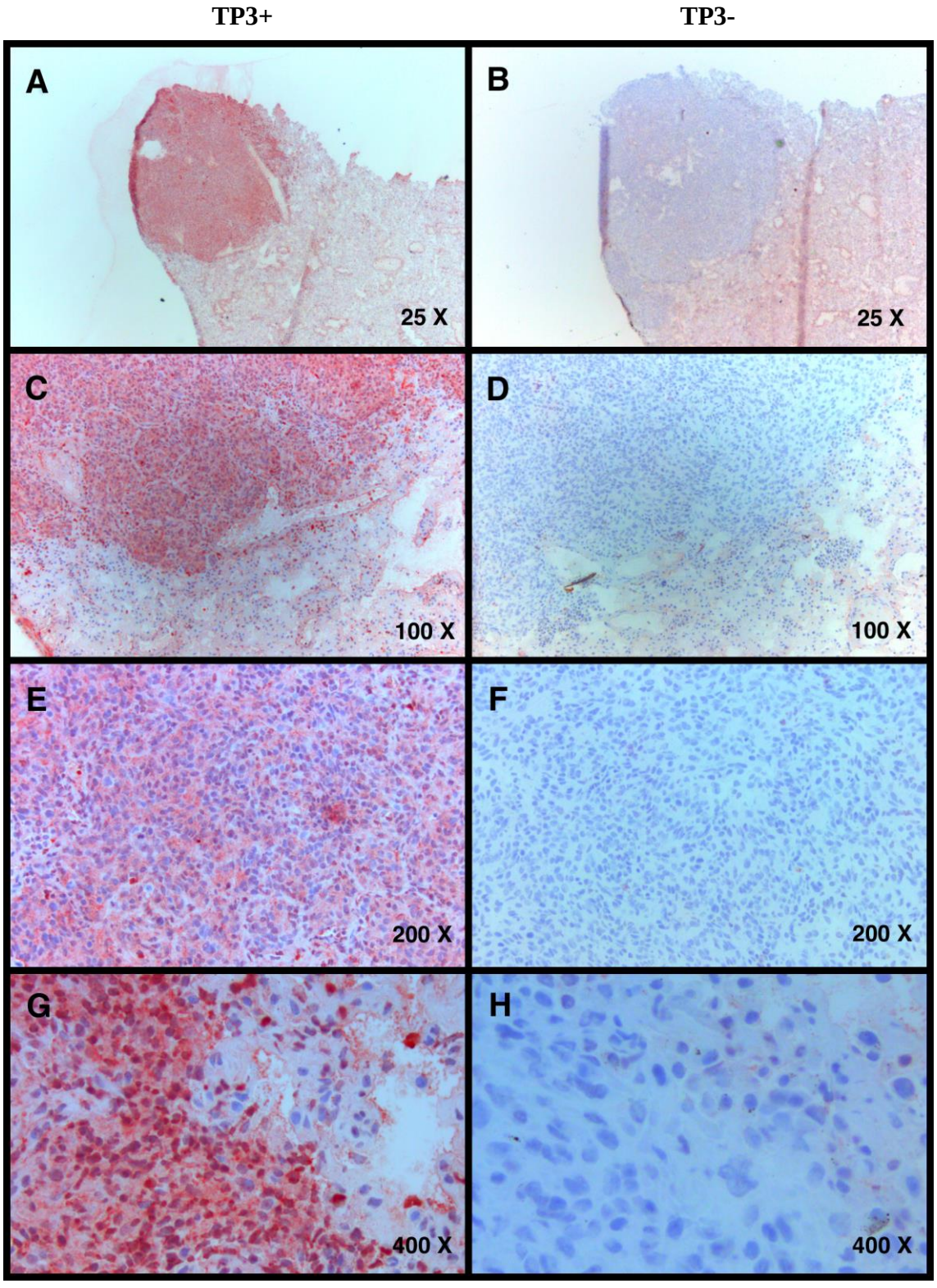

Figure 8: The figure shows the cryosections from the M+ case at different magnifications. Picture A, C, E and G show a metastatic lesion with positive staining following immunolabelling with TP3 antibodies. Picture B, D, F and H show a metastatic lesion with no staining following preparation without TP3 antibodies.

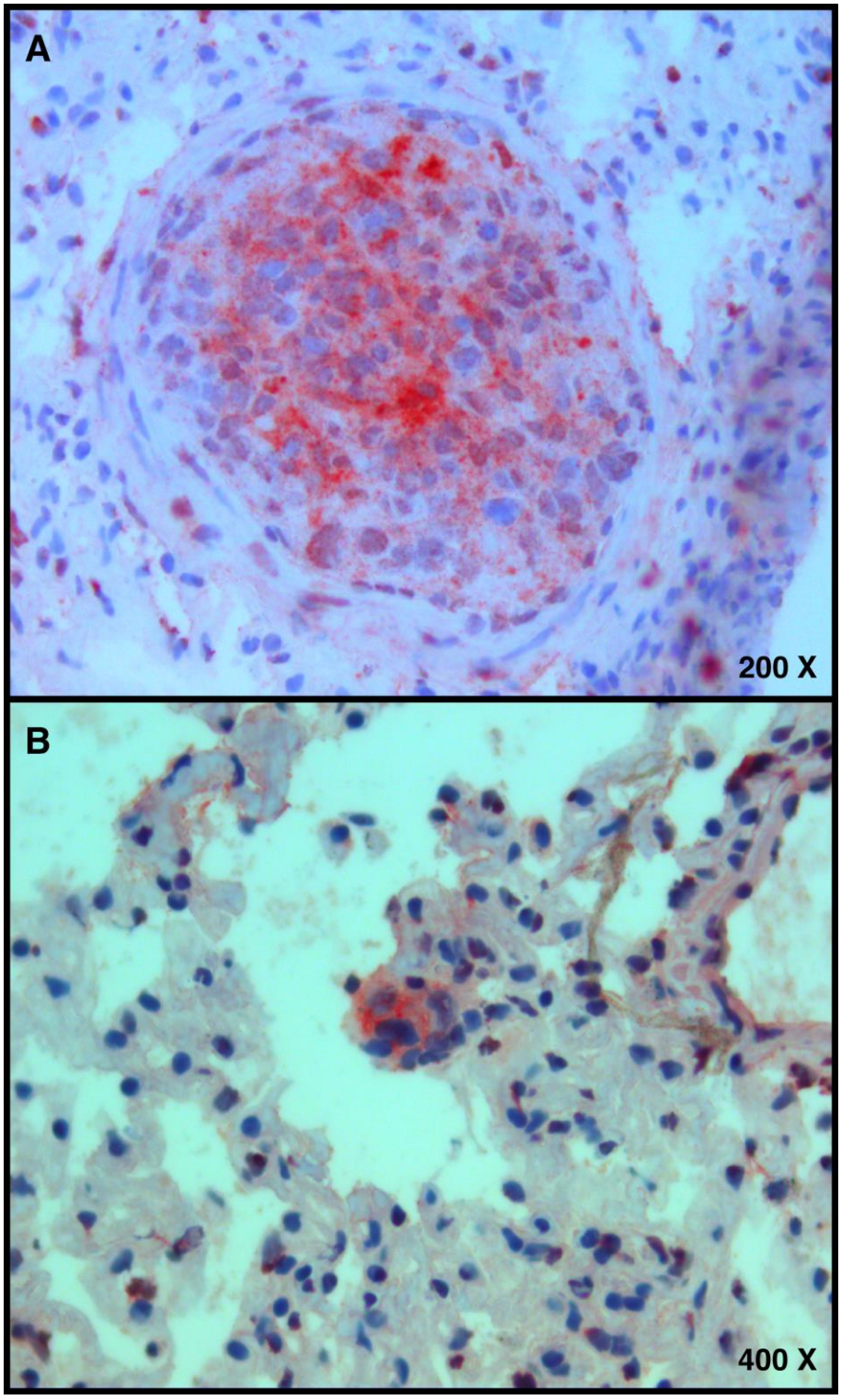

Figure 9: The figure shows the cryosections from the M+ case at different magnifications. Picture A show a small metastatic lesion with positive staining following immunolabelling with TP3 antibodies. Picture B show a small agglomeration of metastatic cells with positive staining following immunolabelling with TP3 antibodies.

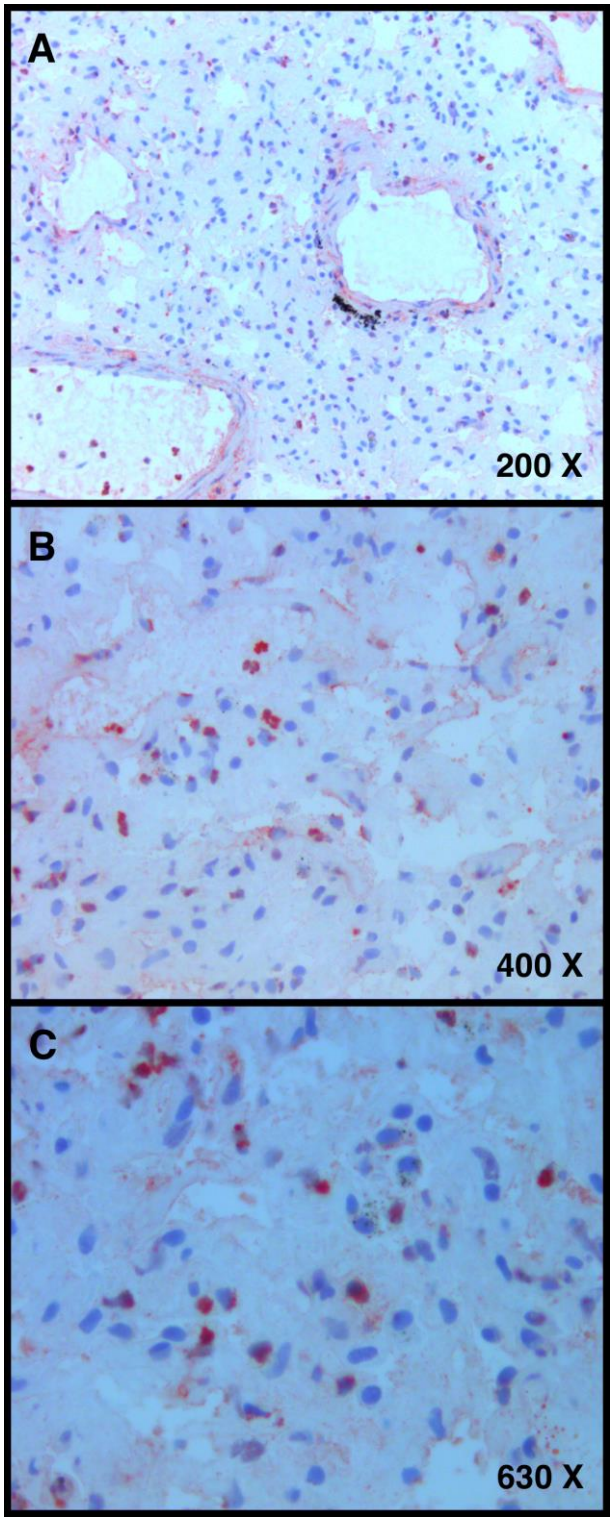

Figure 10: The figure shows the cryosections from the M+ case at different magnifications. Picture A, B and C show alveolar structures with positive staining of single cells following immunolabelling with TP3 antibodies.

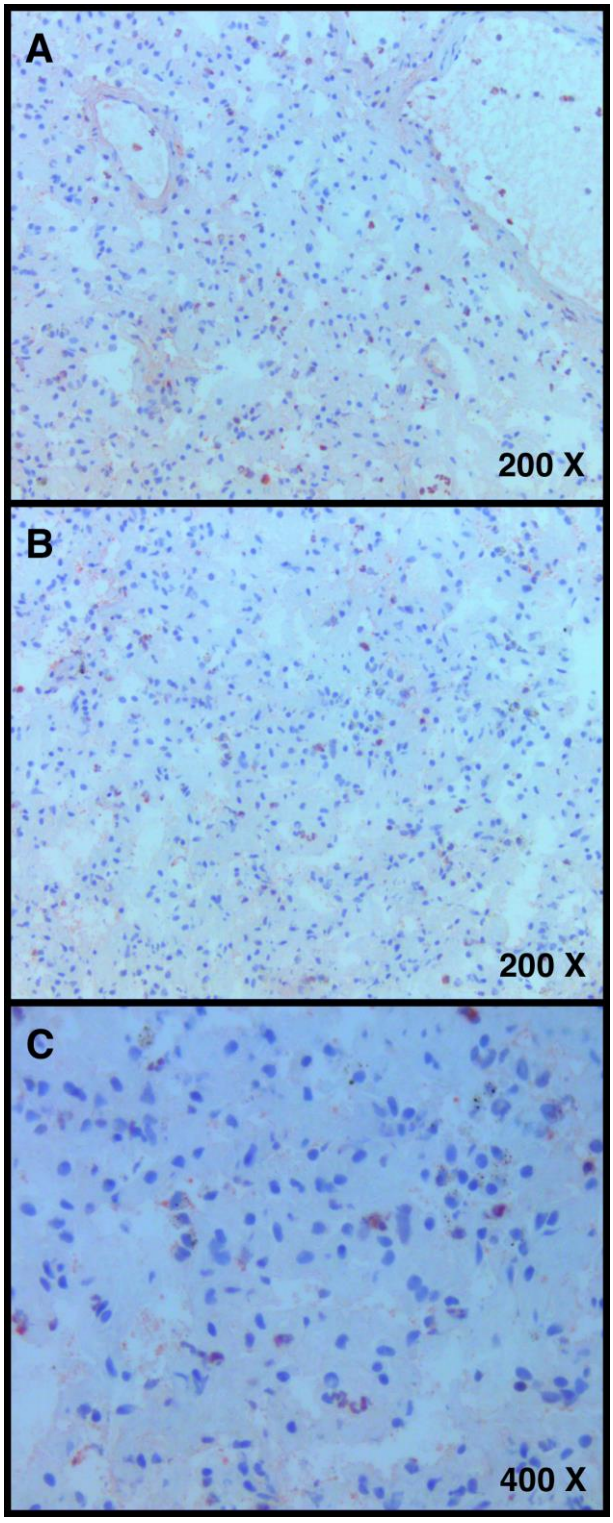

Figure 11: The figure shows the cryosections from the M+ case at different magnifications. Picture A, B and C show alveolar structures with positive staining of single cells following preparation without TP3 antibodies.

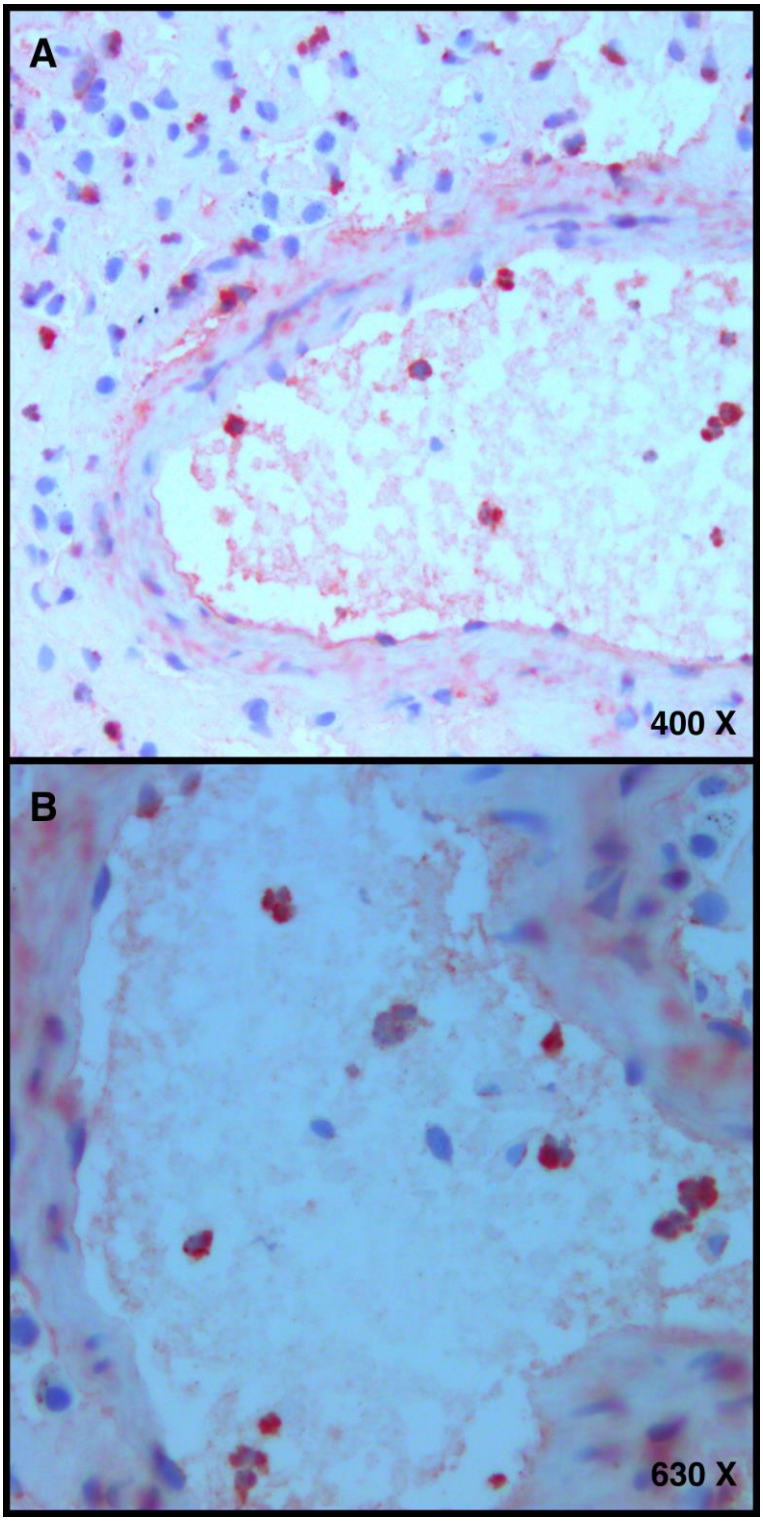

Figure 12: The figure shows the cryosections from the M+ case at different magnifications. Picture A shows endothelial cells in a pulmonary vessel with positive staining following immunolabelling with TP3 antibodies. Picture B shows cells in a pulmonary vessel with positive staining following immunolabelling with TP3 antibodies.

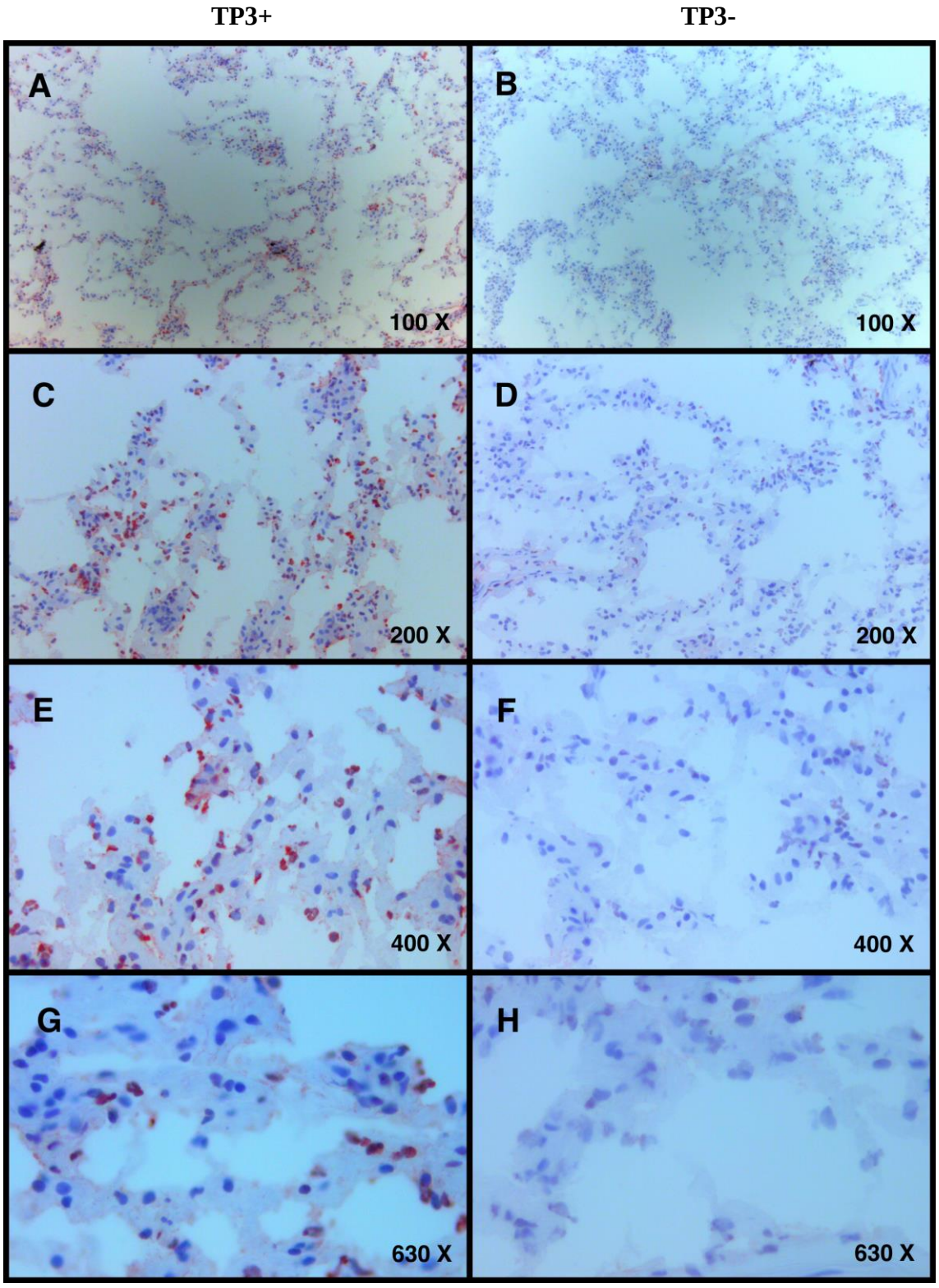

Figure 13: The figure shows the cryosections from the control dog at different magnifications. Picture A, C, E and G show alveolar structures with positive staining of single cells following immunolabelling with TP3 antibodies. Picture B, D, F and H show alveolar structures with no staining following preparation without TP3 antibodies.

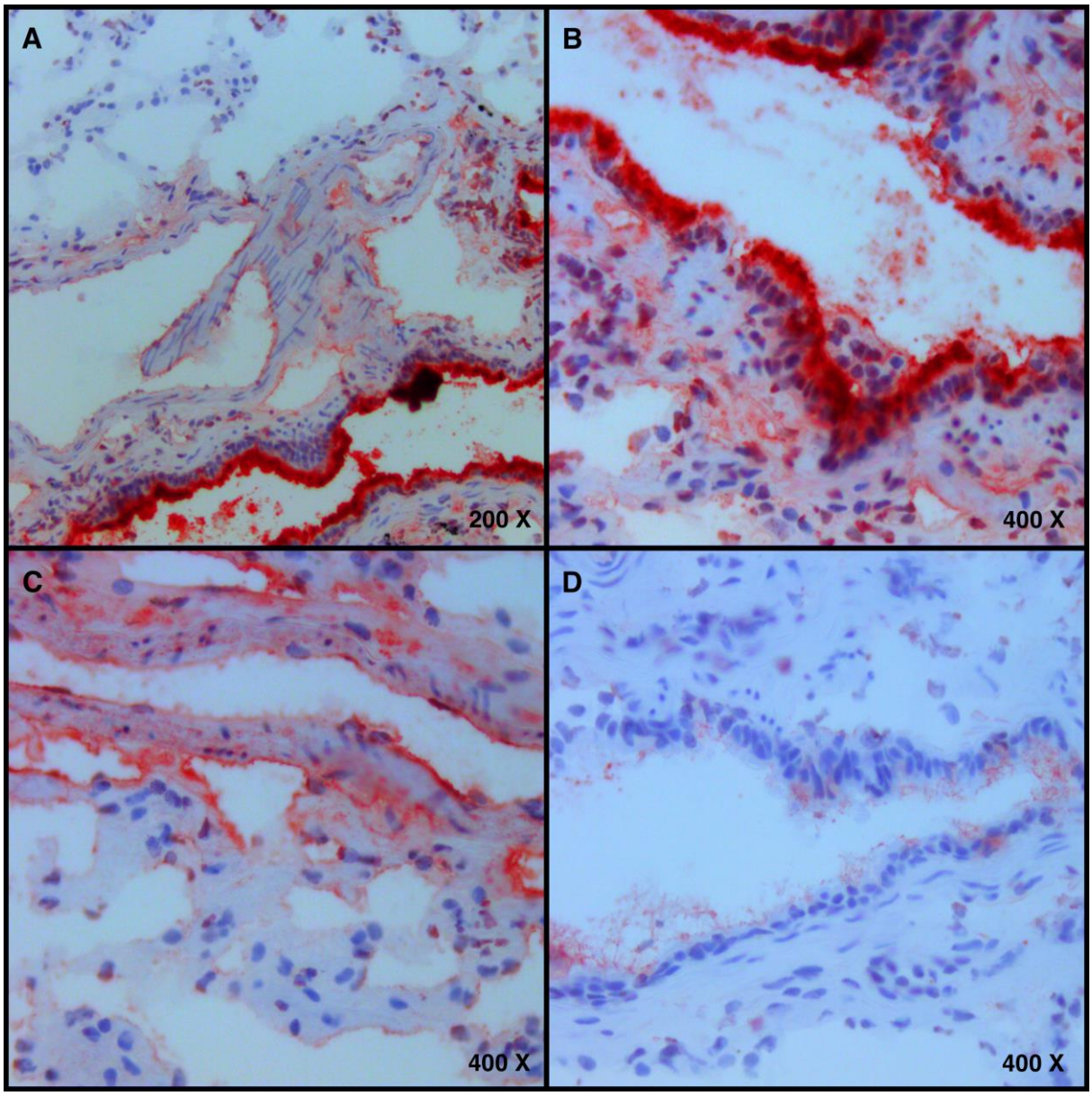

Figure 14: The figure shows the cryosections from the control dog at different magnifications. Picture A and B show a brush border of columnar ciliated epithelium with intense positive staining following immunolabelling with TP3 antibodies. Picture C shows endothelial cells in a pulmonary vessel with positive staining following immunolabelling with TP3 antibodies. Picture D shows the brush border of columnar ciliated epithelium with mild positive staining following preparation without TP3 antibodies.

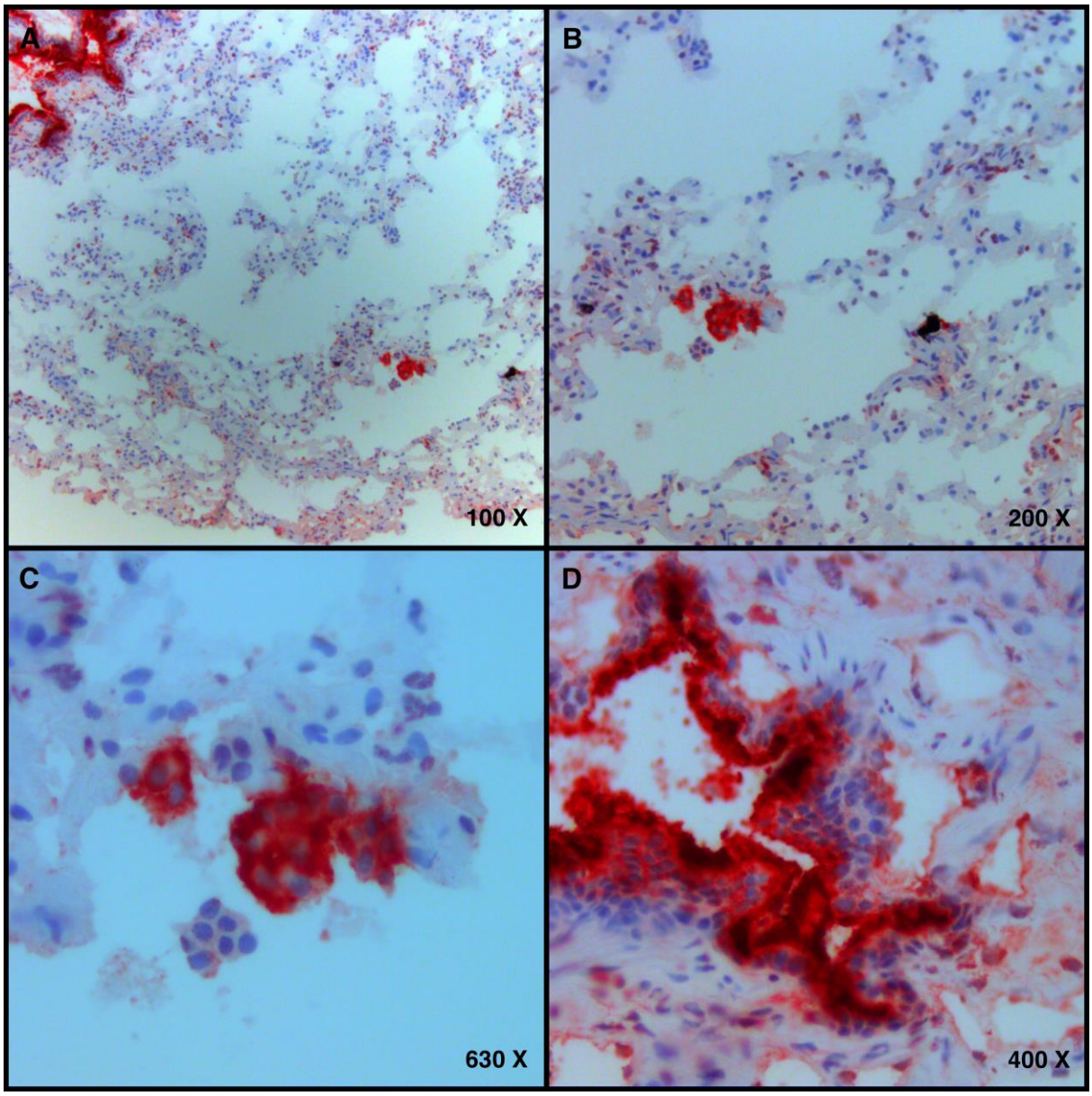

Figure 15: The figure shows the cryosections from the control dog at different magnifications. Picture A, B and C show two clusters of cells with intense positive staining following immunolabelling with TP3 antibodies. Picture D shows the brush border of columnar ciliated epithelium with intense positive staining following immunolabelling with TP3 antibodies, for comparison.

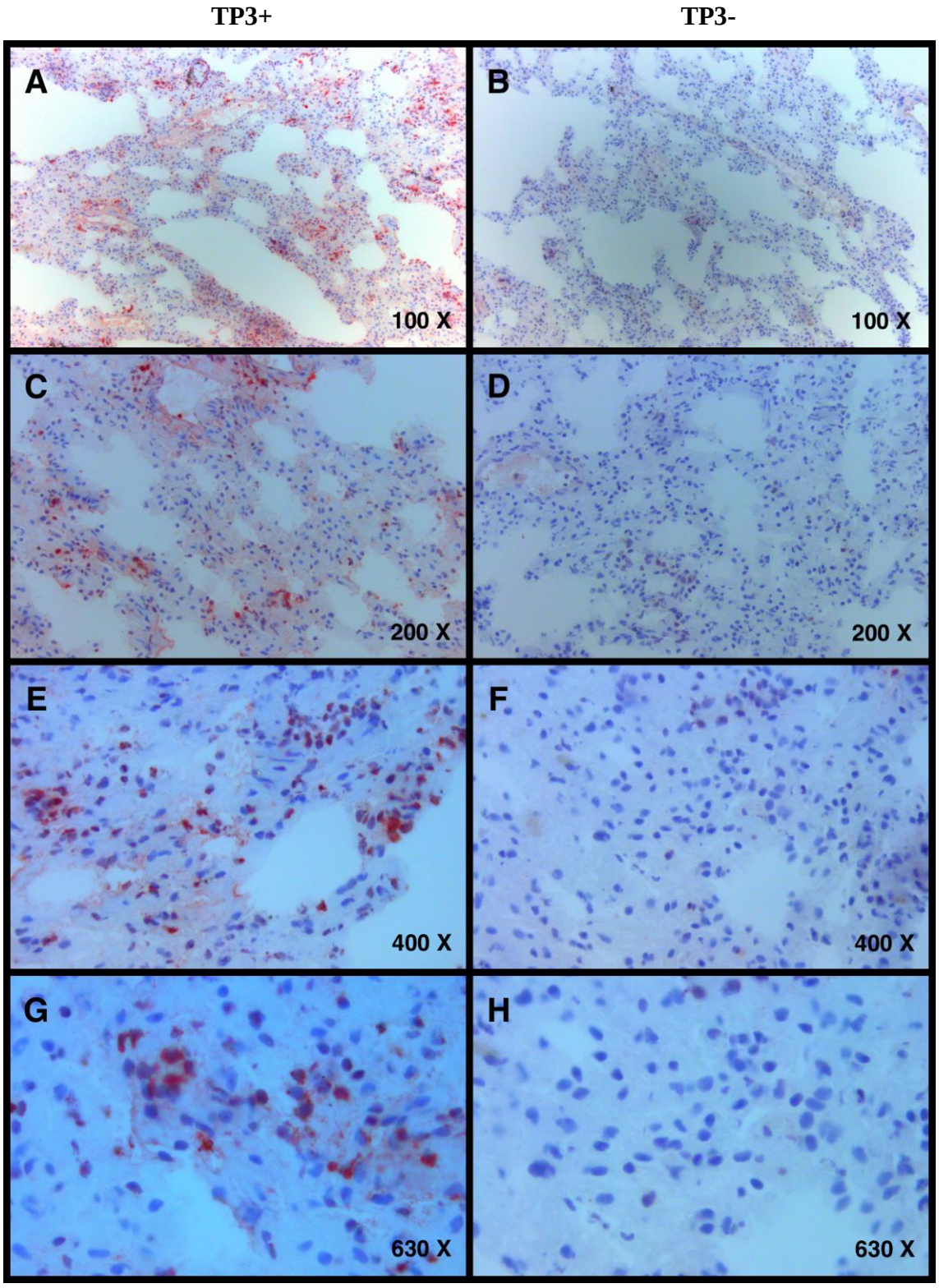

Figure 16: The figure shows the cryosections from the M- case at different magnifications. Picture A, C, E and G show alveolar structures with positive staining of single cells following immunolabelling with TP3 antibodies. Picture B, D, F and H show alveolar structures with no positive staining of single cells following preparation without TP3 antibodies.

Discussion

General

The pathogenesis of metastatic cancer is a complex process, one that has been intensely studied for several decades and that is not yet fully understood. The aim of our study was to assess the distribution of metastatic tumour cells in canine lung tissue and to acquire a better understanding of metastatic osteosarcoma, by comparing the early stages of the disease with more advanced stages. Furthermore, we wanted to assess the viability of this method to be used in the detection of early metastasis. This study was designed as a pilot study to address whether the tested immunohistochemical method could potentially increase sensitivity in detecting lung metastases, and if so, be used in testing a hypothesis on how osteosarcoma cells spread with high frequency to the lungs. Our material did not include many individuals and in order for our results to be generalizable and valid, further studies with a greater number of individuals are warranted.

PCNA

One dog with overt metastatic osteosarcoma and one control dog were used in our study to assess PCNA expression in lung tissue. No sections from the M- case were available for immunohistochemical investigation of PCNA expression. Immunohistochemical staining of FFPE lung tissue with anti-PCNA antibodies was an effective method to assess PCNA expression. Firstly, we found that tumour cells in metastatic lesions showed a high expression of PCNA. We also found that PCNA expression varied between tumour cells, as shown by a variation in staining intensity. This indicates that different tumour cells are in different stages of the cell cycle. Several studies have shown that a high PCNA expression in some canine

and human malignancies is linked to a more aggressive tumour phenotype, a greater potential for metastasis and a worse prognosis (113-116, 122). Anti-PCNA immunolabelling was shown in our study to be useful to visualize small metastatic lesions and that this method could potentially be more sensitive in detecting such lesions than routine histologic examination using HE-slides. We also found that some single cells outside metastatic lesions showed expression of PCNA, as shown by strong staining. The nature of these cells could not be established for sure, but based on cellular and nuclear morphology, they were not thought to represent tumour cells, however this cannot be said for sure. Furthermore, similar staining of single cells was observed in our control dog. Interestingly, the density of stained cells was subjectively higher than that of the M+ case, which further substantiates the claim that these cells were not tumour cells. We have no good explanation why the density of stained cells was higher in the control dog than in the M+ case. Whether the density was higher than normal in the control dog or lower than normal in the M+ case is unclear and further studies are warranted to establish normal levels of PCNA expression in canine lung tissue. Ciliated columnar epithelium in bronchioles in our control dog also showed expression of PCNA, as shown by positive staining. The expression of PCNA varied from cell to cell, which was demonstrated by a variation in staining intensity. This is probably due to the high cellular turnover seen in columnar epithelium, with different cells being in different stages of the cell cycle. Whether the level of PCNA expression observed in the bronchioles was within normal range could not be confirmed, due to the low number of samples included in our study and the lack of published studies evaluating the expression of PCNA in healthy canine lung tissue. In a study evaluating the expression of PCNA in lung tissue of healthy mice, it was shown that ciliated columnar epithelium of the bronchioles stained when immunolabelled with anti-PCNA antibodies (123). In the same study, it was shown that single cells in the alveolar septa in healthy lung tissue stained when immunolabelled with anti-PCNA

antibodies and that these cells were most often characterized as type II-pneumocytes. These findings are similar to those found in our study and may give support to the suspicion that these single cells do not represent tumour cells.

TP3

The expression of TP3 binding antigens was evaluated in lung tissue from the M+ and M- case, as well as from the control dog. Immunohistochemical staining of cryosections immunolabeled with TP3 antibodies was an effective method for assessing the expression of TP3 binding antigens in lung tissue. A trial immunohistochemical staining was performed on FFPE lung tissue. However, results were variable and resulted in significant background staining. In a study involving immunohistochemical staining of FFPE lung tissue from humans using TP3 antibodies, similar findings were made. It was concluded that formalin (formaldehyde) fixation for 5 minutes or longer, and temperatures above 56ºC for 5 minutes abolished antibody-epitope binding (94). Given that sections are exposed to formalin and high temperatures during the preparation of FFPE tissues, it was concluded that this was a poor method to evaluate the expression of TP3 binding antigens. Firstly, we found that tumour cells in metastatic lesions showed a high expression of TP3 binding antigens. Staining intensity was less variable between tumour cells, in comparison to PCNA staining. However, it was observed that cells in the periphery of metastatic lesions stained more intensely than those in the centre and that smaller metastatic lesions often stained more intensely. This variation in staining intensity corresponds to a variation in the expression of TP3 binding antigens, where tumour cells in the periphery of larger metastatic lesions and smaller metastatic lesions have a higher expression of TP3 binding antigens. Furthermore, immunolabelling with TP3 antibodies was useful to visualize small metastatic lesions, and this method could potentially be more sensitive in detecting such lesions than routine

histologic examination using HE-slides. We also found that some single cells in the alveolar septa, alveolar lumen and vascular lumen expressed TP3 binding antigens, as demonstrated by staining following immunolabelling with TP3 antibodies. These cells could not always be characterized, however some of them were classified as neutrophils, lymphocytes and alveolar macrophages, based on cellular and nuclear morphology. None of these cells were considered to be tumour cells. These single cells were present in all slides immunolabelled with TP3 antibodies, in the M+ and M- case, as well as in the control dog. Staining of neutrophils, lymphocytes and macrophages was not evident in slides prepared without TP3 antibodies, demonstrating that staining is a consequence of antibody binding. This can be explained either by the fact that the TP3 antibodies bind to more than one epitope, making it an unspecific antibody, or by the fact that the antigens to which TP3 binds are expressed on these cells as well. This observation has not been made in earlier reports when TP3 antibodies were first evaluated (94, 96). They found that certain normal tissues stained when immunolabelled with TP3 antibodies (Table 2), similarly to what we found, however they did not report staining in leukocytes. In the slides from the M+ case, some staining was observed following preparation without TP3 antibodies. This staining was not a result of antibody-antigen binding and we believe that this staining was a result of endogenous peroxidase reactivity. The small agglomeration of intensely stained cells found in one of the slides from the control dog was believed to be a preparation artefact due to dislodgment of ciliated columnar epithelial cells from one of the bronchioles. No similar findings were found in any of the other slides, nor were there any reason to believe that the control dog had any metastatic disease based on the autopsy and routine histology examination. The density of staining single cells in the parenchyma showed little variation between the dogs included in the study. There is little reason to believe that these single cells represent tumour cells, however this cannot be ruled out based on our findings alone. In order to further assess the

nature of these cells, studies using double staining techniques are warranted. This could be accomplished by using other tumour markers, such as the mesenchymal cell marker vimentin or specific alkaline phosphatase staining (55, 124). Staining against specific immune cells, such as CD3 against T-lymphocytes, CD79a or CD20 against B-lymphocytes and major histocompatibility complex (MHC) class II against antigen presenting cells could be used to classify the different mononuclear cells (125-127). This would make it possible to subtract potential staining leukocytes and evaluate the remaining cells.

Distribution of metastatic tumour cells

As previously mentioned, tumour cells in metastatic lesions showed a high expression of both PCNA and TP3 binding antigens. The distribution of metastases did not follow a particular pattern and they seemed to be randomly distributed throughout the lung tissue. As previously discussed, the nature of the staining single cells that were present in all three dogs could not be determined with certainty. Based on our observations, these cells most likely do not represent tumour cells, due to their morphology and their presence in the control dog. Hence, our observations do not support the idea that the lungs are colonized with tumour cells before macroscopic metastases occur. Whether this finding does in fact reflect the pathogenesis for all cases of osteosarcoma cannot be concluded. This may be due to multiple factors. Firstly, it may be due to limitations of the current method as a tool in detecting early metastatic single cells, due to unspecific staining of non-tumour cells. Secondly, the presence of metastatic single cells may vary depending on what point in the timeline of the disease process the lung samples were harvested. Lastly, it may be that the idea of single cell metastasis to the lungs is in fact not true. This then leads us to the following paradigm: Where do the tumour cells seek shelter if they are not present in the lungs before visible metastases occur, given the fact that most dogs will develop pulmonary metastases, despite surgical

removal of the primary tumour? In an unpublished study where bone marrow samples from dogs with osteosarcoma were evaluated for tumour cells with TP3 antibodies, it was found that some dogs without visible pulmonary metastases had metastatic osteosarcoma cells present in the bone marrow. Our osteosarcoma case without visible metastases was evaluated in the previously mentioned study and it was shown that tumour cells were present in the bone marrow. This could suggest that tumour cells redistribute from the bone marrow to the lungs, which could explain why metastases develop even after the removal of the primary tumour.

Conclusion

We have shown in our study that anti-PCNA and TP3 antibodies bind to tumour cells and that this method could potentially be more sensitive in detecting such lesions than routine histologic examination using HE-slides. Based on our findings, we cannot conclude whether single cell metastases are present in the early stages of osteosarcoma without metastatic disease. We also found that TP3 antibodies bind to antigens on certain leukocytes, which has not been reported earlier.

This study was designed as a pilot study and we believe further investigations are warranted to validate our results in regard to the distribution of metastatic osteosarcoma cells and to investigate the potential for TP3 to be used as a marker for early metastatic disease, as well as a tool to investigate the presence of tumour cells in other tissues.

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**The patent for the TP3 antibodies has now been transferred to Oncoinvent AS.*

Norwegian summary

Tittel: Distribusjonen av lungemetastaser hos hunder med osteosarkom

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Veiledere: Professor Lars Moe, Institutt for sports- og familiedyrmedisin

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NMBU

Osteosarkom er en alvorlig lidelse som forekommer hos både mennesker og hunder. På tross av aggressiv behandling vil de fleste hunder dø kort tid etter at sykdommen er diagnostisert og fortsatt overlever bare halvparten av humane kasus. Flere molekylære og genetiske faktorer er satt i sammenheng med patogenesen ved metastatisk sykdom, og bruk av disse innen diagnostikk har gjort det enklere å kunne forutsi utfallet for pasienten. Mange av disse faktorene har også blitt vurdert som hjelpemiddel for valg av terapi. Det er vist at immunohistokjemi er et effektivt, men komplisert verktøy for å studere protein-uttrykk i ulike vev. For å bedre kunne forstå patogenesen i den tidlige fasen av metastatisk sykdom har vi undersøkt forekomsten av Tumour Protein 3 (TP3)-bindende antigener og Proliferating Cell Nuclear Antigen (PCNA) ved bruk av immunohistokjemi på lungevev fra hunder som ikke hadde postmortelle forandringer. Hundene som ble inkludert i denne studien omfattet en frisk kontrollhund (Dalmatiner, hannhund, 8 år) og to hunder med osteosarkom. En av hundene med osteosarkom (Rottweiler, hannhund, 9 år) hadde metastatisk sykdom, mens den andre (Leonberger, hannhund, 6 år) var fri for synlige metastaser. Hundene i studien ble klassifisert basert på obduksjonsfunn og histopatologisk undersøkelse. Uttrykket av PCNA ble evaluert hos den friske kontrollen, og hos hunden med osteosarkom og synlige metastaser. Uttrykket av TP3-bindene antigener ble evaluert hos alle tre hundene i studien. I vår studie fant vi at

tumorcellene i lungemetastaser uttrykte både TP3-bindene antigener og PCNA. Vi fant også at bruk av TP3 og anti-PCNA i forbindelse med immunohistokjemi var et nyttig verktøy for å påvise små lungemetastaser, og at denne metoden potensielt kan være en mer sensitiv for å detektere slike lesjoner enn rutinemessige histologiske metoder ved bruk av hematoxylin & eosin. Enkeltceller i lungevevet viste også uttrykk av TP3-binende antigener hos alle hundene i studien. Hvilken cellelinje disse tilhørte kunne ikke bestemmes sikkert, men enkelte ble karakterisert som leukocytter. Dette funnet viste at TP3-antistoffer binder antigener på leukocytter, noe som ikke er vist tidligere. For å kunne si med sikkerhet om noen av de øvrige enkeltcellene representer tumorceller, trenger vi videre undersøkelser med dobbeltfarging. Vi mener videre at det er nødvendig med flere studier med fokus på ekstrapulmonær distribusjon av tumor-celler, og deres potensiale for å redistribuere til lungene, og undersøke et større antall lungevevsprøver fra de undersøkte hundene.

References

1. Szewczyk M, Lechowski R, Zabielska K. What do we know about canine osteosarcoma treatment? Review. Vet Res Commun. 2015;39(1):61-7.

2. Morello E, Martano M, Buracco P. Biology, diagnosis and treatment of canine appendicular osteosarcoma: similarities and differences with human osteosarcoma. Veterinary journal (London, England : 1997). 2011;189(3):268-77.

3. Spodnick GJ, Berg J, Rand WM, Schelling SH, Couto G, Harvey HJ, et al. Prognosis for dogs with appendicular osteosarcoma treated by amputation alone: 162 cases (1978-1988). J Am Vet Med Assoc. 1992;200(7):995-9.

4. Selvarajah GT, Kirpensteijn J. Prognostic and predictive biomarkers of canine osteosarcoma. Veterinary journal (London, England : 1997). 2010;185(1):28-35.

5. Trost ME, Kommers GD, Brown CC, Barros CSL, Irigoyen LF, Fighera RA, et al. Primary bone neoplasms in dogs: 90 cases. Pesquisa Veterinária Brasileira. 2012;32:1329-35.

6. Cavalcanti JN, Amstalden EMI, Guerra JL, Magna LC. Osteosarcoma in dogs: clinical-morphological study and prognostic correlation. Brazilian Journal of Veterinary Research and Animal Science. 2004;41:299-305.

7. Knecht CD, Priester WA. Musculoskeletal tumors in dogs. J Am Vet Med Assoc. 1978;172(1):72-4.

8. Brodey RS, Riser WH. Canine osteosarcoma. A clinicopathologic study of 194 cases. Clin Orthop Relat Res. 1969;62:54-64.

9. Langenbach A, Anderson MA, Dambach DM, Sorenmo KU, Shofer FD. Extraskeletal osteosarcomas in dogs: a retrospective study of 169 cases (1986-1996). Journal of the American Animal Hospital Association. 1998;34(2):113-20.

10. Heyman SJ, Diefenderfer DL, Goldschmidt MH, Newton CD. Canine axial skeletal osteosarcoma. A retrospective study of 116 cases (1986 to 1989). Veterinary surgery : VS. 1992;21(4):304-10.

11. Kuntz CA, Dernell WS, Powers BE, Withrow S. Extraskeletal osteosarcomas in dogs: 14 cases. Journal of the American Animal Hospital Association. 1998;34(1):26-30.

12. Patnaik AK. Canine extraskeletal osteosarcoma and chondrosarcoma: a clinicopathologic study of 14 cases. Vet Pathol. 1990;27(1):46-55.

13. Ru G, Terracini B, Glickman LT. Host related risk factors for canine osteosarcoma. Veterinary journal (London, England : 1997). 1998;156(1):31-9.

14. Bergman PJ, MacEwen EG, Kurzman ID, Henry CJ, Hammer AS, Knapp DW, et al. Amputation and carboplatin for treatment of dogs with osteosarcoma: 48 cases (1991 to 1993). J Vet Intern Med. 1996;10(2):76-81.

15. Gellasch KL, Kalscheur VL, Clayton MK, Muir P. Fatigue microdamage in the radial predilection site for osteosarcoma in dogs. American Journal of Veterinary Research. 2002;63(6):896-9.

16. Brodey RS, Abt DA. Results of surgical treatment in 65 dogs with osteosarcoma. J Am Vet Med Assoc. 1976;168(11):1032-5.

17. Cooley DM, Beranek BC, Schlittler DL, Glickman NW, Glickman LT, Waters DJ. Endogenous gonadal hormone exposure and bone sarcoma risk. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2002;11(11):1434-40.

18. Bennett D, Campbell JR, Brown P. Osteosarcoma associated with healed fractures. J Small Anim Pract. 1979;20(1):13-8.

19. Sinibaldi K, Rosen H, Liu SK, DeAngelis M. Tumors associated with metallic implants in animals. Clin Orthop Relat Res. 1976(118):257-66.

20. Stevenson S, Hohn RB, Pohler OE, Fetter AW, Olmstead ML, Wind AP. Fracture-associated sarcoma in the dog. J Am Vet Med Assoc. 1982;180(10):1189-96.

21. Vasseur PB, Stevenson S. Osteosarcoma at the site of a cortical bone allograft in a dog. Veterinary surgery : VS. 1987;16(1):70-4.

22. Burton AG, Johnson EG, Vernau W, Murphy BG. Implant-associated neoplasia in dogs: 16 cases (1983-2013). J Am Vet Med Assoc. 2015;247(7):778-85.

23. Gillette SM, Gillette EL, Powers BE, Withrow SJ. Radiation-induced osteosarcoma in dogs after external beam or intraoperative radiation therapy. Cancer Res. 1990;50(1):54-7.

24. White RG, Raabe OG, Culbertson MR, Parks NJ, Samuels SJ, Rosenblatt LS. Bone Sarcoma Characteristics and Distribution in Beagles Fed Strontium-90. Radiation Research. 1993;136(2):178-89.

25. White RG, Raabe OG, Culbertson MR, Parks NJ, Samuels SJ, Rosenblatt LS. Bone sarcoma characteristics and distribution in beagles injected with radium-226. Radiat Res. 1994;137(3):361-70.

26. Lloyd RD, Taylor GN, Angus W, Miller SC, Bruenger FW, Jee WS. Distribution of skeletal malignancies in beagles injected with 239Pu citrate. Health physics. 1994;66(4):407-13.

27. Miller SC, Lloyd RD, Bruenger FW, Krahenbuhl MP, Polig E, Romanov SA. Comparisons of the Skeletal Locations of Putative Plutonium-Induced Osteosarcomas in Humans with those in Beagle Dogs and with Naturally Occurring Tumors in both Species. Radiation Research. 2003;160(5):517-23.

28. McEntee MC, Page RL, Theon A, Erb HN, Thrall DE. Malignant tumor formation in dogs previously irradiated for acanthomatous epulis. Vet Radiol Ultrasound. 2004;45(4):357-61.

29. Johnson AS, Couto CG, Weghorst CM. Mutation of the p53 tumor suppressor gene in spontaneously occurring osteosarcomas of the dog. Carcinogenesis. 1998;19(1):213-7.

30. Kirpensteijn J, Kik M, Teske E, Rutteman GR. TP53 gene mutations in canine osteosarcoma. Veterinary surgery : VS. 2008;37(5):454-60.

31. Levine RA, Fleischli MA. Inactivation of p53 and retinoblastoma family pathways in canine osteosarcoma cell lines. Vet Pathol. 2000;37(1):54-61.

32. Loukopoulos P, Thornton JR, Robinson WF. Clinical and pathologic relevance of p53 index in canine osseous tumors. Vet Pathol. 2003;40(3):237-48.

33. Mendoza S, Konishi T, Dernell WS, Withrow SJ, Miller CW. Status of the p53, Rb and MDM2 genes in canine osteosarcoma. Anticancer Res. 1998;18(6a):4449-53.

34. Setoguchi A, Sakai T, Okuda M, Minehata K, Yazawa M, Ishizaka T, et al. Aberrations of the p53 tumor suppressor gene in various tumors in dogs. Am J Vet Res. 2001;62(3):433-9.

35. Sagartz JE, Bodley WL, Gamblin RM, Couto CG, Tierney LA, Capen CC. p53 tumor suppressor protein overexpression in osteogenic tumors of dogs. Vet Pathol. 1996;33(2):213-21.

36. Wadayama B, Toguchida J, Shimizu T, Ishizaki K, Sasaki MS, Kotoura Y, et al. Mutation spectrum of the retinoblastoma gene in osteosarcomas. Cancer Res. 1994;54(11):3042-8.

37. Thomas R, Wang HJ, Tsai PC, Langford CF, Fosmire SP, Jubala CM, et al. Influence of genetic background on tumor karyotypes: evidence for breed-associated cytogenetic aberrations in canine appendicular osteosarcoma. Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology. 2009;17(3):365-77.

38. Levine RA, Forest T, Smith C. Tumor suppressor PTEN is mutated in canine osteosarcoma cell lines and tumors. Vet Pathol. 2002;39(3):372-8.

39. Angstadt AY, Motsinger-Reif A, Thomas R, Kisseberth WC, Guillermo Couto C, Duval DL, et al. Characterization of canine osteosarcoma by array comparative genomic hybridization and RT-qPCR: signatures of genomic imbalance in canine osteosarcoma parallel the human counterpart. Genes, chromosomes & cancer. 2011;50(11):859-74.

40. Liao AT, McMahon M, London C. Characterization, expression and function of c-Met in canine spontaneous cancers. Vet Comp Oncol. 2005;3(2):61-72.

41. MacEwen EG, Kutzke J, Carew J, Pastor J, Schmidt JA, Tsan R, et al. c-Met tyrosine kinase receptor expression and function in human and canine osteosarcoma cells. Clin Exp Metastasis. 2003;20(5):421-30.

42. Ferracini R, Angelini P, Cagliero E, Linari A, Martano M, Wunder J, et al. MET oncogene aberrant expression in canine osteosarcoma. J Orthop Res. 2000;18(2):253-6.

43. Fieten H, Spee B, Ijzer J, Kik MJ, Penning LC, Kirpensteijn J. Expression of hepatocyte growth factor and the proto-oncogenic receptor c-Met in canine osteosarcoma. Vet Pathol. 2009;46(5):869-77.

44. Fan TM, Barger AM, Sprandel IT, Fredrickson RL. Investigating TrkA expression in canine appendicular osteosarcoma. J Vet Intern Med. 2008;22(5):1181-8.

45. Flint AF, U'Ren L, Legare ME, Withrow SJ, Dernell W, Hanneman WH. Overexpression of the erbB-2 proto-oncogene in canine osteosarcoma cell lines and tumors. Vet Pathol. 2004;41(3):291-6.

46. Gordon IK, Ye F, Kent MS. Evaluation of the mammalian target of rapamycin pathway and the effect of rapamycin on target expression and cellular proliferation in osteosarcoma cells from dogs. Am J Vet Res. 2008;69(8):1079-84.

47. Kow K, Thamm DH, Terry J, Grunerud K, Bailey SM, Withrow SJ, et al. Impact of telomerase status on canine osteosarcoma patients. J Vet Intern Med. 2008;22(6):1366-72.

48. MacEwen EG, Pastor J, Kutzke J, Tsan R, Kurzman ID, Thamm DH, et al. IGF-1 receptor contributes to the malignant phenotype in human and canine osteosarcoma. J Cell Biochem. 2004;92(1):77-91.

49. Withrow S. J. VDM, Page R. L. Tumors of the skeletal system. Small Animal Clinical Oncology. 1. 5th ed. Missouri Elsevier Saunders; 2013. p. 463-503.

50. Hillers KR, Dernell WS, Lafferty MH, Withrow SJ, Lana SE. Incidence and prognostic importance of lymph node metastases in dogs with appendicular osteosarcoma: 228 cases (1986-2003). J Am Vet Med Assoc. 2005;226(8):1364-7.

51. Boston SE, Ehrhart NP, Dernell WS, Lafferty M, Withrow SJ. Evaluation of survival time in dogs with stage III osteosarcoma that undergo treatment: 90 cases (1985-2004). J Am Vet Med Assoc. 2006;228(12):1905-8.

52. Straw RC, Powers BE, Klausner J, Henderson RA, Morrison WB, McCaw DL, et al. Canine mandibular osteosarcoma: 51 cases (1980-1992). Journal of the American Animal Hospital Association. 1996;32(3):257-62.

53. Dickerson ME, Page RL, LaDue TA, Hauck ML, Thrall DE, Stebbins ME, et al. Retrospective analysis of axial skeleton osteosarcoma in 22 large-breed dogs. J Vet Intern Med. 2001;15(2):120-4.

54. Mehl ML, Withrow SJ, Seguin B, Powers BE, Dernell WS, Pardo AD, et al. Spontaneous regression of osteosarcoma in four dogs. J Am Vet Med Assoc. 2001;219(5):614-7.

55. Barger A, Graca R, Bailey K, Messick J, de Lorimier LP, Fan T, et al. Use of alkaline phosphatase staining to differentiate canine osteosarcoma from other vimentin-positive tumors. Vet Pathol. 2005;42(2):161-5.

56. Britt T, Clifford C, Barger A, Moroff S, Drobatz K, Thacher C, et al. Diagnosing appendicular osteosarcoma with ultrasound-guided fine-needle aspiration: 36 cases. J Small Anim Pract. 2007;48(3):145-50.

57. Neihaus SA, Locke JE, Barger AM, Borst LB, Goring RL. A novel method of core aspirate cytology compared to fine-needle aspiration for diagnosing canine osteosarcoma. Journal of the American Animal Hospital Association. 2011;47(5):317-23.

58. Kirpensteijn J, Kik M, Rutteman GR, Teske E. Prognostic significance of a new histologic grading system for canine osteosarcoma. Vet Pathol. 2002;39(2):240-6.

59. Wrigley RH. Malignant versus nonmalignant bone disease. The Veterinary clinics of North America Small animal practice. 2000;30(2):315-47, vi-vii.

60. Reinhardt S, Stockhaus C, Teske E, Rudolph R, Brunnberg L. Assessment of cytological criteria for diagnosing osteosarcoma in dogs. J Small Anim Pract. 2005;46(2):65-70.

61. Wykes PM WS, Powers BE. Closed biopsy for diagnosis of long bone tumors: accuracy and results. . J Am Anim Hosp Assoc. 1985(21(4)):pp489-94.

62. Powers BE, LaRue SM, Withrow SJ, Straw RC, Richter SL. Jamshidi needle biopsy for diagnosis of bone lesions in small animals. J Am Vet Med Assoc. 1988;193(2):205-10.

63. Armbrust LJ, Biller DS, Bamford A, Chun R, Garrett LD, Sanderson MW. Comparison of three-view thoracic radiography and computed tomography for detection of pulmonary nodules in dogs with neoplasia. J Am Vet Med Assoc. 2012;240(9):1088-94.

64. Eberle N, Fork M, von Babo V, Nolte I, Simon D. Comparison of examination of thoracic radiographs and thoracic computed tomography in dogs with appendicular osteosarcoma. Vet Comp Oncol. 2011;9(2):131-40.

65. Straw RC, Cook NL, LaRue SM, Withrow SJ, Wrigley RH. Radiographic bone surveys. J Am Vet Med Assoc. 1989;195(11):1458.

66. Withrow SJ, Hirsch VM. Owner response to amputation of a pet's leg. Vet Med Small Anim Clin. 1979;74(3):332, 4.

67. Dickerson VM, Coleman KD, Ogawa M, Saba CF, Cornell KK, Radlinsky MG, et al. Outcomes of dogs undergoing limb amputation, owner satisfaction with limb amputation procedures, and owner perceptions regarding postsurgical adaptation: 64 cases (2005-2012). J Am Vet Med Assoc. 2015;247(7):786-92.

68. Jehn CT, Lewis DD, Farese JP, Ferrell EA, Conley WG, Ehrhart N. Transverse ulnar bone transport osteogenesis: a new technique for limb salvage for the treatment of distal radial osteosarcoma in dogs. Veterinary surgery : VS. 2007;36(4):324-34.

69. Covey JL, Farese JP, Bacon NJ, Schallberger SP, Amsellem P, Cavanaugh RP, et al. Stereotactic radiosurgery and fracture fixation in 6 dogs with appendicular osteosarcoma. Veterinary surgery : VS. 2014;43(2):174-81.

70. Berg J, Weinstein MJ, Schelling SH, Rand WM. Treatment of dogs with osteosarcoma by administration of cisplatin after amputation or limb-sparing surgery: 22 cases (1987-1990). J Am Vet Med Assoc. 1992;200(12):2005-8.

71. LaRue SM, Withrow SJ, Powers BE, Wrigley RH, Gillette EL, Schwarz PD, et al. Limb-sparing treatment for osteosarcoma in dogs. J Am Vet Med Assoc. 1989;195(12):1734-44.

72. Thrall DE, Withrow SJ, Powers BE, Straw RC, Page RL, Heidner GL, et al. Radiotherapy prior to cortical allograft limb sparing in dogs with osteosarcoma: a dose response assay. International journal of radiation oncology, biology, physics. 1990;18(6):1351-7.

73. Withrow SJ, Thrall DE, Straw RC, Powers BE, Wrigley RH, Larue SM, et al. Intra-arterial cisplatin with or without radiation in limb-sparing for canine osteosarcoma. Cancer-Am Cancer Soc. 1993;71(8):2484-90.

74. Straw RC, Withrow SJ. Limb-sparing surgery versus amputation for dogs with bone tumors. The Veterinary clinics of North America Small animal practice. 1996;26(1):135-43.

75. Liptak JM, Dernell WS, Straw RC, Jameson VJ, Lafferty MH, Rizzo SA, et al. Intercalary bone grafts for joint and limb preservation in 17 dogs with high-grade malignant tumors of the diaphysis. Veterinary surgery : VS. 2004;33(5):457-67.

76. Liptak JM, Dernell WS, Ehrhart N, Lafferty MH, Monteith GJ, Withrow SJ. Cortical allograft and endoprosthesis for limb-sparing surgery in dogs with distal radial osteosarcoma: a prospective clinical comparison of two different limb-sparing techniques. Veterinary surgery : VS. 2006;35(6):518-33.

77. Pooya HA, Seguin B, Mason DR, Walsh PJ, Taylor KT, Kass PH, et al. Biomechanical comparison of cortical radial graft versus ulnar transposition graft limb-sparing techniques for the distal radial site in dogs. Veterinary surgery : VS. 2004;33(4):301-8.

78. Seguin B, Walsh PJ, Mason DR, Wisner ER, Parmenter JL, Dernell WS. Use of an ipsilateral vascularized ulnar transposition autograft for limb-sparing surgery of the distal radius in dogs: an anatomic and clinical study. Veterinary surgery : VS. 2003;32(1):69-79.

79. Morello E, Vasconi E, Martano M, Peirone B, Buracco P. Pasteurized tumoral autograft and adjuvant chemotherapy for the treatment of canine distal radial osteosarcoma: 13 cases. Veterinary surgery : VS. 2003;32(6):539-44.

80. Moore AS, Dernell WS, Ogilvie GK, Kristal O, Elmslie R, Kitchell B, et al. Doxorubicin and BAY 12-9566 for the treatment of osteosarcoma in dogs: a randomized, double-blind, placebo-controlled study. J Vet Intern Med. 2007;21(4):783-90.

81. Skorupski KA, Uhl JM, Szivek A, Allstadt Frazier SD, Rebhun RB, Rodriguez CO, Jr. Carboplatin versus alternating carboplatin and doxorubicin for the adjuvant treatment of

canine appendicular osteosarcoma: a randomized, phase III trial. Vet Comp Oncol. 2016;14(1):81-7.

82. Phillips B, Powers BE, Dernell WS, Straw RC, Khanna C, Hogge GS, et al. Use of single-agent carboplatin as adjuvant or neoadjuvant therapy in conjunction with amputation for appendicular osteosarcoma in dogs. Journal of the American Animal Hospital Association. 2009;45(1):33-8.

83. Bacon NJ, Ehrhart NP, Dernell WS, Lafferty M, Withrow SJ. Use of alternating administration of carboplatin and doxorubicin in dogs with microscopic metastases after amputation for appendicular osteosarcoma: 50 cases (1999-2006). J Am Vet Med Assoc. 2008;232(10):1504-10.

84. Kent MS, Strom A, London CA, Seguin B. Alternating carboplatin and doxorubicin as adjunctive chemotherapy to amputation or limb-sparing surgery in the treatment of appendicular osteosarcoma in dogs. J Vet Intern Med. 2004;18(4):540-4.

85. Straw RC, Withrow SJ, Richter SL, Powers BE, Klein MK, Postorino NC, et al. Amputation and cisplatin for treatment of canine osteosarcoma. J Vet Intern Med. 1991;5(4):205-10.

86. Berg J, Weinstein MJ, Springfield DS, Rand WM. Results of surgery and doxorubicin chemotherapy in dogs with osteosarcoma. J Am Vet Med Assoc. 1995;206(10):1555-60.

87. Selmic LE, Burton JH, Thamm DH, Withrow SJ, Lana SE. Comparison of carboplatin and doxorubicin-based chemotherapy protocols in 470 dogs after amputation for treatment of appendicular osteosarcoma. J Vet Intern Med. 2014;28(2):554-63.

88. Lane A, Black M, Wyatt K. Toxicity and efficacy of a novel doxorubicin and carboplatin chemotherapy protocol for the treatment of canine appendicular osteosarcoma following limb amputation. Aust Vet J. 2012;90(3):69-74.

89. Coomer A, Farese J, Milner R, Liptak J, Bacon N, Lurie D. Radiation therapy for canine appendicular osteosarcoma. Vet Comp Oncol. 2009;7(1):15-27.

90. Oblak ML, Boston SE, Higginson G, Patten SG, Monteith GJ, Woods JP. The impact of pamidronate and chemotherapy on survival times in dogs with appendicular primary bone tumors treated with palliative radiation therapy. Veterinary surgery : VS. 2012;41(3):430-5.

91. Withrow SJ, Powers BE, Straw RC, Wilkins RM. Comparative aspects of osteosarcoma. Dog versus man. Clin Orthop Relat Res. 1991(270):159-68.

92. Withrow SJ, Wilkins RM. Cross talk from pets to people: translational osteosarcoma treatments. ILAR journal. 2010;51(3):208-13.

93. Mueller F, Fuchs B, Kaser-Hotz B. Comparative biology of human and canine osteosarcoma. Anticancer research. 2007;27(1a):155-64.

94. Bruland Ø, Fodstad Ø, Stenwig E, Phil A. Expression and characterisation of a novel human osteosarcoma associated cell surface antigen. Cancer Res. 1988;48:5302-9.

95. Bruland Ø, Fodstad Ø, Funderud S, Phil A. New monoclonal antibodies spesific for human sarcomas. Int J Cancer. 1986;38:27-31.

96. Haines D, Bruland Ø. Immunohistochemical detection of osteosarcoma-associated antigen in canine osteosarcoma. Anticancer research. 1989;9:903-8.

97. Strzalka W, Ziemienowicz A. Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. Annals of Botany. 2011;107:1127–40.

98. Miyachi K, Fritzler MJ, Tan EM. Autoantibody to a nuclear antigen in proliferating cells. J Immunol. 1978;121:2228–34.

99. Bravo R, Fey SJ, Bellatin J, Larsen PM, Celis JE. Identification of a nuclear polypeptide (“cyclin”) whose relative proportion is sensitive to changes in the rate of cell proliferation and to transformation. Prog Clin Biol Res. 1982;85(Part A):235–48.

100. Celis JE, Bravo R, Larsen PM, Fey SJ. Cyclin: a nuclear protein whose level correlates directly with the proliferative state of normal as well as transformed cells. Leuk Res. 1984;8(143–157).

101. Mathews MB, Bernstein RM, Franza BR, Garrels JI. Identity of the proliferating cell nuclear antigen and cyclin. Nature. 1984;309:374–6.

102. Naryzhny S. Proliferating cell nuclear antigen: a proteomics view. Cellular and Molecular Life Sciences. 2008;65:3789-808.

103. Tan CK, Castillo C, So AG, Downey KM. An auxiliary protein for DNA polymerase-δ from fetal calf thymus. J Biol Chem. 1986;261:12310–6.

104. Prelich G, Tan CK, Kostura M, Mathews MB, So AG, Downey KM, et al. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. Nature. 1987;326:517–20.

105. Bowman GD, O Donnell M, Kuriyan J. Structural analysis of a eukaryotic sliding DNA clamp-clamp loader complex. Nature. 2004;429:724–30.

106. Fukuda K, Morioka H, Imajou S, Ikeda S, Ohtsuka E, Tsurimoto T. Structure-function relationship of the eukaryotic DNA replication factor, proliferating cell nuclear antigen. J Biol Chem. 1995;270:22527–34.

107. Argyle DJ, Khanna C. Tumor Biology and Metastasis. In: Withrow SJ, Vail DM, Page RL, editors. Withrow & MacEwen's Small Animal Clinical Oncology. 5th ed. Missouri: Elsevier Saunders; 2013. p. 30-1.

108. Nussbaum RL, McInnes RR, Willars HF. The Human Genome and the Chromosomal Basis of Heredity. In: Nussbaum RL, McInnes RR, Willars HF, editors. Thompson & Thompson Genetics in Medicine. 7th ed. Philadelphia: Saunders Elsevier; 2007. p. 5-23.

109. Tsurimoto T, Stillman B. Multiple replication factors augment DNA synthesis by the two eukaryotic DNA polymerases, alpha and delta. Embo J. 1989;8(12):3883–9.

110. Bravo R, Frank R, Blundell P, Macdonald-Bravo H. Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. Nature. 1987;326:515-7.

111. Almendral JM, Huebsch D, Blundell PA, Macdonald-Bravo H, Bravo R. Cloning and sequence of the human nuclear protein cyclin: homology with DNA-binding proteins. Proc Natl Acad Sci USA. 1987;84:1575–9.

112. Jaskulski D, Gatti C, Travali S, Calabretta B, Baserga R. Regulation of the proliferating cell nuclear antigen cyclin and thymidine kinasemRNAlevels by growth factors. J Biol Chem. 1988;263:10175–9.

113. Elias JM. Cell proliferation indexes: a biomarker in solid tumors. Biotech Histochem. 1997;72:78–85.

114. Czyzewska J, Guzinska-Ustymowicz K, Lebelt A, Zalewski B, Kemona A. Evaluation of proliferating markers Ki-67, PCNA in gastric cancers. Rocz Akad Med Bialymst. 2004;49:64-6.

115. Morell-Quadreny L, Clar-Blanch F, Fenollosa-Enterna B, Perez-Bacete M, Martinez-Lorente A, Llombart-Bosch A. Proliferating cell nuclear antigen (PCNA) as a prognostic factor in renal cell carcinoma. Anticancer research. 1998;18:677–82.

116. Simoes JP, Schoning P, Butine M. Prognosis of canine mast cell tumors: a comparison of three methods. Vet Pathol. 1994;31(6):637-47.

117. Ehrhart EJ, Kamstock DA, Powers BE. The Pathology of Neoplasia. In: Withrow SJ, Vail DM, Page RL, editors. Withrow & MacEwen's Small Animal Clinical Oncology. 5th ed. Missouri: Elsevier Saunders; 2013. p. 51-67.

118. Mescher AL. Histology & Its Methods of Study. In: Weitz M, Davis K, editors. Junqueira's Basic Histology Text & Atlas. 12th ed: The McGraw-Hill; 2010. p. 1-16.

119. Ramos-Vara JA. Technical Aspects of Immunohistochemistry. Vet Pathol. 2005;42:405–26.

120. Avery AC, Olver C, Khanna C, Paoloni MC. Molecular Diagnostics. In: Withrow SJ, Page RL, Vail DM, editors. Withrow & MacEwen's Small Animal Clinical Oncology. 5th ed. Missouri: Elsevier Saunders; 2013. p. 131-42.

121. Paget S. The distribution of secondary growths in cancer of the breast. The Lancet. 1889;1(133):571-3.

122. Yin S, Li Z, Huang J, Miao Z, Zhang J, Lu C, et al. Prognostic value and clinicopathological significance of proliferating cell nuclear antigen expression in gastric cancer: a systematic review and meta-analysis. Onco Targets Ther. 2017;10(10):319-27.

123. Thaete LG, Ahnen DJ, Malkinson AM. Proliferating cell nuclear antigen (PCNA/cyclin) immunocytochemistry as a labeling index in mouse lung tissues. Cell and Tissue Research. 1989;256(1):167-73.

124. Nagamine E, Hirayama K, Matsuda K, Okamoto M, Ohmachi T, Kadosawa T, et al. Diversity of Histologic Patterns and Expression of Cytoskeletal Proteins in Canine Skeletal Osteosarcoma. Vet Pathol. 2015;52(5):977-84.

125. Pazdzior-Czapula K, Rotkiewicz T, Otrocka-Domagala I, Gesek M, Smiech A. Morphology and immunophenotype of canine cutaneous histiocytic tumours with particular emphasis on diagnostic application. Vet Res Commun. 2015;39(1):7-17.

126. Sapierzynski R. Practical aspects of immunocytochemistry in canine lymphomas. Polish journal of veterinary sciences. 2010;13(4):661-8.

127. Fernandez NJ, West KH, Jackson ML, Kidney BA. Immunohistochemical and histochemical stains for differentiating canine cutaneous round cell tumors. Vet Pathol. 2005;42(4):437-45.

Appendix

Appendix 1: Routine Autopsy Protocol

The following autopsy protocol is based on a metoptic method and consists of an evisceration followed by further investigation of the internal organs.

The skin is examined for wounds, rashes, swellings, asymmetry and nodules. The fur is skinned of the body by making an incision in the ventral midline from the incisura mentalis and all the way to the vulva in female animals, and curved around the prepuce and scrotum in male animals. The incision is extended down to the paws on all four libs. The skin is released from the subcutis.

An incision is made through the medial thigh musculature down to the hip joint on both hind legs and the joints are opened. The ligamentum teres and joint fluid are inspected. The musculature located medially to the scapula is incised to partially release the front legs from the trunk.

The mammary glands are inspected. An incision is made over the spermatic cord through the vaginal tunic, down to the testicle. The gubernaculum is cut over to free the testicle from the scrotum. The penis and scrotum is cut free from the abdominal wall all the way to the arcus ischiadicus. The crus penis is cut on both sides. During this procedure, the position of the testicles is evaluated and the inguinal openings are evaluated and inspected for adherences and signs of fluid.

The abdominal cavity is opened by making an incision from the xiphoid process to the pecten ossis pubis, slightly lateral to the linea alba. The incision is extended from the xiphoid process along the costal arc to the dorsal lumbar region. The incision is also extended from the pecten ossis pubis along the pelvis and up to the groin. The omentum is advanced cranially to expose and permit a good visualisation of the abdominal visceral organs. The positions of the abdominal organs are evaluated, the peritoneal and serosal surfaces are evaluated for hyperemia and inflammation, and the peritoneal space is inspected for signs of fluid.

A double ligature is placed around the rectum and the duodenum at the level of the right pancreatic lobe, and the intestines located between the two ligatures are cut free. The intestines from the rectum to the duodenum are freed from the mesentery near its insertion. During this step, the intestines are inspected for adherences, the position of the intestines is evaluated and the consistency of the faecal contents is evaluated.

The omentum near the greater curvature is cut and is withdrawn along with the spleen. The spleen is investigated for adherences. The distal oesophagus is cut close to the ventricle, to avoid opening the thorax. Both ligamentum triangulare of the liver, the left pancreatic lobe and the vena porta is cut. The caudal vena cava is cut right behind and in front of the liver. The ventricle, liver, pancreas and duodenum are withdrawn together.

A bone cutter is placed in the obturator foramen and the pelvic floor is loosened by cutting in the cranial and caudal direction through the pubic and ischiadic bone. The pelvic floor is removed to expose the pelvic organs.

The adrenal glands are removed. The kidneys are carefully cut free along with the ureters down to the bladder. The kidneys, ureters, bladder and urethra are withdrawn together, along with the genitals and rectum.

The abdominal aorta, caudal vena cava and the dorsal mesentery are withdrawn together. The mesentery lymph nodes are examined.

An incision is made from the most rostro-ventral point of the chin to the manubrium sterni. The trachea, oesophagus and thyroid gland are loosened together. The musculature between the mandibular bones is cut to loosen the tongue. The tongue is pulled caudo-ventrally while cutting free the soft palate and dorsal pharynx. The hyoid bones are cut. The trachea, oesophagus and thyroid glands are freed down to the thoracic inlet, without opening the thorax. The retropharyngeal lymph nodes and thymus (if present) are examined.

The position of the diaphragm is evaluated before cutting it out by laying the incision along the ribs, without opening the pericardial sac. The pericardial sac is loosened from the sternum by hand. The pleural space is examined for fluid.

The thorax is further opened by cutting the ribs at the costochondral junction. The sternum is removed to expose the thoracic organs.

The pericardial sac is further loosened from the sternum by hand. The trachea and oesophagus are released from its attachments at the thoracic inlet before the thoracic organs are removed in one piece. The mediastinum is released from its dorsal attachments during

this process. The thoracic organs are examined for signs of adherences to the parietal pleura, and the parietal and visceral pleura are examined for signs of inflammation.

The superficial cervical, axial, medial iliac, iliofemoral, superficial inguinal and popliteal lymph nodes are carefully examined. These lymph nodes are evaluated for signs of hyperplasia, metastatic neoplasia or abscesses.

The joints in the libs are opened from the medial side starting at the most distal joints. The joint surfaces and joint fluid are carefully examined for signs of erosions in the cartilage, joint mice and osteophyte proliferations. The joint capsule thickness is also evaluated.

Longitudinal incisions are made in the back, neck and psoas-musculature. The cut surfaces are examined for signs of bleeding, colourable changes or signs of parasites.

The carcass is decapitated at the atlanto-ocipital joint in such a manner that the salivary glands are removed with the head. The head is longitudinally cleaved through the centre before the nasal septum is cut out with scissors. The nasal mucosa, concha and sinuses are examined. The brain is carefully withdrawn after carefully cutting the cranial nerves. The meninges are examined for signs of bleeding. The brain ventricles are opened by making medial incisions, and the size and fluid contents are evaluated. The brain tissue consistency is evaluated and the bran is examined for signs of bleeding, neoplasia or abscesses. If necessary, the spinal cord can be evaluated after cutting the vertebral arches in a longitudinal manner.

The mandibular and parotid lymph nodes and the salivary glands are cut and evaluated. Eyes and ears are examined, and both tympanic bullae are opened if signs of otitis are present. The oral cavity, including teeth, is examined.

The pericardial sac is opened and evaluated for signs of fluid. The aorta and arteria pulmonalis are cut as far from the heart base a possible. The shape and size of the heart is evaluated. Incisions are made in the heart to open both ventricles and atries to permit the evaluation of myocardial thickness, changes in the valves, signs of congenital abnormalities or signs of degeneration.

Incisions are made in the tongue. The tonsils are examined for signs of reactivity. The trachea and oesophagus are separated starting at the most caudal end, before the oesophagus is opened by making a longitudinal incision at its dorsal side. The oesophageal mucosa is examined for signs of erosions and bleeding.

The passage through the larynx is evaluated before the larynx and trachea is opened by a dorsal longitudinal incision. The trachea is examined for signs of oedema. The thyroid glands and parathyroid glands are examined.

The lungs are evaluated for changes in shape, size, colour and consistency. The lungs are palpated thoroughly and any areas with altered consistency are incised. These areas are also flotation tested in tap water. Incisions are extended from the trachea to the distal bronchi. Deep cuts are made in each lung lobe. The lungs are examined for signs of oedema, emphysema or atelectasis. The tracheobronchial and mediastinal lymph nodes are incised and evaluated.

Incisions are made through the parietal surface of the spleen. The spleen is examined for signs of infarctions and the size is evaluated. The surrounding lymph nodes are evaluated.

The duodenum is opened anti-mesenteric side and the bile drainage through the duodenal papilla is evaluated by pressing on the gall bladder. The gall bladder and ductus choledocus is opened and examined. The ventricle is opened by cutting along the greater curvature. The jejunum, ileum and colon are opened along the mesenteric attachment. The rectum is separated from the rest of the pelvic organs and opened. The serosal and mucosal sides are examined for signs of hyperemia, bleeding, erosions and inflammatory changes. The faecal content is evaluated.

The liver is examined for changes in size, shape, colour and consistency. The latter is done by pressing a 0,5 cm slice between the fingers. The liver is examined for metastasis and abscesses. Incisions are made in the pancreas, and the cut surfaces are inspected.

Incisions are made in the adrenal glands. The kidneys are divided in two by making a longitudinal incision, which involves the renal pelvis. The kidneys are decapsulated. The kidneys are evaluated for changes in shape, size, colour and texture, and examined for signs of infarction, neoplasia and abscesses. Both ureters are palpated and opened. A v-shaped incision is made over the dorsal aspect of the bladder with the point of the v pointing caudally. The bladder is inspected for signs of inflammation, neoplasia, concrements and polyps.

In male animals, incisions are made in the testis, epididymis and prostate gland. In female animals, the ovaries are inspected. The vulva is cut dorsally, and the cut is extended trough the vestibulum, cervix and uterus.

A rib is broken to evaluate mineralization. A long bone is sawn in half to evaluate the bone marrow.

The mammary glands are palpated for signs of inflammation and neoplasia. Several incisions are made in the mammary glands and the cut surfaces are examined.

Source: University Pathology Department at the Norwegian University of Life Sciences campus Adamstuen

Appendix 2: Protocol for pulmonary tissue sampling

A full body autopsy is performed before the tissue samples are taken. The following samples are to be taken:

Full body:

- Routine incisional tissue samples including from the heart, liver, kidney and lung, fixed in formalin
- Tissue samples from the primary tumour, if present, fixed in formalin
- Tissue samples from the regional lymph node(s) draining the affected limb, if primary tumour is present, fixed in formalin

Lungs:

- Tissue samples from the right-, middle- and left tracheobronchial lymph nodes and pulmonary lymph nodes for both formalin fixation and snap freezing.
- 1 x 1 x1 cm tissue samples from all lung lobes (figure x) with 2 samples taken from the periphery of the lobe (5 cm apart) and 2 samples from the centre (including bronchi less than 5 mm in diameter), for both formalin fixation and snap freezing.
- Tissue samples from lung tissue with palpable findings or gross changes, for both formalin fixation and snap freezing.

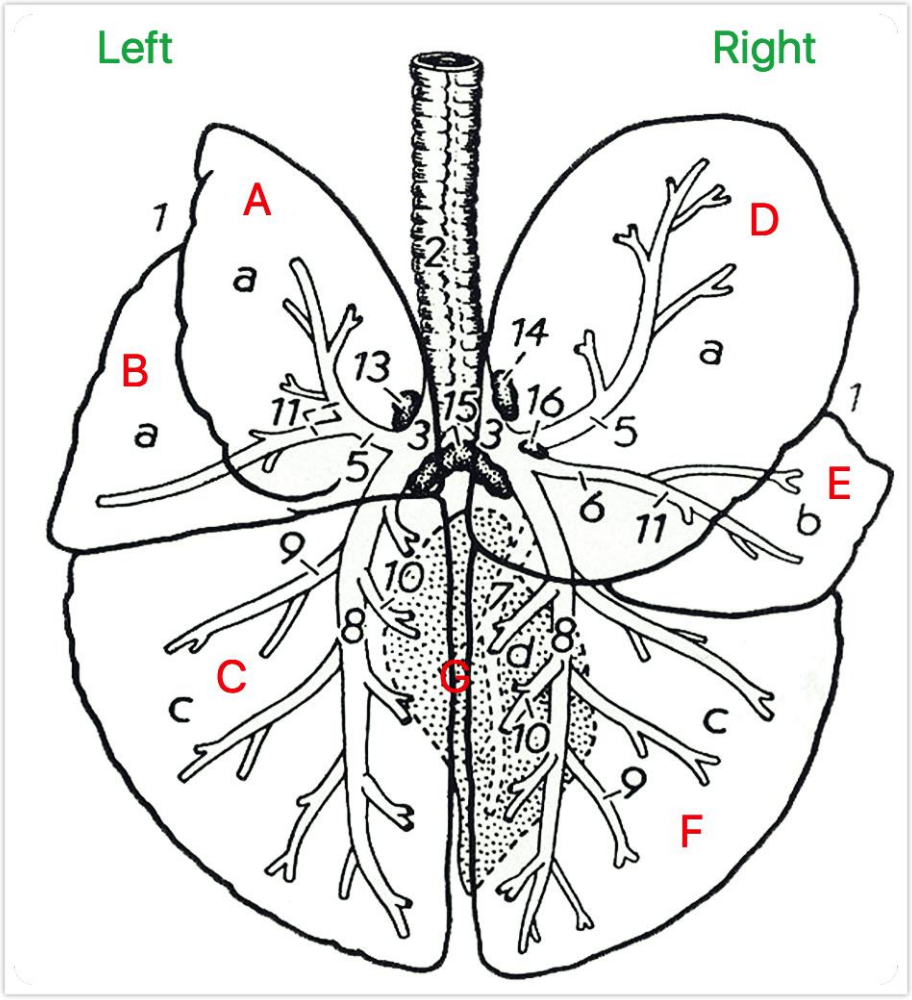

Figure 16: The figure shows the dorsal aspect of a canine lung. The 6 different lung lobes are marked in red from A-G. A: Left cranial lung lobe (cranial part); B: Left cranial lung lobe (caudal part); C: Left caudal lung lobe; D: Right cranial lung lobe; E: Right middle lung lobe; F: Right caudal lung lobe; G: Accessory lung lobe. The lymph nodes are marked from 13-16. 13: Left tracheobronchial lymph node; 14: Right tracheobronchial lymph node; 15: Middle tracheobronchial lymph node; 16: Pulmonary lymph node.

All samples for paraffin embedding are immediately fixed in a 10 % buffered formalin solution and stored at room temperature until further processing.

All samples for cryosections are immediately snap frozen in isopropanol, chilled with liquid nitrogen, before being transferred to liquid nitrogen. The sampled are then transferred and stored at -80 °C until further processing.

Source: Supervisor Lars Moe

Appendix 3: Hematoxylin & Eosin staining protocol

1. Heating chamber at 58°C for 30 minutes
2. 2 changes of xylene, 5 minutes each
3. Re-hydrate in 2 changes of absolute alcohol, 5 minutes each
4. 2 changes of 96% alcohol for 5 minutes each
5. 70% alcohol for 5 minutes
6. Wash thoroughly in running tap water
7. Stain in Meyers Hematoxylin solution for 5 minutes
8. Wash in running tap water for 5 minutes
9. Stain in Eosin solution for 1 minute
10. Wash in running tap water for 1 minute
11. Dehydrate in 70% alcohol for 10 – 20 seconds
12. 2 changes of 96% alcohol for 10 – 20 seconds each
13. 2 changes of absolute alcohol, 10 dips each
14. Clear in xylene for 5 minutes
15. Mount with xylene based mounting medium

Source: University Histology Laboratory at the Norwegian University of Life Sciences campus Adamstuen

Appendix 4: Deparaffinising protocol

1. Heating chamber at 58°C for 30 minutes
2. 2 changes of xylene, 5 minutes each
3. Re-hydrate in 2 changes of absolute alcohol, 5 minutes each
4. 2 changes of 96% alcohol for 5 minutes each
5. 70% alcohol for 5 minutes
6. Wash thoroughly in running tap water
7. The sections are kept in still water until further preparations

Source: University Histology Laboratory at the Norwegian University of Life Sciences campus Adamstuen

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